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A pro-resolving role for Galectin-1 in acute inflammation

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Abstract

Galectin-1 (Gal-1) exerts immune-regulatory and anti-inflammatory actions in animal models of acute and chronic inflammation. Its release into the extracellular milieu often correlates with the peak of inflammation suggesting that it may serve a pro-resolving function. Gal-1 is reported to inhibit neutrophil recruitment and induce surface exposure of phosphatidylserine, an 'eat me' signal on the surface of neutrophils, yet its role in resolution remains to be fully elucidated. We hypothesised that the anti-inflammatory and pro-resolving properties of Gal-1 are mediated through its ability to inhibit neutrophil recruitment and potentiate neutrophil clearance. To investigate this, a murine model of self-resolving inflammation was utilised to uncover the role of both the endogenous and exogenous protein using Gal-1 null mice and recombinant protein respectively. We found that peritoneal macrophages express increased Gal-1 during the resolution phase and enhanced neutrophil recruitment occurs in the early phases of zymosan peritonitis in Gal-1 null mice compared to their wildtype (WT) counterparts. Administration of recombinant Gal-1 following the peak of inflammation led to reduced neutrophil numbers at 24 and 48 hours, shortening the resolution interval from 39 to 14 hours. Gal-1 treatment also enhanced neutrophil apoptosis, indicating a pro-resolving action. Together these results indicate an important role for Gal-1 in the timely resolution of acute inflammation.

1 Introduction

Neutrophil trafficking to the site of inflammation is essential for the clearance of infection and repair of injured tissue. However, excessive inflammation is deleterious to the host and can result in damage to healthy tissue and the development of chronic inflammatory pathologies such as rheumatoid arthritis and atherosclerosis (Nathan and Ding 2010; Perretti et al. 2017; Kasikara et al. 2018). It is now widely accepted that the resolution of inflammation is an active process, driven by the generation of pro-resolving mediators such as resolvins and Annexin A1 by infiltrated neutrophils (Sugimoto et al. 2019). These mediators promote resolution through the inhibition of neutrophil trafficking, induction of neutrophil apoptosis and promotion of neutrophil clearance through efferocytosis, processes that are key to the restoration of tissue homeostasis (Norling and Perretti 2013).

Galectin-1 (Gal-1) is the prototype member of the galectin family, which share a specificity for β-galactoside containing proteins and lipids. Several galectins have been ascribed immunomodulatory functions, with Gal-1 generally regarded as anti-inflammatory due to its inhibitory effects on neutrophil and T cell trafficking and induction of T cell apoptosis (Cooper, Norling, and Perretti 2008; Norling et al. 2008; He and Baum 2006; Earl, Bi, and Baum 2010; Toscano et al. 2007). Evidence is emerging that Gal-1 may also have pro-resolving actions: expression often peaks at the height of the inflammatory response (Iqbal et al. 2011; Ilarregui et al. 2009; Starossom et al. 2012) and Gal-1 was identified in resolving exudates of mice in a model of peritonitis and was down-regulated by the resolution-toxic anaesthetic lidocaine (Chiang et al. 2008). Current findings suggest that Gal-1 links the innate and adaptive immune systems as differentiation of dendritic cells in a Gal-1 rich environment leads to enhanced regulatory functions and suppression of autoimmune disease progression (Ilarregui et al. 2009). However, more recent evidence has expanded the pro-resolving potential of Gal-1 to cells of the innate immune system. Gal-1 has been shown to induce 12/15-lipoxygenase expression in macrophages, switching them to a pro-resolving phenotype (Rostoker et al. 2013). A potential role for Gal-1 in neutrophil clearance has also been suggested due to its ability to induce phosphatidylserine exposure on the surface of neutrophils *in vitro* (Stowell et al. 2007), however evidence of this *in vivo* is currently lacking.

In this study we challenge the hypothesis that alongside its recognised anti-inflammatory actions, Gal-1 also possesses pro-resolving properties through its actions on neutrophil trafficking and lifespan. Using a well characterised model of zymosan-induced peritonitis, we demonstrate that in the absence of Gal-1 there is increased neutrophil recruitment to the peritoneal cavity, whilst administration of recombinant Gal-1 after the peak of inflammation induces neutrophil apoptosis and clearance.

2 Materials and Methods

Galectin-1

Recombinant Human Galectin-1 was provided by GalPharma (Takamatsu, Kagawa, Japan). The recombinant protein is a cysteine-less mutant with all cysteine residues substituted with serine residues, which is resistant to oxidation whilst retaining all known activities of native Gal-1 (Nishi et al. 2008).

Ethics and Regulations

Experiments performed *in vivo* adhered to Home Office regulations (Scientific Procedures Act, 1986) and were additionally approved under the guidelines set down by the Ethical Committee for the Use of Animals, Barts and The London School of Medicine. Additionally, protocols under the supervision of the above were accomplished baring careful consideration to the principles set out by the National Centre for Replacement Refinement and Reduction of Animals in Research (NC3Rs).

Mice

Male wild-type (WT) C57/BL6 mice were purchased from Charles River (Kent, UK). Original breeding pairs of Gal-1 knockout (KO) mice (*Lgals1* null) animals were generously provided by the Consortium for Functional Glycomics (*http://www.functionalglycomics.org*) and were bred and

housed at Charles River (Kent, UK). Where required WT and KO mice were both age and sex-matched. Mice were housed within individually ventilated cages (IVCs), a maximum of six mice per cage in a facility with a 12 hour light-dark cycle and *ad libitum* feeding of a standard laboratory chow diet and water.

Zymosan-induced Peritonitis Model

Zymosan-induced peritonitis was performed with Gal-1 KO mice and WT counterparts or with WT mice administered hrGal-1 (10µg). Mice aged 6-8 weeks (n = 3-8) were administered zymosan (1mg in 500µl Dulbecco's Phosphate Buffered Saline ($DPBS^{+/+}$)) by intra-peritoneal (i.p) injection at the zero hour time point. Mice treated with hrGal-1 were given a dose of 10µg or vehicle (200µl $DPBS^{+/+}$) only control i.p at 8 h post-zymosan (during the peak phase of response). Mice were sacrificed at the indicated time points. Peritoneal cavities were lavaged with ice cold $DPBS^{-/-}$ containing 2mM EDTA. Total cell numbers in the peritoneum were quantified by cell counts using Turk's Solution. Cell free lavage fluid was retained for analysis of Gal-1 or inflammatory cytokine levels by ELISA (R&D systems, Abingdon, UK and Labospace Milan, respectively). For *ex vivo* apoptosis experiments, mice were treated with hrGal-1 at 2h and lavaged at 6h, cells from 1ml peritoneal exudate were then resuspended in RPMI + 0.5% bovine serum albumin (BSA) and incubated for 20h at 37˚C in 5% CO_2 prior to analysis.

