Glycobiology vol. 24 no. 11 pp. 1010–1021, 2014
doi:10.1093/glycob/cwu061
Advance Access publication on June 27, 2014

Galatrox is a C-type lectin in *Bothrops atrox* snake venom that selectively binds LacNAc-terminated glycans and can induce acute inflammation

Marco A Sartim[2], Thalita B Riul[2], Camillo Del Cistia-Andrade[2], Sean R Stowell[3], Connie M Arthur[3], Carlos A Sorgi[2], Lucia H Faccioli[2], Richard D Cummings[4], Marcelo Dias-Baruffi[1,2], and Suely V Sampaio[1,2]

[2]Department of Clinical Analyses, Toxicology and Food Sciences, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto 14040903 São Paulo, Brazil; [3]Department of Pathology and Laboratory Medicine, Center for Transfusion and Cellular Therapies, Emory University School of Medicine, Atlanta 30322, GA, USA; and [4]Department of Biochemistry and The Glycomics Center, Emory University School of Medicine, Atlanta 30322, GA, USA

Received on October 6, 2013; revised on June 14, 2014; accepted on June 21, 2014

Previous studies indicate that snake venom contains glycan-binding proteins (GBPs), although the binding specificity and biological activities of many of these GBPs is unclear. Here we report our studies on the glycan binding specificity and activities of galatrox, a *Bothrops atrox* snake venom-derived GBP. Glycan microarray analysis indicates that galatrox binds most strongly to glycans expressing *N*-acetyllactosamine (LacNAc), with a significant preference for Galβ1-4GlcNAcβ over Galβ1-3GlcNAcβ compounds. Galatrox also bound immobilized laminin, a LacNAc-dense extracellular matrix component, suggesting that this GBP can bind LacNAc-bearing glycoproteins. As several endogenous mammalian GBPs utilize a similar binding LacNAc binding preference to regulate neutrophil and monocyte activity, we hypothesized that galatrox may mediate *B. atrox* toxicity through regulation of leukocyte activity. Indeed, galatrox bound neutrophils and promoted leukocyte chemotaxis in a carbohydrate-dependent manner. Similarly, galatrox administration into the mouse peritoneal cavity induced significant neutrophil migration and the release of pro-inflammatory cytokines IL-1α and IL-6. Exposure of bone marrow-derived macrophages to galatrox induced generation of pro-inflammatory mediators IL-6, TNF-α, and keratinocyte-derived chemokine. This signaling by galatrox was mediated via its carbohydrate recognition domain by activation of the TLR4-mediated MyD88-dependent signaling pathway. These results indicate that galatrox has pro-inflammatory activity through its interaction with LacNAc-bearing glycans on neutrophils, macrophages and extracellular matrix proteins and induce the release of pro-inflammatory mediators.

Keywords: Bothrops atrox / C-type lectin / Inflammatory response / Neutrophil migration / Snake venom

Introduction

Inflammation is critical in the host response to tissue damage and infection, helping the immune system to preserve homeostasis and leading to complex physiological mechanisms characterized by the involvement of vascular and cellular events (Medzhitov and Horng 2009; Soehnlein and Lindbom 2010). Deficiencies in inflammatory responses can increase susceptibility to infectious disease and limit normal wound repair (Netea et al. 2012; White and Mantovani 2013). In contrast, exuberant inflammatory responses are associated with physiopathology of several diseases, leading to tissue damage and organ injury (Buckley et al. 2013; Huang et al. 2013; Strydom and Rankin 2013). Given the critical balance of inflammation in facilitating host immunity while avoiding injury, many factors appear to have evolved unique roles in the regulation of both these mechanisms (Ariel and Timor 2013; Frey et al. 2013; Lu et al. 2013; Rogala and Glück 2013). Among these factors, glycan-binding proteins (GBPs) play a critical role in inflammation by regulating a wide variety of cells involved in the inflammatory process (Dias-Baruffi et al. 1995a,b; Rabinovich et al. 2000; Nishi et al. 2003; Nieminen et al. 2005; Stowell, Qian, et al. 2008; Sato et al. 2009).

While disruptions to normal host homeostasis can significantly alter the inflammatory balance, environmental factors also appear capable of compromising normal homeostatic processes (Ramos and Fernandez-Sesma 2012; Aziz et al. 2013). Several biological active molecules from *Bothrops* spp. snake venoms are responsible for stimulating an intense inflammation during envenomation, triggering immunological events, such as prominent leukocyte infiltration, and promoting local and systemic disorders (Bonavita et al. 2006; Carneiro et al. 2008; Teixeira et al. 2009). As an example of these factors within venom, the C-type β-galactoside-binding lectins have been reported to be involved in inflammatory response (Lomonte et al. 1990; Havt et al. 2005; Panunto et al. 2006; Elifio-Esposito et al.

[1]To whom correspondence should be addressed: Tel: +55-16-36024286; Fax: +55-16-3602-4719; e-mail: suvilela@usp.br (S V S); Tel: +55-16-36024256; Fax: +55-16-3602-4725; e-mail: mdbaruff@fcfrp.usp.br (M D-B)

2011). Given the role of endogenous β-galactoside-binding lectins, such as galectins, within mammals in the regulation of inflammation and immunity (Stowell, Qian, et al. 2008; Sato et al. 2009), these results suggest that snake venom C-type lectins may involve related glycan ligands and also induce changes related to those previously shown for endogenous GBPs.

Galatrox, a C-type lectin isolated from *Bothrops atrox* snake venom, displays significant lactose-binding properties and appears to recognize mammalian glycans (Mendonça-Franqueiro et al. 2011), suggesting that galatrox may engage leukocyte ligands and induce significant inflammatory changes. However, the glycan-binding specificity of galatrox remains undefined. Furthermore, whether galatrox recognizes leukocyte glycan ligands or induces alterations in leukcoyte function is not known. In this study, we examined the glycan-binding specificity and proinflammatory activity of galatrox. Our results indicate that galatrox displays similarity in glycan-binding characteristics to endogenous mammalian GBPs and induces significant changes in leukocyte migration and activation that are likely to contribute to host inflammation following *B. atrox* envenomation.

Results

Galatrox preferentially binds to glycans terminating in N*-acetyllactosamine*

As toxins often utilize host ligands to mediate their activity, we hypothesized that galatrox may induce inflammation through engagement of ligands typically employed by endogenous GBPs. Among mammalian GBPs with β-galactoside-binding activity, several studies demonstrate that members of the galectin family possess remarkable ability to regulate immunity, including the regulation of leukocyte activity (Dias-Baruffi et al. 2003; Nieminen et al. 2005; Garín et al. 2007; Toscano et al. 2007; Stowell, Qian, et al. 2008). However, whether galatrox and galectin family members share similarities in carbohydrate-binding activity remains unknown. To examine this in detail, we turned to the consortium for functional glycomic glycan microarray, which has been previous used to characterize the binding specificity of multiple GBPs. To accomplish this, we directly labeled galatrox with fluorescein isothiocyanate (FITC) and then examined glycan-binding activity on the glycan microarray (Version 3.2) (http://www.functionalglycomics.org/). Of all the carbohydrates represented on the glycan microarray, FITC-galatrox displayed the highest affinity toward glycans expressing non-reducing *N*-acetyllactosamine (LacNAc) sequences, with a significant preference for type 2 (Galβ1-4GlcNAcβ) over type 1 (Galβ1-3GlcNAcβ) LacNAc glycans (Figure 1 and Table I). In addition, although FITC-galatrox appeared to display preference for LacNAc presented on extended glycan structures, it did not bind to LacNAc repeats within glycans, as no increases in binding to polyLacNAc could be detected, and internal modifications to LacNAc failed to alter polyLacNAc binding (Supplementary data, Table S1), similar to previous results with several members of the galectin family (Stowell et al. 2004; Stowell, Arthur, Mehta, et al. 2008). Furthermore, FITC-galatrox failed to bind sialylated LacNAc glycans (in both α2-3 and/or α2-6 linkages) (Figure 1 and Table I), strongly suggesting that sialylation inhibits galatrox LacNAc recognition (Supplementary data, Table S1), similar to previous results on galectin-2 (Stowell, Arthur, Mehta, et al. 2008). Similar inhibition of LacNAc recognition appears to occur if the glycans are α1-2 fucosylated. This weak binding is also observed for either GalNAcα1-3(Fucα1-2)LacNAc (Supplementary data, Table S1) or Galα1-3(Fucα1-2)-LacNAc (Supplementary data, Table S1) glycans, as present in blood group A and B antigens, respectively. In addition, LacNAc-containing glycans expressing Lewis^x, Lewis^a and sialyl-f^x antigens [Galβ1-4(Fucα1-3)GlcNAc-R, Galβ1-3 (Fucα1-4)GlcNAc-R and NeuAcα2-3Galβ1-4(Fucα1-3) GlcNAc-R, respectively] also displayed weak binding to galatrox, as well as LacNAc glycans modified to express 3-O, 4-O or 6-O sulfation (Supplementary data, Table S1), with exception ([6OSO3]GlcNAcb1-3Gal b1-4GlcNAc-b-Sp0) which appeared to be one of the highest bound glycans on the array (Figure 1; Table I). The binding specific displayed by galatrox on the glycan microarray is very similar to the binding specificities of exhibited by many members of the galectin family, which also tend to display a higher preference of extended non-sialylated terminal LacNAc glycans, LacNAc or lactose in the context of blood group antigens and sulfated LacNAc or lactose containing glycans. Given the similarities in binding specificity, these results strongly suggest that galatrox may possess the ability to bind similar cell mammalian glycans as engaged by galectins and therefore directly impact the behavior of cells involved in inflammation.

