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Galectin-1 modulation of neutrophil reactive oxygen species production depends on the cell activation state

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ABSTRACT

Here we report the effects of exogenous and endogenous galectin-1 (Gal-1) in modulating the functional responses of human and murine neutrophils at different stages of activation, i.e. naive, primed, and activated. Exposure to Gal-1 did not induce ROS production in either naive or *N*-formyl-methionyl-leucyl-phenylalanine-primed (fMLP; 10^{-9} M) neutrophils. However, Gal-1 elicited a concentration-dependent ROS production in neutrophils activated with fMLP at concentrations ranging from 10^{-8} M to 10^{-6} M. Additional fMLP (10^{-7} M) stimulation of fMLP-activated neutrophils increased ROS production, whose intensity was inversely related to the fMLP concentration used in the first activation step (10^{-8} M to 10^{-6} M), and was not influenced by the presence of Gal-1. Naive neutrophils treated with Gal-1 and then exposed to fMLP (10^{-6} M) or phorbol-12-myristate-13-acetate (10^{-7} M) produced less ROS, as compared to naive neutrophils not treated with Gal-1. Interestingly, these *in vitro* Gal-1 effects were associated with Gal-1 carbohydrate-binding activity and the ability to decrease FPR-1 (formyl peptide receptor 1) expression in naive human neutrophils. Conversely, positive ROS modulation by Gal-1 in activated neutrophils was not associated with FPR-1 expression but it was related to its carbohydrate recognition. *In vitro*, fMLP stimulation of Gal-$1^{-/-}$ mouse neutrophils produced more ROS than fMLP stimulation of Gal-$1^{+/+}$ neutrophils and this effect may be associated with increased FPR-1 expression. Exogenous Gal-1 induced ROS production in Gal-$1^{-/-}$ mouse neutrophils more effectively than in Gal-$1^{+/+}$ mouse neutrophils. Compared to Gal-$1^{+/+}$ mice, Gal-$1^{-/-}$ mice exhibited lower bacterial load in the peritoneal fluid and peripheral blood, thus indicating a greater bactericidal activity *in vivo*. These findings demonstrate that endogenous Gal-1 restricts ROS generation that correlates with bacterial killing capacity in inflammatory neutrophils. Thus, endogenous and exogenous Gal-1 may either positively or negatively modulate the effector functions of neutrophils according to the cell activation stage.

1. Introduction

Galectin-1 (Gal-1) is a mammalian β-galactoside-binding lectin expressed in various tissues and immunological cell types such as macrophages, neutrophils, and dendritic cells. Its carbohydrate recognition domain (CRD) bears a conserved amino acid sequence that favors its biological functions, such as granulocyte binding to extracellular matrix and regulation of cell migration towards Gal-1 (Barondes et al., 1994; Cooper et al., 2012; Thiemann and Baum, 2016). The participation of Gal-1 in homeostatic signaling is a potential therapeutic target for the resolution of inflammatory, autoimmune, metabolic, and infectious diseases and cancer (Brinchmann et al., 2018; Chou et al., 2018; Laaf et al., 2019). This lectin, associated with anti-inflammatory mediators and resolution-associated molecular patterns, may control innate and

Abbreviations: ANOVA, analysis of variance; CFU, colony forming unit; CL, chemiluminescence; cpm, photon counts per minute; Gal-1, galectin 1; HBSS, Hank's Balanced Saline Solution; fMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; FPR-1, formyl peptide recetor 1; LPS, lipopolysaccharide; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; PBS, phosphate buffered saline; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species; SEM, standard error of the mean; TDG, thiodigalactoside

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adaptive immune responses through macrophage polarization, neutrophil migration, and alteration of the profile of dendritic cells, Treg and T cells viability and function. Considering the modulatory activities of Gal-1, its effector functions comprise a wide range of biological information that connects innate to adaptive immunity (Liu et al., 2012a; Sundblad et al., 2017).

Gal-1 is not constitutively expressed in resting peripheral blood leukocytes and human promyelocytic leukemia (HL-60) cells, but it is present in the cytoplasm, nucleus, and the outer plasma membrane of inflammatory neutrophils (Dias-Baruffi et al., 2010; Elola et al., 2005; Kurihara et al., 2010). Activated neutrophils exhibit increased expression of Gal-1 receptors (Almkvist et al., 2002; Sengelov et al., 1995), and neutrophils that migrate to the inflammatory site after spinal cord injury express large amounts of Gal-1 (Kurihara et al., 2010).

Exogenous Gal-1 triggers ROS production in exudate neutrophils and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-activated neutrophils, but not in naive neutrophils. fMLP-mediated activation drives receptor expression in neutrophils by mobilizing and fusing receptor-storing granules with the plasma membrane (Almkvist et al., 2002; Sengelov et al., 1995). Granule mobilization correlates with Gal-1 binding to neutrophils, in particular to their gelatinase granules and secretory vesicles (Almkvist et al., 2002; Sengelov et al., 1995), and Gal-1 induces the release of myeloperoxidase and gelatinase (Almkvist et al., 2002). Gal-1 also displays a dual effect in neutrophil recruitment by chemoattracting the cells at extremely low concentrations in the absence of inflammation, and negatively regulating cell migration at the inflammation site (Auvynet et al., 2013; Gil et al., 2011). Interestingly, Gal-1 prepares neutrophils for phagocytic recognition by macrophages - an event called “preaparesis” - and helps to downregulate the inflammatory process (Dias-Baruffi et al., 2003; Stowell et al., 2009).

Although Gal-1 induces ROS generation in activated neutrophils, it is not clear which mechanisms control this effector function in naive, primed, and activated neutrophils treated with Gal-1 and how it affects the pathophysiology of various diseases. In the present study, we investigated *in vitro*: (i) at what stage of fMLP-induced neutrophil activation Gal-1 effectively induces ROS production; (ii) how Gal-1 influences the dynamics of ROS generation in naive, primed, and fMLP-activated neutrophils further exposed to fMLP; and (iii) whether Gal-1 affects the dynamics fMLP receptor recycling. In addition, we used a Gal-1-knockout mouse model to assess whether endogenous Gal-1 impacts the neutrophil ROS generation and microbial killing capacity, and whether it contributes to the acute inflammatory response.

2. Materials and methods

2.1. Chemicals

Histopaque®-1077, Histopaque®-1119, iodoacetamide, lactose, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), phorbol-12-myristate-13-acetate (PMA), RPMI-1640 medium, sucrose, thiodigalactoside (TDG), trypan blue, and thioglycollate were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). We acquired the following products from different suppliers: PD-10 desalting column (GE Healthcare, Buckinghamshire, UK), Detoxi-Gel™ endotoxin removing gel (Pierce Biotechnology, Rockford, IL, USA), Complete mini EDTA-free protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany), Difco™ Mueller Hinton agar (BD Biosciences, San Diego, CA, USA), and Romanowsky stain kit (Laborclin, Pinhais, PR, Brazil). All other chemicals and solvents used in this work were of analytical grade and obtained from commercial sources. The aqueous solutions were prepared with water previously purified in a Milli-Q water system (Merck-Millipore, Merck KGaA, Darmstadt, Germany). Sterile and lipopolysaccharide (LPS)-free solutions were used in all the biological assays.

