

# Galectin-1 promotes human neutrophil migration

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Received on September 17, 2011; revised on August 26, 2012; accepted on August 27, 2012

**An important step of innate immune response is the recruitment of polymorphonuclear leukocytes (PMN) to injured tissues through chemotactic molecules. Galectins, a family of endogenous lectins, participate in numerous functions such as lymphoid cell migration, homing, cell–cell and cell–matrix interactions. Particularly, galectin-3 (Gal-3) and -9 have been implicated in the modulation of acute and chronic inflammation by inducing the directional migration of monocytes/macrophages and eosinophils, whereas Gal-1 is considered to function as an anti-inflammatory molecule, capable of inhibiting the influx of PMN to the site of injury. In this study, we assessed the effect of Gal-1 on neutrophil recruitment, in the absence of additional inflammatory insults. Contrasting with its capacity to inhibit cell trafficking and modulate the release of mediators described in models of acute inflammation and autoimmunity, we evidenced that Gal-1 has the capacity to induce neutrophil migration both *in vitro* and *in vivo*. This effect is not mediated through a G-protein-coupled receptor but potentially through the sialoglycoprotein CD43, via carbohydrate binding and through the p38 mitogen-activated protein kinase pathway. These results suggest a novel biological function for CD43 on neutrophils and highlight that depending on the environment, Gal-1 can act either as chemoattractant or, as a molecule that negatively regulates migration under acute inflammatory conditions, underscoring the potential of Gal-1 as a target for innovative drug development.**

**Keywords:** CD43 / chemotaxis / galectin-1 / MAPK neutrophil / polarization

## Introduction

Glycosylation plays a key role in the control of multiple biological activities. Recently, it has taken a central stage in our understanding of the regulation of adaptive and innate immune responses, including cell activation, homing and survival (Daniels et al. 2002; Marth and Grewal 2008; van Kooyk and Rabinovich 2008). Glycosylation modulates cellular responses by generating or masking ligands for endogenous lectins, shaping glycoprotein conformation and controlling intermolecular interactions and intracellular signaling pathways (Demetriou et al. 2001; Amano et al. 2003). In mammals, galectins, a family of 15 conserved  $\beta$ -galactoside-binding proteins, are widely expressed by normal and pathological tissues. Even though members of this family differ in structure and in terms of the multivalent interactions they can establish with specific glycosidic structures, they all share a conserved carbohydrate-recognition domain and most of them have high affinity for poly-*N*-acetyl-lactosamine-enriched glycoconjugates (Brewer et al. 2002). As a result, galectins operate as “readers of the glycode” (Kasai and Hirabayashi 1996; Cooper and Barondes 1999; Leffler et al. 2004) for leukocytes, modulating cell–matrix interactions, trafficking, cytokine secretion, proliferation and survival, thus emerging as important regulatory elements of immunity (Rabinovich et al. 2002a, 2004). Galectin-1 (Gal-1), a 14.5-kDa subunits homodimer, and Gal-3 are probably the most ubiquitously expressed member of the galectin family (Stillman et al. 2006). Gal-1 has been found to regulate multiple facets of adaptive and innate immune responses (Perillo et al. 1995; Rabinovich et al. 2002b; Kuwabara et al. 2003; Norling et al. 2009).

Among innate immune cells, neutrophils are remarkable for their migratory capacity. Upon activation by chemoattractants, chemokines or inflammatory products, these cells rapidly migrate to the site of injury (Burton et al. 1987). Gal-3 and -9 have been characterized as chemoattractants for monocytes/macrophages as well as for neutrophils and eosinophils, respectively (Hirashima 1999, 2000; Sano et al. 2000; Kuwabara et al. 2003; Nieminen et al. 2008; Henderson and Sethi 2009). Although recent reports show that Gal-1 participates in the directional migration of monocytes (Malik et al. 2009) and dendritic cells (Fulcher et al. 2009), less is known about its impact upon neutrophils recruitment. In a mouse model of paw edema, injection of Gal-1 was associated with a reduced PMN influx (Rabinovich et al. 2000). In agreement

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with this, Gal-1 was shown to exert an anti-inflammatory activity as it inhibited IL-8- or TNF $\alpha$ -induced PMN chemotaxis *in vitro*, under acute experimental inflammation (La et al. 2003; Cooper et al. 2008). Interestingly, no published data have assessed the effect of Gal-1 on neutrophils, in the absence of inflammatory stimuli.

The key structure recognized by Gal-1 is the disaccharide unit *O*-linked *N*-acetyl-lactosamine (Gal- $\beta$ 1-4Glc-NAc) present on leukocyte surface molecules such as CD45, CD7, CD43, CD2, CD3, CD4, CD107, glycosaminoglycans, integrins, GM1 ganglioside, glycoprotein 90K/MAC-2BP and pre-B cell receptor, all of which have been identified as potential counter-receptors for Gal-1 (Camby et al. 2006). Of the multiple ligands identified for Gal-1, only CD43 and CD45 are highly expressed on polymorphonuclear leukocytes. However, whether these molecules serve as glycoreceptors for Gal-1 on neutrophils remains to be determined experimentally, as glycosylation patterns are known to differ widely between cell lineages and differentiation stages (Fukuda and Carlsson 1986; Daniels et al. 2002). CD43 (sialophorin, leukosialin) is a prototypic member of cell-associated mucins. It is a transmembrane sialoglycoprotein with a high content of *O*-linked carbohydrates (Remold-O'Donnell et al. 1986, 1987), with multiple functions (reviewed in Aguilar-Delfin et al. 2006). CD43 is one of the most abundant surface proteins on neutrophils and several lines of evidence suggest a possible role for CD43 on neutrophil recruitment. It is rapidly down-modulated in response to cell activation (Campanero et al. 1991; Rieu et al. 1992; Mambole et al. 2008), and CD43-deficient mice have defects in leukocyte rolling and tissue infiltration (Woodman et al. 1998). In addition, cross-linking CD43 induces cell adhesion (Kuijpers et al. 1992; Matsumoto et al. 2008) and regulates neutrophil spreading and oxidative burst (Nathan et al. 1993).

In this study, we evidenced that, *in vitro*, Gal-1 has the capacity to recruit human neutrophils. Moreover, *in vivo*, we show that the injection of Gal-1 into the peritoneal cavity, in the absence of other inflammatory insults, results in neutrophil recruitment. We show that this effect is independent of a G-protein-coupled receptor and that it might instead be mediated by the sialoglycoprotein CD43. These results highlight a dual role for Gal-1, a chemotactic role when acting alone or an anti-inflammatory role by inhibiting migration under acute inflammatory conditions, thus underscoring the potential of this molecule as a target for innovative drug development.

