# Intravascular leukocyte migration through platelet thrombi: directing leukocytes to sites of vascular injury

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**Summary**

Leukocytes recruitment to thrombi supports an intimate cellular interaction leading to the enhancement of pro-coagulant functions and pro-inflammatory responses at site of vascular injury. Recent observations of leukocytes neutrophil extracellular traps (NETs) formation and its mutual reactions with platelet thrombi adds more clinical interest to the growing body of knowledge in the field of platelet-leukocyte cross-talk. However, having considered thrombus as a barrier between leukocytes and injured endothelium, the full inflammatory roles of these cells during thrombosis is still ill defined. The most recent observation of neutrophils migration into the thrombi is a phenomenon that highlights the inflammatory functions of leukocytes at the site of injury. It has been hypothesised that leukocytes migration might be associated with the conveyance of highly reactive pro-inflammatory and/or pro-coagulant mediators to sites of vascular injury. In addition, the evidence of neutrophils migration into arterial thrombi following traumatic and ischaemia-reperfusion injury highlights the already described role of these cells in atherosclerosis. Regardless of the mechanisms behind leukocyte migration, whether these migrated cells benefit normal homeostasis by their involvement in wound healing and vascular rebuilding or they increase unwilling inflammatory responses, could be of interest for future researches that provide new insight into biological importance of leukocyte recruitment to thrombi.

**Keywords**

Platelet-leukocyte crosstalk, NETs formation, intravascular leukocyte migration, neutrophil induced atherosclerosis

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## Introduction

Polymorphonuclear leukocytes (PMN) also called neutrophils are the first and typically the most abundant leukocytes to be recruited to the site of inflammation and vascular injury (1, 2). Traditionally PMNs are well recognised for their capacities in eliminating of pathogens via multiple mechanisms that involve innate immunity (3–5). Nevertheless, there are several lines of evidence which also describe more extended biological roles for neutrophils, the roles that are far beyond their function as simple phagocytes. The pro-inflammatory potentials of PMNs following their recruitment to the site of injury and thrombus formation, their involvements in different stages of primary and secondary homeostasis, wound healing and tissue repairs are important evidences that demonstrate pleiotropic effects of neutrophils (1, 6, 7). The studies of intravascular inflammation induced by infection or sterile injury are well described models which enable scientists to mechanistically discriminate neutrophil functions in infection from their other capabilities (8). During sterile inflammation, cell death and lysis can induce the release and expression of a vast array of pro-inflammatory signals attracting leukocytes to the site of injury (9, 10). More intriguingly, co-occurrence of endothelial injury, thrombus formation and leukocyte recruitment at the site of vascular damage creates a conserved milieu that supports an intimate cellular crosstalk between neutrophils, platelet and endothelial cells. This phenomenon links pro-coagulant state to pro-inflammatory responses that presumably support cellular growth and wound healing at the site of injury (1, 11).

The investigation of cellular crosstalk in thrombi has been hampered by technical limitations. Continuing advances in the development of *in vivo* experimental systems combined with the development of genetically manipulated mouse models, have afforded a much clearer picture into the complex molecular mechanisms regulating neutrophil and platelet crosstalk. Nowadays, *In vivo* studies applying real-time and time-lapse intravital videomicroscopy and confocal microscopy within inflamed or injured microvessels are common methods to investigate the mechanisms behind thrombus formation and leukocytes interaction with thrombi (11). While in regular fluorescent or confocal microscopy, single photon light absorption is used, recent technique of multi-

photon microscopy that applies higher wavelengths to excite fluorescent probes, benefits intravital imaging with its lower photobleaching and phototoxicity, as well as the enhanced tissue penetration depth (12, 13). Now, multiphoton microscopy is widely used for the study of blood rheology and blood flow, shear phenomenon, vascular permeability, thrombus formation, leukocyte adhesion and transmigration in both arteries and veins (14–17).

For years, the adhesion of neutrophils to the site of vascular damage was a main topic of interest for research and today classic multistep pattern of leukocyte recruitment to the inflamed or injured endothelium is well described (11). Nevertheless, the earlier adhesion of platelets and thrombus formation during vascular injury add more complexity to the mechanisms of leukocyte recruitment to the vessel wall. Current observation of NETs formation in adhered leukocytes and its involvement in pro-inflammatory and pro-coagulant functions also encouraged researchers to investigate the clinical importance of leukocytes recruitments to the sites of vascular injury particularly, in deep-vein thrombosis (DVT) (18–21). Most recently, in our studies we showed that leukocyte secondary recruitment to thrombi follows a distinct pattern, termed *Directed Intravascular Conveyance* which leads to an extensive accumulation of leukocytes at sites of vascular injury. The intimate cross-talk between leukocytes and injured endothelium covered by thrombus mass, highlights new concepts for leukocyte function during thrombus formation. Furthermore, the existence of chemotactic gradient induced by activated platelets in thrombi has been shown to be involved in directing of leukocytes migration into the site of injury (2). To discuss leukocytes behaviour during thrombus formation, here, we first provide a brief review on mechanisms of leukocytes recruitment to the site of sterile injury and then focus on some new aspects of thrombi-leukocyte interaction that might be of interest for future research.

## Neutrophil adhesion to the site of inflammation and/or injury

Regardless, whether we discuss the recruitment of leukocytes to the inflamed endothelium or to the site of vascular injury followed by thrombus formation, the classical neutrophil recruitment cascade consists of similar primary stages including "capturing" (tethering), rolling, chemokine arrest, and firm adhesion (11). Additionally, more stages of neutrophil adhesion and trafficking on the endothelium, including crawling and paracellular or transcellular migration have been subsequently described by different groups (22). Ryschich et al. were the first to describe the leukocyte crawling *in vivo* in detail. They divided leukocyte movement on endothelium into some distinct groups, those which firmly adhered including "permanent stickers" (adhesion for more than 30 seconds [s]) and "transient stickers" (adhesion for less than 30 s), those of "rolling" cells which are slowly moving along the endothelium without being arrested and those leukocytes that firmly adhered but still move or "crawl" along the vascular wall (23). In line with these observations, our current published data, for the first time, also showed similar patterns of neutrophil crawling and migration into the body of thrombi (2), the phenomenon that requires being further investigated.