2.2. Purification of recombinant Gal-1

Recombinant human Gal-1 was produced by *Escherichia coli* (M-15 strain) samples and purified as previously described (Dias-Baruffi et al., 2003). Purified Gal-1 was aliquoted and stored at −80 °C in phosphate buffered saline (PBS) supplemented with 100 mM lactose and 14 mm 2-mercaptoethanol. Prior to use, these compounds were removed from Gal-1 samples using gel filtration chromatography. The resulting Gal-1 fractions were treated with iodoacetamide (3.1 mg/g protein) for 12 h – to alkylate the oxidizable sites – and further chromatographed in a PD-10 column. Thereafter, contaminating LPS present in the samples was removed using a Detoxi-Gel™ endotoxin-removing gel column. Finally, Gal-1 samples were filtered through 0.22 μM membranes into sterile vials and kept on ice until use. The absorbance readings at 280 nm and the molar extinction coefficient of Gal-1 (1.17 M^{-1} cm^{-1}) were used to calculate the total protein concentration in Gal-1 samples.

2.3. Healthy subjects

This study enrolled five male and five female healthy adult volunteers who were 20–35 years old. All volunteers signed a written informed consent to participate in this investigation. The local Human Research Ethics Committee approved the study protocol (CEP/FCFRP n° 255), which complied with the 466/2012 Resolution of the Brazilian National Health Council and the Helsinki Declaration of 1975 (revised in 2008).

2.4. Animals

C57BL/6 mice deficient in Gal-1 gene (Gal-$1^{-/-}$) or not (Gal-$1^{+/+}$; wild type) were bred and housed at the Animal House of the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (FCFRP-USP), Ribeirão Preto, SP, Brazil, under the following conditions: no more than four animals per cage of the isolator rack (Alesco, Monte Mor, SP, Brazil), controlled temperature of 20 ± 2 °C, air humidity of 55 ± 2%, 12 h/12 h light/dark cycles, autoclaved water and chow diet *ad libitum*. All experiments were conducted on adult male mice between six and eight weeks of age, and body weight of 25 to 30 g. The Laboratory Animal Care Committee from the University of São Paulo (CEUA), Campus of Ribeirão Preto, Brazil, approved the study protocol (n. 09.1.323.53.5).

2.5. Isolation of human neutrophils

Twelve mL of venous blood was collected from the antecubital vein of each volunteer into heparin-coated vacutainer tubes. Neutrophils were purified as described by Paoliello-Paschoalato et al (2014). Briefly, in a 15-mL conical centrifuge tube, six mL of blood was carefully layered onto the Histopaque®-1119/Histopaque®-1077 density gradient, previously formed according to the manufacturer’s instructions. The tubes were centrifuged (700 ×*g*, 30 min, 22 °C) and the resulting neutrophil layer was transferred to another tube. The remaining erythrocytes were lysed with 0.83% NH_4Cl pH 7.4. Finally, the cells were washed with PBS (400 ×*g*, 10 min, 22 °C), suspended in Hank’s Balanced Saline Solution (HBSS) pH 7.2, and kept on ice until use.

After counting the total number of cells in a Neubauer chamber, concentration of the cell suspension was adjusted to 1×10^6 cells/mL with HBSS. Cell viability was determined by the Trypan Blue exclusion method, using a 0.4% dye solution in 0.15 M NaCl.

2.6. Isolation of murine inflammatory neutrophils

Inflammatory neutrophils were isolated according to the procedure described by Ribeiro-Gomes et al. (2004). Briefly, one mL of 3% thioglycollate solution or PBS (control) was injected intraperitoneally into male Gal-$1^{+/+}$ or Gal-$1^{-/-}$ mice. Six hours later, the peritoneal cavity

was washed with 3 mL of RPMI-1640 medium supplemented with 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Peritoneal cells were transferred to 75 cm^2 flasks (Costar, Cambridge, MA, USA) and incubated in a CO_2 incubator for 60 min, at 37 °C. Afterwards, supernatant of the culture medium containing non-adherent cells was collected to: (a) count the total number of cells in a Neubauer chamber; (b) determine cell viability by the Trypan Blue exclusion method; (c) analyze the sample purity – to this end, small aliquots of the cell suspension were attached to glass slides using cytospin centrifugation, and then stained using the Romanowsky' stain method.

2.7. ROS generation by human neutrophils

ROS production by human neutrophils was assessed using the luminol-enhanced chemiluminescence (CL) method, following the procedure reported by Paula et al. (2009) with slight modifications. Human neutrophils (5×10^5 cells) were incubated with luminol (10^{-4} M) for 2 min, at 37 °C. The cells were sequentially exposed to three groups of agonists during periods of 10 min each, at 37 °C: (1) fMLP (10^{-6} M, 10^{-7} M, 10^{-8} M, and 10^{-9} M), or PMA (10^{-7} M), or HBSS (control); (2) Gal-1 (1.25, 5, 10, and 20 μM) or HBSS (control); (3) fMLP (10^{-6} M) or HBSS (control). We also measured basal ROS production by neutrophils exposed to HBSS in the three steps and considered it as the negative control. The final reaction volume was 0.5 mL.

ROS generation was measured during the whole treatment period (30 min), in the Auto Lumat LB 953 luminometer (EG&G Berthold, Bad Wildbad, Germany), and recorded in photon counts per minute (cpm). The integrated areas of chemiluminescence (CL) corresponding to the following time intervals were calculated (Fig. 1): 0–3 min (CL area 1), 10–13 min (CL area 2), and 20–23 min (CL area 3). These areas comprised nearly 80% of the total ROS production. The last CL value recorded before cell stimulation at 0, 10, and 20 min was considered as the baseline value to calculate the integrated CL area of the respective step. To assess whether the effects of Gal-1 on neutrophil ROS generation were related to its lectin activity, we incubated the control sugars lactose (20 mM), sucrose (20 mM), or TDG (5 mM) with Gal-1 for 10 min, before adding them to the reaction medium.

2.8. ROS generation by murine leukocytes

Murine inflammatory leukocytes (5×10^5 cells) were incubated with luminol (10^{-4} M) during 2 min, at 37 °C, before adding fMLP (10^{-6} M), or Gal-1 (10 μM), or HBSS (negative control) to the reaction medium (0.5 mL). ROS generation was measured in a luminometer during 10 min, at 37 °C, and the integrated CL area produced in the first 3 min of reaction was calculated (CL area 1).

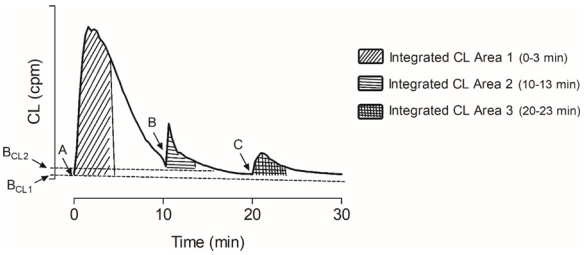

Fig. 1. Kinetics of ROS production by human neutrophils. ROS generation was measured during 30 min, and the integrated areas of chemiluminescence (CL) corresponding to the following time intervals were calculated: 0–3 min (CL area 1), 10–13 min (CL area 2), and 20–23 min (CL area 3); they comprised nearly 80% of the total ROS produced by the neutrophils. The last CL value recorded before cell stimulation at 0, 10, and 20 min (indicated as A, B, and C, respectively) was considered as the baseline value to calculate the integrated CL area of the respective step. B_{CL1}: baseline of the integrated CL Areas 1 and 3; B_{CL2}: baseline of the integrated CL Area 2.