## Results

### *Gal-1 promotes the migration of human neutrophils*

We first evaluated the capacity of Gal-1 to promote the migration of human peripheral blood neutrophils *in vitro*. We found that Gal-1 induced the directional migration of human neutrophils at two concentrations: 25 and 250 nM (Figure 1A). In order to discriminate whether the migration detected reflected a chemotactic or a chemokinetic movement, we performed a checkboard analysis with different concentrations of Gal-1 in the upper and lower wells of the chemotaxis chamber. In the presence of 25 nM Gal-1 in the upper wells of the chamber, neutrophils' migration toward the 25 nM Gal-1-loaded lower wells was abrogated (Figure 1A), suggesting that at that

concentration, neutrophils migration resulted from chemotaxis rather than from chemokinesis (movement in random directions). On the contrary, addition of 250 nM Gal-1 to the upper wells did not cancel the 250 nM Gal-1-induced migration, indicating that 250 nM Gal-1 induced essentially a chemokinetic effect on neutrophils (Figure 1A). As expected, incubation of the neutrophils with fMLP (formyl-methionyl-leucyl-phenylalanine; 1.14 nM) in the upper wells abrogated the fMLP (1.14 nM)-induced chemotaxis. To ascertain that the chemotaxis we observed resulted of the specific interaction of Gal-1 with a putative receptor on the neutrophils' surface, we performed the same assays in the presence of polymyxin B, allowing us to support that this effect was due to Gal-1 and not to contaminating endotoxin (Supplementary data, Figure S1).

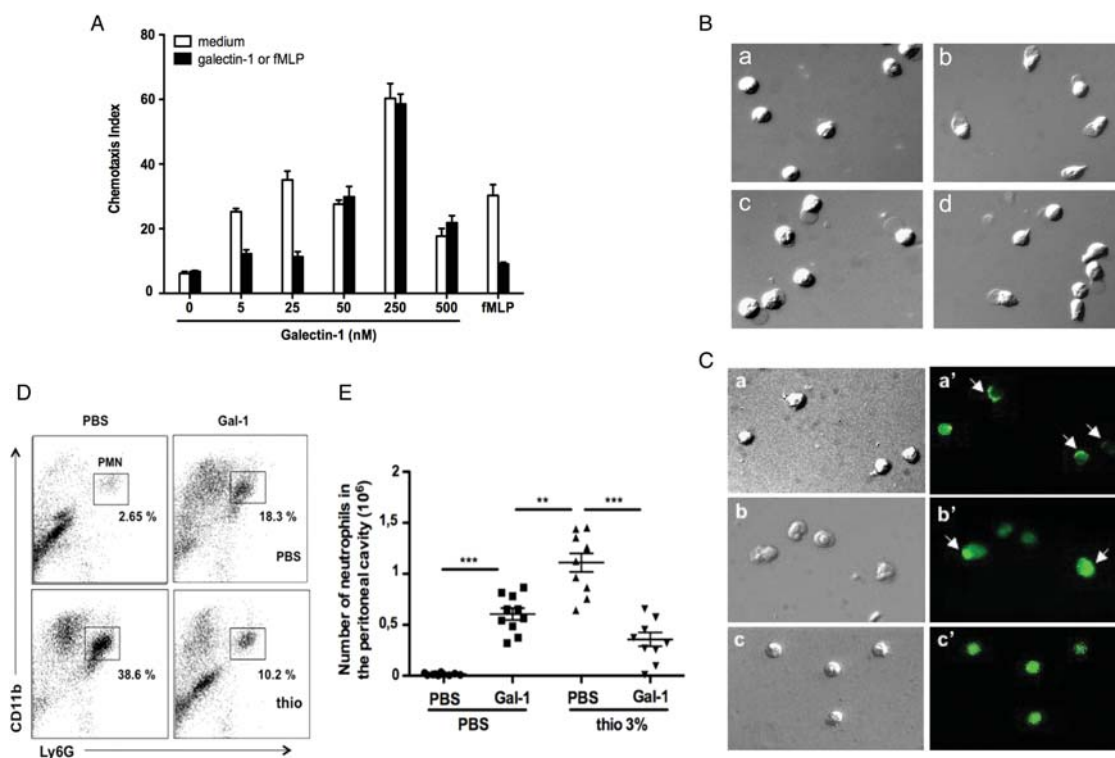
Since cell polarization and actin polymerization are part of the migration process, we evaluated if Gal-1 induced morphological changes in neutrophils. As shown in Figure 1Bb, most neutrophils (75  $\pm$  10%) incubated in the presence of Gal-1 (25 nM) for 5 min exhibited the characteristic uropod of activated cells, with the resulting polarized shape as opposed to the round-shaped control cells (Figure 1Ba). Similar morphological changes were detected when neutrophils were stimulated with fMLP (1.14 nM; Figure 1Bd). Consistent with cell polarization, we found that neutrophils incubated for 2 min with Gal-1 (25 nM) or fMLP (1.14 nM) exhibited localized polymerized actin filaments (Figure 1Ca, a' and b, b', respectively) and increased fluorescence as determined by flow cytometry (data not shown). In neutrophils incubated with medium only, fluorescence was uniformly distributed throughout the cell (Figure 1Cc and c'). Pre-incubating Gal-1 with 1 mM lactose for 10 min prior to stimulate the neutrophils abrogated cell polarization and actin polymerization (data not shown).

To examine more closely the effect of Gal-1 on neutrophils migration, we performed *in vivo* inflammatory studies. We compared the effect of Gal-1 on activated neutrophils after thioglycollate injection, an insult well known to induce peritonitis and thus activation and recruitment of neutrophils, or after a phosphate-buffered saline (PBS) injection, a treatment which should not activate neutrophils. As shown in Figure 1D and as reported previously (La et al. 2003), we can observe that injection of 0.5  $\mu$ g of Gal-1 (equivalent to 35 pmol) strongly inhibited the recruitment of neutrophils to the peritoneal cavity resulting of the thioglycollate injection. On the contrary, in the absence of thioglycollate, i.e. in non-inflamed conditions, the same Gal-1 concentration promoted the recruitment of neutrophils to the peritoneal cavity. Thus, Gal-1 seems to exert opposite functions, pro- or anti-inflammatory, depending on the local environment and the activation state of the neutrophils.

All together, these data indicate that Gal-1 induces actin polymerization, cell polarization and the directional migration of human resting neutrophils.

