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Review article

The pathway of neutrophil extracellular traps towards atherosclerosis and thrombosis

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HIGHLIGHTS

- NETs exist in atherosclerotic lesions of both humans and animal models.
- NETs contribute to atherosclerosis, as well as to arterial and venous thrombosis.
- The proatherogenic and prothrombotic activities of NETs are expressed through various mechanisms.

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ABSTRACT

Neutrophil extracellular traps (NETs) are web-like extrusions of genetic material, which are released upon neutrophil activation. NETs consist of a chromatin substructure, onto which a vast array of proteins with various properties is dispersed. NETs production was initially described as an unrecognized defense mechanism of neutrophils, due to their ability to entrap and possibly eliminate a wide range of pathogens. Nevertheless, growing evidence suggests that NETs are implicated in a multitude of pathophysiological conditions, such as autoimmunity, cancer, diabetes mellitus and Alzheimer's disease. Importantly, NETs may also play a decisive role in atherosclerosis and thrombosis. In this context, it has been demonstrated that NETs are present in atherosclerotic lesions of both humans and animal models and are implicated in various mechanisms leading to atherogenesis. Among others, NETs induce oxidative stress and oxidize high-density lipoprotein particles, thus reducing their beneficial cholesterol efflux capacity. NETs also induce endothelial cell dysfunction and apoptosis and promote the generation of anti-double-stranded-DNA autoantibodies. NETs may also play a prothrombotic role, since they form a fibrin-like base for platelet adhesion, activation and aggregation. Furthermore, NETs promote the accumulation of prothrombotic molecules, like von Willebrand factor and fibrinogen, thus significantly contributing to thrombus formation. Notably, there is vast data linking NETs to arterial and venous thrombosis in animal models, as well as in humans. Future large-scale studies should incorporate NETs and their individual components as disease markers, as well as potential therapeutic targets, to reduce atherosclerosis and to prevent thrombosis.

1. Introduction

Neutrophil extracellular traps (NETs) are web-like extrusions of genetic material, which are released upon neutrophil activation [1]. The procedure of NETs formation is called NETosis. NETs consist of a chromatin substructure, onto which is dispersed a vast array of proteins with various properties. These proteins originate from all types of neutrophil granules (i.e. azurophilic, specific and tertiary), as well as the neutrophil cytoplasm and cytoskeleton [1–3]. However, NETs do not always represent a homogeneous material, concerning their protein content, a fact that may be reflective of different neutrophil subpopulations [4].

At first, NETosis was described as a previously unrecognized defense mechanism of neutrophils, due to the ability of NETs to entrap and possibly eliminate a wide range of pathogens, including Gram-positive and -negative bacteria, along with their virulence factors [1], fungi [5], protozoa [6] and viruses [7]. Nevertheless, ever-increasing evidence suggests the implication of NETs in a multitude of pathophysiological conditions, such as autoimmunity [8], cancer [9], diabetes mellitus [10] and Alzheimer's disease [11]. Additionally, the presence of NETs has been associated with endothelial and tissue impairment [12–14]. This effect can be attributed to the various proteases that NETs harbor,

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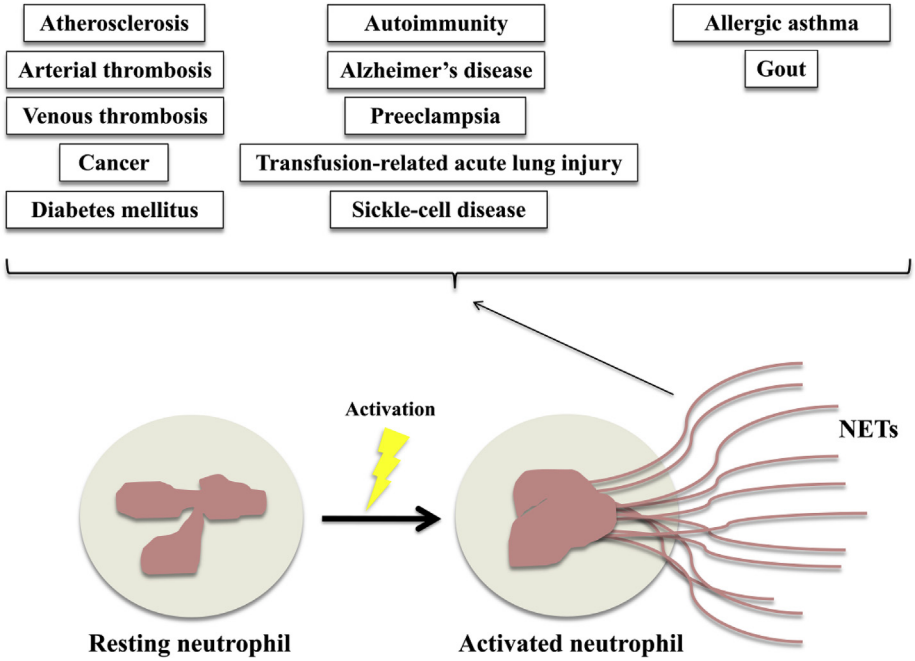