Flow Cytometry

Differential cell counts in the peritoneal cavity as well as intracellular Gal-1 levels were assessed by flow cytometry. Leukocyte infiltrate was quantified using $CD45^+$ cells as the parent population and the subsequent percentage of positive cells by biomarker as follows. Immune cells were labelled with the following panel of fluorescently conjugated antibodies: CD45 PerCP (clone 30-F11, Biolegend), F4/80 BV650 (clone BM8, Biolegend) or F4/80 APC (clone BM8, eBioscience), Ly6G PE (clone 1A8, BD Pharmingen), 7/4 FITC (clone 7/4, Abcam), CD11b BV785 (clone M1/70, Biolegend and SiglecF (clone ES22-10D8, Mitenyl Biotech). In some instances Ly6C PerCP-Cy5.5 (clone HK1.4, eBioscience) was used. Following antibody incubations, cells were washed twice with FACS buffer before fixation in 1% paraformaldehyde (PFA) solution.

For Gal-1 expression cells were stained as above for cell specific markers (Ly6C, Ly6G and F4/80) and then fixed and permeabilised with BD fixation and permeabilisation buffer before addition of anti-Gal-1 antibody (polyclonal, R&D systems) followed by AF488 anti-goat IgG (polyclonal, Invitrogen) secondary antibody to assess Gal-1 levels. In all cases, antibodies or relevant isotype controls were incubated for a minimum of 30 mins at 4°C prior to analysis on a BD LSR Fortessa (BDbiosciences) and analysed post acquisition using FlowJo (v10) software.

For *in vivo* neutrophil apoptosis the peritoneal exudate was analysed immediately post collection. Pelleted cells from 200µl peritoneal exudate were stained with Ly6G (as above). Cells were washed twice, followed by the addition of AnnexinV FITC (BD Pharmingen) and Zombie NIR (Biolegend) in AnnexinV binding buffer (BD Pharmingen). Cells were resuspended, covered and incubated (15 mins, at room temperature (RT)). AnnexinV binding buffer was added to each sample and cells were analysed immediately by flow cytometry. Quadrant gating was applied to determine viable ($AnxV^-NIR^-$), early apoptotic ($AnxV^+NIR^-$), late apoptotic ($AnxV^+NIR^+$) and necrotic ($AnxV^-NIR^+$) neutrophil populations.

Statistical Analysis

Data are expressed as mean ± SEM. Comparisons were analysed for statistical significance. GraphPad Prism (v7) software was used for all analyses and a $P \leq 0.05$ was considered as statistically significant. Levels of Gal-1 profiled across time (0h, 4h, 24h and 48h) were analysed using a one-way ANOVA with Tukey's multiple comparisons test. Leukocyte numbers profiled across time (2h, 6h, 24h, and 48h) were analysed using a two-way repeated measures ANOVA with Sidak's multiple comparisons test. Leukocyte numbers at a single time point (24h) and percentage of neutrophils in each quadrant for apoptosis were analysed using an unpaired t test.

3 Results

Macrophages are a source of Gal-1 in the peritoneal cavity

Given that reports in the literature indicate that Gal-1 levels typically peak at the height of inflammation, we initially sought to characterise endogenous expression of Gal-1 over the course of a self-limiting model of zymosan peritonitis in WT mice. Levels were assessed in both the exudate and the immune cells (monocytes/macrophages and neutrophils) within the cavity. Surprisingly, Gal-1 was readily detected in the peritoneal cavity of naive mice (figure 1A), however levels were comparatively low in the resident macrophage population at this time-point (figure 1B) suggesting a non-immune cell source of the protein in naive mice. Levels of Gal-1 within the exudate decreased following the onset of inflammation with around a 50% reduction at 24 hours post zymosan. A second peak was observed at 48 hours post-zymosan (figure 1A), which correlated with increased levels in the monocyte/macrophage ($Ly6C^+$, $F4/80^+$) (figure 1B). Gal-1 expression was negligible in the neutrophil ($Ly6G^+$) population (data not shown).

Leukocyte recruitment is enhanced in Gal-1 KO mice

Given the increased levels of Gal-1 in the peritoneal cavity during the resolution phase and its expression in the macrophage population within the cavity, we next sought to determine whether the absence of Gal-1 would modulate the inflammatory profile induced by zymosan by utilising Gal-1 KO mice. In WT mice, there was a sharp and significant increase in leukocyte influx into the peritoneal cavity from 2h to 6h as expected, followed by a decline at 24h. A biphasic response was observed with a second increase in total leukocyte number at 48h, as shown in figure 2A. Leukocyte recruitment in Gal-1 KO mice mirrored that observed in WT mice in terms of the temporal nature of the response, however significantly more leukocytes migrated into the peritoneal cavities of Gal-1 KO mice compared to WT at the peak of the inflammatory response (6h time point).

Further analysis of leukocyte subtypes identified neutrophils ($7/4^+Ly6G^+$) as the predominant cell type from 2h onwards, with a decline in number observed at 24h. Whilst this trend was observed in both genotypes, significantly more neutrophils trafficked to the peritoneal cavity of Gal-1 KO mice, compared to WT at the 6h time point as shown in figure 2B.

Monocyte ($7/4^+Ly6G^-$) numbers displayed a bell-shaped trend (figure 2C) with low numbers at 2h, a respective rise at 6 and 24h followed by a decline at 48h in WT mice. No differences were observed between the genotypes. Eosinophil (Siglec F^+) numbers rose sharply at 48h post-zymosan, indicative of their pro-resolving phenotype (Yamada et al. 2011), with no significant differences between genotypes (figure 2D). In WT mice, macrophage ($F4/80^+$) numbers were lowest at 2h, increased from 2 to 6h and remained unchanged at 24h before rising sharply at 48h (figure 2E). At early time points,

there was no significant difference between macrophage numbers in the cavities of Gal-1 KO mice compared to WT, however significantly more macrophages were present in Gal-1 KO mice at 48h. Macrophage phenotype was also assessed and whilst there were no significant differences between genotypes in the number of mature macrophages ($F4/80^{+}CD11b^{high}$), significantly more resolving macrophages ($F4/80^{+}CD11b^{low}$) were present within the cavities of Gal-1 KO mice at 48h compared to their WT counterparts (figures 2F & 2G).

