Vitronectin stabilizes intravascular adhesion of neutrophils by coordinating β2 integrin clustering

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Abstract

The recruitment of neutrophils from the microvasculature to the site of injury or infection represents a key event in the inflammatory response. Vitronectin (VN) is a multifunctional macromolecule abundantly present in blood and extracellular matrix. The role of this glycoprotein in the extravasation process of circulating neutrophils remains elusive. Employing advanced in vivo/ex vivo imaging techniques in different mouse models as well as in vitro methods, we uncovered a previously unrecognized function of VN in the transition of dynamic to static intravascular interactions of neutrophils with microvascular endothelial cells. These distinct properties of VN require the heteromerization of this glycoprotein with plasminogen activator inhibitor-1 (PAI-1) on the activated venular endothelium and subsequent interactions of this protein complex with the scavenger receptor low-density lipoprotein receptor-related protein-1 (LRP-1) on intravascularly adhering neutrophils. This induces p38 mitogen-activated protein kinases (MAPK)-dependent intracellular signaling events which, in turn, regulates the proper clustering of the β2 integrin lymphocyte function associated antigen-1 (LFA-1) on the surface of these immune cells. As a consequence of this molecular interplay, neutrophils become able to stabilize their adhesion to the microvascular endothelium and, subsequently, to extravasate to the perivascular tissue. Hence, endothelial-bound VN-PAI-1 heteromers stabilize intravascular adhesion of neutrophils by coordinating β2 integrin clustering on the surface of these immune cells, thereby effectively controlling neutrophil trafficking to inflamed tissue. Targeting this protein complex might be beneficial for the prevention and treatment of inflammatory pathologies.
Introduction

Vitronectin (VN) is a multidomain macromolecule synthesized by the liver and found in platelets (1). Upon release into the extracellular space (2), VN becomes capable of establishing interactions with different proteins involved in diverse biological processes: Engaging its somatomedin B domain, VN acts as a ligand for the (soluble) urokinase-type plasminogen activator (uPA) receptor or binds to plasminogen activator inhibitor-1 (PAI-1), thereby extending the half life of this protease inhibitor in fibrinolysis. Through its RGD sequence, VN can interact with αvβ3, αvβ5, αIIbβ3, and αvβ1 integrins which are expressed on the surface of leukocytes and platelets. In addition, several other molecules (e.g., proteoglycans, heparin, collagens, or kininogen) serve as binding partners of VN immobilizing this glycoprotein in the extracellular matrix (ECM), ultimately regulating cell adhesion and migration (3-5). Accordingly, enhanced levels of VN have been detected in various inflammatory pathologies including atherosclerosis, glomerulonephritis, or rheumatic disease (6-10). The functional role of VN under these pathological conditions, however, remains largely unclear.

The recruitment of white blood cells (leukocytes) from the microvasculature to the site of inflammation is a fundamental process in the immune response (11-14). In inflamed tissue, circulating leukocytes become captured and start to roll on the microvascular endothelium in a selectin-dependent manner (15). This triggers the intermediate affinity conformation of β2 integrins on the surface of rolling leukocytes which allows them to further slow down in the bloodstream (16, 17). Subsequently, interactions of chemokines presented on the microvascular endothelium with their cognate chemokine receptors on rolling leukocytes as well as of endothelial E-selectin/CD62E with leukocyte P-selectin glycoprotein ligand-1 (PSGL-1/CD168) are supposed to initiate the full activation of leukocyte integrins ultimately facilitating intravascular adhesion of these immune cells to the endothelial surface (15, 18). After stabilizing their adhesion, leukocytes intravascularly crawl to sites of adherent platelets, from where they finally extravasate to the perivascular tissue and migrate to their target destination (19). Whereas a variety of adhesion and signaling molecules have been
characterized to control distinct steps of this highly complex process, the mechanisms underlying the stabilization of leukocyte adhesion to the microvascular endothelium are still poorly understood.

Fibrinolysis is an elementary biological process that maintains blood perfusion by preventing clot formation in the vasculature. Plasmin is the principal effector protease in the fibrinolytic system, which is activated by tissue-plasminogen activator (tPA) and – to a lesser degree – by urokinase-type plasminogen activator (uPA). The activity of these serine proteases is tightly controlled by plasminogen activator inhibitor-1 (PAI-1). Besides these well-known fibrinolytic properties, it has become evident that the components of the fibrinolytic system also considerably contribute to different biological processes such as immune cell trafficking (20-24). With respect to the distinct interactive properties of VN and the substantial involvement of the various binding partners of this glycoprotein in immune cell responses, we hypothesize that VN is critical for leukocyte recruitment to the site of inflammation.
Methods

A detailed description of the methods employed in this study is included in Supplemental Material.

All experiments were performed according to German legislation for the protection of animals and approved by the local government authorities (Regierung von Oberbayern).

