Galectin-3 null mice display defective neutrophil clearance during acute inflammation.

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Summary Sentence: Expression of galectin-3 by exudated neutrophils drives neutrophil apoptosis and clearance in a model of self-resolving peritonitis.

Running Title: Endogenous galectin-3 promotes neutrophil clearance

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**Abbreviations**

Gal-3: Galectin-3

S. Pneumoniae: Streptococcus pneumonia

*E. coli*: Escherichia coli

L. Major: Leishmania major

**Abstract**

Galectin-3 has been attributed with a plethora of pro-inflammatory functions due its abilities, amongst others, to promote neutrophil activation and the reduction of neutrophil recruitment in models of infection in its absence. Conversely, it has also been linked to resolution of inflammation through its actions as an opsonin and ability to promote efferocytosis of apoptotic neutrophils. Using a self-resolving model of peritonitis we have addressed the modulation and role of Gal-3 in acute inflammation. We have shown that Gal-3 expression is increased in neutrophils that traffic to the inflamed peritoneum and that cellular localisation of this lectin is modulated over the course of the inflammatory response. Furthermore, neutrophil recruitment to the inflamed peritoneum is increased in Gal-3 null mice over the course of the response and this correlates with reduced numbers of monocytes/macrophages in the cavities of these mice as well as reduced apoptosis and efferocytosis of Gal-3 null neutrophils. These data indicate a role for endogenous Gal-3 in neutrophil clearance during acute inflammation.

## Introduction

Galectins are a family of beta-galactoside binding proteins that elicit their effects by binding to exposed N-acetyllactosamine residues on cells [1]. Several galectins have been designated key immunomodulatory roles in a range of pathological settings; of these, galectin-3 (Gal-3) is identified as a pro-inflammatory molecule that functions to drive the inflammatory response through the activation of innate immune cells and its chemoattractant actions [2-5]. During infections with pathogens such as *S. Pneumoniae*, Gal-3 appears to be beneficial for the host with enhanced pathogenicity observed in Gal-3 null mice and reduced infection in mice treated with recombinant Gal-3 [6, 7]. Such effects are thought to be due to Gal-3 enhancing recruitment/activation of neutrophils as well as direct bactericidal actions [6]. Administration of recombinant Gal-3 to human neutrophils results in degranulation, release of reactive oxygen species and an increase in phagocytic capability [3, 5, 8]. With regards to neutrophil trafficking, decreased neutrophil recruitment is seen in the lungs of Gal-3 null mice infected with *S. Pneumoniae* and this can be rescued by administration of the recombinant protein [9]; Gal-3 can also act directly as an adhesion molecule and promotes adhesion of neutrophils to endothelial cells *in vitro* [7]. The effects of Gal-3 on neutrophil trafficking appear to be specific to particular pathogens as no differences were observed in neutrophil trafficking to the lungs in Gal-3 null mice infected with *E. coli* or to air pouches inoculated with the *L. Major* substrain Friedlin when compared to substrain LV39 [10]. Interestingly, both *S. Pneumoniae* and LV39 induced release of Gal-3 into the inflammatory exudate whereas *E. coli* and Friedlin did not, suggesting that it is the presence of Gal-3 in inflammatory exudates that functions to drive neutrophil recruitment. This hypothesis is supported by the finding that administration of recombinant Gal-3 into the murine

air pouch results in neutrophil recruitment [10]. Whether endogenous Gal-3 is released and has a role in neutrophil trafficking to other sites of inflammation such as the inflamed peritoneal cavity is not clear. Colnot *et al.* observed reduced recruitment of granulocytes in a model of thioglycollate-induced peritonitis in Gal-3 null mice on day 4 only, whilst Hsu et al observed reduced numbers of neutrophils on day 1 only [11, 12]. Furthermore, it is not clear how Gal-3 promotes neutrophil transmigration when it is released into inflammatory exudates or injected into a cavity such as the air pouch as it is not chemotactic for neutrophils [9] nor does it appear to modulate the levels of major chemokines/cytokines within inflammatory exudates [10].

As well as the aforementioned studies supporting a role for Gal-3 in driving innate immunity, there is evidence to suggest that it may facilitate resolution of inflammation through its actions as an opsonin [13]; furthermore, Gal-3 deficient macrophages are less efficient at phagocytosing apoptotic neutrophils than wild-type cells [6]. Binding of Gal-3 to human neutrophils promotes the exposure of phosphatidylserine without inducing apoptosis, a process known to act as an “eat-me” signal for phagocytes; suggesting a role for Gal-3 in the clearance of neutrophils [14]. However, evidence that treatment of human neutrophils with recombinant Gal-3 delays their spontaneous apoptosis and that the rate of apoptosis of neutrophils from Gal-3 null mice does not differ from that of wild-type cells is at odds with a pro-resolving function [6, 11].

Whilst the studies above have provided evidence that Gal-3 is an important regulator of the innate immune system, the modulation of the endogenous protein in innate immune cells over the course of an inflammatory response has not been

systematically studied. The expression of Gal-3 is reportedly negligible basally in both human and murine neutrophils and whether levels are modulated during inflammation is not clear, as again contrasting reports exist. Gil *et al.,* demonstrated an increase in Gal-3 expression in recruited neutrophils following carrageenan-induced peritonitis in the rat [15]; while Sato and colleagues showed no increased expression of the lectin in murine neutrophils that had been recruited to *S. Pneumoniae* infected alveoli [7]. Human neutrophils express Gal-3 and levels are not modulated upon migration through an endothelial monolayer in an *in vitro* transmigration assay in response to interleukin-8 [16].

With this study, we characterised the regulation and function of Gal-3 in neutrophils over the course of a self-resolving inflammatory response. Our findings demonstrate that Gal-3 expression is increased in neutrophils that have migrated into the inflamed peritoneal cavity and that neutrophil numbers are increased in the peritoneal cavities of Gal-3 null mice. This is likely due to reduced clearance rather than increased trafficking as evidenced by reduced levels of apoptosis of Gal-3 null neutrophils coupled with their reduced efferocytosis.

## Materials and Methods

### Reagents

All antibodies were purchased from eBioscience, Hatfield, UK. Annexin V-FITC apoptosis detection kit was purchased from BD Pharmingen, Oxford, UK. CellTrace™ carboxyfluorescein succinimidyl ester was purchased from Invitrogen, Paisley, UK. Histopaque 1077 and Zymosan were purchased from Sigma-Aldrich, Poole, UK.

### Ethics

All animal studies were conducted with ethical approval from the Queen Mary University of London Local Ethical Review Committee and in accordance with the UK Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986).

### Mice

Male C57BL/6 mice were obtained from Charles River, Margate, UK. Breeding pairs of galectin-3 null mice (B6.Cg-Lgals3tm1Po1/J) were provided by the Consortium for Functional Glycomics (http://functionalglycomics.org) and a colony was established at Charles River, Margate, UK. These mice were on a C57BL/6 background and age- and sex-matched controls were used for all experimental work. All animals were fed standard laboratory chow and water ad libitum and were maintained on a 12 h light-dark cycle under specific pathogen-free conditions. All experiments were performed with mice 6-7 weeks old.