2.9. In vivo bacterial killing

Cecal bacterial culture (4×10^8 CFU/mL) obtained from wild-type mice was injected intraperitoneally into Gal-$1^{+/+}$ and Gal-$1^{-/-}$ mice. Six hours later, the peritoneal cavity was washed with 3 mL of PBS. Bacterial viability in the resulting washes was assessed by serial dilutions followed by plating on Mueller–Hinton agar dishes. After 18 h of incubation at 37 °C, the number of colony forming units (CFU) were counted and the results were expressed as CFU/mL. The appropriate inoculum size was determined using a dose-response survival curve (LD50) and the survival rate of Gal-$1^{+/+}$ and Gal-$1^{-/-}$ mice after intraperitoneal injection of cecal bacteria (10^7 CFU) was analyzed 7 days after infection.

2.10. Analysis of FPR1 expression

2.10.1. Flow cytometry

Human neutrophils (5×10^5 cells) were isolated as reported in Section 2.5 and treated with: (1) HBSS; (2) 10 μM Gal-1 in the presence or absence of 20 mM lactose; or (3) 10^{-6} M fMLP during the first 10 min of reaction at 37 °C. Next, the three groups were treated with 10^{-6} M fMLP for further 10 min. Cells were washed with a 20 mM lactose solution (200 ×*g*, 5 min, 4 °C), blocked with 2% BSA plus 10% fetal bovine serum, incubated with anti-FPR1-PE antibody (Biolegend®) or isotype control (1 h, 4 °C), and fixed with 2% paraformaldehyde. Cells were analyzed using the FACSCanto-I flow cytometer equipped with the BD FACSDiva™ software. Neutrophils were gated based on their forward and side scatter properties, to further determine the percentage of FPR1-PE positive cells. Data are expressed as mean ± SEM of positive FPR1-PE cells from three independent experiments assayed in duplicate.

2.10.2. Immunofluorescence microscopy

Murine inflammatory neutrophils were isolated as reported in Section 2.6, and then plated onto 13-mm coverslips pretreated with Biobond® (EMS, Hatfield, PA). Cells were let to adhere for 1 h, at 37 °C, under 5% CO_2. The attached cells were fixed with 4% formaldehyde plus 50 mM EGTA and 50 μM Taxol for 10 min, at 37 °C, permeabilized with 0.3% Triton X-100 for further 10 min, at room temperature, and blocked with 2% BSA plus 10% fetal bovine serum for 45 min. Next, cells were incubated with primary antibody (rabbit antisera anti-FPR-1 at 1:250 dilution) for 1 h, washed with PBS, and incubated with secondary antibody (goat anti-rabbit_488 at 5.7 μg/mL plus 0.50 ng/ml DAPI) for 45 min. Coverslips were mounted with mount medium and images were acquired using the Leica TCS-SP5 confocal microscope (Mannheim, Germany). The relative quantification of od FPR-1 expression was performed using the LEICA LAS AF Lite software.

2.11. Data analysis

The experimental results were expressed as mean ± standard error of the mean (SEM) of the number of animals or experiments indicated in the legends. Statistical analysis was performed by analysis of variance (ANOVA) followed by the parametric Tukey-Kramer test or unpaired *t* test, with the aid of the GraphPad Prism Software (version 5.01 for Windows, GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered significant.

3. Results

3.1. Gal-1 induces ROS production in activated neutrophils in a concentration-dependent manner

We used the luminol-enhanced CL assay to examine whether Gal-1

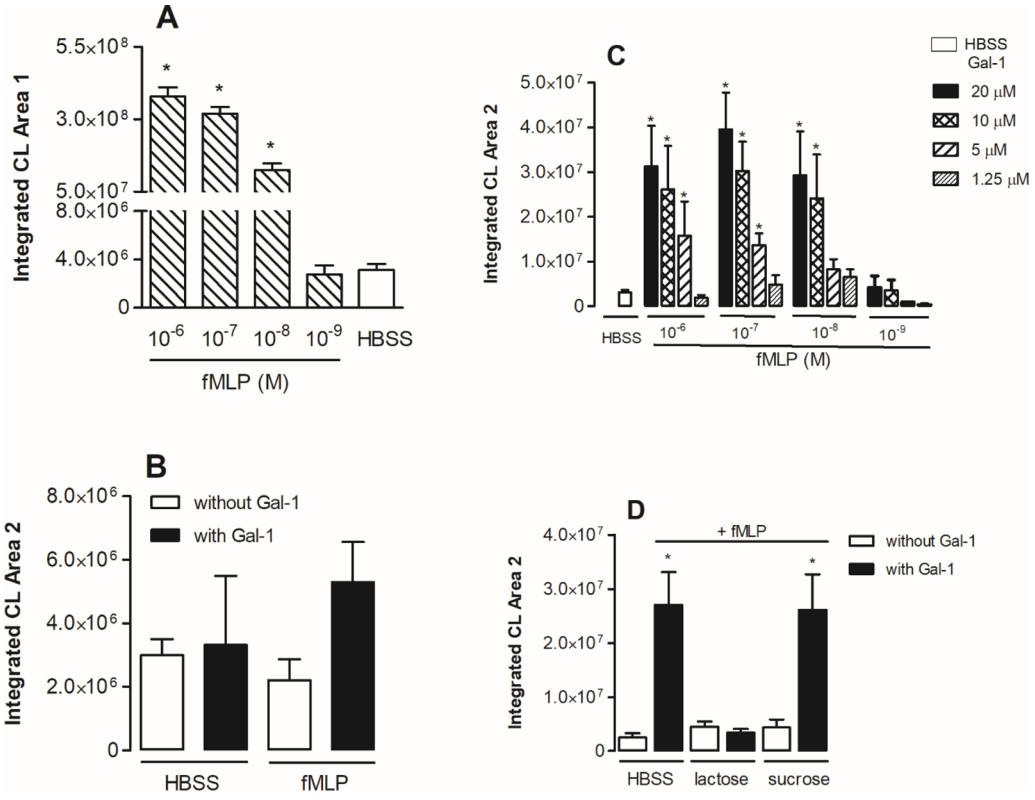

Fig. 2. Gal-1 induced ROS production in human neutrophils pre-activated with fMLP in a concentration-dependent and carbohydrate-sensitive manner. **(A)**: Neutrophils (5×10^5 cells) treated with different concentrations of fMLP during the first 10 min of reaction (CL area 1). **(B)**: Neutrophils sequentially treated with 10^{-9} M fMLP and 10 μM Gal-1. **(C)**: Neutrophils sequentially treated with different concentrations of fMLP (10^{-6}, 10^{-7}, and 10^{-8} M) and Gal-1 (1.25, 5, 10, and 20 μM). **(D)**: Neutrophils sequentially treated with 10^{-7} M fMLP and 10 μM Gal-1, in the presence of lactose (20 mM) or sucrose (20 mM). **(B)**, **(C)**, and **(D)**: fMLP was added to the reaction medium at t = 0 min, but the integrated CL area was calculated only for the second reaction step (CL area 2), after addition of Gal-1 at t = 10 min. **Control**: Cells exposed only to HBSS during the whole treatment period (20 min). Data are expressed as mean ± SEM of the integrated CL areas of three independent experiments assayed in duplicate. * $p < 0.05$ vs. HBSS (ANOVA and Tukey *post-hoc* test).

induces ROS generation in neutrophils at different stages of activation - naive, primed, and activated. To determine what fMLP concentration was capable of inducing ROS production in naive neutrophils, we exposed neutrophils to different concentrations of fMLP for 10 min. fMLP at 10^{-8} M, 10^{-7} M, and 10^{-6} M elicited ROS generation in a concentration-dependent manner, while fMLP at 10^{-9} M did not elicit significant ROS generation (mean CL area 1: 2.7×10^6), which was comparable to that of unstimulated cells (mean CL area 1: 3×10^6) (Fig. 2A).