Along with increased neutrophil recruitment in Gal-1 KO mice we also detected significant increases in the pro-inflammatory cytokine IL-6 as well as the pro-angiogenic growth factor VEGF in KO mice, with a trend towards increased levels of the chemokines CCL2, CCL3 and CXCL1 and the pro-inflammatory cytokine TNF-α (supplementary figures 1G-L).

hrGal-1 administration following the peak of inflammation promotes neutrophil clearance

Given the enhanced neutrophil recruitment observed in Gal-1 KO mice, we next addressed whether Gal-1 is able to drive resolution when administered following the peak of an inflammatory response. To this end, hrGal-1 was administered i.p. at 8h post-zymosan, a time-point where neutrophil trafficking will have peaked in this model. Administration of hrGal-1 at this time-point resulted in significantly fewer leukocytes being recovered 16h later (24h time point) compared to those recovered from vehicle treated mice (figure 3A). As shown in figures 3B-G further analysis of the individual leukocyte subtypes indicated that there was a trend towards an all-round decrease in the numbers of all leukocyte subsets analysed in the cavities of mice treated with hrGal-1 (a trend that was also observed at 48h, see supplementary table 1). Significant reductions in the numbers of neutrophils ($7/4^{+}Ly6G^{+}$; figure 3B), eosinophils ($SiglecF^{+}$; figure 3D) and resolving macrophages ($F4/80^{+}CD11b^{low}$; figure 3G) were observed.

Neutrophils that were collected from the peritoneal cavity at 24h post-zymosan injection were further assessed for apoptosis. The majority of neutrophils were viable (70.69% ± 5.21%), presumably as a result of the rapid clearance of apoptotic cells. Importantly, significantly fewer viable cells were detected within the peritoneal cavities of mice treated with hrGal-1 compared to vehicle (figure 3H), and a significant increase in the percentage of early apoptotic neutrophils ($AnxV^{+}/PI^{-}$) was observed. The percentage of late apoptotic cells was minimal in both genotypes again presumably due to their clearance, with negligible numbers of necrotic neutrophils detected.

To further address the effect of Gal-1 on the apoptotic process, hrGal-1 was administered during the initial recruitment phase (2h) and peritoneal lavages performed at 6h post zymosan. Cells were then incubated *in vitro* overnight to assess apoptosis. Here we found that *ex vivo* late apoptosis of neutrophils was significantly increased following exposure to Gal-1 (as illustrated in supplementary figure 2).

hrGal-1 administration following the peak of inflammation shortened the resolution interval

In order to quantitate the pro-resolving actions of Gal-1, resolution indices were calculated. These are widely utilised to identify mediators that stimulate, as well as those that disrupt or delay resolution (Bannenberg et al. 2005; Schwab et al. 2007; Navarro-Xavier et al. 2010). Parameters calculated included the amplitude and duration of the inflammatory response by monitoring the maximum neutrophil infiltrate (T_{max}), when a 50% loss in neutrophil numbers occurred (T_{50}) and the resolution interval (R_i; time from T_{max} to T_{50}). Treatment with hrGal-1 shortened the resolution interval from

39h observed in vehicle treated mice to 14h, indicating that Gal-1 accelerated neutrophil clearance from the peritoneal cavity (figure 4).

4 Discussion

Leukocyte recruitment is an integral component of the inflammatory response, whereby a mass migration of cells through the vasculature towards the site of inflammation occurs. In this context it is well established that neutrophils are the primary leukocyte subset mobilised. Whilst neutrophil recruitment is critical for a successful inflammatory response, the temporal and spatial confinement of the neutrophilic infiltrate is crucial for resolution. Apoptosis is a critical step in paving the way for successful resolution of acute inflammation, where apoptotic neutrophils play an essential role in reprogramming macrophages towards a pro-resolving state (Fredman et al. 2012). Our findings suggest that Gal-1 should be included in the expanding list of pro-resolving molecules.

Gal-1 was readily detectable in peritoneal lavage fluid, with the highest levels detected at 0h and 48h, suggesting levels are highest in the absence of inflammation and during resolution. Macrophages have previously been identified as a source of Gal-1, with levels highest in M2 macrophages in the peritoneal cavity (Rabinovich et al. 1996; Fredman et al. 2012), which correlates with the significant expression observed in macrophages at 48h in this current study.

Our data indicates the effects of Gal-1 are multi-faceted, with neutrophil trafficking and clearance impacted by either the lack of, or addition of excess (exogenous) Gal-1. Neutrophil trafficking is significantly enhanced in the absence of Gal-1, a result that corroborates with the increased leukocyte emigration observed in the microcirculation of Gal-1 KO mice (Cooper, Norling, and Perretti 2008) and corresponds with reports in the literature of an inhibitory role for Gal-1 on neutrophil recruitment (Iqbal et al. 2011). In line with a heightened inflammatory response in the absence of Gal-1, IL-6 and VEGF levels were also significantly increased in Gal-1 KO mice. IL-6 is a pleiotropic cytokine involved in the acute phase response and its inhibition has been shown to reduce the inflammatory response in a model of zymosan peritonitis (Cuzzocrea et al. 1999) whilst VEGF has been shown to induce rapid recruitment of neutrophils *in vivo* (Massena et al. 2015).

The majority of studies indicating an effect of Gal-1 on neutrophil trafficking have focused on the actions of the exogenous protein (La et al. 2003). Indeed, in a previous study from our laboratory, administration of hrGal-1 reduced neutrophil infiltration in a model of carageenan-induced paw oedema, however the oedema profile was unchanged in the first 24h post-carageenan in Gal-1 KO mice (Iqbal et al. 2011). The enhanced neutrophil infiltration observed in Gal-1 KO in the current study may indicate context specific actions for the endogenous protein with increased neutrophil trafficking also observed in Gal-1 KO mice in the colon in response to infection with *C. rodentium* (Curciarello et al. 2014).

Inflammation induced by zymosan is reliant on the activation of peritoneal macrophages via TLR2; evidence exists in the literature that exogenous Gal-1 reduces pro-inflammatory cytokine production from murine macrophages (Abebayehu et al. 2017; Fredman et al. 2012) and skews towards an M2 (Barrionuevo et al. 2007) and pro-resolving phenotype (Fredman et al. 2012). Additionally, macrophages from Gal-1 KO mice have been shown to have enhanced expression of MHCII (Barrionuevo et al. 2007). We have seen increased levels of pro-inflammatory mediators in the early recruitment phase of the inflammatory response in Gal-1 KO mice despite reduced macrophage numbers in the peritoneal cavity, which may be suggestive of a heightened pro-inflammatory response in macrophages in the absence of Gal-1. To the best of our knowledge, a full

characterization of Gal-1 KO macrophage phenotype has not been performed and the exact role of endogenous Gal-1 in macrophages still requires full characterization.

Whilst significantly more neutrophils were found within the peritoneal cavity of Gal-1 KO mice at 6h, this difference was lost at 24h suggesting that clearance might be enhanced in these mice. Assessment of apoptosis and efferocytosis at this time point, showed no differences in the percentage of cells undergoing either process (data not shown). It is feasible that the apoptotic/efferocytic process is faster or earlier in Gal-1 KO mice, or alternatively these results may simply be a reflection of the efficiency of the resolution process and the ability of the system to regain homeostasis. Exogenous Gal-1 has been demonstrated to enhance macrophage phagocytosis of sheep red blood cells *in vitro*, however interestingly Gal-1 KO macrophages also phagocytosed more effectively than WT cells when cultured *ex vivo* (Barrionuevo et al. 2007). More recently, Gal-1 was shown to induce efferocytic satiation in a model of zymosan-induced peritonitis which is linked to early departure of these macrophages from the resolving cavity. Surprisingly, although Gal-1 is thought to enhance macrophage infiltration into the resolving peritoneum (Malik et al. 2009; Gil et al. 2006), we did not see a reduction in macrophage numbers in the peritoneal cavity of Gal-1 KO animals, in fact the adverse was evidenced. This may be in response to the increased neutrophil infiltrate observed in these mice. These findings propose a divergence in the roles of endogenous and exogenous Gal-1 and thus further work is required to fully understand its role in efferocytosis.