Fig. 1. The implication of NETs in numerous pathophysiological conditions.

as well as the cytotoxic activity of histones [14,15]. Importantly, NETs have also been involved in atherogenesis, as well as arterial and venous thrombosis [16,17]. In this context, it has been proposed that NETs and their components may actively participate in the pathophysiology of atherosclerosis and thrombosis and may also be used as biomarkers, having a prognostic value for the severity of atherosclerosis, cardiovascular risk, coronary infarct size, ischemic stroke, etc [18–20]. The association of NETs with numerous disease states is illustrated in Fig. 1.

The aim of the present review is to briefly describe the intracellular events leading to NETs generation and to provide a comprehensive and critical overview of the data that shapes current knowledge regarding NETs implication in atherosclerosis, as well as in arterial and venous thrombosis.

2. Intracellular events critical for NETs formation

NETosis is considered as a form of cell death mechanism, which is distinct from apoptosis, necrosis or necroptosis [21,22]. Nevertheless, it has been observed that NETosis is not always accompanied by cellular death, since non-lytic NETosis has also been described in response to *Staphylococcus aureus* [23,24].

Lytic NETosis requires delobulation of the neutrophil nucleus, decondensation of chromatin and disintegration of the nuclear and granular membranes [21]. These events are followed by blending of chromatin with the granular and cytoplasmic content. Subsequently, the neutrophil plasma membrane breaches and NETs emerge to the extracellular medium from the dying cell [21]. NETosis relies on the production of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. This is supported by the fact that neutrophils from individuals who suffer from chronic granulomatous disease and lack the NADPH oxidase activity, due to mutations in the enzyme gene, do not undergo NETosis [21]. However, upon neutrophil activation by stimuli that do not involve NADPH oxidase, such as immune complexes and ionomycin, neutrophils rely primarily on mitochondrial ROS to produce NETs [25,26].

Neutrophil elastase (NE) and myeloperoxidase (MPO), which also mediate the antimicrobial activity of NETs [1,27], play an important role in the process of NETosis itself [28–30]. Indeed, downstream of ROS, MPO, being a part of the protein complex termed azurosome, promotes the release of NE from the azurophilic granules [30]. Subsequently, NE translocates from these granules to the nucleus, where it partially degrades histones, thus contributing to chromatin decondensation and the subsequent formation of NETs [28,30]. Moreover, another event during NETosis is the deimination of selected arginine residues of histones, through the peptidyl arginine deiminase 4 (PAD4) activity, which yields citrulline [31,32]. Consequently, the chromatin of NETs contains citrullinated histones [31]. The citrullination of histones leads to loss of electrostatic interactions and hydrogen bonds between the histones and DNA, and consequently leads to chromatin decondensation and, thus, NETosis [31–34]. Histone citrullination via PAD4 is a Ca^{2+}-dependent process, since Ca^{2+} are critical for conformational changes that allow the proper positioning of the PAD4 active site and, thus, it requires an intracellular rise of Ca^{2+} levels, at least *in vitro* [33,34]. It has been reported that distinct protein kinase C (PKC) isoforms are responsible for opposing control of histone deimination via PAD4 and the subsequent NETosis, i.e. PKCα inhibits histone deimination, whereas PKCζ induces histone deimination and, consequently, NETosis [35]. The citrullination of histones takes place downstream of ROS [36].

An additional pivotal event required for NETosis is autophagy. Autophagy occurs independently from ROS production [37] and involves cessation of the mammalian target of rapamycin (mTOR) pathway [38]. More recent data reveal a crucial role for gasdermin D, a protein associated with the form of cell death termed pyroptosis, in NETs formation [39,40]. According to this data, gasdermin D is proteolytically activated by NE and, along with caspase-11, mediates lytic NETosis [39,40].

Overall, neutrophils recruit several intracellular cascades, in order to achieve NETosis, which are summarized in Fig. 2.

3. NETs in atherosclerosis and thrombosis

3.1. The role of NETs in atherosclerosis

NETs are present at sites of atherosclerotic lesions, as revealed by two-photon intravital microscopy and immunohistochemistry in atheromas of both humans and atherosclerotic mice [41]. Importantly,