## The pivotal role of P-selectin in leukocyte recruitment to the site of thrombus formation

Basically, leukocyte tethering on the endothelium is mediated by the interaction of different types of surface-expressed selectins with their specific ligands. Among selectins, E-selectin expression is only restricted to the inflamed endothelium (24) whilst P-selectin is expressed on both endothelial cells and platelets upon activation (25, 26). Interaction of endothelial selectins with their major counter-receptor "PSGL-1" on circulating leukocytes, mediates neutrophil capture and rolling either on inflamed endothelium or on developing thrombi (secondary capturing) (27). Another selectin "L" that is only expressed by leukocytes, is also involved in leukocyte recruitment to the sites of inflammation and/or injury (22) by the mechanism in which L-selectin on free-flowing leukocytes interacts with PSGL-1 presented by adherent leukocytes. This interaction creates leukocyte-leukocyte aggregation representing another model of secondary capturing of leukocyte (28). In our study using real-time intravital microscopy, stable thrombi were established in mouse mesenteric veins or arteries via mechanical injury by needle puncture using a microinjector needle tip. Studies were performed in mesenteric veins (diameter, 120 to 180 μm) and arteries (diameter; 100 μm) with wall shear rates estimated to be 100 $s^{-1}$ and 900 $s^{-1}$, respectively) (29). We showed that localised endothelial injuries are rapidly covered with platelets and a few minutes later, leukocytes were efficiently recruited to platelet thrombi. Leukocyte recruitment increased progressively within a 30-minute (min) period (2) (►Figure 1a). Consistently, Gross et al. showed a delayed recruitment of leukocytes to the surface of developing arteriolar thrombi after laser-induced injury (30). In both studies, leukocyte recruitment commences with a rolling pattern shifting to slow rolling and primary adhesion. Using our model of endothelial injury on mouse mesenteric vein, the complete abolishment of leukocyte recruitment in P-selectin knockout mice, confirmed the pivotal role of this molecule in leukocyte interaction with thrombi (2). Similarly, the results obtained from either P-selectin or PSGL1 null mice in an arterial thrombus model, showed only few rolling leukocytes on thrombi during 15 min. This suggested that leukocyte recruitment on arterial thrombi is also almost entirely dependent to the P-selectin expression on platelets (30). Having declared this, the unique pattern of P-selectin expression through the thrombus mass may also explain the already mentioned a few minutes delay in leukocyte recruitment on thrombi. During thrombus development, P-selectin expression is in line with platelet activation which is first triggered in close vicinity around injury site (thrombus core) and then circumferentially expands to the surface area of thrombus where leukocytes are in direct contact with platelets (2, 30). Hence, efficient leukocyte recruitment occurs after a few minutes, so that P-selectin ex-