There are several studies describing the ability of hrGal-1 to inhibit neutrophil trafficking both *in vitro* and *in vivo* however its effects on neutrophil clearance have not, to the best of our knowledge been assessed *in vivo*. Importantly in this experimental setup we determined neutrophil numbers in response to exogenous Gal-1 administration after the peak of inflammation (at a time point that should not affect neutrophil recruitment) in order to assess neutrophil clearance. *In vitro* studies from Stowell *et al* describe the ability of Gal-1 to induce exposure of the "eat me" signal phosphatidylserine (PS) on the surface of neutrophils (Stowell et al. 2009, 2007). Interestingly, PS exposure normally occurs as part of the early stages of apoptosis, however Gal-1 was not found to induce apoptosis in their studies, rather it was proposed that Gal-1-induced PS exposure is a mechanism to drive clearance of neutrophils in the absence of apoptosis. Here, we detected increased numbers of early apoptotic neutrophils within the peritoneal cavities of mice treated with hrGal-1. Whilst it was not possible to determine whether these cells progressed through the apoptotic process presumably due to the rapid clearance of PS expressing cells *in vivo*, we did uncover a pro-apoptotic role for Gal-1 when assessing the stages of apoptosis *ex vivo*. Further studies are required to elucidate the mechanisms behind this pro-apoptotic effect of Gal-1.

In support of the rapid engulfment of PS exposing neutrophils in this model the lower numbers of resolving macrophages detected 16h post Gal-1 administration is suggestive of their quick retreat to the lymphatics. Findings by Rostoker *et al.* demonstrated that hrGal-1 treatment converts macrophages from a $CD11b^{high}$ to a $CD11b^{low}$ phenotype during resolution. The shift towards a resolving phenotype could be a direct response to the increased numbers of early apoptotic neutrophils that we report within our study. As hrGal-1 increases PS exposure on neutrophils, an action that has been linked to enhanced clearance (Stowell et al. 2009), this 'eat me' signal might stimulate the macrophage phenotypic switch to better enable clearance of these cells. We propose that hrGal-1 treated mice undergo macrophage phenotype switching sooner as a result of the increased apoptotic neutrophils present in the cavity and the requirement of their clearance. We hypothesise that the consequence of the more rapid engulfment of apoptotic neutrophils, is that resolving macrophages in hrGal-1 treated mice reach efferocytic satiation sooner and thus depart the cavity more swiftly.

There is evidence in the literature that Gal-1 can induce apoptosis of human synovial fluid neutrophils (Cedeno-Laurent et al. 2010), which may indicate a divergence between the effects of Gal-1 on neutrophils in the periphery versus those that have trafficked to the inflammatory site. Neutrophils that have trafficked to the inflammatory site are known to express a different repertoire of receptors (Hartl et al. 2008) and it is likely that upon transmigration, Gal-1 is able to bind to a newly expressed receptor on the cell surface. This also correlates with studies indicating increased galectin binding to activated neutrophils and importantly for this study on neutrophils that have trafficked to the inflammatory site (Almkvist et al. 2002). Furthermore, the glycosylation status of neutrophils is modulated upon transmigration, particularly with respect to sialic acid residues (Cross et al. 2003). Sialic acid capping of glycoproteins negatively impacts Gal-1 binding (Toscano et al. 2007), thus a reduction in terminal sialic acids, through the action of sialidases, may also permit the increased binding of Gal-1 to neutrophils post-transmigration. The effects of galectins are indeed highly complex and vary depending on local concentration, intracellular or extracellular localisation, or differentiation status of the target cell, which may account for effects on neutrophils in the periphery as well as in tissue.

The reduction in the resolution interval in response to Gal-1 in this model provides further indication that the protein augments the transition from inflammation to resolution. Gal-1 administration at the 8h time point reduced the resolution interval from 39h to 14h, suggesting that Gal-1 has pro-resolving properties that may be of therapeutic utility. Whilst native Gal-1 is susceptible to oxidation, which can negatively impact its activity, the form utilized in this study has been modified to prevent oxidation and extend the half-life of the protein (Nishi et al. 2008) suggesting that modified forms of the protein may be therapeutically advantageous. However, due to the complexity of galectin biology a further understanding of their mechanism of action is required before galectin-based therapeutics can be utilized clinically.

Collectively, our findings confirm an anti-trafficking role for endogenous Gal-1 and indicate a pro-apoptotic function for the exogenous soluble protein. The clearance of apoptotic cells is a fundamental process following cell damage, ageing and trafficking of leukocytes to an inflammatory site and hence is critical for the resolution of inflammation and preservation of self-tolerance. Our results enhance our understanding of the role of Gal-1 in resolution and sheds light on the promising nature of this protein for the development of a new class of therapeutics that activate resolution.

Figure 1. Macrophages are a source of Gal-1 in the peritoneal cavity. Peritoneal lavages were performed on C57/Bl6 naive mice (0h) or 4h, 24h and 48h following administration of zymosan (1mg in 500µl $DPBS^{+/+}$ i.p.) and cell free supernatants were assayed for Gal-1 concentration by ELISA **(A)**. Total Gal-1 levels were assessed in permeabilised monocytes/macrophages (identified using $Ly6C^{+}$, $F4/80^{+}$) by flow cytometry **(B)**. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparisons test, results are displayed as mean ± SEM, in all cases significant results are considered as *$P < 0.05$, with ***$P < 0.0005$ and ****$P < 0.0001$. n = 4 mice per group.

Figure 2. Leukocyte recruitment is enhanced in Gal-1 KO mice. Mice received zymosan (1mg in 500µl $DPBS^{+/+}$ i.p.) and peritoneal lavage was performed at 2h, 6h, 24h and 48h. Total cell counts were performed **(A)** and the number of neutrophils ($7/4^{+}Ly6G^{+}$) **(B)**, inflammatory monocytes ($7/4^{+}Ly6G^{-}$) **(C)**, eosinophils (Siglec F^{+}) **(D)**, macrophages ($F4/80^{+}$) **(E)**, mature macrophages ($F4/80^{+}CD11b^{high}$) **(F)** and resolving macrophages ($F4/80^{+}CD11b^{low}$) **(G)** were identified by flow cytometry. Representative flow cytometry plots are shown for each subset. Statistical analysis was

performed using a two-way ANOVA with Sidak's multiple comparisons test, results are displayed as mean ± SEM, in all cases significant results are considered as $P < 0.05$. n = 3-8 mice per group.

