Neutrophils as effectors of vascular inflammation

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Abstract

Vascular inflammation underlies most forms of cardiovascular disease, which remains a prevalent cause of death among the global population. Advances in the biology of neutrophils, as well as insights into their dynamics in tissues, have revealed that these cells are prominent drivers of vascular inflammation though derailed activation within blood vessels. The development of powerful imaging techniques, as well as identification of cells and molecules that regulate their activation within vessels, including platelets and catecholamines, have been instrumental to better understand the mechanisms through which neutrophils protect or damage the organism. Other advances in our understanding of how these leukocytes exert detrimental functions on neighboring cells, including the formation of DNA-based extravascular traps, constitute milestones in defining neutrophil-driven inflammation. Here we review emerging mechanisms that regulate intravascular activation and effector functions of neutrophils, and discuss specific pathologies in which these processes are relevant. We argue that identification of pathways and mechanisms specifically engaged within the vasculature may provide effective therapies to treat this prevalent group of pathologies.

Keywords: Neutrophils, cardiovascular disease, vascular inflammation, platelets, adrenergic signaling, neutrophil extracellular traps.

Abbreviations: PSGL-1 (P-selectin glycoprotein ligand 1), AR (Adrenergic Receptor), NET (Neutrophil Extracellular Traps), ROS (Reactive Oxygen Species), I/R (Ischemia Reperfusion), SCD (Sickle Cell Disease), TRALI (transfusion-related acute lung injury)

Introduction: neutrophil lifespan and onset of inflammation

Vascular inflammation is a milestone in the development of subclinical and clinical cardiovascular disease. In fact, damage to the vasculature elicited by immune cells is a leading cause of debilitating disease and death in the world because it critically impairs the function of the irrigated organs. Two examples that best illustrate the relevance of vascular inflammation are acute myocardial infarction and stroke, which combined are responsible for roughly one fourth of deaths worldwide [1]. Other highly prevalent disorders that affect the vascular compartment such as atherosclerosis [2] and sickle cell disease [3], inevitably lead to persistent inflammation.

lsNeutrophils are short-lived innate immune cells, loaded with an arsenal of cytotoxic molecules that are primarily specialized in pathogen clearance during early infection. Upon detection of a variety of danger signals, such as inflammatory cytokines, opsonized pathogens, pathogen-derived molecules (PAMPs, such as viral DNA, bacterial-derived endotoxins, etc.) or cues of host damage (extracellular DNA, adenosine, or other intracellular components), neutrophils become activated and initiate effector functions: they can phagocytose pathogens; produce reactive oxygen species (ROS); release the content of their cytoplasmic granules containing (metallo)proteinases, cationic antimicrobial peptides, defensins, etc.; and produce neutrophil extracellular traps (NETs), which are strings of decondensed chromatin decorated with toxic molecules that limit pathogen dissemination (reviewed in [4]).

The half-life of neutrophils in the circulation is estimated to be about 8-12 hours in the mouse [5, 6]. Similar values are generally accepted in humans [7], although recent analyses have suggested half-lives of over 5 days in humans [6], a value that nonetheless remains controversial [8]. See also the contribution by Koenderman et al. in this issue. The controversy has its roots in the conceptual significance of the neutrophil lifespan when considering the tradeoff between immune defense and tissue health. Indeed, the short life of neutrophils is considered an important control

mechanism that regulates neutrophil numbers, as their cytotoxic capacities –as we discuss below- pose a serious threat to vascular integrity [9].

The interaction of neutrophils with the vasculature during a typical, non-pathogenic inflammatory reaction is fast and highly dynamic. These dynamics have been mostly studied by imaging techniques in living tissues at microscopic resolution. The intravital microscopy techniques use fast scanning devices (e.g., spinning disk technology) combined with either epifluorescence, confocal or two-photon microscopy to track real-time behavior of individual leukocytes *in vivo* (reviewed in [10]) in multiple tissues (**Figure 1**). Indeed, intravital microscopy has been key to dissect leukocyte dynamics and to identify molecular mediators of intravascular interactions both in homeostasis and disease [11]. For example, elegant studies using high-end imaging techniques demonstrated that reverse migration of neutrophils back into the circulation can occur and may contribute to disseminating inflammation systemically [12].

During the first stages of inflammation, the activated endothelium exposes a series of receptors that interact with glycoproteins on the surface of leukocytes, thus promoting their interaction with the vessel wall; this is the case of selectins which mediate leukocyte tethering, rolling and favor firm adhesion [13]. Particularly relevant is endothelial E-selectin, which interacts with PSGL-1 (P-selectin glycoprotein ligand 1), CD44 and ESL-1 (E-selectin ligand 1) on leukocytes [14, 15]. Interactions with PSGL-1 or ESL-1 trigger β2-integrin activation through Syk (spleen tyrosine kinase) or Src-family kinases, respectively. In turn, activated β2 integrins, most notably Mac-1 (also known as αMβ2), display increased affinity for ligands on free-flowing platelets on one hand, and mediate firm adhesion and crawling of neutrophils on the endothelium on the other [16-18].

An important event after the initial adhesion that is often overlooked is the re-organization of the leukocyte cytoskeleton and surface receptors to establish cell polarity. Leukocyte polarization generates a dynamic leading edge enriched in protruding F-actin filaments allowing the

lamellipodia to exert their traction function, and a trailing edge, or uropod, with a prevalent contractile acto-myosin cytoskeleton that allows uropod retraction in migrating cells [19]. After polarization has been established, neutrophils migrate over the vascular endothelium through the integrin Mac-1 binding to ICAM-1 (intercellular adhesion molecule 1). This crawling process has been proposed to be a mechanism that scans for optimal extravasation sites, and allows leukocytes to efficiently migrate through the endothelial wall to the sites of primary inflammation [20]. Additionally, neutrophils crawling over the endothelium during inflammation are able to specifically interact with other cellular blood components, such as platelets and red blood cells, through a process regulated by E-selectin and mediated by Mac-1. These interactions have been shown to be key in the development of thrombotic injury in models of acute inflammation, such as in transfusion-related acute lung injury (TRALI) or during vaso-occlusive crisis in sickle cell disease (SCD) [17]. The molecular mechanisms controlling intravascular neutrophil polarization, crawling and migration remain only partially understood. Similarly, other aspects of the behavior of neutrophils inside vessels prior to their extravasation remain partially obscure, in part due to the lack of high-resolution imaging techniques that allow discrimination between intravascular and perivascular or extravascular spaces. Here, we review emerging mechanisms that regulate the function of intravascular neutrophils in vascular inflammation, including the interplay between platelets and neutrophils, and the effects of adrenergic signaling on their behavior. We also summarize relevant pathologies associated with the toxic activities of neutrophils within vessels.