### *Neutrophil migration toward Gal-1 is mediated by carbohydrate-dependent interactions*

To further characterize the ability of Gal-1 to control the motility of human neutrophils, we evaluated the binding of



**Fig. 1.** Gal-1 promotes neutrophils' migration. (A) Cell migration induced in response to Gal-1 or fMLP (1.14 nM) as a positive control and medium as a negative control (open bars:Gal-1 or fMLP in lower wells only; black bars: Gal-1 or fMLP in upper and lower wells). (B) Neutrophils polarization following stimulation by medium (a), Gal-1 (25 nM) (b), pre-incubation with lactose before adding Gal-1 (25 nM) (c) or fMLP (1.14 nM) (d). (C) Neutrophils were stimulated with Gal-1 (25 nM) (a and a'), fMLP (1.14 nM) (b and b') or medium (c and c') for the times indicated in the text; cells were observed under normal light (a–c) or at 488 nm (a'–c'). Arrows show the polymerized actin at the uropod of polarized neutrophils. Similar results were obtained from three independent experiments. (D) Representative flow cytometry analysis of cell suspensions extracted from the peritoneal cavity of C57Bl/6 mice after injection of 1 mL of thioglycollate 3% or PBS together with 150  $\mu$ L Gal-1 (0.5  $\mu$ g) or PBS. Neutrophils are identified as CD11b<sup>+</sup>Ly6G<sup>+</sup>. (E) Quantification of total number of neutrophils extracted from the peritoneal cavity of C57Bl/6 mice after injection of 1 mL of thioglycollate 3% or PBS together with 150  $\mu$ L Gal-1 (0.5  $\mu$ g) or PBS ( $n = 5$  per group; \*\* $P < 0.001$ ; \*\*\* $P < 0.0001$ ). Data are representative of two independent experiments.

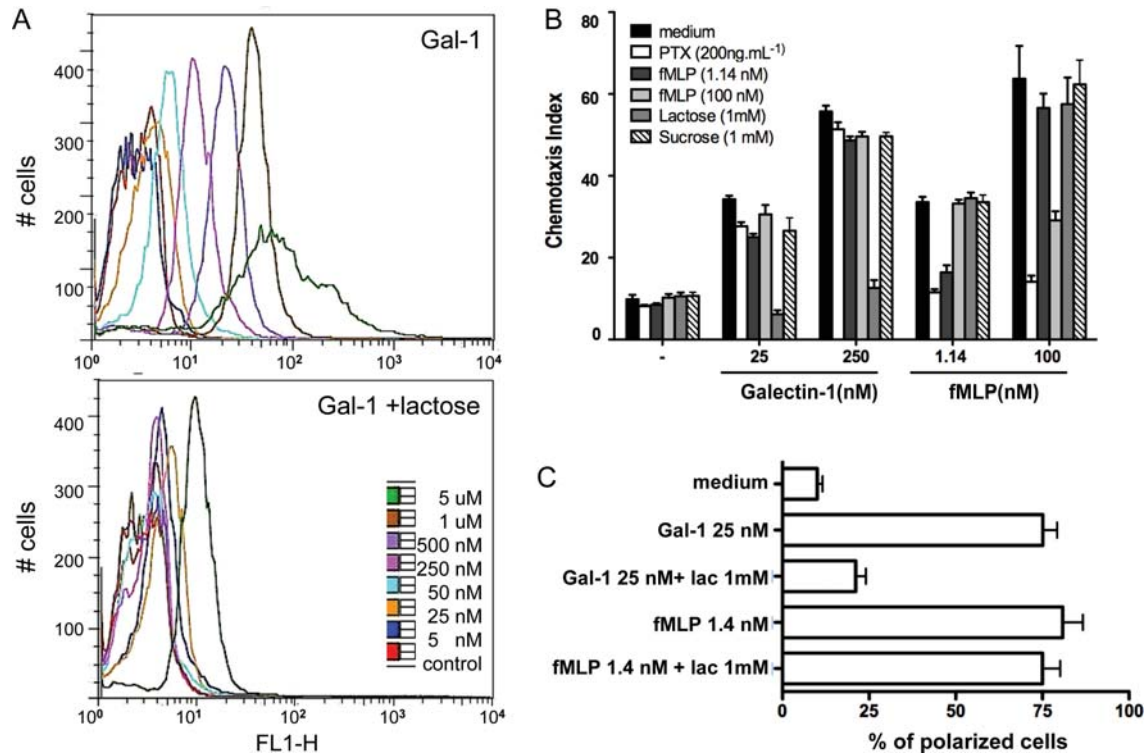
biotinylated-Gal-1 by flow cytometry (Figure 2A). We found that Gal-1 (5 nM–5  $\mu$ M) readily bound to the cell surface of the neutrophils and that this interaction was inhibited by the addition of lactose. These results indicate that neutrophils express on their cell surface carbohydrate ligands specifically recognized by Gal-1.

Most chemoattractants induce cell migration through seven transmembrane  $G_{i\alpha}$ -protein-coupled receptors, as is the case of fMLP that interacts with formyl peptide receptor (FPR) or FPR-like 1 (FPRL-1) expressed on neutrophils. At a concentration of 1.14 nM, fMLP activates FPR while at a concentration of 100 nM it activates FPRL-1 (Selvatici et al. 2006). To determine whether the effect of Gal-1 on neutrophils migration was mediated by galectin–carbohydrate interactions or through a seven transmembrane  $G_{i\alpha}$ -protein-coupled receptor, neutrophils were pre-incubated for 30 min with lactose, sucrose or with Pertussis toxin (PTX), a specific inhibitor of  $G_{i\alpha}$ -protein-coupled receptors, prior to evaluate chemotaxis toward Gal-1. Pre-treatment with the galectin-specific sugar lactose inhibited neutrophil migration in response to either 25 or 250 nM Gal-1 (Figure 2B). In contrast, pre-treating the cells with sucrose, a non-galectin binding sugar, or with PTX did not abolish the Gal-1-induced migration, whereas pre-

incubating the neutrophils with lactose or sucrose did not inhibit the fMLP-induced migration, yet pre-incubation with PTX did. The fact that pre-incubating the cells with fMLP desensitized the fMLP receptor, abolishing fMLP-induced cell migration but not that elicited by Gal-1, further suggested that fMLP and Gal-1 induce cell migration through different receptors (Figure 2B). Accordingly, pre-incubating the cells with lactose (Figures 1Bc and 2C), but not with fMLP (Figure 2C), mostly abrogated Gal-1-induced polarization. Altogether, these results suggest that the Gal-1-induced directional migration of neutrophils is mediated by carbohydrate-dependent interactions and that it does not require the participation of G-protein-coupled receptors.

