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Galectin-8 elicits pro-inflammatory activities in the endothelium

Valentina Cattaneo[2†], María Virginia Tribulatti[2†], Julieta Carabelli[2], Agostina Carestia[3], Mirta Schattner[3], and Oscar Campetella[1,2]

[2]Laboratorio de Inmunología Molecular, Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomus (IIB-INTECH), Universidad Nacional de San Martín-Consejo Nacional de Investigaciones Científicas y Técnicas (UNSAM-CONICET), San Martín B1650HMP, Buenos Aires, Argentina; and [3]Laboratorio de Trombosis Experimental, Instituto de Medicina Experimental (IMEX), CONICET-Academia Nacional de Medicina, C1425AUM Ciudad Autónoma de Buenos Aires, Argentina

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Galectins (Gals), a family of mammalian lectins, play diverse roles under physiological and pathological conditions. Here, we analyzed the tandem-repeat Gal-8 synthesis, secretion and effects on the endothelium physiology. Gal-8M and Gal-8L isoforms were secreted under basal conditions by human microvascular endothelial cells (HMEC-1). However, expression and secretion of the Gal-8M isoform, but not Gal-8L, were increased in response to bacterial lipopolysaccharide (LPS) stimulus and returned to control values after LPS removal. Similarly, cell surface Gal-8 exposure was increased after stimulation with LPS. To evaluate Gal-8 effects on the endothelium physiology, HMEC-1 cells were incubated in the presence of recombinant Gal-8M. Pretreated HMEC-1 cells became proadhesive to human normal platelets, indicating that Gal-8 actually activates endothelial cells. This effect was specific for lectin activity as it was prevented by the simultaneous addition of lactose, but not by sucrose. Endothelial cells also increased their exposition of von Willebrand factor after Gal-8 treatment, which constitutes another feature of cell activation that could be, in turn, responsible for the observed platelet adhesion. Several pro-inflammatory molecules were abundantly produced by Gal-8 stimulated endothelial cells: CXCL1 (GRO-α), GM-CSF, IL-6 and CCL5 (RANTES), and in a lower degree CCL2 (MCP-1), CXCL3 (GRO-γ) and CXCL8 (IL-8). In agreement, Gal-8M induced nuclear factor kappa B phosphorylation. Altogether, these results not only confirm the pro-inflammatory role we have already proposed for Gal-8 in other cellular systems but also suggest that this lectin is orchestrating the interaction between leukocytes, platelets and endothelial cells.

Keywords: endothelial cell / galectin-8 / inflammation / platelets

Introduction

Galectins (Gals), a family of mammalian lectins, are involved in a wide spectrum of biological processes such as proliferation, apoptosis, adhesion, migration and cytokine secretion, among others (Elola et al. 2007; Liu and Rabinovich 2010). Moreover, Gals have immune regulatory functions and play important roles in the homeostasis of the immune system (Norling et al. 2009; Cattaneo et al. 2011). These lectins contain carbohydrate-recognition domains (CRDs) structurally conserved through evolution. Based on their structure, they are classified into: prototype (one CRD, Gal-1, etc.), tandem-repeat (two linked CRDs, Gal-4, -8, etc.) and chimera (one CRD fused to a non-lectin domain, only Gal-3).

Gal-8 belongs to the tandem-repeat group containing two different CRDs joined by a linker peptide of variable length that works as a hinge. Gal-8 is widely expressed by different organs and tissues in both normal and pathological conditions, such as human cancers (Patnaik et al. 2006). Recent results indicate that Gals are also implicated in the pathogenesis of different cardiovascular diseases, particularly in atherosclerosis (Al-Ansari et al. 2009). We have recently shown that Gal-8 is a potent platelet activator, supporting a role for this lectin in thrombosis and inflammation (Romaniuk et al. 2010). The vascular endothelium produces, synthesizes and releases a broad spectrum of soluble substances and expresses adhesion molecules capable of modulating physiopathological responses. Under normal conditions, the vascular endothelium presents an antiadhesive, anticoagulant, antiaggregant and anti-inflammatory surface. Endothelial injuries by physical or chemical agents result in the opposite cellular phenotype. It has been reported that Gal-8 is expressed and exerts angiogenic functions on endothelial cells (Delgado et al. 2011). We have previously shown that human platelets express two splice variants of Gal-8 differing in the linker length (Gal-8M and Gal-8L, for medium and long, respectively), and most important, they expose the lectin on their surface after thrombin stimulation (Romaniuk et al. 2010). These results suggest that activated platelets are a source of Gal-8 at inflammatory processes. In addition, it is reported that Gal-8 exposure on the surface of vascular cells is increased upon activation (Thijssen et al. 2008). Then, endothelial cells could become another source of Gal-8 contributing to platelet activation and inflammation. In this regard, the aim of the present study was to unravel the possible role of Gal-8 in the regulation of inflammatory and hemostatic responses in the vascular endothelium.