Next, we treated neutrophils with fMLP (10^{-9} M to 10^{-6} M) for 10 min before adding Gal-1 to the medium. Gal-1 at 10 μM did not induce ROS generation in naive neutrophils (mean CL area 2: 3.3×10^6), and triggered a small but not significant ROS generation in neutrophils treated with 10^{-9} M fMLP (mean CL area 2: 5.3×10^6) (Fig. 2B), suggesting that 10^{-9} M fMLP may weakly prime neutrophils. On the other hand, Gal-1 elicited ROS production in a concentration-dependent manner (1.25, 5, 10, or 20 μM) in neutrophils pre-activated with fMLP at 10^{-8} M, 10^{-7} M, and 10^{-6} M for 10 min; this effect was independent of the fMLP concentration added to the medium (CL area 2; Fig. 2C). Considering that neutrophil stimulation with fMLP induced a fast and sharp ROS production that lowered to basal levels within 5–10 min, depending on the stimulus concentration, the CL area 2 (10–13 min) from cells treated with fMLP at t = 0 min but not stimulated with Gal-1 at t = 10 min was near to that of non-stimulated cells.

Finally, we preactivated neutrophils with 10^{-7} M fMLP and treated them with Gal-1 in the presence of sucrose or lactose to determine whether the effects of Gal-1 were carbohydrate-dependent. We found that lactose, which inhibits Gal-1 binding through its CRD, but not sucrose, a non-inhibitory sugar, markedly reduced the ability of Gal-1 to elicit ROS generation in pre-activated neutrophils, indicating that the lectin activity of Gal-1 mediated its stimulatory effect (CL area 2; Fig. 2D).

3.2. Pre-treatment of neutrophils with Gal-1 decreases ROS production in response to fMLP and PMA

Although the ability of Gal-1 to trigger the respiratory burst in pre-activated neutrophils may contribute to the host immunological response, activation of these cells in peripheral blood could damage the vascular wall and amplify the inflammatory process. Thus, we examined how Gal-1 affects the dynamics of ROS generation in naive *versus* primed neutrophils.

Naive neutrophils treated with 10 μM Gal-1 during 10 min and further stimulated with 10^{-6} M fMLP (CL area 2; Fig. 3A) or 10^{-7} M PMA (CL area 2; Fig. 3B) produced, respectively, 55% and 40% less ROS than naive neutrophils not treated with Gal-1. To assess whether the CRD of Gal-1 mediates its inhibitory effect on the neutrophil response to fMLP and PMA, naive neutrophils were treated with Gal-1 in the presence of the inhibitory disaccharide TDG or non-inhibitory sucrose before being stimulated with 10^{-6} M fMLP. TDG, but not sucrose, partially (49%) reverted the inhibitory effect of Gal-1 (CL area 2; Fig. 3A). Together, these data indicate that the lectin activity of Gal-1 is essential to it negatively modulate ROS generation in naive neutrophils undergoing further activation with fMLP and PMA.

3.3. Gal-1 does not affect the dynamics of ROS production in neutrophils successively stimulated with fMLP

In the first set of experiments (Section 3.1), we examined how naive neutrophils, and neutrophils primed with 10^{-9} M fMLP or activated with 10^{-8}-10^{-6} M fMLP respond to subsequent stimulation with 10^{-6} M fMLP or 10 μM Gal-1 (CL area 2; Fig. 4A). Analysis of the integrated CL area 2 calculated from the second activation step revealed that the intensity of the cellular response to fMLP was inversely proportional to the concentration of fMLP employed in the first activation step (Fig. 4A). Neutrophils activated with 10^{-8} M fMLP responded more effectively to subsequent stimlation with 10^{-6} M fMLP, as compared with stimulation with Gal-1.

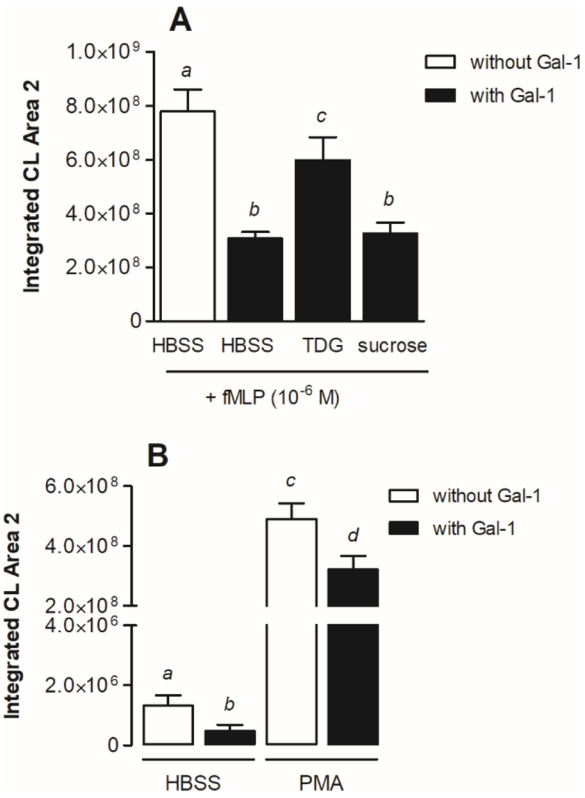

Fig. 3. Gal-1 negatively modulates ROS production in naive human neutrophils and TDG partially reverts its effect. **(A)**: Neutrophils (5×10^5 cells) were treated with Gal-1 (10 μM) for 10 min in the presence of TDG (5 mM) or sucrose (20 mM), and further stimulated with fMLP (10^{-6} M) for 10 min. **(B)**: Neutrophils sequentially exposed to Gal-1 (10 μM) and PMA (10^{-7} M) for 10-min periods. **(A)** and **(B)**: Gal-1 was added to the reaction medium at t = 0 min, but the integrated CL area was calculated only for the second step of reaction (CL area 2), after addition of fMLP or PMA at t = 10 min. **Control**: Cells exposed only to HBSS during the whole treatment period (20 min). Data are expressed as mean ± SEM of the integrated CL areas of three independent experiments assayed in duplicate. Values not sharing the same letter (*a-d*) are significantly different from each other (unpaired *t* test). In **(A)**: $p = 0.045$ (a *vs* b), $p = 0.0309$ (b *vs* c). In **(B)**: $p = 0.0245$ (a *vs* b), $p < 0.0001$ (a *vs* c; a *vs* d), $p = 0.0008$ (b *vs* c), $p = 0.0001$ (b *vs* d).