Galatrox binds to laminin "in vitro"

Although the glycan microarray provides an unprecedented platform to elucidate the binding specificity of GBPs toward defined glycans, we next sought to determine whether galatrox might display high binding toward a previously characterized mammalian glycoprotein ligand. While previous studies suggest that galectins likely mediate leukocyte regulation through engagement of cell surface LacNAc-containing glycans, they appear to do so through formation of complex galectin-mediated lattice formation that may result in the recruitment of many different glycoproteins (Brewer et al. 2002; Belardi et al. 2012) In contrast, early studies demonstrated that more defined glycoproteins, such as laminin, may also serve as glycorprotein ligands for galectins (van den Brûle *et al*. 2003; Camby et al. 2006; Friedrichs et al. 2007). As a result, we next sought to determine whether galatrox might also display high affinity for laminin, which exhibits a large number of *N*-glycans composed of poly-LacNAc$_n$ residues containing β-Gal and α-Gal terminal residues, and forms the major component of the extracellular matrix (ECM; Fujiwara et al. 1988; Jin et al. 1995). FITC-galatrox displayed significant binding to murine laminin in a concentration-dependent manner (Figure 2). Alternatively, we have assessed the capacity of galatrox to interact to other ECM component that lacks of terminal LacNAc motif such as fibronectin. Human plasma fibronectin is a glycoprotein that contains α(2,6)-linked sialic acid at the terminal galactose residues on all seven oligosaccharides (N- and O-linked) and only two have fucosylated glycans (Katnik-Prastowska et al. 2006; Kątnik-Prastowska and Orczyk-Pawiłowicz 2011). Interestingly, FITC-galatrox did not bind to fibronectin, presenting similar binding pattern as bovine serum albumin (BSA; Figure 2). These results suggest that galatrox interaction to laminin is specific and mediated by recognition of LacNAc containing glycans on this component of the ECM.

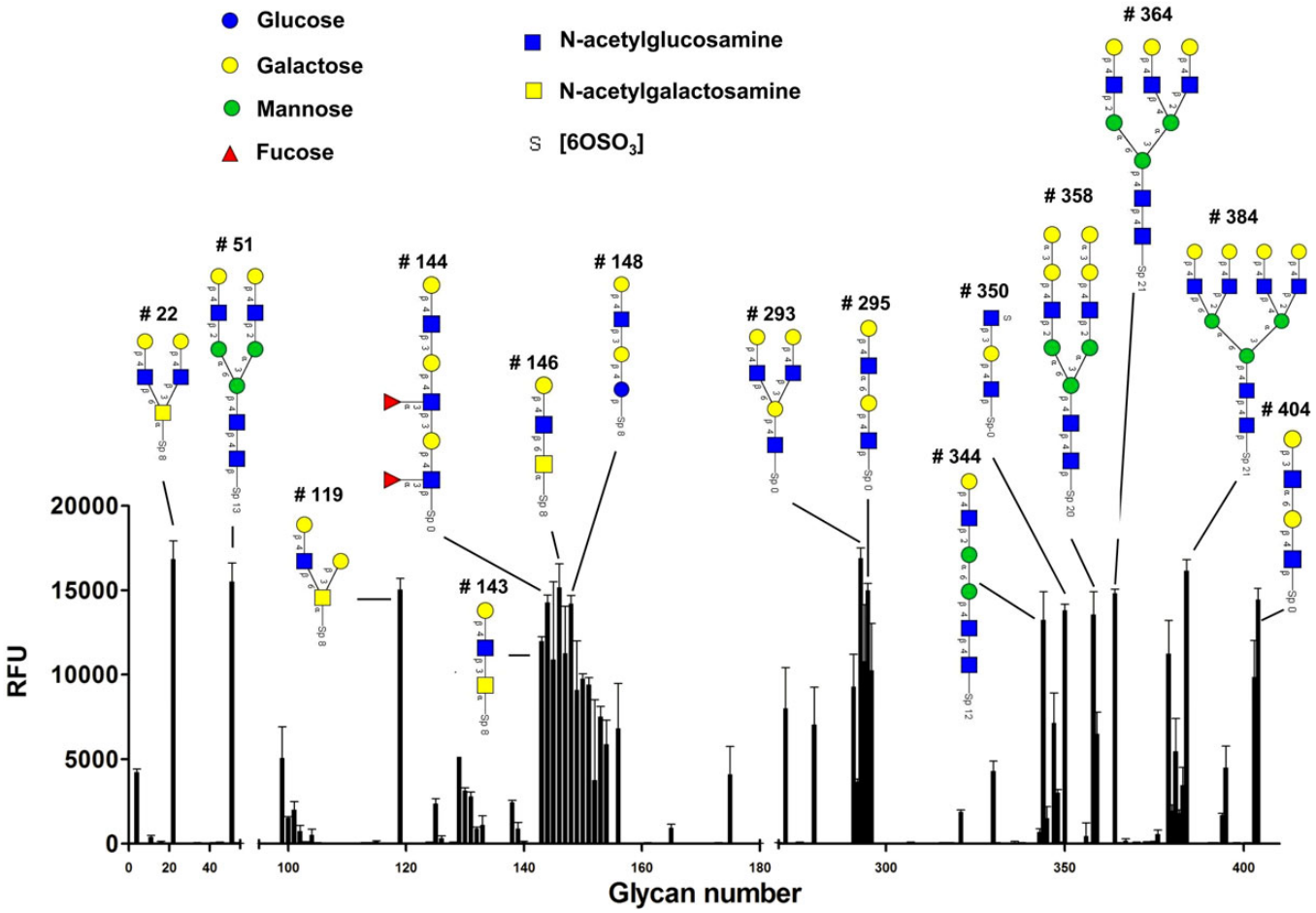

Fig. 1. Galatrox glycan microarray analysis. FITC-galatrox, at concentration of 0.1 μg/mL, was assessed for glycan recognition pattern using mammalian printed array version 3.2 which consists of 406 glycans in replicates of 6. The results are expressed by the mean RFU and SEM and top 15 ranked glycan structures are illustrated with its respective glycan number.

Table I. Top 15 rank of galatrox glycan array recognition

Glycan no.	Glycan structure	Rank[a]
293	Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAc-Sp0	100
22	Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα-Sp8	99.7
384	Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-4)Manα1-3(Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-6)Manα1-6) Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp21	95.6
51	Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp13	91.8
146	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ-Sp0	89.7
119	Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα-Sp8	89.0
295	Galβ1-4GlcNAcα1-6Galβ1-4GlcNAcβ-Sp0	88.7
364	Galβ1-4GlcNAcβ1-2(Galβ1-4GlcNAcβ1-4)Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6) Manβ1-4GlcNAcβ1-4GlcNAc-Sp21	87.7
404	Galβ1-3GlcNAcα1-6Galβ1-4GlcNAcβ-Sp0	85.5
144	Galβ1-4GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ-Sp0	84.5
148	Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Sp8	84.1
350	[6-OSO_3]GlcNAcβ1-3Galβ1-4GlcNAcβ-Sp0	81.7
358	Galα1-3Galβ1-4GlcNAcβ1-2Manα1-3(Galα1-3Galβ1-4GlcNAcβ1-2Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ-Sp20	80.2
344	Galβ1-4GlcNAcβ1-2Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-Sp12	78.4
143	Galβ1-4GlcNAcβ1-3GalNAcα-Sp8	70.9

[a]The rank values were obtained with the following calculation: rank = 100 × (RFU bound/highest RFU) (Heimburg-Molinaro et al. 2011).

Galatrox interacts with human neutrophil surfaces in a glycan-dependent manner and induces neutrophil chemotaxis

Given the remarkable similarities in carbohydrate-binding specificity between galatrox and previously studied members of the galectin family, we next sought to determine whether galatrox might similarly engage leukocytes (Allavena et al. 2004; Camby et al. 2006; Fulcher et al. 2006). We evaluated the ability of galatrox to bind to human neutrophils and examined the involvement of the carbohydrate-recognition domain (CRD) in this interaction, as neutrophils represent a primary cellular mediator of inflammation. Similar to laminin binding, FITC-galatrox displayed significant binding to neutrophils over a range of concentrations (Figure 3A and B). Interestingly, preincubation of FITC-galatrox with lactose (20 mM) inhibited binding, while preincubation with sucrose (20 mM), a hapten sugar which is not recognized by galatrox, failed to alter neutrophil recognition, strongly suggesting that galatrox recognizes neutrophils through a glycan-dependent interaction (Figure 3A and B). The ability of mammalian GBPs to bind ECM and cell surface glycans places them in a unique position to regulate cellular migration and trafficking. As galectins represent the only known soluble mammalian GBPs with LacNAc-binding activity, we next sought to determine whether incubation of neutrophils with galatrox may induce similar alterations in neutrophil function as previously observed following galectin engagement. To accomplish, we first examined the potential impact of galatrox on neutrophil chemotaxis, as several previous studies suggested that galectins might regulate this key feature of neutrophil function (Dias-Baruffi et al. 1995a; de Toledo et al. 2007; Elifio-Esposito et al. 2011; Auvynet et al. 2013). To determine whether galatrox promotes neutrophil migration, we examined potential galatrox-induced chemotaxis through polycarbonate membrane pores using a 48-well microchamber. Galatrox induced significant migration of human neutrophils, with the most significant alterations in leukocyte trafficking occurring following the addition of 32 μg/mL galatrox. Similarly to neutrophil recognition (Figure 3A and B), preincubation of cells with lactose (20 mM) reduced their migration to similar migration levels as observed in the negative control (NC; Figure 3C). However, preincubation with sucrose (20 mM) failed to alter galatrox-induced neutrophil migration (Figure 3C).

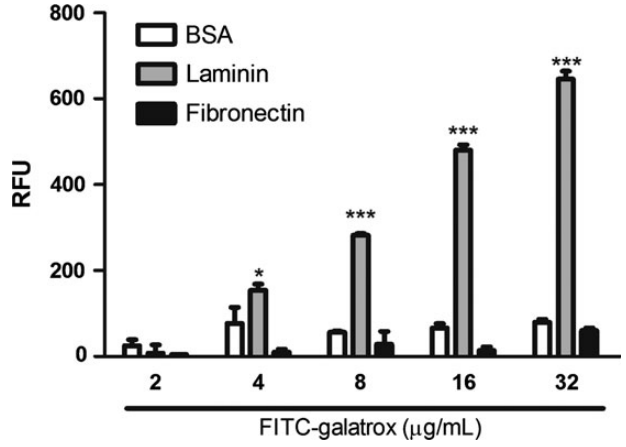

Fig. 2. Analysis of galatrox binding to ECM components. Polystyrene microplate wells were coated with ECM components laminin and fibronectin, and BSA as random binding ($n = 3$ for each component and BSA). After each well was incubated for 1 h with several concentrations of FITC-galatrox (2–32 μg/mL). The analysis was performed by fluorescence reading using a Power Wave X reader on an emission wavelength of 585/20 nm. The results obtained were expressed as mean of RFU ± SEM and are representative of two independent experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with BSA binding.

