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Title page

The Role of Galectin-3 and Galectin-3-Binding Protein in Venous Thrombosis

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Key points

- We determined the location of gal3bp and gal3, their role in promoting VT and leukocyte/endothelial cell interactions for the first time.

- Gal3bp and gal3 have the potential to be used as targets for future VT therapies.
ABSTRACT

Galectin-3 binding protein (gal3bp) and its receptor/ligand, galectin-3 (gal3) are secreted proteins that initiate signaling cascades in several diseases, and recent human proteomic data suggests they may play a role in venous thrombosis (VT). We hypothesized that gal3bp and gal3 may promote VT. Using a mouse stasis model of VT, we found that gal3bp and gal3 were localized on vein wall, red blood cells, platelets and microparticles, while leukocytes expressed gal3 only. Gal3 was dramatically increased during early VT and gal3bp:gal3 co-localized in the leukocyte/endothelial cell interface where leukocytes were partially attached to the vein wall. Thrombus size correlated with elevated gal3, and IL-6 vein wall levels. Recombinant gal3 promoted VT, and increased vein wall IL-6 mRNA. While recombinant gal3 restored the VT size in gal3-/- mice, it had no effect on IL6-/- mice, suggesting gal3:gal3bp promotes VT through IL-6. Moreover, significantly fewer activated neutrophils were present in the gal3-/- vein walls. In a group of human patients, elevated circulating gal3bp correlated with acute VT. In conclusion, gal3bp:gal3 play a critical role in VT, likely via IL-6 and PMN mediated thrombotic mechanisms, and may be a potential biomarker in human VT.
INTRODUCTION

In the United States, an estimated 900,000 people are impacted by venous thrombosis (VT)/pulmonary embolism (PE) annually (1). Causes for VT were originally identified as blood stasis, hypercoagulability, and changes in the vein wall expression of pro-coagulant adhesion molecules (2). However, in the 1970’s inflammation was added as a contributing factor to VT (3). If not treated acutely, VT/PE is a morbid and life-threatening condition and the CDC estimates that up to 30% of those affected will die within 1 month of the initial diagnosis (4). The current standard treatment is anticoagulation but this carries with it significant bleeding risks. Intracranial hemorrhage occurs in 1.15% of patients receiving anticoagulation therapy each year with a case fatality rate (major bleeding) of 13% (5). Therefore, it is imperative that around the clock methods for safe, cost effective therapies for VT be established.

Galectin-3 binding protein (gal3bp), a 60-90kDa protein, and its receptor/ligand, galectin-3 (gal3), a ~35kDa protein, are secreted proteins that can interact with each other to promote cell-to-cell adhesion and initiate pathological, pro-inflammatory signaling cascades (6-9). Both gal3bp and gal3 are found in most normal adult tissues, mainly in epithelial & myeloid/amoeboid cells (10), but to our knowledge, they have never been previously explored in platelets, red blood cells, microparticles, vein wall and venous thrombus, all critical elements that participate in VT. Gal3-is found within the nucleus, cytoplasm, cell surface, and extracellular space as a monomer, multimer, or fragmented as a result of enzymatic cleavage (11). Gal3 multimerization is known to have physiological consequences, such as enhancing the ability of gal3 to facilitate cell-to-cell interactions (12, 13). Gal3bp and gal3, in both its monomeric and multimeric forms, are thought to play pathological roles in a number of diseases, such as cancer and rheumatoid arthritis (8, 9, 14, 15). In cancer, gal3bp and both gal3 monomers
and multimers contribute to metastasis by promoting cell-cell adhesion (8, 10, 14). Exogenous gal3, administered intravenously to mice or directly to cultured cells in doses intended to mimic the amount of circulating gal3 observed in patients with inflammatory diseases (1-10ug/mL), induces the secretion of interleukin-6 (IL-6), CCL2, the mouse version of human MCP1, and TNF-alpha. (10). Both cell-cell adhesion and inflammation are mechanisms deeply involved in VT (2, 3). The importance of IL-6 was recently highlighted in the context of VT in the same animal model as was used in the present work. Wojcik et al. showed that neutralizing IL-6 significantly reduces VT via reductions in CCL2 (16). Although gal3bp and gal3 have been shown to be important in a number of diseases, their role in VT has yet to be investigated.

In prior studies from our laboratory, high levels of gal3bp were detected in pro-coagulant, circulating microparticles from patients with VT (2). These findings led us to investigate gal3bp and gal3 in order to define the role they play in VT (2, 17). We hypothesized that gal3bp and gal3 promote VT. The purpose of this study was to determine: 1) If gal3bp and gal3 are pro-thrombotic; 2) The location of gal3bp and gal3 in solid tissue (vein wall and thrombus) and blood [red blood cells (RBC), white blood cells (WBC), platelets (PLT), and microparticles]; 3) The pro-inflammatory properties of gal3bp and gal3 during VT, and whether or not the induction of IL-6 through gal3 promotes thrombosis; and 4) To determine whether or not gal3bp and gal3 are associated with VT in humans.

