



Insights Into Immunothrombosis: The Interplay Among Neutrophil Extracellular Trap, von Willebrand Factor, and ADAMTS13

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Both neutrophil extracellular traps (NETs) and von Willebrand factor (VWF) are essential for thrombosis and inflammation. During these processes, a complex series of events, including endothelial activation, NET formation, VWF secretion, and blood cell adhesion, aggregation and activation, occurs in an ordered manner in the vasculature. The adhesive activity of VWF multimers is regulated by a specific metalloprotease ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motifs, member 13). Increasing evidence indicates that the interaction between NETs and VWF contributes to arterial and venous thrombosis as well as inflammation. Furthermore, contents released from activated neutrophils or NETs induce the reduction of ADAMTS13 activity, which may occur in both thrombotic microangiopathies (TMAs) and acute ischemic stroke (AIS). Recently, NET is considered as a driver of endothelial damage and immunothrombosis in COVID-19. In addition, the levels of VWF and ADAMTS13 can predict the mortality of COVID-19. In this review, we summarize the biological characteristics and interactions of NETs, VWF, and ADAMTS13, and discuss their roles in TMAs, AIS, and COVID-19. Targeting the NET-VWF axis may be a novel therapeutic strategy for inflammation-associated TMAs, AIS, and COVID-19.

Keywords: neutrophil extracellular traps, von Willebrand factor, ADAMTS13, thrombotic microangiopathy, acute ischemic stroke, COVID-19

INTRODUCTION

Neutrophils, the most abundant leukocyte subset in circulation, act as the critical responders during innate immunity and inflammation (1). Neutrophils have been known to kill and clear invading microorganisms through two strategies: phagocytosis and degranulation. Brinkmann et al. firstly reported a novel antimicrobial strategy of neutrophils, by which neutrophil extracellular traps (NETs), the net-like chromatin structure decorated with histones and granular proteins, are released from neutrophils into extracellular space to catch and kill invading bacteria to protect host from infection (2). However, NETs have a dark side. Uncontrolled NET formation or improper clearance

of NETs may lead to tissue damage and activate inflammatory cells, contributing to the development of multiple diseases, such as fibrosis (3), sepsis (4), cancer metastasis (5), systemic lupus erythematosus (SLE) (3), thrombosis (6), and atherosclerosis (7).

Plasma glycoprotein von Willebrand Factor (VWF), which captures circulating platelets to the sites of vascular injury and mediates subsequent platelet activation and aggregation, is a critical mediator in hemostasis (8). The activity of VWF depends on its size. Ultra-large VWF (UL-VWF) multimers released from endothelial cells may spontaneously recruit excessive circulating platelets and other blood cells, promoting the development of thrombosis (9). Metalloprotease ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motifs, member 13) specifically cleaves the Tyr1605-Met1606 bond within VWF A2 domain to regulate the size and activity of VWF multimers, preventing the formation of thrombus (10).

Increasing studies indicate that NETs, like VWF, play an important role in the formation of thrombus in venous (11), arterial (12), and cancer-associated thrombosis (13). NETs directly interact with VWF *via* electrostatic force (14), and this interaction retains NETs on endothelial surface (15). The colocalization of NETs and VWF has been observed in venous (11) and arterial thrombosis (12). Given that both NETs and VWF have prothrombotic and proinflammatory effects, it therefore is reasonable to speculate that the interactions between NETs and VWF may promote the development of thrombosis and inflammation. Moreover, NET contents indirectly or directly reduce the activity of ADAMTS13, promoting the formation of UL-VWF. NETs, VWF, and ADAMTS13 may form a vicious circle to aggravate the development of thrombosis and inflammation. In this review, we will summarize the biological characteristics of NETs, VWF, and ADAMTS13, and describe the interactions among NETs, VWF, and ADAMTS13. We also discuss the latest findings regarding the role of NET-VWF axis in thrombotic microangiopathies (TMAs), acute ischemic stroke (AIS), and coronavirus disease 2019 (COVID-19), and the therapeutic potential by targeting the NET-VWF axis.

THE MESH-LIKE NEUTROPHIL EXTRACELLULAR TRAP

NET Structure and Components

In 2004, NETs were first described as a form of initial immune defense released from neutrophils (2). The network of NETs is mainly composed of DNA, histones and proteins from both azurophilic and specific granules to virulence factors (2). Neutrophil elastase (NE), myeloperoxidase (MPO), cathepsin G (CG), proteinase 3 (PR3), metalloproteinases 9 (MMP-9), and human neutrophil peptides 1 (HNP1) have been illustrated to present in NETs (5, 16, 17). NET-associated proteins provide a high local concentration of antimicrobial agents that is lethal to bacteria. Urban et al. analyzed 15 NET-associated proteins and demonstrated that 10^{12} neutrophils can release NETs containing, on average, 3.58 ± 0.28 g of protein and 2.24 ± 0.51 g of DNA,

indicating a ratio of 1.67 ± 0.26 g of protein per gram of DNA (18). Of note, the proportion of composition, protein types and post-translational modifications of NETs induced by distinct stimuli are various (19). Combining fluorescence and atomic force microscopy, Pires et al. demonstrated that NETs were organized as branched networks. The topological height of NETs was 3 ± 1 nm and the area of the pores was up to $0.03 \pm 0.04 \mu\text{m}^2$, so that pathogens whose size is within this range can be captured (20).