Results

Gal-8M isoform is released by activated endothelial cells

To analyze Gal-8 expression under stress conditions, HMEC-1 cells were incubated with LPS, a highly pro-inflammatory

[1]To whom correspondence should be addressed: Tel: +54-11-45001600; Fax: +54-11-40061559; e-mail: oscar@iib.unsam.edu.ar; ocampetella@gmail.com
[†]These authors contributed equally to this work.

molecule. Then, Gal-8 content was analyzed in cell lysates and culture supernatants at different time points. While both Gal-8M and Gal-8L were detected in cell culture supernatants, only Gal-8M was detected in cell lysates (Figure 1A and B). The absence of Gal-8L in the pellets fraction might be due to a constitutive production and continuous transport of this isoform without visible accumulation at the cytoplasm.

Interestingly, Gal-8M secretion to the extracellular milieu increased after 16 h of LPS treatment, reaching a maximum value at 24 h that remained after 72 h of culture (Figure 1B; Supplementary data, Figure S1). No changes in secreted Gal-8L levels were registered at any of the analyzed time points. The increase in Gal-8M secretion was LPS dependent because its removal after 24 h of treatment resulted in normalization of the lectin levels. Conversely, no variations of intracellular Gal-8M were observed after LPS removal (Figure 1C). Notably, the rise of extracellular Gal-8M was preceded by a decrease of the cytosolic fraction at 5 h post-LPS treatment that normalized after 16 h (Figure 1A). These observations strongly suggest that activation with LPS promotes both the secretion of Gal-8 that is already stocked at the cytosol, and its synthesis de novo to replenish intracellular protein content.

Gal-8 is exposed at the surface of activated endothelial cells

To analyze if Gal-8 was exposed at the cell surface during its release, cells were treated with LPS, and Gal-8 content was analyzed by flow cytometry under non-permeabilizing conditions. As shown in Figure 2A, Gal-8 content increased at the cell surface after 24 h of LPS addition; however, the intracellular Gal-8 content did not significantly increased at this time point. In order to analyze whether Gal-8 exposure is mediated by lectin–glycan interaction at the cell surface, LPS-stimulated HMEC-1 cells were incubated with lactose as to compete endogenous Gal-8 binding. As shown in Figure 2B, lactose treatment, but not sucrose, significantly reduced Gal-8 exposure suggesting that, after secretion, Gal-8 can be attached at the cell surface by interaction with its glycan ligands. These findings support that the lectin is actively secreted to the extracellular media after LPS stimulus rather than being noticeably accumulated on vascular endothelium surface. Unfortunately, no discrimination among Gal-8 isoforms can be done in these assays because antibodies equally recognize both lectins; however, since Gal-8L content remained unchanged, the Gal-8M isoform is most probably the one exposed at the cell surface.

Gal-8 treatment promotes platelet adhesion to endothelial cells

After LPS stimulus, Gal-8 was exposed and secreted by the endothelial cells, then raising the question whether the lectin is also involved in amplifying the inflammatory signals to the neighboring cells, thus acting in a paracrine way. It is worth to