METHODS

Animals

Male mice (n=244), 8-10 weeks old (20-25 grams - g), all on the C57BL/6 strain background were used. This included wild type (WT) along with gal3 knock-out (KO), and IL-
6KO mice (Jackson Laboratories, Bar Harbor, ME, stock #006338 and stock #002650 respectively).

**Inferior vena cava (IVC) stasis model**

**Surgery:** Mice were anesthetized with isofluorane and IVC ligation was performed as previously described (18). Treatments were given 24 hours before surgery (Day -1) and mice were euthanized at 3hr, 6hr, and 48hrs post-ligation as previously described (18). Non-VT mice did not undergo surgery. The number of animals per experiment is described in figure legends.

**Gal3bp Antibody Treatment:** Mice received one dose of anti-mouse gal3bp antibody (Abcam Cambridge, MA), 20 g diluted in saline or an equal volume of saline via intra-peritoneal (IP) injection.

**Gal3 Treatment:** Mice received one dose of human recombinant galectin-3 (rGal3, Peprotech, Rocky Hill, NJ), 5ug diluted in saline or an equal volume of saline intravenously.

**Harvest:** Blood was collected from the retro-orbital venous sinus, to prevent platelet activation, into a tube containing sodium citrate (3.2%). The IVC and associated thrombi were removed and weighed in grams (thrombus weight). Vein wall samples were either immersed in 1.0mL TRIzol (Invitrogen, Grand Island, NY), homogenized and then frozen in preparation for qRT-PCR analysis or minced finely using a scalpel and placed in 1% SDS preparation for Western Blot analysis. For histology, the IVC, aorta and surrounding tissues were removed as previously described (16).

**Isolation of platelets, Microparticles, and WBC**

**Platelets, Microparticle and Leukocyte Isolation:** (See supplemental data)
**Western Blot**

Westerns blots were performed using 7ug protein per lane, and 4-12% bis-tris NUPAGE gels (Bio Rad Laboratories, Inc, Hercules, CA). Gels were run in 1x MOPs buffer for 60 min at 150 volts, followed by 15 min at 200 volts. Membranes were blocked with 5% BSA in TTBS prior to incubation with antibodies. Membrane bound gal3bp was detected using a Rabbit Anti-Mouse gal3bp antibody (Abcam, Cambridge, MA), 1:500, 5% BSA in TTBS, and gal3 using a Rat Anti-Mouse gal3 antibody (R&D Systems, Minneapolis, MN, USA), 1:100, 5% BSA in TTBS. Membrane bound IL-6 was detected using a Rabbit Anti-Mouse antibody (1:500, 5% BSA in TTBS, Abcam, Cambridge, MA). Membrane bound β-actin was detected using a HRP-conjugated Mouse anti-Bird β-actin antibody (Santa Cruz Biotechnology, Dallas, TX) 1:2000, 5% BSA in TTBS. Appropriate secondary antibodies were used. Membranes were visualized using the BioRad Gel-Doc Imager and band intensity was quantified using the BioRad Gel-Doc Imager’s Volume Analysis Tool.

**Histology**

Hematoxylin and eosin (H&E), immunohistochemistry (IHC) and immunofluorescence (IF) were performed on non-VT, 3hr, 6hr, and 48hr post-ligation IVC/IVC & thrombus samples.  

**Hematoxylin and eosin:** Prepared as previously described (20).

**Immunohistochemistry for Gal3:** Prepared as previously described and stained for gal3. Primary antibodies were diluted, Mouse Anti-Mouse gal3 (1:500, Novus Biologicals, Littleton, CO, USA). A mouse-on-mouse (MOM) kit was used to reduce background noise. Secondary
antibody and ABC steps, followed by color development with DAB substrate and then slides were counterstained with hematoxylin QS (All reagents from Vector Labs, Burlingame, CA).

**Immunohistochemistry for Leukocytes**: Slides were deparaffinized and rehydrated using xylene, graded ethanol, and water. Antigen retrieval was performed using 10 mM Sodium Citrate (1.47g in 500ml dH2O pH to 6.0) for 5 min at 30% power in a microwave. Endogenous peroxide activity was blocked by soaking slides in 3% H2O2 in MeOH for 10 min and then blocked in 2.5% normal goat serum (Blocking buffer) for 30 min. Rat anti-Mouse Ly-6G was diluted (1:1000, 0.5ug/ml) in 2.5% normal goat serum, placed on slides and incubated for 30 minutes. Control tissue received no primary antibody. Impress Anti-Rat Ig solution containing peroxidase was added to all slides (Vector Laboratories, Inc., Burlingame, CA). Slides were color developed with DAB substrate (Vector Laboratories, Inc., Burlingame, CA) for 1 min, then the reaction was stopped by putting slides in distilled water. Slides were counterstained with Hematoxylin QS (Vector Laboratories, Inc., Burlingame, CA) for 45 sec, washed in distilled water, and then cover slipped when dry.