Six hours after intra-peritoneal injection of the chemokines CXCL1 or CCL2, leukocyte recruitment to the peritoneal cavity was studied by flow cytometry in wild-type or VN-deficient mice. The single steps of the neutrophil (visualized by fluorescence-labeled anti-Ly-6G monoclonal antibodies) extravasation process were analyzed by in vivo microscopy in the cremaster muscle of anesthetized mice deficient for distinct proteins or receiving different inhibitors/blocking antibodies. Integrin activation/trafficking was assessed in neutrophils from wild-type mice by flow cytometry and spinning disc confocal microscopy.
Results

Distribution of VN in inflamed tissue

Under a variety of inflammatory conditions, enhanced tissue levels of VN have been observed (6-10). The exact distribution patterns of this glycoprotein in inflamed tissue, however, remained unclear. Employing immunostaining and confocal laser scanning microscopy on tissue whole mounts of the mouse cremaster muscle, VN was barely detected in unstimulated tissue (Fig. 1A). In the acute inflammatory response upon sterile (ischemia-reperfusion (I/R); 30/120 min) injury, however, VN was found to be deposited on the luminal surface of postcapillary venules. This microvascular deposition of VN was nearly absent upon enzymatic degradation of glycosaminoglycans (GAGs).

Role of VN for myeloid leukocyte trafficking

To characterize the role of VN for myeloid leukocyte recruitment to the site of inflammation, we initially used a peritoneal leukocyte trafficking assay. As identified by multi-channel flow cytometry analyses of the peritoneal lavage fluid, six hours of intraperitoneal stimulation with the chemokines CXCL1/KC or CCL2/MCP-1 induced a significant increase in numbers of neutrophils (CD45⁺ CD11b⁺ Gr-1<sup>high</sup> CD115<sup>low</sup>) and classical/inflammatory monocytes (CD45⁺ CD11b⁺ Gr-1<sup>high</sup> CD115<sup>high</sup>; Fig. 1B), but not of non-classical monocytes (CD45⁺ CD11b⁺ Gr-1<sup>low</sup> CD115<sup>high</sup>; not shown) recruited to the peritoneal cavity of wild-type (WT) mice as compared to unstimulated controls. This increase in numbers of neutrophils was almost completely abolished in VN<sup>+/−</sup> or VN<sup>−/−</sup> mice, whereas the recruitment of classical/inflammatory monocytes remained unaffected by VN deficiency. In this context, we found that low density lipoprotein-related receptor protein-1 (LRP-1), which serves as a receptor for the VN binding partner PAI-1 (25-27), is higher expressed on the surface of activated murine neutrophils (mean fluorescence intensity: 1023.0±231.4) than on activated classical monocytes (mean fluorescence intensity: 473.0±128.8). Moreover, LRP-1 was identified to be expressed heterogeneously in murine neutrophils (Fig. S1).
Role of VN for intravascular interactions of neutrophils

To further decipher the role of VN in the extravasation process of neutrophils, we performed multi-channel in vivo microscopy on the mouse cremaster muscle. In these experiments, we observed that Ly-6G+ neutrophils in VN+/− mice are unable to stabilize their adhesion to the endothelial surface of postcapillary venules in inflamed tissue, whereas neutrophils in WT mice properly adhered to the microvascular endothelium (Fig. 2A; video S1, S2). Accordingly, the quantitative analysis of these events revealed no significant differences in numbers of rolling neutrophils or in the rolling velocity of neutrophils between WT and VN-deficient animals upon I/R (30/120 min) or after six hours of intrascrotal stimulation with CXCL1/KC or CCL2/MCP-1 (Fig. 2A). In contrast, the average number of neutrophils adhering to the vessel wall of postcapillary venules for more than 30 s was significantly lower in VN-deficient animals than in WT controls, whereas the average number of neutrophils adhering for less than 30 s was significantly higher in VN-deficient animals than in WT controls. Consequently, the average time of a neutrophil being adherent to the microvascular endothelium was significantly shorter in VN-deficient animals than in WT controls. This defect of neutrophils in VN-deficient mice to stabilize intravascular adhesion resulted in a significantly reduced number of extravasated neutrophils. Noteworthy, a minority of neutrophils in VN-deficient animals was still able to stabilize their adherence to the microvascular endothelium, which might be explained by the heterogeneous expression levels of the VN receptor LRP-1 (Fig. S1) and/or compensatory mechanisms in a subset of these immune cells. Such alternative mechanisms (which might even include cumulative factors) controlling the stabilization of neutrophil adherence are still unclear and subject of future investigations.

To exclude neutrophil-intrinsic effects of VN deficiency on the stabilization of intravascular adhesion of these immune cells, we conducted bone marrow cell transfer experiments (Fig. 2B). In the postischemic mouse cremaster muscle (I/R 30/120 min), the number of accumulated fluorescence-labeled neutrophils isolated from VN-deficient donor mice and
adoptively transferred into WT recipient mice was not significantly altered as compared to the number of accumulated fluorescence-labeled neutrophils isolated from WT donor mice and adoptively transferred into WT mice. In contrast, the number of accumulated fluorescence-labeled neutrophils isolated from WT mice and adoptively transferred into VN-deficient recipient mice was significantly reduced as compared to the number of accumulated fluorescence-labeled neutrophils isolated from WT donor mice and adoptively transferred into WT recipient mice. Thus, a neutrophil-intrinsic effect of VN deficiency was not evident.

