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Galectin-1 induces 12/15-lipoxygenase expression in murine macrophages and favors their conversion toward a pro-resolving phenotype

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ABSTRACT

During the resolution of inflammation macrophages undergo functional changes upon exposure to pro-resolving agents in their microenvironment. Primarily, engulfment of apoptotic polymorphonuclear (PMN) cells promotes conversion of macrophages toward a pro-resolving phenotype characterized by reduced CD11b expression. These macrophages are not phagocytic, do not respond to TLR ligands, and express relatively high levels of the pro-resolving enzyme 12/15-lipoxygenase (LO). Here, we report that the immuno-regulatory lectin galectin-1 is selectively expressed by $CD11b^{high}$, but not $CD11b^{low}$ macrophages. Upon exposure *in vivo* and *ex vivo*, galectin-1 directly promoted macrophage conversion from a $CD11b^{high}$ to a $CD11b^{low}$ phenotype and up-regulated the expression and activity of 12/15-LO. Moreover, galectin-1 treatment *in vivo* promoted the loss of phagocytic capacity (efferocytic satiation) in peritoneal macrophages and down-regulated secretion of TNF-α, IL-1β, and IL-10 upon LPS exposure. Our results suggest that galectin-1 could be an essential mediator in the control of macrophage function during the resolution of inflammation.

1. Introduction

During the active resolution of inflammation [1,2] several components of the immune response are eliminated [3]. Polymorphonuclear (PMN) cells undergoing apoptosis are cleared by macrophages and other phagocytic cells in a non-phlogistic fashion [4–8]. Apoptotic cell engulfment by phagocytes (also termed efferocytosis [4]) is mediated by signals that are expressed on the surface of apoptotic cells and their corresponding receptors (reviewed in [5,6]). Apoptotic cells also serve as resolution cues for macrophages, as their recognition evokes distinct signaling events that block the release of pro-inflammatory mediators and allow further engulfment of apoptotic cells [7,11]. Release of pro-inflammatory mediators is activated by bacterial moieties, and its down-regulation is termed immune-silencing/reprogramming [8,9]. Macrophage reprogramming is accompanied by the production of TGF-β and, in some cases, IL-10 [10–13], cytokines that can promote resolution and wound repair. The engulfment of apoptotic leukocytes by macrophages also leads to inhibition of inducible nitric oxide (NO) synthase (iNOS) expression and stimulates the expression of arginase-1 in the RAW 264 macrophage cell line [14], thereby preventing reactive NO production. In addition, the expression of 15-lipoxygenase (LO)-1, which is involved in the generation of pro-resolving lipid mediators [14–17], as well as the production of angiogenic growth factors [18] by macrophages are consequent to the uptake of apoptotic cells. Of note, molecular entities that are selectively expressed on apoptotic PMN cooperate with soluble bridging molecules in binding distinct receptors on the surface of macrophages. These receptors mediate both apoptotic cell clearance and immune-silencing in resolution-phase macrophages and other phagocytes [13,19–21].

Macrophages are highly diverse cells that adopt various functional phenotypes upon receiving differentiation cues from their surrounding environment [22–24]. Recent reports indicate that macrophages acquire distinct phenotypes during the resolution of acute inflammation [15,25]. A typical phenotype, which distinguishes resolution-promoting macrophages, is characterized

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by low expression of CD11b ($CD11b^{low}$) and is generated upon engulfment of apoptotic PMN [15]. $CD11b^{low}$ macrophages are distinct from either pro-inflammatory, classically-activated (M1) or anti-inflammatory, alternatively-activated (M2) macrophages, since they do not express iNOS or arginase-1 [15]. Macrophage conversion to the $CD11b^{low}$ phenotype results in significant functional changes in addition to the reduction in surface expression of CD11b and F4/80 [15]. Specifically, $CD11b^{low}$ macrophages stop producing TNF-α and IL-1β, do not engulf apoptotic PMN (efferocytic satiation), increase the production of TGF-β and the expression of 12/15-LO, and emigrate to lymphatic vessels [15].

Galectin-1 (Gal-1; encoded by *Lgals1*) belongs to the galectin family of proteins. Members of this lectin family typically act in the extracellular milieu through binding to a myriad of glycosylated receptors on the surface of immune cells [26]. Gal-1 is involved in the regulation of both innate and adaptive immunity. It suppresses antigen presentation and NO production by macrophages [27,28], and de-activates classically-activated microglia during autoimmune neuroinflammation [33]. It also promotes the termination of adaptive immunity by inhibiting pro-inflammatory cytokine production, dampening cell adhesion and trafficking, selectively deleting T helper (Th)1 and Th-17 cells [29–31], and promoting IL-10 secretion [32–34]. Gal-1 also induces the differentiation of tolerogenic dendritic cells and regulatory T cells [31,35]. Importantly, Gal-1 is highly expressed in macrophages during peritonitis and its expression is associated with successful resolution of inflammation [36,37].

The present study reveals that Gal-1 is expressed by a distinct macrophage population and contributes to a pro-resolving macrophage phenotype both *in vivo* and *ex vivo*. Exposure to Gal-1 promotes macrophage conversion from a $CD11b^{high}$ to a $CD11b^{low}$ phenotype characterized by enhanced 12/15-LO expression and activity, reduced efferocytic scores *in vivo*, and diminished cytokine secretion *ex vivo*.

2. Materials and methods

2.1. Reagents

ELISA kits for mouse TNF-α (catalog no. DY210), IL-1β (DY201), and IL-10 (DY417) were obtained from R&D systems. FITC-conjugated anti-mouse Gr-1 (108406), PE-conjugated anti-mouse F4/80 (12216) and PerCP-conjugated anti-mouse CD11b (101230) antibodies were obtained from Biolegend. Goat anti-mouse arginase-1 (ab60176) was from Abcam, rabbit anti-mouse 15-lipoxygenase-1 (160707) was purchased from Cayman chemical, goat anti-mouse MMP-9 (AF909) was from R&D systems, goat anti-mouse CD11b (sc6614), rabbit anti-mouse superoxide dismutase (SOD) 1 (sc11407), goat anti-mouse β-actin (sc1008), goat anti-mouse tubulin (sc9104) and rabbit anti-mouse GAPDH (sc25778) were from Santa Cruz Biotechnology. Recombinant Gal-1 was obtained from Drs. Rabinovich and Lichtenstein and purified according to the protocol described [31]. Rabbit anti-Gal-1 polyclonal antibody was obtained from Dr. Rabinovich's laboratory and used as described [33]. Anti-goat (82462) and anti-rabbit horseradish peroxidase-conjugated IgG (A31573) were obtained from Jackson ImmunResearch laboratories. Zymosan A (Z4250-IG), LPS (L2654), staurosporine (s4400), Carboxyfluorescein succinimidyl ester (CFSE) and PKH2-PCL green fluorescence linker kit were purchased from Sigma–Aldrich. RPMI 1640 (685991) was obtained from GIBCO. Docosahexaenoic acid (DHA) and Resolvin D1 (RvD1) EIA kit were obtained from Cayman chemicals.

