Traps N' Clots: NET-Mediated Thrombosis and Related Diseases

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Insights into Neutrophils and NETs: A Historical Perspective

Vessel wall injury and subsequent blood extravasation activates a series of local biological processes to prevent excess blood loss via the formation of hemostatic plug strictly restricted at the site of vascular injury with minimal or no extension in the vessel lumen.[1] In the vast majority of cases, a catastrophic systemic activation of these processes is contained by specific mechanisms. As opposed to hemostasis, thrombosis is characterized by the deregulated clot formation, various degrees of vessel occlusion, tissue ischemia, and necrosis.[1]

A large body of accumulating experimental and clinical data over the past 20 years has clearly indicated the reciprocal relationship and dynamic interplay between inflammation and thrombosis.[2–5] Today, targeting inflammation to prevent thrombotic events represents a realistic and promising therapeutic approach.[6] Among immune cell subsets that are implicated in multiple molecular pathways during inflammatory response, neutrophils have a crucial role, recruited first to the site of injury following instructive signals from the tissue environment.[7]

The very first observation linking neutrophils with thromboinflammation was reported almost 70 years ago describing granulocytes as a main component of clotted blood in patients suffering from active lupus erythematosus.[8] During the following years, although several studies had described the accumulation of neutrophils at the site of thrombus formation, these cells remained neglected and less studied in many thrombotic diseases. The traditional aspect of neutrophils as dispensable, passive bystanders was dramatically revised after the milestone discovery that they represent a primary source of blood-borne tissue factor (TF), the main in vivo initiator of the extrinsic coagulation cascade, resulting in thrombin generation and ensuing thrombus formation.[9,10] Later on, several studies from our laboratory and others provided evidence for the critical role of neutrophils in thrombosis and inflammation-mediated thrombotic complications.[3,11,12] Intravital microscopy studies in mouse models of venous and arterial thrombosis demonstrated neutrophil recruitment and activation at the site of endothelial damage in the early phase of thrombosis.[13,14] Of note, neutrophils are not only implicated in thrombotic processes, but also seem to be indispensable for thrombosis. Neutropenia induced in vivo by anti-Ly6G or GR-1 antibody abrogated venous[13] and arterial[14] thrombosis, respectively. When purified neutrophils from wild-type mice were injected into transgenic mice that express no mouse TF and only minimal ($< 1\%$) amounts of human TF (low TF mice), the defective fibrin generation in these animals was restored, indicating that TF expressing neutrophils represent the main source of TF during thrombus formation.[14] Similarly, when normal TF expressing mice were transplanted with low-human TF bone marrow cells they did not develop deep vein thrombosis (DVT).[13] However, the mechanisms underlying the activation and delivery of active TF by neutrophils remained unknown.

During the last years, advances in molecular biology provided the most exciting update of neutrophil physiology, in particular their capacity to release neutrophil extracellular traps (NETs). NETs are extracellular web-like structures of chromatin fibers lined with various highly active proteases

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and proteins of nuclear, granular, and cytosolic origin.[15] The release of NETs from activated neutrophils was initially described in 2004 as a novel defense mechanism able to "trap and kill" a wide range of pathogens.[16] However, increasing evidence during the past few years highlighted their fundamental role in the pathogenesis of numerous noninfectious inflammatory disorders, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), autoimmune vasculitis, gout, ulcerative colitis, interleukin (IL)-1β-mediated autoinflammatory syndromes, and thrombosis.[15,17–19]

Activated platelets are able to induce robust NET release within vasculature providing a scaffold for fibrin deposition and stabilization of thrombus.[20–23] Notably, these NETs are decorated with functionally active TF, which explains its extracellular delivery at the site of tissue injury.[13,19,24] Besides TF, NETs were found to deliver several proteins and clot factors involved in thrombosis such as von Willebrand factor (vWF), XII, fibrinogen, and fibronectin.[2,13,25] The thrombogenic potential of NETs was further supported by experimental studies indicating that extracellular histones induce endothelial activation, platelet activation/aggregation, and thrombin generation[26,27] (►**Fig. 1**). Phylogenetically, the capacity of NETs to activate coagulation serves to trap and eliminate pathogens, resembling primitive defense systems operating several millions years ago, and it is conserved today in insects.[28] In these organisms, coagulation and immunity use common mechanisms to prevent fluid loss and pathogen invasion. These systems are tightly interrelated, since NET-associated antimicrobial proteases are able to trigger several coagulation pathways,[2] while activation of the coagulation system supports several immune responses such as bacterial compartmentalization, immobilization, and elimination especially in microvasculature (immunothrombosis). Probably, much of the NET-mediated antimicrobial effect is due to entrapment, rather than direct killing[29,30] (►**Fig. 2**).

