HUMAN PLATELETS EXPRESS AND ARE ACTIVATED BY GALECTIN-8

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Running title: Platelet activation mediated by Galectin-8

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SYNOPSIS

Galectins (Gals) are proteins with glycan affinity that are emerging as mediators of atherosclerosis. Despite the similarities in structure and sequence, different Gals exert distinct effects on their target cells. We have shown that Gal-1 triggers platelet activation suggesting a role for Gals in thrombus formation. Since Gal-8 is expressed upon endothelial activation and also contribute to inflammation, to further understand the role of these lectins in hemostasis, we evaluated the effect of Gal-8 on human platelets. Gal-8 bound specific glycans in the platelet membrane and triggered spreading, calcium mobilization, and fibrinogen binding. It also promoted aggregation, thromboxane generation, P-selectin expression, and granule secretion. Glycoproteins α_{IIb} and Ib-V were identified as putative Gal-8 counter-receptors by mass spectrometry. Studies performed using platelets from Glanzmann's Thromboasthenia, Bernard Soulier patients confirmed that GPIb is essential for transducing Gal-8 signaling. Accordingly, Src, PLC2γ, ERK and PI3K/Akt downstream molecules were involved in Gal-8-signaling pathway. Gal-8 fragments containing either the *N*-or *C*-terminal domains showed that activation is exerted through the *N*-terminal. Western blotting and cytometry showed that platelets not only contain Gal-8 but also expose it after thrombin activation. These data reveal Gal-8 as a potent platelet activator, supporting a role for this lectin in thrombosis and inflammation.

Key words: **inflammation, galectins, thrombosis, integrins, glycans.**

INTRODUCTION

Galectins (Gals) are a family of carbohydrate-binding proteins that are involved in homeostasis and pathological events. Based on their structure they are classified into three groups: prototype such as Gal-1; chimera type with Gal-3 as its only representative; and tandem-repeat type, where Gal-8 is included among other members [1]. Depending on their structure, these proteins are able to act as bivalent or multivalent agents mediating the cross-linking of cellular surface glycoconjugates, thus being involved in biological processes such as apoptosis, differentiation, cytokine secretion, adhesion and lattice formation [1-3]. More recent studies revealed that Gals are also involved in the pathogenesis of cardiovascular diseases in particular atherosclerosis[4]. Gals influence many processes that are important for plaque growth as well as stability/unstability. For example, Gal-3 gene has been found activated in aortas of hypercholesterolemic rabbits, in aortas of rats after balloon injury and in cultured smooth muscle cells (SMC)[5]. Increased levels of Gal-1 and 3 have also been detected in human atherosclerotic lesions [6].

Recent work from our laboratory has shown that Gal-1 triggers platelet activation and promotes platelet-leukocyte mixed aggregates [7]. These observations point out that Gals might be relevant mediators not only of the inflammation/atherosclerotic process but also of thrombus formation. In this regard, Gal-3 binding protein was found up-regulated in a microparticle proteomics analysis of patients with deep venous thrombosis [8].

Gal-8 is composed of two distinct carbohydrate recognition domains (CRDs) with different sugar specificity that are connected by a linker peptide of variable length that defines the isoforms [9]. Like other Gals, Gal-8 can be released to the extracellular compartment and a positive correlation has also been demonstrated between Gal-8 and some human cancers [9]. We have recently shown the presence of two Gal-8 isoforms in murine spleen that play distinctive roles as local enhancers of otherwise borderline immune responses and also stimulate the reactivity at inflammatory foci [10]. Interestingly, also in this work, mass spectrometry studies shown that α_{IIb}, a major integrin of the platelet surface involved in thrombus growth was one of the molecules interacting with Gal-8, suggesting a possible role for this Gal in platelet physiology. Despite the similarities in structure and primary sequence, different Gals exert distinct effects on their target cells. Therefore, to gain a deeper insight about the role of these particular lectins in the thrombotic process, in the present study, we examined the impact of Gal-8 on platelet biology.

MATERIALS AND METHODS

Reagents- Human α-thrombin was purchased from Enzyme research laboratories (Swansea, UK), Apyrase, Acetylsalicylic Acid (aspirin), Luciferin-luciferase, thiodigalactoside (TDG), ATP, and TRITC-phalloidin were from Sigma (St. Louis, MO, USA). NHS-Biotin was from Pierce (Pierce biochemicals, USA). Eptifibatide was kindly provided by Dr. C.Fondevila. FITC-conjugated streptavidin, Alexa488-fibrinogen and trypsin were purchased from Invitrogen (Eugene, OR, USA). Collagen was from Nycomed Pharma (Unterschleibheim, Germany). Ly-294002, U0126, PP1, PP2, U73122 and Wortmannin were purchased from Enzo lifesciences (Plymouth

Meeting, PA, USA). Purified rabbit polyclonal antibody anti-Gal-8 was obtained as described earlier [3] and from Abcam (Cambridge, UK). Rabbit anti-phospho-PLC gamma2 was purchased from AbCam (Cambridge, UK). Lactose and sucrose were obtained from Calbiochem (Darmstadt, Germany). Cytofix/cytoperm kit, FITC-conjugated anti-CD62P, anti-CD41, anti-CD42b, PAC-1, FITC irrelevant IgG_1 and IgM, and anti-rabbit IgG and PE-conjugated anti-CD61 were obtained from BD Biosciences (San José, CA, USA). Mouse anti-phospho ERK1/2 (Tyr 204), rabbit anti-ERK and anti-phospho Akt (Ser 473) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat HRP-anti-mouse IgG, swine FITC-anti-rabbit, rabbit anti-von Willebrand factor (vWF), and HRP-conjugated rabbit anti-vWF were obtained from Dako (Glostrup, Denmark).

Expression of recombinant Gal-8 isoforms - Clones encoding the human Gal-8L and Gal-8M isoforms (GenBank NM_006499 and NM_201543) were synthesized (GeneScript) and subcloned into pTrcHisB (Invitrogen). Murine recombinant *N*-terminal CRD (*N*), *C*-terminal CRD (*C*), and chimeras containing two *N*-terminal CRD (*N-N*) or *C*-terminal CRD (*C-C*) were generated by following different PCR strategies using mouse Gal-8L encoding plasmid [10] as a template. For the *N* cloning, the encoding sequence of the CRD was amplified using the primer set Fwd 5' AAAGCTAGCATGTTGTCCTTAAATAACCTA 3'/Rev 5' AAAGATCTGGCACTTGCCGAGCTGAATCT 3' (sites *Nhe*I and *Bgl*II, underlined). For *C*, the encoding sequence of the CRD was obtained with the primer set Fwd 5' AAGCTAGCAAGCTCCAGCTGAGCCTGCCA 3'/Rev 5´AAAAGATCTCCGGATAAGCCATTTTGTA 3´ (sites *Nhe*I and *Bgl*II, underlined). Both amplicons were cloned into the pTrcHisB expression plasmid (Invitrogen). For *N-N* construction, two CDR amplicons were obtained using the primer sets Fwd 5' AAAGCTAGCATGTTGTCCTTAAATAACCTA 3'/Rev 5' AACTGCAGCTTGCCTGGCTTTTGTATATT 3' (sites *Nhe*I and *Pst*I underlined) and Fwd 5' AACTGCAGCTGAGCCTGATGTTGTCCTTAAATAACCTA 3'/Rev 5' AAAAGCTTTCAGAATCTGAACCCGATGGA 3'(sites *Pst*I and *Hind*III underlined). Amplicons were then sequentially cloned into pTrcHisB. A similar scheme was followed for *C-C*, where two amplicons of the CRD were generated with the primer sets Fwd 5' AAGCTAGCCCATTTGAAGCAAGGTTGAAT 3'/Rev 5' AACTGCAGATCCCCCAGTGTGTGTGGAAGGGCACTTGCCGAGCTCCAGCTC CTTACATCCAGCAA 3' (sites *Nhe*I and *Pst*I underlined) and Fwd 5' AACTGCAGAGTATGGAAACATCTGCTCTG 3'/Rev 5' AAGGTACCTCACCAGCTCCTTACATCCAGCAA 3' (sites *Pst*I and *Kpn*I underlined) and sequentially cloned in pTrcHisB. All constructions were DNA sequenced. The conditions for protein expression in a procariotic system and purification were identical to those described previously for mouse Gal-8. The lectin activity of these proteins was tested by hemagglutination assays [10]. The content of lipopolysaccharide (LPS), as checked by Limulus test, was less than 0.5 ng per mg of the purified protein.