**Peritonitis**

Briefly mice were injected with 1mg zymosan (Sigma) i.p. in 1ml sterile PBS as previously described by Ajuebor et al in 1999 [17], 0 h mice received no treatment. At 4, 24, 48, 72 and 96 h post injection the mice were anaesthetised, along with 0 h controls, with isoflurane and a cardiac puncture was performed to obtain peripheral blood. The mice were then sacrificed by cervical dislocation and peritoneal lavages were performed with 4ml ice-cold PBS to collect peritoneal exudates. Tibias and femurs were collected, cleaned and flushed with sterile PBS using a 25G needle to extract bone marrow. Bone marrow cells were then filtered through a 70μm filter and washed prior to staining for analysis by flow cytometry.

In some experiments peritonitis was induced using 1mg lyophilised *E. coli* (strain K12; Sigma) i.p. in 1ml sterile PBS. Peritoneal cavities were lavaged as described above 4h post-injection.

**Flow Cytometry**

Murine cells were incubated with antibodies for Ly6G (eBioscience, clone 1A8) to identify neutrophils or a combination of F4/80 and Gr-1 (eBioscience, clone BM8 and RB6-8C5 respectively) to identify classical and non-classical monocyte/macrophages. Cells were then fixed and permeabilised with BD fixation and permeabilisation buffer before addition of anti-Gal-3 antibody (eBioscience, clone M3/38) to assess intracellular Gal-3 levels (some cells were incubated with Ly6G and Gal-3 without permeabilisation to assess cell surface levels). To determine cytosolic expression of Gal-3, MFIs for surface expression were deducted from the

MFI values obtained for total expression. In all cases, antibodies or relevant isotype controls were incubated at 4°C prior to flow cytometric analysis using FlowJo.

**Confocal Microscopy**

Peritoneal lavage cells were seeded onto Ibidi 8 well chamber slides in RPMI and allowed to adhere. Following fixation with 1% paraformaldehyde cells were blocked with 5% FBS before permeabilisation with 0.05% Triton X-100. Staining for intracellular Gal-3 was performed using anti-Gal-3-PE (eBioscience) for 1 h at room temperature. Cells were washed three times and then incubated with phalloidin 647 for 20 mins at room temperature. Finally cells were mounted with ProLong Gold Antifade mountant containing DAPI (Thermo Scientific). Immunofluorescence was assessed using a Zeiss LSM 710 confocal microscope. Images were processed using Zen 2012 (Carl Zeiss Microscopy GmbH) and Adobe Photoshop CS6 (Adobe Systems) software.

**Adoptive Transfer**

WT mice were euthanised and tibias and femurs were collected, cleaned and flushed with RPMI (10% FCS; 2mM EDTA) using a 25G needle as described previously [18]. Following centrifugation red blood cells were lysed with 0.2% hypotonic saline for 20 sec. Bone marrow cells were then washed in RPMI and yield of neutrophils was quantified using Turk's solution. To fluorescently label cells for transfer, neutrophils were resuspended in PBS at a concentration of 5 x $10^6$/ml. 5μM CFSE (Thermo Scientific) was added and cells were incubated at 37°C for 8 mins. An equal volume of FCS was then added to quench excess CFSE and the cells were washed. Following two further washes with ice-cold RPMI, the cells were resuspended in ice-

cold PBS at a density of 25 x $10^6$/ml. 5 x $10^6$ neutrophils were administered to each Gal-3 null recipient mouse via the lateral tail vein and 1mg zymosan was injected i.p. 4 h post-zymosan administration, peritoneal lavages were collected as described above and cells were stained with anti-Ly6G to identify neutrophils and Gal-3. Some cells were also permeabilised following surface stain with Ly-6G to assess intracellular expression of Gal-3.

**Gal-3 ELISA**

Gal-3 levels were measured in peritoneal exudates using a commercial ELISA kit (R&D systems, Abingdon, UK) according to manufacturer's instructions.

**Apoptosis Assay**

Exudated neutrophils from WT and Gal-3 null mice were collected following 4, 48, 72 and 96h zymosan-induced peritonitis and labelled with Ly6G. Following this the cells were labelled with Annexin V and propidium iodide as per manufacturer's instructions (BD Biosciences) or Zombie NIR™ (viability dye; 1:400 dilution in PBS; Biolegend) to assess apoptosis. In some experiments 4h exudated neutrophils were cultured overnight in RPMI and assessed the following day at 18h for surface Gal-3 levels and apoptosis. Gal-3 staining was performed as described above and apoptosis was assessed using Annexin V and Zombie NIR™. Viable (Annexin V negative, Zombie negative) and late apoptotic (Annexin V, Zombie double positive) cells were gated and Gal-3 expression assessed.

**Efferocytosis Assay**

Biogel-elicited macrophages were collected from WT mice as previously described [19] and seeded into 24-well plates at 0.5 x $10^6$ cells per well and allowed to adhere. Exudated neutrophils from WT and Gal-3 null mice were collected following 4 h zymosan-induced peritonitis and labelled with BODIPY®FL for 5 mins at 37°C. Following washing, neutrophils were resuspended in RPMI at 2 x $10^6$/ml and 0.5ml was added per well of macrophages and incubated for 1h. Following extensive washing efferocytosis was quantified using ImageJ software. Images were split into their individual red/blue/green channels and the same background threshold was applied to all images prior to quantification of fluorescence.

**Statistical Analysis**

Statistical significance was assessed using SPSS computer software. Data is expressed as mean ± standard error of the mean (SEM) of n experiments. All data were tested for normal distribution and power calculations were performed using G*Power software [20]. Statistical differences were analysed by one or two-tailed T-test for 2 groups, one-way analysis of variance (ANOVA) followed by a Bonferroni or Dunnetts post hoc test (depending on if comparing all values or each value to a control respectively) or two-way ANOVA followed by Bonferroni post hoc test. In all cases a P value < 0.05 was considered significant to reject the null hypothesis and differences were considered significant.

## Results

### Modulation of galectin-3 expression in murine neutrophils during inflammation

To investigate the endogenous levels of Gal-3 over the course of an acute inflammatory response, a zymosan-induced peritonitis was performed and monitored over 96h, by which time the inflammation had resolved. Due to the discrepancy between reports of Gal-3 expression in neutrophils, we fully investigated expression levels in three sources of neutrophils, those from the bone marrow, the peripheral blood and those that had migrated into the inflamed peritoneal cavity. There was a rapid increase in the number of total leukocytes in the peritoneal cavity within 4-24h of zymosan administration and this remained elevated over the course of the response with increased leukocyte numbers still observed at 96 h compared to basal levels (Figure 1A). The neutrophil number in the peritoneal cavity increased sharply over 4-24h and then returned back to basal levels within the 96h time course. In contrast the monocyte/macrophage numbers declined within the first 4h of zymosan treatment and then re-populated the peritoneal cavity over the time course (Figure 1D). The number of leukocytes within peripheral blood decreased rapidly following induction of peritonitis, and then returned to basal levels over the course of the response (Figure 1B). Monocytes account for the decrease in total cells seen at 4h, which return to basal levels over the time course. Whilst there were no significant modulations in the numbers of neutrophils in the peripheral blood, a trend can be seen that mirrors the changes in the peritoneal cavity with a decrease as cells migrate from the peripheral blood to the peritoneal cavity and then an increase as the peripheral blood re-populates (Figure 1E). Although no significant modulation of leukocyte number was observed within the bone marrow (Figure 1C), a similar yet delayed trend followed the peripheral blood neutrophils with a decrease in cell

numbers in the bone marrow approximately 24h later than that seen in the peripheral blood. This is likely to account for leukocyte mobilisation to repopulate the peripheral circulation (Figure 1F).