**Immunofluorescence**: Samples were cryo-preserved, cut into 6-micron sections and probed for gal3bp and gal3. Primary antibodies were diluted for gal3bp and gal3, as described above. Gal3bp was secondarily labeled with a FITC conjugated antibody (Thermo Scientific Pierce, Waltham, MA) and gal3 was secondarily labeled with a Texas Red.

**Quantitative real time-polymerase chain reaction (qRT-PCR): Gal3bp, gal3, IL-6 and CCL2 IVC wall gene expression**

RNA was isolated by homogenizing IVC samples in TRIzol reagent, as per manufacturer’s protocol. Of the total RNA, 5µg was reverse transcribed using Oligo dT,
RNaseOUT and Moloney murine leukemia virus reverse transcriptase (all from Invitrogen, Grand Island, NY). Gene expression determinations used commercially available primers (mouse gal3BP [Lgals3bp]: PPM24925E; mouse gal3 [Lgals3]: PPM06208C; mouse IL-6: PM03015A; mouse CCL2: PPM03151F; USA-QIAGEN Inc., Valencia, CA) in a Rotor-Gene 3000 thermocycler (Corbett Life Science, San Francisco, CA). Relative expression levels were normalized to β-actin (PPM02945B) expression (USA-QIAGEN Inc., Valencia, CA).

Statistics

All statistical analyses were performed using Graph-Pad Prism 6 (GraphPad Software, San Diego, CA). Statistical differences between groups were determined using a Student t-test with Welch's Correction and a one-way analysis of variance (ANOVA) with a Tukey’s multiple comparison test performed between groups for additional comparisons. Spearman correlations between TW, gal3, IL-6 and CCL2 were also performed. A value of $p \leq 0.05$ was considered significant and data are reported as mean ± standard error of the mean (SEM).

RESULTS

Gal3 is increased locally and systemically during VT

Systemic gal3 and gal3bp (Western Blot Analysis)

Red Blood Cells (RBCs): Gal3bp and gal3 were detected in VT and non-VT samples. Gal3bp (64kDa) levels did not significantly vary. A faint ~100kDa band was detected in all samples on the gal3bp blot, which may represent a conjugated form of gal3bp, or gal3bp in association with another protein (data not shown). Multiple bands were also observed on the gal3 blot. Gal3 monomers (gal3m), multimer dimers (gal3MD), and degradation products (gal3d)
were detected in all samples. β-actin was used as a loading control. Individual lanes represent RBC lysate from a single mouse (Figure 1).

**White Blood Cells (WBC):** Only gal3 was detected. Gal3m, gal3MD and multiple trimers ([gal3MT]) were detected and upregulated in the VT group. Gal3m were present in the non-VT condition (2-fold increase), considerably lower levels than in VT. Individual lanes represent WBC lysate pooled from 5 mice (Figure 1).

**Platelets (PLT):** Gal3bp and gal3 were detected in both groups. Gal3bp was abundant in both VT and non-VT and did not vary between the two conditions. Gal3m, gal3MD, and gal3MT and degradation products (~22kDa) were considerably upregulated in the VT condition (gal3m > 2 fold increase). Gal3MT were detected only in VT PLT samples. Individual lanes represent PLT pooled from 3 mice (Figure 1).

**Microparticles (MP):** Gal3bp and gal3 were detected in both VT and non-VT groups. Gal3bp was slightly decreased (<1 fold) in VT group while gal3m and gal3MD were considerably upregulated. The faint gal3MD bands (>35kDa) were detected in the VT group only (Figure 1). Individual lanes represent MP pooled from 3 mice.

*Local gal3 and gal3bp (Western Blot Analysis)*

**Thrombus:** Gal3bp and gal3 were present in large quantities in 48hr thrombi. Only monomeric gal3 (~37kDa, gal3m) was present and/or detectable. Individual lanes represent a protein sample derived from a single 48hr murine thrombus (Figure 1).
IVC: Gal3 and gal3bp were detected in both groups. Gal3 was up-regulated in VT mice as compared to non-VT mice. Gal3bp levels did not differ between VT and non-VT mice (Figure 1). Individual lanes represent IVC sample from a single mouse.

Q-RT-PCR and IHC Analysis

IVC: Gal3bp and gal3 mRNA in IVC was quantified by qRT-PCR for VT and non-VT groups (Figure 2A). Gal3bp mRNA levels were similar in VT and non-VT (0.0150 vs. 0.0118 respectively). A significant increase in gal3 mRNA was observed in VT mice (0.0915 vs. 0.0305, p<0.05) as compared to non-VT. Gal3bp and gal3 mRNA levels were quantified relative to β-actin expression (Figure 2A). To confirm gal3 VT increases, IHC was performed on slides from VT and non-VT groups. These slides showed a qualitative increase in gal3 staining at the lumen/vein wall interface (Figure 2B).

