Novel platelet-neutrophil interaction via activated $\alpha_{IIb}\beta_3$ mediates NETosis under flow

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Abstract

Platelet-leukocyte interactions are important for innate immune responses, but also contribute to the pathogenesis of thrombotic disorders, such as deep vein thrombosis. How these interactions are manifest and how they influence leukocyte function remains poorly understood. Here, we demonstrate that binding to von Willebrand factor under flow through glycoprotein Ibα 'primes' platelets resulting in intracellular Ca^{2+} release and $\alpha_{IIb}\beta_3$ activation. This priming enables platelets to bind leukocytes via a mechanism that is largely independent of P-selectin, but that is inhibited by blocking $\alpha_{IIb}\beta_3$. We show that neutrophils and T-cells bind directly to activated $\alpha_{IIb}\beta_3$ under flow, identifying this platelet integrin as a novel leukocyte receptor. Binding of neutrophils to $\alpha_{IIb}\beta_3$ under flow causes rapid intracellular Ca^{2+} release, and initiates Ca^{2+}- and NADPH oxidase-dependent signaling leading to production of neutrophil extracellular traps (NETs). NET production is itself dependent upon a mechanosensitive mechanism, as NETosis is markedly diminished if neutrophils are captured by $\alpha_{IIb}\beta_3$ under static conditions. Taken together, these data demonstrate a novel mechanism for platelet-neutrophil cross-talk and mechanosensitive NET production.

Key words: NETs/neutrophils/platelets/thrombosis/VWF.

Introduction

To fulfil their hemostatic function, platelets must be recruited from the flowing blood to sites of vessel damage. This process is highly dependent upon von Willebrand factor (VWF) (Springer, 2014). In plasma, VWF adopts a globular fold that conceals its platelet-binding sites (Goto *et al.*, 1998, Goto *et al.*, 1995). Upon vessel injury, exposed subendothelial collagen binds VWF via its A3 domain (Cruz *et al.*, 1995, Huizinga *et al.*, 1997). Elevated shear, or turbulent/disturbed flow, then unravels tethered VWF and exposes its A1 domain, facilitating shear-dependent capture of platelets via glycoprotein (GP) Ibα (Schneider *et al.*, 2007).

As well as capturing platelets under flow, the A1-GPIbα interaction also induces shear-dependent signaling events (Bryckaert *et al.*, 2015). Zhang *et al* proposed a mechanism in which the leucine-rich repeat domain of GPIbα binds the A1 domain of immobilized VWF (Emsley *et al.*, 1998, Huizinga *et al.*, 2002). Rheological forces on the tethered platelet cause mechano-unfolding of the juxtamembrane stalk of GPIbα (Ju *et al.*, 2016, Zhang *et al.*, 2015). This translates the mechanical stimulus into a biochemical signal within the platelet through the actions of phosphatidylinositol-3-kinase and 14-3-3ζ (and other 14-3-3 isoforms) that associates with the GPIbα intracellular tail (Dai *et al.*, 2005, Feng *et al.*, 2000, Gu *et al.*, 1999, Mangin *et al.*, 2004, Mangin *et al.*, 2009). Force-induced signaling leads to release of intracellular Ca^{2+} stores (Du *et al.*, 1994, Gardiner *et al.*, 2010, Kasirer-Friede *et al.*, 2004, Mazzucato *et al.*, 2004, McCarty *et al.*, 2006, Yin *et al.*, 2008) and activation of the platelet integrin, $\alpha_{IIb}\beta_3$ (Kasirer-Friede *et al.*, 2004, Mu *et al.*, 2010).

VWF-mediated signaling is considered to transduce a mild signal that does not lead to robust activation of the platelet. Consequently, these signaling events are often considered redundant within the setting of hemostasis, particularly as platelets respond more dramatically to other agonists present at sites of vessel injury (e.g. collagen, thrombin, ADP, thromboxane A2) (Clemetson, 2012, Jackson *et al.*, 2003, Senis *et al.*, 2014). Full platelet activation involves changes in platelet shape, release of α- and δ-granules, presentation of new cell surface proteins (e.g. P-selectin and CD40 ligand - CD40L), activation of cell surface integrins and alterations in the membrane phospholipid composition (Bevers *et al.*, 1991, Golebiewska *et al.*, 2015, Nieswandt *et al.*, 2009, Stalker *et al.*, 2014). The extent of platelet activation is dependent upon both the concentration, and identity, of the agonist(s) to which the platelets are exposed, which itself is influenced by the location of the platelets relative to the damaged vessel. For example, platelets in the core of a hemostatic plug/thrombus are exposed to higher concentrations of agonists (collagen, thrombin, ADP, thromboxane) and are therefore more highly activated (i.e. P-selectin-positive procoagulant platelets) than those in the surrounding shell (P-selectin-negative) that are exposed to lower concentrations of fewer agonists (ADP, thromboxane A2) (de Witt *et al.*, 2014, Shen *et al.*, 2017, Stalker *et al.*, 2013, Welsh *et al.*, 2014). Thus, platelets exhibit a 'tunable' activation response determined by agonist availability.

Aside from hemostasis, platelets have important roles as immune cells (Kapur *et al.*, 2016, Semple *et al.*, 2011, Sreeramkumar *et al.*, 2014, Wong *et al.*, 2013, Yeaman, 2014). Indeed, several studies have demonstrated the importance of platelets interacting with bacteria in their targeting by leukocytes (Gaertner *et al.*, 2017, Kolaczkowska *et al.*, 2015, Wong *et al.*, 2013). For this, platelets must invariably

interact with leukocytes. Platelet-leukocyte interactions also play key roles in the development of certain inflammatory cardiovascular conditions. In deep vein thrombosis (DVT), the thrombus generally develops over the intact endothelium, meaning that there is no exposure of collagen or extravascular tissue factor to the blood. In the flow restriction murine model of DVT, VWF-dependent platelet recruitment, platelet-neutrophil interactions and the production of highly thrombotic neutrophil extracellular traps (NETs) all contribute to the development of a pathological thrombus (Brill *et al.*, 2011, Brill *et al.*, 2012, Fuchs *et al.*, 2012, Schulz *et al.*, 2013, von Bruhl *et al.*, 2012). Although the precise sequence of events still remains unclear, it appears that during the early stages of DVT, VWF-bound platelets acquire the ability to interact with leukocytes (von Bruhl *et al.*, 2012). Exactly how this is mediated given the lack of vessel damage is unclear. It also remains to be determined precisely how platelet-tethered neutrophils undergo NETosis in DVT in the absence of an infectious agent.

Resting platelets do not interact with unstimulated leukocytes. Known direct platelet-leukocyte interactions involve either P-selectin or CD40L on the surface of platelets binding to P-selectin glycoligand-1 (PSGL-1) and CD40, respectively, on leukocytes (Lievens *et al.*, 2010, Mayadas *et al.*, 1993, Palabrica *et al.*, 1992, Schonbeck *et al.*, 2001). As platelets must be potently activated to facilitate P-selectin/CD40L exposure, such interactions are unlikely to mediate the early platelet-leukocyte interactions that occur in the murine DVT model. Consistent with this contention, lack of platelet P-selectin has no effect upon either leukocyte recruitment or thrombus formation in murine DVT (von Bruhl *et al.*, 2012). Activated leukocytes can also interact with platelets via additional mechanisms. For example, leukocyte activation can lead to activation of the Mac-1 integrin (also termed CD11b/CD18 or $\alpha_M\beta_2$), which can itself mediate direct interactions with GPIbα, or indirectly associate with activated $\alpha_{IIb}\beta_3$ via fibrinogen.(Simon *et al.*, 2000, Weber *et al.*, 1997, Zuchtriegel *et al.*, 2016) Activation of another leukocyte β_2 integrin, lymphocyte function-associated antigen 1 (LFA-1 or $\alpha_L\beta_2$) can also interact with platelet $\alpha_{IIb}\beta_3$ via a fibrinogen bridge. These latter two associations are dependent upon the leukocytes first being activated.

Although it is often assumed that only activated platelets bind leukocytes, a recent study revealed that platelets captured under flow by VWF released from activated endothelial cells could recruit leukocytes (Zheng *et al.*, 2015). Similar observations have also been made in mice (Doddapattar *et al.*, 2018). Although the importance of A1-GPIbα-dependent signaling for hemostasis is questioned, if such signaling promotes leukocyte binding it may be far more relevant for non-hemostatic platelet functions, particularly in scenarios in which other agonists are not available/abundant.