Figure 3. hrGal-1 administration following the peak of inflammation promotes neutrophil clearance. C57/Bl6 mice received zymosan (1mg in 500µl $DPBS^{+/+}$ i.p.) at 0h. At 8h post-zymosan mice were administered hrGal-1 (10µg) or vehicle (200µl $DPBS^{+/+}$) i.p. and peritoneal lavage performed at 24h. Total cell counts were performed **(A)**. Flow cytometry was used to analyse the cell population for leukocyte subsets including neutrophils ($7/4^{+}Ly6G^{+}$) **(B)**, inflammatory monocytes ($7/4^{+}Ly6G^{-}$) **(C),** eosinophils (Siglec F^{+}) **(D)**, macrophages ($F4/80^{+}$) **(E)**, mature macrophages ($F4/80^{+}CD11b^{high}$) **(F)** and resolving macrophages ($F4/80^{+}CD11b^{low}$) **(G)**. Representative flow cytometry plots are inset for each subset. The neutrophil ($Ly6G^{+}$) population was further assessed by AnnexinV (FITC) and Zombie (NIR). Quadrant gating was applied to determine viable ($AnxV^{-}NIR^{-}$), early apoptotic ($AnxV^{+}NIR^{-}$), late apoptotic ($AnxV^{+}NIR^{+}$) and necrotic ($AnxV^{-}NIR^{+}$) neutrophil populations. Results for the percentages of the Neutrophil population within each of the quadrants is shown **(H)** with representative flow cytometry plots inset for vehicle (left) and hrGal-1 (right) treated mice. Statistical analysis was performed using an unpaired t test (with each quadrant for apoptosis), results are displayed as the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n = 5-6 mice per group.

Figure 4. hrGal-1 administration following the peak of inflammation shortened the resolution interval. Peritonitis was initiated in C57/Bl6 mice using zymosan (1mg in 500µl $DPBS^{+/+}$ given i.p.) and mice were treated with hrGal-1 (10µg) or vehicle (200µl $DPBS^{+/+}$) by i.p injection 8h post-zymosan. Peritoneal lavages were performed at 8h, 24h and 48h. Total cell counts were performed and flow cytometry was used to quantify neutrophil ($7/4^{+}Ly6G^{+}$) numbers. Resolution indices were calculated by monitoring the maximum peritoneal neutrophil number (T_{max}), when neutrophil numbers declined by 50% (T_{50}) and the resolution interval (R_i), the time from T_{max} to T_{50}. n = 5-6 mice per group.

Supplementary Figure 1. Inflammatory mediators are increased in the absence of Gal-1. Mice received zymosan (1mg in 500µl $DPBS^{+/+}$ i.p.) and peritoneal lavage was performed at 2h. Exudate was assessed for total cell counts (**A**) and the number of neutrophils ($7/4^{+}Ly6G^{+}$) (**B**), eosinophils (Siglec F^{+}) (**C**), inflammatory monocytes ($7/4^{+}Ly6G^{-}$) (**D**), mature macrophages ($F4/80^{+}CD11b^{high}$) (**E**) and resolving macrophages ($F4/80^{+}CD11b^{low}$) (**F**) were identified by flow cytometry. The cell free supernatant was collected from 1ml of peritoneal exudate and concentrations of analytes measured by ELISA. Results for the concentrations of cell recruitment chemokines CCL2 (MCP-1) (**G**), CCL3 (MIP-1-α) (**H**) and CXCL-1 (KC) (**I**) are shown. Levels of inflammatory cytokines TNF-α (**J**) and IL-6 (**K**) are shown for both genotypes, as well as the growth factor VEGF (**L**). Statistical analysis was performed using an unpaired t-test and significant results considered as $P < 0.05$, n=7-8 mice per group.

Supplementary Figure 2. *Ex vivo* neutrophil apoptosis is increased with hrGal-1. C57/Bl6 mice received zymosan (1mg in 500µl $DPBS^{+/+}$ i.p.) at 0h. At 2h post-zymosan mice were administered hrGal-1 (10µg) or vehicle (200µl $DPBS^{-/-}$) and peritoneal lavage performed at 6h to collect leukocytes from the cavity. Peritoneal exudate (1ml) cells were resuspended in RPMI – 1640 Medium + 0.5% BSA and incubated (20h). Flow cytometry was used to select the neutrophil

($Ly6G^+$) population which was further assessed by AnnexinV (FITC) and Zombie (NIR). Quadrant gating was applied to determine viable ($AnxV^-NIR^-$), early apoptotic ($AnxV^+NIR^-$), late apoptotic ($AnxV^+NIR^+$) and necrotic ($AnxV^-NIR^+$) neutrophil populations in vehicle **(A)** and hrGal-1 **(B)** treated mice. Results for the percentages of the neutrophil population within each of the quadrants is shown **(C)**. Statistical analysis was performed using an unpaired t test (with each quadrant for apoptosis), results are displayed as the mean ± SEM, in all cases significant results are considered as $P < 0.05$. n = 6-7 mice per group.

Supplementary Table 1. Leukocyte numbers in 48h peritoneal exudate with hrGal-1 administered following the peak of inflammation.
Peritonitis was initiated in C57/Bl6 mice using zymosan (1mg, i.p.) and mice were treated with hrGal-1 (10µg) or vehicle (200µl $DPBS^{-/-}$) 8h post-zymosan and peritoneal lavage was performed at 48h. Total cell counts were performed and the number of neutrophils ($7/4^+Ly6G^+$), inflammatory monocytes ($7/4^+Ly6G^-$), eosinophils ($SiglecF^+$), macrophages ($F4/80^+$), mature macrophages ($F4/80^+CD11b^{high}$) and resolving macrophages ($F4/80^+CD11b^{low}$) were identified by flow cytometry. Statistical analysis was performed using an unpaired t test. Results displayed as the mean ± SEM, in all cases significant results were considered as $P < 0.05$. n=5 mice per group.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

DC and LVN planned the project. DC and AJI provided the knockout mice. HLL, RDW and AJI performed and analysed experiments. HLL, RDW, LVN and DC contributed to the design of the experiments. HLL, LVN and DC wrote and edited the manuscript.

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Figure 1.