2.2. Mouse peritonitis

Male C57BL/6 mice (7–8 weeks; protocol approved by the Ethics Committee, The Technion, Israel, authorization no. IL-009-01-2010) were purchased from Harlan Biotech, Israel, and maintained under SPF conditions at the animal facility of the Faculty of Medicine, The Technion, Haifa, Israel. All mice were injected intraperitoneally (i.p.) with freshly-prepared zymosan A (1 mg/ml, 1 mg/25 g body weight) in sterile phosphate-buffered saline (PBS). At 66 h post zymosan A injection, mice were sacrificed, their peritoneal cavity was lavaged with 5 ml of PBS and peritoneal exudates were collected. Exudate cells and supernatants were obtained by centrifugation for further analysis and experimentation. In other experiments resident peritoneal cells were collected from unchallenged mice. In some experiments, recombinant Gal-1 (4–8 μg in 1 ml of PBS) were injected i.p. after 48 h and peritoneal cells were collected at 66 h for analysis. In other experiments PKH2-PCL green (0.25 μM, 1 ml) was injected i.p. alongside Gal-1 (4 μg in one ml of PBS) 48 h post peritonitis initiation and peritoneal or spleen cells were recovered 18 h later, immuno-stained for F4/80 and analyzed by flow cytometry.

2.3. Determination of protein expression by Western blotting

Peritoneal exudates were collected 66 h post zymosan A injection, and exudate cells were immuno-stained with FITC-conjugated rat anti-Ly-6G, PE-conjugated rat anti-F4/80, and PerCP-conjugated rat anti-mouse CD11b (Biolegend). Macrophages were sorted to $CD11b^{high}$ and $CD11b^{low}$ populations (>95% purity) using FACSAria (Beckton-Dickinson) as in [15]. The sorted populations of $CD11b^{high}$ and $CD11b^{low}$ macrophages were washed with PBS and lysed in RIPA buffer containing Protease Inhibitors Cocktail (PIC, Roche). Cell lysates were run by SDS-PAGE followed by Western blotting for Gal-1 or GAPDH as a loading control.

Peritoneal macrophages from Gal-1 or vehicle-treated mice were isolated 66 h post zymosan A injection and lysed. In *ex vivo* experiments, lysates were prepared after incubation of macrophages for 24 h with recombinant Gal-1 (1 μg/ml), AC (1:5 M/AC ratio) or vehicle control. Lysates were run by SDS-PAGE and membranes were immuno-blotted for CD11b, arginase-1, 15-lipoxygenase-1, SOD-1, and MMP-9, or β-actin, tubulin, and GAPDH as loading control (used interchangeably).

2.4. Isolation of mouse peritoneal macrophages

Cells were recovered from peritoneal exudates 66 h after zymosan A challenge. Macrophages were labeled with PE-conjugated rat anti-F4/80 and isolated using EasySep PE selection magnetic beads following the manufacturer's instructions (StemCell Technologies).

2.5. Apoptotic cell preparation

Apoptosis was induced in Jurkat T cells using staurosporine (1 μg/ml; 4 h). Cells were washed twice with PBS, re-suspended in culture medium and added to macrophages plated as described in Sections 2.6 and 2.8.

2.6. FACS analysis

To evaluate leukocyte subsets according to CD11b expression, resident peritoneal cells or exudate cells from Gal-1 (4–8 μg/mouse)- or vehicle-treated mice were blocked with anti-CD16 and anti-CD32 mAb, and then stained (20 min, 4 °C) with FITC-conjugated anti-mouse Gr-1 (0.5 μg/1×10^6 cells),

PE-conjugated anti-mouse F4/80 (0.2 μg/1 × 10^6 cells), and PerCP-conjugated anti-mouse CD11b (0.2 μg/1 × 10^6 cells). Then, cells were washed and analysis was performed by a FACSCalibur flow cytometer (BD Biosciences). Macrophage percentage was determined and used to calculate peritoneal macrophage numbers. For *ex vivo* experiments, macrophages were incubated for 24 h with recombinant Gal-1 (1 μg/ml) or AC (1:5 M/AC ratio) before staining.

2.7. RvD1 production

Macrophages were isolated 66 h post peritonitis initiation and incubated with Gal-1 (4 μg/ml) or vehicle for 24 h. Then, cells were supplemented with DHA (20 μM) or vehicle for additional 24 h. The lipid content of the macrophages was extracted as in [38]. RvD1 content of lipid extraction samples was determined using standard EIA (Cayman chemicals).

2.8. Apoptotic PMN engulfment

Peritoneal macrophages were isolated from exudates 66 h post peritonitis initiation, transferred to poly prep slides (Sigma–Aldrich) and fixed with 2% paraformaldehyde. Then, cells were stained with Hoechst at room temperature for 20 min (Invitrogen). Two areas of two cover slips, each containing at least 50 (overall 200) macrophages were analyzed. The average number of PMN engulfed per macrophage, as well as the number of macrophages with cut-off numbers of engulfed PMN were calculated.

2.9. Apoptotic neutrophil engulfment ex vivo

Neutrophils or macrophages were isolated from peritoneal exudates 24 or 48 h post peritonitis initiation, respectively. Neutrophils were labeled with CFSE (1 μM, 30 min) and washed, and afterwards underwent senescence for 4 h in culture medium. Then, macrophages were incubated for 4 h with the senescent neutrophils (1:5 ratio) in the presence of Gal-1 (4–8 μg/ml) or vehicle. Next, nonadherent cells were washed, and the macrophages were removed to slides, and stained with Hoechst, as above. Efferocytic scores of CFSE-labeled neutrophils in each macrophage were enumerated as in [13].

2.10. LPS responsiveness ex vivo

Exudate macrophages (1 × 10^6 cells in 0.5 ml of culture media) were isolated 66 h post peritonitis initiation from mice treated with recombinant Gal-1 (4–8 μg/ml) or vehicle control and then incubated with LPS (1 μg/ml). After incubation (16 h) supernatants were collected and cytokine levels (TNF-α, IL-1β, and IL-10) were determined by capture ELISA.