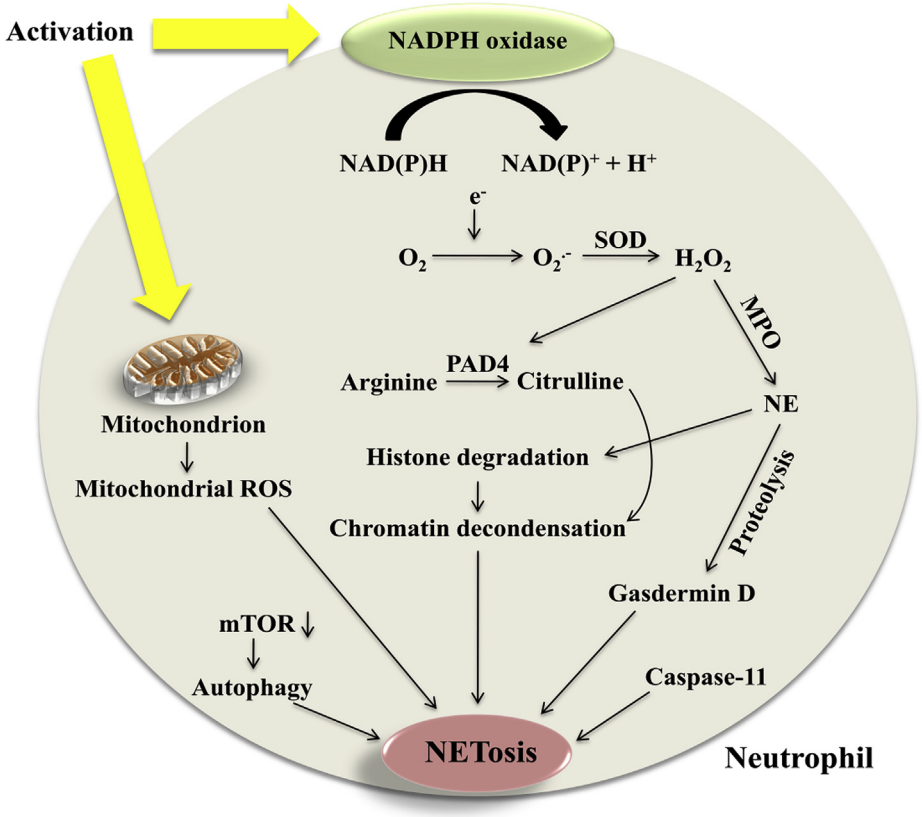

Fig. 2. Critical intracellular events for NETs formation.
Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are produced via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Downstream of ROS, peptidyl arginine deiminase 4 (PAD4) catalyzes the deimination of arginine residues of histones, yielding citrulline. The citrullination of histones leads to chromatin decondensation, a pivotal step for NETs formation. Additionally, downstream of ROS, myeloperoxidase (MPO) promotes neutrophil elastase (NE) release from the azurophilic granules. Subsequently, NE translocates from these granules to the nucleus, where it partially degrades histones, thus contributing to chromatin decondensation and the subsequent formation of NETs. Simultaneously, NE mediates the proteolytic activation of the pyroptosis-associated protein gasdermin D, which, along with caspase-11, also mediates NETosis. Neutrophil activation by stimuli that do not involve NADPH oxidase leads to NETosis via mitochondrial ROS. Independently from ROS production, autophagy also leads to NETosis. mTOR; mammalian target of rapamycin, SOD; superoxide dismutase.

NETs can be generated in the arteries through various mechanisms, in which a crucial role may play oxidized low-density lipoprotein (oxLDL) and cholesterol crystals. In addition, NETs are implicated in the pathophysiology of atherogenesis.

In this regard, oxLDL induces the generation of NETs in a concentration- and time-dependent manner, via the formation of ROS [42]. oxLDL-induced NETosis is probably mediated through its concurrent binding to neutrophil toll-like receptors (TLRs) −2 and −6 [42]. Furthermore, PKC, interleukin-1 receptor associated kinases, as well as extracellular signal-regulated kinase (ERK)1/2 and mitogen-activated protein kinase were identified as intracellular mediators of oxLDL-induced NETosis. Additionally, it was demonstrated that the individual components of oxLDL which are responsible for the production of NETs are oxidized phospholipids, such as lysophosphatidylcholine and oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine [42].

Another inducer of NETosis is cholesterol crystals, which are found in cholesterol-rich areas of atherosclerotic lesions of apolipoprotein E (ApoE)-deficient mice [43]. ApoE/NE/proteinase 3 (PR3)-deficient mice, which are deprived of the ability to make NETs due to deletion of NE and PR3 genes, have reduced atherosclerotic plaque size compared to ApoE-deficient control mice [43]. NETs generated by cholesterol crystal-activated neutrophils prime macrophages to synthesize the precursor molecule of the proinflammatory interleukin (IL)-1β. At the same time, cholesterol crystals are endocytosed by macrophages and promote the production of mature IL-1β, which in turn upregulates the production of another proinflammatory cytokine, IL-17, from T cells. IL-17 enhances the recruitment of immune cells at atherosclerotic sites [43]. Thus, the interrelationship between neutrophils and macrophages in a proatherogenic environment exacerbates atherosclerosis. Very recently, it was demonstrated that deletion of PAD4 (which, as mentioned above, is important for NETosis) in ApoE-deficient mice, was associated with the reduction of NETs formation and aortic inflammation, thus leading to the attenuation of atherogenesis [44].