Role of integrin interaction partners of VN for neutrophil trafficking

Platelets are known to play a critical role in the extravasation process of leukocytes (11-14). Since αvβ3 and αIIbβ3 integrins are expressed on the surface of platelets and serve as binding partners of VN, this glycoprotein might stabilize intravascular adhesion of neutrophils by mediating interactions of these cell populations. Using in vivo microscopy on the postischemic mouse cremaster muscle, however, intravascular firm adherence of platelets or interactions of intravascularly adherent platelets and neutrophils did not significantly vary between WT and VN-deficient mice (Fig. 3A). In contrast, intravascular firm adherence of neutrophils was significantly diminished in VN-deficient animals as compared to WT animals (Fig. 3A). Conversely, antibody-mediated depletion of platelets (by > 95 %) significantly reduced the number of intravascularly adherent (> 30s) and transmigrated neutrophils in the field of view, but did not significantly change intravascular adhesion times of these immune cells in the microvasculature of postischemic tissue (Fig. 3B). These data confirm previous observations documenting that intravascularly adherent neutrophils do not stop crawling in the microvasculature and start their transmigration (which results in less firmly adherent and transmigrated leukocytes quantified in the field of view) until they are captured by intravascularly adherent platelets (19).

In addition to αvβ3 and αIIbβ3 integrins, VN is able to interact with αvβ5, αvβ1, α5β2/LFA-1/CD11a, and αMβ2/Mac-1/CD11b integrins (3-5). In further in vivo microscopy experiments,
we therefore sought to evaluate the effect of these binding partners of VN on the stabilization of neutrophil intravascular adhesion. In the postischemic mouse cremaster muscle, antibody blockade of αv or αIIbβ3 integrins did not significantly alter intravascular adhesion of neutrophils to the endothelium. Although antibody blockade of αβ2/LFA-1/CD11a or of αβ2/Mac-1/CD11b as well as of the endothelial β2 interaction partner ICAM-1/CD54 (Fig. 3C) significantly reduced numbers of intravascularly firmly adherent (> 30s) neutrophils, average adhesion times of neutrophils in the microvasculature were not significantly changed (Fig. 3D). These data indicate that β2 integrins and ICAM-1/CD54 are already involved in the induction of neutrophil intravascular adherence.

Effect of heteromerization of VN with PAI-1 on neutrophil trafficking

PAI-1 is another binding partner of VN that is also involved in neutrophil trafficking (20-24). We here confirm that murine PAI-1 (Fig. S2) binds to VN, but not to fibronectin (FN; Fig. S3A), ultimately forming VN-PAI-1 heteromers (Fig. S3B) as evidenced by (sandwich) ELISA analyses. Moreover, VN-PAI-1 heteromers were found in the peripheral blood of unstimulated mice whose levels slightly increased upon induction of systemic inflammation (Fig. S4). This increase in circulating VN-PAI-1 heteromers might be due to release of VN and PAI-1 from the liver, of PAI-1 by the microvascular endothelium, as well as of VN, PAI-1, and pre-formed complexes of the single proteins by activated platelets (28) in the acute phase of the inflammatory response. Immunostaining and confocal laser scanning microscopy on cremasteric tissue whole mounts further revealed that VN, PAI-1, and adherent neutrophils co-localize on the postischemic venular endothelium (Fig. 4A). To evaluate the role of complex formation of VN with PAI-1 for the stabilization of neutrophil intravascular adhesion, we performed in vivo microscopy experiments in the postischemic cremaster muscle of PAI-1-deficient mice which received different PAI-1 mutant proteins (Fig. 4B). Substitution of PAI-1-deficient mice with active stable PAI-1 (CPAI) or a PAI-1 mutant protein lacking anti-protease activity (PAI-RR) resulted in significantly higher numbers
of intravascularly firmly adherent (> 30 s) neutrophils, significantly lower numbers of intravascularly shortly adherent (< 30 s) neutrophils as well as higher average adhesion times of adherent neutrophils as compared to PAI-1-deficient mice substituted by a PAI-1 mutant protein lacking its VN binding domain (PAI-QR). In this context, substitution of PAI-deficient mice with PAI-QR induced short adhesion, but not of firm adherence of neutrophils to the microvascular endothelium as compared to vehicle-treated PAI-1-deficient animals (which might be due to PAI-1 binding to its receptor LRP-1 (29) without prior interaction of PAI-1 and VN). Further, substitution of VN−/− animals with VN-PAI-1 protein rescued the adhesion defect arising from VN deficiency (Fig. S5). These results strengthen our concept that the interaction of VN and PAI-1 is critical for the stabilization of intravascular adhesion of neutrophils.

In autoperfused flow chambers coated with CD62E/E-selectin and ICAM-1/CD54, an additional coating with VN-PAI-1 (Fig. S3) did not significantly alter rolling and adherence of neutrophils, whereas an additional coating with the chemokine CXCL1/KC induced a significant elevation in numbers of adherent neutrophils (Fig. 4C). These data suggest that chemokines, but not VN-PAI-1 heteromers, are critical for the induction of intravascular adherence of neutrophils.