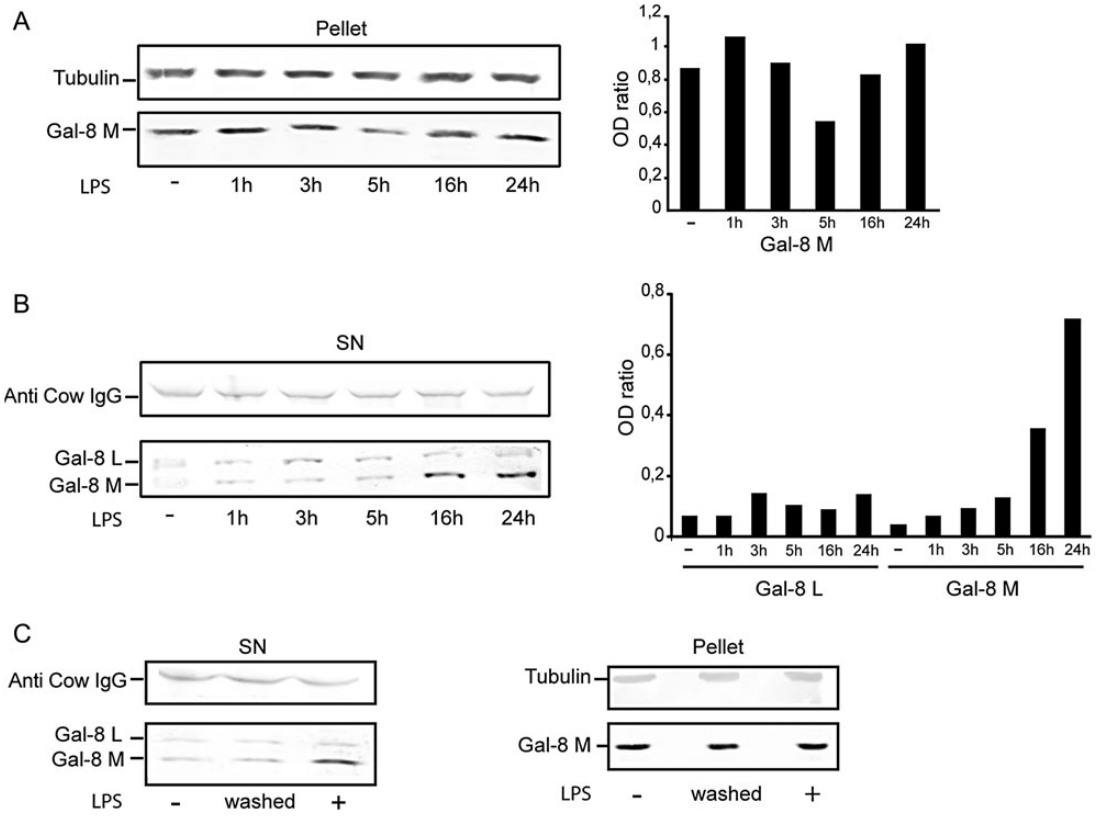

Fig. 1. Gal-8 expression in activated HMEC-1 cells. Western blot shows Gal-8 protein expression in (**A**) pelleted cell lysates (pellet) and (**B**) in culture supernatant (SN). Cells were incubated with 500 ng/mL of LPS for the times indicated. Blots were reacted with affinity-purified anti-human Gal-8 antibodies and signal intensity quantified by ImageJ. Tubulin for cell lysates and cow IgG in culture supernatants were tested to normalize protein load. OD ratio was calculated by dividing OD values from the sample and its corresponding control. (**C**) Western blot assays were carried out as before but LPS was removed after 24 h of incubation (washed), or not (+). In all cases cells were collected at 48 h. Western blots are representative of four independent experiments.

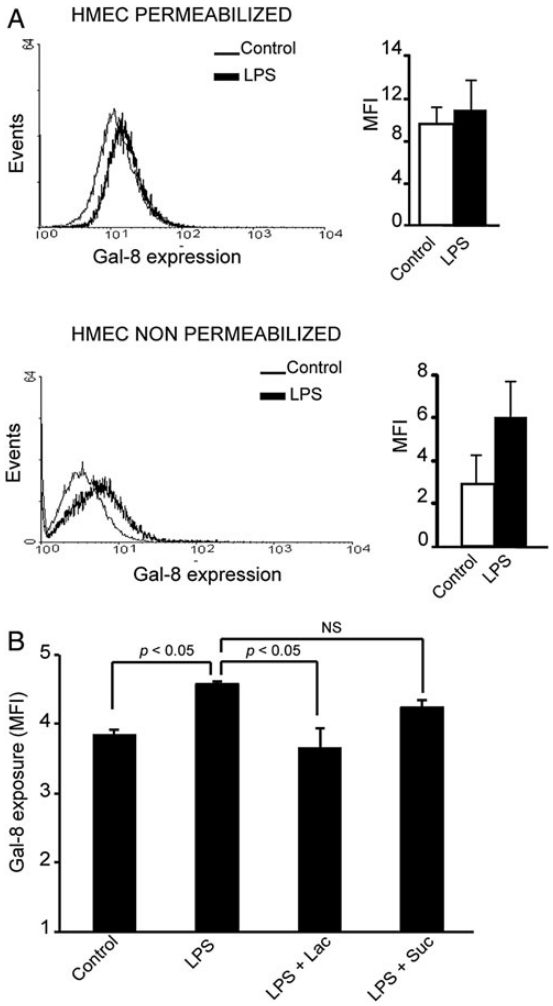

Fig. 2. Gal-8 is exposed at the surface of activated endothelial cells. (**A**) HMEC-1 cells were incubated with 500 ng/mL of LPS for 24 h before flow cytometry assays. Histograms show Gal-8 expression in permeabilized (upper) or at the cell surface (lower). (**B**) HMEC-1 cells were incubated with 500 ng/mL of LPS for 6 h and then washed with 200 mM lactose (Lac) or sucrose (Suc) before flow cytometry assays. Histograms are representative of three independent experiments. MFI, Mean Fluorescence Intensity.

mention that platelets are another source of Gal-8 during inflammation that can account for Gal-8 effects on the endothelium (Romaniuk et al. 2010). Platelets do not adhere to the endothelium under normal conditions, but they bind to activated endothelial cells under several circumstances (Ferroni et al. 2012; Jones et al. 2012). To evaluate whether extracellular Gal-8 promotes endothelial cell activation, we studied platelet-binding capacity to Gal-8-stimulated HMEC-1 cells. For this purpose, endothelial cells were incubated 16 h with increasing amounts of Gal-8M. As shown in Figure 3A, naive platelets readily bound to Gal-8 stimulated HMEC-1 cells while they were unable to adhere to unstimulated cells. The increment in platelet adhesion was evident from 0.1 μM and reached a maximum level at 2 μM of Gal-8. This effect was specific of lectin activity as it was prevented when HMEC-1 cultures where pretreated with 50 mM of lactose, but not with sucrose (Figure 3B). Preincubation of Gal-8 with lactose previous to the addition to cell cultures yielded the same negative results (data not shown). As reported previously, apoptotic endothelial cells become pro-adhesive for non-activated platelets (Bombeli et al. 1999); hence, we tested whether Gal-8 treatment induced apoptosis of HMEC-1 cells. After 48 h of treatment with different concentrations of Gal-8M, no evidence of apoptosis was observed by propidium iodide staining, ruling out this possibility (Figure 3C).