Analysis of peritoneal lavage fluid by ELISA revealed baseline levels of 2.155 ± 0.450 ng/ml Gal-3 within the cavity. These levels increased significantly over the peritonitis time course, with peak levels at 72h (7.293 ± 1.239 ng/ml) (Figure 1G). Endogenous expression of Gal-3 was minimal in monocyte/macrophages within the bone marrow and peripheral blood, yet levels significantly increased in exudate peritoneal cells, with peak expression observed at 72h (Figure 1H). As expected, low levels of Gal-3 were detected in both peripheral blood [7, 9] and bone marrow-derived neutrophils, however total levels were significantly increased in permeabilised, exudated neutrophils over 72h, with peak expression observed at 24h (Figure 1I, J).

**Cellular localisation of Gal-3 is modulated in neutrophils during inflammation**

To determine the cellular localisation of Gal-3, flow cytometry was performed on non-permeabilised versus permeabilised neutrophils (Figure 2A). At 4h post zymosan the Gal-3 expressed by murine neutrophils was predominantly intracellular however at 24h the protein is readily detected on the cell surface, yet by 48h Gal-3 is again expressed predominantly in the intracellular compartment of the cell. Confocal analysis of peritoneal neutrophils taken at the 4h time-point confirmed the intracellular localisation of Gal-3 within migrated neutrophils (Figure 2B).

To determine whether the intracellular Gal-3 detected was derived from the extracellular environment i.e. being taken up from the inflammatory exudate, or being produced by the neutrophils themselves an adoptive transfer experiment was performed. CFSE labelled bone marrow neutrophils isolated from WT mice were injected i.v. into Gal-3 null mice and a zymosan-induced peritonitis performed. CFSE labelled Ly6G positive neutrophils were clearly detectable within peritoneal lavages as shown in Figure 3D. Negligible levels of Gal-3 were detectable on the neutrophil surface (Figure 3E), whilst Gal-3 was readily detectable in permeabilised cells (Figure 3F) indicating the presence of intracellular Gal-3 as shown in Figure 1J.

**The role of galectin-3 in neutrophil clearance**

To further investigate the role of neutrophil derived Gal-3, the zymosan-induced peritonitis was repeated in Gal-3 null mice, initially at the 4h time-point. This time-point was chosen as it represents the peak of neutrophil infiltration into the cavity, which corresponds with low monocyte/macrophage numbers. Surprisingly, significantly higher numbers of leukocytes were observed in the cavities of Gal-3 null mice (Figure 4A). Upon further examination, the percentage of neutrophils within the cavity was significantly higher in KO mice (Figure 4B), whilst the percentage of macrophages was significantly lower (Figure 4C). As was shown in figure 2, surface expression of Gal-3 by peritoneal neutrophils was negligible (Figure 4D) whereas it was readily detected in permeabilised cells by flow cytometry (Figure 4E).

Neutrophil recruitment in Gal-3 null mice appears to vary in its magnitude depending on the inciting stimuli and the site of inflammation. We therefore performed a peritonitis experiment were we used *E. coli* in the place of zymosan. The magnitude

of the response was lower overall in terms of neutrophil recruitment and there was no difference between the two genotypes of mice in terms of numbers of neutrophils recruited to the peritoneal cavity (data not shown). Again Gal-3 was readily detectable within the cytosol of neutrophils that had trafficked to the peritoneum in response to both *E. coli* and zymosan (Supplementary Figure 1) whereas levels were low on the surface of zymosan-elicited neutrophils as well as on those elicited by *E. coli*. To investigate whether neutrophils lacking Gal-3 undergo apoptosis at a different rate to cells from WT animals, neutrophils collected from the peritoneal cavity were cultured overnight and levels of apoptosis were assessed by flow cytometry. Upon removal from the cavity the majority of neutrophils were viable in both genotypes (Figure 5A). However, following overnight culture, significantly fewer neutrophils from the Gal-3 null mice had undergone apoptosis (Figure 5B-E). As Gal-3 has been implicated in the clearance of neutrophils, experiments were conducted to investigate the role of neutrophil-derived Gal-3 on efferocytosis with significantly reduced efferocytosis of Gal-3 null neutrophils observed compared to WT neutrophils (Figure 5F and G). Interestingly surface expression of Gal-3 was found to be increased on apoptotic neutrophils (AnxV/Zombie double positive cells) when compared to viable cells (AnxV/Zombie double negative cells) (Figure 5 H and I).

**Role of Gal-3 during the resolving phase of the peritonitis model.**

As there is evidence in the literature that Gal-3 may be involved in neutrophil clearance, a key facet of the resolution process, leukocyte trafficking was compared in Gal-3 null mice and their WT counterparts at 48-96h post-zymosan. At 48h post-zymosan, total leukocyte counts were comparable for wild type and Gal-3 null mice, however at 72h there was a trend towards more leukocytes in the peritoneal cavities

of Gal-3 null mice although this did not reach statistical significance and levels had declined to those observed in WT mice by 96h (Figure 6A). Interestingly, the percentage of exudated neutrophils was significantly higher in Gal-3 null mice at the 72 and 96h time points (Figure 6B) indicating a potential defect in neutrophil clearance in these mice. Assessment of apoptosis by AnnexinV/PI staining revealed no differences between the two genotypes of mice with regards to levels of apoptotic cells at these later time points (Table 1). As monocytes, particularly non-classical, are key players in the resolution of inflammation, the number of both subsets was compared between the strains of mice. Approximately 40% of cells recovered were non-classical monocytes in both genotypes at 48h and this 40% remained stable in galectin-3 null mice up to 96h. This was in contrast to WT mice in which the number of non-classical monocytes significantly increased at 96h (Figure 6C). In contrast, 20% of the cells recovered from the peritoneum were classical monocytes in both genotypes and this was not significantly altered at 72h although a trend towards a reduced number was seen in the WT mice with approximately 15% of total leukocytes being classical monocytes. By 96h the number of classical monocytes were negligible in both genotypes of mice (Figure 6D).

**Discussion**

The modulation of the inflammatory process, through effects on immune cell biology, by members of the galectin family is an ever-growing area of research. The focus has however by and large been on T cell driven models of auto-immune disease and in the case of Gal-3, cancer. There are several reports describing the effects of Gal-3 on neutrophil activation and its importance in models of infection where it might function as an alarmin by augmenting the inflammatory response [21]. With this

study we have expanded the current knowledge of the role of Gal-3 in acute inflammation, identifying a role for the endogenous protein in neutrophil apoptosis and clearance.

Previous studies have failed to detect expression of Gal-3 intracellularly in murine neutrophils, although it is readily detectable on the cell surface [7, 11]. This has led to the assumption that effects on neutrophil recruitment in Gal-3 null mice are due to a lack of extracellular Gal-3, likely released from cells such as inflammatory macrophages. We have confirmed that bone marrow and naïve peripheral blood murine neutrophils express low levels of Gal-3. However, in contrast to previous findings, we have shown that upon migration to the inflamed peritoneal cavity intracellular Gal-3 levels in neutrophils are significantly increased.