Apart from neutrophils, extracellular traps (ETs) formation has also been described in other types of granulocytes, such as eosinophils and mast cells.[31,32] Very recently data implicate macrophages, mast cells, and eosinophils through ETs generation in atherosclerotic plaque formation and thrombosis.[33,34] However, ETs formation in macrophages is controversial and remains unclear whether it is distinct from pyroptosis.[35]

In view of the above, NETs could be perceived as a double-edged sword during disease processes. They may be beneficial by enhancing the antimicrobial potential in numerous infectious diseases or contributing to normal hemostasis and pathogen curbing in neutrophil clots lattice, but also harmful by amplifying systemic or local inflammation leading to tissue damage and thrombosis[15,36] (►**Fig. 2**). Therefore, NETs and their components emerge today as novel candidate for diagnostic and therapeutic targets of thrombosis in many clinical settings.[12,15,37,38]

NET Formation and Regulation

The exact molecular mechanisms that drive NET release are not clearly defined and still being characterized.[35] Several lines of evidence indicated that NET formation is regulated via multiple, probably interdependent, pathways that can be triggered in vivo by several microbial and noninfectious stimuli, such as cytokines, chemokines, immune complexes, crystals, and inorganic polyphosphate (polyP) or high mobility group box 1 (HMGB1) expressed from activated platelets.[22,24,35,39–41] Stimuli of NET formation may act also synergistically. Thus, it has been suggested, that platelet-induced NET formation requires synchronous neutrophil stimulation by platelet-derived chemokines CXCL4 and CCL5, and Mac-1 integrin.[23] On the other hand, excessive and disproportionate NET formation that leads to tissue damage is homoeostatically regulated by anti-inflammatory mechanisms such as NET degradation by DNase1 and DNase1-like 3, and phagocytic removal of NET remnants by macrophages.[18,42] Interestingly, recent data suggested that an anti-inflammatory action of NETs themselves also exists mainly through the proteolytic modulation of cytokine and chemokine activity by the proteases that decorate aggregated NETs.[43–45]

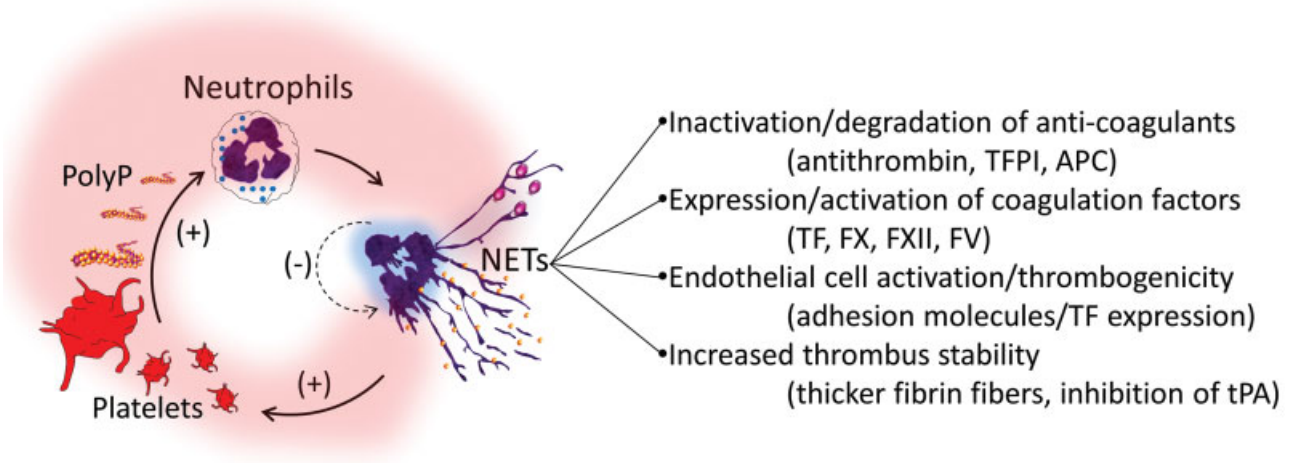

Fig. 1 Mechanisms of neutrophil extracellular trap (NET) thrombogenicity. Left: Platelets-PolyP-neutrophils-NETs interactivation leads to NET generation. (–) denotes NET autoregulation. Right: NETs can deliver thrombogenic signals through many different mechanisms presented here. APC, activated protein C; FX, FXII, FV, coagulation factors; PolyP: polyphosphate; TF, tissue factor; TFPI, TF pathway inhibitor; tPA, tissue plasminogen activator.