Gal-8 activates endothelial cells and induces von Willebrand factor expression

We have already demonstrated that Gal-8 is able to directly mediate platelet adhesion (Romaniuk et al. 2010), thus raising the question whether Gal-8 triggers an activation pathway that renders endothelial cells pro-adhesive for platelets or if it mediates adhesion per se by acting as a linker between platelets and cells. To address this question, we first investigated if the added Gal-8M remained attached to HMEC-1 cells after extensive wash. No differences in Gal-8 signal were observed by flow cytometry between Gal-8M-treated and control HMEC-1 cells (data not shown), thus ruling out a cross-linker role for Gal-8. The release of von Willebrand factor (vWF) is known to mediate the binding between platelets and activated endothelium (Padilla et al. 2004). vWF is stored in the Weibel–Palade bodies of endothelial cells and in the α-granules of platelets. Although vWF is constitutively released to the bloodstream, endothelial cells increase its expression and release in response to inflammatory stimuli. In order to elucidate if Gal-8 was able to induce vWF release, HMEC-1 cells were incubated with Gal-8M for 16 h and vWF surface exposure was tested by flow cytometry. As shown in Figure 3D and E, Gal-8 stimulation resulted in a significant increase in vWF exposure at the cell surface that was prevented in the presence of lactose, but not by sucrose, denoting that lectin activity is involved. As a whole, these findings argue in favor of an activating role for Gal-8 at the endothelium that promotes platelet adhesion and vWF expression.

Gal-8 induces secretion of pro-inflammatory cytokines

In search of other pro-inflammatory mediators, we extended the present study by analyzing the cytokine profile of Gal-8M-stimulated HMEC-1 cells. For this purpose, endothelial cells were incubated for 16 h in the presence of 2 μM of Gal-8M. Lectin concentration and incubation time were selected based on the maximum activity achieved in platelet adhesion assays (see Figure 3A). Data from a representative cytokine-array screening assay is shown in Figure 4A. Quantification of dot signals indicated that Gal-8-treated HMEC-1 cells abundantly produced CXCL1 (GRO-α), GM-CSF, IL-6 and CCL5 (RANTES), and to a lower degree, CCL2 (MCP-1), CXCL3 (GRO-γ) and CXCL8 (IL-8) (Figure 4B).

Growing evidence shows the relevance of nuclear factor kappa B (NFκB) to induce the coordinated expression of pro-inflammatory genes after endothelial cell activation (Ferre et al. 2010). Having found that Gal-8 promoted the release of several inflammatory cytokines, we analyzed the phosphorylation of NFκB p65 subunit, an event required for its translocation to the nucleus. As shown in Figure 4C, a significant increase of p65 subunit phosphorylation was evident after 10 min of Gal-8 stimulation, which decays after 20 min. This effect was