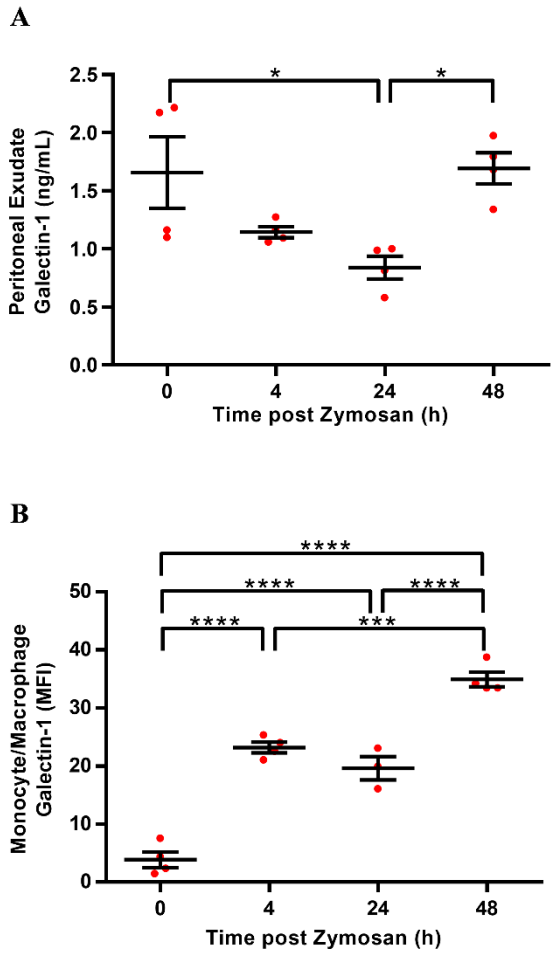

A
Peritoneal Exudate Galectin-1 (ng/mL)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
0
4
24
48
Time post Zymosan (h)
B
Monocyte/Macrophage Galectin-1 (MFI)
50
40
30
20
10
0
****
****
****
****
***
0
4
24
48
Time post Zymosan (h)

Figure 2.

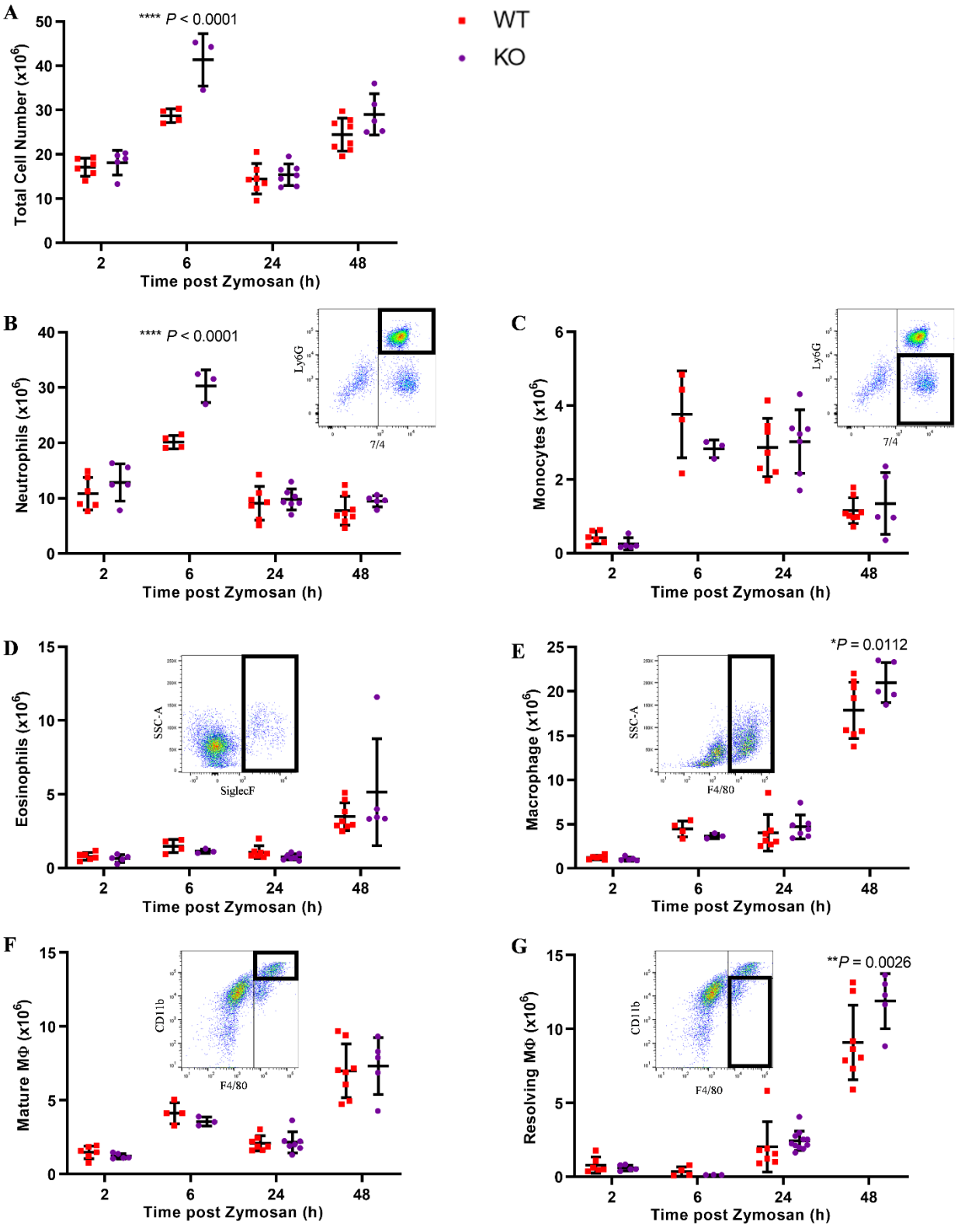

A
**** P < 0.0001
WT
KO
Total Cell Number (x10^6)
Time post Zymosan (h)
B
**** P < 0.0001
Neutrophils (x10^6)
Ly6G
7/4
C
Monocytes (x10^6)
D
Eosinophils (x10^6)
SSC-A
SiglecF
E
*P = 0.0112
Macrophage (x10^6)
F4/80
F
Mature MΦ (x10^6)
CD11b
G
**P = 0.0026
Resolving MΦ (x10^6)

Figure 3.