Effect of VN-PAI-1 on activation of β2 integrins in neutrophils

In inflamed tissue, intravascular firm adherence of neutrophils to the microvascular endothelium is facilitated by interactions of endothelial members of the immunoglobulin superfamily (e.g., ICAM-1/CD54) and neutrophil β2 integrins in higher affinity conformation (16, 17). Employing multi-channel flow cytometry, exposure to VN-PAI-1, but not to VN, uPA, PAI-1, or VN-uPA significantly enhanced the fluorescence signal for the β2 integrins CD11a/LFA-1 and – to a lesser degree – CD11b/Mac-1 on the surface of neutrophils as compared to unstimulated controls (Fig. 5A; Fig. S6).
As a measure of conformational changes of β2 integrins, binding of their interaction partner ICAM-1/CD54 to neutrophils was analyzed in a next step. Similarly to our previous results for expression of β2 integrins, binding of ICAM-1/CD54 to neutrophils was significantly increased upon exposure to VN-PAI-1, but not upon exposure to VN, uPA, PAI-1, or VN-uPA, as compared to unstimulated controls (Fig. 5B). Application of receptor associated protein (RAP; blocking members of the LDL receptor family), application of blocking anti-LRP-1 antibodies, or inhibitors of p38 (but not of JNK or ERK1/2) mitogen-activated protein kinases (MAPK) almost completely abolished VN-PAI-1-elicited ICAM-1/CD54 binding.

To specifically evaluate the effect of VN-PAI-1 on conformational changes of β2 integrins, conformation-specific antibodies for the detection of the intermediate (KIM127) or high-affinity conformation (mAb 24) of β2 integrins (which are only available for human integrins) were used (Fig. 5C). Upon exposure of VN-PAI-1 to human neutrophils, however, binding of ‘KIM127’ and ‘mAb 24’ was not significantly altered as compared to basal antibody binding in unstimulated controls whereas exposure to the chemokine CXCL1/KC significantly increased ‘KIM127’ and ‘mAb 24’ binding. Collectively, these data suggest that the increased ICAM-1/CD54 binding to neutrophils upon exposure to VN-PAI-1 (Fig. 5B) rather is due to enhanced/optimized presentation of β2 integrins on the surface of these immune cells (Fig. 5A; Fig. 6) than to conformational changes in these adhesion and signaling molecules.

Since integrin clustering is thought to be particularly important in postadhesion strengthening of leukocyte-endothelial cell interactions, we employed spinning disc confocal microscopy to study the effect of VN-PAI-1 heteromers on the cell membrane trafficking dynamics of β2 integrins in neutrophils (Fig. 6A, B, C). In these experiments, we found that additional exposure of neutrophils isolated from the peripheral blood of WT mice to VN-PAI-1 heteromers significantly increased the clustering of the β2 integrin CD11a/LFA-1 as compared to exposure to the chemokine CXCL1/KC alone, or to PBS.

Systemic leukocyte counts and microhemodynamic parameters
To assure intergroup comparability in our in vivo microscopy experiments, systemic leukocyte counts and microhemodynamic parameters including blood flow velocity, inner vessel diameter, and wall shear rate were determined in each experiment. No significant differences were detected among experimental groups (Tab. S1).

**Discussion**

The trafficking of circulating leukocytes from the venular microvasculature to the site of injury or infection is a key event in the pathogenesis of inflammatory diseases (11-14). The role of the matricellular protein VN in this fundamental biological process is still unclear. Under homeostatic conditions, VN predominantly circulates in the blood as a monomer. In the acute inflammatory response, however, the binding of activated PAI-1 to VN monomers induces conformational changes in this macromolecule that effectively promote its multimerization. This allows multimeric VN to specifically bind to the surface of endothelial cells and, in turn, to unfold previously cryptic binding sites as evidenced by different in vitro studies (30-32). Accordingly, we found VN to be deposited on the luminal surface of the venular microvasculature in inflamed tissue. Since GAGs cover the luminal aspect of microvascular endothelial cells and have previously been reported to interact with VN through its core polypeptide (33), we hypothesized that these carboanhydrates serve as binding partner for VN on the inner vessel wall. Confirming this assumption, enzymatic degradation of GAGs almost completely abrogated the deposition of VN on the activated microvascular endothelium, thus suggesting that VN is immobilized in inflamed tissue on the surface of microvascular endothelial cells by endothelial GAGs.