Preparation of human platelets- Blood samples were obtained from healthy donors who had not taken nonsteroidal antiinflammatory drugs in the 10 days before sampling. This study was performed according to institutional guidelines (National Academy of Medicine, Buenos Aires, Argentina) and received the approval of the institutional ethics committee and written consent from all the subjects. Blood was drawn directly into

plastic tubes containing ACD (6:1) or 3.8% sodium citrate for aggregation in platelet rich plasma (PRP). PRP from normal donors or from a Glanzmann's Thrombasthenia (GT) patient was obtained by centrifugation of the blood samples (180 x*g* for 10 min) and the cell number was adjusted to 3 x 10^8/ml with platelet poor plasma. PRP from Bernard Soulier (BS) patient was obtained by gravity sedimentation at room temperature placing the tube at 45 degree inclination. BS and GT PRP count were $2x10^8$/ml and $2.1x10^8$/ml respectively.

For washed platelet (WPs) suspensions, PRP was centrifuged in the presence of prostacyclin (PGI_2) (75 nM), and the platelets were then washed in 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, 0.35% BSA, pH 6.5). WPs were resuspended in Tyrode's buffer and the platelet number was adjusted to $3x10^8$/ml, unless otherwise stated. Ca^{2+} (1 mM) was added before platelet stimulation. Highly purified platelets were obtained by depletion of leukocytes by using a high efficiency leukoreduction filter (Purecell PL, PALL Biomedical Products, NY, USA), as previously described [11].

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Gal-8 Binding assay- WPs were incubated for 15 min with biotinilated-Gal-8 and then platelets were washed with PBS/2 mM EDTA and incubated with FITC-conjugated streptavidin for 30 min. The binding of Gal-8 to the platelet surface was analyzed by flow cytometry (FACSCalibur flow cytometer, BD Biosciences, San Jose, CA). In selected experiments Gal-8 binding was measured using platelets whose GPIb was cleaved by incubation during 10 min with trypsin in the presence of apyrase, aspirin and PGI_2. The protease treatment was stopped by the addition of 10% serum and a cocktail containing protease inhibitors.

Platelet spreading- Glass slides were coated with Gal-8 (0.1 μM) and blocked with 2% BSA for two hours. Then, WPs ($5x10^7$/ml) were plated and incubated for 20 min. Adhered platelets were fixed (4% paraformaldehyde), permeabilized (0.1% Triton X-100), and stained with TRITC-Phalloidin. After mounting, the spread platelets were visualized under confocal microscopy (Olympus FV-1000).

Platelet aggregation and ATP release- Aggregation and ATP release were measured in a Lumi-aggregometer (Chrono-Log, Havertown, PA, USA). ATP levels were measured at the end of the assay by adding a known amount of standard ATP (2 μM).

Expression of platelet CD41, CD61, CD42b and P-selectin, fibrinogen or PAC-1 binding - WPs were stimulated, fixed and stained with a FITC-conjugated CD62P antibody (anti-P-selectin) in PBS/0.1% FBS solution or an equivalent amount of isotype FITC-IgG_1 as a negative control. To evaluate $\alpha_{IIb}\beta_3$ integrin activation or fibrinogen binding, platelets were stimulated in the presence of FITC-PAC-1 or Alexa-488 fibrinogen respectively, and then fixed and analyzed by flow cytometry. To evaluate platelet surface expression of CD41, CD61 and CD42b, GT, BS and control platelets were incubated with a FITC-conjugated CD41 or CD42b antibody or with a PE-conjugated CD61 antibody. After fixation, cells were analyzed by flow cytometry.

Intracellular Ca^{2+} mobilization – Intracellular Ca^{2+} concentrations in fluo-3-loaded platelets were assessed under flow cytometry as described previously [12] . In brief, platelets were labeled with 5 µM fluo-3-AM (Sigma) at 37º C for 15 minutes. After washing, 3 x 10^6 platelets in 500 µl of Tyrode´s buffer were subjected to flow cytometry analysis. After the determination for about 10 seconds of baseline fluo-3 fluorescence from the platelet population, cell aspiration into the flow cytometer was briefly paused and Gal-8 was added. The acquisition was then resumed, and changes in fluorescence versus time were recorded. Results are expressed as % of positive cells and represent the events with FL1 values above the threshold.

Measurement of Thromboxane B_2 (TXB_2) release - WPs were incubated for 5 min in a platelet aggregometer stirring at 1000 rpm with Gal-8M or L. The reaction was stopped by the addition of ice-cold PBS containing 2 mM EDTA and 0.5 mM aspirin. The samples were centrifuged, and TXB_2 levels in the supernatants were measured using an ELISA kit from Cayman Chemical (Ann Arbor, MI, USA).

Determination of Gal-8 expression in platelets by FACS- WPs were fixed and permeabilized using a cytofix/cytoperm permeabilization kit (BD, Biosciences). After washing, the cells were first incubated with rabbit polyclonal anti-human Gal-8 or an equivalent amount of rabbit IgG (negative control). Then, the cells were labeled with FITC-conjugated swine anti-rabbit antibody and intracellular Gal-8 expression was determined by flow cytometry.

Determination of vWF release- vWF release was determined by ELISA. Briefly, WPs were stimulated with Gal-8 for 30 min and centrifuged twice (2300 x*g* for 5 min) in the presence of PGI_2 (75 nM). The obtained supernatants were kept at -80°C until assayed. The results are expressed in ng/ml and were extrapolated from serial dilutions of normal pooled plasma, assuming a 10 µg/ml vWF concentration.

Immunoblotting – Highly purified WPs lysates were prepared by solubilizing platelets ($1x10^8$) in loading buffer (62.5 mM Tris-HCl at pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% 2-mercaptoethanol). Equal amounts of proteins were electrophoresed on a 12% SDS-PAGE and electro-transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). After blocking, the membranes were incubated overnight at 4ºC with primary Abs followed by a HRP secondary Ab. Protein bands were visualized by using the ECL reaction. Immunoblotting results were quantified using Gel-Pro Analyzer 3.1 software and the values from blot reprobes were used for monitoring equal protein loads. Western blot assays for Gal-8 detection in WPs lysates were carried out as previously described [10].

Affinity chromatography and mass spectrometry - Human platelets were lysed with 1% Triton X-100 in the presence of a protease inhibitor cocktail and passed through a Gal-8 affinity column made by coupling purified human recombinant Gal-8M to N-hydroxy succinimide-activated HiTrap columns (GE Healthcare, Uppsala, Sweden). Gal-8 binders were eluted with 150mM lactose in PBS and concentrated by ultrafiltration. After alkylation with iodoacetamide, samples were resuspended in cracking buffer and solved by 10% SDS-PAGE. After Coomasie blue staining, discrete bands were cut out, in-gel digested with trypsin, and subjected to peptide mass

fingerprinting to the mass spectrometry service of Institute Pasteur of Montevideo, Uruguay.

Statistical analysis - The results are expressed as mean ± SEM. The Student's paired *t* test was used to determine the significance of differences between the groups. A *P* value lower than 0.05 was considered statistically significant.

RESULTS

Soluble Gal-8 binds to the platelet surface and immobilized Gal-8 promotes platelet adhesion and spreading - To investigate if Gal-8 interacts with glycoconjugates at the platelet surface, we tested the binding ability of Gal-8 M (medium) and L (long), the prominent isoforms in humans [9, 13]. As it is shown in Fig. 1A, both isoforms bound platelets at a similar rate and in a concentration-dependent manner. Addition of lactose, but not sucrose, strongly reduced Gal-8 binding highlighting the specific interaction of this lectin with glycan residues on the platelet surface (Fig. 1B).

Considering that Gal-8 binds to the platelet surface and that in other cell types it induces cell adhesion [14-16], we examined whether immobilized Gal-8 promoted platelet adhesion and spreading. Whereas platelets did not adhere to BSA-coated slides, they effectively attached, spread, and displayed lamellipodia formation on immobilized Gal-8M- or Gal-8L-coated surfaces. Spreading and attachment were significantly reduced by lactose (Fig. 1C).

Gal-8 induces activation of $\alpha_{IIb}\beta_3$ integrin and calcium mobilization - As a consequence of platelet agonist-receptor interaction, $\alpha_{IIb}\beta_3$ integrin suffers conformational changes that lead to the exposure of neoepitopes and allow fibrinogen binding (inside-out signaling) [17]. Flow cytometry studies using PAC-1 antibody that recognizes the activated form of $\alpha_{IIb}\beta_3$ and Alexa 488-fibrinogen showed that both phenomena occurred upon platelet activation induced by the Gal-8 isoforms (Fig. 2A and B). Another feature of inside-out signaling is elevation of cytosolic Ca^{2+} concentrations which is essential for platelet activation. Figure 2C shows that like other platelet agonists, Gal-8 effectively raised intracellular platelet Ca^{2+} levels. Inside-out signaling mediated by Gal-8 was inhibited by lactose (data not shown).

Platelet aggregation mediated by Gal-8 - Conformational changes of $\alpha_{IIb}\beta_3$ integrin allow platelet aggregation to proceed [17]. Figure 3A shows that the two splice variants of Gal-8 induced platelet aggregation both in WPs (EC_{50} 0.19±0.05 μM for Gal-8M and 0.20±0.08 μM for Gal-8L) and in PRP (EC_{50} 0.9±0.06 and 0.8±0.07 μM for Gal-8M and Gal-8L respectively). Because all the evaluated platelet responses triggered by both splice variants were almost identical, the rest of the experiments were performed only with the Gal-8M isoform.