2.11. Statistical analysis

In vitro and *in vivo* experiments were performed at least 2 times with 4 replicates of each data point. Results were analyzed by independent Student's *t-test* and Mann–Whitney *U* test for comparison between two groups and by one-way and Kruskal–Wallis ANOVA, respectively for comparison of more than two groups, with p values less than 0.05 considered statistically significant.

3. Results

3.1. Gal-1 is expressed in CD11b^{high} macrophages, and promotes their conversion toward a CD11b^{low} phenotype

Gal-1 has been shown to promote the differentiation of type 2 macrophages characterized by higher arginase activity and lower iNOS expression [28]. However the functional consequences of this effect remain uncertain. In resolving peritoneal cavities, CD11b^{high} macrophages are the predominant macrophage population. These macrophages display an M2-like phenotype that is abrogated upon conversion to a CD11b^{low} phenotype [15,39]. To determine whether Gal-1 is differentially expressed in these two subpopulations we sorted these cells and analyzed Gal-1 expression in pure CD11b^{high} and CD11b^{low} macrophages. Results indicated selective expression of Gal-1 by CD11b^{high}, as compared to CD11b^{low} macrophages (Fig. 1A and B; relative expression values of 1 and 0.16, respectively). Thus, Gal-1 is preferentially expressed by CD11b^{high} macrophages.

To determine the functional effects of Gal-1 on the conversion of CD11b^{high} to CD11b^{low} macrophages, resolution-phase macrophages were exposed to recombinant Gal-1 *in vivo* and *ex vivo*. Results indicated a statistically-significant and dose-dependent increase in the percentage of peritoneal CD11b^{low} macrophages following *in vivo* exposure to Gal-1 (Fig. 1C and D; max increase 43.25%). Interestingly, *ex vivo* treatment with Gal-1 alone resulted in an increase in the percentage of CD11b^{high} macrophages (max increase 26.74%), whereas a combination of apoptotic cells (AC) and Gal-1 resulted in a reduction of the percentage of CD11b^{high} macrophages, that exceeded the reduction induced by AC alone (Fig. 1E and F; 52.37% max reduction). It is important to note that no significant change in the number of peritoneal F4/80$^+$ macrophages was observed following treatment with Gal-1 *in vivo* (Fig. 1G). Hence, the reduction in CD11b^{high} macrophages is not due to reduced infiltration and differentiation of macrophages following Gal-1 injection. In addition, resident peritoneal macrophages converted to the CD11b^{low} phenotype following incubation *ex vivo* with apoptotic cells, but this was not enhanced by Gal-1 treatment (Fig. 1H). This finding underscores the specific effect of Gal-1 on phenotype transitions in inflammatory and resolving settings. Thus, Gal-1 promotes resolution-phase macrophage conversion from a CD11b^{high} toward a CD11b^{low} phenotype when combined with efferocytosis *in vivo* or *ex vivo*.

3.2. Gal-1 promotes the expression and activity of 12/15-LO in vivo and ex vivo

Macrophage conversion from a CD11b^{high} to a CD11b^{low} phenotype is characterized by an increase in the expression of 12/15-LO and a decrease in the expression of CD11b, arginase-1, and MMP-9 [15]. We determined whether the expression of these signature proteins is modulated by Gal-1. Exposure of resolution-phase macrophages to recombinant Gal-1 *in vivo* resulted in an increase in the expression of 12/15-LO, arginase-1, and CD11b, but not MMP-9 (Fig. 2A and B; max relative increase of 1.47, 1.25, and 1.29, respectively). Importantly, the increase in 12/15-LO and arginase-1 expression was evident at a dose of 4 μg/mouse, but not at 8 μg, suggesting that the biological activity of Gal-1 is self-limited. To determine whether the effect of Gal-1 on the expression CD11b^{low} macrophage signature proteins is direct and comparable to the effect of AC we treated resolution-phase macrophages with both agents *ex vivo*. The results in Fig. 2C and D indicate that Gal-1 acted directly on macrophages and induced a greater increase in 12/15-LO expression than AC (1.63 and 1.36 relative increase for Gal-1 and AC, respectively). Notably, Gal-1 did not induce a significant change in arginase-1, CD11b, or MMP-9.