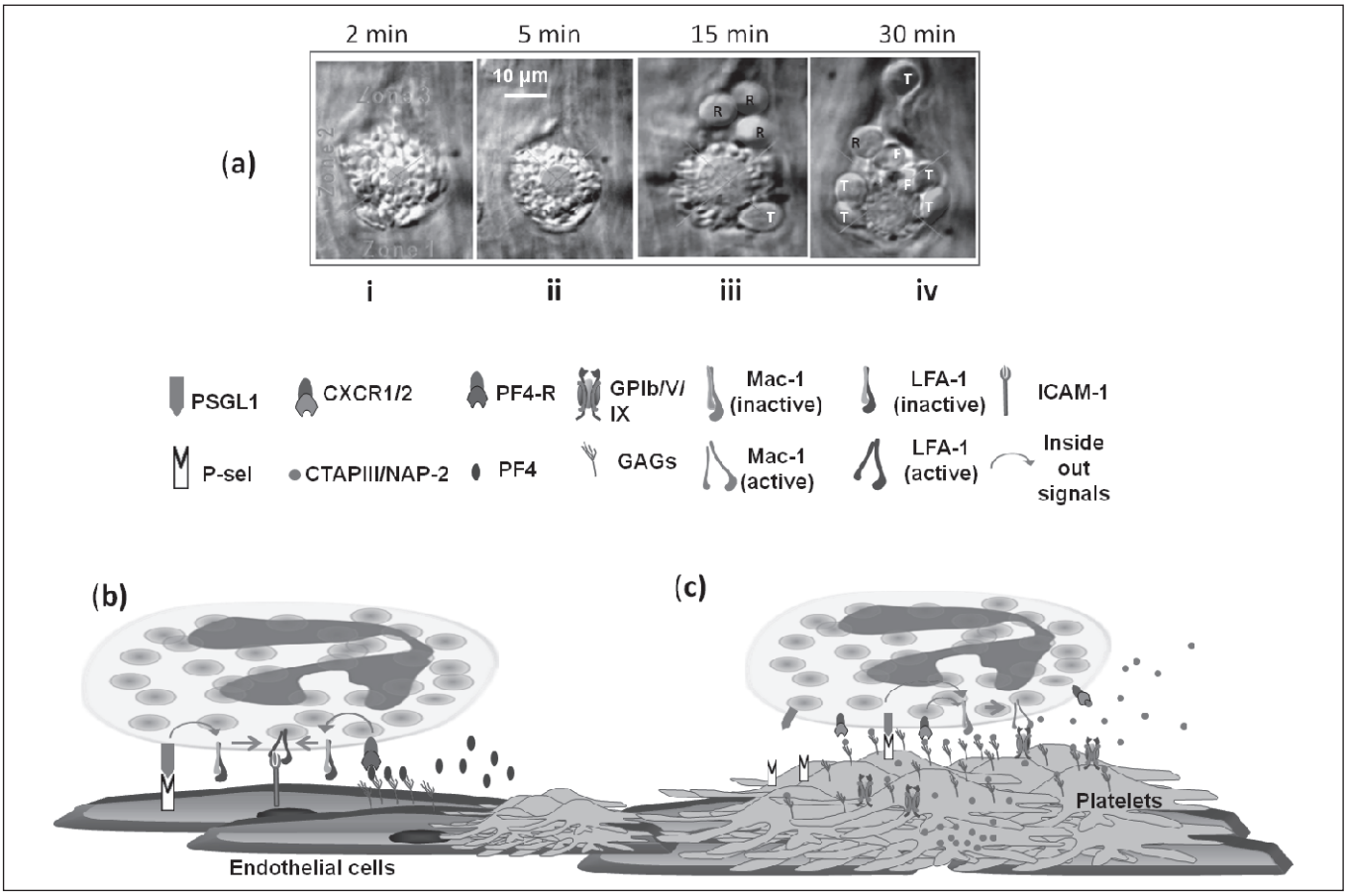


**Figure 1: Leukocyte recruitment to the site of vascular inflammation and /or injury.** a) DIC images illustrating leukocytes recruitment to the indicated thrombus created by needle injury on a mouse mesenteric vein. (i) Newly stabilised thrombi with the majority of discoid platelets around a small solid core at the side of injury (shaded area). (ii, iii) Upon further activation of platelets, solid core of thrombus extends and leukocyte recruitment gets started. (iv) Leukocytes tether and firmly adhere on a mature thrombus. Note: leukocytes have been letter-coded to reflect their adhesive behaviour; R: rolling; T: transient adhesion; F: firm adhesion. Zones (1: upstream; 2: lateral; 3: downstream). b) Leukocyte recruitment to inflamed endothelium. Leukocyte rolling along the inflamed endothelium is mediated by the interaction of endothelial P-selectin with PSGL1 on leukocytes. This interaction is a prerequisite step for leukocyte firm adhesion which is supported by leukocyte LFA-1 and endothelial ICAM-1 engagement. PF4 released from activated platelets resides on endothelium by the binding surface GAGs. PF4 and PSGL-1 interaction with their specific receptors induces inside-out signals leading to integrin activation. c) Secondary leukocyte capture by platelets. Activation and accumulation of platelets at the site of vascular injury leads to the creation of a stable thrombus that provides an alternative adhesive surface for leukocyte rolling via the interaction of platelet P-selectin and PSGL-1 on leukocytes. The rolling events support subsequent leukocyte firm adhesion mediated by the interaction of β2 Integrin Mac-1 with their specific ligands on platelets including immobilised fibrin (ogen) and/or GPIb. CTAPIII is released from activated platelets and remains membrane associated by the binding to platelet surface GAGs. Cathepsin G released from recruited leukocytes then converts CTAPIII to a potent chemokine, NAP-2. NAP-2 and PSGL-1 interactions by their specific receptors induce inside-out signals leading to integrin activation. Note: PF4 and CTAPIII/NAP-2 can be expressed on the cell surface by the aide of GAGs. This figure was originally prepared by M. Ghasemzadeh.

pression reaches the surface area of thrombi and this might be the reason of the observed delay in leukocyte recruitment to thrombus.

## Neutrophil integrins involved in adhesion process

Integrins are important player of neutrophil adhesion and trafficking during inflammatory reactions, and act as both adhesion receptors and signal transducers. They mediate cell adhesion, migration, phagocytosis and oxidant production in neutrophils. The binding of activated integrins to multimeric ligands triggers integrin crosslinking which induces the clustering of integrins in the plasma membrane. This process enhances substrate-binding avidity and signaling capacity (31). Neutrophils mainly express the $\beta_1$ and $\beta_2$ integrin subfamilies (32). $\beta_2$ integrins have crucial roles in various neutrophil inflammatory functions (**1**, 33). This family of integrin consists of heterodimers which composed of a unique α (CD11) subunit non-covalently associated with a common $\beta_2$ (CD18) subunit. $\beta_2$ integrins include four members, CD11a/CD18 [lymphocyte function-associated antigen-1 (LFA-1), ($\alpha L\beta_2$)],

CD11b/CD18 [macrophage antigen-1 (Mac-1), ($\alpha M\beta_2$), (CR3)], CD11c/CD18 ($\alpha X\ \beta_2$, gp150/95, CR4) and CD11d/CD18 ($\alpha D\beta_2$) (34).

$\beta_2$ integrins mediate leukocyte adhesion and transmigration across the endothelium through their interactions with Intercellular Adhesion Molecule 1 (ICAM-1) on the activated endothelium. In addition, Mac-1 and CD11c/CD18 have been shown to play important roles in promoting neutrophil phagocytosis by either direct recognition of pathogens (35), enabling the incorporation of microbes into phagosomes or acting as a opsonin receptor for complement factor C3bi. Mac-1 is also responsible for several other adhesion-dependent neutrophil functions, including binding to fibrinogen, immune complexes and platelets (through GPIb). It is a unique receptor that has the ability of interaction with a large variety of ligands while at the same time can crosstalk functionally with other surface receptors such as FCγ receptors, Toll-like receptor 2 (TLR2) and CD14 (36, 37). In line with other groups, using Mac-$1^{-/-}$ mice in our *in vivo* experimental model of thrombus formation, we confirmed the major role of this integrin in leukocyte firm adhesion to thrombi (2, 11). Several lines of evidence suggest that neutrophil activation is even initiated from the first step of leukocyte recruitment following P-selectin binding to PSGL-1 (38, 39). Inside out signals induced by P-selectin binding, lead to increased affinity of $\beta_2$ integrins to their ligands (40). We demonstrated that this increased affinity might be important for leukocyte slow rolling and their transient adhesion to the thrombi. In our *in vivo* study, we showed that blockade of chemokines or chemoattractants activities by either PTX, platelet activation factor (PAF) inhibitor or CXCR1 and 2 antagonist, cannot affect leukocyte slow rolling and transient adhesion (2) . Noteworthy, these data are in line with other studies that showed P-selectin binding can only prime the neutrophil integrin, a process which is not sufficient for the full arrest of the cell (41–43).

## Chemokine-induced leukocyte arrest on thrombi

A synergistic corporation between P-selectin stimulation and pro-inflammatory stimuli such as cytokines, chemokines and chemoattractants is necessary to induce full integrin activation in leucocytes, leading to their firm adhesion to the inflamed endothelium or platelet thrombi (41, 44, 45). The majority of chemokines mediate their effects through the G protein-coupled receptors (GPCRs) expressed on target cells. Using $G\alpha_{i2}^{-/-}$ neutrophils or lethally irradiated mice reconstituted with $G\alpha_{i2}^{-/-}$ bone marrow, Zarbock et al. has shown almost complete loss of chemokine-induced neutrophil arrest *in vivo* and *in vitro* (44). Accordingly, in our *in vivo* experimental model we showed that blocking GPCRs-related signalling pathway by PTX can significantly reduce the firm adhesion of leukocyte to developing thrombi (2). However, having considered that the majority of these mediators are released as soluble materials, being washed away by blood flow, their curtail role for cellular adhesion in vasculatures especially under a high shear conditions, may still be a matter of debate. Nonetheless, during leukocyte recruitment to endothelium and/or to thrombi, released materials that either trapped in a conserved inflammatory milieu, or anchored on the surface of reactive cells, can be preserved from that "washing off" effect (46).