To evaluate the functional relevance of VN for leukocyte migration to the site of inflammation, we employed a peritoneal leukocyte trafficking assay. In these experiments, neutrophil extravasation to the peritoneal cavity was severely compromised in VN-deficient mice as compared to WT controls. Notably, this impairment in neutrophil recruitment reached similar levels in heterozygous and homozygous VN-deficient animals suggesting that comparatively large amounts of endothelially deposited VN are required for the induction of neutrophil responses. Moreover, we found that extravasation of classical/inflammatory monocytes
remained unaffected by VN deficiency collectively indicating that VN particularly mediates the trafficking of neutrophils to inflamed tissue. This might be explained by higher expression of the scavenger receptor LRP-1 (which serves as a receptor of the VN binding partner PAI-1) on activated neutrophils as compared to activated classical monocytes, hence extending previous observations on the involvement of VN in leukocyte recruitment under inflammatory conditions (34-38).

With respect to the deposition of VN on the surface of postcapillary venules in inflamed tissue, we hypothesized that this glycoprotein contributes to the regulation of intravascular interactions of neutrophils in the extravasation process of these immune cells. Here, we show that VN deficiency does not alter the rolling behavior of neutrophils in inflamed postcapillary venules, suggesting that VN does not influence the "selectin-dependent phase" of the neutrophil extravasation process. Most interestingly, however, the average time of a neutrophil being adherent to the endothelial surface was significantly lower in VN-deficient mice than in WT controls. Furthermore, adoptively transferred neutrophils from WT donor mice accumulated less efficiently in the inflamed tissue of VN-deficient recipient mice as compared to neutrophils isolated from VN-deficient or WT donor mice transferred into WT recipient animals. Hence, our findings clearly demonstrate that endothelially deposited VN stabilizes intravascular adherence of neutrophils on the microvascular endothelium in inflamed tissue. To our knowledge, this is the first description of a protein specifically regulating this critical step in the extravasation cascade of neutrophils. Further, our observations indicate that stable intravascular adherence of neutrophils is prerequisite for (and does not interfere with) the subsequent transmigration of these immune cells into the perivascular space, a process that is facilitated by sequential heterophilic (e.g., between neutrophil LFA-1/CD11a and endothelial ICAM-2/CD102 or JAM-A as well as between neutrophil Mac-1/CD11b and ICAM-2/CD102 or JAM-C) and homophilic (between neutrophil and endothelial PECAM-1/CD31 or CD99) molecular interactions between neutrophils and endothelial cells as well as by endothelial molecules such as VE-cadherin, ESAM, or CD99L2 (11-14).
In addition to interactions with endothelial cells, the interplay between leukocytes and platelets considerably contributes to the extravasation of leukocytes (39-42). In this context, platelets have recently been demonstrated to guide intravascularly crawling neutrophils and monocytes to their site of transmigration into the interstitial tissue (19). Since VN is able to bind to platelets through αvβ3 and αIIbβ3 integrins via its RGD motif (43, 44), these cellular blood components might also participate in VN-dependent neutrophil trafficking. In further experiments, however, VN deficiency neither altered intravascular adherence of platelets nor interactions of intravascularly adherent platelets and neutrophils. In line with these observations, platelet depletion did also not significantly change intravascular adhesion times of neutrophils in inflamed tissue collectively indicating that platelets do not contribute to the stabilization of neutrophil adherence in the microvasculature.

Besides platelet integrins, integrins expressed on the surface of leukocytes including αvβ3, αvβ5, αvβ1, αLβ2, and αMβ2 represent potential interaction partners of VN (3-5). Similar to our results for platelet integrins, however, blockade of these integrins did not significantly alter the average time of neutrophils resting on the endothelial surface of postcapillary venules in the inflamed mouse cremaster muscle.

Beyond their established role in fibrinolysis, the components of the fibrinolytic system are increasingly recognized as mediators of immune cell migration (20-24). Recently, PAI-1 has been implicated in leukocyte trafficking to the site of inflammation by regulating intravascular adherence and (subsequent) transmigration of these immune cells (29). Since VN is capable of binding to PAI-1 via its somatomedin B domain (thereby extending the half-life of this protease inhibitor in fibrinolysis (3-5)), heteromerization of VN with PAI-1 might promote intravascular adherence of neutrophils to the endothelium in inflamed tissue. To prove this hypothesis, we reconstituted PAI-1-deficient animals with different PAI-1 mutant proteins. Substitution with active stable PAI-1 or a PAI-1 mutant protein lacking anti-protease activity completely rescued the adhesion and extravasation defect of neutrophils observed in PAI-1-deficient mice. Substitution of PAI-1-deficient animals with a PAI-1 mutant protein lacking its VN binding domain, however, significantly diminished the average intravascular adhesion
time of neutrophils as compared to active stable PAI-1-substituted mice resembling our observations in VN-deficient animals. Conversely, substitution of PAI-1-deficient mice with this non-VN-binding PAI-1 mutant protein induced short, but not firm adherence of neutrophils to the inflamed vessel wall which might be due to VN-independent binding of PAI-1 to its receptor LRP-1 (29). Finally, substitution of VN-deficient animals with VN-PAI-1 heteromer protein completely rescued the adhesion defect of neutrophils observed in animals lacking VN. Consequently, complex formation of VN with PAI-1 is needed for the stabilization of intravascular adherence of neutrophils in the acute inflammatory response.