Moreover, deficiency of ATP-binding cassette transporter A1/G1 in myeloid cells of mice, which normally mediates cholesterol efflux to high-density lipoprotein (HDL), leads to excessive cholesterol accumulation, NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome activation, and the subsequent recruitment of neutrophils in atherosclerotic plaques and NETs generation, probably during the early stages of atherogenesis [45]. Interestingly, it has been shown that secretory phospholipase A_2 ($sPLA_2$)-modified HDL inhibits neutrophil activation and the subsequent formation of NETs [46]. This NETosis-preventing effect may be due to the higher cholesterol efflux capacity of $sPLA_2$-modified HDL, which reduces the cholesterol content of the cell, as well as due to the suppression of intracellular Ca^{2+} rise [46]. However, another study showed that neutrophil cholesterol reduction leads to increased NETosis [47]. Consequently, the inhibition of NETosis by $sPLA_2$-modified HDL may be attributed only to the suppression of intracellular Ca^{2+} rise and not to the reduction of the neutrophil cholesterol levels. The contradictory results of these studies warrant further investigation, in order to elucidate the role of the neutrophil cholesterol content in the induction of NETosis.

NETs can also promote atherogenesis. In this regard, while oxLDL functions as an inducer of NETosis, components of the NETs, such as nitric oxide synthase, NADPH oxidase and MPO, oxidatively modify HDL, thus reducing its beneficial cholesterol efflux capacity [48].

Additionally, it has been reported that the NET-derived DNA, in combination with high levels of the antimicrobial peptide LL37 in atherosclerotic lesions, may trigger a pathway of plasmacytoid dendritic cells (pDCs)-driven autoimmunity, thus leading to the generation of anti-double-stranded-DNA antibodies, which are critical in early lesion development [49]. Furthermore, pDCs have been identified in both murine and human atherosclerotic plaques, whereas elevated levels of anti-double-stranded-DNA antibodies were detected in patients with symptomatic, but not in those with asymptomatic carotid artery stenosis [49].

A hallmark of the early stages of atherogenesis is endothelial dysfunction and injury. In this context, it has been demonstrated that

neutrophils and NETs accumulate preferentially in smooth muscle cell-rich atheromatous plaques, where they induce oxidative stress and endothelial cell apoptosis via a TLR2-dependent mechanism, rendering them more susceptible to superficial erosion [50]. PAD4 may be implicated in the thrombotic complications that take place in superficially eroded plaques [51]. According to these authors, NETs induce the apoptosis of human carotid artery endothelial cells, an effect which is enhanced in the presence of complement [51]. Furthermore, in the setting of superficial erosion, NETs are associated with endothelial cell activation via the concomitant action of IL-1α and cathepsin G [52]. In a recent work, it was shown that the reduction of NETosis was linked to decreased obesity-induced endothelial dysfunction in mice [53].

Another factor which links atherosclerosis and NETosis is advanced age. In this setting, it was demonstrated that atherosclerosis and NETs were promoted in aged mice [54]. Social factors also greatly contribute to the development of atherosclerosis. In this regard, it is of interest that a new study suggested that socially defeated mice exhibit higher levels of intraplaque NETosis, which leads to increased atherosclerotic lesion formation [55].

A very recent study has showed that, in atherosclerotic mice, lesional smooth muscle cells become activated and induce the migration of neutrophils, which in turn form NETs [56]. Subsequently, histone H4, which is contained within the NETs, impairs smooth muscle cells, resulting in atheromatous plaque instability [56]. The above data is further corroborated by the fact that treatment of mice with anti-histone H4 antibody inhibits smooth muscle cell death, leading to plaque stabilization [56].

The involvement of NETs in the pathogenesis of atherosclerosis has been translated to human subjects, since, as mentioned above, increased levels of NETosis markers are related to severe coronary atherosclerosis in patients [18]. In a small study, extracellular traps were detected within the lipid core of carotid arteries with advanced atherosclerotic lesions [57]. Moreover, neutrophils may produce NETs in response to periodontal bacteria within atherosclerotic carotid arteries with hemorrhage, which is a feature of plaque instability [58]. More recently, it was shown that NETs may offer a diagnostic potential in atherosclerosis [59]. In this regard, patients with rheumatoid arthritis, as well as healthy volunteers, were tested for markers of NETosis in relation to early atherosclerosis development. According to the study, cell-free nucleosomes, a NETosis marker, could identify rheumatoid arthritis patients versus healthy volunteers with a sensitivity of 84% and specificity of 86%. Furthermore, in these patients, cell-free nucleosomes could identify pathologic versus normal carotid artery intimae-media thickness, a marker of early atherosclerosis manifestation, with a sensitivity of 68% and specificity of 72%, demonstrating that the above marker could be a useful tool in detecting atherosclerosis development [59]. In a current study, autopsied plaques with complications, such as superficial erosion or hemorrhage, from patients who had acute coronary syndromes, exhibited significantly more NETs compared to autopsied plaques with no such features [60]. The specimens of complicated plaques had NETs in the thrombus and hemorrhagic area, as well as in the perivascular tissue [60].