## Lipid chemoattractants

PAF is one of the most important chemoattractant pro-inflammatory lipids that acts cooperatively with other extracellular stimuli to induce full integrin activation and leukocyte arrest (44, 47, 48). Activated endothelial cells and platelets generate considerable amount of PAF (49–51). Likewise other chemoattractants, PAF has been detected in the circulation; however, this molecule is mostly cell membrane-associated and operates in a paracrine manner on GPCRs of neighbouring cells (52, 53). Previous studies have indicated that platelets regulate neutrophil activation not only by the release reaction, but also through the generation of inflammatory lipids such as PAF which is selectively produced during the formation of sustained calcium-induced platelet morphology (SCIP) (11, 54). This fact is supported with the observation that SCIP formation in platelets is associated with significant generation of PAF which leads to enhanced neutrophil adhesion and spreading. The level of platelet stimulation, impacts directly on neutrophil adhesion to platelets monolayer on which neutrophils activity is spatially regulated by PAF generation (55).

Inconsistent with these results, in our experimental model of thrombus formation we systemically administered PAF inhibitors to mice, and demonstrated a critical role for PAF in leukocyte firm adhesion to thrombi. However, whether PAF mostly originates from activated platelets or leukocytes is not well defined yet (2). Notably, this observation is clearly supported by already published data that report the significant effects of PAF in leukocyte Mac-1 activation and expression (56).

## Non-lipid chemoattractants

Likewise PAF, non-lipid chemoattractants which are usually considered being soluble materials, can be also presented on the reactive surfaces by the aide of different molecules contributing to their immobilisation. Of those, the Duffy antigen receptor for chemokines (DARC) is a well-defined molecule expressed on the surface of endothelial cells and can interact with a variety of chemokines (57) keeping them linked to endothelium. Elimination of DARC by gene targeting reduces neutrophil recruitment into the tissues following injury (58). Similarly, negatively charged polysaccharides, glycosaminoglycans (GAGs), are also known to bind to chemokines (59, 60) and present them on endothelium. This in direct expression of chemokines is shown to be necessary for efficient recruitment of leukocytes on endothelium (61). Likewise, activated platelets secrete GAGs that contribute to immobilisation of chemokines on thrombus, inducing leukocyte adhesion and extravasation. One example is chondroitin sulfate A which is effi-

ciently expressed on activated platelets surface and contributes to the binding of CCL5 to endothelial cells (62).

Chemoattractant molecule, platelet factor 4 (PF4) which is continuously released from activated platelets, can also bind to GAGs including chondroitin sulfate, heparin and dermatan sulfate (63, 64). It has been shown that PF4 enables to activate neutrophil $\beta_2$ integrin, LFA-1, which has important role in leukocyte adhesion to endothelial cells (65, 66). Hence, it seems that the immobilisation of PF4 on the surface of endothelial cells via interaction with expressed GAGs plays an important role in leukocytes recruitment on endothelium in close vicinity of thrombus (►Figure 1b).

In line with these data, our recent published work also demonstrated the immobilisation of CXCL7 [neutrophil activating peptide (NAP)-2)], a potent chemoattractant molecule, on the surface of thrombi where its target cells, leukocytes, get recruited. As the only source of NAP-2, platelets store large quantities of its precursor molecule, CTAP-III (67), which upon release is proteolysed to NAP-2 (68). Although this molecule may be considered a soluble chemokine, our real-time intravital study showed a time-dependent increase in the indirect expression of NAP-2 on the surface of thrombi, similar to the pattern that has been already reported for P-selectin expression (30). Like P-selectin, the highest level of NAP-2 deposition also exists in the middle core of thrombi around the injury site where the first adherent platelets get fully activated. NAP-2 deposition then extends circumferentially around the thrombi (2). The idea of this experiment originated from several *in vitro* studies that demonstrated a high affinity of NAP-2/CTAPIII to heparin (69), and also the fact that activated platelets provide a considerable expression of GAGs on the surface of developing thrombi (70). Hence, the released CTAPIII and other beta-thromboglobulins can be potentially trapped and presented by the aid of GAGs expressed on platelet thrombi. The trapped CTAPIII and other beta thromboglobulins then convert to the NAP-2 by the proteolytic function of proteases like cathepsin G that release from already recruited leukocytes at sites of vascular injury and thrombus formation (2, 68) (►Figure 1c).

Our *in vitro* experiment also confirmed NAP-2 as the main platelet-released chemokine that can activate neutrophil Mac-1 (2), the major integrin that supports leukocyte adhesion to thrombi (11). Concurrently, in our *in vivo* experiment, we also confirmed the effect of NAP-2 on the enhancement of leukocytes firm adhesion to thrombi by injecting *rec* NAP-2 upstream of thrombus formed in murine mesenteric vessels. However, using specific antagonists of NAP-2, we did not find any significant inhibition of leukocyte recruitment to the site of thrombus formation, while PAF inhibitors significantly attenuated leukocyte adhesion (2).

## Platelet activation induces neutrophil extracellular traps (NETs) formation

Neutrophil activation has been shown to be associated with the significant membrane alterations that form an extracellular trap so-called NETs. This compartment consists of extracellular chromatin fibres with a backbone of histones. Neutrophil NETosis was first reported to be induced by close contact of pathogens and leukocytes, especially in sepsis condition. It acts as an antimicrobial mechanism that facilitates killing of ensnared micro-organisms. Several other factors, including activated platelets, inflammatory stimuli or chemical compounds can also induce NETs formation following neutrophil activation (71, 72). Massberg et al. have shown that the incubation of neutrophils with activated platelets *in vitro* can induce a rapid release of NETs (14, 73). It has been already revealed that platelet-induced NETosis is mechanistically beyond a classical P-selectin-dependent interaction of platelets and neutrophils. Several lines of evidence suggest that platelet Toll-like receptor 4 (TLR4) is involved in inducing NETs (11, 74). LPS engages platelet TLR4 to promote the formation of platelet-neutrophil aggregates and NETs generation (74, 75). However, plasma from septic patients has been shown to enhance NETs in a process that was only partly platelet TLR4-dependent, suggesting that TLR4 ligands are not the only mediators involved in NET formation (76). An experimental model of DVT using IL4-R/Iba mice showed a significant reduction in the number of NETs released per recruited leukocyte. This observation proposed a dual role for GPIbα which can be involved in the induction of NET formation by neutrophils in addition to its well known effects in leukocyte recruitment to developing thrombi (14). On the leukocyte side, the study by Neeli et al. suggested that the Mac-1 integrin may be involved in the initiation of neutrophil cytoskeletal changes that facilitate the release of NETs (77). Thus, considering platelets GPIbα one of the major ligands for leukocyte Mac-1, its cumulative contribution in the induction of NETs during thrombosis will be of interest for future studies (11, 14).