Results

To explore the influence of platelet binding to VWF under flow upon platelet function, we used a microfluidic system. Full length (FL-) VWF was absorbed directly onto microchannel surfaces, or the isolated recombinant VWF A1 domain, or an A1 domain variant (Y1271C/C1272R, termed A1*) that exhibits a 10-fold higher affinity for GPIbα (**Supp Fig 1**) (Blenner *et al.*, 2014), were captured via their 6xHis tag. Fresh blood anticoagulated with D-phenylalanyl-prolyl-arginyl chloromethyl ketone (PPACK) and labelled with $DiOC_6$, was perfused through channels at $1000s^{-1}$ for 3.5 minutes. On all substrates

(FL-VWF, A1 and A1*), a similar time-dependent increase platelet recruitment (i.e surface coverage) was observed (**Fig 1A** & **Supp Fig 2**).

Platelets were rapidly recruited to the surface and rolled prior to attaching more firmly. Median initial platelet rolling velocity on VWF A1 was 1.76 μms^{-1}, whereas on A1* this was significantly slower (median 0.23μms^{-1}) (**Fig 1B** & **C**), consistent with its 10-fold higher affinity for GPIbα (**Supp Movie 1**)

Platelet binding to VWF under flow induces intraplatelet signaling and activation of $\alpha_{IIb}\beta_3$

After ~2 minutes, platelets bound to either FL-VWF, A1 or A1* started to form small aggregates (**Fig 2Ai**) due to activation of $\alpha_{IIb}\beta_3$ and binding of plasma fibrinogen. Consistent with this, when plasma-free blood (i.e. RBCs, leukocytes and platelets resuspended in buffer) was used, platelets remained as a uniform monolayer, and did not coalesce into microaggregates (**Fig 2Aii**). Similarly, when whole blood containing either eptifibatide or GR144053 that block activated $\alpha_{IIb}\beta_3$, aggregation was also greatly attenuated (**Fig 2 Aiii** & **iv**). Irrespective of the surface (VWF, A1 or A1*), platelet aggregation was markedly reduced if plasma-free blood was used, or if $\alpha_{IIb}\beta_3$ was blocked (**Fig 2B-E**). These results demonstrate that the A1-GPIbα interaction transduces a signal that leads to activation of $\alpha_{IIb}\beta_3$. In support of this, fluorescent fibrinogen bound to platelets tethered via FL-VWF, but not to platelets captured to channel surfaces using an anti-PECAM-1 antibody (**Fig EV 1A**).

To investigate the effect of A1-GPIbα-dependent signaling, platelets were preloaded with the Ca^{2+}-sensitive fluorophore, Fluo-4 AM. Platelets that bound to A1* under flow exhibited repeated transient increases in fluorescence, corresponding to Ca^{2+} release from platelet intracellular stores in response to A1-GPIbα binding under flow (**Movie EV 1**). (Kasirer-Friede *et al.*, 2004, Mu *et al.*, 2010) Despite intracellular Ca^{2+} release, this did not lead to appreciable P-selectin exposure (i.e. α-granule release) (**Fig EV 1B**), or exposure of negatively-charged phospholipids detected by annexin V binding (de Witt *et al.*, 2014, Deng *et al.*, 2016). We therefore propose that flow-dependent VWF-GPIbα signaling 'primes', rather than activates, platelets. Priming is characterized by activation of $\alpha_{IIb}\beta_3$, but minimal α-granule release, platelet spreading, or negatively-charged phospholipid exposure.

Platelets 'primed' by VWF interact with leukocytes.

To explore the influence of platelet 'priming' upon their ability to interact with leukocytes, platelets were captured and 'primed' on VWF for 3 minutes at $1000s^{-1}$. Thereafter, leukocytes in whole blood (also labelled with $DiOC_6$) were perfused at $50s^{-1}$ and seen to roll on the platelet-covered surface (**Movie EV 2** & **Supp Fig 3A**). Conversely, platelets bound to channel surfaces using an anti-PECAM-1 antibody failed to capture, or interact with, any leukocytes under flow (**Fig 3A**), demonstrating the dependency of the observed leukocyte binding on prior A1-GPIbα-mediated platelet 'priming'.

As VWF-'primed' platelets present activated $\alpha_{IIb}\beta_3$, we first hypothesized that 'outside-in' integrin signaling might be important for platelet-leukocyte interactions to occur (Durrant *et al.*, 2017). We therefore performed experiments using plasma-free blood to severely deplete the plasma fibrinogen (**Fig 3B**). Contrary to this hypothesis, we observed a significant (~2-fold) increase in the number of leukocytes interacting with the VWF-bound platelets in plasma-free conditions. Addition of purified

fibrinogen to plasma-free blood to 50% normal plasma concentration significantly reduced platelet-leukocyte interactions to levels very similar to those seen using whole blood (**Fig 3B**). These results suggest that leukocytes compete with fibrinogen to bind 'primed' platelets, potentially via the same receptor. To test this, we blocked $\alpha_{IIb}\beta_3$ using eptifibatide or GR144053 (**Fig 3A-B** & **Supp Fig 3A**), which caused a significant decrease in platelet-leukocyte interactions. Blocked $\alpha_{IIb}\beta_3$ caused a very similar effect irrespective of whether platelets were captured on FL-VWF or A1*, or whether experiments were performed in whole blood or plasma-free blood (**Fig 3A-C)**. Together, these results demonstrate that leukocytes can interact with platelets in an activated $\alpha_{IIb}\beta_3$-dependent manner.

To further confirm this contention, platelets were first captured onto anti-PECAM-1 coated channels before perfusing through an anti-β_3 antibody (ligand induced binding site – LIBS) that induces activation of $\alpha_{IIb}\beta_3$. (Du *et al.*, 1993) Antibody-mediated activation of $\alpha_{IIb}\beta_3$ caused a significant increase in the number of leukocytes binding in a manner that could be blocked with GR144053 (**Fig 3D**).

The best characterized platelet-leukocyte interaction is mediated by P-selectin on activated platelets binding to PSGL-1 on leukocytes (Vandendries *et al.*, 2004). Although we detected little/no P-selectin presentation on the surface of VWF-primed platelets, this did not formally exclude a role for P-selectin in leukocyte adhesion. Therefore, we explored the influence of antibody-mediated P-selectin blockade upon leukocyte binding to VWF-primed platelets. We first established the efficacy of P-selectin blockade through the marked reduction of leukocyte binding to collagen captured/activated platelets (**Supp Fig 3B-C**). Blockade of P-selectin on FL-VWF-bound platelets from whole blood or plasma-free blood had no effect, suggesting that the recruitment of leukocytes is independent of P-selectin (**Fig 3E**). However, interestingly, analysis of the speed of leukocyte rolling over VWF-primed platelets revealed that leukocytes rolled faster after blocking P-selectin in plasma-free blood (**Fig 3F** & **Movie EV 2**) or whole blood (**Supp Fig 3D**). This suggests that whereas leukocyte capture is highly dependent on activated $\alpha_{IIb}\beta_3$ and not P-selectin, once recruited, P-selectin may potentially interact with PSGL-1 to further slow leukocyte rolling.

Leukocytes bind directly to activated $\alpha_{IIb}\beta_3$.

To more specifically test the leukocyte interaction with activated $\alpha_{IIb}\beta_3$ (and to exclude other platelet receptors), purified $\alpha_{IIb}\beta_3$ was covalently coupled to microchannels and activated with Mn^{2+} (Litvinov *et al.*, 2005). Isolated PBMCs and PMNs were perfused through $\alpha_{IIb}\beta_3$-coated channels at $50s^{-1}$. Cells from both PBMCs and PMNs (**Fig 4Ai-ii** & **B**) directly attached to the activated $\alpha_{IIb}\beta_3$ surface. Microchannels coated in the same way with BSA did not bind cells from either preparation (**Fig 4Aiii** & **B**). When activated $\alpha_{IIb}\beta_3$-coated channels were pre-incubated with eptifibatide, or PMNs were perfused in the presence of purified fibrinogen, there was a large reduction in PMN binding to the channels (**Fig 4Aiv-v** & **4B**).

In addition to covalent coupling of purified $\alpha_{IIb}\beta_3$ to the channel surface, we also captured purified $\alpha_{IIb}\beta_3$ using the activating anti-β_3 (LIBS) antibody. Leukocytes were again efficiently captured to this surface in a manner that could be inhibited by blocking $\alpha_{IIb}\beta_3$ (**Fig 4B**).

Previous studies have suggested that activated leukocytes can interact with platelets via different activated leukocyte integrins, Mac-1 ($\alpha_M\beta_2$) or LFA-1 ($\alpha_L\beta_2$) (Simon *et al.*, 2000, Weber *et al.*, 1997, Zuchtriegel *et al.*, 2016). Both of these integrins in their activated form can bind fibrinogen, which may potentially bridge with $\alpha_{IIb}\beta_3$ (Simon *et al.*, 2000, Weber *et al.*, 1997, Zuchtriegel *et al.*, 2016). Although we found that fibrinogen can compete for leukocyte binding to platelets, we explored a potential role for Mac-1 and/or LFA-1 through the use of a polyclonal anti-β_2/CD18 antibody capable of functionally blocking β_2 integrins. In these experiments the anti-β_2/CD18 had no effect upon leukocyte binding to VWF-'primed' platelets (**Fig 4C**), consistent with the fact that the leukocytes are not activated prior to platelet binding and do not require fibrinogen for platelet binding. In summary we show leukocytes can bind $\alpha_{IIb}\beta_3$ directly that is dependent upon its RGD-binding groove, but independent of Mac-1 or LFA-1.