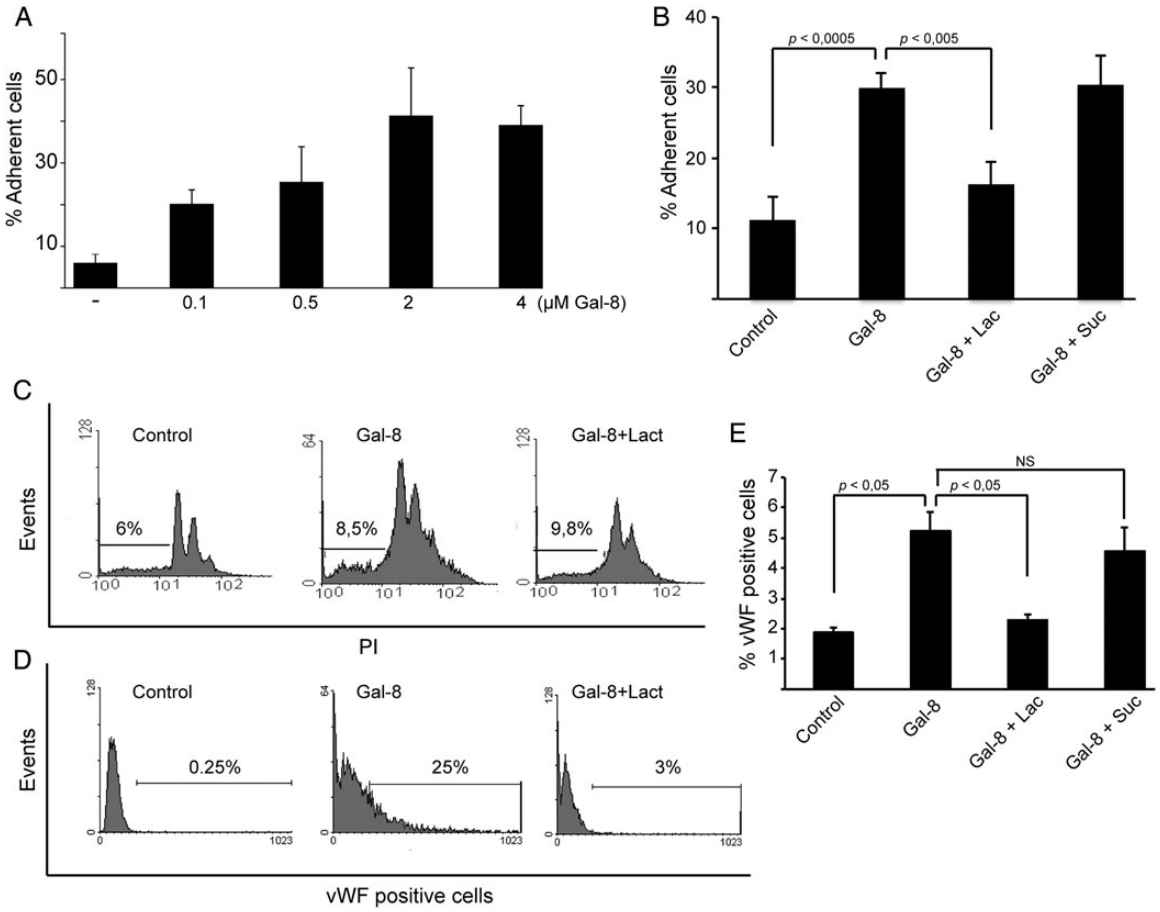

Fig. 3. Gal-8 activates HMEC-1 cells. Non-activated washed platelets were allowed to adhere for 1 h to HMEC-1 cells stimulated with the indicated amount of Gal-8 (**A**) or 2 μM of Gal-8 in the presence of lactose or sucrose (**B**). Platelet adhesion is shown as the percentage of the total platelet added. (**C**) Flow cytometry histograms showing propidium iodide (PI) staining of HMEC-1 cells cultured for 48 h in the presence of 2 μM of Gal-8. (**D**) Flow cytometry histograms showing vWF surface expression of HMEC-1 cells after 16 h incubation with 2 μM of Gal-8. (**E**) Histograms showing lack of inhibition of vWF expression in the presence of sucrose. In all cases, lactose (Lac) and sucrose (Suc) were added at 50 mM, 30 min before addition of Gal-8. Control, cells assayed in the absence of Gal-8 addition. Adhesion assays are representative of four independent experiments.

prevented by preincubation with 50 mM lactose, denoting specificity for lectin activity. Altogether, these results confirm that Gal-8 actually triggers an activation state in endothelial cells, thus promoting platelet adhesion, increased exposure of vWF and release of several pro-inflammatory cytokines.

Discussion

Gals are involved in endothelial cell activation (Thijssen et al. 2008) and tumor angiogenesis (Thijssen et al. 2006). In this context, a key function for Gal-8 in angiogenesis has been demonstrated (Delgado et al. 2011) together with its increased expression at endothelial cells of blood vessels surrounded by perivascular inflammatory infiltrates (Stancic et al. 2011). Our previous studies show that Gal-8 behaves as a pro-inflammatory-like molecule in different cells of the immune system and platelets (Tribulatti et al. 2009; Romaniuk et al. 2010; Cattaneo et al. 2011; Tribulatti et al. 2012). Gal-8 has been also proposed to play a role in a mouse model of joint inflammation and in synovial cells from rheumatoid arthritis patients, where high concentrations of Gal-8 are found (Eshkar Sebban et al. 2007). Here, we report that LPS stimulation of endothelial cells induced secretion of the Gal-8M isoform, which persisted as long as LPS was present in culture. Coincidently, Gal-8 exposure at the cell surface was increased after LPS treatment, an event probably required as a previous step in the secretion process that might be dependent on transient binding to glycoconjugates. In contrast, the intracellular levels of Gal-8M remained constant despite of its secretion to the milieu, indicating that it was replenished by synthesis de novo. Unlike the inducible increase of Gal-8M secretion under LPS stimulus, Gal-8L isoform remained unchanged in culture supernatants and undetectable in the intracellular fraction. Interestingly, Thijssen et al. (2008) have found that culturing human umbilical endothelial cells in the presence of 20% human serum resulted in a 3-fold increment in Gal-8 protein exposure at cell surface, without an obvious increment in total protein synthesis together with a decrease in mRNA levels. These results partially agree with ours; however, we cannot directly contrast findings due to the different nature of the stimuli used and also because the secretion of the protein is not considered by those authors. There is evidence of other members of the