## NETs induce platelet activation and thrombosis propagation

In addition to the aforementioned roles of platelets in neutrophil activation and NETosis, several lines of evidence also support the involvement of leukocyte NETs in the initiation and propagation of thrombosis, especially in DVT as well as in ischaemic conditions in vessels that lead to thrombotic events. During DVT, hypoxia and changes in shear forces may drive the release of von Willebrand factor (vWF) from Weibel-Palade bodies of activated endothelial cells (14, 78). Released vWF might help the recruitment of platelets and leukocytes, leading to the initiation of thrombosis. Furthermore, Weibel-Palade body secretion up-regulates endothelial P-selectin expression that promotes leukocyte adhesion (21, 79). It has been shown that P-selectin–dependent leukocyte recruitment is a critical step in DVT formation. In a mice model of DVT induced by flow restriction in the inferior vena cava (IVC), neutrophil depletion after injection of an anti-Ly6G antibody (neutropenic mice) was associated with no or significantly less thrombi formation compared to isotype-treated controls (14). Ischaemic condition induced by DVT enhances the production of IL-8 (80) and reactive oxygen species (ROS) (81) by recruited leukocytes. While IL-8 enables leucocytes to induce NETs (71) and is considered to be a risk factor for venous thrombosis (82), *in vitro*

stimulation of neutrophils with exogenous ROS was also shown to be capable of inducing NETs (19, 20). Recent observations indicate that hypoxia can induce hypoxia-inducible factor 1a (HIF-1a), which is involved in neutrophil activation and NETosis (83). In a mouse model with IVC stenosis, histone infusion was shown to be associated with significantly accelerated thrombus formation and vWF release (84). Another study also reported the correlation of histone infusion and microthrombosis in mice (85). These observations suggest an important role for NETs originated histone in further enhancement of endothelial activation that may develop thrombosis (21).

Upon NETs formation, its fibrous meshwork provides a scaffold for platelet binding and aggregation (20, 86). The first step of platelet binding may be mediated by Toll- like receptors (87) or by electrostatic interaction between histones located in NETs and phospholipids and/or carbohydrates of platelets (72). NETs can also concentrate several adhesion molecules including VWF, fibronectin, and fibrinogen that promote platelet adhesion and activation (20, 84). NETs interact with fibrin strands which are involved in thrombus organisation and stability. Furthermore, the pro-coagulant activity of NETs components such as nucleic acids (88) and polyphosphates (89) may suggest a role for this molecular structure in the promotion of coagulation (20). It has been shown that the negatively charged surfaces supported by NETs can elicit FXII activation which propagates the intrinsic coagulation pathway. This is confirmed by a DVT model of thrombogenesis in which thrombus formation is significantly reduced either after injection of the FXII inhibitor or in mice lacking FXII (14).

Thrombosis can also be initiated and propagated by the release of tissue factor (TF) (90, 91). TF binding to factor VII activates factor X that initiates the coagulation cascade (92) . Recent studies showed the generation of TF by neutrophils and its presentation during NET formation (14, 91). The presence of neutrophil elastase (NE) on NETs can favour thrombosis through the inactivation of tissue factor pathway inhibitor (TFPI), resulting in increased pro-coagulant activity (73). Neutrophil proteases also proteolytically activate the platelet receptors (93) leading to further neutrophil activation and NETs release that increases endothelial permeability (20, 84).

Besides DVT, the evidence of NET formation was shown in large arteries where NETs in addition to fibrin may play a role in stabilisation of the thrombus against arterial shear. The presence of NETs in the carotid lumen proximal to atherosclerotic lesion also supports the clinical observations that suggest NETs contribution to coronary atherosclerosis (18, 21).

Regardless of NETs involvement in thrombosis, several lines of evidence have also shown NETs cytotoxicity towards endothelial and epithelial cells, thereby potentiating bystander injury. This phenomenon is primarily mediated by histones, MPO, NE and cathepsin G as the main NETs-related components (1, 72, 94) that might be involved in tissue destruction, remodeling and wound healing mechanisms.

## Monocyte involvement in thrombosis

In our *in vivo* model of leukocyte-thrombus interaction, the majority of recruited leukocytes were Gr-1 positive (primarily neutrophils) (2). Consistently, von Brühl et al. also showed $Ly6G^{hi}$ myeloperoxidase (MPO)-expressing neutrophils as the predominant leukocyte accumulated on thrombi (~70 % of total) in a DVT model in mice whereas $Ly6G^{-}F4/80^{+}$ monocytes represented the remainder of all recruited leukocytes(~30 %). They did not virtually detect Lymphocytes in thrombi, suggesting the active accumulation of myeloid leukocytes in mice model of DVT (14).

Amongst leukocytes, monocytes are the major source of blood cell–derived TF; however, platelet-leukocyte conjugation may enhance thrombin generation by the transfer of TF between monocytes/neutrophils and platelets/PDMPs (95–97). Falati et al. showed the accumulation of monocyte-derived microparticles enriched by TF at the site of injury (98). In comparison with monocytes, $Ly6G^{+}$ neutrophils were found to express weak TF expression in DVT; however, in this model of thrombosis, neutropenic mice developed no or significantly smaller thrombi compared with isotype-treated controls. This observation proposes that monocytes and monocyte-derived TF cannot fully compensate impaired thrombosis in neuropenic condition, i. e. it suggests that TF per se is not a main player in the initiation and propagation of thrombosis. Thereby pointing out to important role of neutrophils in thrombosis, it seems NET formation and FXII activation mediated by these cells might have a more predominant role in thrombogenesis in a DVT model. However, this cannot rule out neutrophil-derived TF activity that might still contribute to DVT progression (14).

In addition to monocyte, macrophages may also interact with thrombus. Macrophages can contribute to thrombus resolution. These cells are capable of NETs degradation with their high lysosomal DNase II contents (99). Macrophages also phagocytose intact NETs and/or remove fragmented NETs. Monocytes/macrophages also present plasminogen activator that is involved in fibrinolysis helping thrombus resolution mechanisms (100).

## Distinct mechanisms of arterial and venous thrombosis

Although wall shear rate particularly in arteries is expected to act against cell adhesion, thrombus can form in seconds to minutes at either venous or arterial flow rates (101, 102).

Altered flow can significantly affect the morphology and adhesive properties of platelets and leukocytes (103, 104). It also has significant effect on the morphology and function of endothelial cells (105). These alterations in flow can affect the atherogenic process through the induction of shear gradients, turbulence, flow separation, and eddy formation. On the other hand, the progression of the atherosclerotic lesion may lead to arterial stenosis that aggravates flow disturbances, thereby inducing a potential cycle of shear-dependent pathology (106). It is postulated that high shear stress rates (> 5,000 $s^{-1}$) in stenosed atherosclerotic arteries can di-

rectly induce platelet activation (107) which is dictated by the interaction of VWF with GPIbα and integrin $\alpha_{IIb}\beta_3$, as well as the subsequent release of ADP (108).