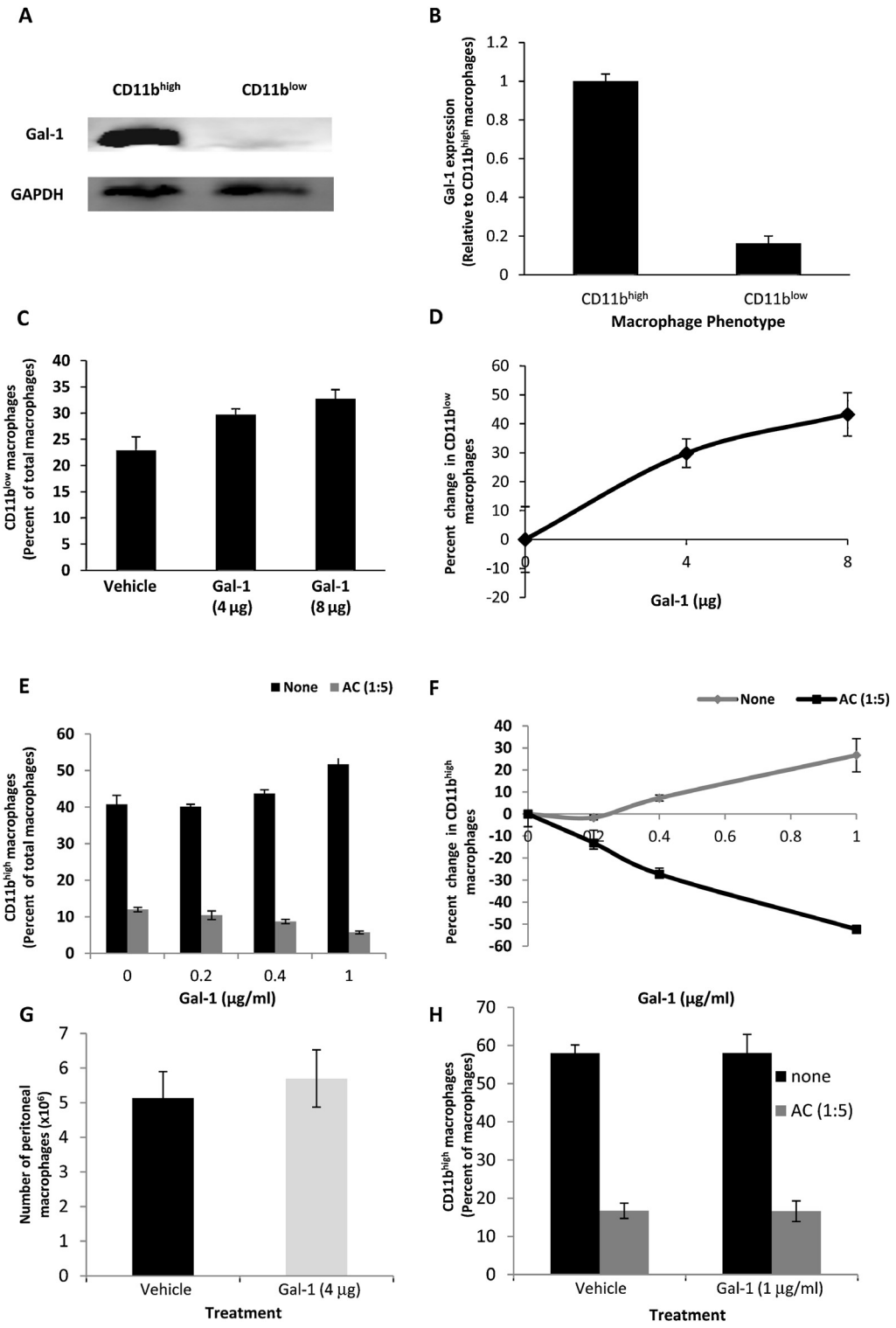

Fig. 1. Gal-1 is expressed in CD11b^{high} macrophages, and promotes their conversion toward a CD11b^{low} phenotype. A and B: Macrophages were isolated from peritoneal exudates of mice undergoing zymosan A-induced peritonitis for 66 h and sorted to CD11b^{high} and CD11b^{low} subpopulations by FACSAria. Cell lysates from each subpopulation were subjected to SDS-PAGE and Western blotting for Gal-1 and GAPDH (A). Gal-1 expression was quantified by densitometric analysis, normalized to GAPDH expression, and difference from the expression in CD11b^{high} macrophages was determined. Results are representative (A) and average (B) of 3 independent experiments. C and D: Mice were injected i.p. with zymosan A. After 48 h Gal-1 (4 or 8 μg), or vehicle control, were introduced into the peritoneum. After additional 18 h, exudate cells were recovered, immunostained for Ly-6G, F4/80 and CD11b and analyzed by flow cytometry using FACSCalibur. The results show the percentage of CD11b^{low} macrophages from total macrophages (C), and percent change in CD11b^{low} macrophages in Gal-1-treated mice (D). Data are presented as the mean ± SD from 2 independent experiments. E and F: Murine peritoneal macrophages were isolated 66 h post initiation of peritonitis, incubated with the indicated concentrations of Gal-1 alone or alongsides AC (1:5 M/AC ratio) for 24 h. Then, cells were immuno-stained for the leukocyte markers F4/80 and CD11b and the percentage of (E) or percent change in (F) CD11b^{high} macrophages were determined. G: Peritoneal exudate cells were recovered, enumerated and immuno-stained as in C. Then, the number of peritoneal macrophages was calculated for vehicle or Gal-1 (4 μg)-treated mice, as indicated. H: Resident peritoneal macrophages were incubated with vehicle, apoptotic cells (AC; 1:5 ratio), Gal-1 (1 μg/ml) or both. After 24 h the macrophages were immuno-stained as above and the percentage of CD11b^{high} and CD11b^{low} macrophages was determined. Data are presented as the mean ± SD from 3 independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$.

