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# Platelets, NETs and cancer

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ABSTRACT

In addition to the central role of platelets in hemostasis, they contribute to pathological conditions such as inflammation and tumor progression. Aberrant expression and/or exposure of pro-coagulant factors in the tumor microenvironment induce platelet activation and subsequent release of growth factors from platelet granules. Cancer patients are commonly affected by thrombotic events, as a result of tumor-induced platelet activation. A novel player potentially contributing to cancer-associated thrombosis is the formation of neutrophil extracellular traps (NETs). NETs are composed of externalized DNA of nuclear or mitochondrial origin, bound to histones and granular proteases such as neutrophil elastase (NE) and myeloperoxidase (MPO). These extracellular traps help neutrophils to catch and kill pathogens such as bacteria, virus and fungi. It is now clear that NETs form also under conditions of sterile inflammation such as cancer and autoimmunity and can promote thrombosis. Recent data show that platelets play a key role in determining when and where NETs should form. This review will highlight our current insight in the role of platelets as regulators of NET formation, both during infection and sterile inflammation.

## 1. The procoagulant tumor microenvironment

Cancer patients frequently display an increased risk of thrombotic occlusion of vessels, as a result of tumor-induced activation of platelets and the coagulation system. This phenomenon is mainly due to elevated expression of prothrombotic factors, either directly by the tumor cells or by normal cells present in the tumor microenvironment [1–3]. In addition to their pro-thrombotic effect, activated platelets can also promote tumor progression in several ways [4,5]. Platelet alpha-granules carry large amounts of growth factors like PDGF, VEGF and TGFβ, which are released upon activation and contribute to wound healing and tissue regeneration at sites of tissue injury. However, when released in the tumor microenvironment, these growth factors can support processes like proliferation, angiogenesis and invasiveness, thereby fueling tumor progression. Furthermore, platelets have been shown to promote metastasis through distinct mechanisms [6]. Platelets can protect tumor cells from immune recognition and shear stress in the circulation and also facilitate tumor cell adherence and subsequent extravasation through the vessel wall [7]. Recently a novel mechanism was added to this list when it was demonstrated that platelet-derived TGFβ, in synergy with NF-κB-dependent signaling induced by direct contact between tumor cells and platelets, promote an epithelial-mesenchymal-like transition (EMT) of tumor cells [8]. EMT is a sign of invasiveness and believed to be a prerequisite for epithelial cells to metastasize into distant organs.

Intravascular clotting is prevented under physiological conditions when the vessel wall integrity is maintained. The leaky tumor vasculature, however, contributes to exposure of sub-endothelial platelet activators such as tissue factor (TF) and collagen, and stimulates platelet activation locally in the tumor. Cancer cells frequently produce and secrete pro-coagulant factors that stimulate platelet activation and thrombosis both in the tumor microenvironment and systemically. TF can either be expressed on the surface of tumor cells or secreted and has been associated with cancer progression and thrombosis. For example, the expression level of TF in the tumor tissue was found to correlate with poor prognosis in patients with pancreatic cancer [9,10]. Tumors can also shed TF-containing microparticles (MPs) [11]. Elevated numbers of these MPs has been associated with the prothrombotic state in cancer patients [12–16]. Besides TF, cancer procoagulant (CP) expressed by malignant cells can activate the extrinsic coagulation pathway independent of TF [17]. While CP has been detected in both solid and hematological tumors, no expression has been reported in

*Abbreviations:* NETs, neutrophil extracellular traps; VTE, venous thromboembolism; PE, pulmonary embolism; DIC, disseminated intravascular coagulation; DVT, deep vein thrombosis; TF, tissue factor; MP, microparticle; CP, cancer procoagulant; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; MPO, myeloperoxidase; NE, neutrophil elastase; LPS, lipopolysaccharides; TLR, Toll like receptor; poly (I:C), polyinosinic:polycytidylic; TRALI, transfusion-related acute lung injury; ADP, adenosine diphosphate; vWF, von Willebrand Factor; HMGB1, high-mobility group box 1; RAGE, Receptor for Advanced Glycation End products; PSGL-1, P-selectin glycoprotein ligand-1; ED-A, extra domain-A

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healthy tissues. Furthermore, a number of reports suggest a correlation between malignant progression and CP expression in various types of cancer [18,19]. Several additional factors in the tumor microenvironment contribute further to the pro-thrombotic state in cancer, such as heparanase, matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF) [3]. Heparanase promotes coagulation by enhancing TF activity [20], and MMP-2 can bind to the fibrinogen receptor on activated platelets and promote platelet aggregation [21,22]. VEGF, expressed at high levels in tumors, contributes to the leaky tumor vasculature, resulting in elevated exposure of pro-thrombotic factors as described above. Furthermore, VEGF functions as a chemo-attractant for innate immune cells such as neutrophils and monocytes, which promotes an inflammatory tumor microenvironment but also contributes to coagulation [23].

Hemostasis and inflammation were traditionally considered as separate processes, but recent data suggest that they are closely connected [24]. For example, neutrophils can contribute to immunothrombosis and thus have a significant impact on thrombotic disease. A few years ago, a novel player was implicated in these processes when neutrophil extracellular traps (NETs; described below) were found to activate platelets and promote deep vein thrombosis (DVT) in mice [25–27].