The aggregation response was associated with the ability of Gal-8 to bind to specific glycan molecules in the platelet surface since it was completely suppressed by preincubation of platelets with lactose or its related sugar TDG (Fig 3B I and II) but not by sucrose (Fig 3B III). Because lectins are known to induce cell agglutination, to determine whether this phenomenon accounted for the Gal-8-induced response, platelet stimulation was performed in the presence of EDTA or eptifibatide, an $\alpha_{IIb}\beta_3$ antagonist.

Under these conditions, platelet aggregation induced by Gal-8M was almost completely suppressed (Fig. 3B IV). Moreover, Gal-8 was not able to induce aggregation of fixed platelets (Fig. 3B IV) confirming that the observed response was not an agglutination phenomenon and that, similar to classical agonists, Gal-8 mediated platelet aggregation is ultimately dependent on fibrinogen binding to $\alpha_{IIb}\beta_3$.

Role of ADP and TXA_2 in platelet activation mediated by Gal-8- Most classical agonists promote the release of ADP and TXA_2, which through the interaction with specific surface platelet receptors, amplify the aggregation response [18]. The ability of Gal-8 to generate TXA_2 was evaluated by the production of its stable metabolite, TXB_2, and the ADP secretion was measured as the surrogate dense body constituent, ATP. Figure 4 A and B depicts that Gal-8 stimulated both TXB_2 formation and the release of ATP. To explore the contribution of ADP and TXA_2 to Gal-8 mediated platelet activation, we monitored platelet aggregation induced by Gal-8 in the presence of selective inhibitors. The effect of secreted ADP was ablated using the ADP scavenger enzyme, apyrase and the effect of TXA_2 generation was suppressed by the cyclooxygenase inhibitor, aspirin. The presence of the ADP-scavenger inhibited the aggregation response mediated by low Gal-8 concentrations but the inhibition was bypassed by higher concentrations (Fig 4C). Aspirin treatment showed almost a similar pattern of inhibition than apyrase (Fig. 4C) and although the combination of both apyrase and aspirin exerted a stronger inhibition than each drug alone, increasing the Gal-8 concentration was able to overcome the suppression mediated by both drugs (Fig 4C). Thus, our results suggest that platelet aggregation mediated by low concentrations of Gal-8 requires ADP and TXA_2 though higher concentrations are independent of these mediators.

Platelet secretion mediated by Gal-8 – The role of platelets in inflammation and vascular repair is mainly associated to the release of alpha granule content. [18]. Having demonstrated that Gal-8 effectively triggered secretion of dense granules, we next analyzed whether a similar effect was exerted on alpha granule release. Fig. 5A and B shows that both vWF and P-selectin were detected in the supernatants and in the membrane from stimulated platelets respectively, reinforcing the notion of Gal-8 as a proinflammatory molecule.

Identification of putative Gal-8 counter receptors – To screen for possible platelet receptors that interact with Gal-8 in a carbohydrate-dependant manner, a Gal-8-affinity-chromatography step followed by mass spectrometry analysis was performed using platelet lysates. After column elution with lactose and SDS-PAGE resolution, a discrete number of bands was observed. The subunit α_{IIb} from the $\alpha_{IIb}\beta_3$ integrin and glycoprotein (GP) Ib and V from GPIb-IX-V complexes respectively, were the only membrane proteins identified that could eventually act as counter-receptors for Gal-8 (Table 1). In addition to these surface proteins, other relevant molecules that are released after platelet activation such as vWF, coagulation factor V or multimerin were also identified. In fact, vWF is one of the few ABO glycosylated proteins, being this sugar signature a known target of Gal-8 [13, 19]. In addition, other internal proteins seem to be indirectly co-purified with the Gal-8 counter-receptors as might be the case of actin and filamin, both known to interact with integrins. To further characterize the possible role of $\alpha_{IIb}\beta_3$ integrin and GPIb-IX-V as Gal-8 counter-receptors, functional studies were performed using platelets from a patient with Glanzmann Thrombasthenia (GT), deficient in $\alpha_{IIb}\beta_3$ integrin (Fig. 6A), from a patient with Bernard Soulier (BS)

syndrome whose platelets lack GPIb (CD42b) (Fig. 6A) or platelets whose GPIb was cleaved by pretreatment with trypsin. Consistently with mass spectrometry results, significant decrease in the mean fluorescence intensity (MFI) was observed at all Gal-8 concentrations employed either in GT, BS or trypsin-treated platelets As expected, due to the absence of $\alpha_{IIb}\beta_3$ integrin, collagen and Gal-8 induced platelet aggregation were profoundly decreased in GT platelets (Fig. 7AI). However, P-selectin exposure mediated by either collagen or Gal-8 was not affected despite the absence of $\alpha_{IIb}\beta_3$ (Fig. 7BI) pointing out that $\alpha_{IIb}\beta_3$ integrin is dispensable for Gal-8-induced platelet activation. Notably, platelet aggregation (Fig. 7AII) or the expression of P-selectin mediated by Gal-8 (Fig. 7BII) was almost absent in BS platelets. The mild aggregation response observed at the highest Gal-8 concentration employed in both GT and BS platelets, was not inhibited by EDTA (Fig. 7 AI and AII) indicating that it might be an agglutination effect instead.

Together, these results suggest that platelet GPIb but not α_{IIb} is essential for Gal 8-dependent signal transduction and therefore GPIb represents a biologically functional/relevant Gal-8 counter-receptor.

Signaling pathways involved in Gal-8 mediated platelet activation-Because GPIb appears to be at least one of the Gal-8 counter-receptors, we next explored by western blotting and pharmacological approaches several downstream signaling molecules known to be involved in GPIb. Although the model of how GPIb-IX-V signals in human platelets is currently incomplete, the activation of Src, PI3K, PLCg2, ERK appears to be involved in GPIb activation mediated by vWF binding [20, 21]. In agreement, we found that platelet stimulation by Gal-8 resulted in PLCg2, ERK1-2 and Akt phosphorylation (Fig. 8 A,B,C).

The phosphorylation of all these proteins but ERK, appears to be relevant for platelet activation induced by Gal-8 since platelet aggregation was significantly impaired in the presence of specific inhibitors of PLC (U73122), PI3K (Ly-294002 and Wortmannin) but not of MAPKK (U0126) [22, 23] (Fig. 8D). The activity of U0126 was confirmed by the observation that it completely inhibited ERK phosphorylation induced by thrombin or Gal-8 (data not shown). As Src kinases are also involved in GPIb sigaling [21] two specific inhibitors (PP1 and PP2) were tested. Preincubation of platelets with either of these drugs strongly inhibited Gal-8-induced aggregation (Fig. 8D). Altogether these data demonstrate that most of the GPIb downstream signaling molecules are activated by Gal-8 giving further support for this GP as a major counter-receptor for Gal-8 in platelets.

The N-terminal CRD triggers platelet activation - The absence of platelet agglutination in the presence of Gal-8 (Fig.3B IV) raises the idea that this lectin may works as a monomer. To gain a deeper insight into the structural-function relationship of Gal-8 on platelet physiology, the effects of fragments containing only the *N*-terminal (*N*) or the *C*-terminal (*C*) CRDs were tested. Since human and murine Gal-8 structures are highly related [3] and showed almost identical activity on human platelets (Table 2), recombinant *N* and *C* CRDs from murine Gal-8 were used in the following experiments. Notably, only the *N*-terminal domain was able to trigger platelet activation responses including P-selectin exposure, fibrinogen binding and aggregation (Table 2). Protein chimeras containing two *N*-CRD (*N-N*) or *C*-CRD (*C-C*) domains connected by a linker peptide were also tested. Consistent with the results obtained with each separate domain, platelets incubated with the *N-N* chimera but not with the *C-C* displayed a strong activation response (Table 2). These results demonstrate that the activation of

platelets mediated by Gal-8 is due to the *N*-terminal CRD domain and, moreover, that the lectin bivalency is not essential to achieve its activity on platelets.

Human platelets contain Gal-8 isoforms M and L and platelet activation leads to Gal-8 exposure - Gal-8 is a secreted protein that, like other galectins, lacks a signal peptide and uses a non-conventional secretion pathway [24]. Considering that exogenous Gal-8 triggers several platelet effector responses, we investigated whether platelets could also be a source of this lectin. The two principal splice variants of human Gal-8 (M and L isoforms) were detected on resting platelets from healthy donors by western blot (Fig. 9A). These findings were confirmed by detecting Gal-8 in permeabilized platelets by flow cytometry (Fig. 9B). These results indicate that platelets, like other vascular cells such as endothelial cells [25], also express Gal-8, which might account for its local effects. Notably, Gal-8 was detected in non-permeabilized platelets only after thrombin stimulation (Fig. 9C). However, we were unable to detect the presence of secreted Gal-8 in the supernatants of activated platelets even after washing with lactose (data not shown). These results are in agreement with previous observations of Hadari *et al.* [26] where Gal-8 remains attached to the surface of human carcinoma cells after secretion being released after treatment with trypsin.