#### *Gal-1- and fMLP-induced chemotaxis involve different signaling pathways*

The lipid products of PI3K, several members of the protein kinase C (PKC) family as well as of the mitogen-activated protein kinase (MAPK) family control key signaling pathways that regulate cell polarity and motility. In order to characterize the signaling pathways regulating the Gal-1-mediated cell motility, we performed chemotaxis assays in the presence of



**Fig. 2.** Gal-1 and fMLP are sensed through different receptors. (A) Binding of biotinylated Gal-1 to neutrophils was evaluated by flow cytometry after incubating with different concentrations of Gal-1 for 30 min prior to incubation with avidin-FITC for 30 min. (B) Chemotaxis was assessed after pre-incubating neutrophils with medium, PTX (200 ng/mL), fMLP (1.14 nM), fMLP (100 nM), lactose (1 mM) or sucrose (1 mM) before adding 25 nM or 250 nM Gal-1. (C) The percentage of polarized neutrophils was determined after incubation with medium, fMLP (1.14 nM) or 25 nM Gal-1 with or without pre-treatment with lactose (1 mM). Data shown are representative of at least three identical experiments.

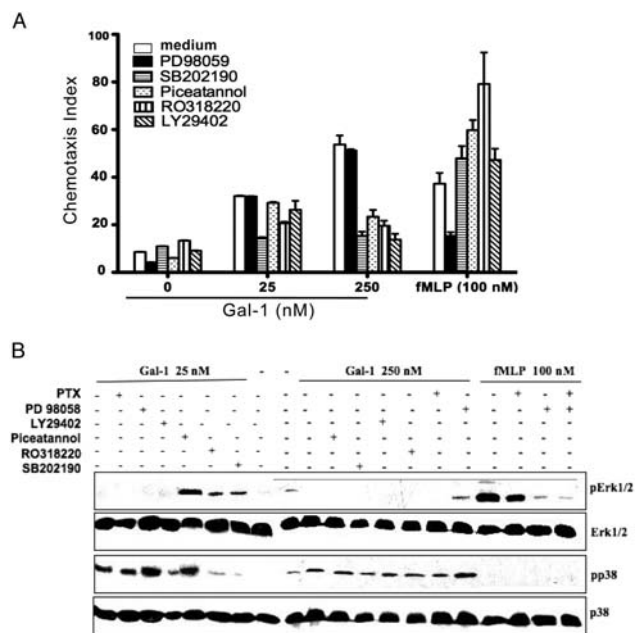
inhibitors for PI3K, spleen tyrosine kinase (Syk), PKC, extracellular signal-regulated kinase (ERK) and p38, since all these kinases have been implicated in the directional migration of neutrophils (Selvatici et al. 2006; Heit et al. 2002). We found that different signaling pathways controlled the directional migration and the chemokinetic movement of neutrophils. The directional migration induced by 25 nM Gal-1 was impaired when cells were pre-incubated with the p38 inhibitor and to a lesser extent with the PKC inhibitor, whereas the chemokinetic motility induced by 250 nM Gal-1 was clearly dependent on functional Syk, PKC, PI3K and p38. The 100 nM fMLP-induced migration was highly impaired when cells were incubated in the presence of the ERK inhibitor and augmented in the presence of the Syk, PKC, PI3K and p38 MAPK inhibitors (Figure 3).

Consistent with this, when evaluating the phosphorylation of ERK and p38 in neutrophils exposed to 25 or 250 nM Gal-1 for 5 min, we found that p38, but not ERK1/2, was phosphorylated when compared with control cells. Pre-incubation with the Syk/Zap70 (zeta-chain-associated protein kinase 70) inhibitor, the PKC inhibitor or the PI3K inhibitor before activation with 250 nM Gal-1 slightly diminished p38 phosphorylation. In contrast, when cells were stimulated with 25 nM Gal-1, p38 phosphorylation was dependent on PKC and to a lesser extent on PI3K activity. Surprisingly, blocking ERK1/2 resulted in enhanced phosphorylation of p38, suggesting that inhibition of the ERK1/2

pathway led to p38 activation. Pre-incubation with PTX had no effect upon ERK 1/2 or p38 phosphorylation; as expected, SB202190, a p38 MAPK inhibitor, inhibited the phosphorylation of p38 (Figure 3B). These results suggest that different signaling pathways control the chemokinetic or chemotactic movement induced by Gal-1. Both, the directional migration and the random migration are p38 MAPK- and PKC-dependent, but contrary to the chemokinetic movement, the chemotactic one is Syk/Zap70, PI3K-independent. As expected (Selvatici et al. 2006), stimulation with 100 nM fMLP resulted in ERK1/2 phosphorylation but not of p38 MAPK, and this was inhibited in the presence of the mitogen-activated protein extracellular kinase 1/2 inhibitor (Figure 3B). Collectively, these data support the idea that the response of neutrophils to different chemoattractants involves the participation of different signaling pathways.

#### *Galactin-1 binds to CD43*

CD43 and CD45, two abundant neutrophil cell surface molecules have been described to function as ligands for Gal-1 (Perillo et al. 1995; Nguyen et al. 2001). As a first step to assess the possibility that CD43 may be a receptor for Gal-1 on neutrophils, we evaluated whether pre-incubating the cells with different anti-CD43 antibodies that recognize different epitopes of the molecule would modify the binding of 5  $\mu$ M biotin-labeled Gal-1 to neutrophils. Pre-treatment of cells with



**Fig. 3.** Gal-1-induced migration of neutrophils is mediated through the p38 MAPK pathway. (A) Chemotaxis assay in response to Gal-1 in the presence of PD98058, SB202190, piceatannol, RO318220 or LY29402.

(B) Phosphorylation of the MAPK p38 and ERK1/2 of neutrophils pretreated with medium, PD98058, SB202190, piceatannol, RO318220 or LY29402 before stimulating with Gal-1 (25 or 250 nM) or fMLP (100 nM). The same membrane was stripped and blotted with anti-ERK1/2 or anti-p38. Experiments were done three times independently.

DFT.1 or the CBF.78 mAbs did not modify the binding of biotin-labeled Gal-1, whereas pre-incubation with MEM59, MC7 or L10 mAbs interfered with Gal-1 binding. Gal-1 binding was inhibited by 30% in the presence of L10, by 40% in that of MEM59 or MC7 and by around 60% when L10 and MEM59 were combined (Figure 4A), when compared with cells incubated only with Gal-1.