Neutrophil activation response during inflammation can be also regulated by haemodynamic shear forces (109). Darbousset et al. have recently shown that stable attachment of neutrophil to the vessel wall can occur in the seconds under arteriole flow condition after a laser-induced injury (110). Neutrophils ability to roll at high shear stress *in vivo* was already demonstrated by different publications (111, 112). This ability may be supported by the formation of slings which is described as an important mechanism involved in high-shear rolling of neutrophils (113). However, in some high-shear conditions like in brain neutrophil recruitment, ((which)) may just be dependent on the presence of platelets that adhere to the injured endothelium (leukocyte secondary recruitment) (114, 115), platelets are capable of expressing much higher levels of P-selectin than endothelial cells (116). This may be particularly important for their capacity to promote neutrophil recruitment in a high shear environment (1).

A flow-based method in lower shear condition showed an increased neutrophil activation in a shear stress magnitude- and time-dependent manner. It seems that the exposure with fluid shear stress can increase L-selectin shedding, Mac-1 integrin activation and morphological changes in neutrophils that already primed with PAF (109). This evidence may support neutrophil adhesion to thrombi in venules where the low-shear provides a condition for more efficient cross-talk between circulating leukocytes and inflamed endothelium. In addition, the presence of different subpopulations of circulating neutrophils supports this suggestion that pre-activated neutrophils such as those which experienced a reverse transmigration, are able to adhere rapidly at the site of injury even in higher shear stress existed in arteries (110). This phenomenon may also be attributable to the arterial thrombi where the rolling neutrophils interact with adherent platelets and inflamed endothelial cells in a low shear pocket downstream of thrombus (2).

Regardless of the shear effect, the type and the intensity of the injury also seemed to be important in the nature of thrombus formation. In our model of needle injury or with the other methods like the ferric chloride-induced vascular injury, in which subendothelial matrix is exposed (117, 118), the progressive accumulation of platelets at the site of injury is the first event leading to thrombus formation and subsequent recruitment of leukocytes. Based on the intensity of injury in an artery or vein, the extensive thrombosis may eventually lead to vessel occlusion (119).

Platelet P-selectin plays an important role in arterial thrombogenesis by the formation of large stable platelet-leukocyte aggregates. Using ferric chloride-induced carotid arterial thrombosis model in mice, Yokoyama et al. showed a longer time to thrombotic occlusion in P-selectin-deficient ($P^{-/-}$) mice than in wild-type ($P^{+/+}$) mice. They also observed spontaneous reflow after total thrombotic occlusion in eight of 10 $P^{-/-}$ mice but not in any $P^{+/+}$ mice. They confirmed large aggregates as platelet-leukocyte aggregates where in which the number of leukocytes within thrombi was significantly less in $P^{-/-}$ mice than in $P^{+/+}$ mice (120).

During arterial injury, thrombin also plays an important role in platelet activation and thrombus propagation. Some studies showed reduced platelet accumulation following ferric chloride injury in PAR4-null mice and the formed thrombi were also unable to occlude the mesenteric arterioles (119, 121, 122). Several lines of evidence have suggested that neutrophils per se maybe able to provide blood-borne TF (110) or act as a primary activator of the TF cascade (73). In addition, platelet membranes enriched of phosphatidyl serine also provide an active site for coagulation factor complexes formation and thrombin generation (123, 124) which lead to the production of fibrin. Fibrin fibres at the platelet surface stabilise and consolidate the thrombus, resulting in platelet-dependent clot retraction (125, 126) where full-vessel occlusion may ultimately occur via the trapping of flowing erythrocytes and leucocytes in fibrin network. In addition to the blood-borne TF, inflamed endothelium and vascular smooth muscle cell – derived TF are also considered to be involved in arterial thrombosis in mice (127). However, using laser induced injury, Falati et al. did not show TF expression at the site of injury followed by platelet localisation in mice deficient in P-selectin or PSGL-1 (98). This may highlight the role of leukocyte-borne TF in the model of injury that lack of sub-endothelial matrix exposure. It seems that increased TF expression by endothelial and blood cells exposed to inflammatory mediators plays an important role in the pathogenesis of arterial and venous thromboem-bolism in inflammatory disorders (128). This is an important fact that suggests a potential triggering role of inflammation in thrombosis (129). Several studies showed that either in arteries or in veins, the inflammatory condition may induce injuries with local activation of the endothelium, without exposure of the sub-endothelial matrix to the bloodstream (117, 130).

The pivotal role of platelet-leukocyte crosstalk in a mouse model of DVT has been shown by von Brühl et al. in a recent study where they demonstrated how blood monocytes and neutrophils, crawling along and adhering to the venous endothelium, provide the initiating stimulus for DVT development (14). In this model, neutrophils are essential in the initiation of venous thrombosis as well as in the propagation of the clotting cascade by the interaction with factor XII (FXII) and its subsequent activation through the release of NETs. In this model of venous thrombosis, leukocyte recruitment and neutrophil-dependent coagulation are promoted further by platelets following thrombosis (110).

Similarly, in arterial model of injury without the exposure of sub-endothelial matrix, neutrophils seemed to be initially involved in triggering thrombus formation. A proposed model of laser-induced arterial injury was introduced by Atkinson et al. who determined endothelial cells activation after the use of a dye laser (131). Darbousset et al. confirmed these results by the detection of both LAMP-1 (CD107a) and ICAM-1 (CD54) on endothelial cells immediately after the laser-induced injury. They suggested endothelial cells activation as a prerequisite for leukocytes interaction with endothelium because the fluorescently labelled circulating leukocytes did not show to be recruited by resting endothelium *in vivo*. They showed that either the blockade of ICAM-1 or its ligand, LFA-1 (CD11a), expressed by leukocytes, eliminated the accumu-

lation of leukocytes at the site of laser-induced injury. They also showed that neutrophils are the exclusive circulating cells that bound to the activated endothelium in the seconds after the arterial injury prior to platelets recruitment (110).

Similar to aforementioned ferric chloride-induced injury, when the arterial sub-endothelial matrix is not exposed to the microcirculation, platelet activation by thrombin is also crucial for the propagation of thrombus formation (119). Using laser-induced injury in cremaster arterioles, Vandendries et al. showed that thrombi formed in PAR4-null mice rapidly become unstable and loss their sizes (132). Similarly, in a laser-induced arteriole injury model, Dubois et al. also showed that the infusion of a thrombin inhibitor such as lepirudin in wild-type mice significantly decreases the size of the thrombus formed (133). These observations indicate that in arterial laser-induced injury where the sub-endothelium is not exposed, thrombin, but not collagen, plays main role in thrombus formation and propagation (119).