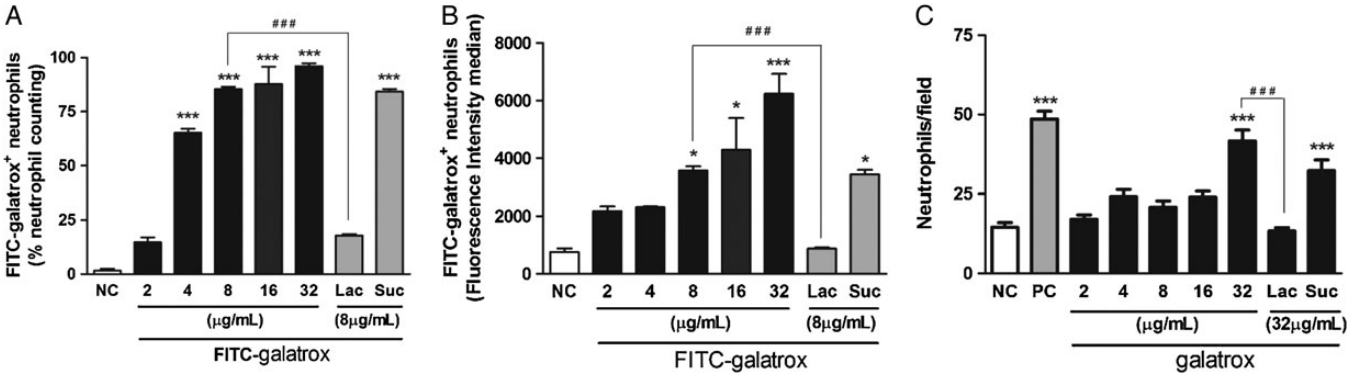

Fig. 3. Galatrox human neutrophil surface interaction and chemotaxis. Human neutrophils (1×10^6 cells) were incubated with different concentrations of galatrox-FITC (2–32 μg/mL) for 30 min at 4°C. The CRD involvement was assessed by previous incubation of FITC-galatrox samples (8 μg/mL) with α-lactose or α-sucrose (20 mM). Cells treated with HBSS only were used as NC. After incubation, the suspension was washed with PBS and cells analyzed by flow cytometry. The treatment was performed in triplicate for each condition and the results were reported as (**A**) percentage ± SEM and (**B**) fluorescence intensity median ± SEM of galatrox-FITC^{+} neutrophil population from two independent experiments. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with NC and $^{\#\#\#}P < 0.001$ when compared with galatrox 8 μg/mL preincubated with α-lactose 20 mM. (**C**) Human neutrophil chemotactic activity in vitro was performed using a 48-well Boyden microchamber with galatrox concentration ranging from 2 to 32 μg/mL. Also, galatrox (32 μg/mL), preincubated with 20 mM of α-lactose (Lac) or α-sucrose (Suc), was assessed for CRD involvement. NC, represented by the random migration, and fMLP (10^{-7} M) were used as the reference chemoattractant for PC. The cells that migrated from the upper chamber to the lower through the membrane were counted and recorded as the mean ± SEM of total neutrophil per field (**C**). The assay was performed in triplicate for each condition, in two independent experiments. $^{***}P < 0.001$ compared with NC. $^{\#\#\#}P < 0.001$ when comparing Galatrox 32 μg/mL which was pre-incubated or not with α-lactose 20 mM.

Galatrox induces in vivo neutrophil migration and the release of inflammatory cytokines

Since galatrox induced neutrophil migration in vitro and is re leased into tissues following envenomation, we explored whether galatrox could induce neutrophil migration in vivo. Injection of galatrox induced significant leukocyte migration into the peritoneal cavity 4 h postadministration in a concentration-dependent manner with neutrophils representing the dominant leukocyte population (Figure 4A and B). In contrast, mononuclear cell migration profile did not change during all treatments, with recruitment values remaining statistically equivalent to the NC (Figure 4A). A progressive reduction of cell infiltrate was observed following 12 h. Significant levels of leukocytes/neutrophils continued to be observed up to 12 h following administration, following which a decline in neutrophil numbers occurred, eventually reaching similar values as the NC from 24 up to 72 h following injection (Figure 4B).

In addition to examining the cellular infiltrate following galatrox injection, we also determine whether alterations in cytokines levels might accompany leukocyte recruitment. Galatrox injection induced significant levels of interleukin (IL)-1α and IL-6 in a concentration- and time-dependent manner (Figure 4C and D). In contrast, no different in the levels of tumor necrosis factor (TNF)-α and IL-10 were observed in comparison with NC (data not shown). Taken together, these results suggest that galatrox not only possesses the capacity to engage similar ligands as endogenous mammalian galectin GBPs but also appears to induce similar changes in neutrophil function an overall cytokine secretion of proinflammatory cytokines. Thus, galatrox may in part mediate the proinflammatory activity of *B. atrox* envenomation by engaging similar pathways normally utilized by endogenous GBPs (Escocard Rde et al. 2006; Stowell, Qian, et al. 2008; Moreira et al. 2012).

Galatrox binds to glycoconjugates on BMDM surface and induces proinflammatory cytokine production mediated by Toll-like receptor 4

In addition to neutrophils, macrophages can significantly impact inflammatory changes following injury or infection and are likewise mediators of the proinflammatory activity of many galectin

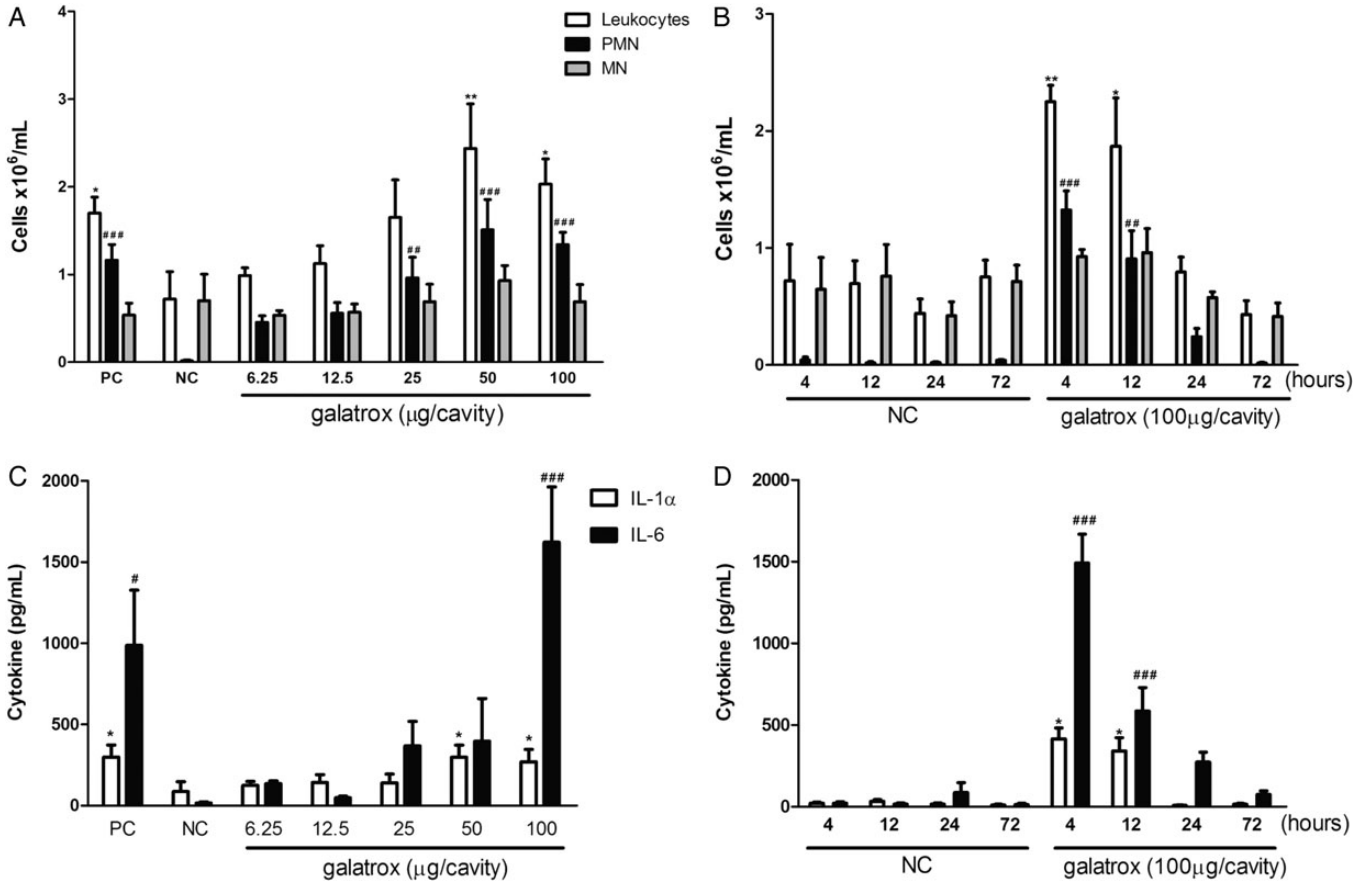

Fig. 4. Mice peritoneal leukocyte migration. In vivo leukocyte migration was performed by administration of galatrox (6.25–100 μg/cavity) into male BALB/c mice peritoneum, and the cavity washed after 4 h administration. NC animals received PBS alone, and PC group carrageenan (500 μg/cavity). For time-course evaluation, mice were treated with galatrox (100 μg/cavity) and leukocyte count was evaluated 4–72 h after administration. The peritoneal wash from dose (**A**) and the time-course (**B**) assay were assessed for total and differential cell counting. The results were expressed by the mean ± SEM of leukocyte, polymorphonuclear neutrophils (PMN) and mononuclear cell (MN) concentration (cells/mL). Peritoneal wash cytokine levels from dose (**C**) and time-course (**D**) in vivo leukocyte migration were evaluated by ELISA. Each treatment group consisted of five animals ($n = 5$) in two independent experiments. In cell counting (**A** and **B**), $^{*}P < 0.05$ and $^{**}P < 0.01$ when total leukocytes groups compared with respective control. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ when PMN groups compared with respective control. For cytokine level detection (C and D), $^{*}P < 0.05$ for IL-1α compared with respective control, $^{\#}P < 0.05$, and $^{\#\#\#}P < 0.001$ for IL-6 compared with respective control.

family members (Garcia-Ramallo et al. 2002; Gordon and Taylor 2005). As a result, we next determine whether galatrox may similarly engage macrophages cell surface carbohydrate receptors and induce alterations in cytokine secretion. To accomplish this, we prepared a differentiated bone marrow-derived macrophage (BMDM) cell culture suspension that was composed of >90% of $CD11b^{+}F4/80^{+}$ macrophage population (data not shown). As observed for neutrophil surface binding (Figure 2), FITC-galatrox was capable to interact to BMDM surface in a dose-dependent manner (Figure 5A and B). Preincubation of FITC-galatrox with lactose (20 mM) drastically inhibited binding, but not with sucrose (20 mM), suggesting that galatrox recognition of glycoconjugate(s) on BMDM surface (Figure 5A and B), similar to previous studies examining galectin function.