Vein wall expression of gal3 correlates with thrombus weight

Thrombus weight was measured and gal3 expression in the IVC was quantified by q-RT-PCR for VT (3hr, 6hr, 48hr) and non-VT groups (Figure 3A & B). Thrombus weight increased between 3hrs and 48hrs, as did the expression of gal3 in the IVC. A statistically significant, positive correlation was found between gal3 expression in the IVC and thrombus weight (r²=0.77, p<0.05) (Figure 3C).

Vein wall expression of gal3 correlates with vein wall expression of IL-6
The expression of gal3 and IL-6 was measured in IVC samples collected from VT (48hr) and non-VT mice (Figure 4A). Significant increases in gal3 (p<0.05) and IL-6 (p<0.05) expression were observed in VT mice. A significant positive correlation was found between gal3 expression and IL-6 expression (r²=0.81, p<0.05) (Figure 4A).

**Vein wall expression of IL-6 correlates with vein wall expression of CCL2**

The expression of IL-6 and CCL2 was measured in IVC samples collected from VT mice (48hrs) that either received rGal3, or saline prior to surgery, and also from non-VT mice. IL-6 expression was significantly increased in mice that underwent surgery compared to non-VT mice (0.00715 vs. 0.00054, p<0.05) (Figure 4B). Furthermore, IL-6 expression was significantly increased in mice that received rGal3 before undergoing surgery as compared to mice that received a control injection before undergoing surgery (0.02382 vs. 0.00715, p<0.05) (Figure 4B). CCL2 expression was increased in mice that underwent surgery as compared to non-VT mice, but this increase failed to reach statistical significance (Figure 4B). CCL2 expression was also increased in mice that received rGal3 before undergoing surgery compared to mice that received a saline, but this increase too failed to reach statistical significance as well (Figure 4B). A strong positive correlation was found between IL-6 expression and CCL2 expression (r²=0.79, p<0.05) (Figure 4B).

**Exogenous gal3 does not increase thrombus weight in IL-6 deficient mice**

Thrombus weight was evaluated at 48hrs post-ligation in WT, Gal3KO, and IL-6KO mice that either received rGal3 or saline prior to surgery (Figure 5). Gal3KO and IL-6KO mice treated with saline had significantly smaller thrombi than WT mice that received saline.
(0.0210±0.0025g vs. 0.0320±0.0009g and 0.0211±0.0031g vs. 0.0320±0.0009g respectively, both p<0.05) (Figure 5). Statistically significant increases in thrombus weight were observed in WT and Gal3KO mice that received rGal3 compared to saline controls (0.0402±0.0034g vs. 0.0320±0.0009g and 0.0391±0.0020g vs. 0.0210±0.0025g respectively, both p<0.05). No increase in thrombus weight was observed in IL-6KO mice that were given rGal3 versus saline controls (0.0211±0.0031g vs. 0.0248±0.0019g, NS) (Figure 5). Thrombi harvested from IL-6KO mice that received rGal3 remained significantly smaller than thrombi harvested from WT mice treated with saline (0.0248±0.0019g vs. 0.0320±0.0009g, p<0.05) (Figure 5).

**Leukocyte migration during VT is dependent on gal3**

IVC and Thrombus Ly6G+ cells were assessed in in WT and gal3KO mice at 3hrs, 6hrs, and 48hrs post-ligation (Figure 6 A & B). A significant reduction in leukocyte extravasation was observed in samples collected from gal3KO mice at 48hrs post-ligation as compared to those collected from WT mice (p<0.05) (Figure 6B).

**Gal3-gal3bp interactions at the lumen-vein wall interface**

To determine whether gal3-gal3bp contribute to thrombosis, we examined the locations of gal3bp and gal3 in non-VT and VT IVC samples using immunofluorescence. Endothelial cells, lining the IVC, stained positively for gal3bp (Figure 7B,F, FITC). Gal3bp endothelial cell staining was absent in areas of the IVC that were flush against thrombus (Figure 7J). Neutrophils and endothelial cells stained positively for gal3 (Figure 7C,G,K, Texas Red). Samples in which the endothelial cell layer was well visualized showed pro-inflammatory leukocytes interacting with “sockets” of gal3bp in the vein wall (Figure 7D,H; Figure 8D,H,L). Neutrophils circulating
(Figure 8A-D), or migrating into the IVC (Figure 8E-L), were negative for gal3bp. Leukocytes partially attached to the vein wall (Figure 8E-H) were observed in contact with one socket of gal3bp at the vein wall surface. Leukocytes totally attached to the vein wall were observed in contact with at least two sockets of gal3bp (Figure 8I-L). Strong RBC auto-fluorescence at the red and green wavelengths made it impossible to identify any specific gal3bp or gal3 staining therein.