T-cells and neutrophils interact with VWF-'primed' platelets via activated $\alpha_{IIb}\beta_3$.

We used cell-type-specific antibodies to ascertain the identify the leukocytes interacting with VWF-primed platelets. We found no evidence of either $CD14^+$ monocytes or $CD19^+$ B-cells in isolated PBMCs interacting with activated $\alpha_{IIb}\beta_3$. Moreover, anti-CD16 staining of PBMC showed no obvious $CD14^{dim}CD16^+$ monocyte subset interaction. Interestingly, T-cells were the only cell type amongst the PBMCs capable of binding activated $\alpha_{IIb}\beta_3$ (**Fig 4D**). This was confirmed in experiments in which PBMCs were perfused over VWF-primed platelets (**Fig 4E**).

Using isolated PMNs, we found that cells stained with anti-CD16 bound to activated $\alpha_{IIb}\beta_3$-coated channels and also to VWF-'primed' platelets (**Fig 4F** & **4G**). Based on multi-lobulated segmented nuclear morphology (**Fig 4F**), these cells were indicative of $CD16^+$ neutrophils. These cells only interacted with platelets and not with the VWF on the channel surface. Neutrophils were also seen to scan the platelet- or $\alpha_{IIb}\beta_3$-coated surfaces (**Fig 4G** & **Supp Movie 2**) suggesting that the binding of neutrophils to $\alpha_{IIb}\beta_3$ under flow may itself initiate signaling events within neutrophils.

Binding to $\alpha_{IIb}\beta_3$ under flow induces intracellular Ca^{2+} release within neutrophils.

To explore the effect of neutrophil binding to platelets, we preloaded PMNs with Fluo-4 AM before perfusion over either VWF-'primed' platelets (**Fig 5A**) or activated $\alpha_{IIb}\beta_3$ (**Fig 5B**). In both cases, bound neutrophils exhibited a gradual increase in fluorescent intensity corresponding to release of intracellular Ca^{2+} (**Supp Movie 3**). Neutrophil fluorescence intensity reached a maximum after 200-300 seconds (**Fig 5C**-**D**). Neutrophils captured to the channel surface using an anti-CD16 antibody exhibited no change in fluorescence signal (**Fig 5C**), demonstrating that the release of intracellular Ca^{2+} stores was dependent upon the interaction with $\alpha_{IIb}\beta_3$.

Binding of neutrophils to $\alpha_{IIb}\beta_3$ under flow induces Nox- and Ca^{2+}-dependent NETosis.

Recent studies have revealed that platelets assist in the targeting of intravascular bacterial pathogens through stimulation of the release of NETs (Gaertner *et al.*, 2017, Wong *et al.*, 2013, Yeaman, 2014) (Brinkmann *et al.*, 2004). However, the physiological agonists or mechanisms that drive NETosis are not fully resolved (Nauseef *et al.*, 2016, Sorensen *et al.*, 2016). We therefore examined whether the binding

of neutrophils to $\alpha_{IIb}\beta_3$ might induce NETosis. Neutrophils were stained with Hoechst dye to visualize their nuclei, and also with cell impermeable Sytox Green that stains extracellular DNA. Isolated PMNs were perfused through channels coated with either activated $\alpha_{IIb}\beta_3$ or anti-CD16 (negative control) at $50s^{-1}$ for 10 minutes and NETosis was subsequently analyzed under static conditions (**Fig 6A** & **Movie EV 3**). After ~60 minutes, nuclear lobuli became less defined in neutrophils bound to activated $\alpha_{IIb}\beta_3$. Sytox Green fluorescence, indicative of extracellular DNA/NETosis, was detected from ~90 minutes. Very similar results were obtained when neutrophils were captured by $\alpha_{IIb}\beta_3$ bound to the activating anti-β_3 antibody, or by platelets 'primed' by A1* or FL-VWF. NETosis was quantified through the whole microchannel after two hours. On activated $\alpha_{IIb}\beta_3$, 69% ±14% of neutrophils formed NETs after 2 hours, compared to minimal NETosis events (8% ±8%) when neutrophils were captured by anti-CD16 (**Fig 6B**). Interestingly, when neutrophils were captured on $\alpha_{IIb}\beta_3$ in the absence of flow, neutrophils still attached, but the proportion of cells that underwent NETosis (17% ±17%) was significantly reduced (**Fig 6B**). This suggested that the signaling mechanism from the platelet to the neutrophil may itself be mechano-sensitive. These results also demonstrated that NETosis does not require other platelet receptors or releasate components.

As a positive control, neutrophils captured by anti-CD16 and stimulated with phorbol 12-myristate 13-acetate (PMA) for two hours led to 100% ±0.5% of neutrophils releasing NETs (**Fig 6C**). PMA-induced NETosis was not significantly inhibited in the presence of TMB-8 (an antagonist of intracellular Ca^{2+} release; 90% ±9%), but was effectively inhibited in by DPI (NADPH oxidase inhibitor; 16% ±13%), similar to previous reports (Gupta *et al.*, 2014). NETosis of neutrophils captured by $\alpha_{IIb}\beta_3$ under flow was significantly inhibited to 17% ±9% in the presence of TMB-8 and to 25% ±10% with DPI (**Fig 6D**). Together, these results highlight the dependency of intracellular Ca^{2+} release and NADPH oxidase signaling pathways in NETosis in response to binding $\alpha_{IIb}\beta_3$ under flow, and that there may be some synergy between these pathways.

Discussion

Although VWF/GPIbα-mediated platelet signaling has been known for many years, the role that it fulfils is still not well understood (Du *et al.*, 1994, Goto *et al.*, 1995). Using FL-VWF and recombinant VWF A1/A1* immobilized to microchannel surfaces, we isolated platelet signal transduction to the GPIbα-A1 interaction (**Fig 1**). We propose that under flow GPIbα-A1 binding 'primes', rather than activates, platelets, based on the rapid activation of $\alpha_{IIb}\beta_3$, but the lack of significant P-selectin exposure (**Fig 2** & **Fig EV 1**). Although some studies have reported that GPIbα-VWF-mediated signaling can induce modest α-granule release, it is widely accepted that when compared to other platelet agonists degranulation and P-selectin exposure are very low (de Witt *et al.*, 2014, Deng *et al.*, 2016, Depraetere *et al.*, 1998).

That platelet binding to VWF under flow 'primes', rather than activates, the platelets is consistent with *in vivo* observations. At sites of vessel damage, VWF is important for platelet accumulation through all layers of the hemostatic plug (Joglekar *et al.*, 2013, Lei *et al.*, 2014, Verhenne *et al.*, 2015). All platelets within a thrombus/hemostatic plug likely form interactions with VWF. Despite this, it is only the platelets

in the 'core' of the thrombus that become P-selectin-positive, procoagulant platelets, whereas the more loosely bound platelets that form the surrounding 'shell' remain essentially P-selectin-negative (Welsh *et al.*, 2014). If VWF-binding alone were sufficient to fully activate platelets, such differential characteristics of the 'core' and 'shell' would not be observed.

It is frequently implied that VWF is only important for platelet capture under high shear conditions. This is, in part, associated with the ability of platelets to bind collagen directly at low shear. However, murine models of venous thrombosis with no collagen exposure have repeatedly revealed an important role for VWF-mediated platelet accumulation (Bergmeier *et al.*, 2008, Brill *et al.*, 2011, Chauhan *et al.*, 2007). Using linear microchannels, platelet binding to VWF occurs most efficiently at arterial shear rates but does still occur under lower linear venous shear (Goto *et al.*, 2002, Miyata *et al.*, 1999, Yago *et al.*, 2008, Zheng *et al.*, 2015). However, linear channels do not mimic the distorted and branched paths of the vascular system that cause more disturbed and turbulent flow patterns, particularly around valves. Using bifurcated channels, or channels with changing geometry under lower shear conditions, we and others have noted that VWF captures platelets appreciably more efficiently in areas of turbulent flow or where flow rates change due to channel narrowing than under comparable uniform shear rates in linear channels (Zheng *et al.*, 2015). Indeed at venous flow rates through bifurcated channels (**Fig EV 2A**), we detected platelet capture on FL-VWF with concomitant 'priming' and leukocyte binding (**Fig EV 2B**). This was appreciably augmented at bifurcation points where turbulent flow exists. Consistent with our earlier findings, leukocyte binding was almost completely inhibited when $\alpha_{IIb}\beta_3$ was blocked (**Fig EV 2C**). This implies that VWF can function in platelet recruitment within the venous system, particularly in areas of turbulence (e.g. branch sites, valves), which are frequently the nidus for thrombus formation in DVT.