Cell surface glycosylation can impact the function of many cell surface receptors. For example, the activity of several cell surface receptors, such as Toll-like receptors (TLRs) family members and Dectin-1 receptor, can be modulated following engagement by members of the galectin family and others lectins to induce proinflammatory cytokine secretion (da Silva Correia and Ulevitch 2002; Amith et al. 2010; Esteban et al. 2011; Galván-Moroyoqui et al. 2011). In order to identify possible galatrox glycan targets on the macrophages surface, we pre-incubated BMDM with galatrox to determine whether galatrox possessed the capacity to inhibit interactions with anti-TLR-4, anti-TLR-2 and anti-Dectin1 (Figure 5C and D). Preincubation with galatrox significantly inhibited anti-TLR-4, anti-TLR-2 and anti-Dectin1 binding, strongly suggesting that

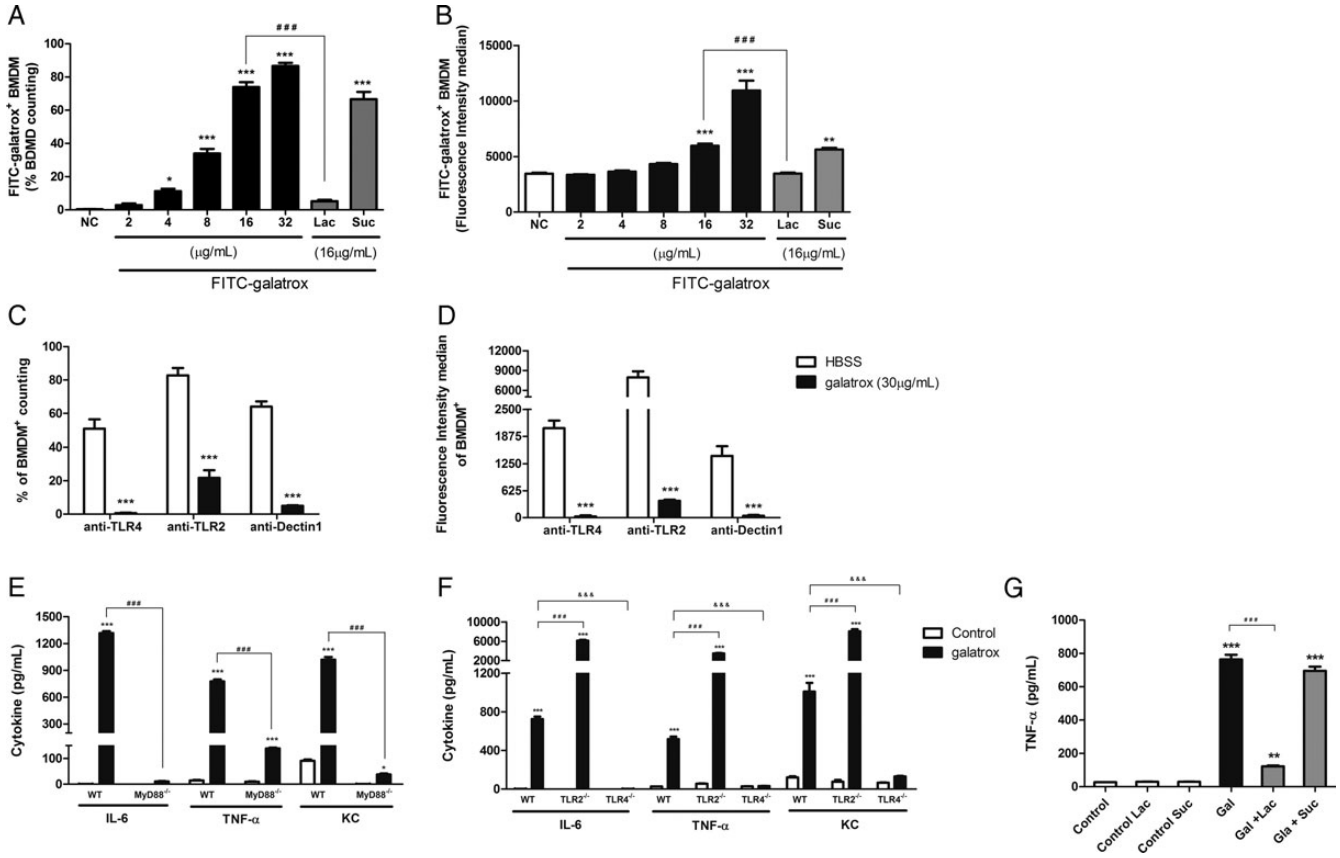

Fig. 5. BMDM-galatrox binding and cell culture inflammatory cytokine release. BMDM (1×10^6 cells/mL) was incubated with different concentrations of FITC-galatrox (2–32 µg/mL) in the presence of α-lactose or α-sucrose (galatrox 16 µg/mL + 20 mM of respective sugar). Cells treated with HBSS only were used as NC (**A** and **B**). BMDM treated previously with non-conjugated galatrox (30 µg/mL) or HBSS only was incubated with conjugated anti-TLR-4 (PE), anti-TLR-2 (PE-Cy7) and anti-Dectin 1 (Alexa Fluor 647) (**C** and **D**). After incubation, the suspensions were washed with PBS and cells analyzed by flow cytometry. The results were reported as percentage ± SEM of $BMDM^{+}$ (**A** and **C**) and fluorescence intensity median ± SEM $BMDM^{+}$ (**B** and **D**) from two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control and $^{\#\#\#}P < 0.001$ when compared with galatrox 16 µg/mL preincubated with α-lactose 20 mM. Cytokines KC, IL-6 and TNF-α concentrations were determined from BMDM cell suspension isolated from wt, *tlr4*$^{-/-}$, *tlr2*$^{-/-}$ and *myd88*$^{-/-}$ animals and treated with galatrox 30 µg/mL for 24 h. Control represents cells treated with medium only. CBA system kit assay was used to detect IL-6 and TNF-α and ELISA for detection of KC production in the supernatants. Alternatively, BMDM cells isolated from wt mice were treated galatrox (30 µg/mL) preincubated or not with α-lactose (20 mM) or sucrose (20 mM), for 24 h. Culture medium supplemented or not with carbohydrates were used as controls. Cell supernatants were used to determine the TNF-α level by ELISA (**G**). The results were reported as mean ± SEM of cytokine concentration (pg/mL) of BDMD supernatant treated with galatrox ($n = 7$). $^{***}P < 0.001$ when compared with Control group and $^{\#\#\#}P < 0.001$ or $^{\&\&\&}P < 0.001$ when comparing wt BMDM against knockout groups and $^{***}P < 0.001$ when compared Galatrox groups with Control groups and $^{\#\#\#}P < 0.001$ when compared Galatrox sample with Galatrox sample plus α-lactose.

galatrox may interact with BMDM cell surface TLR-4, TLR-2 and Dectin-1 ligands.

Although the ability of galatrox to inhibit anti-TLR-4 and anti-TLR-2 binding suggests that TLRs may serve as functional ligands, we next sought to determine whether galatrox-induced proinflammatory activity indeed requires TLR signaling. While galatrox induced significant IL-6-, TNF-α- and keratinocyte-derived chemokine (KC) in wt BMDM (Figure 5E), similar incubation with BMDM from MyD88$^{-/-}$ deficient mice, which are insensitive to TLR-2 and TLR-4 signaling (Sorgi et al. 2012; Shin et al. 2008), failed to result in similar increases in these same cytokines, strongly suggesting that galatrox mediates its proinflammatory activity through a TLR MyD88-dependent pathway. Although Dectin-1 may serve as a potential ligand for galatrox, the significant inhibition observed following galatrox incubation with MyD88$^{-/-}$ BMDM suggests that galatrox signals through a Dectin-1-independent pathway, as Dectin-1 signaling through a MyD88-independent Syk tyrosine kinase-dependent pathway (Saijo et al. 2007; Leal et al. 2010). As a result, we next sought to determine whether galatrox may preferentially signal through TLR-2 and TLR-4. Importantly, TLR-4$^{-/-}$ BMDM displayed significantly reduced sensitivity to galatrox-induced cytokine secretion than wt BMDM (Figure 5F). In contrast, galatrox induced increased cytokine secretion from TLR-2$^{-/-}$ macrophages when compared with the same wt BMDM (Figure 5F), similar to other TLR-4 agonists (Sorgi et al. 2012). Interestingly, the capacity of galatrox to promote the release of inflammatory cytokines from macrophages, such as TNF-α, is dependent on their ability to recognize glycans on the surface of this phagocyte, since its proinflammatory activity was dramatically inhibited by lactose (Figure 5G). Interestingly, Park et al. (2010) described a lectin named Korean mistletoe lectin-C that interacts with TLR-4 and promotes macrophage activation as the same as demonstrated to galatrox (Figure 5). These results resolutely suggest that TLR-4 likely plays the primary role in mediating galatrox-induced cytokine secretion and support a major participation of TLR-4 as a mediator of galatrox macrophage stimulation by a carbohydrate-dependent mechanism.