Next, we performed a three-step activation experiment: first, cells were activated with fMLP at 10^{-6}, 10^{-7} or 10^{-8} M; second, cells were treated with Gal-1 at concentrations ranging from 1.25 to 20-μM; third, cells were activated with 10^{-6} M fMLP. The neutrophil ROS generation was measured during the third activation step (CL area 3; Fig. 4B). The fMLP concentration used in the first activation step determined the level of ROS production by neutrophils in the third stimulation step: the amount of ROS produced in the final step was inversely proportional to the fMLP concentration used in the first activation step, but it was independent of the Gal-1 concentration used in the second step (CL area 3; Fig. 4B). In addition, successive neutrophil stimulation with fMLP and Gal-1 during 30 min did not induce cell apoptosis or necrosis, under the conditions assessed (data not shown).

Together, our findings suggest that (i) the level of fMLP-induced neutrophil activation determines its ability to subsequently respond to the same stimulus, and (ii) Gal-1 does not affect the dynamics of fMLP sensitivity in neutrophils previously activated with fMLP.

3.4. The lack of endogenous Gal-1 increases ROS production and favors bacterial killing by inflammatory neutrophils

Peritoneal neutrophils from Gal-$1^{-/-}$ and Gal-$1^{+/+}$ mice were used to examine how endogenous Gal-1 affects the cell ability to produce ROS and kill microbes. Neutrophils were collected from the peritoneal cavity six hours after injection of 3% thioglycolate. Cell preparations contained around 95% of neutrophils with viability greater than 95% (data not shown).

fMLP-stimulated Gal-$1^{-/-}$ mouse neutrophils released more ROS *in vitro* than fMLP-stimulated Gal-$1^{+/+}$ mouse neutrophils (mean CL area of 1.95×10^7 and 1.29×10^7, respectively) (Fig. 5A). Exogenous Gal-1 improved ROS production in Gal-$1^{-/-}$ mouse neutrophils more effectively than in Gal-$1^{+/+}$ mouse neutrophils (mean CL area of 8.3×10^5 and 5×10^5, respectively), when compared with their respective controls (Fig. 5B). Hence, neutrophils from mice lacking endogenous Gal-1 are more sensitive to the action of exogenous Gal-1, and endogenous Gal-1 may be involved in the negative regulation of neutrophil oxidative burst.

Compared with Gal-$1^{+/+}$ mice, Gal-$1^{-/-}$ mice exhibited stronger bactericidal activity against intraperitoneal cecal bacteria. Bacterial load in the peritoneal fluid and peripheral blood of Gal-$1^{+/+}$ mice (5.2×10^6 and 5.4×10^6 CFU/mL, respectively) was tenfold greater than bacterial load in the same fluids of Gal-$1^{-/-}$ mice (5.7×10^5 and 5.8×10^5 CFU/mL, respectively) (Fig. 6A). These findings demonstrate that the lack of endogenous Gal-1 improves ROS generation and the bactericidal activity of peritoneal mouse neutrophils.

3.5. FPR-1 expression in naïve neutrophils is negatively modulated in the presence of Gal-1

To examine whether Gal-1 negatively modulated ROS generation by altering the levels of FPR-1 expression in neutrophils, we performed immunostaining of neutrophil FPR-1 receptors using an anti-FPR-1-PE antibody. Naive neutrophils were isolated from human peripheral blood and treated with 10 μM Gal-1 in the presence or absence of 20 mM

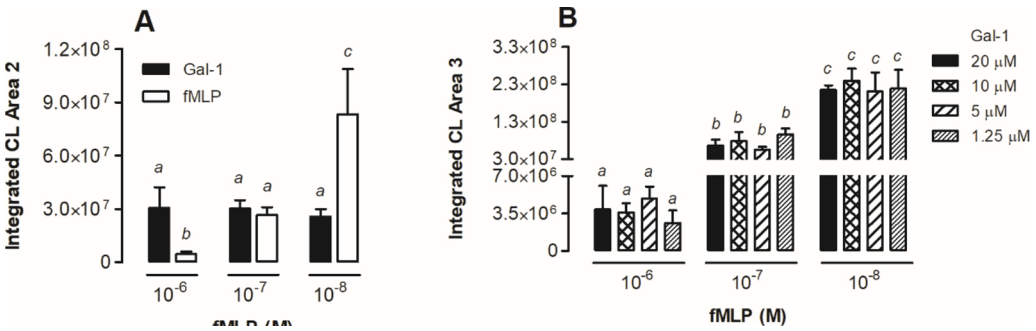

Fig. 4. Gal-1 induces ROS production in activated human neutrophils independently of fMLP receptor desensitization. **(A)**: Neutrophils (5×10^5 cells) were treated with fMLP (10^{-8}-10^{-6} M) and further stimulated with 10 μM Gal-1 or 10^{-6} M fMLP; the integrated CL area 2 (after addition of Gal-1 or 10^{-6} M fMLP at t = 10 min) was analyzed. **(B)**: Neutrophils (5×10^5 cells) were sequentially treated with fMLP (10^{-8}-10^{-6} M), Gal-1 (1.25–20 μM), and 10^{-6} M fMLP for 10-min periods; the integrated CL area 3 (after addition of 10^{-6} M fMLP at t = 20 min) was analyzed. Results are representative of six independent experiments. *Statistical analysis*: values not sharing the same letter (*a-c*) are significantly different from each other. In **(A)**: $p < 0.0005$ (a *vs* b), $p < 0.0001$ (a *vs* b; b *vs* c), ANOVA combined with Tukey *post-hoc* test. In **(B)**: $p = 0.0095$ (a *vs* b), $p = 0.0239$ (a *vs* c), $p = 0.0144$ (b *vs* c), unpaired *t* test.