In venous thrombosis, the thrombus generally forms over the intact endothelium, in the absence of vessel damage. This poses the question of how VWF might contribute to DVT if subendothelial collagen is not exposed. It is thus likely that this reflects the function of newly-secreted ultra-large VWF released from endothelial Weibel-Palade bodies. Under low turbulent shear, ultra-large VWF may tangle to form strings/cables over the surface of the endothelium, even in the presence of ADAMTS13 (Crawley *et al.*, 2011). Tangled VWF strings are appreciably more resistant to ADAMTS13 proteolysis than VWF that is simply unraveled. In the murine stenosis model of DVT, complete VWF-deficiency prevents platelet binding to the endothelium, demonstrating that endothelial secreted VWF is capable of platelet binding in the venous system (Bergmeier *et al.*, 2008, Brill *et al.*, 2011, Chauhan *et al.*, 2007). Similarly, blocking GPIbα binding to VWF also completely blocks platelet accumulation and thrombus formation in the stenosis model of DVT. Thus, when platelets bind to VWF under flow in such setting, platelets may become 'primed' facilitating both aggregation and neutrophil binding through activated $\alpha_{IIb}\beta_3$ and fibrinogen, but without activating them into procoagulant platelets.

Our study reveals, for the first time, that T-cells and neutrophils can bind directly to activated $\alpha_{IIb}\beta_3$ on platelets. This contention is based on our data showing that blocking $\alpha_{IIb}\beta_3$ with eptifibatide or GR144053 significantly diminished leukocyte binding, whereas performing experiments in plasma-free/fibrinogen-free conditions appreciably increased leukocyte binding (**Fig 3A-C**). T-cells and neutrophils also bound directly to activated $\alpha_{IIb}\beta_3$ that was either covalently coupled to microchannels or captured by an

activating anti-β_3 antibody (**Fig 3D** & **Fig 4A-B**). In both cases, the interaction was inhibited by either eptifibatide or GR144053 suggesting that both cell types may share the same receptor, involving an RGD, or RGD-like epitope. The most frequently reported interaction between platelets and leukocytes involve platelet P-selectin. However, we showed that P-selectin is not responsible for the leukocyte capture that we observe as; *1)* we detected little/no P-selectin on VWF-'primed' platelets (**Fig EV 1**), *2)* P-selectin blockade had no effect upon the number of leukocytes binding (**Fig 3E**), and *3)* only T-cells and neutrophils bind VWF-'primed' platelets (**Fig 4**) - given that all leukocytes express PSGL-1 (Laszik *et al.*, 1996), if the capture of leukocytes were entirely P-selectin-mediated, such cell-type selectivity would not be observed. We did however measure an influence of P-selectin upon the rolling speed of leukocytes over VWF-primed platelets (**Fig 3F** & **Supp Fig 3D**). This observation suggests that small quantities of P-selectin present following VWF-priming is insufficient to facilitate leukocyte capture, but may synergize to slow rolling of leukocytes that are first captured by $\alpha_{IIb}\beta_3$.

There are several studies that provide support for P-selectin independent interactions of neutrophils and T-cells with platelets. Guidotti *et al* demonstrated the interaction of T-cells with small intrasinusoidal platelet aggregates in the liver during hepatotropic viral infections was independent of both P-selectin and CD40L in platelets (Guidotti *et al.*, 2015). Thus, the function of T-cell-platelet binding via $\alpha_{IIb}\beta_3$ requires further investigation and may be associated with roles of platelets in anti-viral and interferon responses.

Using a murine model of peritonitis, Petri *et al* demonstrated that neutrophil recruitment and extravasation was highly dependent upon VWF, GPIbα, and platelets, but largely independent of P-selectin (Petri *et al.*, 2010). Two further studies also corroborate the contention that VWF/GPIbα-bound platelets are capable of promoting neutrophil recruitment/extravasation in murine models of ischemia/reperfusion via P-selectin-independent mechanisms (Gandhi *et al.*, 2012, Khan *et al.*, 2012). Together, these studies support the idea that both VWF and platelets can function beyond hemostasis to fulfil a role in leukocyte recruitment at sites of inflammation.

If T-cells and neutrophils (and not B-cells or monocytes) are capable of interacting with platelets via $\alpha_{IIb}\beta_3$, this suggests that a specific receptor exists on these cells that is absent on B-cells or monocytes. We excluded a role for the β_2 integrins, Mac-1 and LFA-1 (**Fig 4C**), both of which in their activated form can interact with fibrinogen and potentially form a bridge with $\alpha_{IIb}\beta_3$. However, not only did we find that fibrinogen competes for the platelet-leukocyte interaction, but also Mac-1 and LFA-1 activation would be required prior leukocyte binding for such a mechanism to occur. Moreover, the expression pattern of Mac-1 and LFA-1 could not account for the T-cell and neutrophil selectivity that we observe. Studies to identify the leukocyte receptor through analysis of transcriptomic and proteomic databases searching for cell surface proteins expressed by T-cells and neutrophils, but not by monocytes or B cells from, and also through pull-down assays are on-going.

Although NET production is an established mechanism through which neutrophils control pathogens (Brinkmann *et al.*, 2004), many questions remain as to how NETosis is regulated (Nauseef *et al.*, 2016). It was recently reported that the binding of platelets to Kupffer cells in the liver of mice following infection with *B. cereus* or *S. aureus* is mediated by VWF (Wong *et al.*, 2013). This binding augments the

recruitment of neutrophils, NET production and the control of infection (Kolaczkowska *et al.*, 2015). Mice lacking VWF or GPIbα do not form aggregates and so have diminished neutrophil recruitment and, therefore, decreased survival (Wong *et al.*, 2013). How NETosis is initiated following platelet binding remains uncertain. Alone, lipopolysaccharide (LPS) is not a potent activator of NETosis (Clark *et al.*, 2007). However, LPS-stimulated platelets can robustly activate NETosis, independent of P-selectin on their surface (Clark *et al.*, 2007), but it does stimulate binding of fibrinogen (i.e. induces $\alpha_{IIb}\beta_3$ activation) (Lopes Pires *et al.*, 2017) and promote platelet-neutrophil interactions. Whether activated $\alpha_{IIb}\beta_3$ contributes to neutrophil recruitment to LPS-exposed platelets warrants further investigation, particularly as platelets appear essential for intravascular NET production (Looney *et al.*, 2009, McDonald *et al.*, 2012). Previous studies have reported that blocking platelet binding to VWF, or blocking $\alpha_{IIb}\beta_3$ are capable of diminishing NET production *in vivo* (Carestia *et al.*, 2016a, Caudrillier *et al.*, 2012). Although, each of these observations are entirely consistent with our data, it must be remembered that both of these approaches influence platelet function in ways that influence the normal hemostatic response and are not specific for just blocking platelet 'priming', or neutrophil binding, respectively.

The dependency of NET production upon platelets and VWF in murine models of infection led us to explore NETosis in neutrophils bound to $\alpha_{IIb}\beta_3$ under flow. We detected rapid release of intracellular Ca^{2+} within bound neutrophils (**Fig 5** & **Supp Movie 3**). This preceded the release of NETs after 90-120 minutes (**Fig 6**). This process was dependent upon neutrophils being captured under flow, suggesting that signal transduction through ligation of $\alpha_{IIb}\beta_3$ may be mechanosensitve, which is consistent with the recent report suggesting a major influence of shear upon NETosis in the presence of platelets (Yu *et al.*, 2018). As NETosis can be induced following binding to purified $\alpha_{IIb}\beta_3$ under flow alone, this suggests that this process does not require a component of the platelet releasate (e.g. mobility group box 1, platelet factor 4, RANTES and thromboxane A2) which have been reported to be capable of driving NETosis (Carestia *et al.*, 2016b).

We propose a model in which platelets have a tunable response that can distinguish their role in hemostasis and immune cell activation (**Fig 7**). The 'priming' of platelets by binding to VWF under flow (in the absence of other platelet agonists) may assist in the targeting of leukocytes to resolve pathogens or mediate vascular inflammatory response. The activated $\alpha_{IIb}\beta_3$ integrin can then mediate neutrophil (and T-cell) recruitment (**Fig 7**). We do not exclude a supporting role for P-selectin in maintaining T-cell/neutrophil recruitment, but this is not essential. Under flow this transduces a mechanical stimulus capable of promoting NETosis via a pathway involving synergy between NADPH oxidase and Ca^{2+} signaling (**Fig 7**). Homeostatically, this may be beneficial for immune responses. However, during chronic infection or vascular inflammation NET production may promote intravascular thrombosis.