To further analyze the role of endogenous Gal-8 on platelet activation, aggregation was triggered by different agonists in the presence of lactose or TDG. Figure 9 D shows that the aggregation response induced by submaximal collagen, PAR1-AP or ADP concentrations was inhibited by preincubation of platelets with both related sugars.

Our findings indicate that platelet Gal-8 is translocated to the outer membrane after activation, allowing us to postulate an amplifying role for this lectin at the site of inflammation/injury.

DISCUSSION

The results reported here show that two splice variants of Gal-8 bind to the platelet surface and activate different platelet functional responses including spreading, activation of integrin $\alpha_{IIb}\beta_3$, aggregation, and secretion of the content of both dense and alpha granules. Inhibition of Gal-8 binding and platelet aggregation by lactose or TDG, but not by sucrose, indicated that the interaction of Gal-8 with the platelet surface involves recognition of specific glycans in the platelet membrane.

The observation that immobilized Gal-8 functions as an extracellular matrix protein that mediates cell adhesion was previously reported by other groups in other cell types [14-16]. We have now extended these findings showing for the first time that immobilized Gal-8 promotes platelet adhesion and spreading. Interestingly, the ability to artificially trigger these responses independently of agonist-induced integrin activation, is usually restricted to $\alpha_{IIb}\beta_3$ ligands, including fibrinogen, vWF, or CD40L [17, 27] .

The affinity of $\alpha_{IIb}\beta_3$ for fibrinogen and other ligands is modulated through conformational changes. Like most traditional platelet agonists, soluble Gal-8 promoted the transition of this integrin from a resting state to a high-affinity state, resulting in the unmasking of neoepitopes in the $\alpha_{IIb}\beta_3$ complex, and allowing fibrinogen binding. Furthermore, we also demonstrated that both Gal-8 isoforms were able to induce platelet aggregation as well as TXB_2 release. Although the aggregation response triggered by low Gal-8 concentrations was inhibited in the presence of aspirin and/or an

ADP-scavenger, a full response was restored at higher concentrations showing that Gal-8 is a strong agonist that can activate platelets independently from TXA_2 formation or ADP release.

Stimulation of platelets with Gal-8 also led to the release of both dense and alpha-granule content such as ATP and vWF as well as P-selectin exposure. Platelet secretion is critical for hemostasis and vascular repair [18]. Besides, the ability of platelets to interact with other vascular cells through the expression of P-selectin has been implicated in the progression of inflammatory conditions, the metastatic spread of malignancies, and the immune response to bacterial challenge [28, 29]. Thus, our results suggest that Gal-8 might play a relevant role, not only in the physiopathology of thrombus formation, but also in fueling the inflammatory response or in metastasis development.

Different cell surface glycoconjugates, as well as extracellular matrix glycoproteins, appear to be primary receptors for Gals [30]. Among the different receptors, integrins are known to be involved in Gal-mediated biological responses [31, 32]. In agreement with these data, mass spectrometry studies showed that among the different platelet surface GPs, subunit α_{IIb} from the $\alpha_{IIb}\beta_3$ integrin and GPIb and V from GPIb-IX-V complex, appear to be Gal-8-counter-receptors. The decreased binding of Gal-8 in either GT, BS or platelets whose GPIb was cleaved by trypsin confirmed that both GPIb and $\alpha_{IIb}\beta_3$ integrin are involved in Gal-8 interaction with the platelet surface. However, the observation that Gal-8 mediated platelet activation was only impaired in BS platelets, revealed that GPIb is essential for Gal 8-dependent signal transduction and therefore represents a Gal-8 functional major counter-receptor. Moreover, our findings showing that Src, PI3K/Akt and PLCγ2 (well known downstream signaling molecules related to GPIb-IX-V [20, 21], are involved in platelet activation induced by Gal-8, gave additional support to the notion that GPIb is an essential receptor for transducing Gal-8 signaling.

Since all the evaluated platelet activation responses were exerted by different concentrations of the two splice variants of Gal-8 (M and L) with a similar potency, it could be assumed that the isoforms would function likewise *in vivo*. Besides, as the isoforms share identical CRDs differing only in the length of the linker region, our results also indicate that platelet activation triggered by Gal-8 does not depend on a specific spacing of the CRDs or does not rely upon lectin bivalency. The importance of the presence of multiple isoforms of human Gal-8 has still not been addressed; however, some authors speculate that they may be tissue specific, or correspond to certain pathologies like cancer [9]. Interestingly, single *N*-terminal CRD-containing fragments were able to trigger platelet activation, indicating that lectin bivalency is not essential and that the clustering of surface receptors is not required. This observation was further supported by the fact that Gal-8 failed to induce platelet aggregation in the presence of EDTA or in fixed platelets, precluding agglutination.

Although Gal-8 is a secreted protein, it is mainly found in the cytosol of different cell types [33] . Western blot and flow cytometry studies revealed that human platelets express the two splice variants of Gal-8. Moreover, whereas Gal-8 was absent on the surface of resting platelets, like P-selectin, it was exposed on the membrane of thrombin-stimulated platelets. In addition, the observation that lactose and TDG inhibited the aggregation induced by classical agonists, indicates that platelet-derived Gal-8 could be a mediator of platelet activation. Therefore, in the vascular system, platelets are another source of Gal-8 that would be accessible upon platelet activation not only to eventually promote further thrombus growth but also to activate endothelial cells and/or leukocytes. In fact, it has been demonstrated that Gal-8 promotes adhesion

and superoxide production in neutrophils [34], and we have recently shown that Gal-8 induces lymphocyte proliferation in the presence or absence of antigen [10]. In addition, high amounts of this Gal were found in the inflamed synovia [35].

Regarding the biological implication of this novel role of Gal-8 as a platelet agonist, it is reasonable to propose that Gal-8 might be relevant in the physiopathological hemostatic response after vessel injury. In fact, the exposure of Gal-8 in the subendothelium or on activated endothelial cells [25] would trigger platelet adhesion, spreading and thrombus formation; the latter event being reinforced by the Gal-8 exposed on platelet membrane after activation. In addition, the effects of Gal-8 on platelets and inflammatory cells could also contribute to the fate of the atherosclerotic plaque. Although the expression of Gal-8 in human atherosclerotic lesions was not yet explored, the expression of Gal-1 and Gal-3 has been recently shown [5, 6]. Among the mechanisms proposed to explain the effects of Gal-1 in the atherogenic process are the ability of this lectin to induce SMC proliferation through a lipoprotein(a) dependent mechanism and to trigger T cell apoptosis [36]. Regarding Gal-3, it has been recognized a role for this protein in the transformation of macrophages into foam cells as well as in chemotaxis for monocytes and macrophages [37]. Besides, it has been shown that Gal-3 exacerbates vascular inflammation by stimulating macrophages to release superoxide anion [37] and express a range of chemokines and other proinflammatory molecules [38]. Our present data together with our previous observation that Gal-1 triggers platelet activation [7], suggests that Gals not only might be involved in the fate of atherosclerotic plaque but also that they could act as potent thrombogenic molecules that are exposed after plaque rupture. Although both Gals are capable of promoting similar platelet functional responses, Gal-8 is ten times more potent than Gal-1. Perhaps, the differences in the concentration required to achieve a similar effect, could reflect different downstream molecular signals triggered by each lectin and/or the relative abundance of Gal-1, compared with Gal-8, in some cells/tissues [3]. The observed increased levels of Gal-1 and Gal-8 in tumoral endothelial cells as well as in other malignant cells [9, 39] could represent a pathogenic mechanism involved in thrombosis and disseminated intravascular coagulation complications, commonly present in cancer patients [40, 41]. Furthermore, the formation of mixed-cell aggregates between tumor cells expressing high levels of Gal-8/Gal-1 and platelets might also contribute to tumor progression and metastasis. Nevertheless, further experiments are required to test these hypotheses.

In conclusion, our results reveal Gals as a new family of endogen platelet agonists capable of triggering activation in either its soluble or immobilized form.

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ABBREVIATIONS

Gal, Galectin; SMC, smooth muscle cells; CRD, carbohydrate recognition domain; TDG, thiodigalactoside; PFA, paraformaldehyde; PRP, platelet rich plasma; WP, washed platelets; TXB_2, thromboxane B_2; vWF, von Willebrand Factor; GP, glycoprotein.

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DISCLOSURE

None

FIGURE LEGENDS

Fig. 1. Gal-8 binds to platelet surface and immobilized Gal-8 promotes platelet spreading. (A) WPs were incubated with biotinilated Gal-8M or L isoforms for 15 min and stained with FITC-labelled streptavidin. The percentage of Gal-8 positive cells was analyzed by flow cytometry (n=3). (B) WPs were incubated with Gal-8 (0.1 μM) in the presence or absence of lactose or sucrose (30mM). The histograms are representative of three independent experiments. (C) WPs were plated on 0.1 μM Gal-8M- or L-coated slides and platelet spreading was visualized by confocal microscopy. The images are representative of five independent experiments.