Struck by this significant increase in Gal-3 expression upon migration to the inflammatory site, we have sought to address the role of neutrophil Gal-3 through investigation of the profile of neutrophil recruitment, apoptosis and clearance in the zymosan peritonitis model. Increased levels of neutrophil recruitment into the peritoneal cavity of Gal-3 null mice were observed at 4, 72 and 96 h. This is in contrast to the reported response to thioglycollate in previous studies, as well as in models of pneumonia in which neutrophil migration to the peritoneal cavity and lungs respectively was reduced in Gal-3 null mice [6, 9, 11, 12]. Such discrepancies may be due to the inducing inflammatory stimuli as like zymosan, *E. coli* infection also resulted in a significant enhancement of neutrophil trafficking to the lungs of Gal-3 null mice [9]. The finding that *E. coli* increases neutrophil migration to the lungs in Gal-3 null mice is in contrast to our findings in the peritoneal cavity. This highlights

differences between distinct anatomical sites and might be due to the effects of Gal-3 on stromal cells at different sites. Although it should also be noted that a different strain of mice was used in this study, which may also account for the observed differences. Further similarities exist between these inciting stimuli, which may account for the differing responses seen. *S. Pneumoniae* infection increases Gal-3 levels in bronchoalveolar lavage fluid as high as 50μg/ml, likewise cutaneous infection with L. Major LV39 results in increased levels of extracellular Gal-3 and is associated with enhanced neutrophil recruitment. In contrast, infection with *E. coli* or the L. Major substrain Friedlin failed to significantly induce Gal-3 release and a role for Gal-3 dependent neutrophil recruitment was not observed. Whilst we observed Gal-3 within the peritoneal exudate and levels were significantly raised over the course of the response the amount detectable was in the low ng/ml range and as was observed following *E. coli* infection, neutrophil recruitment was increased rather than decreased in Gal-3 null mice. Another important factor might be the mechanism by which neutrophils traffic to the inflammatory site. Migration in response to zymosan and *E. coli* is known to be dependent on β2 integrins [22], which function to allow neutrophils to adhere to and crawl on the endothelium [23]. In contrast, neutrophil trafficking to the lungs in response to *S. Pneumoniae* is independent of β2 integrins and it is thought that under these circumstances Gal-3 is able to act as an adhesion molecule to facilitate neutrophil trafficking, which likely explains the requirement for high levels of the protein extracellularly in these models. Our data suggests that the observed enhancement of neutrophil numbers within the peritoneal cavity of Gal-3 null mice is due to alterations in neutrophil clearance rather than a direct role on neutrophil trafficking per se, although this cannot be ruled out.

It is important to consider the cellular localisation of galectins when studying their actions. Published reports on the effects of Gal-3 have shown cellular localisation impacts its function with regards to induction of apoptosis in T cells. Extracellular Gal-3 induces T cell apoptosis [24] whereas intracellular Gal-3 inhibits apoptosis [25]. With regards to neutrophils, extracellular Gal-3 delays their spontaneous apoptosis, whilst no role has been identified for the intracellular protein in murine neutrophils with similar rates of apoptosis observed between WT and Gal-3 null cells [6]. The present study demonstrated intracellular expression of Gal-3 at 4h post zymosan administration, and importantly our data shows an increased surface expression of Gal-3 on apoptotic neutrophils. These findings, together with the delayed apoptosis observed in Gal-3 null neutrophils recovered from the peritoneal cavity, leads us to hypothesise that Gal-3 is externalized by neutrophils and acts as an “eat me” signal. As efferocytosis peaks in this model at 6h post-zymosan administration [26] it would be expected that more neutrophils would be retained in the peritoneal cavity of Gal-3 null mice at the 4h time-point. The role of Gal-3 as an “eat-me” signal also explains why there are fewer cells in the peritoneal cavity with surface Gal-3 at later time-points (48h onwards) as these cells will have been cleared from the peritoneal cavity. Our findings are in contrast to the aforementioned study by Farnworth and colleagues [6] and the study of Colnot et al [11] in which no defect in apoptosis was observed in neutrophils from Gal-3 null mice. There are however differences between the studies; importantly in our study apoptosis was assessed following incubation of neutrophils that had emigrated to the inflamed peritoneal cavity whilst bone marrow neutrophils were used in the study of Farnworth *et al*. Although Colnot *et al* assessed levels of apoptosis in neutrophils taken from the peritoneal cavity, this was in a model of thioglycollate-induced peritonitis and

cells were taken 24h post-administration, whereas we observed reduced levels of apoptosis in cells taken at 4h. In-line with the findings of Colnot *et al*, we did not observe differences in the levels of apoptosis of cells collected from the peritoneum at later time-points (48-96h). Over the course of the inflammatory response we found that the cellular localisation of Gal-3 was altered. As mentioned above, Gal-3 levels were increased in the cytosol of neutrophils present within the peritoneal cavity at the 4h time-point. Our data from the adoptive transfer experiment indicates that the neutrophils themselves are a source of Gal-3 although it does not rule out the possibility that neutrophils can bind Gal-3 present within the extracellular environment. In fact Karlsson et al [13] have shown that apoptotic neutrophils readily bind recombinant Gal-3. At 24h Gal-3 was detectable on both the cell surface and intracellularly; we hypothesise that satiated neutrophils might translocate Gal-3 to the cell surface in order to signal to incoming phagocytes that they need to be cleared. This is supported by the observed reduction in efferocytosis of Gal-3 null neutrophils by WT biogel-elicited macrophages and the lack of neutrophils with surface Gal-3 detectable at 48h post-zymosan.

Macrophages are crucial to the resolution process, with essential roles in clearance of apoptotic neutrophils [27]. Previous studies have demonstrated defective resolution processes in Gal-3 null mice with reduced alternative monocyte activation and consequently reduced phagocytic capabilities of these cells [28, 29], this correlates with our data showing reduced numbers of non-classical monocytes within the peritoneal cavity of Gal-3 null mice during the resolution phase.

Together our data highlight a role for endogenous Gal-3 in host phagocyte function, enhancing neutrophil apoptosis and clearance. Failure to eliminate dying neutrophils leads to tissue damage and dissemination of cellular contents, which can have major pathological consequences, particularly with regards to infection.

## Authorship

R.D.W performed experiments, analysed data and wrote the manuscript, P.S., P.T. and M.B.F. performed experiments and analysed data, L.V.N. performed experiments, analysed data and wrote manuscript, D.C. designed and performed experiments, analysed data and wrote manuscript.


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## Conflict of interest disclosure

The authors declare no conflict of interest.

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**Figure Legends**

Figure 1: Expression of Gal-3 by murine neutrophils during a resolving inflammatory response. Total leukocyte counts in the peritoneal exudate (A), blood (B) and bone marrow (C) following zymosan (1mg) challenge in the peritoneal cavity. Differential leukocyte counts in the peritoneal exudate (D), blood (E) and bone marrow (F) following zymosan (1mg) challenge in the peritoneal cavity. Gal-3 concentration in peritoneal exudate fluid following zymosan-induced peritonitis (G). Total Gal-3 expression in monocyte/macrophages (H) and neutrophils (I) following zymosan (1mg) challenge in the peritoneal cavity. Representative dot plot showing neutrophils double-stained for Ly6g and Gal-3 at 4h post zymosan in the bone marrow, peripheral blood and peritoneal cavity (J). N = 12 per group for peritoneal exudate, 8 per group for peripheral blood and 4 per group for bone marrow. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs peripheral blood and bone marrow at same time point or 0hr control.