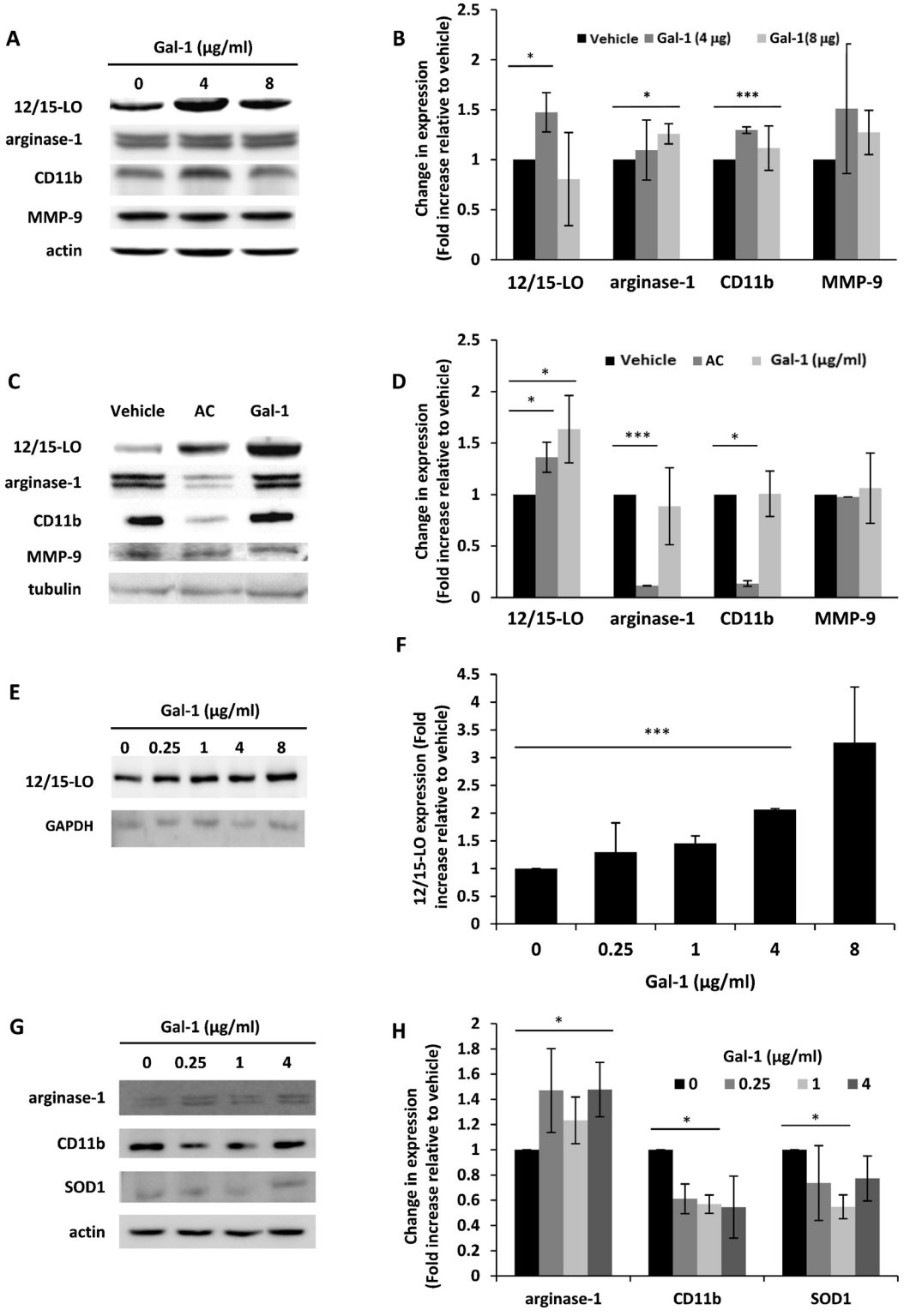

Fig. 2. Gal-1 increases 12/15-LO expression in macrophages during the resolution of inflammation. A and B: Mice were injected i.p. with zymosan A. After 48 h recombinant Gal-1 (4 or 8 μg), or vehicle control were introduced into the peritoneum. After an additional 18 h, peritoneal macrophages were isolated and lysed. Protein extracts were subjected to SDS-PAGE and Western blotting for the indicated proteins (A). Protein expression was quantified by densitometric analysis, normalized relative to actin expression, and fold increase from the expression in vehicle-treated mice was determined (B). Results are representative (A) and average (B) of 3 independent experiments. C and D: Murine peritoneal macrophages were isolated 66 h post initiation of peritonitis and incubated with AC (1:5 M/AC ratio) or recombinant Gal-1 (1 μg/ml) for 24 h. Then, macrophages were lysed and their protein content was analyzed by Western blotting for the indicated proteins (A). Protein expression was quantified by densitometric analysis, normalized to tubulin expression, and fold increase from the expression in vehicle-treated mice was determined (B). Results are representative (A) and average (B) of 3 independent experiments. E and H: Peritoneal macrophages were isolated 24 h post zymosan A injection and incubated with the indicated concentrations of Gal-1 for 24 h. Then macrophages were lysed and their protein content was analyzed by Western blotting for the indicated proteins. Protein expression was quantified by densitometric analysis, normalized to GAPDH expression (E and F) or actin expression (G and H), and fold increase from the expression in vehicle-treated macrophages was determined. Results are representative (E and G) and average (F and H) of 3 independent experiments.*$P < 0.05$, ***$P < 0.001$.

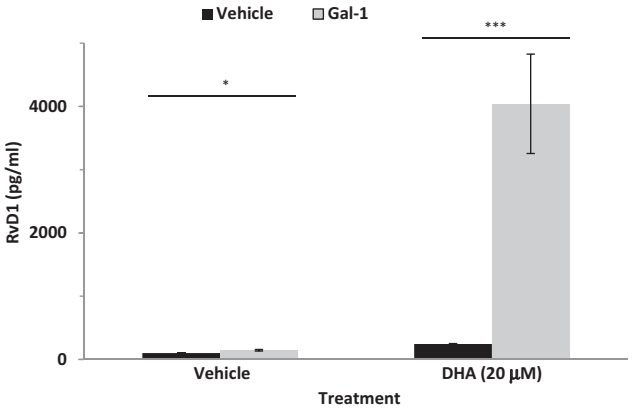

Fig. 3. Gal-1 promotes the generation of RvD1 by resolution-phase macrophages. Peritoneal macrophages were recovered 66 h post peritonitis initiation and incubated with Gal-1 (4 μg/ml) for 24 h. Then, the cells were supplemented with DHA (20 μM) and incubated for additional 24 h. Next, lipid extraction was performed and the levels of RvD1 in the extracts were determined using standard EIA. Results are representative of 3 independent experiments.*$P < 0.05$, ***$P < 0.001$.

Since resolution-phase macrophages express relatively high amounts of 12/15-LO, we wished to determine whether macrophages recovered at earlier time points during peritonitis also up-regulated 12/15-LO in response to Gal-1. Our results indicate that macrophages recovered 24 h post initiation of peritonitis show increased expression of 12/15-LO following exposure to recombinant Gal-1 *ex vivo*. This effect was dose-dependent but not self-limiting (Fig. 2E and F; 2.06 relative increase from control and statistically significant at 4 μg/ml). Of interest, these macrophages also responded to Gal-1 by increasing the expression of arginase-1 (1.477 max relative increase) and reducing the expression of CD11b (0.54 max relative decrease) and SOD-1 (0.548 max relative decrease; Fig. 2E and F).

Lipoxygenases produce various lipid mediators pending on substrate availability and intracellular signaling. Hence we determined whether the increase in 12/15-LO expression following *ex vivo* Gal-1 exposure led to an increase in activity and production of an important pro-resolving lipid mediator, namely RvD1. Notably, Gal-1-treated macrophages produced higher levels of RvD1 (101.01 and 144.51 pg/ml for vehicle and Gal-1, respectively; Fig. 3). This effect was significantly enhanced when exogenous docosahexaenoic acid (DHA), the substrate for RvD1 production, was added to cultures (244.11 and 4042.19 pg/ml for vehicle and Gal-1, respectively). Thus, Gal-1 regulates the expression and functionality of 12/15-LO in macrophages obtained from different phases of inflammation (including the resolution phase) while Gal-1-mediated control of other inflammation-related proteins is exerted in a temporal fashion.

3.3. Gal-1 promotes macrophage efferocytosis and efferocytic satiation

Macrophage conversion from a CD11b^{high} toward a CD11b^{low} phenotype is associated with the loss of their efferocytic potential, a process termed efferocytic satiation [15]. This important function of macrophages is regulated by pro-resolving mediators, such as resolvin D1 and E1, dexamethasone and the atypical chemokine receptor D6 [13,15]. We sought to determine whether Gal-1 modulates macrophage efferocytosis during the resolution of inflammation. Our results indicate a significant reduction in the numbers of apoptotic neutrophils engulfed by peritoneal macrophages during the resolution of inflammation (Fig. 4A; efferocytic scores of 5.65 and 4.6 for vehicle and 4 μg/mouse Gal-1, respectively). According to engulfment thresholds, the most significant difference following Gal-1 treatment was observed in the percentage of macrophages that engulfed more than 7 neutrophils (Fig. 4B; 35.55 and 20.65% of macrophages for vehicle and 4 μg/mouse Gal-1, respectively). These findings suggest a reduction in the satiation threshold that lead to an enhanced conversion of macrophages toward a CD11b^{low} phenotype and an earlier departure of the resolving inflammation site. Of interest, an increase in the percentage of macrophages that engulfed less than 2 apoptotic neutrophils was also found (Fig. 4B). Although this parameter did not reach statistical significance it suggests that in agreement with previous reports [40,41] Gal-1 promotes the infiltration of new macrophages to the resolving site to reinforce efferocytosis.