Intravascular firm adherence of leukocytes to microvascular endothelial cells is facilitated by interactions between endothelially expressed members of the immunoglobulin superfamily (e.g., ICAM-1/CD54, VCAM-1/CD106) and leukocyte β2 integrins in higher affinity conformations (11-14). We therefore proposed that VN-PAI-1 heteromers stabilize intravascular adhesion of neutrophils in the inflamed microvasculature by activating neutrophil β2 integrins. In our experiments, endothelial-bound VN and PAI-1 were identified to co-localize with intravascularly adherent neutrophils pointing to interactions of complexes of VN and PAI-1 with adhering neutrophils in the inflamed venular microvasculature. Importantly, however, exposure of neutrophils to the VN-PAI-1 complex (but not to VN, PAI-1, and uPA alone or to the VN-uPA complex) induced a significant increase in the expression of CD11a/LFA-1 and – to a lesser degree – of CD11b/Mac-1 on their cell surface, but did not further promote affinity changes in these β2 integrins. Although the induction of conformational changes in integrins enhances their affinity for their individual binding partners, the formation of multiple bonds to multivalent substrates in a process termed ‘integrin clustering’ is thought to be prerequisite for sustained cell adhesion. In leukocyte adhesion, integrins initially form transient microclusters on the surface of these immune cells that confluent into large focal adhesions. This leads to the establishment of complex clusters of adhesion and signaling molecules that trigger outside-in signaling, thus representing a critical event in post-adhesion strengthening (45-47). Here, we demonstrate that VN-PAI-1 heteromers substantially regulate the clustering of β2 integrins on the surface of neutrophils.
With respect to our *in vivo* microscopy observations, these findings conversely suggest that the stabilization of neutrophil intravascular adherence particularly requires integrin clustering on these immune cells rather than the induction of conformational changes of integrins. Inactive integrins exhibit a bent-closed conformation, not extended (E−) and not high affinity (H+), thus being unable to bind a ligand. In contrast, fully activated integrins (E+H+) bind to ICAMs expressed on opposing cells in *trans* (e.g., supporting neutrophil arrest). Integrins transitioning from the resting (E−H−) to the fully activated (E+H+) conformation through E+H− or E−H+ conformations, however, bind to ICAMs on the same cell in *cis*. In (intravascularly rolling) neutrophils, this transition process prohibits the arrest of these immune cells on the microvascular endothelium (48). Recently, it has been found that not only E+H+ integrins, but already E−H+ and E+H− integrins are forming clusters on the surface of neutrophils during cell activation (49). Since endothelially presented VN-PAI-1 heteromers specifically mediate the stabilization of neutrophil adherence on the microvascular endothelium, these protein complexes are thought to promote the binding of clustering integrins in *trans*.

Importantly, however, we are not able to exclude that binding of VN to PAI-1 concomitantly interferes with the inhibitory action of PAI-1 on tissue plasminogen activator (tPA), a proteolytic enzyme that has also been demonstrated to promote leukocyte trafficking to the site of inflammation (50). Furthermore, previous *in vitro* studies proposed a mechanism in which uPA cleaves the RGD motif of VN thereby reducing pro-adhesive properties of VN in an uPAR-dependent manner. Binding of PAI-1 to uPA in this protein complex effectively interferes with these events and subsequently restores the pro-adhesive properties of VN (51). In addition, binding of VN to uPAR has been shown to induce integrin signaling in cells by transmitting a mechanical stimulus to the integrin through the plasma membrane ultimately facilitating cell adhesion (52). Since uPAR has recently been demonstrated to be dispensable for the extravasation of neutrophils in the acute inflammatory response (at least in the model systems employed in the present study) (53), we cannot clearly state to which extent these uPAR-dependent mechanisms contribute to VN-dependent stabilization of intravascular adherence of neutrophils in the inflamed microvasculature. Along the same line,
fibrin(ogen) has been described to facilitate interactions between leukocyte integrins and endothelial ICAM-1/CD54 (54). In this context, however, fibrin(ogen) has been identified to specifically promote the transmigration of neutrophils to postischemic tissue (50).

To restore homeostasis, VN-PAI-1 heteromers are cleared from the circulation through the scavenger receptor LRP-1 (25-27). Recently, we have demonstrated that PAI-1-dependent leukocyte responses in sterile injury are mediated via this receptor protein and subsequent MAPK-dependent intracellular signaling events (29). VN-PAI-1 heteromer-dependent activation of neutrophil β2 integrins might therefore also rely on such molecular processes. In line with this hypothesis, we show that VN-PAI-1 heteromer-elicited surface trafficking of β2 integrins in neutrophils requires LRP-1 and p38 (but not JNK or ERK1/2) MAPK-dependent signaling events. Noteworthy, also selectin- and integrin-triggered signals contribute to the activation of p38 MAPK (15) collectively suggesting that these selectin- and integrin-mediated molecular events (already occurring at the initial stages of the leukocyte recruitment process) are not sufficient for the stabilization of intravascular adhesion of neutrophils, but might prepare this critical step in the extravasation cascade of these immune cells.