DISCUSSION

Gal3bp and gal3 are known to play important roles in a number of pathologies, such as cancer, infections, diabetes, atherosclerosis, wound healing, asthma, and rheumatoid arthritis, but their role in VT has not been defined (12, 13, 21-27). This study shows that gal3 and gal3bp are associated with murine thrombogenesis, that gal3 and gal3bp interact at the thrombus-vein wall interface, and that gal3 may be contributing to thrombosis through pro-inflammatory, IL-6 dependent mechanisms. Our findings suggest that gal3bp and gal3 maybe a potential therapeutic target as well as biomarkers in human VT.

Animal model

We acknowledge that the IVC ligation model employed in this study has been shown to drive thrombus formation through an endothelial cell-derived tissue factor dependent mechanism as opposed to leukocyte-derived tissue factor dependent mechanism (28). That said, there is evidence that venous thrombogenesis is a complex and multi-factorial driven process that involves coagulation, inflammation, and fibrinolysis. Thus, tissue factor, VWF, NETs, the coagulation cascade, P and E-selectin, leukocyte migration, cytokine production, including IL-6,
PAI-1, and plasminogen/plasmin among others play important roles in VT and were studied using several animal models including the IVC ligation model. The result of this work shows that gal-3 contributes to the inflammation and leukocyte-driven components of VT.

**Gal3bp and gal3 location**

Gal3bp and gal3 were found in all tissue and blood elements pertinent to the thrombi that were examined except leukocytes.

**Gal3bp:** Consistent with Ramacciotti *et al.*’s observations in patients, large quantities of gal3bp were found in mouse microparticles (2). Although gal3bp was abundant in all elements studied except leukocytes, gal3bp was not significantly increased in any elements in the VT condition.

**Gal3 monomer and multimers:** In contrast, gal3 was markedly increased in the VT condition in all blood and tissue samples. As the concentration of gal3 protein increases, gal3 is known to form multimers, from dimers to pentamers (6, 11, 29, 30). Multimerization of gal3 occurs via the N-terminal domain, and does not interfere with gal3’s ability to bind carbohydrate ligands though its carbohydrate recognition domain (CRD) in its C-terminal domain (12). In our study, gal3 was upregulated not only at the monomer level in IVC and thrombus, but also at the multimer (dimers and trimers, ~67, 80kDa) level in RBC, WBC, PLT, and microparticles, indicating high levels of gal3 within these elements. Gal3 multimerization is known to have physiological consequences, such as enhancing the ability of gal3 to facilitate cell-cell interactions (12, 13). Cell-to-cell adhesion plays a major role in thrombogenesis, both at the thrombus-vein wall interface and within the thrombus itself (31, 32). The adhesive properties of gal3 multimers may contribute to gal3’s apparent pro-thrombotic function. We suspect that the
increase in gal3 protein observed during VT in PLTs may be due to in situ translation of pre-existing gal3 mRNA, as PLTs, although lacking in nuclei, have been shown to possess translational machinery (33, 34). To the best of our knowledge, RBC and microparticles do not possess translational machinery so the increase in gal3 observed is likely due to the transport of pre-existing gal3 protein to their surface or cytosol (35).

**Gal3 degradation products:** In addition to observing an abundance of gal3 monomers and multimers, we observed an increase in gal3 degradation products (<36kDa) in circulating RBCs, PLTs, and MPs. Gal3 is a known substrate for matrix metalloproteinases (MMPs) 2 and 9 (11). These enzymes cleave the Ala62-Try63 bond within gal3, yielding a C-terminal domain fragment (~22kDa) and an N-terminal domain fragment (~10kDa). Cleavage of gal3 significantly alters its function. Interestingly, after cleaved, gal3 can no longer multimerize, but the C-terminal domain fragment has a higher binding capacity for ligands than intact gal3 (10, 11). Thus, it is possible that cleavage of gal3 decreases its ability to promote cell-cell interactions while enhancing its ability to stimulate cell-signaling cascades. The relevance of gal3 degradation fragments to thrombosis has yet to be determined.

**Gal3 plays a pro-inflammatory role in the context of VT:**

Evidence has mounted showing that a number of inflammatory cytokines have pro-thrombotic effects (36). Inflammatory cytokines implicated in thrombosis include IL-6, IL-8, P-selectin, TNFα, CCL2, and CRP (36). Several studies have shown that gal3 up-regulates IL-6 in pathological conditions (8, 9, 37). We have previously shown that IL-6 plays a key role in promoting VT (16). We therefore decided to investigate the relationship between gal3 and IL-6 during VT. The strong correlation we observed between gal3 and IL-6 expression in the vein
wall led us to examine if these were mechanistically involved in venous thrombogenesis. The WT mice that were given rGal3 had significantly elevated thrombus weights and increased vein wall expression of IL-6. Conversely, IL-6KO mice had smaller VT, and recombinant gal3 did not restore the thrombogenic phenotype. These data suggest that gal3 is promoting thrombosis in an IL-6 dependent manner. We also found an increase in CCL2 expression in the vein wall of VT mice treated with rGal3, further supporting a role of gal3 in the inflammatory component of VT. Based on the data we gathered in this study and Wojcik et al.’s (16), we believe gal3bp and gal3 are upstream elements of a pathway that up-regulates IL-6 and ultimately CCL2 during VT. Understanding the pathway(s) by which gal3 and gal3bp up-regulate inflammatory cytokines is imperative points along this pathway could serve as targets for future therapies to prevent VT.