Fig. 2. Soluble Gal-8 promoted inside-out signaling in platelets. (A) $\alpha_{IIb}\beta_3$ integrin activation of resting and Gal-8 **or thrombin**-stimulated WPs was determined by flow cytometry, using FITC-conjugated PAC-1 Ab (n=4, * $P < 0.05$ vs. unstimulated (unst). (B) Fibrinogen binding of unstimulated and Gal-8 or thrombin-stimulated WPs was determined by flow cytometry using Alexa 488-conjugated fibrinogen (n=4, * $P < 0.05$ vs. unst). For comparison, thrombin (0.1 U/ml) and Gal-8 (0.5 μM) were the minimal agonists concentration that induced 100% of PAC or Fibrinogen binding (C) Intracellular Ca^{2+} concentration was determined by flow cytometry using fluo-3-AM (n=3).

Fig. 3. Platelet aggregation induced by Gal-8. (A) WPs were stimulated with different concentrations of Gal-8M or Gal-8L. (B) WPs were incubated with 30 mM (I) lactose, (II) TDG, (III) sucrose or (IV) 2 mM EDTA, 4% PFA or 0.5 μM Eptifibatide (Epti) for 1 min prior to stimulation with Gal-8M (0.5 μM). The figures show the representative tracings of four separate experiments.

Fig 4. Role of ADP and TXB_2 on Gal-8 induced aggregation. (A) WPs were stimulated with Gal-8 for 5 min under stirring conditions and TXB_2 levels in supernatants were determined by ELISA (n=3, * $P < 0.05$ vs. unst). (B) ATP release was measured by using a lumi-aggregometer. (C) Aggregation induced by a range of concentrations of Gal-8 was measured in the presence of apyrase (2 U/ml) and aspirin (0.5 mM), individually and in combination. Results are shown as the mean ± SEM of three independent experiments. The % aggregation plotted is the maximal aggregation achieved (n=4, * $P < 0.05$ vs. control).

Fig. 5. Gal-8 triggered alpha granule secretion. WPs were stimulated with Gal-8 **or thrombin** at the indicated concentrations. (A) vWF levels in the supernatants were quantified by ELISA. (B) Surface expression of P-selectin was detected by flow cytometry (n=6, * $P < 0.05$ vs. unstimulated).

Fig. 6. Binding of Galectin-8 to platelets from Glanzmann's Thrombasthenia (GT) and Bernard Soulier (BS) patients and to trypsin-treated normal platelets. (A) CD41, CD61 and CD42b expression on platelet surface was determined by flow cytometry using FITC or PE-labelled monoclonal antibodies. (B) Gal-8 binding on platelet surface was determined in PRP by flow cytometry using different biotinilated Gal-8 concentrations (histograms show binding induced by 0.3 uM Gal-8) and FITC-labeled streptavidin. No Gal: No Gal-8 added; C: control (normal donor).

Fig 7. Gal-8-mediated activation of platelets from Glanzmann's Thrombasthenia (GP) or Bernard Soulier (BS) patients. (A) The percentage of platelet aggregation induced by the indicated agonist's concentrations was determined in PRP (B) Surface expression of P-selectin induced by Gal-8 or Collagen (Coll) was detected by flow cytometry.

Fig. 8. Involvement of Src, PLCg2, ERK and PI3K/Akt in Gal-8-mediated platelet activation. WPs were stimulated with Gal-8 (0.5μM) at 37 ºC, and the reaction was stopped by adding loading buffer. PLCg2 (A), ERK (B) or Akt (C) phosphorylation was determined in cell lysates. Total ERK and actin antibodies were used to monitor protein loading in all lanes. The images are representative of three independent experiments (* $P < 0.05$ vs. unst). (D) WPs were pretreated with vehicle (DMSO), Ly-294002, Wortmannin, U0126, PP1, PP2 or U73122 for 1 min and then aggregation was induced by Gal-8. Aggregation induced by Gal-8 (0.5 μM) in the presence of vehicle was considered as 100% (n=3, * $P < 0.05$ vs. control).

Fig. 9. Gal-8 isoforms M and L are expressed in human platelets. (A) Resting platelets from three different donors (I, II and III) were highly purified and lysed, and the expression of both isoforms of Gal-8 was determined by Western blot (lane 3). Recombinant Gal-8M (lane 1) and L (lane 2) isoforms were used as positive control. (B) The percentage of cells positive for Gal-8 in permeabilized WPs was determined by flow cytometry. The histograms are representative of three independent experiments. (C) Non-permeabilized WPs were stimulated with 1 U/ml thrombin for 20 min and the percentage of positive cells for Gal-8 in permeabilized WPs was determined by flow cytometry. The histograms are representative of four independent experiments. (D) PRP was preincubated with vehicle (PBS), Lactose (30mM) or TDG (30mM) for 1 min and then aggregation was induced by submaximal concentrations of PAR-1-AP (2μM), coll (0.5 μg/ml) and ADP (1.25 μM). Tracings are representative of three independent experiments.

Table 1. Gal-8 protein binders identified by MALDI-TOF from platelets. WPs were lysed and passed through a Gal-8 affinity column. Gal-8 binders were eluted in the presence of lactose and solved by 10% SDS-PAGE. The bands were cut out, and subjected to MALDI-TOF mass spectrometry.

Common name	ID
von Willebrand factor	vwf human
Transforming growth factor beta-1 binding protein	gi|339548
Multimerin-1	gi|45269141
Integrin alpha 2b	gi|119571979
Glycoprotein Ib alpha	gi|121531
Amyloid-beta protein	gi|209915573
Coagulation factor V	gi|60416383
FLNA protein	gi|15779184
Glycoprotein V	gi|4758460
Actin beta	gi|14250401

Table 2. Platelet activation mediated by chimeric and truncated forms of Gal-8. WPs were stimulated with human Gal-8, murine Gal-8, the *N* and *C* CRD domains from murine Gal-8 or by protein chimeras containing two *N*-CRD (*N*-*N*) or two *C*-CRD (*C*-*C*) domains connected by a linker peptide (n=5, * $P < 0.05$ vs. unst).

	Fibrinogen binding (% of positive Cells)	**P-selectin exposure** (% of positive Cells)	**Aggregation** (% of light transmission)
Unstimulated	3 ± 1	5 ± 1	0
Human Gal-8 0.5 μM	92 ± 5*	81 ± 6*	90 ± 6*
Murine Gal-8 1 μM	91 ± 3*	68 ± 2*	89 ± 3*
N-CRD 2 μM	97 ± 2*	59 ± 1*	61 ± 4*
C-CRD 2 μM	2 ± 1	7 ± 3	0
N-*N* 2 μM	96 ± 4*	65 ± 2*	66 ± 5*
C-*C* 2 μM	5 ± 4	5 ± 4	0