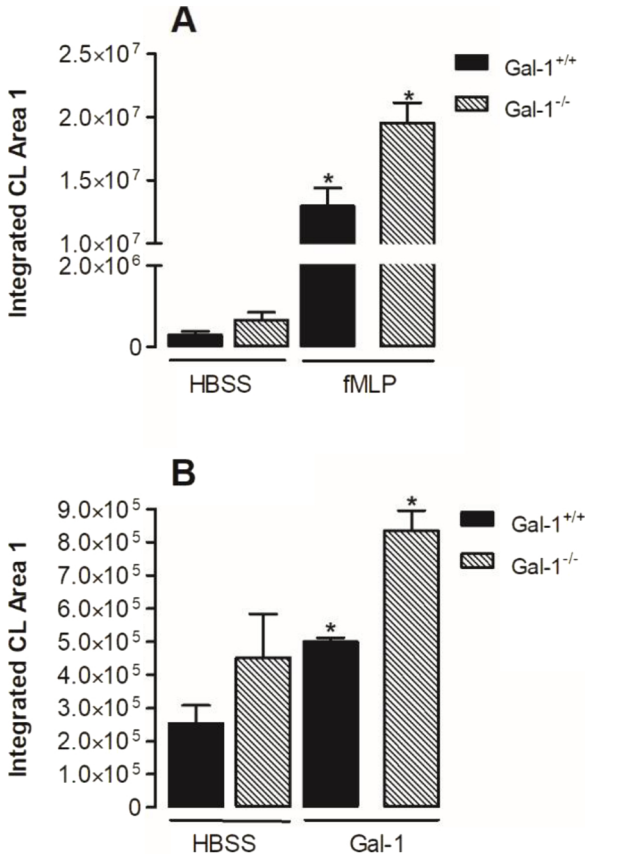

Fig. 5. fMLP and Gal-1 induce stronger ROS production in Gal-$1^{-/-}$ (knockout) mouse neutrophils than in Gal-$1^{+/+}$ (wild-type) mouse neutrophils. Peritoneal neutrophils were obtained from mice treated with thyoglycollate i.p. for 6 h. The luminol-enhanced CL assay was used to measure ROS production by mouse neutophils (5×10^5 cells) treated with 10^{-6}M fMLP **(A)** or 10 μM Gal-1 **(B)** for 10 min at 37 °C. **Control**: Cells exposed only to HBSS during the whole treatment period. Data represent the mean ± SEM of three animals per group. *$p < 0.05$ vs. HBSS (ANOVA combined with Tukey's *post-hoc* test).

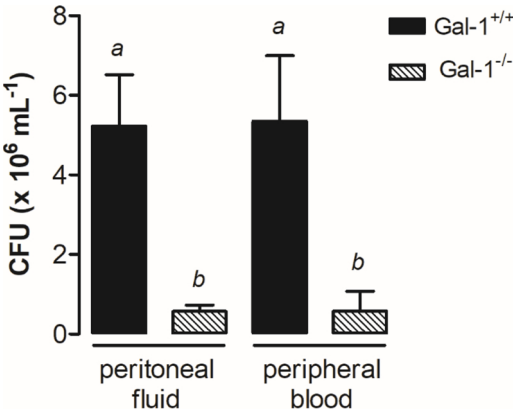

Fig. 6. Gal-$1^{-/-}$ mice exhibit reduced bacterial load in peripheral blood and at the infection site. The peritoneal wash and peripheral blood from Gal-$1^{-/-}$ mice was collected six hours after intraperitoneal injection of cecal bacteria (4×10^8 CFU/mL) from Gal-$1^{+/+}$ (wild-type) mice. Bacterial viability was determined by CFU counting after serial dilution and plating on Mueller-Hinton agar dishes. Data represent the mean ± SEM of six animals per group. Values not sharing the same letter are significantly different from each other ($p = 0.0116$ (a *vs* b; peritoneal fluid); $p = 0.0323$ (a *vs* b; blood); unpaired *t* test).

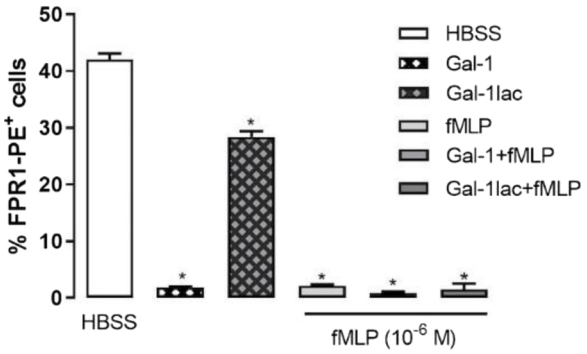

Fig. 7. Gal-1 reduces FPR1 expression in naïve human neutrophils. Neutrophils (5×10^5 cells) were treated with 10 μM Gal-1 in the presence or absence of 20 mM lactose, and with 10^{-6} M fMLP during the first 10 min of reaction. Next, the cells were treated with 10^{-6} M fMLP for further 10 min, stained with anti-FPR1-PE or isotype control antibody for 1 h, at 4 °C, and analyzed by flow cytometry. HBSS (*Hanks balanced saline solution*; control): Unstimulated cells, exposed only to the reaction medium during the whole treatment period (20 min). Data are expressed as mean ± SEM of positive FPR1-PE cells from three independent experiments assayed in duplicate. * $p < 0.05$ *vs.* HBSS (ANOVA and Tukey *post-hoc* test).

lactose, and with 10^{-6} M fMLP, as described in Section 2.10.1.

The percentage of FPR1-PE-positive cells decreased after treatment with Gal-1 and fMLP alone, as well as after the sequential treatment with Gal-1 plus fMLP. Lactose partially reversed downregulation of FPR-1 expression by Gal-1, indicating that the lectin effect depended on carbohydrate recognition (Fig. 7). This finding suggests that Gal-1 downmodulates fMLP-dependent ROS production in naïve neutrophils by decreasing the levels of FPR-1 expression.

3.6. Gal-$1^{-/-}$ mice express higher levels of FPR1 receptor than wild-type mice

To analyze how endogenous Gal-1 affects FPR1 expression, we collected peritoneal neutrophils from Gal-$1^{-/-}$ and Gal-$1^{+/+}$ mice six hours after injection of 3% thioglycolate, and stained the cells using anti-FPR-1 primary antibody and a secondary fluorescent-conjugated goat anti-rabbit_488 antibody, associated with DAPI to stain nuclei.

Immunofluorescence images evidenced stronger staining for FPR-1 in Gal-$1^{-/-}$ mice neutrophils than in Gal-$1^{+/+}$ mice neutrophils (Fig. 8A-D). The respective negative control groups are depicted in Fig. 8E-H. Fluorescence quantification confirmed the increased RFU of Gal-$1^{-/-}$ mice neutrophils when compared with the wild-type mice neutrophils (Fig. 8I, $p < 0.05$). Hence, the higher levels of FPR-1 receptor expression in Gal-$1^{-/-}$ mice neutrophils may be associated with the increased fMLP-dependent ROS generation and the stronger bacteria killing capacity of these mice reported in Section 3.4.

4. Discussion

Gal-1 is a multifunctional glycan-binding protein that modulates several immune cell functions, including biological properties of neutrophils such as migration towards inflammatory sites and ROS production (Almkvist et al., 2002; Arthur et al., 2015; Auvynet et al., 2013; Camby et al., 2006; Gil et al., 2011; La et al., 2003; Liu and Rabinovich, 2010). To date, the mechanisms by which Gal-1 controls neutrophil ROS production are not fully understood.