Discussion

Snake venom and other toxins often utilize endogenous cellular receptor systems and signaling pathways to exert their effects. While early studies suggested that several key mediators of snake venom might possess carbohydrate-binding activity, the fine specificity of venom GBPs remained unknown. As elucidation of carbohydrate-binding activity can often provide key insight into potential ligands and function, we utilized recently developed glycan microarrays to evaluate galatrox-binding specificity (Coombs et al. 2006; Stowell, Arthur, Mehta, et al. 2008; Heimburg-Molinaro et al. 2011). The results obtained following glycan microarray analysis provided critical insight into galatrox function and suggest that galatrox may engage similar ligands as endogenous β-galactoside GBPs to exert a proinflammatory response (Stowell, Arthur, Slanina, et al. 2008; Stowell et al. 2009, 2010, 2014; Cerliani et al. 2011).

The results obtained from glycan microarray analysis show that galatrox displays similar glycan interaction characteristics to others β-galactoside-binding snake lectins, such as BJcuL, from *Bothrops jararacussu* snake venom (Elifio-Esposito et al. 2011) and RSVL from *Crotalus atrox* snake venom (Young et al. 2011) and mammalian lectins such as galectins (Karmakar et al. 2008; Stowell, Arthur, Mehta, et al. 2008; Stowell et al. 2009), SRCL (human scavenger receptor C-type lectin), rat Kupffer cell receptor, rat macrophage galactose lectin and rat hepatocytes asialoglycoprotein receptor lectin (Coombs et al. 2006). The assay also shows that galatrox does not bind to glycans with non-reducing terminal LacNAc modified by sialic acid residues, strongly suggesting that sialylated biological receptors resist galatrox–LacNAc interaction. Similar to galatrox, RSVL interaction to LacNAc glycans is also inhibited by sialylation (Young et al. 2011), as well as the mammalian galectin, galectin-2 (Stowell, Arthur, Mehta, et al. 2008). While other mammalian and snake β-galactoside-binding proteins appear to bind LacNAc following blood group or sialylic acid modification (Leppänen et al. 2005; Coombs et al. 2006; Toscano et al. 2007; Stowell, Arthur, Mehta, et al. 2008; Stowell et al. 2009; Young et al. 2011), galatrox binding appeared to be more sensitive to various LacNAc modifications. These results suggest that while galatrox may possess some glycan cell surface receptor overlap with other snake and mammalian β-galactoside containing ligands, galatrox appears to possess a more restricted LacNAc profile and therefore may only engage a subset of the ligands bound by similar GBPs.

The preference for terminal LacNAc glycans by galatrox is most similar to mammal galectin-1 (Stowell, Arthur, Mehta, et al. 2008), the first described galectin family member. Early studies suggested that galectin-1 displays significant affinity laminin, a basement membrane ECM glycoprotein expressing high amounts of LacNAc-containing glycans and also known to participate as a substrate chemoattractant interaction involved in leukocyte migration (Jin et al. 1995; Lauffenburger and Horwitz 1996; Sánchez-Madrid and del Pozo 1999; Camby et al. 2006). Similar to previous studies, galatrox also bound laminin, resolutely suggesting that glycan microarray results accurately predict potential interactions with LacNAc bearing glycoproteins and suggested that galatrox may share similar ligands as members of the galectin family. In contrast, galatrox failed to recognize fibronectin, a heavily sialylated glycoprotein that can also fail to serve as a suitable ligand for galectin family members (Carsons et al. 1987). Given the role of ECM components in galectin-mediated inflammatory regulation (Camby et al. 2006; Elifio-Esposito et al. 2011), galatrox may mediate some of its proinflammatory activities through similar interactions, where GBP gradients cannot only facilitate activation, but play a significant role in leukocyte chemotaxis (Hauzenberger et al. 1995; Lauffenburger and Horwitz 1996; Sánchez-Madrid and del Pozo 1999). Taken together, these results demonstrated that the glycan microarray can accurately predict interactions with intact glycoproteins and that glycan microarray results of toxin may predict potential ligands shared by endogenous mammalian GBPs.

Given the similarities between galatrox- and galectin-binding specificity, we determined whether galatrox may similar engage human neutrophils, as previous studies demonstrated that galectins can induce neutrophil activation. Our results demonstrate that galatrox can induce significant neutrophil activation, similar to mammalian lectins, galectin-3 (Nieminen et al. 2005; Farnworth et al. 2008), galectin-1 (Almkvist et al. 2002;

Dias-Baruffi et al. 2003) and galectin-8 (Nishi et al. 2003; Stowell, Arthur, Slanina, et al. 2008). Although the glycosylation patterns of leukocyte glycoproteins have been studied (Wenzel-Seifert and Seifert 2003; Wang et al. 2004; Sun et al. 2006; Babu et al. 2009), the carbohydrate compositions of most individual neutrophil receptor glycans have not fully been characterized. Indeed, the actual receptors for galectin-mediated neutrophil activation remain to be elucidated despite early studies describing this activity nearly 20 years ago. Although a more clear understanding of the glycosylation pattern of leukocyte receptors will be necessary to better define the biological role of GBPs in neutrophil-mediated immune responses, galatrox may induce lectin lattice formation of multiple counter receptors to signal a response, as has been recently reported for several members of the galectin family (Brewer et al. 2002; Boscher et al. 2011; Earl et al. 2011)

During innate immunity, the role of tissue resident macrophages is pivotal for initiation and resolution of inflammatory response (Davies et al. 2013). Therefore, to better understand the involvement of macrophages on galatrox proinflammatory behavior, we assessed the ability of galatrox to stimulate macrophages. BMDM-binding assays suggested that galatrox may interact with proinflammatory cell surface glycoproteins TLR-2, TLR-4 and Dectin-1. Moreover, galatrox stimulation of macrophages, observed by the expression of IL-6, TNF-α and KC, appears to be predominantly mediated by TLR-4 activation through a MyD88 cell signaling pathway. Recent results suggest that other snake GBPs may signal through similar pathways (Moreira et al. 2013). Protazoan β-galactoside-binding GBPs also appear to engage a similar pathway, suggesting that other exogneous proteins may engage TLR-4 through their CRDs (Galván-Moroyoqui et al. 2011). da Silva Correia and Ulevitch (2002) showed that glycosylation of TLR-4 and MD2, a TLR-4 adapter protein, is necessary for cross-linking and the functional integrity of this receptor. Taken together, our results suggest that galatrox likely interacts with LacNAc-containing glycans on TLR-4 to engage MyD88-dependent proinflammatory cytokine secretion by macrophages and subsequent neutrophil recruitment. As a result, galatrox may belong to the venom-associated molecular pattern, a description of venom-associated molecules that possess the capacity to engage and activate pattern recognition receptors (Zoccal et al. 2014).

The results from the present work suggest that galatrox possesses high selectivity for LacNAc-containing glycans. Engagement of LacNAc cell surface glycans appears to induce neutrophil activation and migration, which may be further enhanced by galatrox-induced macrophage activation and subsequent cytokine secretion. Therefore, investigation into the carbohydrate-binding specificity of galatrox provided significant insight into its potential ligands and function. This understanding will likely enable the development of novel approaches to inhibit the deleterious effects of evonomation in patients suffering from a snake bite.

Materials and methods

Animals

Male BALB/c and C57BL/6 mice weighting 18–22 g were provided by Isogenic Animal Facility from the School of Pharmaceutical Sciences of Ribeirão Preto. Male TLR-2, TLR-4 and MyD88 knockout mice (*tlr4*$^{-/-}$, *tlr2*$^{-/-}$ and *myd88*$^{-/-}$ from C57BL/6 genetic background strain) were provided by the Animal Care Facility at the Ribeirão Preto Medical School, University of Sao Paulo. Animal care procedures were performed according to COBEA (Brazilian College of Animal Experimentation) guidelines and experimental protocols approved by The Committee for Ethics on Animal Use (CEUA) from the Ribeirão Preto Campus Prefecture of the University of São Paulo (Protocol number: 07.1.1495.53.2).

Peripheral blood sample from human donors

Peripheral blood sample were donated from the Hemotherapy Center of Ribeirão Preto. The samples were collected from both male and female health donors, belonging to the 19- to 40-year-old age group, not undergoing any form of pharmacological therapy. All care procedures were approved by the local Ethical Committee/School of Pharmaceutical Sciences of Ribeirão Preto of the University of São Paulo (protocol number: 118).

Isolation of galatrox

Galatrox was prepared from *B. atrox* crude venom according to Mendonça-Franqueiro et al. (2011). Briefly, ~150 mg of *B. atrox* whole venom was suspended in phosphate-buffered saline (PBS) buffer and applied into a lactosyl-Sepharose column (10 × 1 cm—Sigma–Aldrich). The column was washed with PBS only to elute the unbound material, and the bound material was eluted with PBS combined with α-lactose (100 mM). A desalting procedure on a Sephadex G-25 column (8 × 1 cm—Amersham Pharmacia Biotech) was performed to remove lactose from galatrox preparations. The homogeneity of galatrox samples was evaluated by SDS–PAGE and the preservation of lectin activity was confirmed by hemagglutinating assay, as described by Nowak et al. (1976). The protein concentrations were determined using a microassay based on the Bradford dye-binding procedure (Bradford 1976). Before the assays, galatrox samples were treated in polymyxin-B resin to remove remaining endotoxin residues and filtered through 0.22 μm pore filters.