In conclusion, our experimental data indicate that the heteromerization of VN with PAI-1 on the microvascular endothelium of inflamed tissue represents a critical event for the extravasation process of neutrophils that substantially controls the transition of dynamic to static endothelial interactions of these immune cells. To this end, VN-PAI-1 heteromers promote the surface clustering of β2 integrins on adhering neutrophils, thus enabling effective neutrophil responses (Fig. 7). Targeting this process might be beneficial for the prevention and treatment of inflammatory diseases.
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Figure legends

Figure 1. Role of VN for leukocyte trafficking to inflamed tissue. A: Using confocal laser scanning microscopy on tissue whole mounts of the cremaster muscle of WT mice, deposition of VN (green) in the PECAM-1/CD31+ microvasculature (blue) was analyzed, representative images are shown (scale bar: 20 µm). Panels show quantitative results from relative fluorescence intensity measurements for VN in sham-operated WT mice as well as in WT mice undergoing I/R (30/120 min) and intravenous application of GAG digesting enzymes or vehicle (mean±SEM for n=4 animals per group; #p<0.05 vs. sham; *p<0.05 vs vehicle). B: Employing multi-channel flow cytometry, the recruitment of neutrophils and classical monocytes to the peritoneal cavity was analyzed 6h after intraperitoneal injection of CXCL1 or CCL2, the gating strategy is shown. Panels show results for LPS-treated WT control mice as well as for WT, VN+/−, or VN−/− mice receiving an i.p. injection of CXCL1 or CCL2 (mean±SEM for n=4 animals per group; #p<0.05 vs. control; *p<0.05 vs WT).

Figure 2. Role of VN for interactions of neutrophils and endothelial cells. A: Using multi-channel in vivo microscopy on the inflamed mouse cremaster muscle, interactions of Ly-6G+ neutrophils (red) with endothelial cells were analyzed in postcapillary venules, representative still images are shown (scale bar: 20 µm). Panels show quantitative results for rolling flux, rolling velocity, short adhesion, firm adherence, adhesion time, and transmigration of neutrophils in WT, VN−/−, or VN+/− mice (mean±SEM for n=4 animals per group; *p<0.05 vs WT). See also Tab. S1. B: Accumulation of calcein AM-labeled bone marrow leukocytes were quantified in the postischemic cremaster muscle using multi-channel in vivo fluorescence microscopy. Panel shows results for WT recipient mice receiving leukocytes from WT or VN-deficient donors as well as for VN-deficient recipient mice receiving leukocytes from WT donors (mean±SEM for n=5 animals per group; *p<0.05 vs WT→WT).
Figure 3. Role of platelets and integrins for the stabilization of intravascular adhesion of neutrophils. A: Interactions of Ly-6G⁺ neutrophils (red) and GP-Ibβ⁺ platelets (green) were analyzed in postcapillary venules (perfused by FITC dextran; grey) of the postischemic mouse cremaster muscle of WT or VN-deficient mice by multi-channel in vivo microscopy, representative images from a WT mouse are shown (scale bar: 10 µm). Panels show quantitative results for intravascular adherence of neutrophils or platelets as well as for the colocalization of these two cell populations (mean±SEM for n=3 animals per group; *p<0.05 vs WT). Further panels show quantitative results for rolling flux, rolling velocity, short adhesion, firm adherence, adhesion time, and transmigration of neutrophils in WT mice receiving platelet-depleting antibodies, blocking antibodies directed against the β2 integrins LFA-1/CD11a and Mac-1/CD11b (B), or ICAM-1/CD54 (C), or blocking antibodies directed against αv/CD51 integrins and αIIbβ3/CD41/CD61 integrins (D; mean±SEM for n=4 animals per group; *p<0.05 vs isotype control). See also Tab. S1.

Figure 4. Effect of VN-PAI-1 heteromers on the stabilization of neutrophil intravascular adhesion. A: Using confocal laser scanning microscopy on tissue whole mounts of the postischemic cremaster muscle of WT mice, PAI-1 (blue) and VN (green) deposited on the endothelium of postcapillary venules was detected to co-localize with intravascularly adherent Ly-6G⁺ neutrophils, representative images are shown (scale bar: 10 µm). B: Using multi-channel in vivo microscopy on the postischemic mouse cremaster muscle, interactions of Ly-6G⁺ neutrophils and endothelial cells were analyzed in postcapillary venules. Panels show quantitative results for short adhesion and adhesion time of neutrophils in PAI-1-deficient mice receiving vehicle or the PAI-1 mutant proteins PAI-RR (active stable mutant), PAI-QR (unable to bind VN), or CPAI (proteolytically inactive; mean±SEM for n=4 animals per group; *p<0.05 vs PAI-RR). See also Tab. S1. C: Employing an autoperfused flow chamber assay, intravascular rolling and firm adherence of neutrophils was analyzed in chambers coated with E-selectin/CD62E and ICAM-1/CD54 as well as with or without VN-
PAI-1 heteromers or the chemokine CXCL1/KC, panels show quantitative results (mean±SEM for n=7–14 per group; *p<0.05 vs E-selectin/CD62E + ICAM-1/CD54 coating).