To directly determine whether Gal-1 promotes macrophage satiation and departure from the peritoneum, we used a fluorescent phagocytic labeling procedure. The injection of PKH2-PCL-green i.p. 48 h post induction of peritonitis resulted in the labeling of macrophages in the peritoneum and allowed tracking of these cells in the spleen. The mean fluorescence intensity (MFI) of labeled cells indicated their phagocytic activity in the peritoneum, whereas the percentage of labeled cells in each site indicated the extent of departure/emigration at each site. As shown in Fig. 3C–F, Gal-1 treatment resulted in a significant decrease in the labeling of peritoneal macrophages (4129.17 and 3182.78 MFI for vehicle and 4 μg/mouse Gal-1, respectively), whereas labeling of spleen macrophages was significantly increased (1688.49 and 2061.43 MFI for vehicle and 4 μg/mouse Gal-1, respectively). Of interest no significant changes in the percentage of labeled cells were observed in either site. These results suggest that the highly phagocytic macrophages in the peritoneum underwent enhanced satiation and departed this cavity thus resulting in the reduced MFI values in the peritoneum. Consequently, once these macrophages reached the spleen they increased the MFI of spleen macrophages. The lack of Gal-1 effect on macrophage percentage (Fig. 4D) and numbers in the peritoneum (data not shown, $N = 3$) could be explained by the previously reported chemotactic properties of Gal-1, when acting on macrophages [40,41]. The lack of Gal-1 effect on macrophage percentage in the spleen suggests that Gal-1 promotes macrophage emigration to other organs, in addition to the spleen.

The increase in the phagocytic activity of peritoneal macrophage prior to departure to the spleen suggests that like RvE1, RvD1 and dexamethasone [15,16,42], Gal-1 might enhance efferocytosis *ex* vivo. To determine whether Gal-1 modulates efferocytosis of macrophages *per se*, peritoneal macrophages were incubated with senescent peritoneal neutrophils in the presence or absence of Gal-1, and the uptake of apoptotic neutrophils was recorded. As shown in Fig. 4G, Gal-1 promoted neutrophil efferocytosis by macrophages, with the most significant effect observed at 8 μg/ml (0.6 and 3.04 N/M for vehicle and 8 μg/ml Gal-1, respectively). Thus, like other pro-resolving mediators, Gal-1 seems to promote both efferocytosis and efferocytic satiation with the net outcome being a reduction in the peritoneal efferocytic scores.

3.4. Gal-1 promotes macrophage immune-silencing

CD11b^{low} macrophages are characterized by lack of responsiveness to LPS. This phenomenon, termed immune-silencing, is manifested by reduced secretion of pro-inflammatory cytokines and chemokines, and production of the suppressive cytokine IL-10, following efferocytosis [8,15]. To determine whether Gal-1 promotes immune-silencing, resolution-phase macrophages from Gal-1-treated mice were stimulated with LPS and their secretion of TNFα, IL-1β, and IL-10 was determined. Our results (Fig. 5A–C) showed a diminished production of all cytokines by peritoneal macrophages recovered from Gal-1-treated mice. The differences were statistically significant in unstimulated as well as LPS-stimulated macrophages. Thus, Gal-1 induces macrophage