Overall, the aforementioned data provides strong evidence that NETs actively participate in the development of atherosclerosis through various mechanisms, which are depicted in Fig. 3.

3.2. The role of NETs in thrombosis

Recent data has provided strong evidence that NETs are involved in arterial, as well as in venous thrombosis. The cells that are crucial participants in hemostasis and thrombosis are platelets. Accumulated data suggests that there is a complex interaction among platelets, neutrophils and NETs (Fig. 4).

In this regard, it has been demonstrated that NETs form a backbone not only for platelet adhesion, activation and aggregation, but for erythrocyte adhesion as well [61]. Furthermore, NETs components promote the gene expression of coagulation factors [62], whereas the NETs scaffold promotes the accumulation of prothrombotic molecules, like von Willebrand factor (vWF) and fibrinogen, the latter of which can then be converted to fibrin [61]. However, even without fibrin, the NETs scaffold supports clot formation [61]. The accumulation of prothrombotic factors, as well as platelet aggregation, are possibly attributed to the NET histones, since treatment of platelets with histones alone is sufficient to trigger aggregation [61]. However, newer evidence suggests that the NET DNA and histones are not required for NET-induced platelet activation [63]. Instead, according to these authors, platelets are rather activated by cathepsin G embedded on NETs [63]. Indeed, in a previous publication it was demonstrated that neutrophil-derived cathepsin G activates platelets via protease-activated receptor 4 [64]. NET-induced platelet activation seems to involve the adenosine diphosphate (ADP) receptor, $P2Y_{12}$, and the integrin-receptor $\alpha_{IIb}\beta_3$, as well as the Syk kinase [63]. Several lines of evidence suggest that NETs from septic patients or from patients who suffered a myocardial infarction (MI) bear tissue factor (TF) [65,66]. Once exposed in the bloodstream, TF can trigger the coagulation cascade and lead to the production of thrombin, thus activating platelets.

Interestingly, a recent article supported a regulatory role of NETs in the production and molecular integrity of thrombospondin-1 (TSP-1), a platelet- and endothelial cell-released protein which, among others, participates in hemostasis [67]. The neutrophil-derived proteases, NE and cathepsin G, induce the proteolysis of TSP-1 from a 185-kDa to a 160-kDa isoform, which exhibits better hemostatic properties than its precursor molecule [67]. NETs enhance the production of TSP-1 and prevent its complete degradation after exposure to excessive protease concentrations [67].

Except for the important role of NETs in platelet activation and thrombotic processes, it has been suggested that activated platelets trigger NETosis, thus implicating the platelet-neutrophils interaction not only in thrombosis, but also in inflammation and related disorders. Previously published results provided for the first time evidence that stimulated platelets induce NETosis in septic patients. According to these results, platelets become activated via TLR4 and subsequently bind to endothelium-adherent neutrophils, which in turn generate NETs [12]. This procedure takes place in the microvasculature of the liver and lungs, presumably its ultimate aim being to increase the effectiveness of bacterial entrapment [12]. Platelets are crucial components in the aforementioned mechanism, since, in their absence, NETosis does not take place [12]. In another study, activated platelets were shown to trigger NETosis in the setting of transfusion-related acute lung injury [68]. Hence, platelet activation seems to be important for NETosis in both non-sterile and sterile inflammatory conditions. Indeed, a more recent work supported that neutrophils may search for activated platelets in order to initiate inflammation [69].

Importantly, activated platelets, in response to various classic platelet agonists, such as collagen, ADP, thrombin and arachidonic acid, cause the production of NETs in a platelet number-dependent manner [70,71]. Both membrane-bound and soluble mediators are involved in platelet-mediated NETs formation. The classic platelet ligand that mediates the interaction between platelets and leukocytes is the adhesion molecule P-selectin, which binds to the leukocyte receptor P-selectin glycoprotein ligand-1 (PSGL-1). Although P-selectin seems to mediate NETosis in mice [69,72,73], some publications indicate that P-selectin does not mediate NETosis in human samples [70,71]. Platelet glycoprotein Ib (GPIb), of the complex GPIb/IX/V, neutrophil cluster of differentiation 18, as well as the release from platelets of vWF and platelet factor 4 were also found to mediate platelet-induced NETosis [71]. The signaling pathways that lead to platelet-mediated NETs formation seem to involve ERK, phosphatidylinositol 3-kinase and Src kinases, but not the generation of ROS via NADPH oxidase [71]. Platelet-derived high mobility group box 1 (HMGB1), a nuclear, non-histone protein which extracellularly functions as a damage-associated molecular pattern with proinflammatory properties [74], was found to