This study identifies activated $\alpha_{IIb}\beta_3$ as a novel receptor and agonist for T-cells and neutrophils. This process may provide an additional mechanism of how platelet-neutrophil cross-talk is manifest in innate immunity; it may also provide an explanation for how VWF and platelet dependent neutrophil recruitment and NETosis may occur in thrombotic disorders such as DVT, despite the absence of vessel damage and pathogen-associated molecular patterns. Identification of the counter-receptor for $\alpha_{IIb}\beta_3$ and

mechanisms of mechanosensing on neutrophils may provide opportunities to target this process without influencing the hemostatic function of platelets.

Materials and Methods

Preparation of VWF A1 domain and multimeric VWF

The human VWF A1 domain and A1 mutant Y1271C/C1272R (termed A1*) (Blenner *et al.*, 2014) both with a C-terminal V5 and polyhistidine tag were expressed in S2 insect cells and purified to homogeneity (see Supplementary Methods). Full length, multimeric VWF was isolated from Haemate P by gel filtration and quantified by a specific VWF ELISA, as previously described (O'Donnell *et al.*, 2005).

Blood collection and processing

Fresh blood was collected in 40μM PPACK (for whole blood experiments), 3.13% citrate (for leukocyte isolation) or 85mM sodium citrate, 65mM citric acid, 111mM D(+) glucose, pH 4.5 (1x ACD, for plasma-free blood preparation). For reconstituted plasma-free blood, red blood cells (RBCs) and leukocytes were pelleted and washed twice. Separately, platelets were washed twice in 1x HT buffer containing 0.35% BSA, 75mU apyrase and 100nM prostaglandin E1 (Sigma). RBCs, leukocytes and platelets were resuspended in 1x HT buffer supplemented with 0.35% BSA. In some experiments, 1.3mg/ml purified fibrinogen (Haem Tech) was added. For Ca^{2+} assays, PRP was incubated with 5μM Fluo-4 AM (Thermo Fisher Scientific) for 30 minutes at 37°C prior to washing, and plasma-free blood was recalcified with 1mM $CaCl_2$ (final concentration) immediately prior to flow experiments.

Polymorphonuclear cells (PMNs) and peripheral blood mononuclear cells (PBMCs) separated using Histopaque1077 and Histopaque1119 were resuspended in 1x HT, supplemented with 1.5mM $CaCl_2$. For Ca^{2+} assays, PMNs were preloaded with 1μM Fluo-4 AM for 30 minutes at 37°C, before washing.

Flow experiments

VenaFluoro8+ microchips (Cellix) were coated with VWF, NTA PEGylated microchips (Cellix) with isolated VWF A1 and A1* domains and NHS-microchannels (Cellix) with purified $\alpha_{IIb}\beta_3$ (ERL), anti-PECAM-1 (BioLegend), anti-β_3 (LIBS2, Millipore), anti-CD16 (eBiosciences) or BSA by amine-coupling (see Supplementary Methods). For $\alpha_{IIb}\beta_3$-coated channels, 1mM $MnCl_2$ was used to cause $\alpha_{IIb}\beta_3$ to favor its ligand binding conformation, as previously reported.(Litvinov *et al.*, 2005)

Whole blood or plasma-free blood was perfused through channels coated with either FL-VWF, A1, A1* or anti-PECAM-1 at shear rates of 500-1500s^{-1} for 3.5 minutes, followed by 50s^{-1} for 15 minutes using a Mirus Evo Nanopump and Venaflux64 software (Cellix). In separate experiments, 2.4μM eptifibatide (Sigma), 2μM GR144053 (Tocris), or 50μg/ml anti-P-selectin blocking antibody (clone AK4; BD Biosciences) were supplemented to whole blood or plasma-free blood. $DiOC_6$ (2.5μM) was used to label platelets and leukocytes. Cells were monitored in real-time using an inverted fluorescent microscope (Zeiss) or a SP5 confocal microscope (Leica). Leukocytes and platelets were distinguished by their larger size. For presentation and counting purposes, leukocytes were pseudo-colored to distinguish them.

Isolated PMNs and PBMCs were perfused through channels coated either directly or indirectly with $\alpha_{IIb}\beta_3$, or BSA at $50s^{-1}$ for 15 minutes. Antibodies specific to the different types of leukocytes were added to isolated leukocytes, i.e. anti-CD16 (eBiosciences) conjugated to allophycocyanin (APC) to identify neutrophils, anti-CD14-APC for monocytes, anti-CD3-APC for T-cells and anti-CD19-APC for B-cells (BioLegend).

To visualize NETosis, neutrophils were labelled with 8μM Hoechst dye (cell permeable) and 1μM Sytox Green (cell impermeable) and monitored for 2 hours. As indicated, isolated PMNs were preincubated with 20μM TMB-8 (Ca^{2+} antagonist and protein kinase C inhibitor; Sigma), for 15 minutes, or 30μM DPI (NADPH oxidase inhibitor; Sigma) for 30 minutes at 37°C prior to NETosis assays. In some experiments, neutrophils were captured on microchannels coated with anti-CD16 and stimulated with 160nM PMA prior to analysis of NETosis in the presence and absence of inhibitors.

Quantitation of platelet rolling, aggregation and intracellular Ca^{2+} release was achieved using SlideBook 5.0 software (3i). The number of leukocytes rolling/attaching per minute at $50s^{-1}$ was derived by counting the number of cells in one field of view over a period of 13 minutes. NETosis was quantified by determining the proportion of all neutrophils in the microchannel that had undergone NETosis after 2 hours.

Statistics

Statistical analysis was performed using Prism 6.0 software (GraphPad). Differences between data/samples was analyzed using unpaired two-tailed Student's t-test or Mann-Whitney, as appropriate and as indicated in figure legends. Data are presented as mean ±standard deviation, or median ± 95% confidence interval. The number of individual experiments performed (n) is given in each legend. Values of $p<0.05$ were considered statistically significant.

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Author contributions

A.C-B designed and performed the experiments, analyzed the data, prepared the figures and wrote the manuscript; I.I.S-C designed and performed the experiments, analyzed the data, and wrote the manuscript K.J.W designed experiments, analyzed the data, and wrote the manuscript; J.T.B.C designed experiments, analyzed the data, prepared the figures and wrote the manuscript.

Conflict of Interest Statement

The authors report no conflict of interest.

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Figure Legends

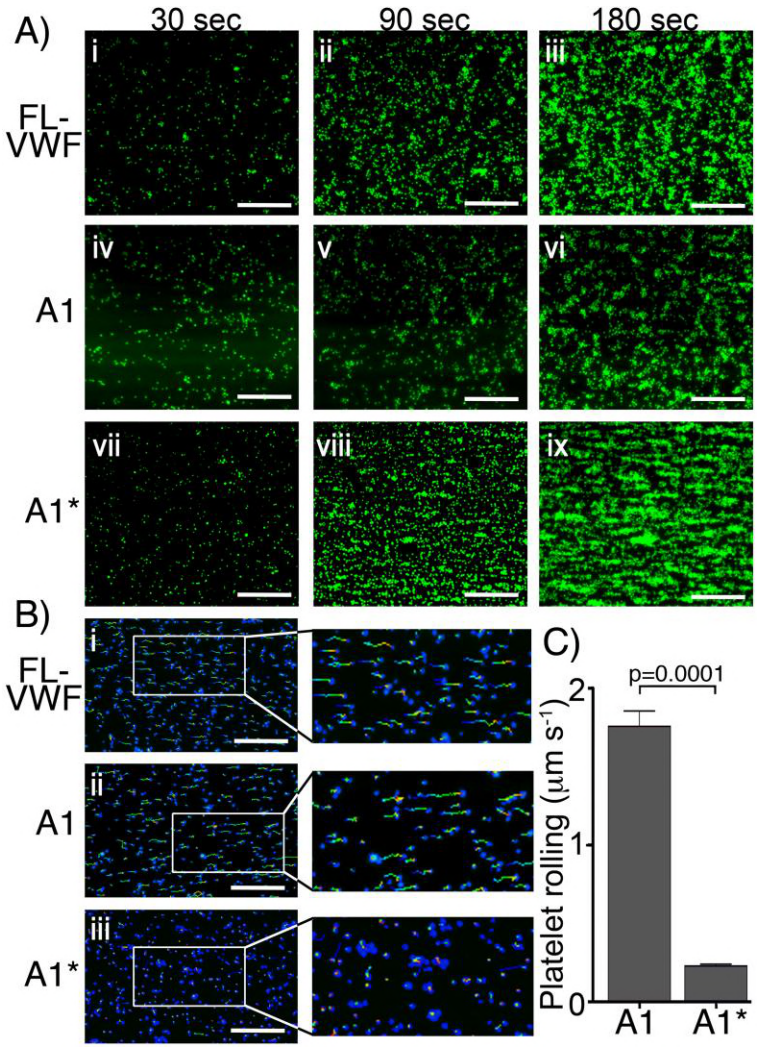

Figure 1: Platelet rolling and attachment to VWF under flow. A) Vena8 microchannels were coated with either full-length VWF (FL-VWF; i-iii), VWF A1 (iv-vi) or A1* (vii-ix). Whole blood labelled with $DiOC_6$ was perfused at $1000s^{-1}$. Representative images (n=3) of platelets (green) after 30, 90 and 180 seconds are shown. Scale; 50μm (see also **Supp Movie 1**). **B)** Experiments performed as in A), bound platelets (blue) were tracked (depicted by multi-coloured lines) representing distance travelled in the first 30 sec of flow. Scale bar; 50μm. **C)** Platelet rolling velocity on channels coated with A1 and A1*. Data plotted are median ±95% CI. n=3562 platelets from 3 different experiments (A1) and n=4047 platelets from 3 different experiments (A1*). Data were analyzed using the Mann-Whitney test.