Galatrox glycan array

To determine the glycan recognition pattern of galatrox, the glycan microarray was performed by the Consortium for Functional Glycomics Core H (Emory University School of Medicine, Atlanta, GA, USA). For the analysis, different concentrations of galatrox (0.05, 0.1, 1 and 144 μg/mL) were assessed using mammalian printed array version 3.2 which consists of 406 glycans in replicates of 6. The galatrox–glycan interaction was detected by an FITC-galatrox conjugate. The lectin was conjugated to FITC (Pierce, Rockford, IL, USA) by incubating both protein and fluorophore according to manufacture instructions. In an attempt to remove the excess of unbound FITC from conjugated FITC-galatrox, the incubated sample was submitted to a Sephadex G-25 column. The results obtained from the glycan microarray analysis relate to the analysis of FITC-galatrox at a concentration of 0.1 μg/mL, expressed by both the mean relative fluorescence unit (RFU) plus the standard error of the mean (SEM). A ranking of the

ligands was also indicated with the following calculation according to Heimburg-Molinaro et al. (2011): rank = 100 × [RFU bound/highest RFU]. The full table of glycan recognition patterns is available in Supplementary data, Table S1.

Extracellular matrix components binding assay

The binding efficiency of galatrox to ECM components, laminin and fibronectin, was evaluated by solid-phase assay. Solid white polystyrene microplate wells (MaxiSorp FluroNunc) were coated with murine sarcoma laminin, human plasma fibronectin or BSA (5 μg/well—Sigma–Aldrich) in PBS overnight at 4°C. After incubation, the plate was washed three times with PBS-Tween 20 0.05%, and then blocked with PBS–BSA 1% for 1 h at 37°C. Each well was incubated for 1 h with several concentrations of galatrox-FITC (2–32 μg/mL) in a final volume of 50 μL. After washing procedure, the analysis was performed by fluorescence reading using a Power Wave X reader (Bio-Tek Instruments, Inc.) on a emission wavelength of 585/20 nm. The assay was performed in triplicate of each condition. The results were expressed as mean ± SEM of the RFU.

Neutrophil surface binding assay

Human neutrophils (1×10^6 cells), obtained according to the *Human neutrophil migration* "in vitro" section, were incubated with different concentrations of galatrox-FITC (2–32 μg/mL) for 30 min at 4°C. Aiming to evaluate the role of the lectin CRD on cell surface receptor recognition, galatrox-FITC samples (8 μg/mL) were previously incubated with α-lactose or α-sucrose (20 mM) for 30 min at 4°C. Cells treated with Hank's balanced salt solution (HBSS) only were used as a NC. After incubation, the suspension was washed twice with PBS (200 × *g* for 10 min), and resuspended to a final volume of 0.5 mL. The samples were analyzed by flow cytometry in a FACSCalibur (BD Biosciences). The results were expressed as the percentage of FITC-galatrox$^+$ population and fluorescence intensity median of the FITC-galatrox$^+$ population.

Human neutrophil chemotaxis

Chemotatic activity in vitro was performed according to Bignold et al. (1988) using a 48-well acrylic Boyden microchamber (Neuroprobe). The microchamber consisted of two distinct compartments separated by a polycarbonate filter membrane (Millipore) with 5 μm diameter pores.

In order to obtain human neutrophils, heparinized human peripheral blood was layered on a density gradient of a neutrophil isolation medium Monopoly Resolving Medium (density: 1.114—ICN Pharmaceuticals) as described by manufacture instructions. A final neutrophil suspension in HBSS was obtained at a concentration of 1×10^6 neutrophils/mL (~94% of polymorphonuclear cells). Galatrox samples in several concentrations (2, 4, 8, 16 and 32 μg/mL) were placed in the lower chamber and neutrophil suspension placed in the upper chamber. In order to evaluate the involvement of lectin CRDs in the event, galatrox at its most effective concentration was preincubated with α-lactose or α-sucrose (20 mM) at 4°C for 30 min and consequently placed in the lower chamber. Random migration was assessed by using HBSS in the lower chamber. fMLP (10^{-7} M) was used as the reference chemoattractant. After incubation, the membrane was removed, fixed and stained with Panotic stain. Cells that migrated through the polycarbonate membrane pore during the 1 h incubation at 37°C in 5% CO_2 were counted with a light microscope using a 40 × objective in a total of five fields for each sample.

Mice peritoneal cavity leukocyte migration

Male BALB/c mice were injected intraperitoneally with galatrox (6.25–100 μg/cavity) in 200 μL of sterile PBS. NC animals received PBS alone, and positive controls (PCs) were given carrageenan (500 μg/cavity). After 4 h, animals were anesthetized and the peritoneal cavity was washed with 3 mL PBS supplemented with EDTA (1 mM). For time-course evaluation, mice were treated with galatrox (100 μg/cavity) and peritoneal wash was collected 4, 12, 24 and 72 h after administration. The exudate materials from animals were recovered for total and differential cell counting using a Neubauer chamber and Rosenfeld-staining cytospin preparations, respectively. Each treatment group constituted of five animals. In order to determinate the concentrations of inflammatory mediators, IL-1α, IL-6, IL-10 and TNF-α, peritoneal wash fluid was submitted to centrifugation at 200 × *g* for 10 min at 4°C and the supernatant was collected and stored at −80°C for further measurement.

Macrophage cell culture

BMDM cell suspension were obtained from pathogen-free C57BL/6 mice (wild type—wt) and *tlr4*$^{-/-}$, *tlr2*$^{-/-}$ and *myd88*$^{-/-}$, as described by Marim et al. (2010). Briefly, mice bone marrow from both femurs of three different animals from each strain were removed with RPMI medium supplemented with 10 mM of L-glutamine, 30% L929-cell conditioned medium containing macrophage colony stimulating-factor and 10% FBS. Cells were cultured in BD Optilux polyestirene Petri dishes (4×10^6 cells/mL) for 7 days (37°C and 5%CO_2) in the aforementioned medium, with subsequent medium addition after 4 days. The primary BMDM obtained were then detached from the dishes, washed with sterile PBS and treated with galatrox (30 μg/mL) for 24 h, with or without α-lactose (20 mM), in the same incubation conditions as described above. NC represents cells not treated with galatrox or galatrox plus sucrose (20 mM). After incubation, cell suspensions were centrifuged at 200 × *g* for 10 min at 4°C and the supernatant collected and stored at −80°C for inflammatory mediators (IL-2, IL-4, IL-6, IL-10, IL-17a, TNF-α, IFN-γ and KC).

Determination of inflammatory mediator concentrations

Cytokines detection. Enzyme-linked immunosorbent assay. The material collected from peritoneal cavity was assessed for inflammatory cytokine release using commercially available enzyme-linked immunosorbent assay (ELISA) kits to measure IL-1α, IL-6, IL-10 and TNF-α according to the manufacturer's instructions (BD Bioscience). KC detection was performed for samples of macrophage cell culture material using the same technique as above. The detection sensitivities were >10 pg/mL.

Flow cytometry cytokine bead array analysis

Levels of cytokines released into the cell medium supernatant of macrophage culture treated with galatrox were determined using the TH1/TH2/TH17 Cytometric Bead Array (CBA) Kit

(BD Biosciences) according to the manufacturer's instructions. Briefly, cell culture supernatants were incubated with the anti-cytokine beads and then added to the samples and incubated with the included PE-detection reagent. Beads and detection reagent were also mixed with a serially diluted standard of all cytokines (IL-2, IL-4, IL-6, IL-10, IL-17a, TNF-α and IFN-γ) provided by the manufacturer. Samples and standards were incubated in the dark at room temperature for 3 h. The samples were submitted to flow cytometry (FACSCalibur—BD Biosciences) and the events were analyzed using CBA analysis software (BD Biosciences) applying the five-parameter logistic fit for cytokine standard curve.

Statistical analysis

Results were expressed as mean ± SEM employing one-way ANOVA, followed by Turkey test. Differences with $P < 0.05$ were considered statistically significant.

Supplementary data

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

Funding

This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP—N° 2005/544855 and 2011/2323604). The Protein-Glycan Interaction Resource (Core H) of the Consortium for Functional Glycomics is supported by grants from the NIH (GM62116) and the National Center for Functional Glycomics NIH grant to RDC (P41GM103694). The research leading to these results has received support and funding from the Núcleo de Apoio à Pesquisa em Doenças Inflamatórias [NAPDIN] under grant agreement n° 11.1.21625.01.0.

Acknowledgements

We thank João José Franco and Rubens Eduardo da Silva for the excellent assistance with animal handling and technical support. Also, we thank Dr Adélia Cristina O. Cintra for the support in isolation of galatrox from *B. atrox* snake crude venom.

Abbreviations

BMDM, bone marrow-derived macrophages; BSA, bovine serum albumin; CRD, carbohydrate-recognition domain; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; HBSS, Hank's balanced salt solution; IL, interleukin; KC, keratinocyte-derived chemokine; LacNAc, *N*-acetyllactosamine; NeuAc, 5-*N*-acetylneuraminic acid; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLRs, Toll-like receptors; TNF, tumor necrosis factor.

References

Allavena P, Chieppa M, Monti P, Piemonti L. 2004. From pattern recognition receptor to regulator of homeostasis: The double-faced macrophage mannose receptor. *Crit Rev Immunol*. 24:179–192.

Almkvist J, Dahlgren C, Leffler H, Karlsson A. 2002. Activation of the neutrophil nicotinamide adenine dinucleotide phosphate oxidase by galectin-1. *J Immunol*. 168:4034–4041.

Amith SR, Jayanth P, Franchuk S, Finlay T, Seyrantepe V, Beyaert R, Pshezhetsky AV, Szewczuk MR. 2010. Neu1 desialylation of sialyl alpha-2,3-linked beta-galactosyl residues of TOLL-like receptor 4 is essential for receptor activation and cellular signaling. *Cell Signal*. 22:314–324.

Apostolakis S, Lip GY, Shantsila E. 2010. Monocytes in heart failure: Relationship to a deteriorating immune overreaction or a desperate attempt for tissue repair? *Cardiovasc Res*. 85:649–660.

Ariel A, Timor O. 2013. Hanging in the balance: Endogenous anti-inflammatory mechanisms in tissue repair and fibrosis. *J Pathol*. 229:250–263.

Auvynet C, Moreno S, Melchy E, Coronado-Martínez I, Montiel JL, Aguilar-Delfin I, Rosenstein Y. 2013. Galectin-1 promotes human neutrophil migration. *Glycobiology*. 23:32–42.

Aziz M, Jacob A, Yang WL, Matsuda A, Wang P. 2013. Current trends in inflammatory and immunomodulatory mediators in sepsis. *J Leukoc Biol*. 93:329–342.