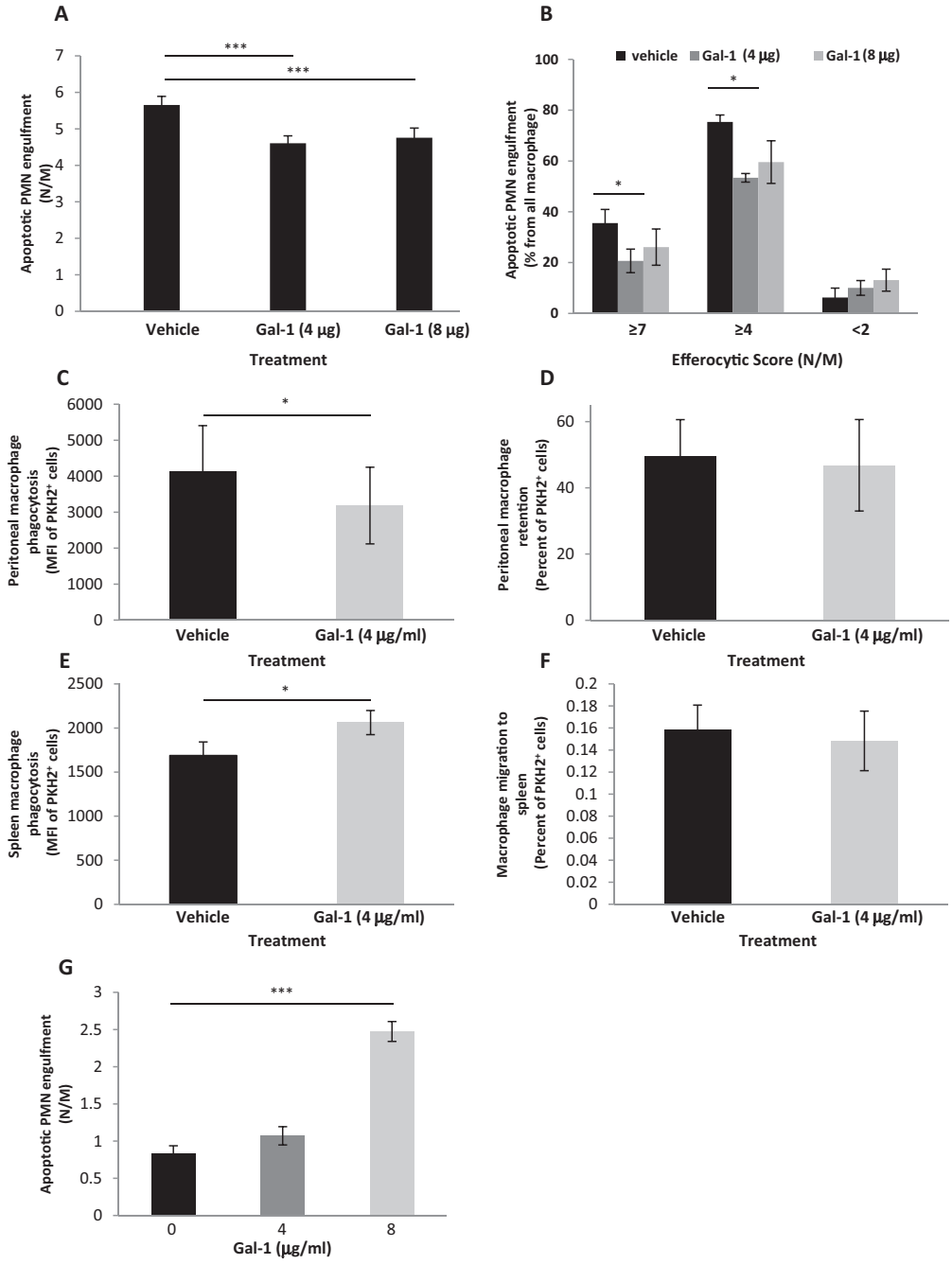

Fig. 4. Gal-1 enhances efferocytic satiation and peritoneal departure of macrophages *in vivo*. A and B: Mice undergoing zymosan A-induced peritonitis were injected i.p. with recombinant Gal-1 (4 or 8 μg), or vehicle at 48 h. After an additional 18 h, peritoneal macrophages were recovered, laid on slides and stained with Hoechst. Then, the macrophages were enumerated for efferocytic scores using fluorescence microscopy. The analysis shows average neutrophils engulfed per macrophage (A; N/M), and percentage of cells reaching engulfment thresholds (B). C–F: Mice undergoing zymosan A-induced peritonitis were injected i.p. with recombinant Gal-1 (4 μg), or vehicle alongside PKH2-PCL-green. After an additional 18 h, peritoneal (C and D) or spleen (E-F) cells were immunostained for F4/80 and analyzed by flow cytometry. Results show the MFI (C and E) and percentage (D and F) of PKH2-positive macrophages. G: Peritoneal macrophages were isolated 48 h after zymosan A injection and incubated *ex vivo* with CFSE-labeled senescent peritoneal neutrophils (1:5 ratio) and Gal-1 (4–8 mg/ml) or vehicle. After 4 h, unbound neutrophils were washed and macrophages were recovered, stained with Hoechst and observed under a fluorescent microscope. Macrophages were enumerated for labeled neutrophil uptake (efferocytic scores) and the average values per macrophage are presented. Data are presented as the mean ± SD from 3 independent experiments. $^{*}P < 0.05$, $^{***}P < 0.005$.

conversion toward a CD11b^{low} phenotype which contributes to immune-silencing.

4. Discussion

Gal-1 has emerged as a novel regulator of immune tolerance and inflammation [26]. This β-galactoside-binding protein suppresses acute and chronic inflammation in several experimental models through modulation of innate and adaptive immunity [30]. In macrophages and microglia this endogenous lectin induces a switch toward a type 2 – anti-inflammatory phenotype [28,43]. In this study we found Gal-1 contributes to a pro-resolving functional state in macrophages. These properties include the efferocytosis-dependent conversion of macrophages toward a CD11b^{low} phenotype *in vivo* and *ex vivo* (Fig. 1), hallmarked by the expression of 12/15-LO (Fig. 2), as well as the promotion of