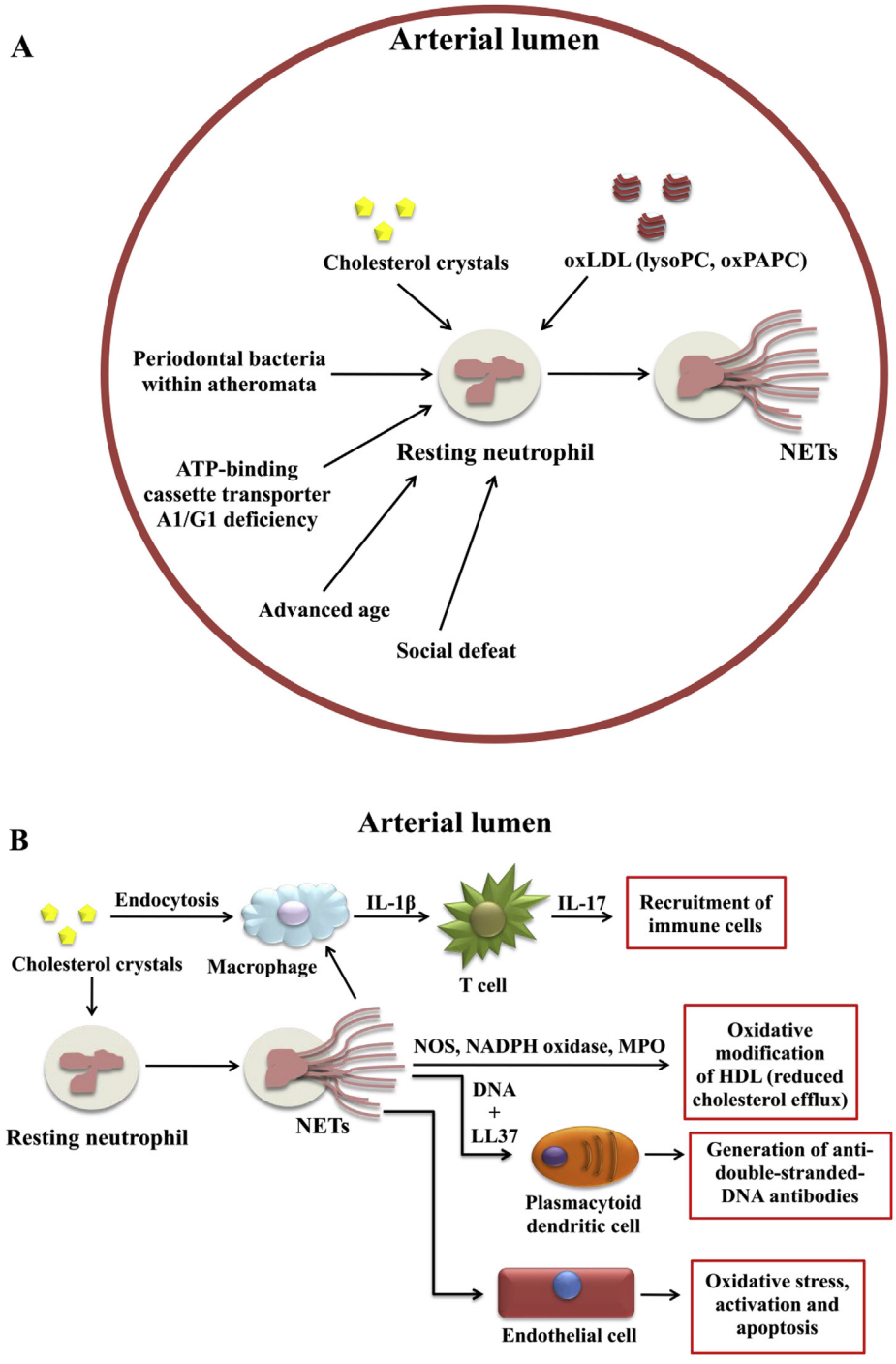

Fig. 3. NETs participation in the development of atherosclerosis.
(A) Triggers of NETosis within atheromatic arteries. (B) Molecular mechanisms associated with increased NETs production, leading to atherogenesis. Cholesterol crystal-induced NETs, as well as cholesterol crystals themselves, contribute to the production of interleukin (IL)-1β, which in turn upregulates the production of IL-17 from T cells. Subsequently, IL-17 promotes the recruitment of immune cells at atherosclerotic sites, exacerbating atherogenesis. Moreover, nitric oxide synthase (NOS), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and myeloperoxidase (MPO), embedded on NETs, oxidatively modify high-density lipoprotein (HDL), reducing its cholesterol efflux effect. Furthermore, the NETs DNA, in combination with the antimicrobial peptide LL37, induce the production of anti-double-stranded-DNA antibodies, a process that is driven by plasmacytoid dendritic cells. Finally, NETs induce oxidative stress in endothelial cells and are implicated in their activation and apoptosis. lysoPC; lysophosphatidylcholine, oxLDL; oxidatively modified low-density lipoprotein, oxPAPC; oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine.