**Gal3 and Gal3bp participate in leukocyte migration:**

Inflammation was first linked to VT in a study published by Stewart et al. in 1974 (3). Gal3bp and gal3 promote inflammation not only by up-regulating the expression of inflammatory cytokines, but also by attracting and facilitating the migration of WBC into tissue (22, 38-40). Sato et al. found that gal3 accumulation in the alveolar space of infected lungs correlated strongly with the onset of neutrophil infiltration (22), and Hsu et al. found that gal3KO mice had less inflammatory cell infiltration into the peritoneal cavity upon thioglycollate broth injection than gal3+/+ mice (39). To the best of our knowledge, prior to this study no investigations had been performed on the role of gal3bp and gal3 in leucocyte migration during VT. Our IHC and quantification of leukocyte extravasation in WT and Gal3KO mice demonstrated that gal3 contributes to the inflammatory component of VT. In addition, in IVC samples harvested from mice without VT, we observed “socket” like positive signals for gal3bp
at the vein wall surface, interacting with gal3-rich leukocytes. This pattern was also observed in samples harvested from mice with VT in areas where the thrombus was not attached to the IVC, but disappeared in areas where the thrombus was strongly adhered. It is likely that vein wall areas that were flush against the thrombus suffered hypoxia and tissue damage, even perhaps cell death, which could explain the lack of gal3bp and gal3 staining in these regions. The observation that the vein wall stained positively for both gal3bp and gal3 is consistent with our western blot data, which showed both gal3bp and gal3 bands in blots performed using homogenized vein wall samples. In all observed cases of neutrophil attachment to and migration into the vein wall, gal3 rich neutrophils were interacting with gal3bp rich “sockets” in the endothelium. To the best of our knowledge, no previous studies have shown galectin or galectin-binding protein “sockets” in the vein wall. Moreover, leukocyte extravasation into the vein wall of VT positive mice was significantly reduced in gal3 deficient mice 48 hours post-ligation. Based on these observations, we strongly suspect that a gal3 mediated pathway contributes to the leukocyte-driven component of venous thrombosis.

**Gal3 and Gal3bp as biomarkers candidates**

In the present work, we present two biomarker candidates, circulating gal3bp and gal3, both of which are elevated in humans and mice during VT (Figure 1A&B supplemental data). Our gal3 values were clearly below the levels of the circulating levels range in humans. Our human blood samples were collected using sodium citrate and not EDTA. In addition, we used bank samples stored for more than 6 months (41). Storage of samples for this long can cause gal3 levels to decrease in samples. None of these factors affected gal3bp assay performance, but they likely did affect gal3 assay performance. Future studies should evaluate the levels of gal3bp and
gal3 in a larger cohort of VT positive and VT negative individuals immediately after their blood is drawn. The ability of gal3bp and gal3 to accurately rule in/rule out VT should then be evaluated and compared against the ability of currently used biomarkers (such as D-dimer) to do so.

**Gal3 and Gal3bp as therapeutic candidates**

Both blocking gal3bp and knocking out gal3 reduced thrombus weight (Figure 1C supplemental data). The significant decreases observed in the thrombus weights of mice deficient in gal3 or treated with a gal3bp depleting antibody confirm that both of these proteins are pro-thrombotic and suggest that both are potential therapeutic targets. Although gal3KO mice had lower thrombus weights than anti-gal3bp treated mice, the difference between the two groups was not significant. Whether or not the therapeutic effect of eliminating gal3 or blocking gal3bp was due to the prevention of an interaction between the two proteins, between the individual proteins and some of their alternative ligands, or some combination of both, still remains to be evaluated. Regardless of the mechanism, our data suggest that reducing gal3 and gal3bp can have protective effects against VT.

**Conclusion**

In conclusion, this is the first study to demonstrate a role for gal3bp and gal3 in experimental VT. We have shown that gal3bp and gal3 possess pro-thrombotic and pro-inflammatory properties in the context of experimental VT, and that these proteins may be ideal targets for therapies that seek to prevent VT. Furthermore, based on our findings, we believe that
gal3 and/or gal3bp should be rigorously investigated to determine their quality as potential biomarkers of VT.