Babu P, North SJ, Jang-Lee J, Chalabi S, Mackerness K, Stowell SR, Cummings RD, Rankin S, Dell A, Haslam SM. 2009. Structural characterisation of neutrophil glycans by ultra sensitive mass spectrometric glycomics methodology. *Glycoconj J*. 26:975–986.

Belardi B, O'Donoghue GP, Smith AW, Groves JT, Bertozzi CR. 2012. Investigating cell surfasse galectin-mediated cross-linking on glycoengineered cells. *J Am Chem Soc*. 134:9549–9952.

Bignold LP. 1988. Kinetics of chemo-attraction of polymorphonuclear leukocytes towards N-formyl peptide studied with a novel polycarbonate (Nucleopore) membrane in the Boyden chamber. *Experientia*. 44:518–521.

Bonavita AG, da Costa AS, Pires AL, Neves-Ferreira AG, Perales J, Cordeiro RS, Martins MA, e Silva PM. 2006. Contribution of mast cells and snake venom metalloproteinases to the hyperalgesia induced by Bothrops jararaca venom in rats. *Toxicon*. 47:885–893.

Boscher C, Dennis JW, Nabi IR. 2011. Glycosylation, galectins and cellular signaling. *Curr Opin Cell Biol*. 23(4):383–392.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72:248–254.

Brewer CF, Miceli MC, Baum LG. 2002. Clusters, bundles, arrays and lattices: Novel mechanisms for lectin-saccharide-mediated cellular interactions. *Curr Opin Struct Biol*. 12:616–623.

Buckley CD, Gilroy DW, Serhan CN, Stockinger B, Tak PP. 2013. The resolution of inflammation. *Nat Rev Immunol*. 13:59–66.

Camby I, Le Mercier M, Lefranc F, Kiss R. 2006. Galectin-1: A small protein with major functions. *Glycobiology*. 16:137R–157R.

Carneiro AS, Ribeiro OG, Cabrera WH, Vorraro F, De Franco M, Ibañez OM, Starobinas N. 2008. Bothrops jararaca venom (BjV) induces differential leukocyte accumulation in mice genetically selected for acute inflammatory reaction: The role of host genetic background on expression of adhesion molecules and release of endogenous mediators. *Toxicon*. 52:619–627.

Carsons S, Lavietes BB, Slomiany A, Diamond HS, Berkowitz E. 1987. Carbohydrate heterogeneity of fibronectins. Synovial fluid fibronectin resembles the form secreted by cultured synoviocytes but differs from the plasma form. *J Clin Invest*. 80:1342–1349.

Cerliani JP, Stowell SR, Mascanfroni ID, Arthur CM, Cummings RD, Rabinovich GA. 2011. Expanding the universe of cytokines and pattern recognition receptors: Galectins and glycans in innate immunity. *J Clin Immunol*. 31:10–21.

Coombs PJ, Taylor ME, Drickamer K. 2006. Two categories of mammalian galactose-binding receptors distinguished by glycan array profiling. *Glycobiology*. 16:1C–7C.

da Silva Correia J, Ulevitch RJ. 2002. MD-2 and TLR4 N-linked glycosylations are important for a functional lipopolysaccharide receptor. *J Biol Chem*. 277:18.45–18.54.

Davies LC, Jenkins SJ, Allen JE, Taylor PR. 2013. Tissue-resident macrophages. *Nat Immunol*. 14:986–995.

de Toledo KA, Bernardes ES, Baruffi MD, Roque-Barreira MC. 2007. Neutrophil haptotaxis induced by mouse MNCF: Interactions with extracellular matrix glycoproteins probably contribute to overcoming the anti-inflammatory action of dexamethasone. *Inflamm Res*. 56:368–376.

Dias-Baruffi M, Roque-Barreira MC, Cunha FQ, Ferreira SH. 1995a. Biological characterization of purified macrophage-derived neutrophil chemotactic factor. *Mediators Inflamm*. 4:263–269.

Dias-Baruffi M, Roque-Barreira MC, Cunha FQ, Ferreira SH. 1995b. Isolation and partial chemical characterization of macrophage-derived neutrophil chemotactic factor. *Mediators Inflamm*. 4:257–262.

Dias-Baruffi M, Zhu H, Cho M, Karmakar S, McEver RP, Cummings RD. 2003. Dimeric galectin-1 induces surface exposure of phosphatidylserine and phagocytic recognition of leukocytes without inducing apoptosis. *J Biol Chem*. 278:41282–41293.

Earl LA, Bi S, Baum LG. 2011. Galectin multimerization and lattice formation are regulated by linker region structure. *Glycobiology*. 21(1):6–12.

Elifio-Esposito S, Tomazeli L, Schwartz C, Gimenez AP, Fugii GM, Fernandes LC, Zishler LF, Stuelp-Campelo PM, Moreno AN. 2011. Human neutrophil migration and activation by BJcuL, a galactose binding lectin purified from Bothrops jararacussu venom. *BMC Immunol*. 12:10.

Escocard Rde C, Kanashiro MM, Petretski JH, Azevedo-Silva J, Queiroz de Carvalho EC, Dias da Silva W, Kipnis TL. 2006. Neutrophils regulate the expression of cytokines, chemokines and nitric oxide synthase/nitric oxide in mice injected with Bothrops atrox venom. *Immunobiology*. 211:37–46.

Esteban A, Popp MW, Vyas VK, Strijbis K, Ploegh HL, Fink GR. 2011. Fungal recognition is mediated by the association of dectin-1 and galectin-3 in macrophages. *Proc Natl Acad Sci USA*. 108:14270–14275.

Farnworth SL, Henderson NC, Mackinnon AC, Atkinson KM, Wilkinson T, Dhaliwal K, Hayashi K, Simpson AJ, Rossi AG, Haslett C, et al. 2008. Galectin-3 reduces the severity of pneumococcal pneumonia by augmenting neutrophil function. *Am J Pathol*. 172:395–405.

Frey H, Schroeder N, Manon-Jensen T, Iozzo RV, Schaefer L. 2013. Biological interplay between proteoglycans and their innate immune receptors in inflammation. *FEBS J*. 280:2165–2179.

Friedrichs J, Torkko JM, Helenius J, Teräväinen TP, Füllekrug J, Muller DJ, Simons K, Manninen A. 2007. Contributions of galectin-3 and -9 to epithelial cell adhesion analyzed by single cell force spectroscopy. *J Biol Chem*. 282:29375–29383.

Fujiwara S, Shinkai H, Deutzmann R, Paulsson M, Timpl R. 1988. Structure and distribution of N-linked oligosaccharide chains on various domains of mouse tumour laminin. *Biochem J*. 252:453–461.

Fulcher JA, Hashimi ST, Levroney EL, Pang M, Gurney KB, Baum LG, Lee B. 2006. Galectin-1-matured human monocyte-derived dendritic cells have enhanced migration through extracellular matrix. *J Immunol*. 177: 216–226.

Galván-Moroyoqui JM, Del Carmen Domínguez-Robles M, Meza I. 2011. Pathogenic bactéria prime the induction of Toll-like receptor signalling in human colonic cells by the Gal/GalNAc lectin Carbohydrate Recognition Domain of Entamoeba histolytica. *Int J Parasitol*. 41:1101–1112.

Garcia-Ramallo E, Marques T, Prats N, Beleta J, Kunkel SL, Godessart N. 2002. Resident cell chemokine expression serves as the major mechanism for leukocyte recruitment during local inflammation. *J Immunol*. 169:6467–6473.

Garín MI, Chu CC, Golshayan D, Cernuda-Morollón E, Wait R, Lechler RI. 2007. Galectin-1: A key effector of regulation mediated by CD4+CD25+ T cells. *Blood*. 109:2058–2065.

Gordon S, Taylor PR. 2005. Monocyte and macrophage heterogeneity. *Nat Rev Immunol*. 5:953–964.

Hauzenberger D, Klominek J, Bergström SE, Sundqvist KG. 1995. T lymphocyte migration: The influence of interactions via adhesion molecules, the T cell receptor, and cytokines. *Crit Rev Immunol*. 15:285–316.

Havt A, Toyama MH, do Nascimento NR, Toyama DO, Nobre AC, Martins AM, Barbosa PS, Novello JC, Boschero AC, Carneiro EM, et al. 2005. A new C-type animal lectin isolated from Bothrops pirajai is responsible for the snake venom major effects in the isolated kidney. *Int J Biochem Cell Biol*. 37:130–141.

Heimburg-Molinaro J, Song X, Smith DF, Cummings RD. 2011. Preparation and analysis of glycan microarrays. *Curr Protoc Protein Sci*. Chapter 12: Unit12.10.

Huang EY, Devkota S, Moscoso D, Chang EB, Leone VA. 2013. The role of diet in triggering human inflammatory disorders in the modern age. *Microbes and infection / Institut Pasteur*. 15:765–774.

Jin F, Chammas R, Engel J, Reinhold V. 1995. Structure and function of laminin 1 glycans: Glycan profiling. *Glycobiology*. 5:157–158.

Karmakar S, Stowell SR, Cummings RD, McEver RP. 2008. Galectin-1 signaling in leukocytes requires expression of complex-type N-glycans. *Glycobiology*. 18:770–778.

Katnik-Prastowska I, Kratz EM, Faundez R, Chełmońska-Soyta A. 2006. Lower expression of the alpha2,3-sialylated fibronectin glycoform and appearance of the asialo-fibronectin glycoform are associated with high concentrations of fibronectin in human seminal plasma with abnormal semen parameters. *Clin Chem Lab Med*. 44:1119–1125.

Kątnik-Prastowska I, Orczyk-Pawiłowicz M. 2011. Expression and potential biological role of α(1,2)fucosylated glycotopes on amniotic and seminal fibronectins. *Biochem Soc Trans*. 39:355–359.

Lauffenburger DA, Horwitz AF. 1996. Cell migration: a physically integrated molecular process. *Cell*. 84:359–369.

Leal SM, Jr, Cowden S, Hsia YC, Ghannoum MA, Momany M, Pearlman E. 2010. Distinct roles for Dectin-1 and TLR4 in the pathogenesis of Aspergillus fumigatus keratitis. *PLoS Pathog*. 6:e1000976.