Figure 2: Cytosolic and cell surface expression of galectin-3 in murine neutrophils taken from the peritoneal exudate at 4, 24 and 48h post zymosan ip. Surface Gal-3 expression was measured by flow cytometry on Ly6G positive neutrophils and total Gal-3 expression was measured following permeabilisation. Cytosolic expression was calculated by subtracting the cell surface expression from the total expression (A). Gal-3 expression in neutrophils taken from the peritoneal exudate at 4h post zymosan as detected by confocal microscopy (B). Scale bar = 5μm. N= 8-12 per group. *** $P < 0.001$ vs cytosol.

Figure 3: WT neutrophils exhibit increased Gal-3 expression upon transmigration to the peritoneal cavities of Gal-3 null mice. Bone marrow neutrophils were isolated from WT mice, labelled with CFSE and administered i.v. to Gal-3 null mice, which subsequently underwent a zymosan (1mg) induced peritonitis. Peritoneal cavities were lavaged at 4 h and analysed by flow cytometry. Neutrophils were identifiable by their characteristic FSC/SSC profile (A). Following doublet exclusion (B), Ly6G positive neutrophils were identified (C). WT Ly6G positive neutrophils were identified by their positive CFSE staining (D). Surface (E) and total (F) Gal-3 expression was measured on CFSE negative (neutrophils from Gal-3 null mice) and CFSE positive cells (neutrophils from WT mice) by flow cytometry and representative histograms are shown.

Figure 4: Gal-3 null mice demonstrate altered leukocyte trafficking. Total leukocyte counts (A), % neutrophils (B) and % macrophages (C) in the peritoneal exudate at 4h post zymosan (1mg) challenge. Surface (D) and total (E) Gal-3 expression was measured on Ly6G positive neutrophils by flow cytometry and representative histograms are shown. N= 4-7 per group, * = $P < 0.05$;*** $P< 0.0001$.

Figure 5: Gal-3 null mice demonstrate reduced levels of apoptosis. % viable (A), early apoptotic (B) and late apoptotic (C) cells following overnight culture of peritoneal exudate cells. Representative dot-plots, following overnight culture of peritoneal exudate cells (taken 4h post-zymosan), showing cell viability (zombie NIR) and phosphatidylserine exposure (AnxV) in KO (D) and WT (E) of exudated leukocytes. WT biogel-elicited macrophages were incubated with BODIPY®FL - labelled peritoneal exudate cells following overnight culture. Efferocytosis was

quantified using ImageJ software (F). Representative images used for the data analysis are shown (G). Gal-3 levels were assessed on the surface of viable ($AnxV^-$/$zombie^-$) and late apoptotic ($AnxV^+$/$zombie^+$) cells by flow cytometry (H and I). N= 4-7 per group, * = $P < 0.05$;*** $P< 0.0001$.

Figure 6: Gal-3 null mice demonstrate dysregulated resolution of inflammation compared to wild type controls. Gal-3 null mice and age- and sex-matched wild type controls were injected with 1mg zymosan ip and peritoneal exudates were collected during the resolution period (48-96 h). Total leukocyte counts (A), % neutrophils (B), % non-classical monocytes (C) and % classical monocytes (D) in peritoneal exudates following zymosan (1mg) administration. N= 5 per group, * = $P < 0.05$.

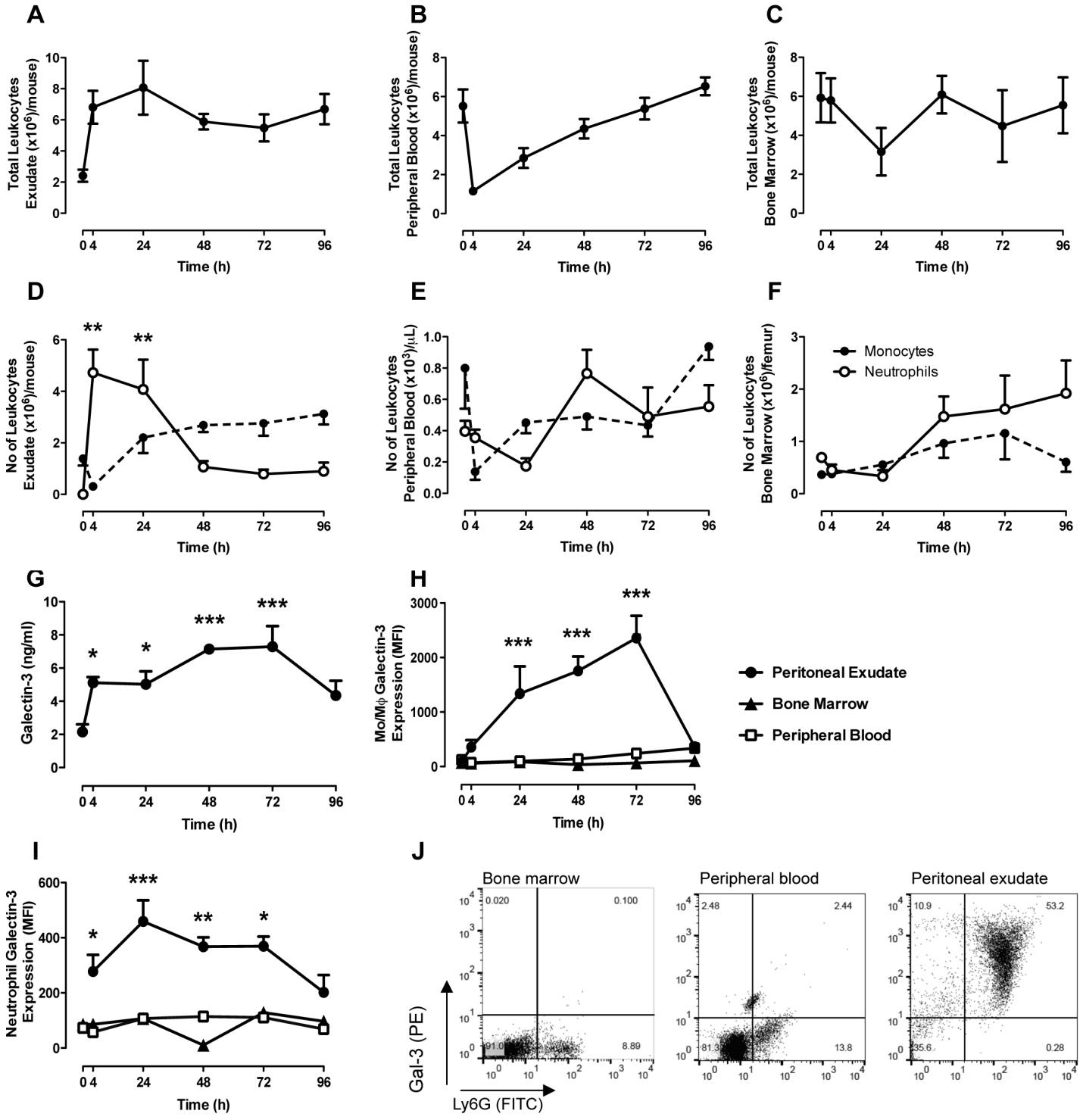
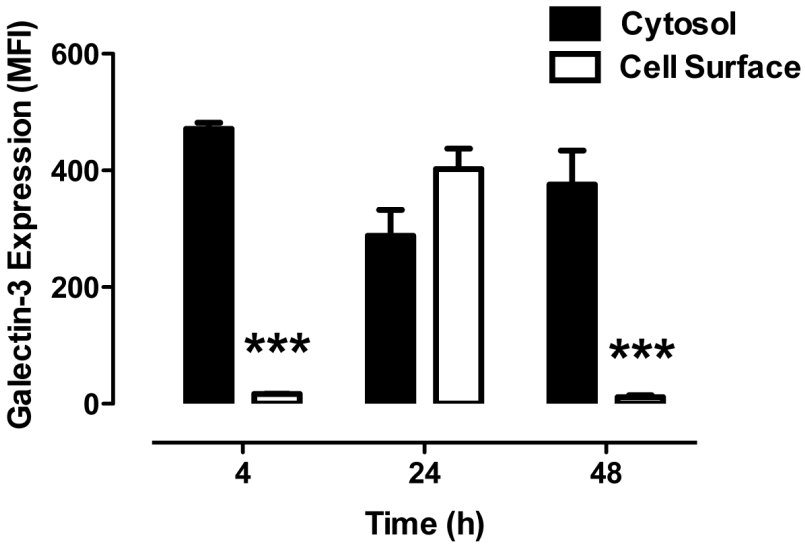