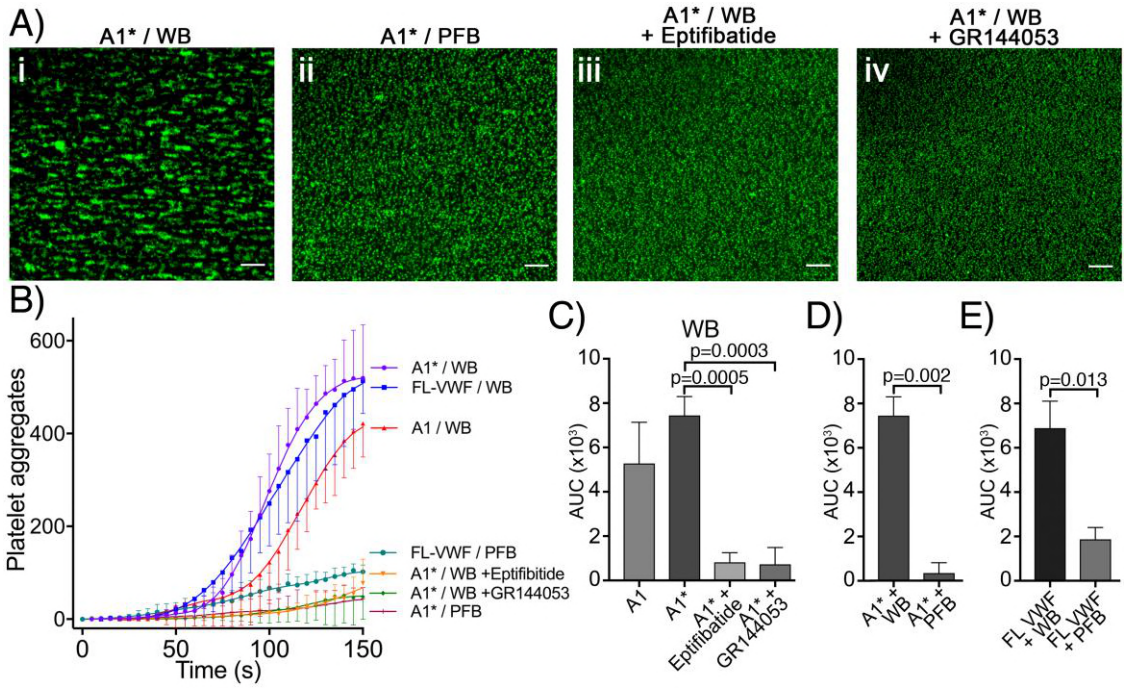

Figure 2: Platelet binding to VWF under flow induces $\alpha_{IIb}\beta_3$-dependent aggregation. A) Vena8 microchannels were coated with A1* via its 6xHis tag. i) Whole blood (WB) or ii) plasma-free blood (PFB), iii) WB containing eptifibatide or iv) WB containing GR144053 were perfused through channels at $1000s^{-1}$. Representative images acquired after 3 minutes. Scale bar; 50μm. **B)** Graph measuring platelet aggregation over time in WB perfused through channels coated with A1 (red, n=3), A1* (purple, n=4) and FL-VWF (blue, n=3), WB pre-incubated with eptifibatide (orange, n=3) or GR144053 (green, n=4) over channels coated with A1* and PFB over channels coated with A1* (magenta, n=3) or FL-VWF (teal, n=3). Data plotted are mean ±SD. **C-E)** Bar charts comparing area under the curve (AUC) of the data presented in B). **C)** WB perfused over A1 or A1* with or without eptifibatide or GR144053. **D)** WB or PFB perfused over A1*. **E)** WB or PFB perfused over FL VWF. Data presented are mean ±SD, n=3 or 4 as indicated in B). Data were analyzed using the Mann-Whitney test.

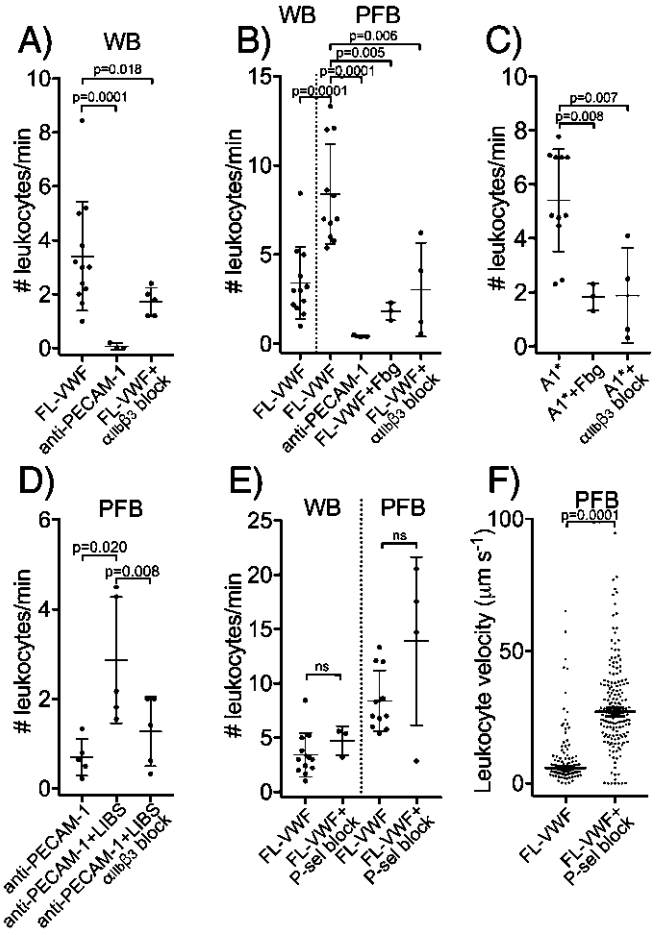

Figure 3: Leukocytes bind to VWF-bound platelets under flow (see also **Movie EV 2**). **A)** Graph of the number of leukocytes/minute in WB interacting with platelets bound to FL-VWF in the absence (n=12) or presence of eptifibatide/GR144053 (n=5), or binding to platelets bound to anti-PECAM-1 antibody (n=3). **B)** Graph of the number of leukocytes/minute in WB or PFB interacting with platelets bound to FL-VWF in the absence (n=12) or presence of 1.3mg/ml fibrinogen (n=3) or eptifibatide/GR144053 (n=4), or binding to platelets bound to anti-PECAM-1 antibody (n=3). **C)** Graph of the number of leukocytes/minute in PFB interacting with platelets bound to A1* in the absence (n=11) or presence of fibrinogen (n=3) or eptifibatide/GR144053 (n=4). **D)** Graph of the number of leukocytes/minute in PFB interacting with platelets bound to anti-PECAM-1 antibody in the absence (n=5) or presence of LIBS/anti-β_3 activating antibody (n=5) ± GR144053 (n=5). **E)** Graph of the number of leukocytes/minute in WB or PFB, as shown, interacting with platelets bound to FL-VWF in the absence or presence of a blocking anti-P-selectin antibody. **F)** Graph of leukocyte rolling velocity on platelets bound to FL-VWF in PFB in the absence or presence of a blocking anti-P-selectin antibody. Data shown are individual leukocyte rolling velocities (n=121 and 178, respectively) for 3 separate experiments. In all graphs, data plotted are mean ±SD. Data were analyzed using unpaired, two-tailed Student's t test; ns not significant.