Exogenous monocytes and monocyte-derived microparticles were previously shown to contribute to thrombus formation after a laser-induced injury by delivering TF to the site of injury and interacting with accumulating platelets (98, 134). However, in their model of arterial thrombosis, Darbousset et al. showed that neutrophil participation in coagulation via TF occurs through the extrinsic pathway which is independent of FXII and the presence of platelets and monocytes at the site of injury (110).

## Neutrophil crawling and migration into thrombi

Although the initial adhesion of leukocytes is an important step prior to leukocyte emigration, usually the adhesion site is not the optimal position for leukocytes to emigrate (135). Thus, crawling around, leukocytes survey the endothelium to find the best place for transmigration.

Several lines of evidence have shown the main contribution of $\beta_2$ integrin to the leukocyte crawling and transmigration across endothelium (136, 137). The sequential adhesive function of $\beta_2$ integrin was first demonstrated with an early LFA-1-dependent adhesion of leukocyte to ICAM-1 transfected cells, that follows a sustained adhesion dependent on Mac-1 (138). Accordingly, using digital time-lapse intravital microscopy, Ryschich et al. showed an essential role for the interaction between leukocyte $\beta_2$ integrin and endothelial ICAM-1 in intravascular leukocyte adhesion followed by crawling (23).

Consistently, Phillipson et al. discriminated between $\beta_2$ integrins based on their functional mechanisms involved in leukocyte recruitment. They defined the crawling stage of leukocyte recruitment as a Mac-1 dependent event, molecularly distinct from leukocyte adhesion which is mediated by LFA-1 (135). Recently, employing a developed confocal imaging system, scientists found a role for the interaction of lumenal ICAM-2 and neutrophil MAC-1 in efficient crawling and transendothelial migration through the endothelium (139).

Most recently, Sreeramkumar et al. indicated that following endothelial activation and leukocyte arrest, neutrophils polarisation on endothelium is associated with PSGL-1 clustering that enable neutrophils to scan for circulating activated platelets. Subsequently, PSGL-1 transduced signals originated from these interaction lead to the redistribution of receptors such as Mac-1 and CXCR2 that drive neutrophil crawling and migration. They concluded that neutrophils which unable to polarise or to transduce signals through PSGL-1, display abnormal crawling and the blockade of this domain protects mice against thromboinflammatory injury (140).

To highlight platelet thrombi effectiveness in leukocyte recruitment, we induced endothelial inflammation without thrombi formation by the intralumenal injection of LTB4 into murine mesenteric veins. We then showed that the number of recruited leukocytes to inflamed endothelium was around 20 times less than what was usually observed to be recruited to thrombi with the same size surface area (unpublished data by M. Ghasemzadeh). Accordingly, in an ischaemia-reperfusion (IR) injury model, using real-time confocal microscopy, similar results were also obtained where the number of polarised/spread leukocytes per square millimeter on the surface of endothelium was around 20-fold less than that observed on spontaneous platelet thrombi (2). In our study, stable thrombi were established in C57Bl/6 or GFP murine mesenteric veins or arteries via mechanical injury alone (103, 141). We also showed different stages of leukocytes recruitment to thrombi (2), similar to that already described for endothelium (22). We indicated that higher shear rate condition in arteries lowered both size of thrombi and the number of recruited leukocytes. The inhibitory effect of high shear stress on leukocyte recruitment to arterial thrombi was expected as already described by different groups in their experimental models (142–144). However, leukocyte rolling, adhesion and crawling showed to be more stable on the lateral sides of thrombi and especially more prominent in low shear pocket downstream of thrombi (2). The lower shear condition may also enhance leukocytes activation in these areas as already shown in model of low fluid shear stress (109, 145). In our study, using a developed epifluorescence and confocal microscopy, for the first time we demonstrated both steps of crawling and migration of leukocytes into thrombi (►Figure 2a and b). These events were significantly Mac-1-dependent (2). Given the fact that Mac-1 activation and expression are mainly influenced by PAF (56), our further experiments showed that the observed number of crawling and migrated leukocytes was also significantly PAF-dependent similar to that we already found in neutrophil adhesion to thrombi. Using NAP-2 antagonist (CXCR1/2 antagonist MSGA 8–73) or mice lacking CPIII/NAP-2 also showed a reduced number of migrating leukocytes into thrombi whereas these conditions had no effects on leukocytes adhesion. Regardless of the molecular mechanisms, most intriguingly, we found that a large number of leukocytes followed a directional migration pattern toward the site of injury within thrombi, where has the highest level of platelet activation, P-selectin expression and CTAPIII/NAP-2 deposition (2).

The observed association between direction of migration and the gradual increase of CPIII/NAP-2 deposition creates a hypoth-

 