Platelet signaling in vascular inflammation

Platelets are well known for their role during hemostasis and coagulation, but they are also important providers of inflammatory signals in blood [21]. A wide range of cues is able to trigger platelet activation, mostly by engaging danger signals such as collagen, thromboxane A_2 (TxA_2), or ADP or thrombin [22]. Platelet activation induces the release of granule contents – including

proteins that regulate leukocyte function, such as IL-1β, PF4, RANTES, CD154, TGFβ1 and CD40L [23, 24]. Circulating platelets can also cooperate with leukocytes during pathogen recognition and clearance through mechanical capture of bacteria and debris [25]; and can also generate platelet-derived microvesicles. These particles have been involved in the development of cardiovascular disease, by enhancing expression of endothelial adhesion molecules and recruitment of blood leukocytes to inflamed areas, thereby increasing tissue damage during acute myocardial infarction, atheroma plaque development and atherosclerotic-related stroke [26].

Platelets not only generate soluble mediators, they are also able to physically interact with neutrophils to form heterotypic aggregates. These aggregates have been associated with vascular inflammation, thereby establishing a link between hemostasis and inflammation [27, 28]. In addition to these circulating aggregates, neutrophils adhering to the endothelial wall during inflammation are able to "capture" circulating platelets (as well as other blood components, as discussed below) through the different microdomains that form upon polarization. Interactions between a neutrophil's leading edge and circulating platelets are dependent on ESL-1 signaling, which regulates the capture of platelets and red blood cells through activation of the integrin Mac-1. This type of interaction has been shown to be important for binding subsets of circulating erythrocytes [17]. On the other hand, engagement of platelet P-selectin with its ligand PSGL-1 displayed at the uropod, regulates the intravascular crawling of neutrophils. When this interaction is impaired, neutrophils remain stationary at their initial adhesion spot and are unable to crawl normally. Consistently, inhibition of these interactions ameliorates thrombo-inflammatory injury in murine models of acute lung injury or stroke [29]. Therefore, we speculate that platelet-neutrophil interactions (at least those mediated by the leukocyte's uropod and inhibited by blockade of PSGL-1) act as a safeguard mechanism that provides a second signal before neutrophils become fully activated, and prevents uncontrolled vascular inflammation upon a single, mild stimulus [29]. Consistent with this possibility, depletion of neutrophils or platelets were shown to protect from

vascular damage in various forms of lung injury [17, 30, 31]. It is unclear how neutrophils integrate these external signals; for example, it is unknown whether transduction of signals from platelets through PSGL-1 occurs through the kinases involved in integrin activation during the rolling phase, or through alternative pathways; defining these pathways may be relevant to identify pathogenic intravascular mediators. Interestingly, engagement of P-selectin on the surface of activated platelets with neutrophil PSGL-1 is not only required to regulate crawling over the endothelium, it is also a signal to trigger NETosis [32], a major neutrophil-derived mediator driving intravascular inflammation (discussed below).

Although platelet-neutrophil interactions are clearly major drivers of thrombo-inflammatory injury in the vasculature, crosstalk with platelets is also required for neutrophils to properly exert their pathogen-clearing functions. In a model of experimental pneumonia, neutrophils provided arachidonic acid to platelets through extracellular vesicles, and these lipids were in turn substrate for the generation of TxA_2, which enhanced endothelial expression of adhesion molecule ICAM-1 to further amplify leukocyte recruitment [33].

In summary, bidirectional signals between platelets and neutrophils are key in the initiation, regulation and amplification of inflammation (summarized in **Figure 2**). Thus, balancing this crosstalk may be a central regulatory mechanism of both innate immunity and vascular injury.

Adrenergic signaling during vascular inflammation

Sympathetic innervation has been described as a key regulator of innate immunity, for example by controlling key homeostatic features such as circadian recruitment of leukocytes to tissues [34]. The overall contribution of adrenergic signaling during the acute phases of inflammation remains, however, poorly characterized. Although catecholamine levels may be increased in plasma through the release of epinephrine and norepinephrine by adrenal glands or sympathetic

nerves, innate immune cells may also regulate the levels of plasma catecholamines (**Figure 3**). Neutrophils and macrophages have been reported to synthesize and secrete cathecolamines upon *in vitro* activation with bacterial lipopolysaccharide (LPS) through upregulation of the enzymes tyrosine hydroxylase and dopamine β-hydroxylase. The relevance of this myeloid-dependent production of cathecolamines was initially underscored in experimental models of acute lung injury, in which this cathecholamine production controlled the secretion of pro-inflammatory cytokines by alveolar macrophages through α2 adrenergic receptors in response to initial inflammatory signals, including LPS or IgG complexes [35]. In addition, phagocyte-derived catecholamines have been shown to trigger the secretion of pro-inflammatory cytokines by macrophages and neutrophils in an NF-κB dependent fashion. Studies in adrenectomized rats demonstrated that neutrophils are alternative sources of catecholamines that regulate basal susceptibility to acute inflammatory injury [36]. It is thus intriguing that the innate immune system may function as an alternative and "diffuse" source of catecholamines. As such, immune cells would integrate pathogen-derived signals (endogenous) with environmental (exogenous) cues generated by the neural and endocrine systems. The contributions of this integrative signaling during inflammatory responses remain, however, poorly defined.

Adrenergic signals may regulate inflammation by directly acting on vascular cells. It has been shown that β-adrenergic stimulation increases the expression of adhesion molecules in coronary vessels, and the increase in VCAM-1 (vascular cell adhesion molecule 1), ICAM-1, E-selectin and L-selectin correlates with accumulation of leukocytes in the coronary vasculature [37]. This feature of adrenergic signaling may be specifically relevant for the pathological mechanisms underlying acute myocardial injury, in which a series of stressors associated with heart attack, such as anxiety and pain, may systemically increase plasma levels of catecholamines, and contribute to the recruitment of neutrophils to the ischemic area, therefore propagating tissue damage [38]. Despite this deleterious effect of adrenergic signaling on acute myocardial injury, it has been

shown that expression of β2-Adrenergic Receptors (AR) on monocytes can mediate healing and survival after myocardial injury. Monocytes lacking β2-AR are unable to be recruited to inflamed areas of the heart and are retained in the spleen, thereby preventing healing and amplification of the inflammatory response after infarction [39].

Surprisingly, studies in murine models of myocardial injury have shown that adrenergic signaling on leukocytes modulates inflammation. Although different forms of β-adrenergic receptors are expressed on neutrophils [40], β1-AR was found to be critical in early stages of myocardial injury: an antagonist for this receptor impaired the migration of neutrophils towards chemotactic stimuli in vitro, and hematopoietic-specific deficiency in the receptor protected from acute injury during the early stages of infarction [41]. Interestingly, inhibition of this receptor leads to aberrant morphology of intravascular neutrophils and reduced motility, similar to that found upon disruption of platelet-neutrophil interactions [29]. Blunted activation of neutrophils both by disruption of β1-AR signaling or by blocking platelet interaction with the uropod suggests a possible crosstalk between both pathways in the intravascular neutrophil, and the existence of dedicated mechanisms that regulate the behavior of the cell specifically inside the vasculature.