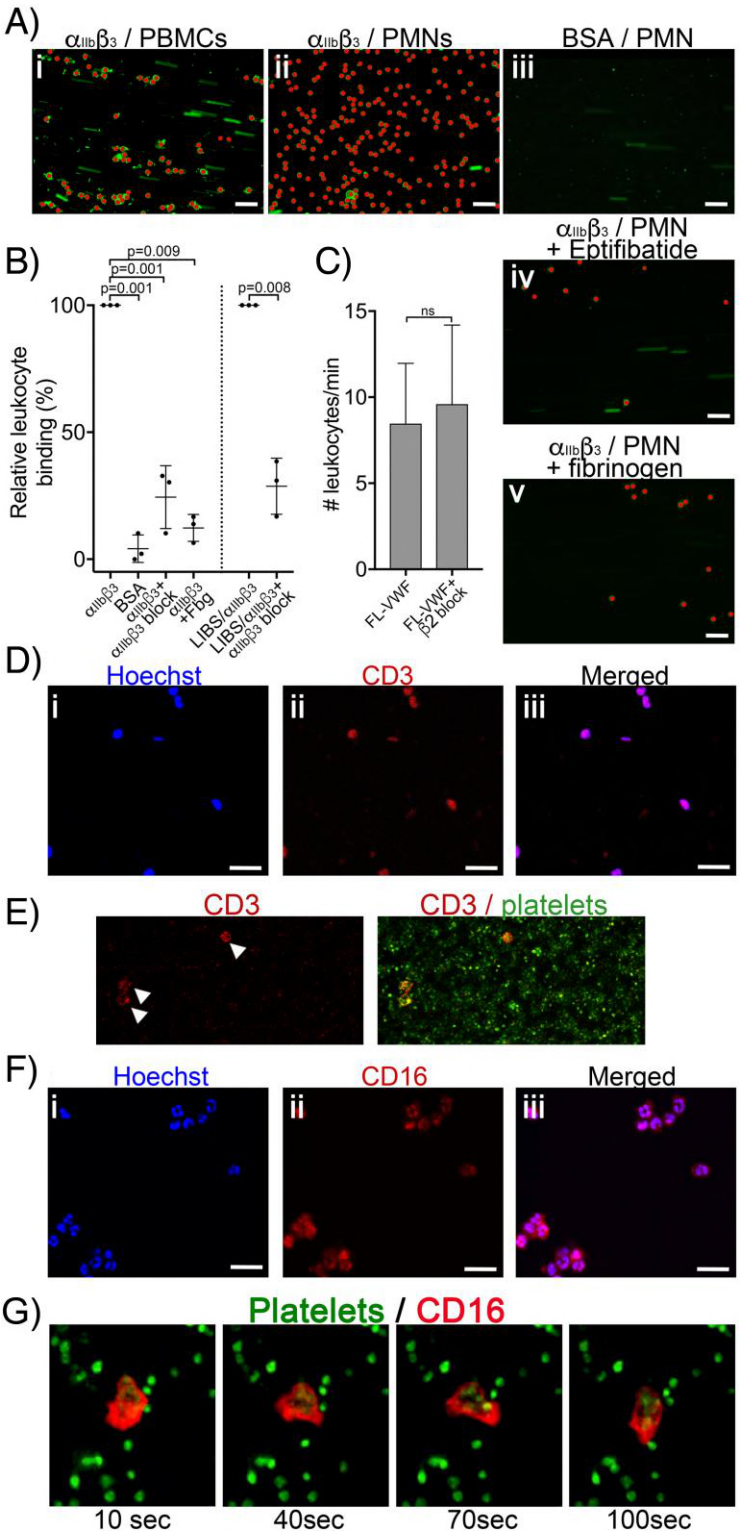

Figure 4: Leukocytes bind to activated $\alpha_{IIb}\beta_3$ under flow. A) Purified $\alpha_{IIb}\beta_3$ or BSA, as noted, were covalently coupled to microchannel surfaces by amine coupling. $\alpha_{IIb}\beta_3$ was activated using Mn^{2+} and Ca^{2+} in all buffers. PBMCs (i) or PMNs (ii-v) labelled with $DiOC_6$ were perfused through channels in the presence and absence of eptifibatide (iv) or 1.3mg/ml purified fibrinogen (v). Bound leukocytes (as opposed to flowing) are pseudo-colored red to aid visualization and to distinguish from leukocytes in transit. Scale bar; 50μm. **B)** Graphical representation of relative leukocyte binding to activated $\alpha_{IIb}\beta_3$ in the presence and absence of eptifibatide or 1.3mg/ml purified fibrinogen, or to BSA after 15 minutes of leukocyte perfusion (n=3), or to $\alpha_{IIb}\beta_3$ captured and activated by LIBS/anti-β_3 activating antibody in the absence and presence of GR144053 (n=3). Data plotted are mean ±SD. Data were analyzed using unpaired, two-tailed Student's t test. **C)** Graph of the number of leukocytes/minute in PFB interacting with platelets bound to FL-VWF in the absence (n=12) or presence of a blocking anti-β2 integrin polyclonal antibody (n=5) capable of blocking both LFA-1 or Mac-1 on leukocytes. **D)** PBMCs stained with Hoechst dye (i - blue), anti-CD3 (ii - red) and merged (iii). Representative of n=4. Scale bar; 20μm. **E)** PFB stained with DiOC6 was perfused over FL-VWF at $1000s^{-1}$ followed by $50s^{-1}$. T-cells labelled with anti-CD3 (red - arrows) were seen to attach to 'primed' platelets **F)** PMNs stained with Hoechst dye (i - blue), anti-CD16 (ii - red) and merged (iii). Representative of n=4. Scale bar; 20μm (see also **Supp Movie 2**). **G)** Images depicting a neutrophil stained with anti-CD16 (red) 'scanning' the 'primed' platelets stained with $DiOC_6$ (green). Images shown were taken 10, 40, 70 and 100 seconds after neutrophil attachment note the movement of the neutrophil shown - see also **Supp Movie 2**.

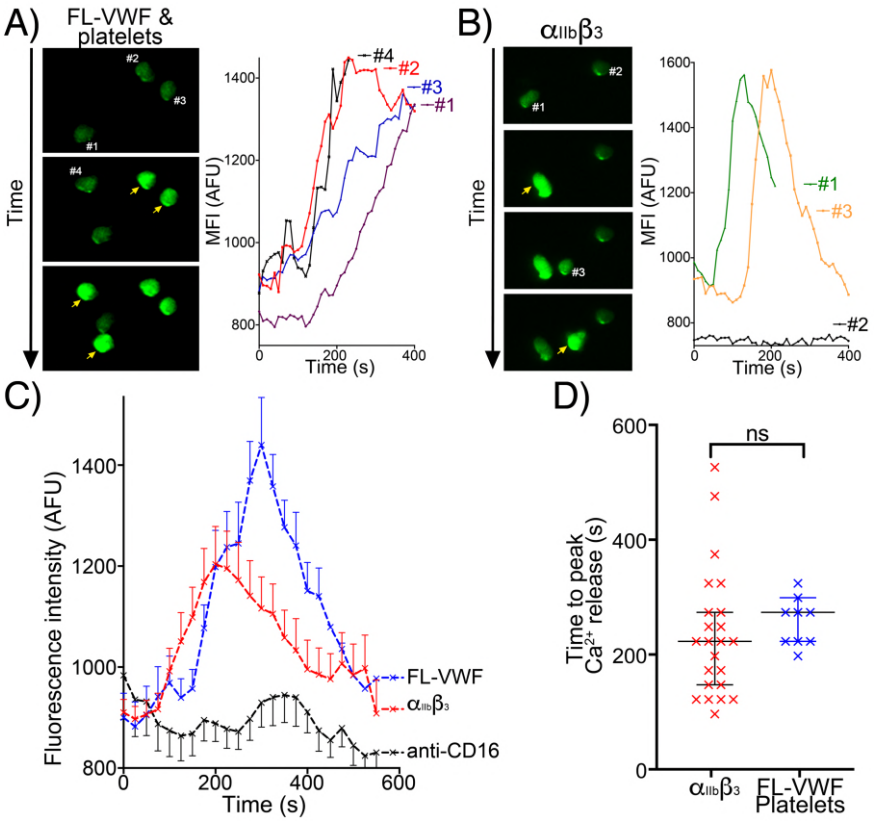

Figure 5: Binding to $\alpha_{IIb}\beta_3$ induces intracellular Ca^{2+} release in neutrophils. A) Representative images of neutrophils pre-loaded with Fluo-4 AM bound to VWF-'primed' platelets captured (**Supp Movie 3**). Neutrophils are numbered #1-#4. The yellow arrow highlights a frame in which the fluorescence has increased in the attached neutrophil. For each neutrophil shown, intracellular Ca^{2+} release is quantified by measurement of cellular mean fluorescent intensity (MFI) over time. **B)** As in A) except neutrophils were perfused over activated $\alpha_{IIb}\beta_3$. MFI increased for neutrophils #1 and #3, but not for neutrophil #2. **C)** Graph depicting the change in MFI as a function of time after neutrophil attachment to microchannels coated with activated $\alpha_{IIb}\beta_3$ (n=24 neutrophils from 3 different experiments), VWF-'primed' platelets (n=9 neutrophils from 1 experiment) or anti-CD16 (n=13 neutrophils from 2 different experiments). Data plotted are mean ±SEM. **D)** Dot plot presenting the time between neutrophil attachment and maximum MFI of neutrophils binding to purified $\alpha_{IIb}\beta_3$ (red), or VWF-'primed' platelets (blue). Data plotted are median ±95% confidence interval. Data were analyzed using the Mann-Whitney test.