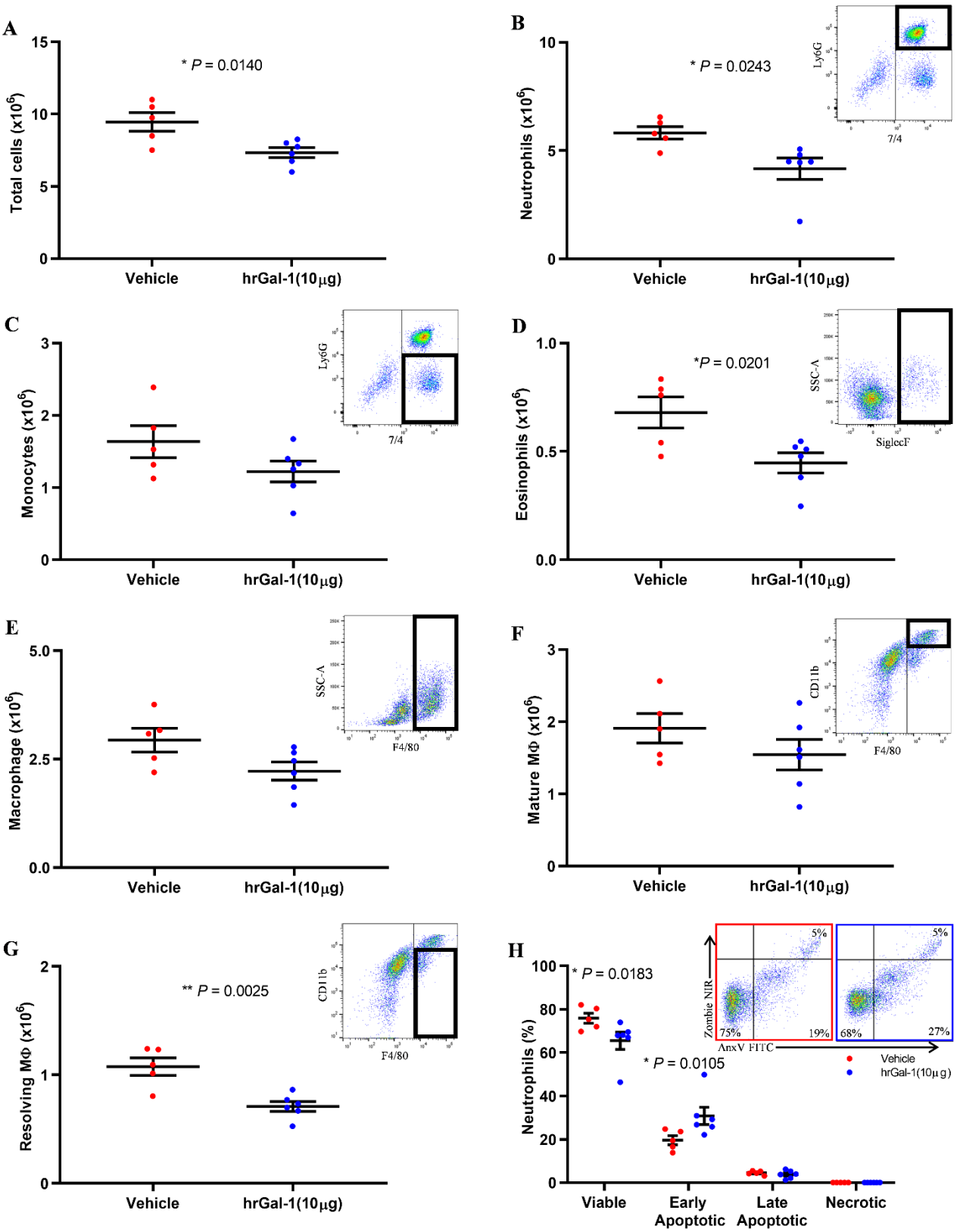
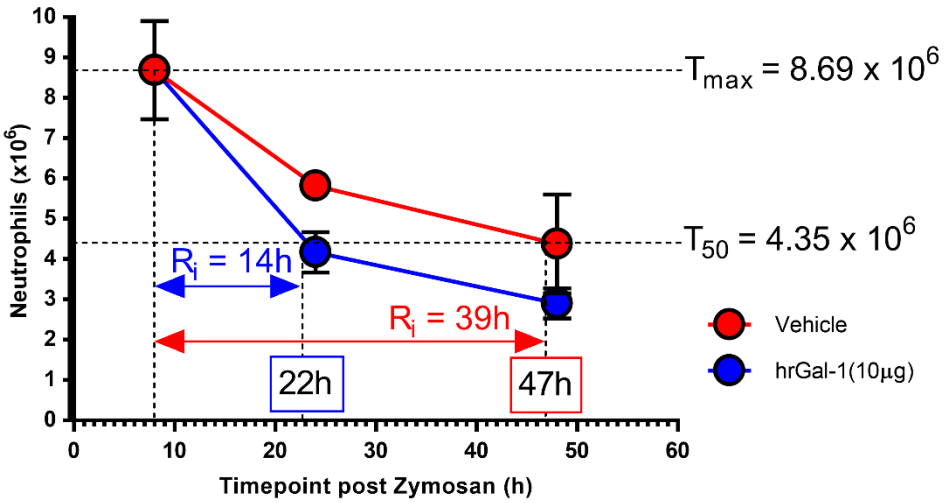
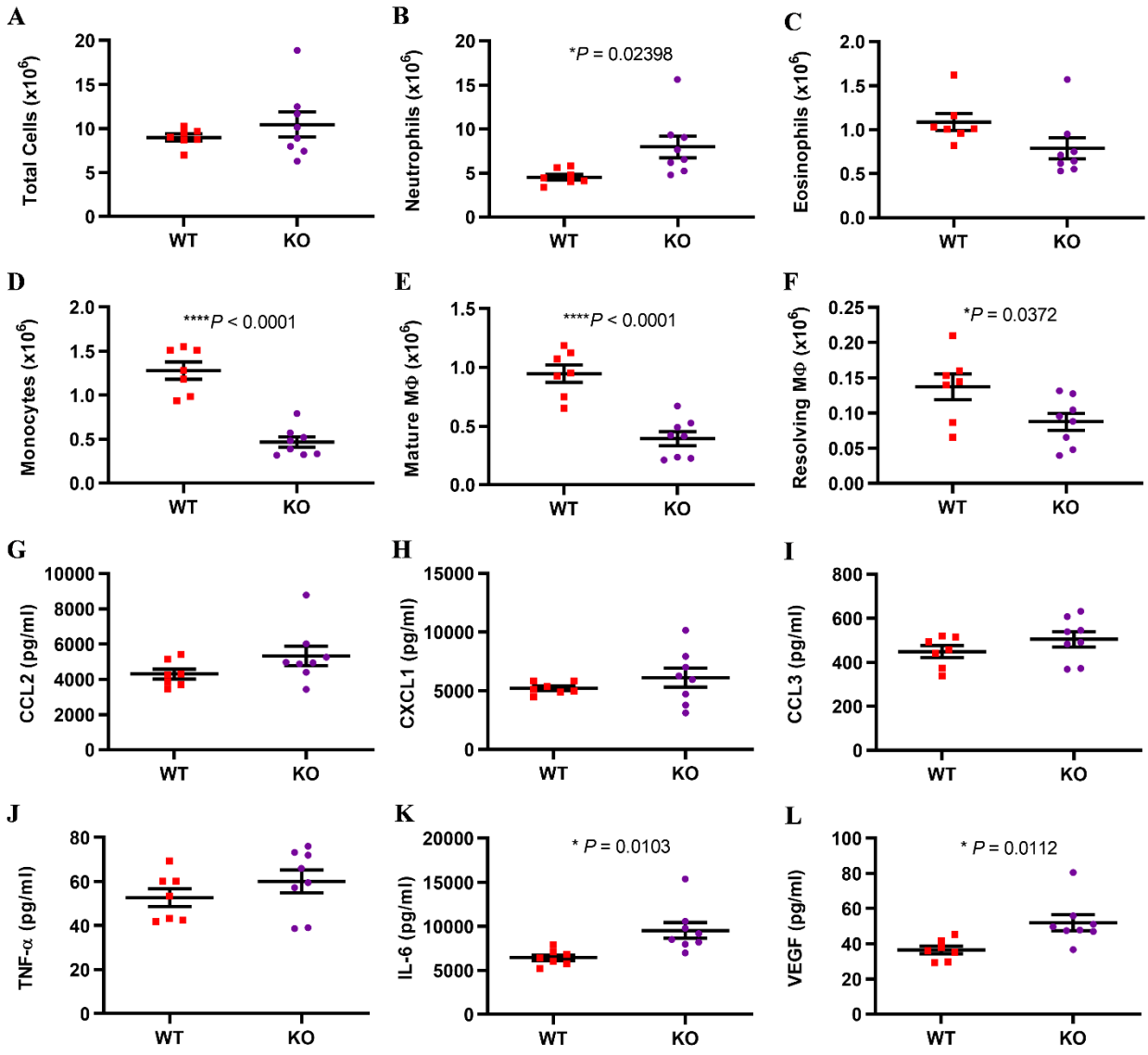