Adrenergic signals do not only modulate inflammation through local effects on inflamed areas. They can remotely regulate bone marrow progenitor cells, which are able to distally influence inflamed areas. For example, studies under chronic stress showed that release of catecholamines from sympathetic nerves in the bone marrow affects megakaryopoiesis, leading to increased release of platelets into the circulation that are prone to activation in a α2-AR- extracellular signal-regulated kinase (ERK1/2)-dependent manner. This enhanced thrombopoiesis of highly reactive platelets was shown to contribute to the aggravation of the atherosclerosis in ApoE-deficient mice [42]. In a somewhat similar way, sympatho-adrenergic signaling triggered by a heart attack incites inhibitory effects on the distal bone marrow, through β3-AR-dependent adrenergic signaling in hematopoietic niche cells. Niche inhibition after myocardial injury in turn results in mobilization of

myeloid progenitors which then seed the spleen. These extramedular progenitors sustain increased myelopoiesis after myocardial infarction and generate an elevated supply of monocytes and neutrophils to the atheroma plaque, cause plaque instability, and can create susceptibility for new ischemic events [38].

In summary, adrenergic signaling modulates intravascular activation of several types of innate immune cells, including neutrophils (**Figure 3**). This effect varies, depending on the target population and its display of receptors, and thus changes in the panel of adrenergic receptors of immune cells can shape the inflammatory response. It is conceivable that alterations in the baseline levels of catecholamines, a feature of chronic stress [43], or in the levels of their receptors, influence the susceptibility to inflammatory disease. More work will be needed to precisely define how catecholaminergic signals and their effects on neutrophils or other myeloid cells influence vascular inflammation.

Mechanisms of neutrophil-induced injury

The activity of neutrophils has been extensively studied *ex vivo* and during tissue infiltration, in which it is considered a hallmark of inflammation. Neutrophils are also recognized culprits of vascular injury, as discussed below. Paradoxically, few reports have focused on the specific features of neutrophil activation within the vessels, which may be relevant to minimize vascular and tissue damage. Below we discuss common mechanisms by which neutrophils can cause damage to the vasculature.

Reactive Oxygen Species

One key mechanism that neutrophils use for pathogen clearing is phagocytosis. Once the pathogens are engulfed in the phagosome, several reactive compounds, peptides and enzymes are used to kill the pathogen. Among these, superoxide anion ($O_2^{-\cdot}$) is the initial step in the

generation of a collection of highly reactive oxygen species, including hydrogen peroxide (H_2O_2), hydroxyl radical (OH·) and hypochlorous acid (HOCl). These compounds, collectively known as Reactive Oxygen Species (ROS), contribute to pathogen inactivation and death. The initial superoxide generation is mediated by the NADPH oxidase, a molecular complex that produces ROS by rapid association of its components upon neutrophil activation [44]. Interestingly, not only pathogen cues are responsible for the induction of NADPH oxidase, but integrin receptors prime neutrophils and drive ROS production in a Src-kinase dependent fashion [45]. Therefore, neutrophils attached to the inflamed vasculature are capable of becoming activated and generate ROS within blood vessels, which can be deleterious. Although this issue has not been directly addressed, some recent reports show that neutrophils are able to handle ROS in a variety of ways, depending on the threat, and are even capable of secreting ROS by fusing ROS-generating vesicles to their plasma membrane [46]. Most reports on ROS-driven vascular damage come from models of ischemia-reperfusion injury and focus mostly on mitochondrial ROS production. When blood flow is restored after an ischemic event, the myocardial tissue suffers a fast re-oxygenation, which leads to mitochondrial electron transport chain dysfunction, oxidative stress and increased susceptibility to the opening of the mitochondrial permeability transition pore. These events lead to further increases in ROS production, enhanced leukocyte recruitment and vascular damage, although these are likely mediated by non-neutrophil derived ROS [47].

Indirect evidence supporting the importance of neutrophil-derived ROS in vascular injury has been obtained from intravital imaging of neutrophils as they contact the vascular wall [12, 17]. As neutrophils prepare to leave the circulation, they upregulate ICAM-1, and this ICAM-1^{HI} subset of neutrophils concomitantly produce higher levels of ROS. In models of ischemia-reperfusion, this ROS-producing subset displays impaired extravasation, and their retention within vessels can cause local or distal damage to the pulmonary microcirculation [12]. Similarly, a report showed that subsets of neutrophils that have “aged” in the circulation are prone to produce ROS, and their

abnormal retention within the vasculature can precipitate vascular injury in the context of sickle cell disease [17, 48].

Neutrophil extracellular traps

Out of the many immune defense mechanisms that neutrophils have at their disposal, the formation of extracellular traps is perhaps the most striking from an evolutionary standpoint. These neutrophil extracellular traps, or NETs, are filamentous extracellular DNA structures coated with histones and granule-derived proteins that help ensnare and kill oversized [49] or supernumerary pathogens [50]. In most cases, NET formation leads to the lytic death of the neutrophil, thus configuring a novel type of cell death that has been termed NETosis [51]. As such, it may seem a last-resort option to contain an ongoing infection. However, on the contrary, extracellular DNA traps may also be an evolutionary old defense mechanism that could have evolved in the verge of multicellular life [52] and be a common response to infection in mammals, since NETs are formed in response to many different types of pathogens [50], and even in sterile injuries [53]. For a review on the role of NETs in the defense of neutrophils against infections and in the generation of autoimmune and autoinflammatory phenomena, see the article of Van Avondt and Hartl in this issue.

Although there are other means of extrusion of nuclear DNA, NETosis is the best known way of NET formation. It involves the blockade of cytoskeletal dynamics [54], and requires ROS production by either the NADPH-oxidase [51] or other sources, like the mitochondria. Upstream pathways to NADPH oxidase are unclear, and may include RAF/MEK/ERK pathways [51] and require calcium influx to the cell [55]. Once engaged, ROS from the NADPH oxidase induce myeloperoxidase (MPO) activity, releasing neutrophil elastase (NE) from azurophillic granules to the cytoplasm and finally to the nucleus. Once in the nucleus, NE disassembles the chromatin. It is unclear, however, whether NE is an absolute requirement for NET formation, since NE-deficient neutrophils have been reported to form NETs in models of deep vein thrombosis [56]. Another

hallmark of NETosis is the citrullination of histone 3, which is often used in combination with MPO as a marker of NETs in tissues (**Figure 4A**). This process is dependent on the enzyme peptidyl arginine deiminase 4 (PAD4), which lies downstream of ROS signaling, but the extent to which histone citrunillation is needed for NET formation is again unclear, since some stimuli do not depend on PAD4 activity [57]. Nonetheless, PAD4 inhibition with Cl-amidine has been successfully shown to reduce NET formation in many disease models, including lupus, atherosclerosis, diabetic wound-healing or deep vein thrombosis, which highlight the pathogenic role of NETs in various forms of vascular inflammation (reviewed in [53]).