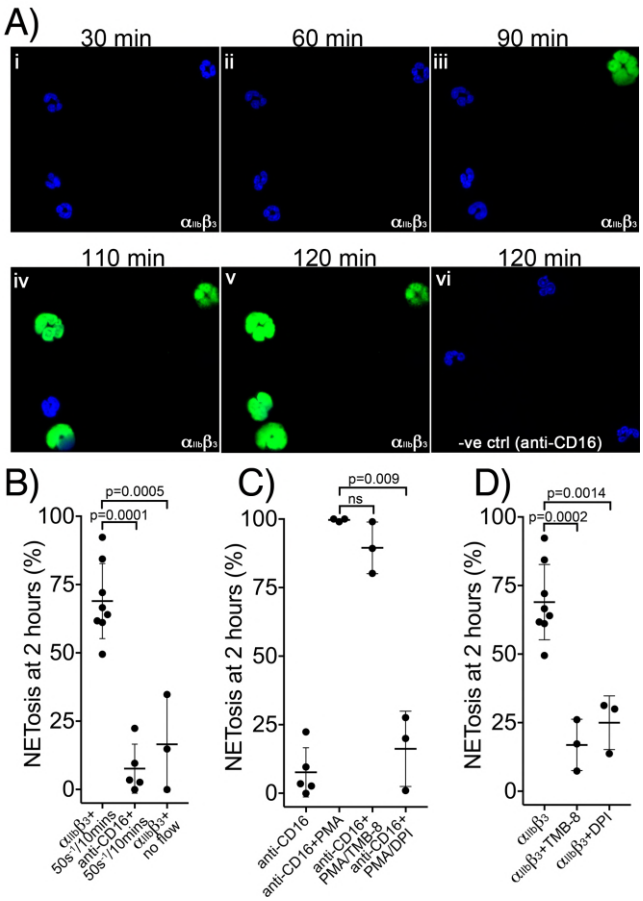

Figure 6: Binding of neutrophils to $\alpha_{IIb}\beta_3$ under flow induces NETosis. A) Isolated PMNs labelled with Hoechst (blue) and cell-impermeable Sytox Green were perfused over $\alpha_{IIb}\beta_3$-coated microchannels (i-v), or anti-CD16, (vi, -ve ctrl) at $50s^{-1}$ for 10 minutes and then monitored under static conditions. Representative composite images after 30, 60, 90, 110 and 120 minutes of attachment. Neutrophils bound to $\alpha_{IIb}\beta_3$ underwent NETosis. After about 90 minutes, Sytox Green staining appears, indicative of DNA becoming extracellular/NETosis (see **Movie EV 3**). Neutrophils bound to surfaces using an anti-CD16 antibody did not undergo NETosis or did so very rarely (vi). **B)** Graph showing the mean % of neutrophils ±SD in the entire microchannel that formed NETs after 2 hours of attachment on $\alpha_{IIb}\beta_3$ (n=8) or anti-CD16 (n=5), captured in the presence of flow ($50s^{-1}$/10 minutes), or captured on $\alpha_{IIb}\beta_3$ under static/no flow conditions (n=3). **C)** Graph showing the mean % of neutrophils ±SD in the entire microchannel that formed NETs after 2 hours of attachment on anti-CD16 antibody in the presence of flow ($50s^{-1}$/10 minutes) (n=5) in the presence of PMA (n=3), PMA and TMB-8 (n=3) or PMA and DPI (n=3). **D)** Graph showing the mean % of neutrophils ±SD in the entire microchannel that formed NETs after 2 hours of attachment on $\alpha_{IIb}\beta_3$ in the presence of flow ($50s^{-1}$/10 minutes) (n=8) and in the presence of TMB-8 (n=3) or DPI (n=3), as noted. Data were analyzed using unpaired, two-tailed Student's t test; ns not significant.

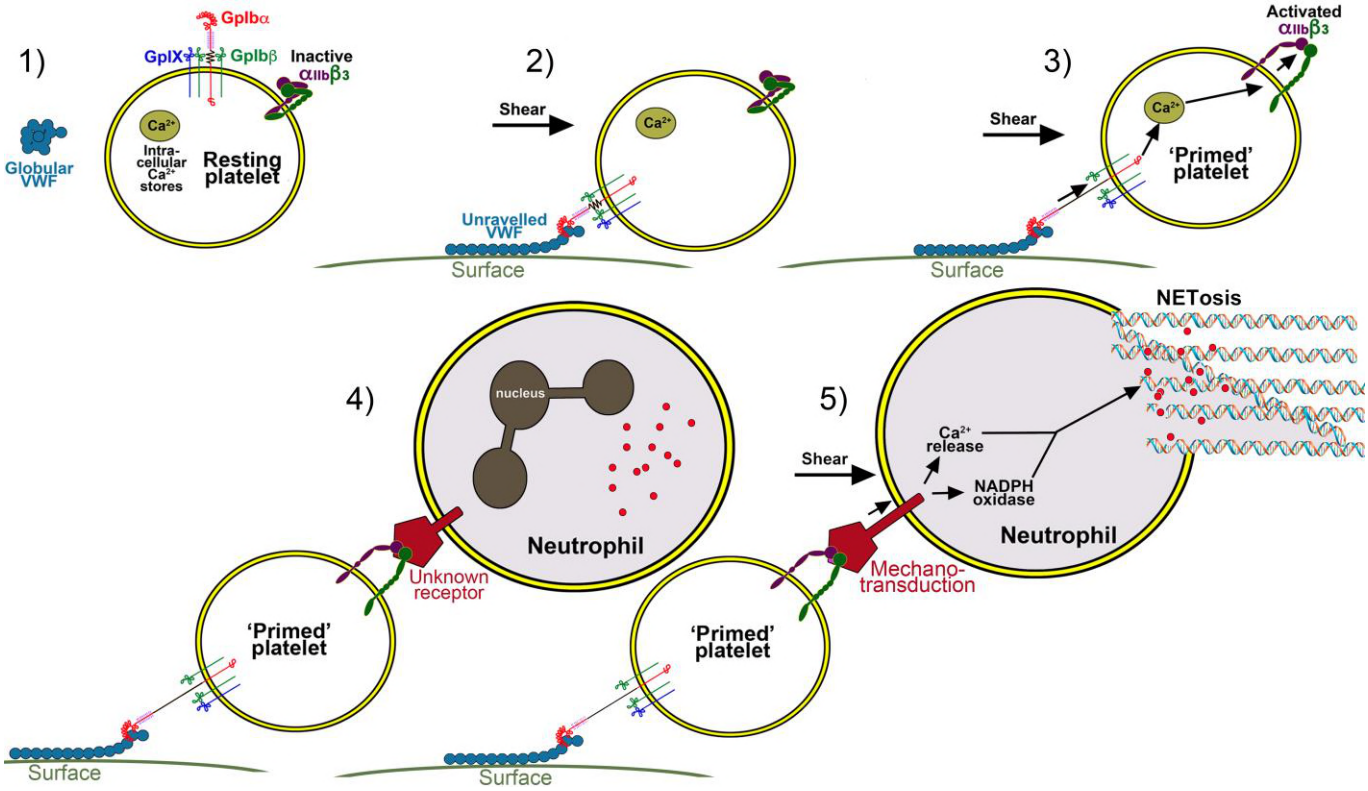

Figure 7: Model of platelet priming, neutrophil binding and NETosis. 1) Under normal conditions, VWF circulates in plasma in a globular conformation that does not interact with platelets. Resting platelets present GPIbα on their surface - in complex with GPIbβ, GPIX and GPV - and also $\alpha_{IIb}\beta_3$ in its inactive conformation. **2)** When VWF is attached to a cell surface (e.g. activated endothelial cell/Kuppfer cell or to a bacterial cell) or to an exposed collagen surface under flow, VWF unravels to expose its A1 domain enabling capture of platelets via GPIbα. **3)** Binding of platelets to VWF under flow induces mechano-unfolding of the juxtamembrane stalk of GPIbα leading to intraplatelet signaling, release of intraplatelet Ca^{2+} stores and activation of integrin $\alpha_{IIb}\beta_3$. **4)** Neutrophils can bind to activated $\alpha_{IIb}\beta_3$ under flow via an unknown receptor. **5)** Shear forces on the neutrophil induce mechanosensitive signaling into the neutrophil causing intracellular Ca^{2+} release and NADPH oxidase-dependent NETosis.