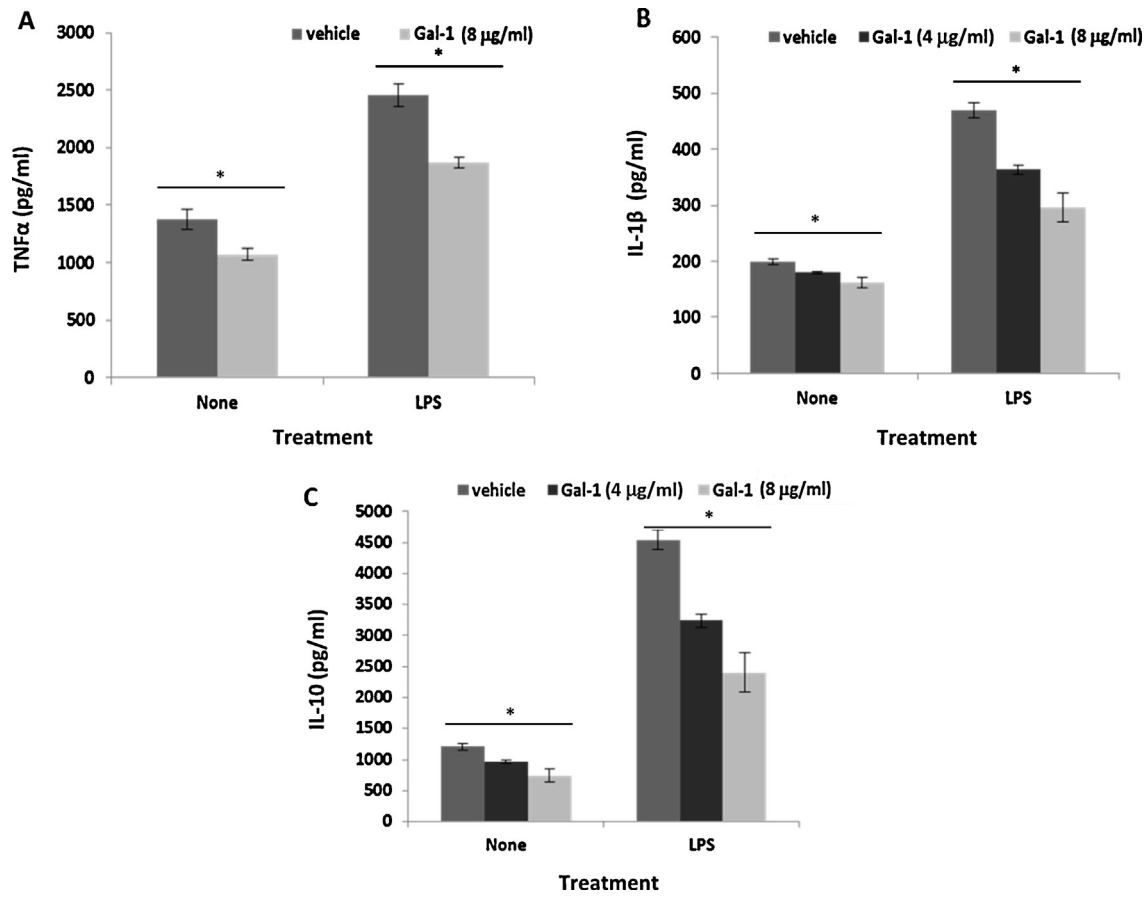

Fig. 5. Gal-1 inhibits TNF-α, IL-1β and IL-10 secretion from LPS-treated macrophages. Mice undergoing zymosan A-induced peritonitis were injected i.p. with recombinant Gal-1 (4 or 8 μg), or vehicle control at 48 h. After an additional 18 h, peritoneal macrophages were recovered, activated with LPS (1 μg/ml) and evaluated for the secretion of TNF-α (A), IL-1β (B) and IL-10 (C) using standard ELISA. Data are presented as the mean ± SD from two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

efferocytosis and efferocytic satiation (Fig. 4) and the switch toward a state of immune-silencing (Fig. 5). Similar actions were reported for specialized pro-resolving lipid mediators, such as resolvin (Rv) E1 and D1, as well as the synthetic glucocorticoid dexamethasone [15]. Importantly, the effect of Gal-1 on macrophages fluctuated during the transition from the inflammatory to the resolving phases of peritonitis. Initially (24 h post induction), *ex vivo* Gal-1 treatment promoted arginase-1 and reduced CD11b expression in macrophages (Fig. 2B). However, these actions were not evident during the later phase of resolution (66 h post induction; Fig. 2D). Moreover, the conversion of resolution-phase macrophages from the CD11b^{high} (M2-like) to the CD11b^{low} (denoted M_{res} [39]) phenotype was promoted by Gal-1 *in vivo* and *ex vivo*, in the presence of apoptotic PMN. Thus, Gal-1 seems to promote the generation of M2-like macrophages, which in turn could favor tissue repair during early resolution of inflammation. However, during late resolution this lectin promotes the generation of M_{res} from M2 macrophages, and favors the generation of pro-resolving lipid mediators, macrophage efferocytic satiation and departure from resolving peritoneal cavities, as well as return to tissue homeostasis [44].

Gal-1 was previously reported to be expressed in macrophages [37,45] and to promote pro-resolving functions in T cells and neutrophils [30,45–47]. Moreover, the resolution-delaying anesthetic lidocaine was found to diminish the amounts of Gal-1 as well as anti-inflammatory cytokines in cell-free exudates 24 h after initiation of peritonitis [36]. We found Gal-1 to be selectively expressed in CD11b^{high} macrophages, and its expression declined significantly once these cells converted toward a CD11b^{low} phenotype (Fig. 1A). Since CD11b^{low} macrophages are the predominant subtype to depart the peritoneum [15], Gal-1 expression seems to be limited to peritoneal macrophages during the resolution of inflammation and could potentially prevent non-specific immune suppression within lymphoid organs.

Expression and function of 12/15-LO in peritoneal macrophages was reported in both non-inflammatory and resolving inflammatory settings [15,48,49], as well as in certain inflammatory conditions associated with lipid peroxidation [50,51]. This enzyme and its orthologs are key mediators in the resolution of inflammatory pathologies and wound repair as well as in tolerance and autoimmunity [17,52–57]. Most of their actions are attributed to specialized pro-resolving lipid mediators, such as lipoxins, resolvins, protectins, and maresins [58]. 15-LO expression in human and murine monocytes/macrophages is up-regulated by the type-2 cytokines IL-4 and IL-13, as well as the engulfment of apoptotic leukocytes [14,15,38,59,60]. Our findings indicate for the first time that Gal-1 directly promotes 12/15-LO expression and activity in macrophages during the inflammatory and resolving phases of peritonitis (Figs. 2 and 3), thus facilitating the resolution of macrophage-mediated inflammation. Moreover, our results suggest that Gal-1 expression and function are essential components of the early reprogramming system of macrophages from the M1 to the M2/CD11b^{high} phenotype [39]; yet it also promotes the transition from the M2/CD11b^{high} to the M_{res}/CD11b^{low} phenotype at later periods of resolution. Both phenotype transitions are probably supported by 12/15-LO products, such as RvD1, that promote the resolution of murine peritonitis [15]. Our results suggest that Gal-1 is a key

mediator at different phases of the resolution of inflammation.

Gal-1 was previously reported to inhibit pro-inflammatory cytokines, chemokines and iNOS expression by microglia and macrophages [28,43]. Importantly, within the T cell compartment the regulatory actions of Gal-1 are mostly mediated by IL-10 secretion [35,61]. However, in our experimental setting macrophages recovered from Gal-1-treated mice synthesized lower amounts of pro-inflammatory (TNF-α and IL-1β) as well as anti-inflammatory (IL-10) cytokines following exposure to LPS (Fig. 4). Of note, these findings are consistent with the increased conversion of macrophages toward a $CD11b^{low}$ phenotype since $CD11b^{low}$ macrophages are poor producers of IL-10 as well as pro-inflammatory cytokines and chemokines [15]. Along these lines, human mesenchimal stromal cells in which Gal-1 expression was knocked down displayed increased secretion of IL-10 and inflammatory cytokines upon exposure to activated T cells [62]. Thus, the immunosuppressive effects of Gal-1 do not necessarily involve IL-10 production and might be replaced by 12/15-LO products within the macrophage compartment.

Collectively, our findings indicate unique patterns of expression and action for Gal-1 as an important mediator involved in the resolution of acute inflammation. Particularly, the control of 12/15-LO expression and the promotion of a $CD11b^{low}$ phenotype in macrophages seem to be key modules in the pro-resolving effect of Gal-1. These findings add a significant element to our understanding of the molecular machinery that governs the resolution of inflammation.

Disclosure statements

R. Rostoker and H. Yaseen performed the experiments, analyzed the data, prepared the figures and assisted in writing the manuscript. S. Schif-Zuck assisted in animal experimentation and FACS analysis. R. G. Lichtenstein provided Gal-1 protein. G. A. Rabinovich provided polyclonal anti-Gal-1 antibodies and recombinant Gal-1 protein, and assisted in the writing of the manuscript. A. Ariel planned the experiments, assisted in data analysis and presentation and wrote the manuscript. All authors read and approved the manuscript. All authors declare they have no conflict of interest.

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