After chromatin de-condensation, the nuclear membrane disassembles, and DNA is mixed with cytoplasmic contents, even with granule-held proteins [51]. Finally, in most cases the plasma membrane loses its integrity and DNA is pushed outwards through a poorly defined process, thus creating NETs (**Figure 4B**). NETs are coated with granule-derived proteins that are highly cytotoxic, i.e. cationic antimicrobial peptides able to permeabilize cells, MPO and NE. Histones by themselves are also cytotoxic [58]. Thus, NET release helps contain infections, but can also promote severe damage to the host. Within blood vessels, NETs are able to resist degradation and shear flow for hours [59] because their degradation by plasmatic DNases is slow [60]. Intravascular NETs have been shown in several conditions, including sepsis, thrombosis and atherosclerosis, and a recent study found low levels of basal NETosis in the steady state [61], perhaps hinting to a homeostatic role for NETs. So far, nonetheless, NETs have only been reported in pathological states. In sepsis, NETs are present in the liver sinusoids, where they enhance bacteria capture compared to Kupffer cells alone [62], thus helping to control bacteremia, but at the same time causing liver damage. These studies illustrate that NET formation inside vessels is useful to prevent the spread of ongoing infections, but also hints at wrongly timed or exacerbated intravascular NET production as harmful to the host.

Intravascular NETs also promote thrombosis, and coagulation, in turn, promotes NET formation. Upon damage to the endothelium, neutrophils can be recruited within seconds, even before platelets [63]. There, the release of NETs provides a scaffold for platelet activation and deposition [64], thus promoting thrombus formation and growth through several mechanisms (**Figure 5**). For instance, NETs provide pro-coagulant substances like nucleic acids or polyphosphates, and Toll-like receptor (TLR) ligands that promote the activation and adhesion of platelets. NETs support the electrostatic interaction between platelets and histones that promote their adhesion, or the activation of Factor XII, leading to the intrinsic coagulation cascade. NETs also present tissue factor [65] and contain NE, which deactivates the tissue factor pathway inhibitor, thus promoting coagulation. Recently a mechanism of NET-driven thrombosis independent of platelets has been described [61] that is able to fully obstruct blood vessels and cause organ damage, indicating that intravascular neutrophil-derived products can cause vaso-occlusion.

Thrombi also promote NET formation, as neutrophils are efficiently recruited in a platelet-borne P-selectin-dependent manner [66]. Once recruited, the interaction of neutrophils with activated platelets drives NETosis [67], especially in the lower-shear region downstream of the thrombus [68]. NET production in the growing thrombus, in turn, promotes further immune recruitment and the activation of coagulation cascades as discussed above, ultimately leading to increased thrombus size. In a model of deep vein thrombosis, the NET-inhibitor Cl-amidine greatly reduced thrombus formation [69], further supporting a role for intravascular NETs in thrombosis and organ failure.

Intravascular NETs may also play a role in ischemic injury, as HIF-1α induced under low oxygen conditions promotes NET formation [70]. In atherosclerosis, cholesterol crystals induce NETs and contribute to arterial damage [71], and in the late-stages of atherothrombosis NET inhibition leads to a decline in arterial IFNα expression and a reduced number of intimal macrophages [72].

Furthermore, elevated levels of circulating DNA and chromatin correlate with the severity and pro-thrombotic state of coronary atherosclerosis [73].

Even though NETs form a growing and very active area of research, technical caveats are still hindering progress on intravascular NET biology: NETs are difficult to visualize *in vivo* and different laboratories use different definitions of NETs and different methods to visualize them, rendering some results difficult to compare. In addition, although inhibitors or NET-degrading compounds have been developed, these lack specificity and controversy remains as to the extent to which NETs contribute to pathophysiological processes. While more specific inhibitors are sorely needed, recent advances indicate that NETs are key players in several pathologic conditions, in particular as instigators of vascular inflammation [53].

Other neutrophil mediators

Although the main effector molecules produced by intravascular neutrophils during vascular inflammation have already being discussed, these cells can also release other toxic mediators. While most of the granule proteins exert their vascular-damaging effect through the association with NETs, there are reports showing that neutrophil granule proteins by themselves are able to seed the endothelium on atherosclerotic lesions. There, granule proteins are able to recruit other leukocytes (mostly monocytes) by increasing the expression of adhesion molecules and cytokines by endothelial cells, thus generating chemoattractants for circulating monocytes [74].

During degranulation, neutrophil proteases are also released together with other granule proteins. Neutrophil serine proteases have been shown to participate in the disruption of the endothelial cell structure, leading to increased endothelial permeability and subsequent tissue damage in a model of glomerulonephritis [75]. Interestingly, neutrophil proteases display a range of regulatory functions in inflammation, such as cytokine release, receptor activation and integrin activity modulation [76]. In a model of atherosclerosis, neutrophil proteases have been involved in the

regulation of endothelium-borne inflammatory signals. Neutrophil elastase was shown to be responsible for the processing of pro-IL-1β in endothelial cells independently from caspase-1 activity, and to mediated mature IL-1β secretion by the endothelium in the atherosclerotic plaque, which helped to sustain chronic inflammation [77].

Vascular inflammation and Disease

As discussed earlier, the uncontrolled activation of neutrophils within vessels and in the perivascular space can trigger irreversible damage to vessels and tissues. These processes are relevant in the progression of in a number of disorders, the most prominent of which are discussed below.

Ischemia-reperfusion injury

Vascular occlusion and reduced blood supply is the triggering event of major debilitating conditions, including myocardial infarction and stroke. During ischemia, several danger signals can induce neutrophil activation. In a model of microvascular inflammation, activated neutrophils increase the affinity of integrins for their ligands in an AKT2-dependent fashion, resulting in binding of GP-Ibα (platelet glycoprotein Ib, alpha chain) on the surface of platelets in cooperation with P-selectin/PSGL-1 interactions. This generates shear-resistant platelet-neutrophil aggregates that ultimately promote thrombotic events [78]. Upon reperfusion of occluded vessels, smaller thrombi are released and cause secondary microvascular obstructions that further extend the ischemic area. This process, known as no-reflow phenomenon, contributes to aggravating tissue damage [79].

The complement system appears to contribute to the development of I/R injury. Cleavage of C3, a hallmark of complement activation, was associated with leukocyte activation in a model of myocardial injury [80]. Different components of the complement system have been identified to

have variable roles and relevance in different I/R models, and pharmacological inhibitors have been developed in order to limit damage derived from direct lytic or pro-inflammatory capacities of complement fragments [81]. C3 activation on the surface of activated platelets was shown to allow platelet-neutrophil interactions *in vitro* [82], and also mediates neutrophil-erythrocyte aggregates *in vivo* via the integrin Mac-1, which is a receptor for C3 [17]. These C3-mediated interactions could potentially modulate intravascular neutrophil activity and promote tissue damage, although this possibility remains to be formally confirmed.