In the present study, we reported several unique aspects of Gal-1 functions: (i) Gal-1 interfered with ROS production in neutrophils repeatedly stimulated with fMLP; (ii) Gal-1 exerted paradoxical effects on neutrophil ROS production; iii) exogenous and endogenous Gal-1 modulated the cell surface expression of FRP1; (iv) endogenous Gal-1 restricted ROS generation and suppressed the bacterial killing capacity

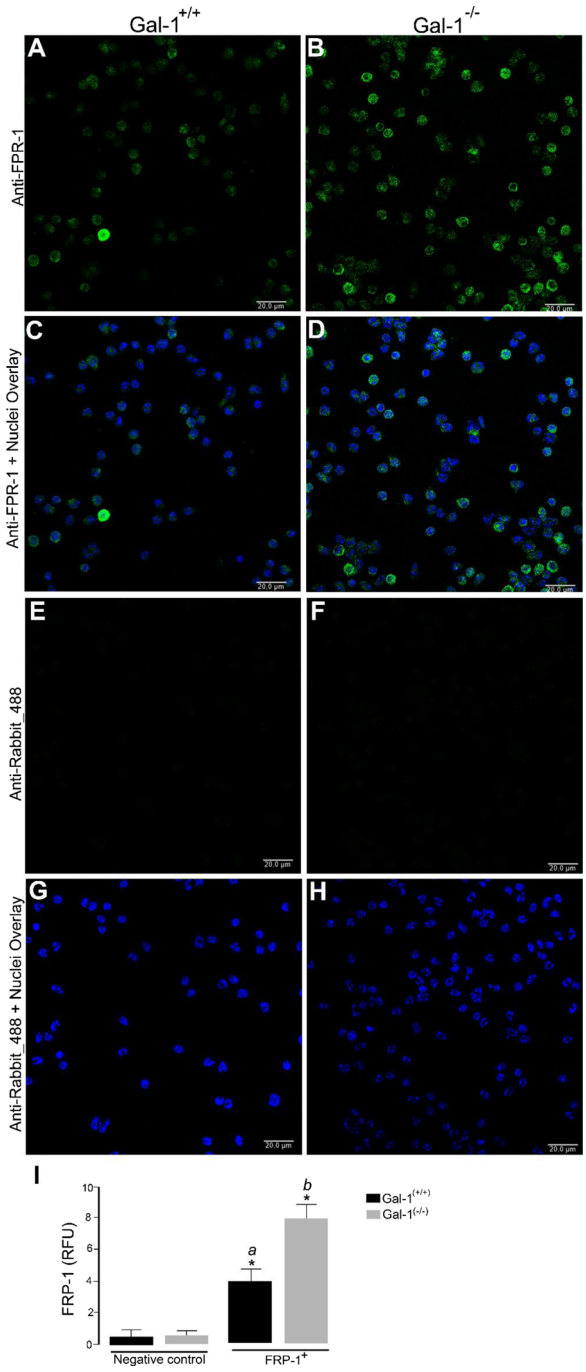

Fig. 8. Lack of endogenous Gal-1 improves FPR1 expression. Peritoneal neutrophils were colected from Gal-$1^{-/-}$ and Gal-$1^{+/+}$ mice treated with thyoglycollate i.p. for 6 h, plated onto glass coverlips, and immunostained with anti-FPR-1 primary antibody, fluorescent-conjugated goat anti-rabbit_488 secondady antibody, and DAPI (to stain nuclei). The confocal microscopy immunofluorescence images shown are representative of two indepent experiments with similar results (scale bar: 20 μm). **(A)** and **(C)**: overlay from Gal-$1^{+/+}$ mice neutrophils stained with anti-FPR-1 antibody alone or combined with DAPI, respectively. **(B)** and **(D)**: overlay from Gal-$1^{-/-}$ mice neutrophils stained with anti-FPR-1 antibody alone or combined with DAPI, respectively. **(E)** and **(G)**: Gal-$1^{+/+}$ mice neutrophils stained only with the secondary antibody alone or combined with DAPI, respectively. **(F)** and **(H)**: Gal-$1^{-/-}$ mice neutrophils stained only with the secondary antibody alone or combined with DAPI, respectively. **(I):** Quantification of RFU (Relative Fluorescence Unit) of od FPR-1 expression. $p < 0.05$, *a* versus *b*; $^{*}p < 0.05$ *vs.* negative control (ANOVA and Tukey *post-hoc* test).

of inflammatory neutrophils.

To date, there are no literature reports on the interference of Gal-1 on ROS production in neutrophils repeatedly stimulated with fMLP. The neutrophil ROS production capacity decreases after repeated exposure to fMLP and its effect can be associated with the fMLP receptor endocytosis and/or recycling (Panaro and Mitolo, 1999).

As we found that Gal-1 did not affect the dynamics of fMLP sensitivity in neutrophils previously activated with fMLP, when these cells were sequentially activated with fMLP, Gal-1, and fMLP, we suggest that the role that Gal-1 plays in the positive regulation of ROS production is not associated with fMLP receptor self-regulation and recycling in fMLP-activated neutrophils. In contrast to Gal-1, Gal-3 induces ROS generation and fMLP receptor desensitization in activated neutrophils by favoring fMLP oxidation by myeloperoxidase (Forsman et al., 2008). On the other hand, naive and fMLP-primed neutrophils pretreated with exogenous Gal-1 and further exposed to fMLP produced less ROS, as compared with naive neutrophils not treated with this lectin.

After analyzing the expression levels of FPR1 – a formylpeptide receptor that mediates neutrophil ROS production (Liu et al., 2012b; Tsai et al., 2019) –, in Gal-1-pretreated neutrophils, we demonstrated that the negative effect of this lectin on ROS production could be associated with reduction of FPR1 expression on the cell surface (Figs. 7 and 8). Furthermore, non-activated neutrophils from Gal-$1^{-/-}$ mice produce ROS more effectively when stimulated with fMLP. This phenomenon can be explained by the higher levels of FPR1 receptor expression in Gal-$1^{-/-}$ mice than in Gal-$1^{+/+}$ mice. Interestingly, Gal-1 also reduced ROS production in neutrophils exposed to PMA, which is known to activate neutrophil functions independently of fMLP receptor (DeCoursey and Ligeti, 2005; Huang et al., 2008).

Gal-1 effectively induced ROS production in neutrophils pretreated with fMLP (10^{-8} to 10^{-6}M) but not in naive and fMLP-primed neutrophils (10^{-9} M fMLP), corroborating the findings reported by (Almkvist et al., 2002). However, differently from such report, we demonstrated that Gal-1 decreased the fMLP-induced ROS production in naive and fMLP-primed neutrophils.

Galectin-3 (Gal-3) exerts a similar effect, and likewise requires neutrophil priming to effectively activate cell functions (Feuk-Lagerstedt et al., 1999). TNF-α-primed neutrophils exhibit increased expression of the Gal-3 receptors CD66a and CD66b and of the granule marker CD11b (Feuk-Lagerstedt et al., 1999), while cytochalasin B-treated neutrophils exhibit enhanced ROS production in the presence of Gal-3 (Yamaoka et al., 1995). The recombinant form of Gal-3 lacking the N-domain (Gal-3C) suppresses induction of ROS production by the full-length Gal-3. Possibly, Gal-3C binds to neutrophil surface and hinders full binding of Gal-3, which thereby downregulates ROS production in primed neutrophils. In this sense, it is possible that modulation of ROS production by Gal-3/Gal-3C and/or Gal-1 control inflammation and prevent tissue damage mediated by overproduction of oxidant species (Sundqvist et al., 2018).

The negative modulatory effect of Gal-1 on ROS production was partially reverted by TDG, a Gal-1 inhibitor. In cells treated with Gal-1 before stimulation with PMA or fMLP, inhibition of ROS production may result from the lectin ability to regulate (i) intracellular signaling pathways that mediate ROS generation, such as the NF-κB and RAS pathways (Suire et al., 2006; Toscano et al., 2011), and (ii) the distribution of signaling molecules in lipid rafts of the neutrophil membrane (Prior et al., 2003). In contrast, in neutrophils treated with fMLP before stimulation with Gal-1, the increased number of glycoproteins present in the membrane of gelatinase granules and secretory vesicles may explain the enhancement of ROS production through the lectin ligands (Almkvist et al., 2002; Elola et al., 2005).