Figure 5. Effect of VN-PAI-1 heteromers on activation of neutrophil β2 integrins. A: Using multi-channel flow cytometry, expression of the β2 integrins LFA-1/CD11a and Mac-1/CD11b was analyzed on the surface of neutrophils isolated from the peripheral blood of WT mice undergoing exposure to VN, PAI-1, uPA, VN-PAI-1, or VN-uPA, panels show quantitative results (mean±SEM for n=4 per group; #p<0.05 vs unstimulated). B: Binding of ICAM-1/CD54-Fc to neutrophils isolated from the peripheral blood of WT mice was analyzed upon exposure to VN, PAI-1, uPA, VN-PAI-1, or VN-uPA, panels show quantitative results (mean±SEM for n=6 per group; #p<0.05 vs unstimulated). VN-PAI-1-elicited binding of ICAM-1/CD54 to neutrophils isolated from the peripheral blood of WT mice was analyzed after application of receptor associated protein (RAP; blocking receptors of the LDL receptor family), blocking anti-LRP-1 antibodies, or different MAPK inhibitors (mean±SEM for n=4-6 per group; #p<0.05 vs unstimulated). Binding of conformation-specific antibodies 'KIM127' (intermediate and high affinity conformations of β2 integrins) or 'mAB 24' (high affinity conformation of β2 integrins) to human neutrophils was analyzed after application of human VN-PAI-1 (mean±SEM for n=3 per group).

Figure 6. Effect of VN-PAI-1 heteromers on clustering of neutrophil β2 integrins. A: Representative images of LFA-1/CD11a re-distribution and clustering in single neutrophils perfused over surfaces coated with E-selectin/CD62E, ICAM-1/CD54, and CXCL1 or CXCL1/VN-PAI-1 at indicated shear stress levels. LFA-1/CD11a expression is shown in pseudocolors as indicated by the lookup table (scale bar: 10 µm). B: Scatter plot analysis of co-localized actin sum intensities versus co-localized LFA-1/CD11a sum intensities of single cells at indicated shear stress levels. C: Quantification of actin co-localization and LFA-1/CD11a co-localization per clustered area at indicated shear stress levels.
Selectin/CD62E + ICAM-1/CD54 coating: light green/yellow; E-selectin/CD62E + ICAM-1/CD54 + CXCL1 coating: dark green/brown; E-selectin/CD62E + ICAM-1/CD54 + CXCL1 + VN-PAI-1: grey; mean±SD for n=3 independent experiments, n=6 flow chambers/coating, n=40-56 single cells/coating; * p<0.05 vs. E-selectin/CD62E + ICAM-1/CD54 coating).

Figure 7. Graphical synopsis. In inflamed tissue, heteromers of vitronectin (VN) and plasminogen activator inhibitor-1 (PAI-1) are deposited on the activated venular endothelium via glycosaminoglycans (GAGs). Subsequently, this protein complex interacts with LRP-1 on intravascularly adhering neutrophils and induces p38 MAPK-dependent intracellular signaling pathways. As a consequence of these events, β2 integrins form clusters on the surface neutrophils ultimately allowing these immune cells to stabilize their adhesion via endothelial ICAM-1/CD54 in *trans*.
Figure 1

A

PECAM-1

VN

merge

unstimulated

I/R

PECAM-1

VN

merge

I/R + vehicle

PECAM-1

VN

merge

I/R + GAG digest

vitronecint [MFI]

unstimulated

I/R

vitronecin [MFI]

I/R + vehicle

I/R + GAG digest

B

CD45^+ cells

F4/80^+ cells

SSC

CD11b^+ cells

GR-1^+ PE

WT, unstimulated

WT, stimulated

VN^+/−, stimulated

VN^+ stimulated

neutrophils [10^3/μl]

CXCL1

cMOs [10^3/μl]

CXCL1

neutrophils [10^3/μl]

CCL2

cMOs [10^3/μl]

CCL2
Figure 2

A

I/R

rolling flux

rolling velocity

short adherence

firm adherence

adherence time

transmigration

WT

VN^{-/-}

I/R

CXCL1

neutrophil rolling flux [n/0.03s]

neutrophil rolling velocity [n/m/s]

neutrophil adherence [n/10^4 μm^2 < 30s]

neutrophil adherence [n/10^4 μm^2 > 30s]

neutrophil time of adherence [s]

neutrophil transmigration [n/ml/mm^2]

I/R

CCL2

neutrophil rolling flux [n/0.03s]

neutrophil rolling velocity [n/m/s]

neutrophil adherence [n/10^4 μm^2 < 30s]

neutrophil adherence [n/10^4 μm^2 > 30s]

neutrophil time of adherence [s]

neutrophil transmigration [n/ml/mm^2]

B

isolation of BM leukocytes

WT

i.v.

WT

i.v.

VN^{-/-}

isolation of BM leukocytes

WT

i.v.

WT

accumulation of fluorescent cells [n/HPF]

donor: recipient

WT VN^{-/-} WT VN^{-/-}