Leukocytosis is an independent risk factor associated with poor outcomes after acute myocardial infarction (AMI). Interestingly, far from being a passive actor, the infarcted tissue is also able to modulate circulating leukocyte numbers and tissue damage. Cardiac fibroblasts from infarcted areas secrete GM-CSF (granulocyte-macrophage colony-stimulating factor), which activates bone marrow myeloid progenitors, driving the production of new neutrophils and monocytes, which are then recruited to infarcted areas to further aggravate tissue damage [83]. In the context of AMI, interference with the intravascular activity of neutrophils has proven to be of potential therapeutic value. For example, genetic or pharmacological inhibition of β1-AR dampened intravascular neutrophil crawling and dramatically limited inflammation and myocardial damage [41]. Likewise, inhibition of neutrophil-platelet aggregation by inhibition of PSGL-1 [29] significantly reduced the infarcted areas [41], altogether suggesting that mechanisms that specifically drive intravascular activation of neutrophils aggravate tissue injury. The exact mechanisms by which inhibiting neutrophil activation confers protection during AMI, however, are still unclear. It is possible that neutrophils that have been “stunned” upon β1-AR inhibition additionally display reduced NETosis and clot-forming capabilities, and indeed NET burden in coronary arteries has been positively associated with infarct size in AMI patients [84], and PAD4-deficient mice are partially protected in experimental AMI [85]. Neutrophil recruitment and activity dynamics within infarcted vasculature is therefore one of the key steps controlling the outcome of

the affected myocardium and should be regarded as an attractive therapeutic target for patients undergoing AMI.

Brain injury is another consequence of vascular ischemia that is also dependent on early neutrophil recruitment and intravascular activation. Although it is clear that neutrophils are recruited to the ischemic sites during stroke, the kinetics of this recruitment remain controversial. Current consensus is that neutrophils appear during the first few minutes after the onset of local ischemia, although some reports suggested that their influx is preceded by the recruitment of monocytes [86]. During the first 24 hours, neutrophils actively contribute to neuronal death, and inhibition of their interaction with circulating platelets alleviates ischemic injury, suggesting that early intravascular activation of neutrophils elicits vascular damage and drives brain injury [29]. Once neutrophils are recruited to the ischemic area, they aggravate the thrombotic event by accumulating in the thrombus and forming NETs, which have been shown to be critical in maintaining thrombus architecture [87]. In later stages of inflammation, a subpopulation of neutrophils displaying features of M2-macrophages and dependent on PPARγ (peroxisome proliferator-activated receptor gamma) signaling (the so-called “N2” neutrophils) is recruited to the ischemic site. This population displays neuroprotective features, limits inflammation, and promotes resolution [88]. Alternative neutrophil subsets with iron-scavenging and neuroprotective properties during intracerebral hemorrhage have also been reported [89]. Thus, neutrophils perform dual roles in the progression of ischemic brain injury: during the early stages they drive inflammation, and at later stages they promote resolution. This duality suggests that targeting bulk neutrophil infiltration into the ischemic core may not be an optimal strategy towards the treatment of stroke. Developing new strategies that instead modulate neutrophil function or phenotype, or their early intravascular activation may provide a more effective therapeutic strategy.

Atherosclerosis

Atherosclerosis underlies the majority of thrombotic events that cause vascular occlusion and tissue infarction, including AMI and stroke. The formation of atherosclerotic lesions in large arteries is multifactorial and biologically complex, and is precipitated by exogenous factors including hyperlipidemia, elevated blood pressure and the presence of inflammatory cytokines that favor endothelial dysfunction and atheroma plaque development. In the initial phases, neutrophils are recruited to the dysfunctional endothelium, and the production of ROS, granule proteins and pro-inflammatory cytokines increases vascular permeability [2]. NETs have also been involved in the early stages of atherosclerosis. Cholesterol crystals and inflammatory cytokines have been shown to be sufficient to trigger NETosis, and some authors postulate that these represent a key mechanism by which neutrophils contribute to atherogenesis, in part because this allows localized deposition of granule proteins and enzymes over the affected vascular bed [90].

Once the initial lesion has been established, neutrophils further aggravate and perpetuate the disease. The number of local neutrophils directly correlates with lesion size, and neutropenic mice are protected from atherosclerosis [91]. Mechanistically, intravascular neutrophils and platelets cooperate to enhance the recruitment of circulating monocytes by seeding the vascular surface with different chemokines and granule proteins that act as chemoattractants [90, 92]. After recruitment, monocytes take up oxidized lipids and transform into foam cells that dominate the atherosclerotic core. Intravascular neutrophils additionally produce NETs that stimulate IL-1β production by lesional macrophages, and sustain chronic inflammation of the arterial wall [93].

In advanced stages of the disease, thrombotic events are caused by rupture of the fibrous cap. Although the role of neutrophils in plaque instability has yet to be firmly established, correlations have been drawn between the number of circulating neutrophils and the onset of ischemic events (e.g., AMI or stroke) [94]. Indeed, neutrophil activity likely underlies plaque rupture, since they accumulate in damaged areas prone to rupture during late stages of atherosclerosis, and

metalloproteinases and ROS production are associated with plaque instability and erosion of the endothelial and fibrous cap covering the lesion [95]. Neutrophils are thus important drivers of inflammation at all stages of atherosclerosis, from plaque development and chronification to rupture.

Sickle cell disease

A single point mutation in the hemoglobin beta-globin gene leads to aberrant polymerization of the deoxygenated mutant hemoglobin, and leads to the deformation and acquisition of the "sickle" red blood cell phenotype. Within the vasculature, these damaged erythrocytes are prone to directly adhere to the endothelium through VCAM-1, and to neutrophils through the β2-integrin Mac-1 [17]. Lysis of sickle erythrocytes in blood and physical damage to the endothelium incite a chronic inflammatory state, which may explain why leukocytes and platelets from sickle cell disease (SCD) patients display a constitutively activated phenotype. Furthermore, interactions of neutrophils with sickle erythrocytes and platelets, which are mediated by Mac-1 and P-selectin [17, 96], ultimately lead to the development of vaso-occlusive events and acute crises in SCD patients, which are the main cause of morbidity and mortality in these patients [97].