Plant lectins that bind to carbohydrates similar to those recognized by Gal-1 can interact with the gp91phox component of the neutrophil NADPH oxidase and favor ROS generation (Gorudko et al., 2011). Both the positive and negative modulatory effects of Gal-1 on ROS

generation by activated, primed, and naive neutrophils were related to the lectin activity of Gal-1. Hence, the paradoxical effect of Gal-1 depends on the cell activation state and its carbohydrate recognition property, as described for other dual biological functions of this lectin (Camby et al., 2006). Based on our data and considering the association between cell activation level and differential expression of Gal-1 ligands on the neutrophil surface (Almkvist et al., 2002; Elola et al., 2005), we hypothesize that Gal-1 modulates, positively or negatively, the neutrophil ROS generation through the interaction with distinct carbohydrate ligands on this leukocyte surface.

The complex effects of Gal-1 on neutrophils at different stages of activation are related to the ability of galectins to modulate the immune response through different mechanisms, according to the site of action and composition of the surrounding milieu (Rubinstein et al., 2004). Calcium influx is one of the mechanisms that mediate the action of Gal-1 on activated and non-activated neutrophils (Stowell et al., 2007). Our results demonstrated that Gal-1 participated in the fine regulation of ROS production, disfavoring free radical-mediated tissue damage in the absence of infection, but favoring the elimination of infectious agents in the inflammatory site by optimizing ROS production by activated neutrophils.

In our study, peritoneal neutrophils from Gal-1$^{-/-}$ mice released more ROS *in vitro* in response to fMLP and exogenous Gal-1 than neutrophils from wild-type animals. Endothelial cells lacking Gal-1 favor neutrophil adhesion, rolling, and transmigration to the inflamed tissue (Cooper et al., 2012). Gal-1 also induces the release of myeloperoxidase, gelatinase, and other granule components that act in concert with ROS to kill the phagocytosed microbes (Almkvist et al., 2002; Mayer-Scholl et al., 2004).

Although the cecal bacterial load used to promote infection in wild-type and Gal-1$^{-/-}$ mice resulted in 80% survival rate in both groups (data not shown), peritoneal and blood neutrophils from Gal-1$^{-/-}$ infected mice exhibited stronger microbicidal activity, as demonstrated by the number of CFU recovered 6 h after bacterial injection. These results explain, at least in part, the participation of ROS in microbial killing and suggest that the lack of Gal-1 leads to increased ROS production, which is associated to less bacterial spreading and more effective control of infection. Another study has demonstrated that Gal-3 prevents sepsis mortality in a (CLP) sepsis model using a bacterial load 10 times greater than that used in the present experiment; the survival rate of the groups are clearly distinct: 100% and 40% of wild-typ and Gal-3 knockout mice survived, respectively (Ferreira et al., 2018). In addition, our findings indicate that the higher levels of FPR-1 receptor expression in Gal-1$^{-/-}$ mice neutrophils may be associated with the increased fMLP-dependent ROS generation and the stronger bacteria killing capacity of these mice. Similarly, the FPR1 deficiency has negative impacts on neutrophil ROS production and bactericidal activity (Liu et al., 2012b).

Gal-1$^{-/-}$ mice are more resistant to *Yersinia enterocolitica* infection than Gal-1$^{+/+}$ mice, probably due to the Gal-1-mediated control of NF-κB activation and TNF production. The selective iNOS inhibitor reverses the protective effect of lack of Gal-1 in Gal-1$^{-/-}$ mice and increases the *Y. enterocolitica* load. The lack of Gal-1 also results in increased production of IFN-γ and IL-17 (Davicino et al., 2017). Therefore, the type of interaction between Gal-1 and the infectious agent can determine their elimination or the establishment of infection in the host (Garner et al., 2010; Ouellet et al., 2005). Other galectins, such as Gal-4 and Gal-8, are known to kill bacteria that express carbohydrates similar to the antigens that characterize the human B type from ABO blood group system, through mechanisms that are independent of the participation of neutrophils and the complement system (Stowell et al., 2010).

The increased neutrophil infiltration and ROS production associated with the decreased bacterial load in peripheral blood indicate that the acute inflammatory response was effective and prevented the systemic infection in Gal-1$^{-/-}$ mice. Together, our results suggest that endogenous Gal-1 participates in the control of the infection severity in wild-type animals with moderate sepsis, and probably modulates the initial inflammatory process. Gal-1 regulates various events of the acute inflammatory response by inhibiting neutrophil migration and release of pro-inflammatory cytokines (Sundblad et al., 2017; Toscano et al., 2011). Gal-1 diminishes the arachidonic acid release and iNOS expression in LPS-stimulated macrophages (Correa et al., 2003), and can contribute to resolution of sepsis by acting as a damage-associated molecular pattern molecule and as a pathogen-associated molecular pattern receptor (Sato et al., 2009).

The fine regulation of ROS generation in neutrophils at different stages of activation may be one of the mechanisms underlying the anti-inflammatory and immune modulating properties of Gal-1. The production of adequate amounts of ROS is essential to maintain the cellular and tissue redox balance, which affects the expression of a variety of molecules that regulate the course of acute and chronic inflammatory responses and autoimmune inflammation (Hultqvist et al., 2009). Under some circumstances, oxidative burst-inducing compounds have been successfully employed to prevent and treat rheumatoid arthritis in rats (Hultqvist et al., 2006). Thus, the ROS-inducing ability of Gal-1 in activated neutrophils can be beneficial to treat autoimmune and inflammatory diseases (Liu and Rabinovich, 2010).

The treatment of zymosan-induced peritonitis in mice with Gal-1 lowers the expression levels of the adhesion molecules L-selectin and β2-integrin on the neutrophil surface, as well as lowers the levels of TNF-α and IL-1β in the peritoneal fluid of these animals (Gil et al., 2011). Gal-1 inhibits the release of arachidonic acid and prostaglandin E2 in the bee venom phospholipase A_2-induced paw edema model (Rabinovich et al., 2000), as well as negatively modulates neutrophil recruitment to the peritoneal cavity of rodents (Gil et al., 2006), degranulation of mastocytes (Rabinovich et al., 2000), and neutrophil chemotaxis induced by IL-1β, IL-8, and TNF-α (La et al., 2003).

In summary, Gal-1 can up- or down modulate the neutrophil ROS generation in response to fMLP. The prevalence of one or the other effect depends on the cell activation stage and the composition of the surrounding milieu. The carbohydrate-binding property of Gal-1 significantly contributes to its immune modulating action. Taken together, our results help to understand how Gal-1 participates in the homeostatic production of ROS by favoring their bactericidal effects in activated neutrophils, as well as by protecting from tissue damage caused by unnecessary ROS production by non-activated neutrophils. In addition to shedding light on the roles that Gal-1 plays in the course of the inflammatory response and resolution of infectious processes, these findings shall help to develop therapeutic strategies based on carbohydrate recognition to treat inflammatory and infectious diseases.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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