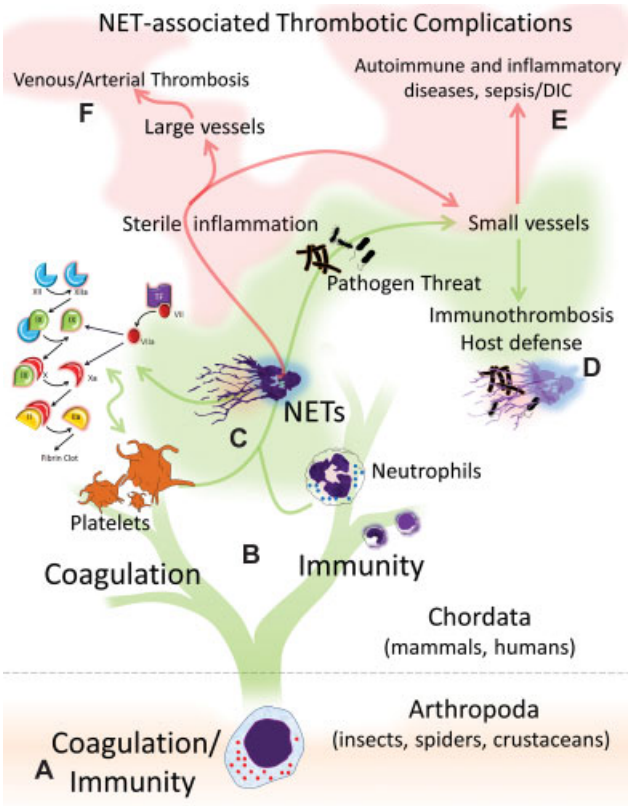

Fig. 2 Skewing of immunothrombosis (tissue protection) to pathological thrombosis (tissue damage). In primitive organisms (bottom, **A**) immunity and coagulation use a common, hemocyte-based system to prevent fluid loss and pathogen invasion. In mammals including humans (**B**) hemostasis and immunity are operated by distinct systems (platelets and coagulation, left; and white blood cells such as neutrophils, right, respectively). Interaction between platelets/coagulation and neutrophils leads to neutrophil extracellular trap (NET) formation (**C**). In cases of a pathogen invasion, NETs are involved in pathogen entrapment and elimination in microcirculation (green path, host defense, **D**). On the contrary, inappropriate NET formation in cases of sterile inflammation (autoimmune or inflammatory environment) leads to thrombotic complications (red path, tissue damage) in microcirculation (**E**) or large vessels (**F**). DIC, disseminated intravascular coagulation.

Recent clinical and experimental studies suggested that, in the context of different diseases, neutrophils are able to release NETs that are qualitatively different and coated with disease-specific bioactive proteins determined by the disease inflammatory environment such as TF, IL-1β, IL-17, and LL-37.[17,19,46–52] It has been suggested that the systemic inflammatory environment of each disease leads to transcriptional reprogramming in circulating neutrophils inducing the de novo expression of disease-related proteins (first-hit), and an additional trigger (second-hit) enables NET formation leading to the extracellular delivery of these bioactive proteins via NETs.[19,49]

NETs and Local Thrombotic Manifestations

Experimental Arterial Thrombosis

In mice, following endothelial injury and activation, neutrophils are recruited first and promote thrombosis by forming NETs and by delivering active TF and neutrophil proteases such as elastase (NE) and cathepsin G (CG).[2,14,53] In mice lacking NE and CG ($Elane^{-/-}$; $Ctsg^{-/-}$), $FeCl_3$ carotid injury resulted in lower fibrin formation, smaller and unstable thrombi leading to rapid recanalization, possibly due to defective TF pathway inhibitor degradation from NET-bound NE and CG.[2] Extracellular histones, an essential NET component, also aggravated ischemic injury in a mouse model of transient occlusion and reperfusion of middle cerebral artery when injected during the reperfusion phase, resulting in larger infarcts and worse functional outcomes. Significant attenuation of these effects was observed with administration of histone-neutralizing antibody or recombinant human DNase1.[54] NETs also operate in myocardial reperfusion injury. In a left anterior descending artery ligation and reperfusion model in rat, treatment with DNase1 and recombinant tissue plasminogen activator (tPA) reduced ischemic injury, no reflow and infarct size, and improved long-term left ventricular remodeling and systolic function compared with control and to either treatment alone.[55] Recently, in models of experimental atherosclerosis it has been also demonstrated that NET-derived extracellular histone H4 induces smooth muscle cells (SMCs) lysis leading to plaque destabilization, while neutralization of histone H4 prevents cell death of SMCs and stabilizes atherosclerotic lesions.[56]

Human Arterial Thrombosis

Atherothrombosis is triggered by strong platelet activation following plaque erosion or rupture end exposure of thrombogenic material to the blood stream. Initial platelet-rich thrombus is potentiated by activation of blood coagulation through vascular and blood-borne TF (►**Fig. 3**). Neutrophils and NETs have been implicated in coronary thrombosis induced by endothelial activation,[57] plaque erosion,[58,59] and rupture.[33,60] Neutrophils and NETs are abundantly present in thrombi derived from patients with ST-elevation myocardial infarction (STEMI)[19,22,61] and thrombosed stents.[62] Coronary thrombus-resident NETs express IL-17A/F and TF, both of which promote platelet and coagulation activation.[19,63,64] Mechanistically, TF-bearing NETs are induced by polyP secreted from thrombin-activated platelets, while IL-29/interferon λ1 attenuates NET formation by inhibiting polyP/mammalian target of rapamycin (mTOR)/autophagy pathway.[24]