induce NETosis via autophagy [70]. HMGB1 binds to the receptor for advanced glycation end products and/or TLR4 [70,75,76]. Very recently, platelet-derived microparticles (PMPs) were shown to induce NETosis in both control subjects and patients suffering from systemic sclerosis [77,78]. The PMPs from the latter may also express HMGB1 [78]. Some authors identified platelet-derived, inorganic polyphosphate as a novel mediator of platelet-dependent NETosis, in thrombin-activated platelets from patients with a ST-segment MI [79]. Polyphosphate-mediated NETosis requires mTOR inhibition, in addition to autophagy induction [79]. Polyphosphate-mediated NETosis is inhibited by IL-29, which also suppresses neutrophil migration [79].

Consequently, platelets, neutrophils and NETs are involved in a perpetual cycle, in which activated platelets stimulate neutrophils to produce NETs, which in turn activate more platelets, either via the direct contact of platelets with NETs, or via the production of thrombin [66].

NETs are involved in both arterial and venous thrombosis. Regarding arterial thrombosis, several publications have demonstrated that NETs are present in the culprit artery of MI patients and that the constituents of coronary thrombi from such patients are predominantly activated platelets, neutrophils, as well as NETs in close contact with platelets [66,70]. NETs were identified in both fresh and lytic, but not in organized coronary thrombi [60,80]. Recently, it was suggested that extracellular traps derived from many leukocyte types (macrophages, mast cells, eosinophils and neutrophils) are all found in the various stages of coronary atherothrombosis progression, with NETs and macrophage extracellular traps being more prominent than extracellular traps from mast cells and eosinophils [81]. Moreover, in a large,

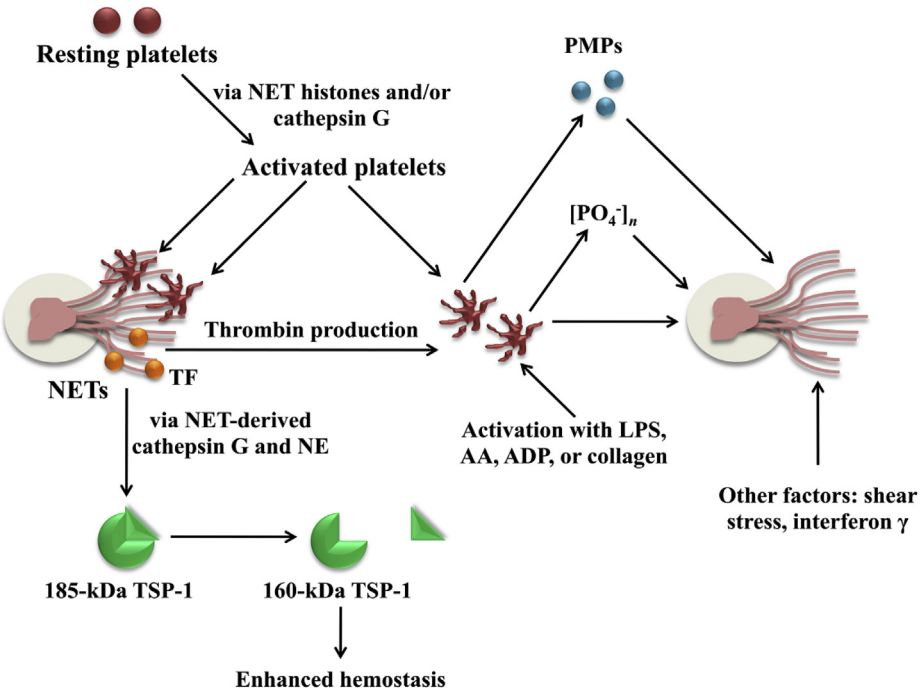

Fig. 4. The interaction between platelets, neutrophils and NETs involves a complex cycle of continuous interactivation.
NETs can activate platelets via two pathways, i.e. through the NET histones and/or cathepsin G, which is embedded on the NETs scaffold, or through thrombin produced by NET-derived tissue factor (TF). Meanwhile, cathepsin G and neutrophil elastase (NE), embedded on the NETs scaffold, induce the proteolysis of thrombospondin-1 (TSP-1), thus enhancing its hemostatic properties. Conversely, platelets activated by lipopolysaccharide (LPS), or the physiological agonists arachidonic acid (AA), adenosine diphosphate (ADP) or collagen, induce NETosis. Platelet microparticles (PMPs) or inorganic polyphosphate derived from activated platelets have also been shown to trigger NETosis. Other factors that induce NETosis include physical stimulation of neutrophils by shear stress, as well as interferon γ produced by natural killer cells.