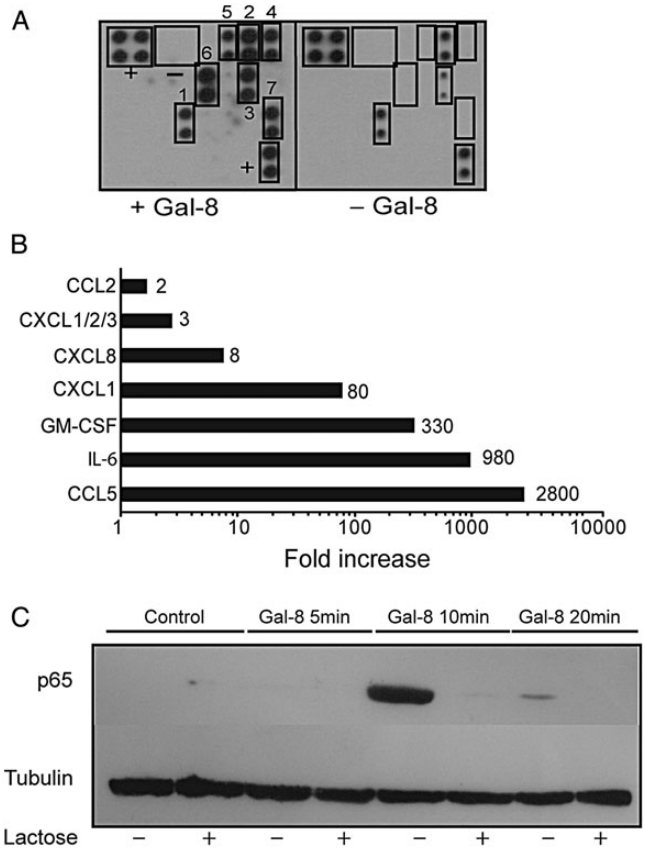

Fig. 4. Pro-inflammatory cytokines are expressed in Gal-8-stimulated HMEC-1 cells. Human cytokine-array analysis was used to determine the release of cytokines from Gal-8-treated cells. (**A**) Representative arrays filters incubated with conditioned media of control cells (right) or treated with 2 μM of Gal-8 (left). Numbers indicate the position of different cytokines: (1) CCL2, (2) CXCL3; (3) CXCL8, (4) CXCL1, (5) GM-CSF, (6) IL-6, (7) CCL5 . (+) Positive and (−) negative controls. (**B**) Arrays were scanned to determine the signal of the protein spots. Fold increases for reacting cytokines are shown. The numbers on the right end of each bar represents the fold increase value. (**C**) NFκB p65 subunit phosphorylation. HMEC-1 cells were incubated with Gal-8 2 μM for 5, 10 or 20 min. Western blots were reacted with anti-human NFκB p65 antibodies. Lac, 50 mM lactose was added 30 min before Gal-8. The cytokine array shown is a representative one of two independent experiments. Western blots are representative of three independent experiments.

galectin family whose levels are increased upon activation of cultured endothelial cells, such as surface Gal-1 and Gal-3, albeit they seemed to act as anti-inflammatory molecules (He and Baum 2006; Dabelic et al. 2012).

After demonstrating its secretion to the milieu, we searched for Gal-8 participation in the platelet–endothelium interaction. Platelet adhesion is a complex process that includes interactions between components of the extracellular matrix and receptors expressed at the platelet surface. A variety of inflammatory states may result in platelet adhesion to endothelial cells in the absence of denudation, and without evidence of significant alterations in the endothelial integrity (Chen and Lopez 2005). Here, we demonstrate that platelets adhered to Gal-8-treated HMEC cells, strongly suggesting that Gal-8 is actually triggering an activation state on endothelium in an inflammatory context. In addition, NFκB p65 subunit phosphorylation was observed in Gal-8-treated HMEC cells (Figure 4C). Platelet adhesion to the endothelium may result from inhibition of the endogenous mechanisms preventing platelet adhesion and/or by inducing endothelial release of molecules like vWF and P-selectin from the Weibel–Palade bodies that binds to platelet GPIb to promote adhesion (Wagner 1993). According to this, vWF exposition was found increased after Gal-8 treatment (Figure 3C), suggesting that this molecule is probably responsible for the platelet proadhesive phenotype observed on Gal-8-activated endothelial cells. Interestingly, it has been recently shown that Gal-1 and Gal-3 are associated with vWF in endothelial cells, where they modulate vWF-mediated thrombus formation (Saint-Lu et al. 2012). Additional proadhesive roles have been proposed for human Gal-8, since we have shown that immobilized Gal-8 triggered platelet adhesion and spreading (Romaniuk et al. 2010); and also it induced a firm but reversible adhesion of peripheral blood neutrophils (Nishi et al. 2003). Moreover, Gal-8 induces adhesion of different peripheral blood leucocytes such as eosinophils, monocytes, T and B cells to human umbilical vein endothelial cells by acting as a linker between the cells (Yamamoto et al. 2008). These results contrast with those reported here where a platelet proadhesive phenotype was induced on Gal-8 activated endothelium cells, probably mediated by vWF exposition, but not by acting as a linker between cells. Endothelial activation markers were prevented in the presence of lactose, but not sucrose, indicating that specific lectin–glycan interactions were involved. Unfortunately, no neutralizing antibodies specific against Gal-8 are available and attempts to silence the Gal-8 expression by shRNAi were unsuccessful (not shown) due to the low cell passages number allowed by HMEC-1. Then the question about the existence of an additive effect of endogenous galectin on the added recombinant protein still remains, as well as the participation of endogenous Gal-8 in the LPS activation pathway.