The presence of NETs was associated with more stable coronary thrombi, sustained ischemia, and increased enzymatic and cardiovascular magnetic resonance-measured infarct size in 111 patients with STEMI.[61] In a prospective, cross-sectional study of 282 individuals with clinically suspected coronary artery disease, plasma markers of NETosis citH4 and myeloperoxidase (MPO)-deoxyribonucleic acid (DNA) complexes, were positively associated with increase thrombin–antithrombin complex and vWF levels as well as revascularization, acute coronary syndrome, and cardiac death after a median follow-up period of 545 days.[65] Significantly elevated concentrations of DNA, nucleosomes, and citH3 as markers of enhanced NETotic burden were also measured in stroke patients.[66–68] In patients with acute ischemic stroke, NETotic markers

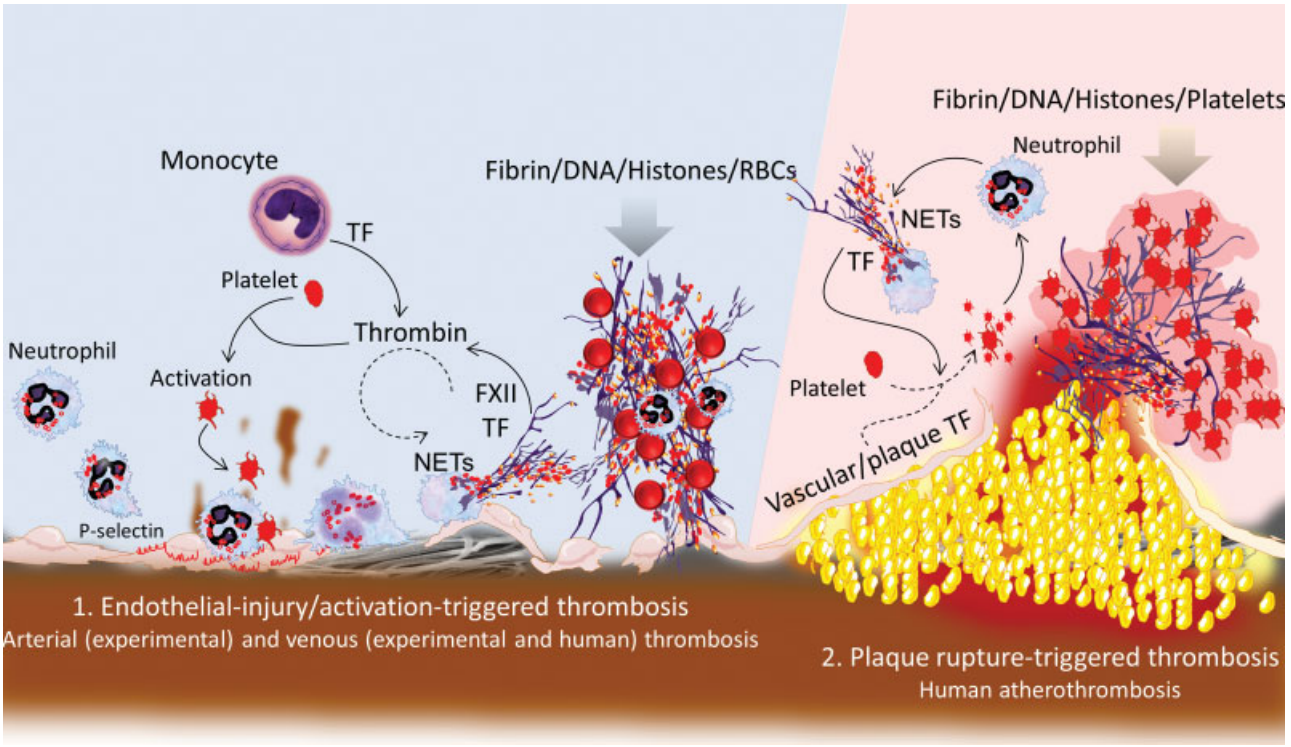

Fig. 3 Left (1): In case of endothelial damage/activation, neutrophils adhere through P-selectin and release neutrophil extracellular traps (NETs) upon interaction with neighboring activated platelets. NETs support tissue factor (TF) and FXII activation and thrombin generation (together with TF derived by monocytes). The result is the formation of a thrombus composed of fibrin, deoxyribonucleic acid (DNA), histones, red blood cells (RBCs), and some platelets. Right (2): Following plaque rupture, vascular TF activates locally recruited platelets which interact with TF-loaded neutrophils to release TF-bearing NETs. Active NET-bound TF supports thrombin generation which further activates more platelets in a vicious cycle. The result is a robust platelet activation and the formation of a thrombus composed of fibrin, DNA, histones, and platelets.

were positively correlated with clot stability and resistance to endovascular therapy,[69] and with stroke severity scores and all-cause mortality at 1 year.[68] MPO, cell-free DNA, and MPO-DNA complexes were also detected in intraplaque hemorrhage segments of carotid atherosclerosis specimens,[70] and in intraluminal thrombi derived from patients with abdominal aortic aneurysms.[53,71]