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Figure 1

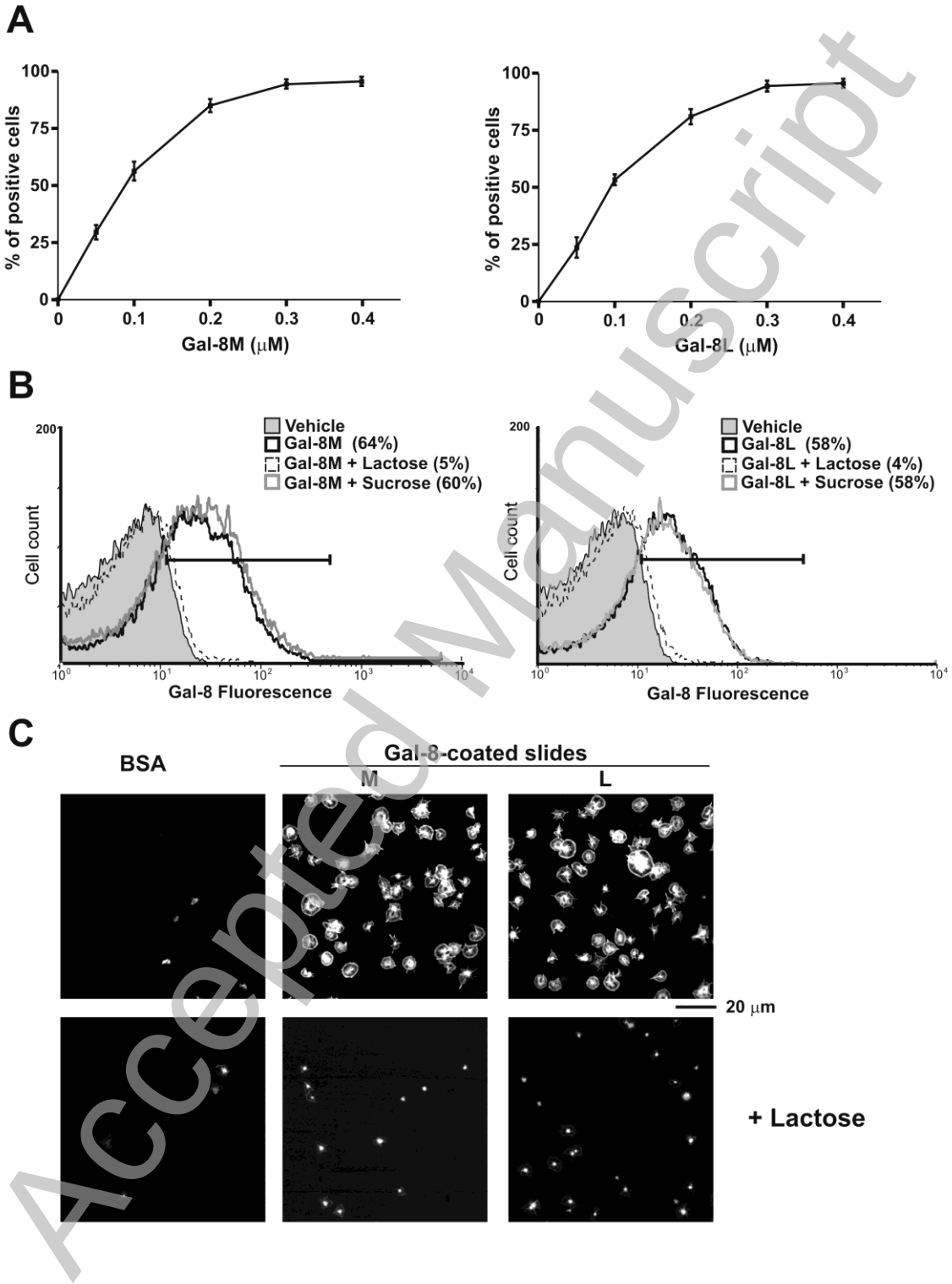

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Figure 2

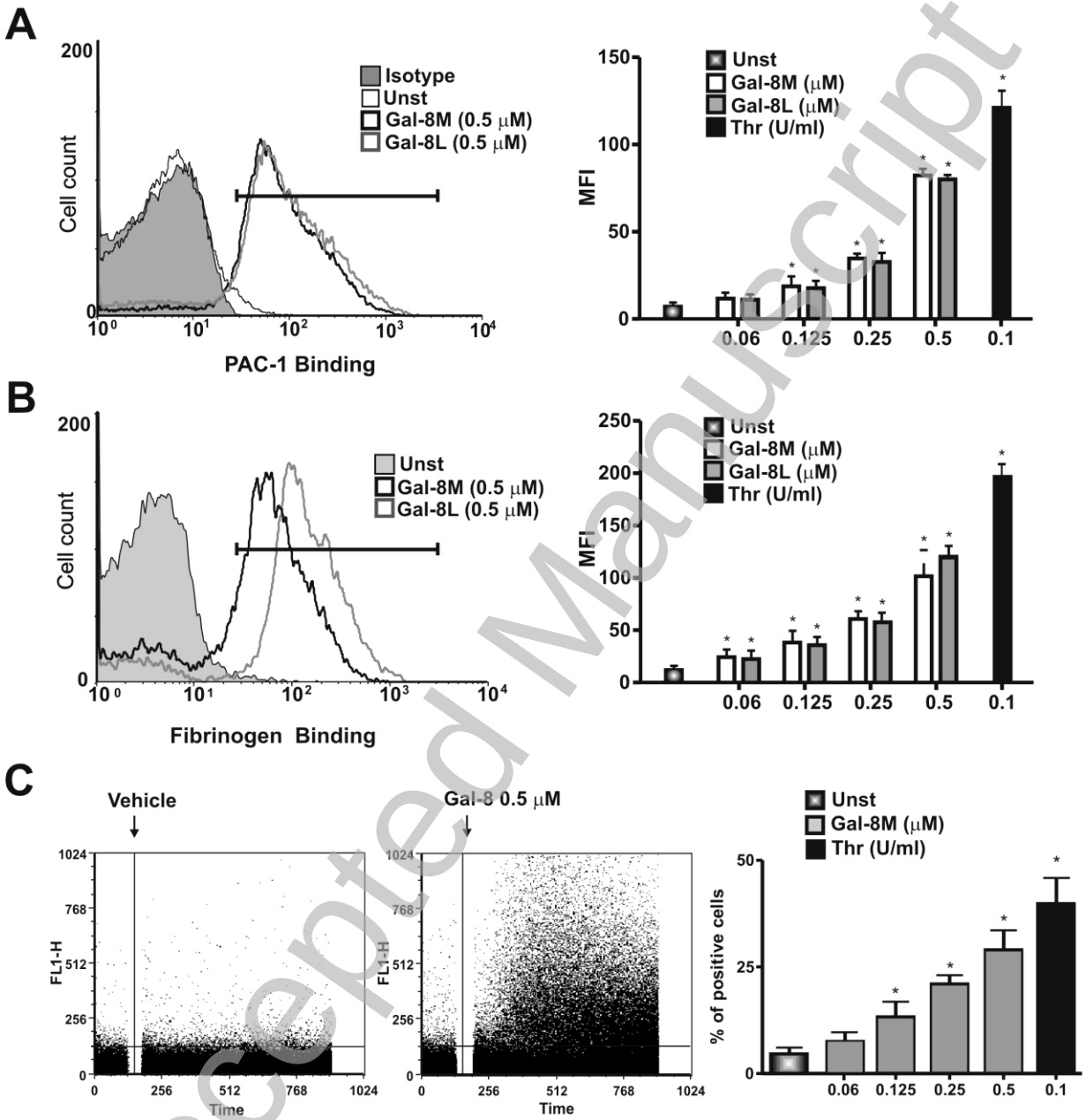

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Figure 3