NETs have been demonstrated to have a critical role on development of vaso-occlusive crises in SCD. In a mouse model of SCD, iron-loaded heme groups generated by erythrocyte lysis were shown to induce NET formation in primed neutrophils within the pulmonary circulation, and were critical in the development of vaso-occlusive events [98]. Intravascular neutrophils activated by endothelial E-selectin additionally contribute to vascular occlusion by capturing sickle erythrocytes and platelets from the circulation [17]. GMI-1070, a sialyl—Lewis-x glycomimetic inhibitor of all selectins that is currently in a phase-II clinical trial, has been demonstrated to be effective in reverting acute crises in a mouse model of SCD by impairing neutrophil adhesion and interaction with erythrocytes, resulting in improved blood flow and extended survival [99]. These combined findings have identified neutrophils as central elements in the vascular complications

associated with SCD, and justify current efforts to target neutrophil activity to alleviate SCD symptoms. Because inhibition of neutrophil activity can impair recruitment and cause susceptibility to infections, SCD represents a prime example in which specifically targeting intravascular activation would be therapeutically optimal.

Deep Vein Thrombosis

Deep Vein Thrombosis is a major cause of cardiovascular disease that is usually initiated by turbulent blood flow and thrombotic occlusion in large veins of the body, resulting in blood stasis, edema and ischemia, and necrosis of the surrounding tissues. Thrombi can be released from the initial lesion and generate an associated pulmonary embolism [100]. After initial stasis or inflammation of the vasculature, leukocytes are recruited independently of pre-existing clots, and crawling neutrophils and monocytes activate the coagulation cascade [101]. Neutrophils are also primed to generate NETs due to a pro-inflammatory environment, and changes in flow and blood pressure [102], propagate thrombosis by activating factor XII [101] and recruit platelets that generate thrombi and occlusion of the vascular lumen. Thus, neutrophils can initiate and propagate thrombotic events. In this scenario, NET-degrading enzymes (DNAses) in plasma may be essential to confer protection from thrombosis, as in their absence life-threatening thrombi form rapidly [61].

Conclusion

Vascular damage is a common feature of several highly prevalent forms of cardiovascular disease. Upon an inflammatory or ischemic insult, a combination of mechanisms trigger the rapid recruitment of neutrophils to the vessel wall. In many instances, the recruited neutrophils are detrimental rather than protective as they release a toxic cargo that compromises vascular integrity or induces thrombosis. Essential to understanding these processes, which are

responsible for the death of millions of patients every year, is to understand that the damaging action of neutrophils occurs intravascularly, while they are in intimate contact with the endothelium. We propose that defining the signaling mechanisms that regulate intravascular crawling, degranulation and NETosis at these early stages of inflammation, and identifying the mediators that are distinct from those involved in the classic recruitment of neutrophils that combat infections, will be key to developing effective therapies for the treatment of vascular disease.

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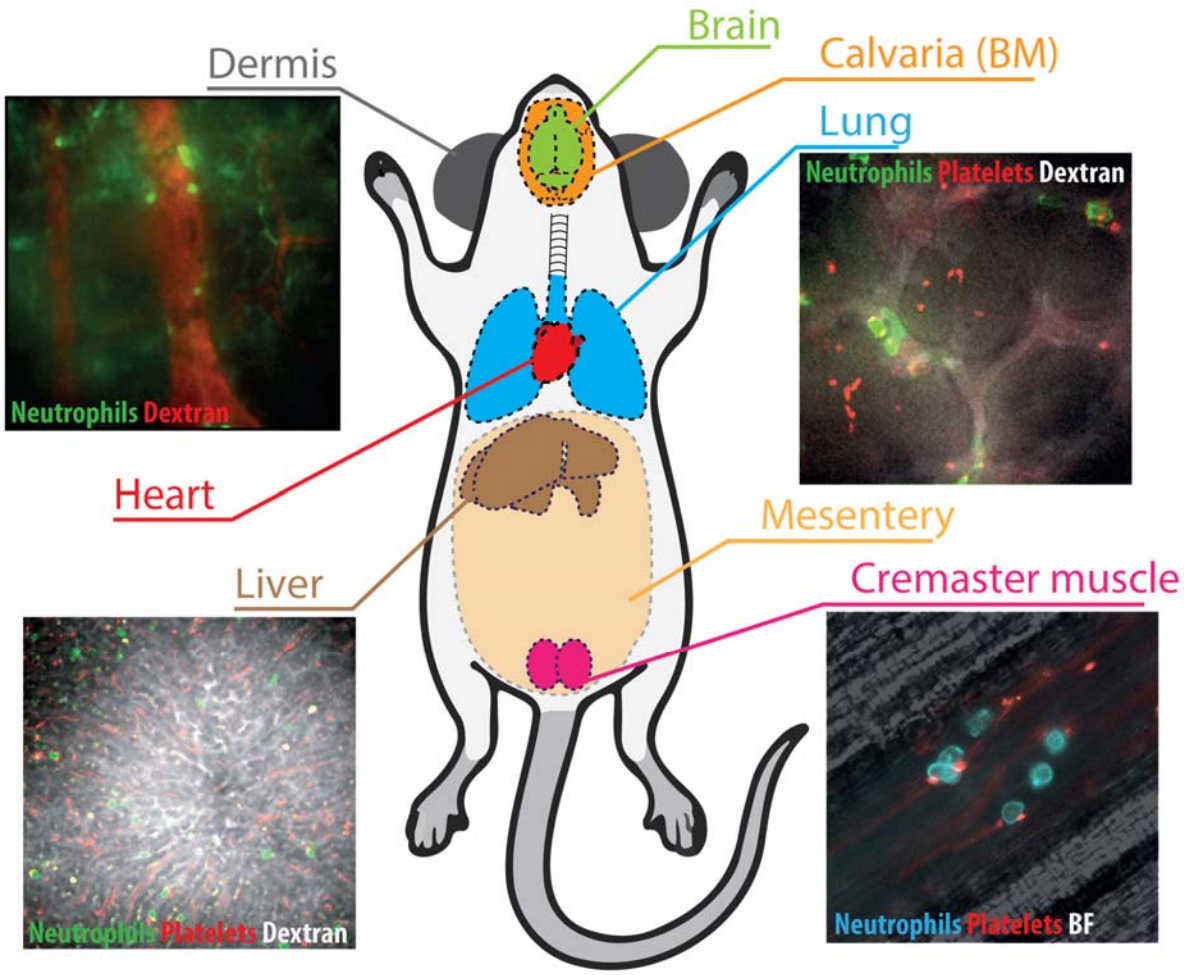

Figure 1: Intravital microscopy to study the intravascular behavior and dynamics of leukocytes. The development of intravital microscopy techniques has allowed the study of leukocyte dynamics in both homeostasis (e.g., dermis and calvaria) or during inflammation (e.g., cytokine-treated cremaster muscle or liver injury). Legends in each image indicate which cells or structures have been labeled. BF, brightfield.