Experimental and Human venous Thrombosis

A massive platelet aggregation is not essential for venous thrombosis (►**Fig. 3**). Low shear venous thrombosis is triggered by stasis-induced endothelial activation, rapid neutrophil recruitment, and adhesion via endothelial P-selectin. Both neutrophils and NETs were found in large amounts in venous thrombi in a mouse model of inferior vena cava flow restriction-induced thrombosis. Adherent neutrophils interact with platelets locally and expose thrombogenic elements such as TF and FXII through NET release.[13,72] Elevated NETosis markers in plasma were measured in experimental iliac vein thrombosis in baboons (extracellular DNA) and humans (circulating nucleosomes and NE/α1-antitrypsin complexes).[27,73–75] In a case–control study with 345 participants, high levels of NETotic markers increased the risk of DVT by threefold even after adjustment for known confounders (malignancy, smoking, recent immobilization, recent hospitalization) in a dose-dependent manner.[73]

Thrombus Stabilization and Lysis

Beyond their contribution to thrombus propagation, extracellular DNA and NETs affect also thrombus structure and stability with clinical implications related to thrombus lysis. Inhibition of leukocyte infiltration in a baboon model of DVT resulted in unstable thrombi suggesting a role for neutrophils/NETs in thrombus stabilization.[76] NETs can directly degrade fibrin via NET-bound NE and CG while histone H2B can serve as a receptor for plasminogen, recruiting plasminogen from the plasma.[77] However, neutralization of NET-associated proteases by plasma antiproteases in vivo results in a net inhibitory effect of NETs on clot lysis in the blood.[78] Addition of DNA, histones, or both, on clotting plasma, resulted in thicker fibrin fibers, while NETs incorporated into plasma clots significantly delayed clot lysis in vitro by reducing tPA activity. In quantitative terms, the addition of histone and DNA to clots produced 50% thicker fibers and doubled the critical shear stress for loss of fibrin viscosity.[79] Moreover, we have observed absence of NETotic neutrophils and NETs in thrombus remnants aspirated from STEMI patients with spontaneous thrombus resolution.[19] These observations suggest that the formation of NETs is imperative for thrombus stability and lytic resistance.

The characteristic of chronic thromboembolic pulmonary hypertension (CTEPH) is extreme persistence and lytic resistance of pulmonary thromboemboli leading to chronic obstruction of pulmonary arteries and in small vessel arteriopathy.[80]

Since the presence of NETs and DNA has been associated with thrombus stability, it is possible that NETotic infiltration of pulmonary thrombi (inflamed thrombi) causes resistance to endogenous lytic mechanism and thrombus persistence. Indeed, in two independent cohorts of CTEPH patients a 10-fold increase of MPO plasma levels, 7-fold increase in plasma NE, and more than 2-fold increase in circulating MPO/DNA levels compared with healthy volunteers were measured.[81] Moreover, analysis of endarterectomy specimens obtained from several independent cohorts of CTEPH patients consistently revealed abundant CitH3-positive cells, protein arginine deiminase-4 (PAD4)+ NETotic neutrophils, MPO/DNA colocalization, and NETs in the organizing stage of thrombus development and maturation.[75,81]

NETs and Systemic Inflammation-Mediated Thrombosis

Apart from locally occurring thrombosis in the context of cardiovascular diseases, several lines of evidence demonstrate that NETs mediate thrombosis in various systemic thromboinflammatory conditions, such as sepsis, autoimmunity, and malignancy. These disorders are characterized by the presence of activated neutrophils and NETs fragments in the bloodstream and affected tissues, and a high propensity for serious, life-threatening thrombotic events.

Autoimmune and Inflammatory Diseases

It is well established that NET formation is implicated in the induction of autoimmunity and correlated with the severity of related diseases.[17,38,82] Accordingly, NET-dependent coagulation has been suggested as a common mechanism that might explain the increased incidence of thromboembolic events in patients suffering from systemic inflammatory diseases, especially during periods of exacerbation.[11,12]

The key role of NETs in the pathogenesis of autoimmune-related thrombosis was first demonstrated in antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV). Excessive NETosis has been observed in pulmonary and venous thrombi from autopsy specimens in severe AVV.[83,84] In line with these, TF-bearing NETs were released by circulating and bronchoalveolar lavage neutrophils, and were found in nasal and renal biopsies of active AVV patients. Additionally, in vitro stimulation of neutrophils with serum or ANCA derived from these patients induced the expression of TF in neutrophils and its extracellular delivery via NETs. Interestingly, disease remission or ANCA depletion attenuated these effects suggesting the pathogenic role of ANCA in AVV.[85,86] The presence of thrombogenic TF on NETs has also been demonstrated in peripheral blood and inflamed tissues from active SLE and ulcerative colitis patients in a suggestive mechanism that is triggered by disease inflammatory microenvironment and is regulated by mTOR/autophagy pathway.[48,52] Besides thrombosis, signaling through protease-activated receptors that results in the activation of endothelial, epithelial, and other tissue cells could be an additional pathogenic pathway for the involvement of neutrophils/NETs-expressed TF in organ damage of several chronic inflammatory diseases.[87,88]