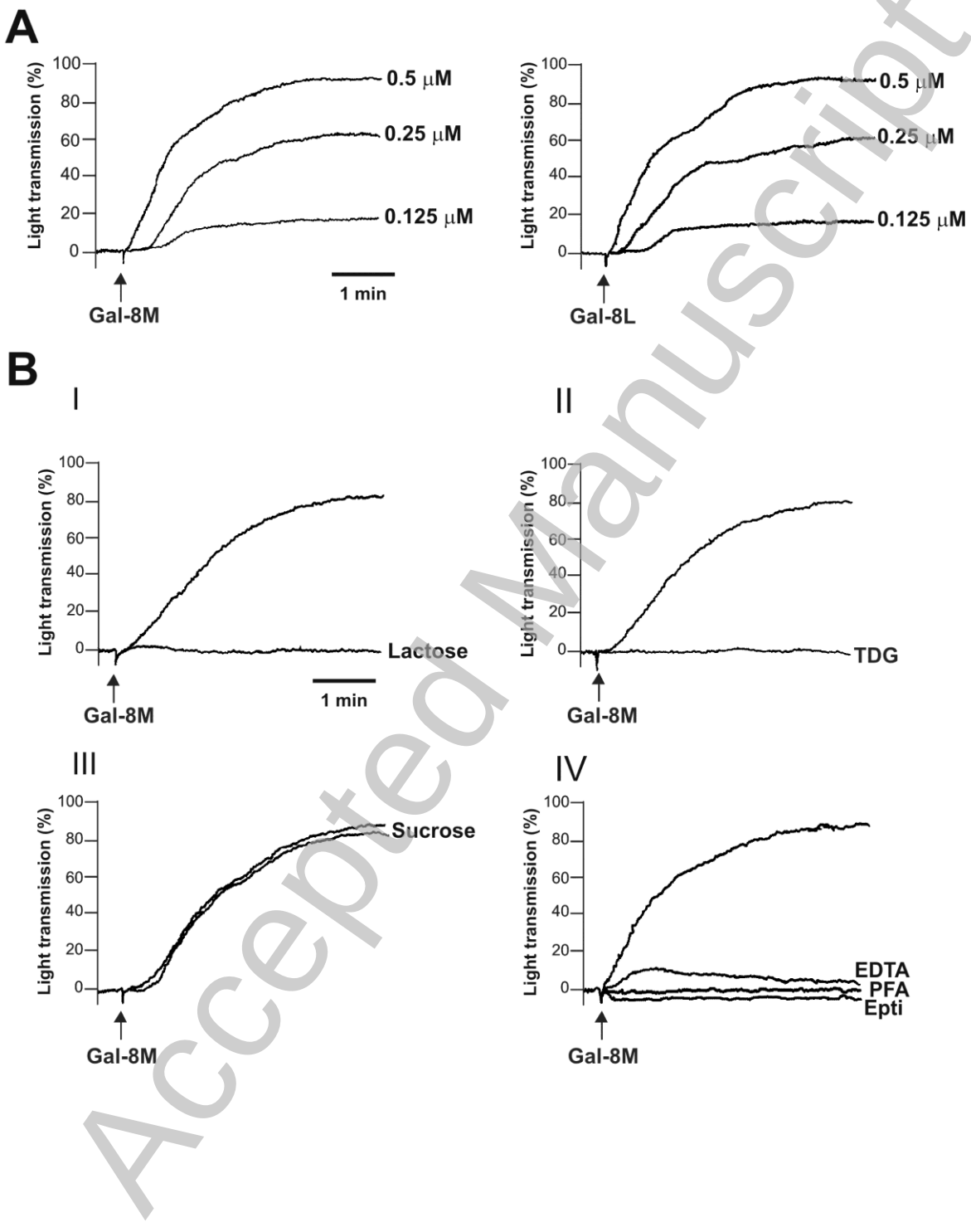

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Figure 4

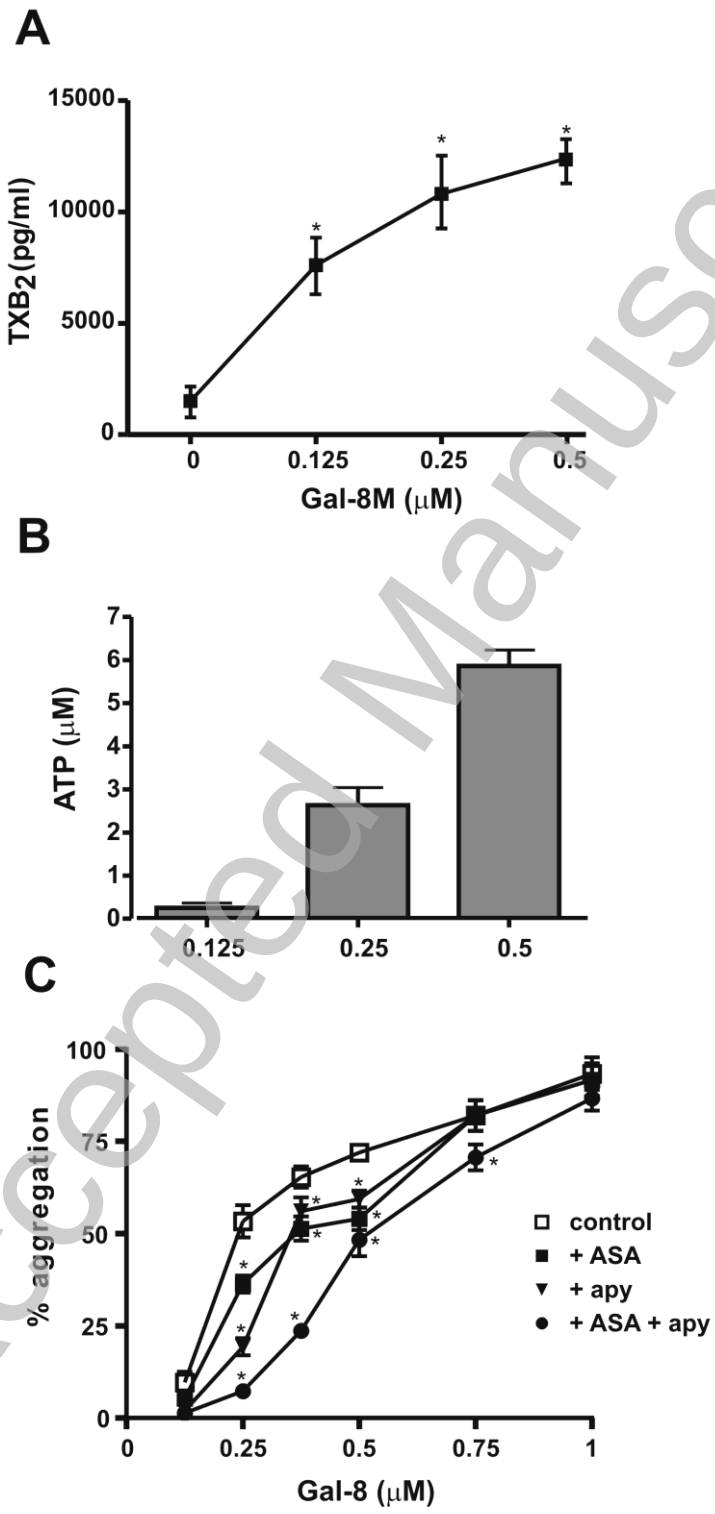

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Figure 5

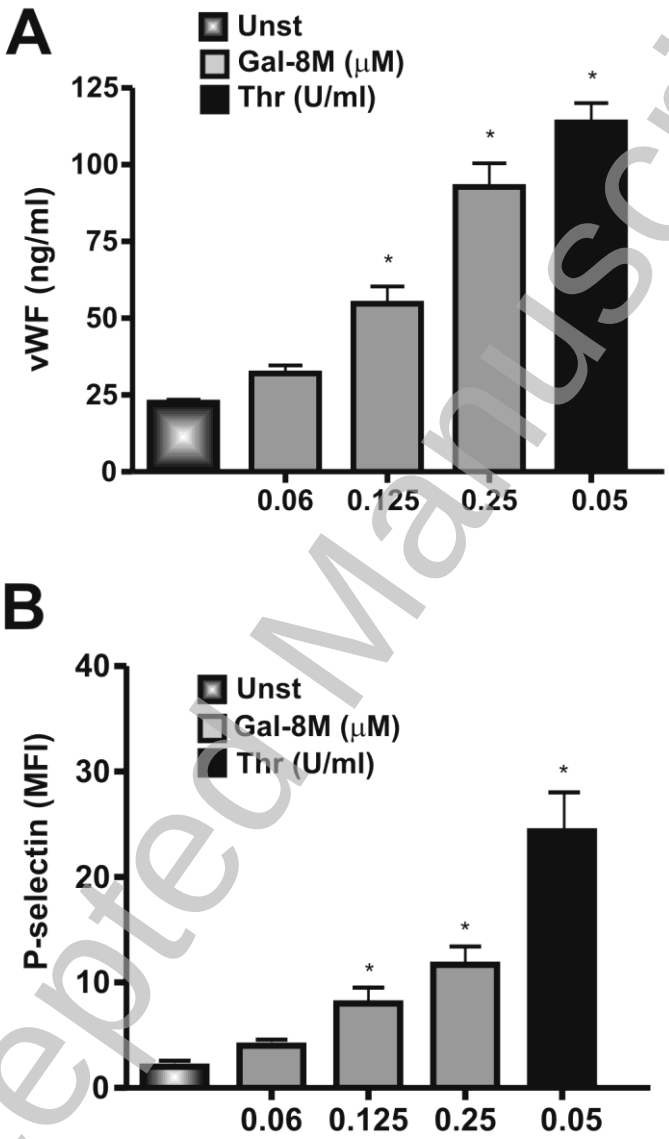

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Figure 6