A
Total Leukocytes Exudate (x10^6)/mouse
Time (h)
B
Total Leukocytes Peripheral Blood (x10^6)/mouse
Time (h)
C
Total Leukocytes Bone Marrow (x10^6)/mouse
Time (h)
D
No of Leukocytes Exudate (x10^6)/mouse
Time (h)
E
No of Leukocytes Peripheral Blood (x10^3)/μL
Time (h)
F
No of Leukocytes Bone Marrow (x10^6)/femur
Monocytes
Neutrophils
Time (h)
G
Galectin-3 (ng/ml)
Time (h)
H
Mo/Mφ Galectin-3 Expression (MFI)
Time (h)
Peritoneal Exudate
Bone Marrow
Peripheral Blood
I
Neutrophil Galectin-3 Expression (MFI)
Time (h)
J
Bone marrow
Peripheral blood
Peritoneal exudate
Gal-3 (PE)
Ly6G (FITC)
0.020
0.100
91.0
8.89
2.48
2.44
81.3
13.8
10.9
53.2
35.6
0.28

Figure 1

A


Cytosol
Cell Surface
Galectin-3 Expression (MFI)
600
400
200
0
***
***
4
24
48
Time (h)

B

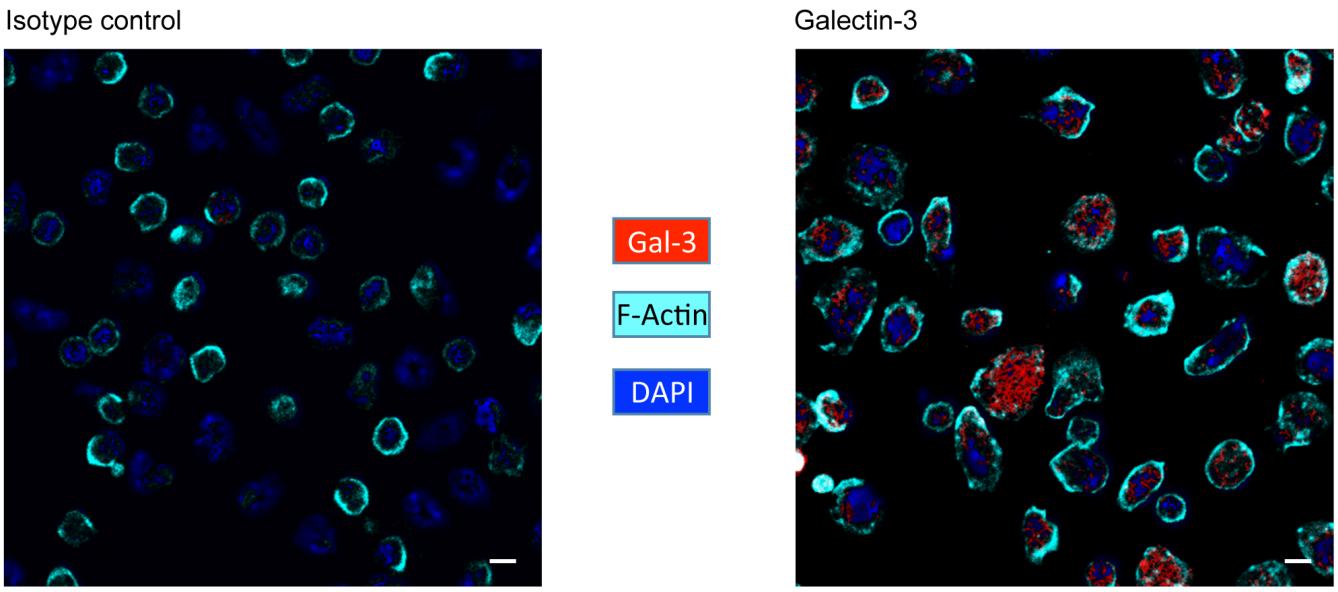

Isotype control
Galectin-3
Gal-3
F-Actin
DAPI

Figure 2

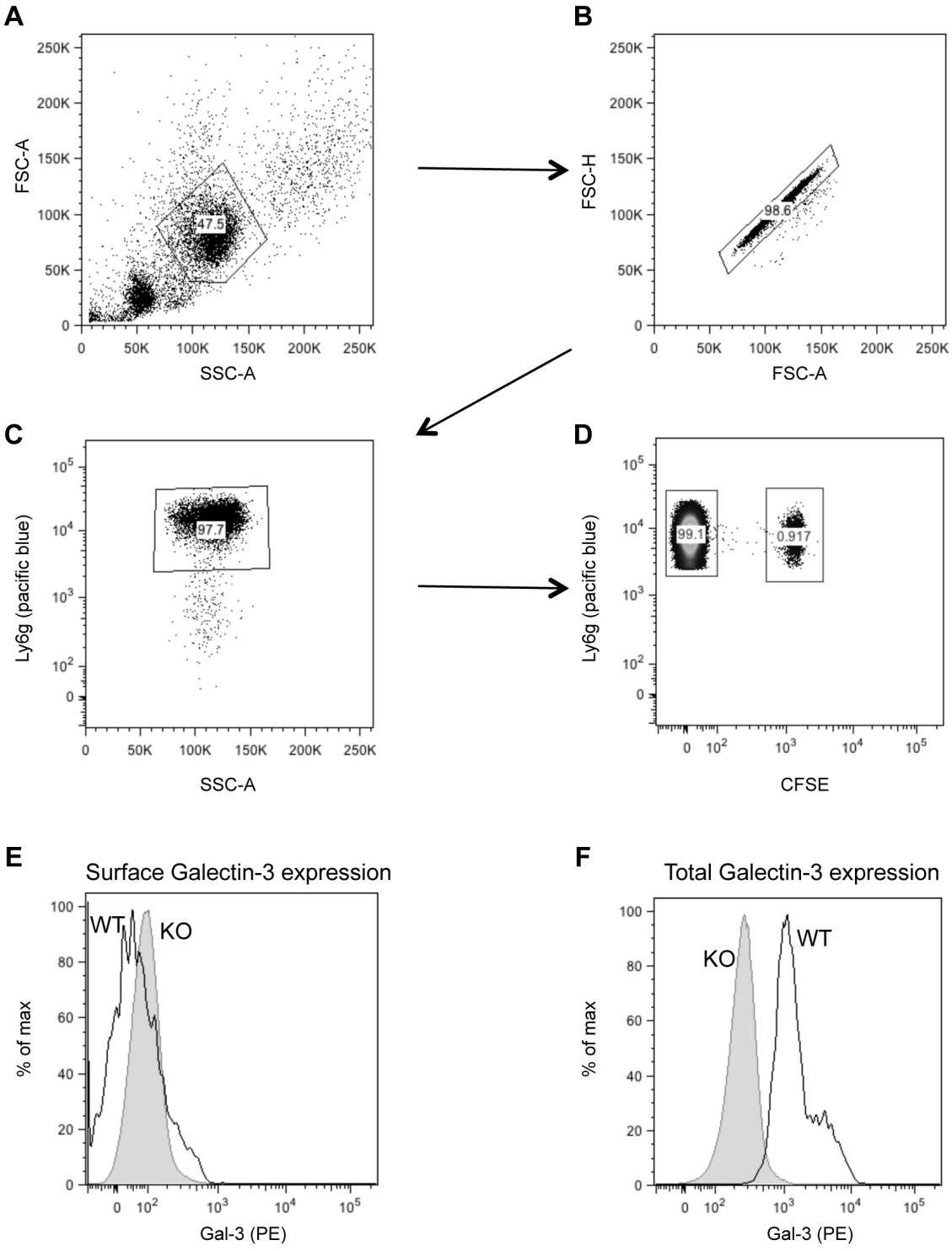

A
FSC-A
SSC-A
47.5
B
FSC-H
FSC-A
98.6
C
Ly6g (pacific blue)
SSC-A
97.7
D
Ly6g (pacific blue)
CFSE
99.1
0.917
E
Surface Galectin-3 expression
WT
KO
% of max
Gal-3 (PE)
F
Total Galectin-3 expression
KO
WT
% of max
Gal-3 (PE)

Figure 3

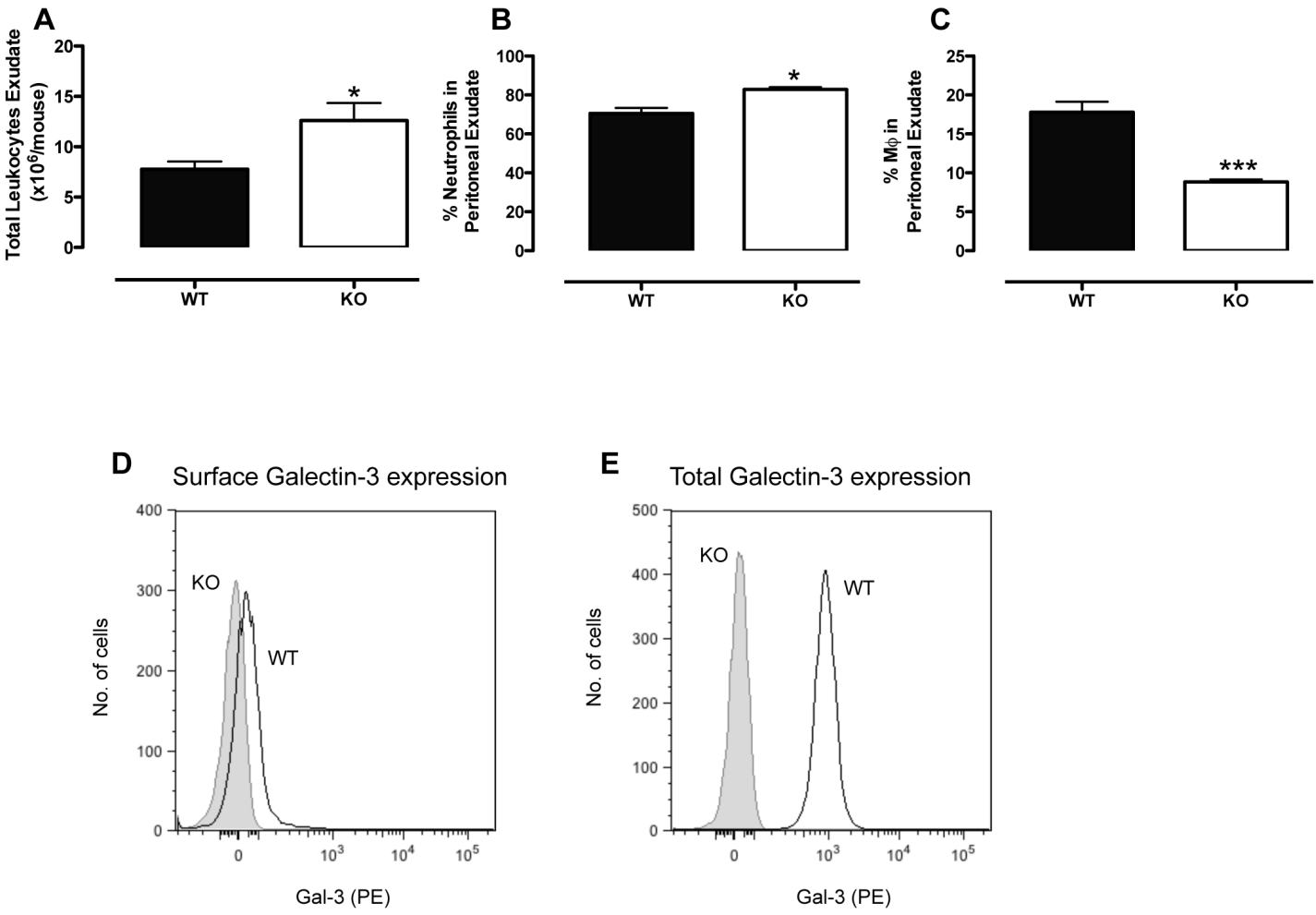

A
Total Leukocytes Exudate (x10^6/mouse)
20
15
10
5
0
*
WT
KO
B
% Neutrophils in Peritoneal Exudate
100
80
60
40
20
0
*
WT
KO
C
% Mϕ in Peritoneal Exudate
25
20
15
10
5
0
***
WT
KO
D
Surface Galectin-3 expression
No. of cells
400
300
200
100
0
KO
WT
0
10^3
10^4
10^5
Gal-3 (PE)
E
Total Galectin-3 expression
No. of cells
500
400
300
200
100
0
KO
WT
0
10^3
10^4
10^5
Gal-3 (PE)