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REFERENCES


FIGURE LEGENDS

Figure 1.
The effects of venous thrombosis on Gal3BP and gal3 in blood circulating elements (Systemic), thrombus and vein wall (Local). Western blot and quantification.

Western blot analysis of gal3BP and gal3 in red blood cells (n=1 per band), white blood cells (n=5 per band), platelets (n=3 per band), pro-coagulant microparticles (n=3 per band), thrombus (n=1 per band), and vein wall (n=1 per band) collected from non-VT (-) (n=21) or VT (+) (n=25) mice 48 hours post thrombosis. Actin was used as a loading control. Quantification of blot intensity relative to loading control shown below. Abbreviations: gal3BP: galectin 3 binding protein; gal3: galectin 3; gal3m: gal3 monomer; gal3MD: gal3 multimer dimer; gal3MT: gal3 multimer trimer; gal3d: gal3 degradation product; red blood cells (RBC), white blood cells (WBC), platelets (PLT), pro-coagulant microparticles (MP), vein wall (IVC); VT: venous thrombosis.

Figure 2.
The effects of venous thrombosis on Gal3BP and gal3 in vein wall and thrombus (Local).

Gene expression and Histology
A) Analysis of gal3BP and gal3 in vein wall by qRT-PCR: gal3BP and gal3 mRNA isolated from the IVC (Vein Wall) of non-VT (n= 8) or 48hr VT (n=12) WT mice were quantified using qRT-PCR. Gene expression was quantified relative to β-actin.

B) H&E and IHC for gal3. H&E stain and IHC for gal3 in both non-VT and VT conditions.

Figure 3.

Gal3 correlation with IVC+Thrombus weights: Time course.
A) IVC or IVC+ Thrombus weight 3hrs, 6hrs, or 48hrs (n=5 per time point) post ligation, and in non-VT mice (n= 5). B) Expression of gal3 in the vein wall of non-VT and VT mice 3hrs, 6hrs, or 48hrs post-ligation. C) Correlation between gal3 gene expression in the IVC and IVC+Thrombus weight. VT: venous thrombosis; IVC: inferior vena cava.

Figure 4.

Gal3/IL-6 and IL-6/CCL2 Correlations in VT.
A) Gal3 and IL-6 gene expression in the IVC of non-VT (n=4) and 48hrs VT (n=3) mice. Shown below is the correlation between gal3 and IL-6 gene expression in individual mice. B) IL-6 and CCL2 gene expression in the IVC of non-VT (n=4), 48hrs VT (n=3), and 48hrs VT mice that were treated with recombinant human gal3 (n=4). Shown below is the correlation between IL-6 and CCL2 expression in individual mice.

Figure 5.

The effect of recombinant gal3 (rGal3) on 48 hours IVC+TW in WT, Gal3KO, and IL-6KO mice. Columns from left to right: IVC or 48hrs post-ligation IVC+TW in non-VT (black column,
n=4), WT (white, n=10), rGal3 treated WT (white, n=4), Gal3KO (gray, n=7), rGal3 treated Gal3KO (gray, n=4), IL-6KO (gray with dots, n=7), and rGal3 treated IL-6KO (gray with dots, n=3) mice.

**Figure 6.**

**Leukocyte infiltration into the vein wall of WT and Gal3KO mice.**

A) Representative pictures of 48 hours post thrombosis initiation showing leukocyte infiltration in C57Bl/6 mice and Gal3KO mice (20x). B) Quantification of leukocytes migration in the vein walls of WT and Gal3KO mice at 3hrs, 6hrs, and 48 hours (n=5 per time point) post-IVC ligation, and non-VT (n=5) were examined under high power oil immersion light microscopy. Note that in Gal3KO mice, leukocyte extravasation was significantly depressed compared to WT mice, at 48hrs after thrombosis was initiated, the time point used for most of the experiments described in this manuscript.

**Figure 7.**

**Gal3BP and gal3 at the lumen/IVC interface.** Non-VT images: Top raw (A-D); VT images 48 hours after thrombosis was initiated from an area where there was not thrombus/vein wall contact, middle raw (E-H); and VT images 48 hours after thrombosis was initiated from an area where there was thrombus/vein wall fusion, bottom raw (I-L). Note that vein wall gal3 and gal3bp are abundant in areas where the thrombus is not contacting the vein wall (F and G), but disappear in areas where the thrombus contacts the vein wall (J and K). Red blood cell fluorescence is predominately non-specific auto-fluorescence.

**Figure 8.**

**Gal3BP and gal3 mediated leukocyte interactions with the IVC.** Sockets distribution of gal3bp appears to be interacting with gal3 rich leukocytes (H, L). Red blood cell fluorescence is predominately non-specific autofluorescence. IVC lumen indicated with an asterisk in DAPI images. Yellow arrows indicate WBC that is not attached to the vein wall. White arrows indicate gal3bp “sockets” on the vein wall. (A,E,I) Nuclear DAPI staining. (B,F,J) Gal3bp localization (FITC). (C,G,K) Gal3 localization (Texas Red). (D,H,L) Merge of images.
Gal3BP and gal3 analysis in blood circulating elements (Systemic), thrombus and vein wall (Local). Blots and quantification.