Thirteen years after the discovery that purified immunoglobulin G from patients with antiphospholipid antibody syndrome (APS) is able to induce TF expression in human neutrophils,[9] recent experimental and clinical data demonstrate that NET formation and regulation constitutes a new pathogenic mechanism in typical (auto)immune-mediated thrombotic diseases associated with a severe prothrombotic state, such as APS, heparin-induced thrombocytopenia/thrombosis, and thrombotic thrombocytopenic purpura, further supporting the crucial role of neutrophils in immunothrombosis.[89–94]

Apart from TF, the contribution of histones and other neutrophils/NETs-derived components, such as phosphatidylserine positive microparticles (MPs) or autoantigens, in the hypercoagulability of clinical active autoimmune/inflammatory diseases have also been recognized.[85,95,96] Recently, the role of platelet/neutrophil interaction in autoimmune vascular damage is further reinforced. HMGB1 on platelets-derived MPs was found to be key inducer of autophagy-mediated NET release in patients suffering from systemic sclerosis suggesting that neutrophil autophagy and NETs are tightly implicated in disease vasculopathy and microthrombosis.[97]

Besides their direct prothrombotic action, NETs are major contributors to endothelial dysfunction and atherosclerotic plaque formation.[98] NET formation provides a reasonable pathophysiological link between inflammation and atherothrombosis in patients with chronic rheumatic diseases such as SLE and RA.[99–101] Consistent with this, blocking of NET formation using PAD4 inhibition in a murine model of lupus that is characterized by accelerated vascular damage and prothrombotic tendency, significantly improved endothelial dysfunction and thrombosis risk supporting an important role for NETs in the atherosclerosis and hypercoagulability of systemic autoimmunity.[102]

Taken together, functionally diverse neutrophil subsets are apparently attractive targets for "omics" studies that may/will unveil novel anti-inflammatory strategies in several devastating, difficult-to-treat autoimmune thromboinflammatory conditions.[103–106] For example, RNAseq analysis of whole blood from patients with AAV has previously linked low-density granulocytes (LDGs), a population functionally characterized by high propensity to form NETs, with disease activity and decreased response to treatment. This probably implies that more aggressive treatment and close follow-up are needed in AAV patients with increased frequency of LDGs.[103] Similarly, transcriptome analysis of neutrophils revealed P-selectin glycoprotein ligand-1 (PSGL-1) adhesion molecule as a potential therapeutic target in primary APS. Interestingly, PSGL-1 deficient mice were characterized by reduced NET formation.[104] Moreover, genome-wide DNA methylation analysis of neutrophils showed an entirely distinct DNA methylation profile between primary APS and SLE patients.[105] Whether this heterogeneity is related with NETosis or/and therapeutic response in different phenotypes of APS remains to be studied.

NETs and Cancer-Associated Thrombosis

Malignancy is characterized by neutrophil accumulation in tumor environment and high thrombotic risk, whereas thrombosis is a main cause of morbidity and mortality in

Table 1 Important findings on NET-associated thrombosis in different diseases bearing therapeutic potential

Disease		Reference
STEMI	NETs abundance in coronary thrombi of patients NETs are present in coronary stent thrombus NETs promote thrombus organization Platelet–neutrophil interaction through polyP triggers NET generation Functionally active TF on NETs of culprit coronary artery	19,22,61 62 63 24 19
Stroke	NETs in thrombi are positively correlated with resistance to endovascular therapy	69
DVT/PE	TF positive neutrophils adhere to activated vein endothelium in vivo NETs promote thrombus organization and maturation Activated neutrophils and plasma nucleosomes/DNA as DVT risk factors NETotic neutrophils in endarterectomy specimens from VTE patients	13 72,75 73,74 75
CTEPH	NETs activate pulmonary arterial endothelial cells and proliferation of pulmonary smooth muscle cells NETs in plasma and intrapulmonary thrombi of patients	81 81
AAV	LDGs may be associated with disease activity and response to treatment NETs in pulmonary and venous thrombi in severe disease Functionally active TF on peripheral and tissue NETs	103 83,84 85,86
SLE	Functionally active TF on peripheral and tissue NETs REDD1/mTOR/autophagy pathway is related to thrombogenic NETs	52 52
APS	Decreased degradation of NETs Antiphospholipid antibodies induce NETs Neutrophil PSGL-1 as a putative therapeutic target Adenosine receptor agonism protects against NETosis and thrombosis	89 90 104 106
HIT	Neutrophil activation leads to NETs-induced thrombosis	92,93
TTP	Impaired DNase1-mediated degradation of NETs	94
UC	NETs enhance procoagulant activity Functionally active TF on peripheral and intestinal NETs REDD1/mTOR/autophagy pathway is related to thrombogenic NETs	48,96 48 48
Sepsis	Functionally active TF on peripheral NETs NETs are associated with DIC, VTE, and impaired fibrinolysis	47 123–127
Cancer	NETs as prediction marker of cancer-associated VTE Tumor environment primes neutrophils to release procoagulant NETs NETs-bound TF in colon cancer and metastatic lymph nodes NETs promote cancer-associated venous and arterial thrombosis	37 109,111 118 115–117 119,120