As a second step, neutrophils were treated with *Vibrio cholerae* sialidase, a exoglycosidase that catalyzes the hydrolysis of  $\alpha$ -2-3 and, at a much lower rate,  $\alpha$ -2-6-linked *N*-acetyl-neuraminic acid residues from oligosaccharides and that would thus uncover the binding sites for Gal-1 (Cross and Wright 1991; Remold-O'Donnell and Parent 1994). Although this enzyme is not specific for CD43, the relatively high abundance of CD43 on the cell surface allows evaluating the contribution of CD43 to the binding of Gal-1 on neutrophils. Following treatment with sialidase, cells were incubated with medium, several anti-CD43 mAbs or with the anti-CD45RO UCHL1 mAb (CD45RO being the most abundant isoform of CD45 on neutrophils) before assessing the binding of Gal-1 to the cells. Similar to a previous report (Dias-Baruffi et al. 2003), we found a 4.6-fold increase of Gal-1 binding when neutrophils were pre-treated with sialidase, suggesting that sialidase uncovered Gal-1-binding sites. This was partially inhibited (30%) in the presence of L10, MEM59 + L10 or MC7, suggesting that the anti-CD43 mAbs interfered with Gal-1 binding to the neutrophils' surface. On the contrary, UCHL1, the anti-CD45RO antibody and

MEM59 did not have any impact on Gal-1 binding to sialidase-treated neutrophils, consistent with the fact that both mAbs recognize sialyl-dependent epitopes (Alvarado et al. 1995; Hamann et al. 1996; Figure 4B).

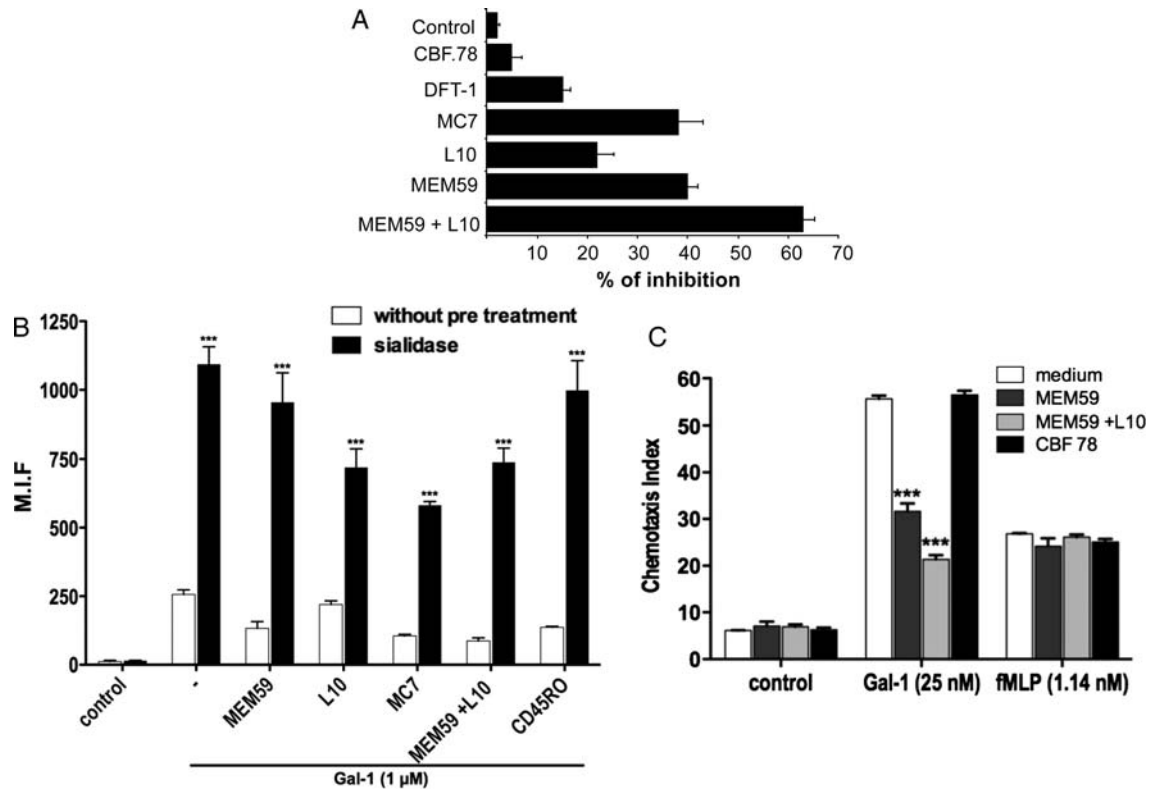
In order to evaluate if the Gal-1-induced chemotaxis was mediated through CD43, neutrophils were pre-incubated with the anti-CD43 mAbs MEM59, MEM59 + L10 or CBF78. While pre-incubating the cells with MEM59 or MEM59 + L10 clearly diminished the chemotactic migration toward Gal-1, the pre-incubation with the CBF78 mAb did not (Figure 4C). As expected, none of the CD43 antibodies affected the fMLP-induced chemotaxis, confirming the fact that fMLP and Gal-1 acted through different receptors.

Altogether, these data suggest that CD43 functions as a receptor for Gal-1 on human peripheral blood isolated neutrophils.

## Discussion

Galectins participate in multiple biological events including immune cell response and homeostasis. Notably, Gal-1, -3 and -8 have been shown to participate in the directional migration of monocytes, macrophages and neutrophils (reviewed in Rubinstein et al. 2004; Norling et al. 2009; Sato et al. 2009). Gal-1 is present in inflamed tissue and its secretion is up-regulated in inflammatory and activated rat peritoneal macrophages (Rabinovich et al. 1998). Most studies point at an anti-inflammatory role of Gal-1, possibly by modifying the physiological turnover of activated neutrophils and by inhibiting the chemotaxis and transmigration of PMN under acute inflammation conditions (La et al. 2003; Stowell et al. 2007; Cooper et al. 2008). Recent evidence has revealed that Gal-1 down-regulates T cell responses by contributing to the immunosuppressive capacity of CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> regulatory T cells (Garin et al. 2007) as well as by inducing apoptosis of thymocytes and T cells (Perillo et al. 1995, 1997), which is likely the mechanism by which it suppresses autoimmune diabetes (Perone et al. 2009). However, other studies suggest that Gal-1 could also have a pro-inflammatory role. Human Gal-1 activates NADPH-oxidase on primed neutrophils, but not on resting neutrophils (Almkvist et al. 2002). Consistent with this, and contrary to T lymphocytes, Gal-1 induces the exposure of phosphatidylserine to the membrane of fMLP-activated neutrophils, but not of resting neutrophils, independently of alterations in mitochondrial potential, caspase activation or cell death (Stowell et al. 2007, 2009).