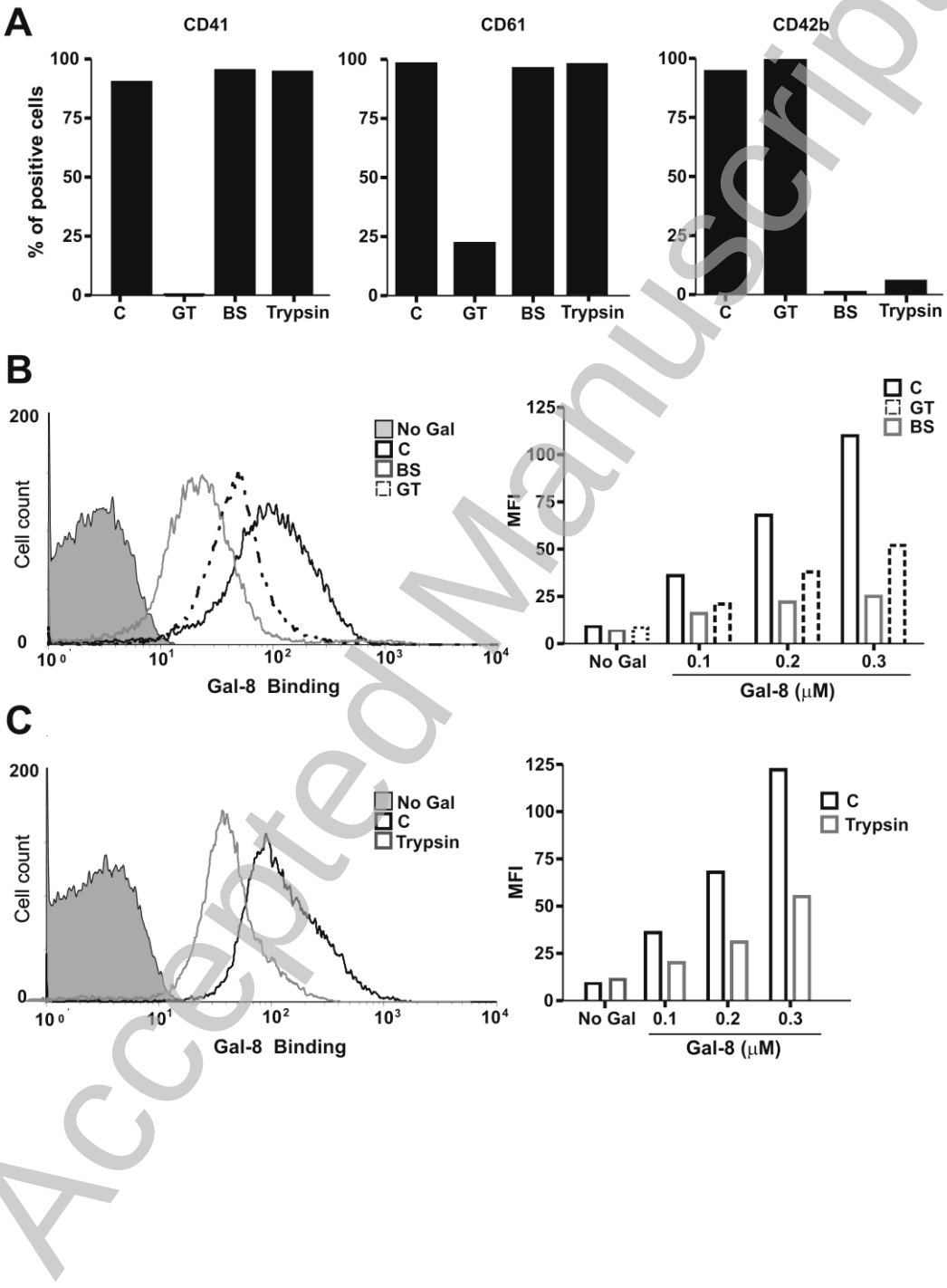

Figure 7

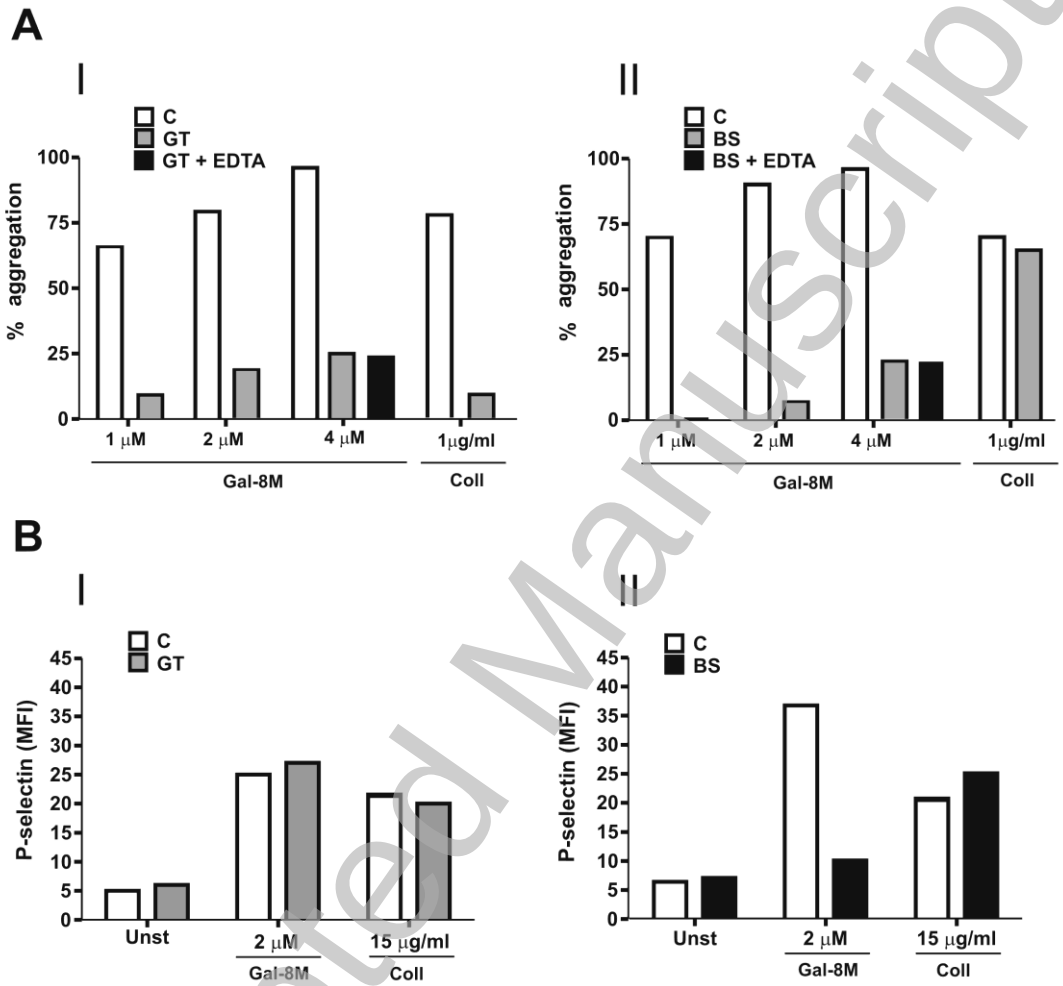

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Figure 8

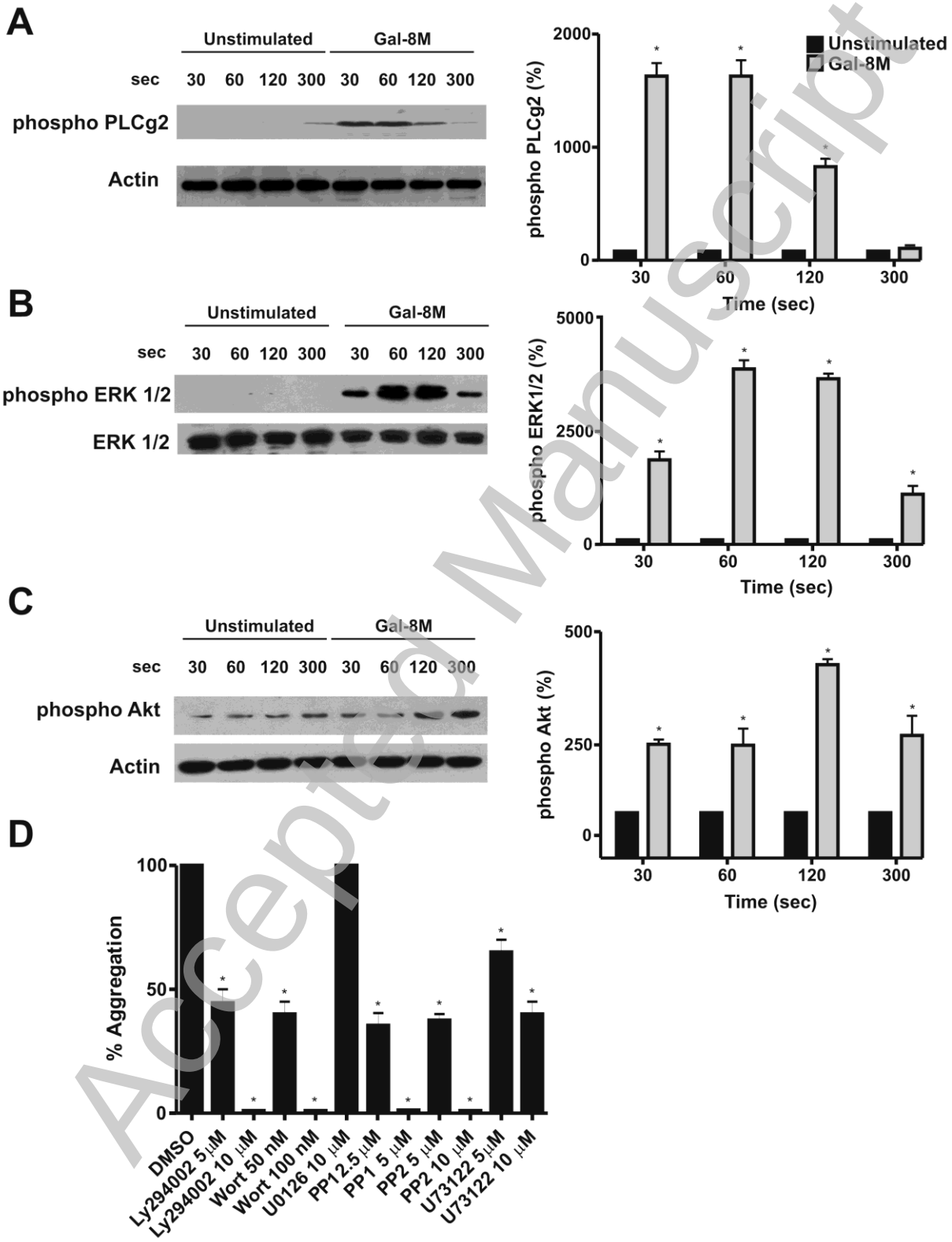

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Figure 9