Another characteristic of the vascular endothelium is the expression and release of diverse inflammatory molecules. Our results show that Gal-8 induces the production of various chemokines and cytokines, namely CCL2, CXCL3, CXCL8, CXCL1, GM-CSF, IL-6 and CCL5. The most dramatically upregulated chemokine induced by Gal-8 was CCL5. Interestingly, this molecule is involved in chronic inflammation by the recruitment of inflammatory cells (Suffee et al. 2011) which is in agreement with the pro-inflammatory function of Gal-8. GM-CSF is secreted by macrophages and endothelial cells, among others, and stimulates stem cells to produce granulocytes and monocytes (Di Gregoli and Johnson 2012). The IL-6 signaling pathway in endothelial cells and smooth muscle cells alters leukocyte recruitment, which also could contribute to the inflammatory role proposed for Gal-8 in this endothelial context (McPherson and Davies 2012). CXCL1 has neutrophil chemoattractant activity and is involved in inflammation processes and wound healing (Devalaraja et al. 2000). CXCL8 induces chemotaxis in neutrophils and granulocytes, primarily secreted by monocytes, macrophages and dendritic cells. CCL2 is involved in the pathogenesis of several diseases characterized by monocytic infiltrates, such as rheumatoid arthritis and atherosclerosis (Xia and Sui 2009), while CXCL3 is involved in monocytes adhesion and migration. Therefore, Gal-8 induces a plethora of pro-inflammatory molecules in the endothelium that might not only trigger the local platelet adhesion/activation processes but also fuels a given

pathological abnormality such as atherosclerosis. In this context, Gal-8 may orchestrate the inflammatory interaction among leukocytes, platelets and endothelial cells and provides a direct linkage among hemostasis, infection, inflammation and the development of atherosclerosis.

Materials and methods

Cell culture

Human microvascular endothelial cells (HMEC-1) were cultured in MCDB 131 medium (Invitrogen), supplemented with 10 ng/mL epidermal growth factor (Becton-Dickinson) and 1 μg/mL hydrocortisone (Sigma). Cells were cultured in 5% CO_2 at 37°C in the presence of 10% fetal bovine serum (FBS) (Invitrogen), 2 mM glutamine, and gentamycin (complete media), and used for <16 passages.

Expression of recombinant proteins

The human Gal-8M isoform-encoding gene was subcloned into pTrcHisB (Cattaneo et al. 2011). Conditions for protein expression and purification by Lactosyl-Sepharose (Sigma) followed by immobilized metal affinity chromatography were identical to previously described for mouse Gal-8 (Tribulatti et al. 2007). The lectin activity of the recombinant protein was tested by hemagglutination assays (Tribulatti et al. 2007). Endotoxin was quantified by Pyrotell *Limulus polyphemus* amebocyte lysate assay (Cape Cod). Sample endotoxin levels were considered acceptable at values <0.25 U/mL. All experiments using recombinant Gal-8 were done in parallel with the same concentration of the recombinant protein plus 7 μg/mL of Polymixin B (USB) as control.

Western blot

HMEC-1 cells (7.5×10^5) were cultured in 1500 μL of complete medium in 6-well plates and incubated for 24 h or until reaching confluence. Cells were cultured with 1% FBS for 24 h before adding 500 ng/mL of LPS from *Escherichia coli* O111: B4 (Sigma) and collected at 1, 3, 5, 24, 48 or 72 h thereafter. Cells were scrapped, washed with cold phosphate-buffered saline (PBS) and pellets resuspended in 150 μL cracking buffer 1× and sonicated for 15 s in the presence of a cocktail of protease inhibitors (Sigma). For Gal-8 detection, the total protein content in the culture supernatants was precipitated with trichloroacetic acid (Sigma) overnight at 4°C. After spinning for 30 min at 13,000 rpm at 4°C, the pellet was resuspended in cracking buffer 1× and pH was adjusted. Cell extracts and concentrated supernatants were run in 10% SDS–PAGE and then electrotransferred to nitrocellulose membranes (GE, Life Technologies). Blots were probed with an in house-made affinity-purified rabbit immunoglobulin G (IgG) anti-human Gal-8 followed by horseradish peroxidase (HRP)-labeled goat secondary antibodies 1/5000 (Sigma), and developed by chemiluminescence (Pierce).

Immunofluorescence

HMEC-1 cells were cultured and stimulated as described above. After culture, cells were harvested, washed with cold PBS cells and fixed with 2% *p*-formaldehyde in PBS for 20 min at room temperature (RT). Where is stated, cells were permeabilized in the presence of 0.1% Triton X-100 in PBS for 2 min. Then cells were washed and incubated with 0.5% BSA on ice for 30 min before the addition of affinity-purified rabbit anti-human Gal-8 antibodies. After 1 h, cells were washed and incubated with Alexa 488-labeled anti-rabbit secondary antibody (Invitrogen). In order to remove Gal-8 from the surface, cells were washed with cold PBS and incubated in the presence of 200 mM lactose (Calbiochem) or sucrose (Sigma) for 15 min on ice, before fixation. A FlowMax cytometer PASIII (Partec, Münster, Germany) and WinMdi 2.9 software were used to analyze data.

p-NFκB detection

An identical procedure as for western blot was followed, except that after serum starvation, HMEC-1 cells were incubated with 2 μM Gal-8 for 5, 10 or 20 min and blots were probed with polyclonal rabbit anti-human p-NFκB p65 (Ser_{311}) (Santa Cruz) 1/200, followed by HRP-labeled goat anti-rabbit IgG 1/5000 (Sigma).