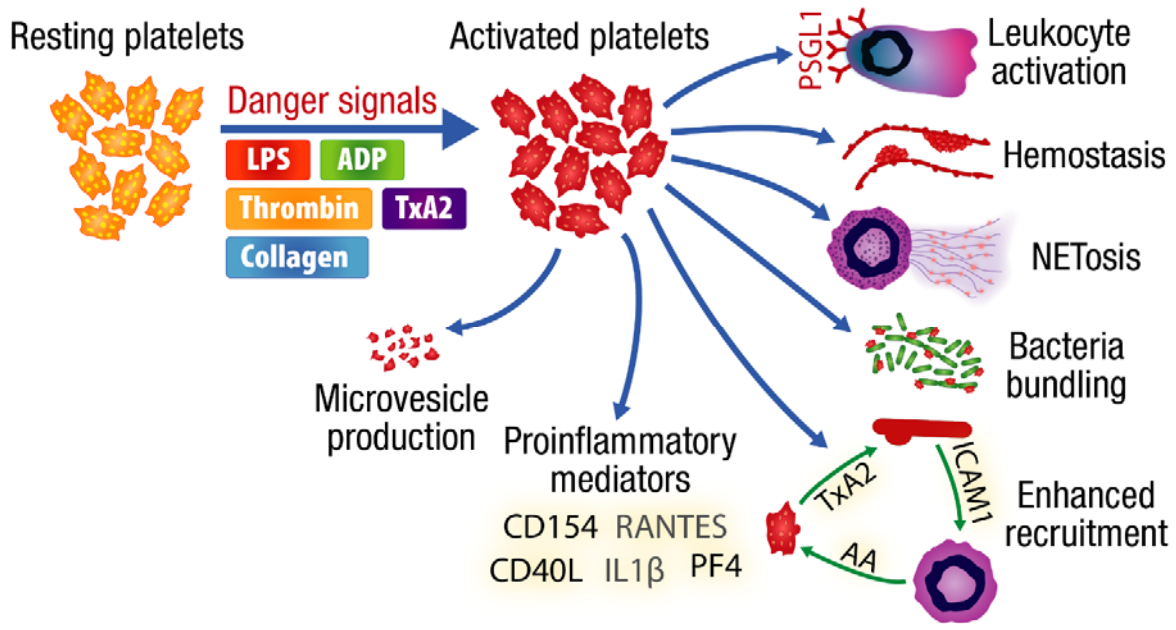

Figure 2: Platelet functions during vascular inflammation.
Platelets are endowed with a wide range of immune modulatory functions that affect vascular inflammation. Upon exposure to a series of danger signals, such as bacterial lipopolysaccharides (LPS), pro-inflammatory mediators like thromboxane A_2 (TxA2) or ADP, clotting signals (thrombin) and basal lamina collagen, activated platelets can produce soluble inflammatory mediators or directly interact with leukocytes (e.g., through PSGL-1 at the uropod of neutrophils) to control neutrophil activation. Platelets also participate in hemostasis and clot formation, trigger NETosis, bind and clear bacteria, and enhance neutrophil recruitment by secreting thromoboxane A_2 (TxA_2) generated from neutrophil-derived arachidonic acid (AA) that enhances endothelial ICAM-1 expression. Platelets are also capable of generating microvesicles that have additional functions.

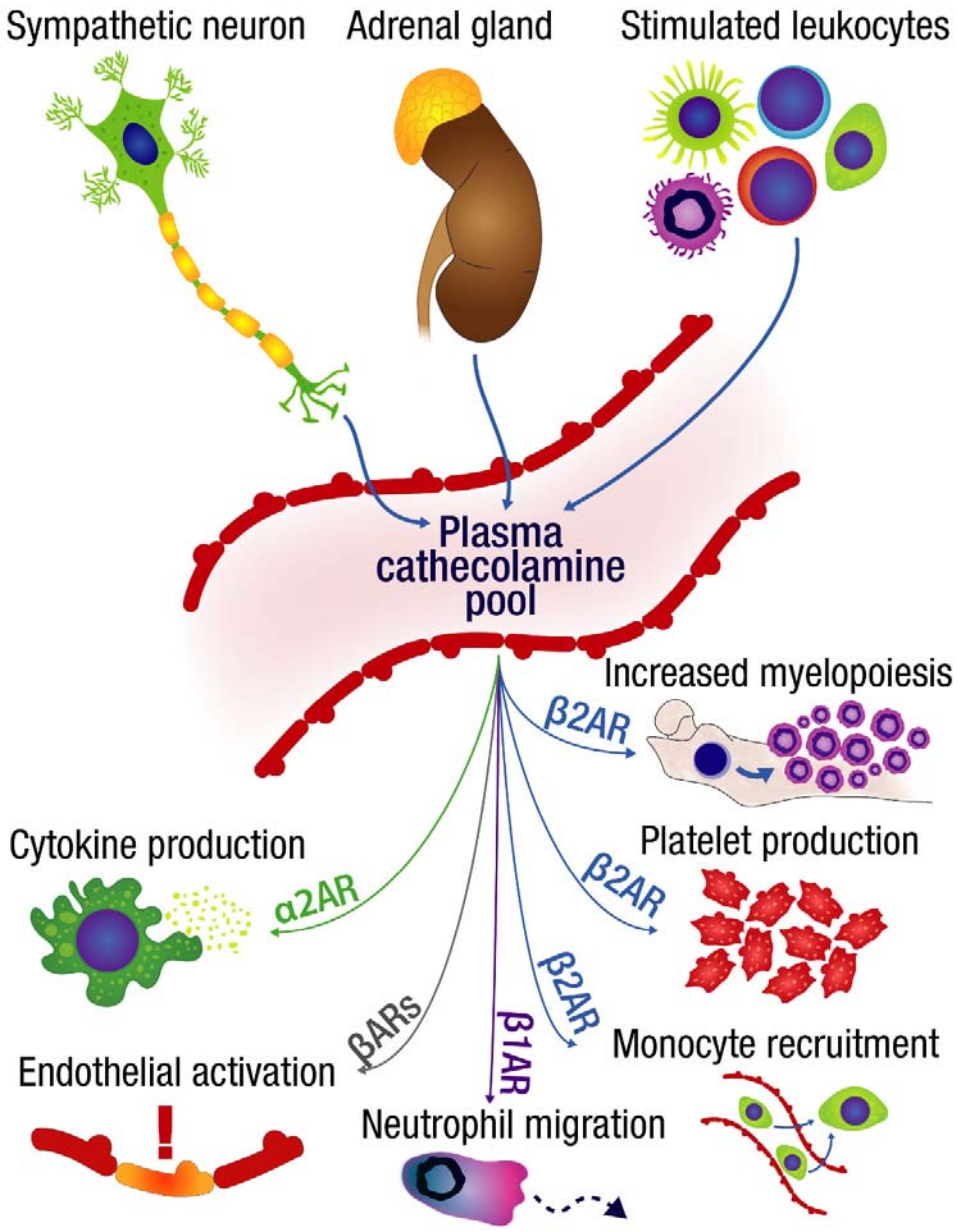

Figure 3: Adrenergic signaling in the regulation of vascular inflammation.
Catecholamine levels in plasma depend on the function of the sympathetic nervous system, adrenal glands and diffuse populations of leukocytes. Plasma catecholamines, through different α– and β-adrenergic receptors (AR), in turn regulate the generation of new leukocytes and platelets in the bone marrow, recruitment of leukocytes to sites of inflammation, control neutrophil intravascular migration, induce the expression of adhesion molecules by the endothelium (endothelial activation) and enhance cytokine production by leukocytes.