Our study evidenced that, in vitro, Gal-1 promoted the directional migration of human peripheral blood neutrophils at 25 nM, while inducing random movement at 250 nM. Interestingly, we found that these different biological effects were under the control of distinct signaling pathways: the chemotactic effect was found to depend on MAPK p38 and PKC, whereas the chemokinetic movement of the cells depended on MAPK p38, PKC and Syk/Zap70 and PI3K. Moreover, we found that the intraperitoneal injection of Gal-1 (35 pmol), in the absence of additional inflammatory insults, had the capacity to attract neutrophils to the peritoneal cavity. However, as described previously (La et al. 2003), we found that the same concentration of Gal-1 inhibited PMN infiltration resulting of the injection of thioglycollate. Thus,



**Fig. 4.** Gal-1 promotes neutrophils migration presumably through CD43. (A) Cells were pre-incubated with the anti-CD43 mAbs (1  $\mu$ g/mL) in medium for 30 min prior to assess the binding of biot-Gal-1. (B) Neutrophils were pre-incubated with medium, sialidase, anti-CD43 (MEM59, L10, MC7, MEM59 + L10) or anti-CD45 (CD45RO) mAbs, and binding of biot-Gal-1 was assessed by flow cytometry. (C) Neutrophils were pre-incubated with medium or the indicated anti-CD43 mAbs before measuring the chemotactic response to Gal-1 (25 nM) or fMLP (1.14 nM). Similar results were obtained from two independent experiments.

depending on the environment, Gal-1 has a dual capacity: attract neutrophils in the absence of inflammatory signals or negatively regulate the migration of neutrophils to the inflammation site. It is conceivable that the neutrophils that migrate to the peritoneal cavity in response to Gal-1 alone have different capacities than those that migrate in response to thioglycollate and Gal-1. In addition, the possibility that Gal-1 and other galectins work in concert to balance the influx of specific populations of neutrophils to specific sites, ultimately regulating the amplitude of an inflammatory response cannot be excluded.

Gal-1 is not exclusively chemotactic for neutrophils, as it has also been shown to influence the migration of dendritic cells, monocytes as well as of retinal pigment epithelial cells in human proliferative vitreoretinopathy (Alge et al. 2006; Fulcher et al. 2009; Malik et al. 2009). Consistent with a role in promoting directional cell migration, we found that Gal-1 induced neutrophils polarization and actin polymerization, two indispensable events for chemotaxis (Wallace et al. 1984; Howard and Oresajo 1985), in a GPCR-independent manner. We confirmed that Gal-1-induced locomotion and polarization were carbohydrate-dependent. In accordance with a study indicating that high concentrations (10  $\mu$ M) of porcine Gal-1 induced neutrophil degranulation and  $O_2^-$  formation (Elola et al. 2005), we found that 5  $\mu$ M Gal-1 induced a reduction in neutrophils size, a change in cellular complexity/granulosity

and hexosaminidase release when compared with cells incubated with chemotactic doses of Gal-1 or control cells (Supplementary data, Figure S2).

Although many glycoproteins have lactosamine sequences, Gal-1 has been shown to preferentially bind different proteins of the extracellular matrix such as fibronectin, 90K/Mac-2BP as well as the cell surface receptors CD7, CD3, CD45 and CD43 (reviewed in Elola et al. 2005), of which only CD43 and CD45 are present on neutrophils. Gal-1 is known to bind all four isoforms of CD45, through both *N*- and *O*-glycans. Among those, CD45RO is the most abundant on neutrophils (Thomas 1989) but, different to the other isoforms, it expresses only *N*-glycans (Perillo et al. 1995). On the contrary, either glycoform of CD43 (the 115-kDa glycoform with the core 1 *O*-glycans and the 130-kDa glycoform with the core 2 *O*-glycans) exhibits around 80 *O*-glycans and only one *N*-glycan, suggesting that *O*-glycans would be primarily responsible for Gal-1 affinity for CD43 (Nguyen et al. 2001; Amano et al. 2003). In addition, studies with purified or synthetic oligosaccharides have evidenced that Gal-1 interacts with either core 2 or core 1 *O*-glycans, suggesting that Gal-1 could bind all isoforms of CD43 (Leffler and Barondes 1986; Leppanen et al. 2005; Sangeetha and Appukuttan 2005; Seelenmeyer et al. 2005; Hernandez et al. 2006; Baum et al. 1995).

Neutrophil CD43 membrane expression has been reported to be down-modulated in response to pro-inflammatory stimuli (TNF $\alpha$  or fMLP) by shedding the extracellular domain of the molecule by a proteolytic process, apparently regulated by cathepsin G and presenilin-gamma secretase (Campanero et al. 1991; Remold-O'Donnell and Parent 1994; Mambole et al. 2008). The diminished binding of biotinylated Gal-1 following the incubation of the neutrophils with the MEM59, MC7 and L10 anti-CD43 mAbs could be explained by the fact that the epitopes recognized by these antibodies on the CD43 molecule participate in Gal-1 binding and hence that the CBF.78 and DFT.1 recognize epitopes that are not involved in the Gal-1 binding. Alternatively, and although experiments were carried out all through in the presence of sodium azide, it is possible that the MEM59, MC7 and L10 mAbs induced specific intracellular signals that activate a proteolytic process that results in shedding the extracellular domain of CD43, and the loss of the binding sites for Gal-1 to human neutrophil's surface. Under this scenario, the fact that pre-incubating the cells with CBF.78 and the DFT.1 mAbs does not alter the binding of the biotin-labeled Gal-1 is indicative that these mAbs do not induce the intracellular signals that activate the shedding mechanism and that the epitopes they recognize are different from the ones recognized by Gal-1. Although additional experiments are needed to determine whether certain anti-CD43 mAbs mask the binding sites for Gal-1 or induce shedding of the extracellular domain, data shown here point that the CD43 molecule is a target for Gal-1.