Abbreviations: AAV, anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis; APS, antiphospholipid antibody syndrome; CTEPH, chronic thromboembolic pulmonary hypertension; DIC, disseminated intravascular coagulation; DNA, deoxyribonucleic acid; DVT/PE, deep venous thrombosis/pulmonary embolism; HIT, heparin-induced thrombocytopenia/thrombosis; LDGs, low-density granulocytes; mTOR, mammalian target of rapamycin; NET, neutrophil extracellular trap; PSGL-1, P-selectin glycoprotein ligand-1; REDD1, regulated in development and DNA damage responses 1; STEMI, ST-elevation myocardial infarction; TF, tissue factor; TTP, thrombotic thrombocytopenic purpura; UC, ulcerative colitis; VTE, venous thromboembolism.

cancer patients.[107,108] First, Demers et al using various animal models of cancer showed an increase in neutrophils count and their propensity for NET formation in combination with the appearance of venous thrombi in tumor affected tissues.[109] This effect is probably through a systemic effect on the host that involves tumoral granulocyte colony-stimulating factor and other tumor microenvironment such as IL-8 and exosomes.[109–111]

Over the last years, many studies in tumor experimental models and patients suffering from hematological or solid malignancies strongly implicate NETs in cancer-associated venous and arterial thrombosis, as well as tumor growth and metastasis.[112–117] NETs were found in colon tumor biopsies and the respective metastatic lymph nodes representing a prominent source of TF in cancer microenvironment.[118] Mechanistically, intratumor NET-mediated TF expression may promote tumor thrombosis and necrosis, or contrariwise, may enhance neoangiogenesis offering metabolic support to rapidly growing malignant cells. Furthermore, tumor-associated NETs could be an important source of TF that contributes to systemic thrombosis. Tumor cells can also promote platelet aggregation by stimulating endothelial production of vWF, another coagulation factor that is able to bind NET scaffold contributing to thrombosis.[119] In another experimental study, tumorigenesis was associated with coagulation induced by low-density neutrophils that displayed spontaneous NET formation in a complement-dependent manner.[120] Consistent with the abovementioned data, in a prospective observational study that comprises almost 1,000 patients with newly diagnosed cancer or cancer progression after remission, levels of CitH3, a NET formation biomarker, are independently associated with the occurrence of venous thromboembolism (VTE).[37] The same group has currently reported that NET biomarkers in patients with cancer were not associated with arterial thromboembolism, but with higher mortality.[121]

Systemic Inflammatory Response Syndrome

Besides noninfectious inflammatory conditions, systemic inflammatory response syndrome in the context of severe infection/sepsis is able to trigger excessive NET release in the vasculature causing endothelial tissue damage, intravascular coagulation, and organ dysfunction.[18,122–124] Septic patients spontaneously release NETs carrying functionally active TF in an autophagy-depended manner, whereas the inflammatory environment of sepsis induces the in vitro formation of thrombogenic TF-bearing NETs.[47] In line with this, several studies have indicated that increased levels of NET releasing in septic patients were significantly correlated with disseminated intravascular coagulation development, VTE risk, and impaired fibrinolysis.[123,125–127]

Translational Impact and Future Perspectives

Although effective antithrombotic approaches have been developed to combat this cardiovascular epidemic, current regimens target both pathological thrombosis and physiological hemostasis, and therefore almost inevitably increase the risk of bleeding. Therapeutic approaches aimed at inflammatory mechanisms of thrombosis induction rather than directly in the coagulation system may be used in the future alternatively or synergistically with conventional treatments, enhancing therapeutic benefit and potentially reducing the risk of hemorrhagic complications.

The above described findings regarding the important role of NETs in the generation and stabilization of thrombus, offers the potential for new therapeutic options targeting neutrophil-driven thromboinflammation in several diseases (►**Table 1**). For example, therapies against: (1) inductive/regulatory mechanisms of NET formation (e.g., complement, autophagy, anti-polyP), (2) NETs themselves (chromatin scaffold integrity, histones), and (3) specific NET-bound proteins (e.g., TF/clot factors, IL-1β/cytokines), without affecting NET release per se (►**Fig. 4**), may prove more effective and/or safer antithrombotic agents provided that they will not interfere with normal hemostasis or immunocompetence.[24,128–136]

Taken in consideration the experimental and human data obtained since today, and without ignoring traditional risk factors and classical antithrombotic regimens, clinical trials that will investigate the anti-NETotic effect on thrombotic