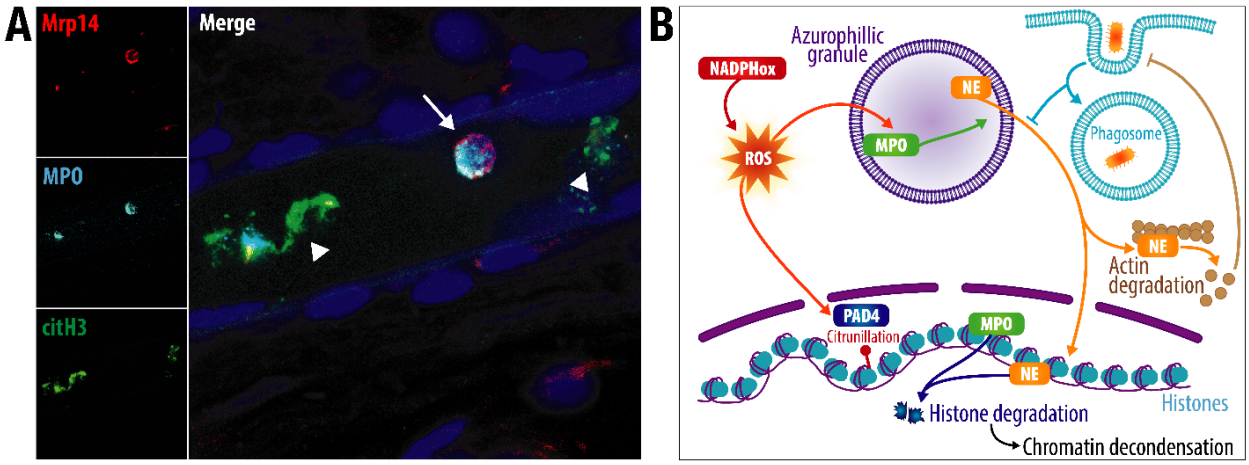

Figure 4: Intravascular NET formation.
A) Confocal microscopy image showing an intact MRP14+ intravascular neutrophil (arrow) and two intravascular NETs (arrowheads), detected by means of myeloperoxidase (MPO) and citrullinated histone 3 (citH3), two common markers used to visualize these structures. Note that Mrp14 is an intracellular protein, and is lost upon NET formation, while MPO is maintained attached to the NET. **B)** General scheme showing the pathway leading to NETosis. ROS production by the NADPH oxidase promotes histone citrullination and the release of neutrophil elastase (NE) to the cytoplasm, where it promotes actin degradation, and finally NE migration to the nucleus, where it promotes histone degradation and chromatin decondensation.

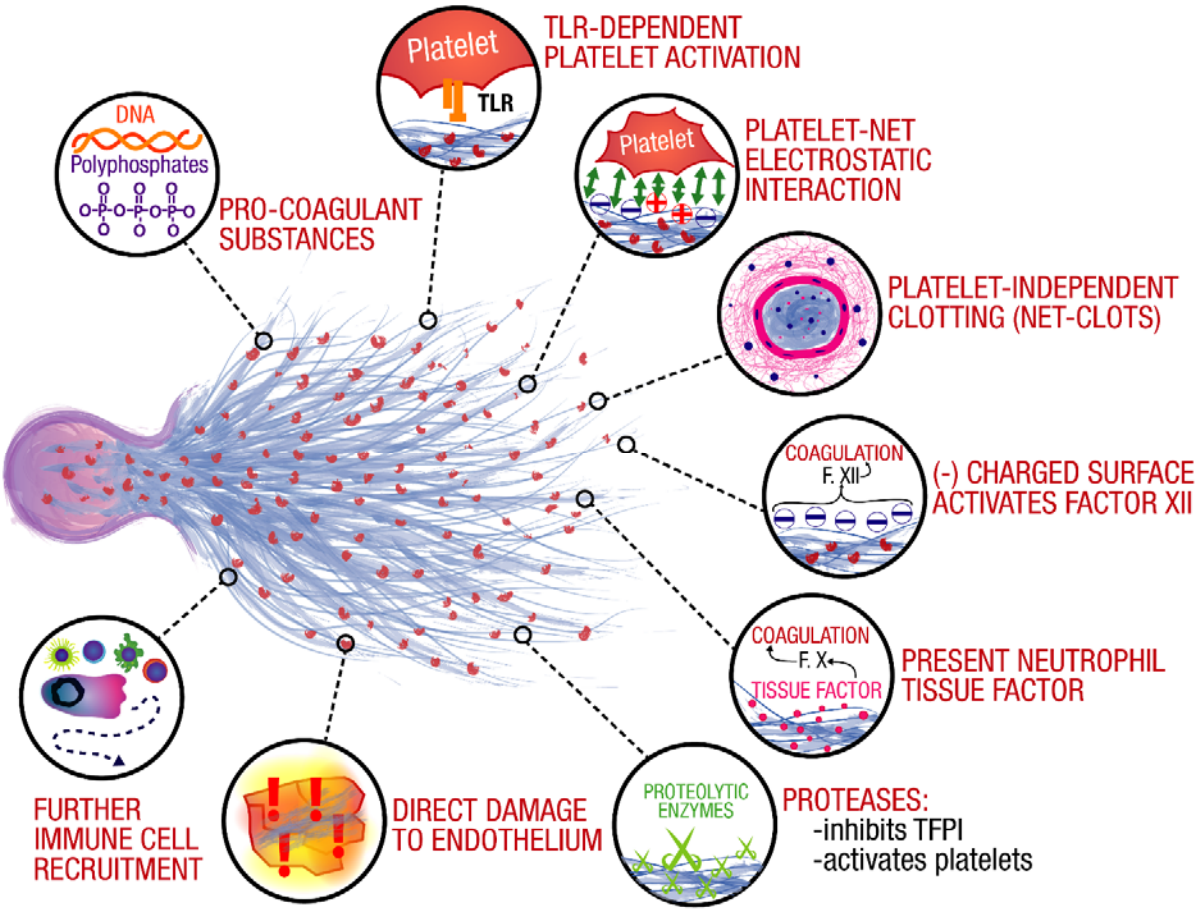

Figure 5: Mechanisms of NETs in vascular disease
Intravascular NETs exert a variety of effects related to immune cell recruitment, platelet adhesion and activation, direct damage to the endothelium, and thrombosis. TFPI: tissue factor pathway inhibitor. F: factor.