multicenter study, in which the composition of thrombi from early and late stent thrombosis was examined, neutrophils were the main leukocyte component and NETs were found in about one quarter of all thrombus specimens [82]. Very recently, the presence of NETs has been implicated in the pathogenesis of aortic stenosis [83]. Notably, levels of NETosis markers, such as citrullinated histone H3, MPO and NE, were significantly higher within the aortic valves, as well as in the plasma of patients suffering from aortic stenosis, compared with control subjects [83]. Furthermore, the above markers were correlated with parameters, such as aortic valve area and mean transvalvular gradient [83]. In a very recent paper it was suggested that NETosis can be generated by shear stress, at levels found in the arteries, but not in veins [84]. According to the authors, neutrophils entrapped within thrombotic occlusions can be physically stimulated by shear stress, in the absence of neutrophil- and platelet-derived agonists and mediators [84]. Further work by the same group demonstrated that NETosis induced by shear stress is abolished in the presence of fibrin, whereas, conversely, the absence of fibrin promotes NETosis [85].

The adhesion of erythrocytes to NETs results in a red thrombus, similar in appearance to venous thrombi [61]. Interestingly, there is vast data linking NETs with venous thrombosis in animal models [61,86–89], as well as in human subjects [90,91]. vWF, TF and platelets, via GPIb, are also implicated in NET-mediated venous thrombosis [86,87]. Moreover, data suggests that synergistic signaling through PSGL-1 and the chemokine receptor CXCR2 in neutrophils results in the formation of NETs, an event that promotes deep vein thrombosis in mice [92]. Similarly to coronary thrombi, NETs are present in organizing, but not in organized venous thrombi [91]. PAD4, but not NE, seems to be critical for NETosis in mouse models of deep vein thrombosis [88,89]. Of importance, platelet-derived delivery of HMGB1, and the subsequent generation of NETs, has been implicated in venous thrombosis in mouse models [93,94]. The presence of TF in NETs, which was mentioned above (References [65,66]), could contribute to the pathogenesis of NET-inflicted venous thrombosis by triggering the coagulation cascade. Some authors proposed that platelet-triggered neutrophil necroptosis also contributes to venous thrombosis [95]. Furthermore, there is evidence proposing that interferon γ produced by natural killer cells triggers NETosis in a mouse model of venous thrombosis [96]. NETs also seem to participate in venous thrombosis development, in heparin-induced thrombocytopenia (HIT) inflicted in mice [97]. During HIT induction, neutrophils bind to venous endothelium and incorporate into the developing thrombi. NETs generated by these neutrophils form complexes with platelet factor 4 and HIT antibodies, becoming resistant to resolution and, thus, contributing to thrombus propagation [97]. Inhibition of NETosis via PAD4 abolishment reduces thrombosis [97]. Finally, it has been demonstrated that NETs are associated with a hypercoagulable state and venous thrombosis in the setting of cancer [98–100]. More specifically, neutrophils from patients who suffer from some types of cancer, such as oral squamous cell carcinoma and pancreatic cancer, are more prone to NETosis compared to control subjects [98–100]. These NETs lead to increased production of thrombin and fibrin compared to controls [98–100]. Conversely, depletion of neutrophils, as well as disruption of the NETs structure, inhibits the aforementioned procoagulant activity [98,99].

The compelling evidence provided above, establish firmly a crucial role for NETs in the multiple facets of thrombosis, which is mostly attributed to the interaction of neutrophils and NETs with platelets.

4. Conclusions

The discovery of NETs has opened new horizons in the understanding of neutrophil biology and the role of these cells in immunity and disease. The use of chromatin, in combination with intracellular proteins, as an effective antimicrobial weapon has ancient roots [101] and annuls the function of chromatin solely as the carrier of genetic information. Interestingly, in addition to packaging DNA into nucleosomes, in the nucleus of eukaryotic cells, histones seem to bear antibacterial [102] and cytotoxic properties [14], whereas they may induce platelet aggregation [61].

Of note, since their first description, vast bibliography links NETosis to atherosclerosis, as well as to arterial and venous thrombosis. The presence of NETs in these conditions gives rise to the opportunity for NETs and their individual components to be used as potential biomarkers for these diseases, by their qualitative and quantitative determination [18–20].

NETs should also be viewed as attractive therapeutic targets. For example, in addition to their hypolipidemic effect, statins could also be

used to prevent NETosis [103]. Since platelet activation is indispensable for platelet-mediated NETosis, antiplatelet therapy, such as aspirin [68], could also prevent indirectly the formation of NETs. Interestingly, the anticoagulant heparin dismantles the NETs scaffold as effectively as deoxyribonuclease [61]. It is worth mentioning that the phosphodiesterase 4 inhibitor, roflumilast, abrogates the interaction of neutrophils with activated platelets and endothelial cells, as well as NETosis [104].

Future large-scale studies should take into account the importance of NETs in the pathogenesis of arterial and venous thrombosis and, hence, incorporate NETs as disease markers and investigate the effect of hypolipidemic and antithrombotic drugs on the reduction of NETosis.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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