A
Total cells (x10^6)
* P = 0.0140
Vehicle
hrGal-1(10μg)
B
Neutrophils (x10^6)
* P = 0.0243
Ly6G
7/4
C
Monocytes (x10^6)
D
Eosinophils (x10^6)
*P = 0.0201
SSC-A
SiglecF
E
Macrophage (x10^6)
F4/80
F
Mature MΦ (x10^6)
CD11b
G
Resolving MΦ (x10^6)
** P = 0.0025
H
Neutrophils (%)
* P = 0.0183
* P = 0.0105
Zombie NIR
AnxV FITC
5%
75%
19%
68%
27%
Vehicle
hrGal-1(10μg)
Viable
Early Apoptotic
Late Apoptotic
Necrotic

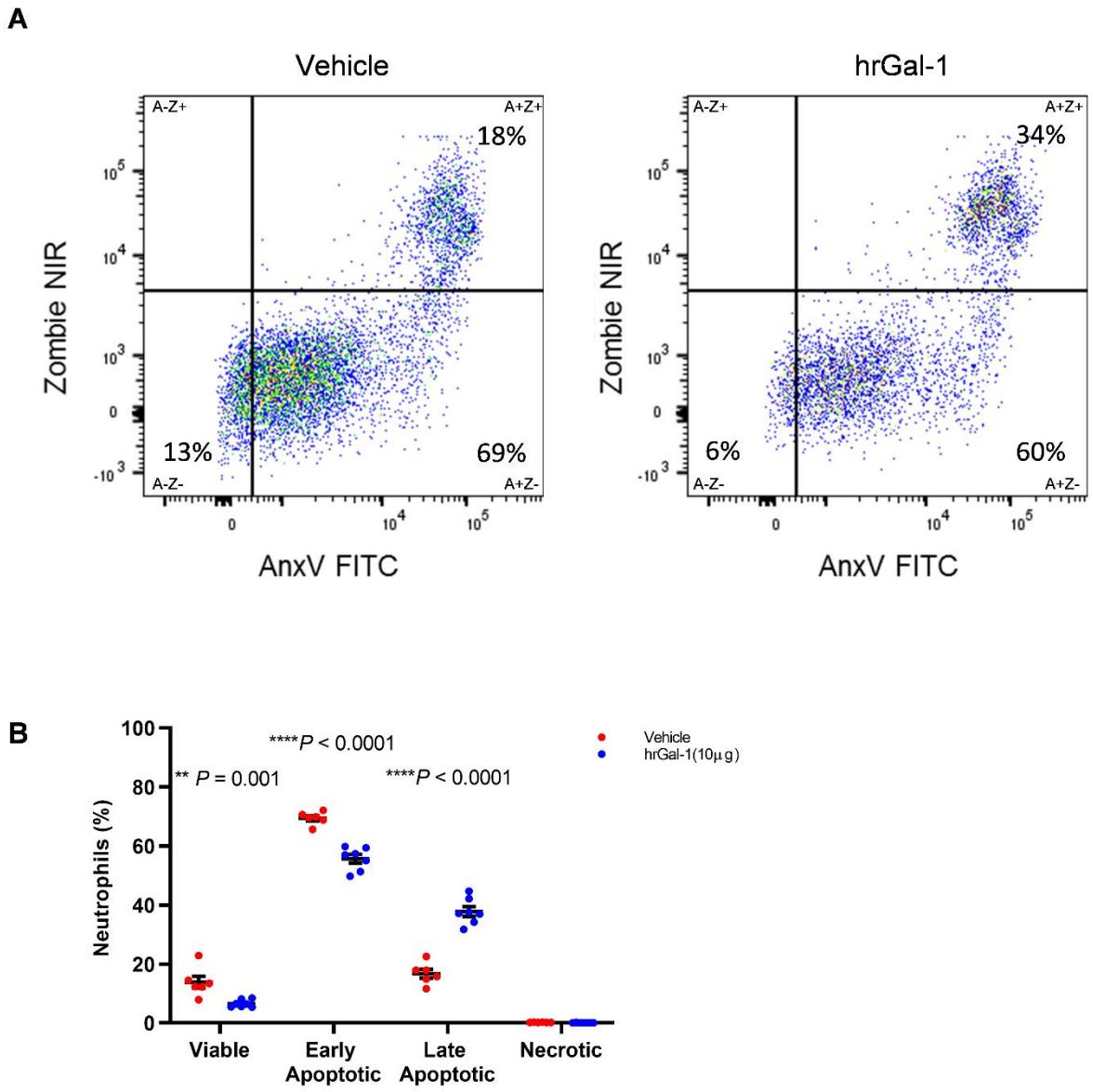
Figure 4.


Neutrophils (x10^6)
Timepoint post Zymosan (h)
T_max = 8.69 x 10^6
T_50 = 4.35 x 10^6
R_i = 14h
R_i = 39h
22h
47h
Vehicle
hrGal-1(10μg)

Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Table 1.

Leukocytes (x 10^6)	**Vehicle**	**hrGal-1 (10µg)**
Total Cells	12.9 ± 2.50	7.05 ± 1.28
Neutrophils	4.38 ± 1.23	2.91 ± 0.38
Monocytes	2.17 ± 0.57	0.98 ± 0.18
Macrophages	5.33 ± 1.57	2.32 ± 0.46
Mature Macrophages	3.90 ± 1.26	1.43 ± 0.26
Resolving Macrophages	1.45 ± 0.32	1.05 ± 0.23
Eosinophils	2.12 ± 0.55	1.44 ± 0.14