One characteristic of CD43 is its high content in sialic acid residues at the extremity of *O*-glycan chains (Remold-O'Donnell and Rosen 1990). Addition of  $\alpha$ 2,6-linked sialic acid to the Gal $\beta$ 1,4GlcNAc sequence, the preferred ligand for Gal-1, inhibits the recognition of this saccharide ligand by Gal-1 (Amano et al. 2003). In line with previous studies (Perillo et al. 1995, Dias-Baruffi et al. 2003), we show that the binding of Gal-1 to the cells was augmented following sialidase treatment of the cells as the removal of the sialic acid residues uncovers Gal-1-binding sites, promoting the association of Gal-1 molecules to the cell surface, presumably to CD43. Our data suggest that Gal-1 binds CD43 on neutrophils and that this interaction is likely to be involved in cell migration. Whether CD43 relocates to the uropod in an Ezrin-Radixin-Moesin (ERM)-dependent fashion as described in response to chemotactic stimuli other than Gal-1, favoring adhesion and migration (Serrador et al. 1998; Yonemura et al. 1998; Seveau et al. 2000; Savage et al. 2002; Dehghani Zadeh et al. 2003), remains to be determined. Additional experiments are required to unequivocally determine if CD43 is implicated in the Gal-1-induced chemotaxis and degranulation processes as well as to better understand the *in vivo* implications for the dual function of Gal-1.

In conclusion, our study demonstrates that in the absence of inflammatory stimuli that will promote neutrophil's infiltration, Gal-1 induces migration and degranulation of non-activated neutrophils in a carbohydrate-dependent manner, possibly through the CD43 receptor. These results suggest that Gal-1 could have a dual function: it could act as an endogenous activator of innate immune cells and a pro-inflammatory molecule

under physiological conditions, yet, in acute inflammation, it would serve as an anti-inflammatory molecule. This is in agreement with previous work which demonstrated that multiple factors, such as activation status of the target cells, concentration and subcellular compartmentalization, would allow Gal-1 to function as either a pro- or a anti-inflammatory mediator (Rabinovich et al. 2004). This dual role may be exploited for immune intervention as Gal-1 could contribute to either boost immune responses or down-modulate undesirable responses as is the case of acute inflammation.

## Materials and methods

### *Galectin-1*

Human recombinant Gal-1, kindly provided by Dr Ishiro Kuwabara (School of Medicine of the University of California, Davis), was produced in *Escherichia coli* and purified by affinity chromatography on a lactosyl-sepharose column as described previously (Pace et al. 2003), with pyrogen-free water-based reagents. Gal-1 was biotinylated with NHS-Biotin (Pierce-Thermo Scientific, Rockford, IL), following the manufacturer's instructions. Endotoxin levels were found to be <0.5 EU, as determined by the limulus lyase assay. Gal-1 was stored in sterile PBS, in the absence of DTT (dithiothreitol).

### *Antibodies*

Several clones of anti-human CD43 mAbs were used: L10 (directed toward a sialic acid-independent epitope; Remold-O'Donnell et al. 1984), MEM59 (directed toward a sialic acid-dependent epitope; Stefanova et al. 1988), MC7 (directed toward a sialic acid-dependent epitope, kindly provided by Dr Sanchez-Madrid, Hospital de la Princesa, Spain), DFT-1 (Stross et al. 1989; directed toward a sialic-acid independent epitope, Santa Cruz Biotechnology Santa Cruz, CA), CBF.78 (directed toward a sialic acid-independent epitope; Tkaczuk et al. 1999). Additionally, rabbit anti-mouse IgG-Alexa-488 (Molecular Probes Life Technologies, Grand Island, NY), anti-human CD16-PE (3G8 clone, Caltag, Carlsbad, CA) and anti-CD45RO-FITC (UCHL1 clone, Caltag) were used.

### *Additional reagents*

The additional reagents used are PTX (Sigma-Aldrich, St. Louis, MO), PD98059 (Calbiochem-EMD4Biosciences, Merck KGaA, Darmstadt, Germany), Piceatannol (Calbiochem), SB202190 (Calbiochem), LY29402 (Calbiochem) and RO318220 (Calbiochem). Tissue culture medium RPMI 1640 (Hyclone, ThermoScientific), Ficoll 400, Dextran T-500 (Amersham Biosciences Piscataway, NJ), D-lactose (Sigma-Aldrich), sucrose (Sigma-Aldrich), avidin-FITC streptavidin-PE, BODIPY-phalloidin (Molecular Probes), calcein-AM (Molecular Probes) and  $\alpha$ 2-3,6,8-neuraminidase from *V. cholerae* (Calbiochem).

### *Cell preparation*

Neutrophils were isolated from heparinized peripheral blood from healthy adult donors using dextran sedimentation, Ficoll-Hypaque gradient centrifugation and hypotonic lysis

(Boyum 1968). All cells were washed and resuspended in the appropriate medium, according to the experiment. Purity of the neutrophils (>98%) was determined by flow cytometry as the percentage of CD16-positive cells. The local Bioethics Committee approved this procedure.

#### *Chemotaxis assays*

Chemotaxis assays were performed using a 48-well microchemotaxis chamber (Neuro Probe, Gaithersburg, MD) as described previously (Deng et al. 1999). Briefly, different concentrations of Gal-1 diluted in chemotaxis medium (RPMI 1640, 1% BSA) were placed in the bottom wells of the chamber. Neutrophils ( $50 \times 10^3$ /well) pre-treated for 30 min with  $10 \mu\text{g mL}^{-1}$  calcein AM were added to the upper wells, separated by a 5- $\mu\text{m}$  pore uncoated polycarbonate membrane (Neuro Probe) from the lower wells. fMLP (1.14 or 100 nM) was used as a positive control and chemotaxis medium alone as a negative control. After incubating at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  in a humidified chamber for 45 min, the membranes were rinsed with PBS and non-migrating cells were scrapped from the upper side. Cell migration was assessed by measuring the calcein fluorescence of cells on the lower side of the membrane with a digital image analyzer (Alpha Innotech, Fluorchem 8800 software). Results are shown as chemotaxis index relative to fluorescence levels in wells where no chemoattractant was added. To differentiate between chemotaxis and chemokinesis, experiments were carried out where the same concentrations of Gal-1 were added simultaneously to the upper and lower wells. For some experiments, neutrophils were pre-incubated at  $37^\circ\text{C}$  with lactose (1 mM), sucrose (1 mM), fMLP (1.14 or 100 nM), PTX ( $200 \text{ ng mL}^{-1}$ ), PD98059 (30  $\mu\text{M}$ ), Piceatannol (10  $\mu\text{M}$ ), SB202190 (10  $\mu\text{M}$ ), LY29402 (25  $\mu\text{M}$ ), RO318220 (10  $\mu\text{M}$ ) for 30 min or sialidase (0.02 U) for 10 min, or the different anti-CD43 antibodies and anti-CD45RO, prior to evaluate their migration toward Gal-1.

#### *Animals*

Ten-week-old male or female C57BL/6 mice (Janvier, Le Genest Saint-Isle, France) were kept in pathogen-free conditions with food and water available *ad libitum* and housed in a 12 h light/12 h dark (100–500 lux) cycle. The local institutional Animal Care and Use Committee approved animal experiments.