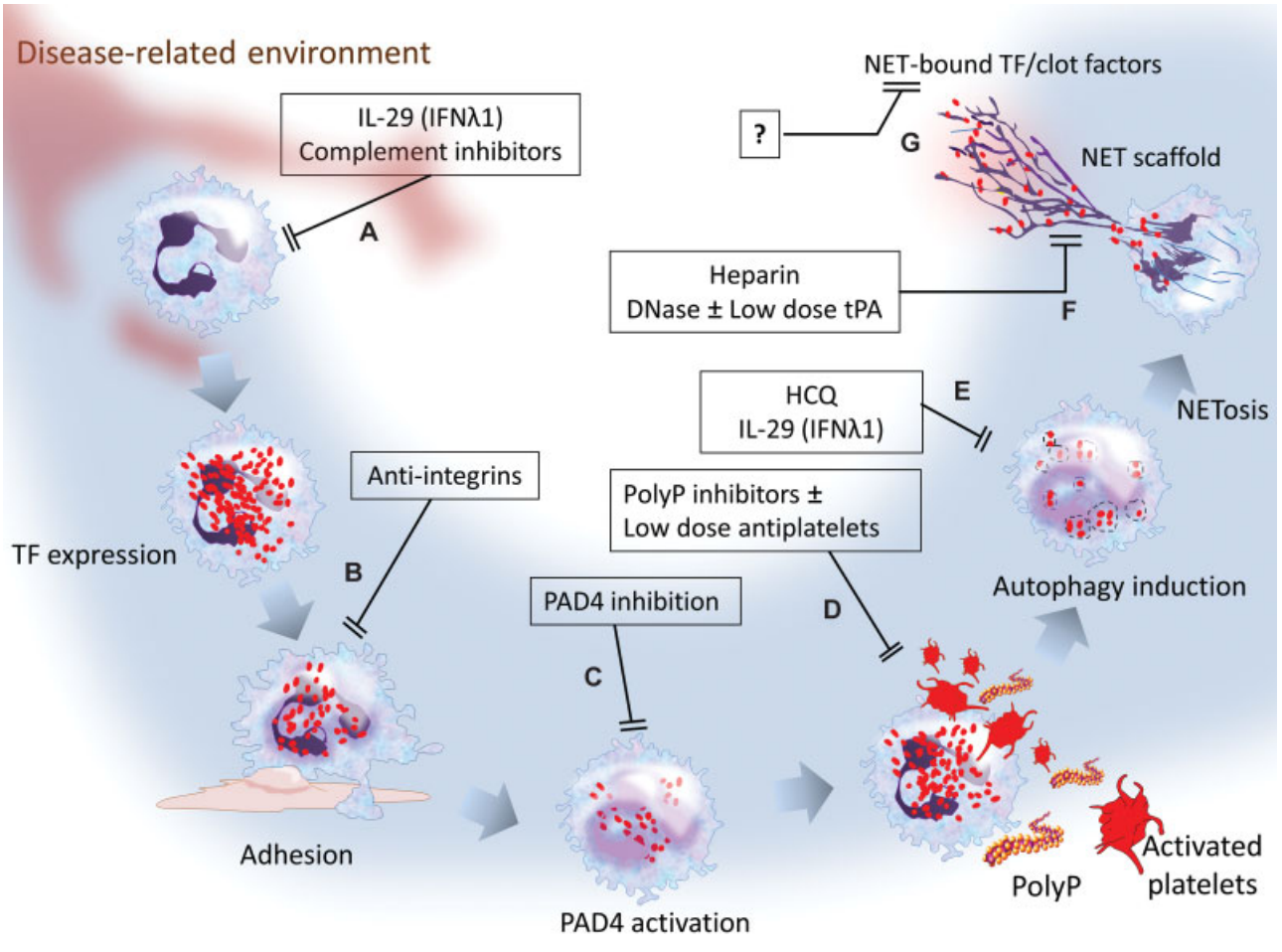

Fig. 4 Possible targets of neutrophil/neutrophil extracellular trap (NET)-driven thromboinflammation. Inhibition of the intracellular expression of disease-related proteins in circulating neutrophils (**A**); inhibition of adhesion and early NETotic steps (**B**, **C**); inhibition of late NETotic steps and autophagy (**D**, **E**); dismasting of NET scaffold (**F**); blocking of specific NET-associated proteins (**G**). HCQ, hydroxychloroquine; IFNλ, interferon-λ; IL-29, interleukin-29; PAD4, protein arginine deiminase-4; PolyP, inorganic polyphosphate; tPA, tissue plasminogen activator.

diseases are needed for the development of novel therapeutics. Recently, the CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcome Study) trial demonstrated that anti-inflammatory therapy targeting IL-1β significantly reduced cardiovascular events in patients with previous myocardial infarction and residual inflammatory risk, without affecting lipid levels. This large randomized, double-blind trial, represents the first "proof-of-principle" of the inflammation hypothesis of atherothrombosis paving the way for further research into this field.[6] In a similar way, large international trial HIBISCUS has been launched to examine the efficacy of hydroxychloroquine (HCQ) in the secondary prevention of thrombotic events of primary APS.[131] Interestingly, HCQ inhibits autophagy[129] and several human studies have linked IL-1β and autophagy with NET formation and activity.[22,24,43,47–49,52,97,132,137]

Further investigation of the neutrophil/NET/coagulation axis is expected to unveil novel candidate diagnostic, prognostic, and therapeutic targets in inflammatory and thrombotic conditions, but also to redefine the clinical indications of old drugs through repositioning.[12,37,68,121,130–132] In parallel, new, NETosis-based, coagulation assays are on the way.[138,139]

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Conflict of Interest
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