<table>
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| VT   |      |      | VT  |          | VT   |
| Gal3BP | VT |      | VT  |          | VT   |
| Gal3MD | VT | vt   | VT  |          | VT   |
| Gal3m | VT | VT   | VT  |          | VT   |
| Gal3d | VT | VT   | VT  |          | VT   |
| Actin | VT | VT   | VT  |          | VT   |

| VT   |      |      | VT  |          | VT   |
| Gal3BP | VT |      | VT  |          | VT   |
| Gal3MD | VT | vt   | VT  |          | VT   |
| Gal3m | VT | VT   | VT  |          | VT   |
| Gal3d | VT | VT   | VT  |          | VT   |
| Actin | VT | VT   | VT  |          | VT   |

| VT   |      |      | VT  |          | VT   |
| Gal3BP | VT |      | VT  |          | VT   |
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| Gal3m | VT | VT   | VT  |          | VT   |
| Gal3d | VT | VT   | VT  |          | VT   |
| Actin | VT | VT   | VT  |          | VT   |

| VT   |      |      | VT  |          | VT   |
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| Gal3d | VT | VT   | VT  |          | VT   |
| Actin | VT | VT   | VT  |          | VT   |

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| gal3MD | VT | vt   | VT  |          | VT   |
| gal3m | VT | VT   | VT  |          | VT   |
| gal3d | VT | VT   | VT  |          | VT   |
| Actin | VT | VT   | VT  |          | VT   |

| VT   |      |      | VT  |          | VT   |
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| gal3d | VT | VT   | VT  |          | VT   |
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| VT   |      |      | VT  |          | VT   |
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| gal3MD | VT | vt   | VT  |          | VT   |
| gal3m | VT | VT   | VT  |          | VT   |
| gal3d | VT | VT   | VT  |          | VT   |
| Actin | VT | VT   | VT  |          | VT   |

| VT   |      |      | VT  |          | VT   |
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| gal3MD | VT | vt   | VT  |          | VT   |
| gal3m | VT | VT   | VT  |          | VT   |
| gal3d | VT | VT   | VT  |          | VT   |
| Actin | VT | VT   | VT  |          | VT   |
Figure 2

Gal3BP and gal3 analysis in vein wall and thrombus (Local)
Gene expression and Histology

A  Vein wall gene expression of gal3BP and gal3

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mRNA expression Rel. to B-Actin

* p<0.05

B  Gal3 interaction at the lumen/IVC interface

Non-VT
- Lumen
- Vein wall

VT
- Vein wall
- Thrombus

H&E

100x
Figure 3

Gal3 correlation with IVC+Thrombus weights: Time course

A  IVC+Thrombus weight

B  Vein wall Gal3 gene expression

C  IVC+Thrombus weight and Gal3 gene expression correlation

\[ r^2 = 0.77 \]

\[ p < 0.05 \]
Figure 4

Gal3/interleukin-6 and interleukin-6/CCL2 correlation

A

Gal3 gene expression

IL-6 gene expression

p < 0.05

Gal3/IL-6 correlation

r² = 0.81

p < 0.05

B

IL-6 gene expression

CCL2 gene expression

p < 0.05

IL-6/CCL2 correlation

r² = 0.79

p < 0.05
Figure 5

**IVC + Thrombus weight**

- **Non-VT**
- **WT**
- **WT + rGal3**
- **Gal3 KO**
- **Gal3 KO + rGal3**
- **IL-6 KO**
- **IL-6 KO + rGal3**

Weight in grams

<table>
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<td>WT vs. WT + rGal3</td>
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</table>

48 hours VT
Figure 6

A

WT mice

Leukocyte extravasation (staining)

Gal3KO mice

20X – 48 hours VT

B

Leukocyte extravasation (quantification)

WT

Gal3 KO

* \( p < 0.05 \)

Cell count per 5 HPF

Non-VT

Hours VT

3 6 48

Non-VT

Hours VT

3 6 48
Figure 7

Gal3 and gal3bp interaction at the lumen/IVC interface
Non-VT and VT conditions
Figure 8

Gal3 and gal3bp interaction at the lumen/IVC interface
Leukocytes: A-D) non attached to any other cell, E-H) partially attached and I-L) totally attached to the endothelial cell line
The role of galectin-3 and galectin-3-binding protein in venous thrombosis

Elise P. DeRoo, Shirley K. Wrobleski, Evelyn M. Shea, Ramsey K. Al-Khalil, Angela E. Hawley, Peter K. Henke, Daniel D. Myers Jr., Thomas W. Wakefield and Jose A. Diaz