Leppänen A, Stowell S, Blixt O, Cummings RD. 2005. Dimeric galectin-1 binds with high affinity to alpha2,3-sialylated and non-sialylated terminal N-acetyllactosamine units on surface-bound extended glycans. *J Biol Chem*. 280:5549–5562.

Lomonte B, Rojas G, Gutiérrez JM, Ramírez G. 1990. Isolation of a galactose-binding lectin from the venom of the snake Bothrops godmani (Godmann's pit viper). *Toxicon*. 28:75–81.

Lu M, Varley AW, Munford RS. 2013. Persistently active microbial molecules prolong innate immune tolerance in vivo. *PLoS Pathog*. 9:e1003339.

Marim FM, Silveira TN, Lima DS, Zamboni DS. 2010. A method for generation of bone marrow-derived macrophages from cryopreserved mouse bone marrow cells. *PLoS ONE*. 5:e15263.

Medzhitov R, Horng T. 2009. Transcriptional control of the inflammatory response. *Nat Rev Immunol*. 9:692–703.

Mendonça-Franqueiro EeP, Alves-Paiva ReM, Sartim MA, Callejon DR, Paiva HH, Antonucci GA, Rosa JC, Cintra AC, Franco JJ, Arantes EC, et al. 2011. Isolation, functional, and partial biochemical characterization of galatrox, an acidic lectin from Bothrops atrox snake venom. *Acta Biochim Biophys Sin (Shanghai)*. 43:181–192.

Moreira R, Balseiro P, Romero A, Dios S, Posada D, Novoa B, Figueras A. 2012. Gene expression analysis of clams Ruditapes philippinarum and Ruditapes decussatus following bacterial infection yields molecular insights into pathogen resistance and immunity. *Dev Comp Immunol*. 36:140–149.

Moreira V, Teixeira C, Borges da Silva H, D'Império Lima MR, Dos-Santos MC. 2013. The crucial role of the MyD88 adaptor protein in the inflammatory response induced by Bothrops atrox venom. *Toxicon*. 67:37–46.

Netea MG, Wijmenga C, O'Neill LA. 2012. Genetic variation in Toll-like receptors and disease susceptibility. *Nat Immunol*. 13:535–542.

Nieminen J, St-Pierre C, Sato S. 2005. Galectin-3 interacts with naive and primed neutrophils, inducing innate immune responses. *J Leukoc Biol*. 78:1127–1135.

Nishi N, Shoji H, Seki M, Itoh A, Miyanaka H, Yuube K, Hirashima M, Nakamura T. 2003. Galectin-8 modulates neutrophil function via interaction with integrin alphaM. *Glycobiology*. 13:755–763.

Nowak TP, Haywood PL, Barondes SH. 1976. Developmentally regulated lectin in embryonic chick muscle and a myogenic cell line. *Biochem Biophys Res Commun*. 68:650–657.

Panunto PC, da Silva MA, Linardi A, Buzin MP, Melo SE, Mello SM, Prado-Franceschi J, Hyslop S. 2006. Biological activities of a lectin from Bothrops jararacussu snake venom. *Toxicon*. 47:21–31.

Park HJ, Hong JH, Kwon HJ, Kim Y, Lee KH, Kim JB, Song SK. 2010. TLR4-mediated activation of mouse macrophages by Korean mistletoe lectin-C (KML-C). *Biochem Biophys Res Commun*. 396:721–725.

Rabinovich GA, Sotomayor CE, Riera CM, Bianco I, Correa SG. 2000. Evidence of a role for galectin-1 in acute inflammation. *Eur J Immunol*. 30:1331–1339.

Ramos I, Fernandez-Sesma A. 2012. Cell receptors for influenza a viruses and the innate immune response. *Front Microbiol*. 3:117.

Rogala B, Glück J. 2013. The role of interleukin-33 in rhinitis. *Curr Allergy Asthma Rep*. 13:196–202.

Saijo S, Fujikado N, Furuta T, Chung SH, Kotaki H, Seki K, Sudo K, Akira S, Adachi Y, Ohno N, et al. 2007. Dectin-1 is required for host defense against Pneumocystis carinii but not against Candida albicans. *Nat Immunol*. 8:39–46.

Sánchez-Madrid F, del Pozo MA. 1999. Leukocyte polarization in cell migration and immune interactions. *EMBO J*. 18:501–511.

Sato S, St-Pierre C, Bhaumik P, Nieminen J. 2009. Galectins in innate immunity: Dual functions of host soluble beta-galactoside-binding lectins as damage-associated molecular patterns (DAMPs) and as receptors for pathogen-associated molecular patterns (PAMPs). *Immunol Rev*. 230:172–187.

Shin OS, Isberg RR, Akira S, Uematsu S, Behera AK, Hu LT. 2008. Distinct roles for MyD88 and Toll-like receptors 2, 5, and 9 in phagocytosis of Borrelia burgdorferi and cytokine induction. *Infect Immun*. 76: 2341–2351.

Soehnlein O, Lindbom L. 2010. Phagocyte partnership during the onset and resolution of inflammation. *Nat Rev Immunol*. 10:427–439.

Sorgi CA, Rose S, Court N, Carlos D, Paula-Silva FW, Assis PA, Frantz FG, Ryffel B, Quesniaux V, Faccioli LH. 2012. GM-CSF priming drives bone marrow-derived macrophages to a pro-inflammatory pattern and downmodulates PGE2 in response to TLR2 ligands. *PLoS ONE*. 7:e40523.

Stowell SR, Arthur CM, Dias-Baruffi M, Rodrigues LC, Gourdine JP, Heimburg-Molinaro J, Ju T, Molinaro RJ, Rivera-Marrero C, Xia B, et al. 2010. Innate immune lectins kill bacteria expressing blood group antigen. *Nat Med*. 16:295–301.

Stowell SR, Arthur CM, McBride R, Berger O, Razi N, Heimburg-Molinaro J, Rodriques LC, Gourdine JP, Noll AJ, von Gunten S, et al. 2014. Microbial glycan microarrays define key features of host-microbial interactions. *Nat ChemBiol*. 10:470–476.

Stowell SR, Arthur CM, Mehta P, Slanina KA, Blixt O, Leffler H, Smith DF, Cummings RD. 2008. Galectin-1, -2, and -3 exhibit differential recognition of sialylated glycans and blood group antigens. *J Biol Chem*. 283:10109–10123.

Stowell SR, Arthur CM, Slanina KA, Horton JR, Smith DF, Cummings RD. 2008. Dimeric Galectin-8 induces phosphatidylserine exposure in leukocytes through polylactosamine recognition by the C-terminal domain. *J Biol Chem*. 283:20547–20559.

Stowell SR, Cho M, Feasley CL, Arthur CM, Song X, Colucci JK, Karmakar S, Mehta P, Dias-Baruffi M, McEver RP, et al. 2009. Ligand reduces galectin-1 sensitivity to oxidative inactivation by enhancing dimer formation. *J Biol Chem*. 284:4989–4999.

Stowell SR, Dias-Baruffi M, Penttilä L, Renkonen O, Nyame AK, Cummings RD. 2004. Human galectin-1 recognition of poly-N-acetyllactosamine and chimeric polysaccharides. *Glycobiology*. 14:157–167.

Stowell SR, Qian Y, Karmakar S, Koyama NS, Dias-Baruffi M, Leffler H, McEver RP, Cummings RD. 2008. Differential roles of galectin-1 and galectin-3 in regulating leukocyte viability and cytokine secretion. *J Immunol*. 180:3091–3102.

Strydom N, Rankin SM. 2013. Regulation of circulating neutrophil numbers under homeostasis and in disease. *J Innate Immun*. 5:304–314.

Sun J, Duffy KE, Ranjith-Kumar CT, Xiong J, Lamb RJ, Santos J, Masarapu H, Cunningham M, Holzenburg A, Sarisky RT, et al. 2006. Structural and functional analyses of the human Toll-like receptor 3. Role of glycosylation. *J Biol Chem*. 281:11144–11151.

Teixeira C, Cury Y, Moreira V, Picolob G, Chaves F. 2009. Inflammation induced by Bothrops asper venom. *Toxicon*. 54:988–997.

Toscano MA, Bianco GA, Ilarregui JM, Croci DO, Correale J, Hernandez JD, Zwirner NW, Poirier F, Riley EM, Baum LG, et al. 2007. Differential glycosylation of TH1, TH2 and TH-17 effector cells selectively regulates susceptibility to cell death. *Nat Immunol*. 8:825–834.

van den Brûle F, Califice S, Garnier F, Fernandez PL, Berchuck A, Castronovo V. 2003. Galectin-1 accumulation in the ovary carcinoma peritumoral stroma is induced by ovary carcinoma cells and affects both cancer cell proliferation and adhesion to laminin-1 and fibronectin. *Lab Invest*. 83:377–386.

Wang J, Babcock GJ, Choe H, Farzan M, Sodroski J, Gabuzda D. 2004. N-linked glycosylation in the CXCR4 N-terminus inhibits binding to HIV-1 envelope glycoproteins. *Virology*. 324:140–150.

Wenzel-Seifert K, Seifert R. 2003. Critical role of N-terminal N-glycosylation for proper folding of the human formyl peptide receptor. *Biochem Biophys Res Commun*. 301:693–698.

White ES, Mantovani AR. 2013. Inflammation, wound repair, and fibrosis: Reassessing the spectrum of tissue injury and resolution. *J Pathol*. 229:141–144.

Young NM, van Faassen H, Watson DC, Mackenzie CR. 2011. Specificity analysis of the C-type lectin from rattlesnake venom, and its selectivity towards Gal- or GalNAc-terminated glycoproteins. *Glycoconj J*. 28: 427–435.

Zoccal KF, Bitencourt Cda S, Paula-Silva FW, Sorgi CA, de Castro Figueiredo Bordon K, Arantes EC, Faccioli LH. 2014. TLR2, TLR4 and CD14 recognize venom-associated molecular patterns from Tityus serrulatus to induce macrophage-derived inflammatory mediators. *PLoS ONE*. 9:e88174.