## 2. Neutrophil extracellular traps

The most abundant type of leukocyte in humans is the neutrophil, which constitutes 60–70% of the total pool. Neutrophils are an essential part of the innate immune system. They can protect the host from pathogens by phagocytosis of microbes and secretion of anti-microbial peptides. The identification of neutrophil extracellular traps (NETs) in 2004 [28] added a novel player to the neutrophil's defense strategies against microbes. NETs consist of externalized DNA (nuclear or mitochondrial), decorated by histones and granular proteases such as neutrophil elastase (NE) and myeloperoxidase (MPO) [29]. Formation of these extracellular traps, which creates a high local concentration of proteases, helps neutrophils to catch and kill pathogens such as bacteria, virus and fungi [30–34].

In addition to stimulation with microbes or substances like lipopolysaccharides (LPS), it is now clear that NETs form also under conditions of sterile inflammation such as thrombosis, cancer and autoimmunity [35–37]. The first description of tumor-induced NET formation was published in 2012 [38]. It was demonstrated that neutrophils from tumor-bearing mice were more prone to form NETs than neutrophils from healthy donors, and that NETs in tumor-bearing mice were associated with formation of thrombi in the lungs. Data from our own group show that NETs also accumulate in the peripheral circulation in mice with cancer, and cause systemic inflammation and significantly reduced vascular function in organs that are not sites for either primary or metastatic tumor growth [39]. In agreement, a recent study identified NET formation as a previously unknown cause of cancer-associated renal dysfunction [40].

An association between NETs and thrombosis was first demonstrated a few years ago when Fuchs et al. showed that neutrophil-derived extracellular DNA provides a scaffold for platelet activation [26]. Since then, NETs have been implicated in cancer-associated thrombosis and NETs were suggested as potential targets to prevent thrombosis in cancer patients [38]. The pro-thrombotic effect of NETs can be explained by their high content of nucleic acids and histones, rendering the NETs highly pro-coagulant and with a capacity to activate and aggregate platelets [26]. NETs have even been suggested to be indispensable during propagation of deep-vein thrombosis due to their binding and activation of factor XII, an inducer of the intrinsic coagulation pathway [27]. The connection between inflammation and thrombosis is increasingly recognized and it is possible that NETs provide this link.

Although NETs help to control an infection, formation of NETs also comes at a price since they can cause tissue damage [41]. Extracellular histones were shown to mediate tissue injury already in 1987 [42] and have since then been implicated as main inducers of NET cytotoxicity. Histones largely contribute to endothelial dysfunction as well as organ failure and mortality in septic mice [43]. *In vitro* data shows that histones have a direct damaging effect on endothelial cells. More recently, NETs were also confirmed to be cytotoxic not only for endothelial cells, but also for epithelial cells in the lungs [44]. The same study demonstrated that the cytotoxic effect of NETs on endothelial, as well as epithelial cells, is not solely mediated by histones but also by MPO, a protease secreted upon NET formation.

Formation of NETs through distinct mechanisms has been described. NETs can form both through a lytic, suicidal mechanism and through a mechanism that enables the neutrophil to survive and perform its other immune functions [45]. In addition, both nuclear and mitochondrial DNA have been identified in NETs [46–48]. A model that would incorporate both of these mechanisms is that the mitochondrial DNA is externalized first in a quick process, followed by a suicidal event where the neutrophil lyse and release its nuclear DNA content [49]. There is clearly room for more extensive research addressing the physiological stimuli and mechanism/s of NET formation *in vivo* and it will, without doubt, be an exciting field to follow.

This review will not scrutinize the different mechanisms of NET formation but will instead focus on the current knowledge regarding the contribution of platelets to this process, both during infection and in sterile inflammation.

## 3. Contribution of platelets to NET formation during infection and sterile inflammation

As described above, NETs constitute a scaffold for platelet activation and thrombus formation. At the same time, activated platelets can provide signals that promote formation of NETs, giving the impression of a self-sustaining process. A role for platelets in promoting NET formation was first described in 2007, when Clark et al. demonstrated that platelets work as sensors for the severity of an infection in mouse blood. LPS binds to Toll-like receptor 4 (TLR4) on the platelet membrane and triggers neutrophil activation and NET formation when the LPS concentration reaches sufficient levels [50]. This mechanism would be consistent with the idea of NET formation representing a last resort for the immune system to control an infection, since formation of NETs also induces tissue damage. A role for platelets in LPS-stimulated NET formation has also been demonstrated using human cells [51]. In addition to LPS, which represents stimulation by gram-negative bacteria, platelets were also found to promote NET formation induced by Pam3-cysteine-serine-lysine 4 (Pam3CSK4), a TLR1/2 agonist and mimetic of the main lipopeptide in gram-positive bacterial cell walls, using human cells *in vitro* [51]. In a similar way, platelets and neutrophils also interact to facilitate release of NETs in response to viruses. In a study using the TLR3 agonist polyinosinic:polycytidylic (poly (I:C)), as a double-stranded viral analog, or a live virus infection model in mice, platelets and neutrophils were found to interact within the liver vasculature and NETs formed. When mice deficient for the platelet surface molecule CD41 (integrin αIIb) were subjected to the same treatment, platelets were not recruited to the liver and NETs did not form [52].

In addition to situations with microbial stimuli interacting with TLRs, platelets have also been shown to promote formation of NETs in sterile inflammation. In a mouse model of transfusion-related acute lung injury (TRALI), the leading cause of death after transfusion therapy, activated platelets were found to induce NET formation [53]. In this model NETs were found to contribute to lung endothelial injury. Moreover, NETs were found in the lungs and plasma of human TRALI patients. Targeting platelet activation in the TRALI mouse model with either aspirin or tirofiban, an inhibitor of the platelet fibrinogen receptor, decreased NET formation and reduced lung injury. These data suggest that platelets activated by classical agonists such as collagen,