Figure 4

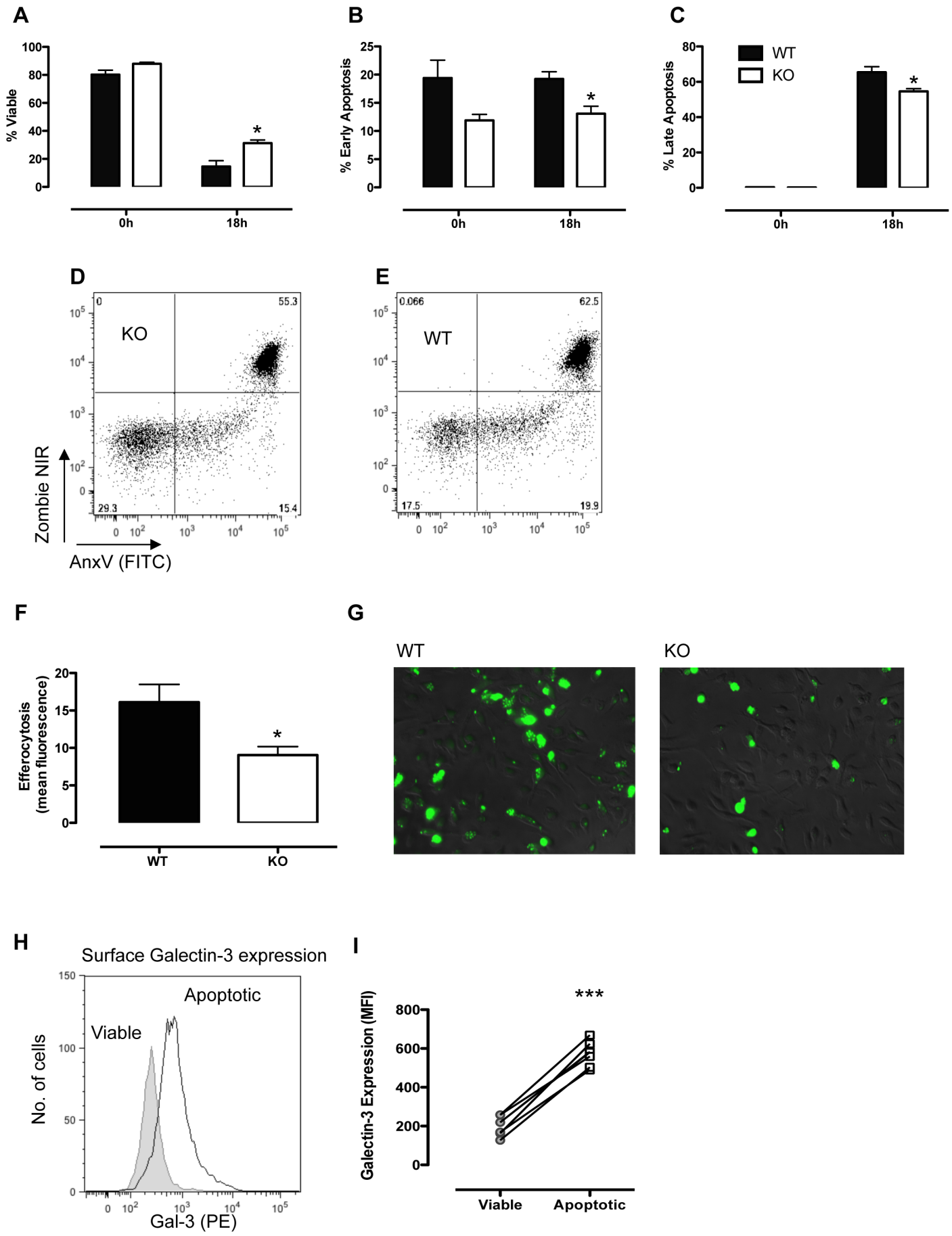

A
% Viable
0h
18h
B
% Early Apoptosis
C
% Late Apoptosis
WT
KO
D
KO
55.3
29.3
15.4
Zombie NIR
AnxV (FITC)
E
WT
0.066
62.5
17.5
19.9
F
Efferocytosis (mean fluorescence)
WT
KO
G
WT
KO
H
Surface Galectin-3 expression
Apoptotic
Viable
No. of cells
Gal-3 (PE)
I
Galectin-3 Expression (MFI)
***
Viable
Apoptotic

Figure 5

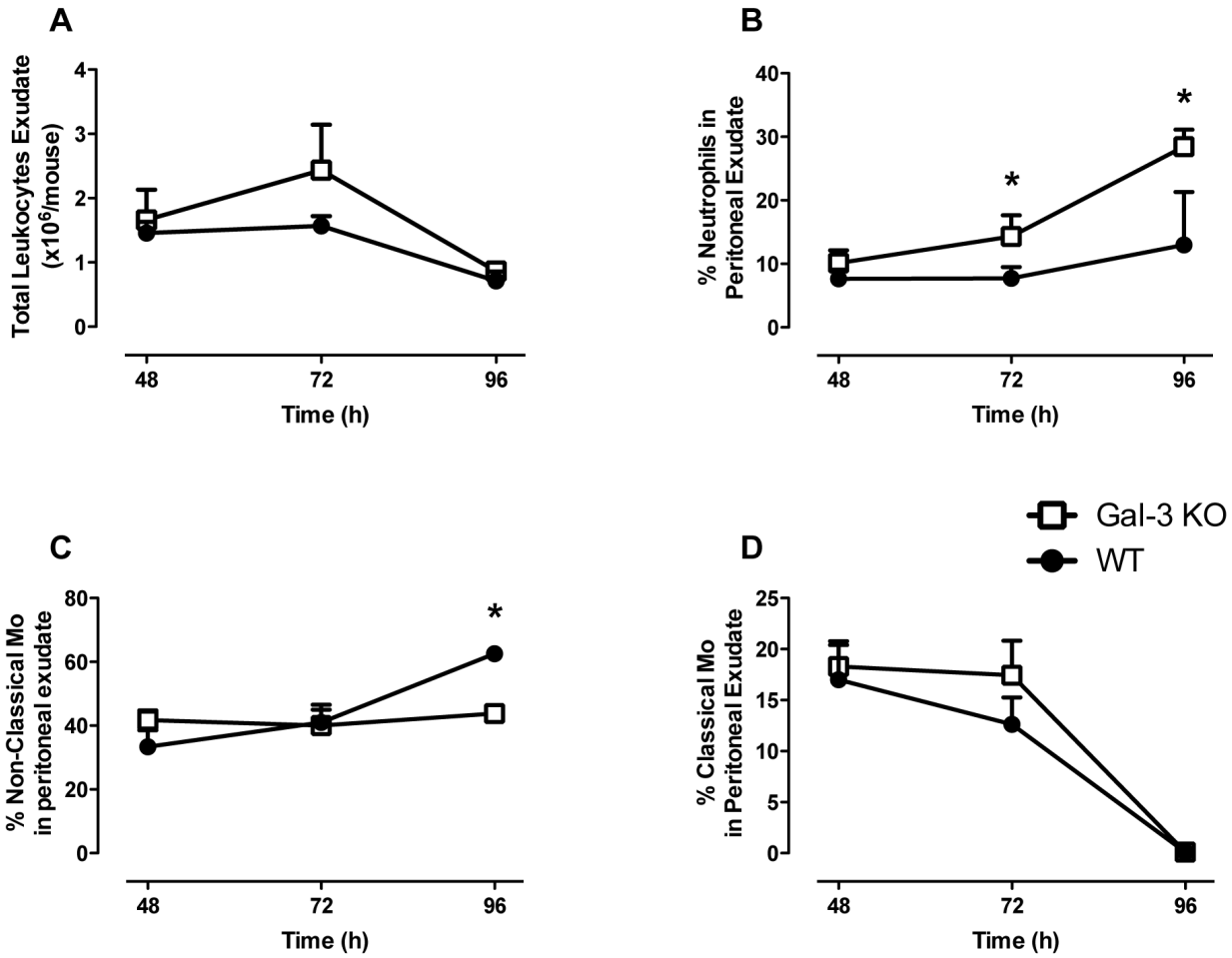

A
Total Leukocytes Exudate (x10^6/mouse)
4
3
2
1
0
48
72
96
Time (h)
B
% Neutrophils in Peritoneal Exudate
40
30
20
10
0
*
*
48
72
96
Time (h)
C
% Non-Classical Mo in peritoneal exudate
80
60
40
20
0
*
48
72
96
Time (h)
D
% Classical Mo in Peritoneal Exudate
25
20
15
10
5
0
48
72
96
Time (h)
Gal-3 KO
WT

Figure 6