Preparation of human platelets

Blood samples were obtained from healthy donors who had not taken non-steroidal anti-inflammatory drugs for 7 days prior to sampling. This study was performed according to institutional guidelines (Academia Nacional de Medicina, Buenos Aires, Argentina) and approved by the Institutional Ethics Committee. All participants provided written consents. Blood was drawn directly into plastic tubes containing 3.8% sodium citrate (9:1). For washed platelets suspensions, platelet-rich plasma was centrifuged in the presence of prostacyclin [PGI2 (prostaglandin I2)] (75 nM), and platelets were then washed with washing buffer (140 mM NaCl, 10 mM $NaHCO_3$, 2.5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgCl_2$, 22 mM sodium citrate, 0.55 mM glucose and 0.35% BSA, pH 6.5). Washed platelets were resuspended in Tyrode's buffer and the platelet number was adjusted to 1×10^8 cells/mL.

Platelet adhesion to endothelial cells assay

HMEC-1 cells were seeded at 2.5×10^4 cells in 96-well plates in 150 μL of complete medium. When cells reached confluence, they were incubated with recombinant Gal-8 for 16 h. Then, cells were blocked with 100 μL heat-inactivated BSA (5 mg/mL) for 1 h at RT. After three washes, 50 μL of a suspension of washed platelets was added to each well and incubated for 1 h at 37°C. Then, the plate was washed four times with PBS to remove all non-adherent platelets, and 150 μL of acid phosphatase substratum (5 mM *p*-nitrophenyl phosphate, 0.1 M citrate, 0.1% Triton X-100, pH 5.4) was added. After incubation for 1 h in the dark at RT, the reaction was stopped by adding 100 μL of 2 N NaOH to each well. The colorimetric signal was registered at 405 nm (Microplate Reader, Bio-Rad). The adhesion values are expressed as percentages of the total adherent platelets. The total activity of those wells containing unwashed cells and platelets represents 100%.

vWF exposure

HMEC-1 cells (7.5×10^5) were plated in 500 μL of complete medium in 24-well plates. Cells were allowed to adhere and grow to confluence and then incubated for 16 h with Gal-8 2 μM. vWF surface exposure of HMEC-1 cells was analyzed

by flow cytometry. Rabbit anti-vWF monoclonal antibody was used followed by an FITC-conjugated secondary antibody anti-rabbit IgG (both from DAKO).

Human cytokine array

HMEC-1 cells were seeded at 7.5×10^5 in 6-well culture plates and allowed to reach confluence. Cells were serum-starved for 24 h before adding 2 μM of Gal-8 and incubated for another 16 h. After incubation, conditioned medium was removed and cell debris pelleted by centrifugation at $500 \times g$, for 15 min at 4°C. Cytokine detection was carried out on membranes spotted with an array of specific antibodies following manufacturer's recommendations (RayBiotech). Modern names of cytokines are indicated and used along the article with the old name in brackets for quick reference. The anti-CXCL1/2/3 (GRO) included, is a pan antibody detecting CXCL1 (GRO-α), CXCL2 (GRO-β) and CXCL3 (GRO-γ) with ∼1000× greater affinity to CXCL3 (GRO-γ) and then its reactivity was considered as indicative of CXCL3 (GRO-γ) presence. Briefly, membranes were blocked before 1 mL of conditioned medium was added and incubated at RT for 2 h. Membranes were washed and 1 mL of diluted primary biotin-conjugated antibody was added. After 30 min at RT membranes were washed and HRP–streptavidin added for 30 min at RT. Membranes were developed by chemiluminiscence (Pierce). Densitometric plots were quantified with the ImageJ software (NIH). Signal from each spot was normalized to the signal of the positive control spots on each array. Then the normalized signal from the treated sample array was compared with the normalized signal from untreated control arrays to obtain the relative values. When spots were absent, the background was quantified instead.

Statistical analysis

Student's *t*-test was used.

Supplementary data

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

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Abbreviations

CRD, carbohydrate-recognition domain; CCL2, chemokine C–C motif ligand 2 (MCP-1); CCL5, chemokine C–C motif ligand 5 (RANTES); CXCL1, chemokine CXC motif ligand 5 (GRO-α); CXCL3, chemokine CXC motif ligand 3 (GRO-γ); CXCL8, chemokine CXC motif ligand 8 (IL-8); FBS, fetal bovine serum; Gal, galectin; GM-CSF, granulocyte macrophage colony stimulating factor; HMEC-1, human microvascular endothelial cells; HRP, horseradish peroxidase; IgG, immunoglobulin G; IL, interleukin; LPS, lipopolysaccharide; NFκB, nuclear factor kappa B; PBS, phosphate-buffered saline; RT, room temperature; vWF, von Willebrand factor.

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