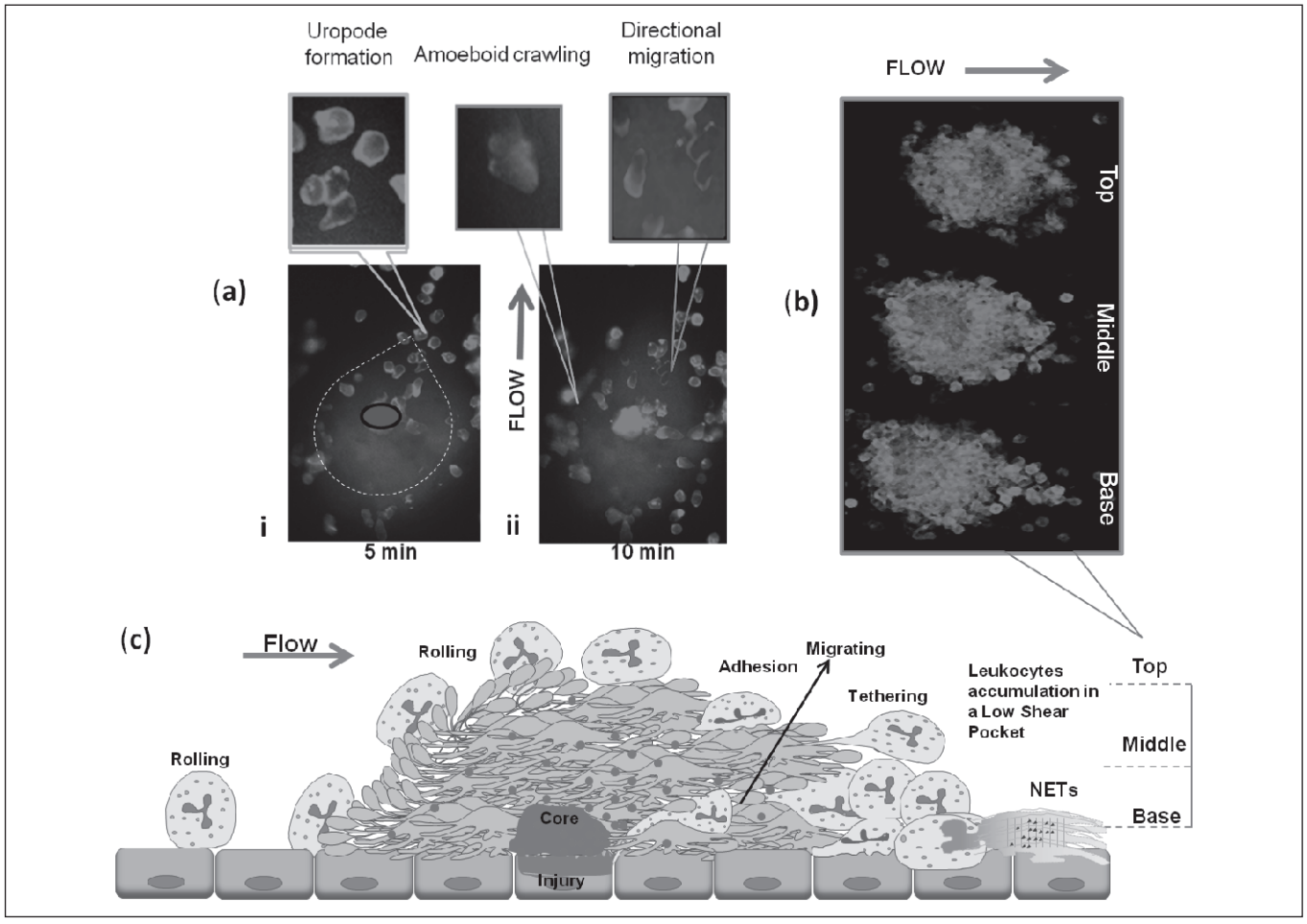


**Figure 2: Directed intravascular leukocyte migration.** a) Leukocyte adhesion, crawling and directional migration into a developing thrombus. C57Bl6 control mice were administered DiOC6 and anti-Gr-1 antibody prior to vessel injury. Mesenteric veins were externalised and injury was made by needle puncture under epifluorescence microscope. Real-time imaging performed on the base of thrombi. Note: the site of injury was depicted as circle shaded area. (i) Representative image at 5 min post injury depicting adhered leukocytes on thrombus (dashed circle area). Left inset demonstrates the polarised morphology of adhered leukocytes including surface protrusion and uropode formation. (ii) Representative image at 10 min post injury depicting migrating leukocytes at different Positions between the margin and the centre of a thrombus; the right inset demonstrates individual migrating leukocytes directed to the site of injury while in the middle inset crawling leukocytes with irregular movement have been shown. b) Confocal images of leukocyte migration through a thrombus. GFP-NOD mice were injected systemically with an anti Gr-1 Ab, prior to induction of vascular injury via needle puncture. Thrombus formation was monitored by confocal microscopy over 40 min. The figure shows confocal sections through the Top, Middle and Base of a representative thrombus (30 µm height), as schematically depicted on the top. c) Schematic model of directed intravascular leukocyte migration into thrombi. Leukocytes tether and roll on the surface of platelet thrombi via a P-selectin-dependent mechanism. Rolling leukocytes typically accumulate on the sides and downstream margin of thrombi in a low shear pocket where they meet lower "rheological stress" and experience easier adhesion and migration. A chemotactic gradient of CTAPIII/NAP-2 originates from the core (in close vicinity of the injury site) to the platelets resided on the surface of thrombi. This gradient dictates directional cues for leukocyte migration into the thrombi. Note: the blue shaded circles represent released CTAPIII/NAP-2 molecules. This figure was originally prepared by M. Ghasemzadeh, panels aii and b are adapted from Ghasemzadeh et al. (2).

esis that whether this directional migration is dictated by a chemotactic gradient within the thrombus body or not. This idea was then confirmed by the absence of directional migration in mice lacking CPIII/NAP-2. This observation demonstrates the importance of chemokine gradient for directional migration of neutrophils in an *in vivo* model of leukocyte recruitment. ►Figure 2c illustrates a schematic model that has summarised the multistep stages of leukocytes recruitment to thrombi, with more focusing on leukocyte directional migration. Several lines of evidence have also shown that neutrophils play role in atherosclerosis. They proposed that neutrophils may be involved in atherogenesis and plaque destabilisation, monocyte attraction into atherosclerotic lesions as well as macrophage activation to promote foam cell formation (146). Notably, the evidence of neutrophil migration into platelet thrombi in arteries following traumatic and IR injury (2), may highlight the described role of these cells in both induction and promotion of atherosclerosis. Furthermore, our unpublished data also evidenced NET formation in migrating neutro-

phils, especially of those settled in low shear pocket downstream of thrombi. Although more experiments are required to further recognise this phenomenon. Whether the focal presentation of protease by NETs as well as its cytotoxic function (1) can affect thrombi and endothelial permeability, or transporting NETs-related material to the site of injury by migrating leukocytes could be important in endothelial remodelling and wound healing, all might be matter of interest for future studies.

## Conclusion

Followed by thrombus formation, the recruitment of leukocytes on the surface of developing thrombi is a subsequent event that has been mechanistically well discussed by numerous researches. However, the biological importance of this phenomenon is not fully defined yet. Studies that investigated the recruitment of neutrophils to thrombi have focused more attention on the role of these cells in the enhancement of pro-coagulant function or thrombi stabilisation. In line with these studies, the observation of neutrophils' directional migration into thrombi and their accumulation in close vicinity of the injury site are the most recent findings that raise further questions about other possible functional roles for neutrophil in homeostasis. What mediators are mainly involved in neutrophil migration and by which mechanisms this phenomenon is induced, whether migrated neutrophils have a beneficial role in wound debridement and vascular rebuilding or whether they may enhance unwilling pro-inflammatory events, are all important questions that might be of interests for future research. We hope that further advances in this area of research will result in the development of new pharmacological agents that would either maintain vascular integrity and normal homeostasis or reduce unwanted risk factors during thrombotic events.

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**Author contributions**

MG provided intellectual input and wrote the manuscript. EH provided intellectual input and co-wrote the manuscript.

**Conflicts of interest**

None declared.

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