#### *In vivo model of inflammation*

C57BL/6 mice were injected i.p. with 1 mL 3% (wt/vol) thioglycollate (Sigma-Aldrich) dissolved in sterile PBS or with 1 mL of PBS, together with Gal-1 (0.5  $\mu\text{g}$  in 150  $\mu\text{L}$ ) or PBS (150  $\mu\text{L}$ ). Six hours after, mice were killed and 3 mL of cold PBS was injected i.p. to harvest peritoneal cells, which were then stained with anti-mouse Ly6G-PE and anti-mouse CD11b-PerCP (BD Biosciences, San Jose, CA). Cells and beads were then counted by FACSCalibur flow cytometry and data were analyzed with FlowJo (Ashland, OR). Cells being CD11b<sup>hi</sup>Ly6G<sup>+</sup> were considered to be neutrophils.

#### *Flow cytometry and binding experiments*

To evaluate the binding of Gal-1 to neutrophils, cells were incubated 30 min with biotinylated Gal-1 at the indicated concentrations, washed and incubated with avidin-FITC for 30 min. For the analysis with antibodies, Fc receptors were blocked by pre-incubating the cells with 10% human serum for 15 min, following which neutrophils were incubated with the different antibodies for 30 min at  $4^\circ\text{C}$ , washed with RPMI–2% FCS to remove excess antibody and further incubated with a rabbit anti-mouse IgG-Alexa-488 for an additional 30 min, on ice. Cells were washed, resuspended in fluorescent activated cell sorter (FACS) solution (2% FCS, 0.026%  $\text{NaN}_3$ , PBS, pH 7.4) and fixed with 2% paraformaldehyde. Analysis was performed on a FACSort (Becton Dickinson, San Jose, CA) using the CellQuest or FlowJo softwares.

#### *Cell polarization and actin polymerization*

To evaluate neutrophil polarization,  $1 \times 10^6$  neutrophils were incubated with Gal-1 at different concentrations, fMLP (1.14 nM) or chemotaxis medium at  $37^\circ\text{C}$  under gentle agitation for different time periods (1–30 min). Cells were then fixed with 2% *p*-formaldehyde for 30 min at room temperature, washed and resuspended in PBS/glycerol (1:1) and mounted on a microscope slide. To visualize actin polymerization,  $1 \times 10^7$  neutrophils were incubated with Gal-1, fMLP (as a positive control) or chemotaxis medium as a negative control for 3 min at  $37^\circ\text{C}$  under gentle agitation. Cells were then fixed with 2% *p*-formaldehyde for 15 min at room temperature, permeabilized with 3% saponin for 30 min, washed and incubated with BODIPY FL phalloidin (165 nM, Molecular Probes) on ice for 30 min; after washing, neutrophils were fixed again with 2% *p*-formaldehyde, spinned and resuspended in PBS/glycerol (1:1) to mount on a glass slide for microscopy. Pictures were taken at  $40\times$  on a Nikon Eclipse E600 microscope ( $40\times$ ) and the IPLAB software (Scanalytics, Rockville, MD).

#### *Cell activation and immunoblot*

Purified human neutrophils ( $2 \times 10^6$ /sample) resuspended in RPMI 1640 were incubated with Gal-1 (25 or 250 nM), fMLP (100 nM) or chemotaxis medium for the indicated periods of time at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ . In some cases, neutrophils were pre-treated for 30 min at  $37^\circ\text{C}$  with PTX ( $200 \text{ ng mL}^{-1}$ ) and/or with PD98059 (30  $\mu\text{M}$ ) or fMLP (100 nM), with Piceatannol (10  $\mu\text{M}$ ), SB202190 (10  $\mu\text{M}$ ), LY29402 (25  $\mu\text{M}$ ) or RO318220 (10  $\mu\text{M}$ ). Activation was stopped by adding ice-cold PBS and spinning the cells at 10,000 rpm for 10 s. The supernatant was then removed and the cell pellet was lysed by adding 50  $\mu\text{L}$  of lysis buffer (25 mM HEPES, pH 7.7, 150 mM NaCl, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM ethylenediaminetetraacetic acid, 0.5% Triton X-100, 0.5 mM DTT, 20 mM  $\beta$ -glycerophosphate, 1 mM  $\text{Na}_3\text{VO}_4$ , 5 mM NaF, 4 mM phenylmethylsulfonyl fluoride, 1  $\mu\text{g/mL}$  leupeptin, 1  $\mu\text{g/mL}$  aprotinin). Tubes were left under agitation at  $4^\circ\text{C}$  for 15 min and spun for 10 min at 10,000 rpm at  $4^\circ\text{C}$ . Total cell lysates (cellular equivalents) were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, proteins were transferred to nitrocellulose membranes and the phosphorylation of ERK1/2 and p38 were detected with anti-phospho-ERK1/2 (Santa Cruz Biotechnology)



and anti-phospho-p38 (Cell Signaling Technology, Danvers, MA), respectively, by chemiluminescence with enhanced chemiluminescence (Amersham Biosciences). Membranes were re-probed for ERK1/2 (Santa Cruz Biotechnology) and p38 (Cell Signaling).

#### Enzymatic treatment

Sialidase treatment of neutrophils was performed as described previously. Cells were then washed, centrifuged and resuspended either in FACS solution or chemotaxis medium.

#### Supplementary data

Supplementary data for this article is available online at <http://glycob.oxfordjournals.org/>.

#### Funding

This work was supported by grants from the Consejo Nacional de Ciencia y Tecnología (CONACyT) (Mexico) (U46505-M and 100275-M) to Y.R. and from the Ministère des Affaires Étrangères (France) and the Secretaría de Relaciones Exteriores (Mexico) to C.A.

#### Conflict of interest

None declared.

#### Abbreviations

BSA bovine serum albumin; DTT, Dithiothreitol; ERM, Ezrin Radixin Moesin; ERK, extracellular signal-regulated kinase; FACS, fluorescent activated cell sorter; FCS, fetal calf serum; FITC, fluorescein thiocyanate; mAb, mouse antibody; fMLP, formyl-methionyl-leucyl-phenylalanine; FPR, formyl peptide receptor; FPRL-1, formyl peptide receptor like-1; GPCR, G protein coupled receptor; HEPES, (4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid); IL, interleukin; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol-3-phosphate kinase; PKC, protein kinase C; PMN, polymorphonuclear leukocytes; PTX, Pertussis toxin; Syk, spleen tyrosine kinase; TNF, tumoral necrosis factor Zap70, zeta-chain-associated protein kinase 70.

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