Mechanisms of NET Formation

Various stimuli, such as bacteria, fungi, viruses, parasites, activated platelets, and some chemical substances, are able to induce NET formation termed as NETosis (16). Recently, the cellular events of NET formation were demonstrated (21, 22) and extensively reviewed elsewhere (23). Briefly, NET formation is initiated by actin disassembly, followed by plasma membrane microvesicles shedding, vimentin remodeling, microtubule disassembly, endoplasmic reticulum vesiculation, chromatin decondensation, and nuclear rounding, progressively increased plasma membrane and nuclear envelope permeability, nuclear lamin meshwork, and then nuclear envelope rupture to release chromatin into the cytoplasm, and finally plasma membrane rupture and discharge of extracellular chromatin (21). However, the mechanism of NET formation is complicated and still not fully understood. The molecular events leading to NET formation is intensively studied with the stimulation of phorbol 12-myristate 13-acetate (PMA). PMA activates the Raf-MEK-ERK pathway, resulting in the assembly of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and the generation of reactive oxygen species (ROS). ROS triggers the activation of the azurosome proteases and MPO, allowing these proteases to pass through the intact membrane (24). NE, which functions as an antimicrobial protease, translocates to the nucleus, and drives the degradation of histones inducing chromatin decondensation (25). In addition, the increased levels of intracellular calcium activate peptidylarginine deiminase 4 (PAD4) that converts arginines in histones to citrullines, reducing the positive charge of histones and contributing to chromatin decondensation as well (26). Sequentially, the nucleus loses the characteristic nuclear lobuli and rounds up, nuclear envelope ruptures, cell membrane ruptures, and chromatin is released into extracellular space (22). This process takes more than 2 h, and is termed as suicidal NETosis (**Figure 1A**). Notably, the requirement of NADPH or PAD4 is not universal to all NETotic pathways. For instance, NETosis can be induced in a NADPH oxidase-independent manner (27, 28) or in a PAD4-independent manner (29, 30).

Apart from suicidal NETosis, increasing evidence demonstrates that NETs could be induced in a rapid way. Stimulated by *Staphylococcus aureus* (*S. aureus*), neutrophils release NETs within 5–60 min independent of ROS produced by NADPH oxidase (NOX) (31) but depend on Toll-like receptor 2 (TLR2) and complement-mediated opsonization (32). This process starts with the separation of the inner and outer nuclear membranes and budding of vesicles. These vesicles containing

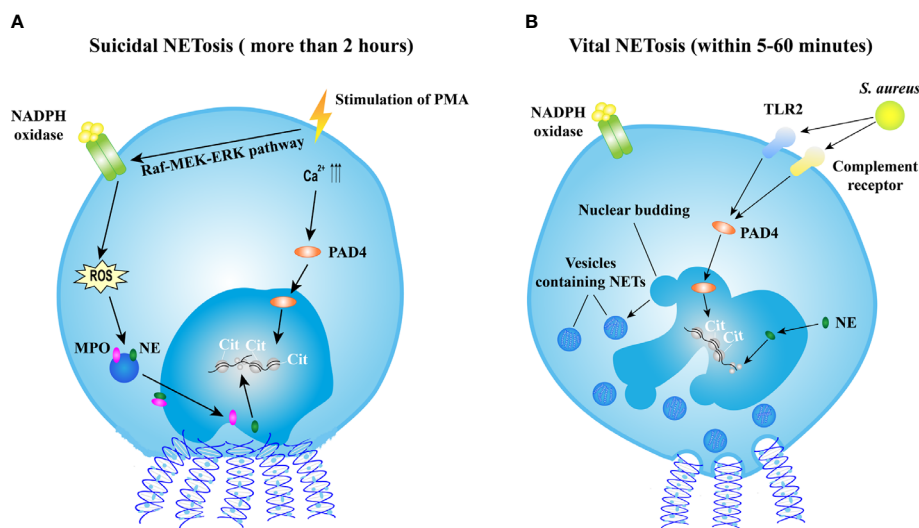


FIGURE 1 | Mechanisms of NET formation. **(A)** The mechanism of suicidal NETosis. Suicidal NETosis induced by PMA takes approximately 2 h. Stimulation of PMA induces the assembly of NADPH oxidase (NOX) via the Raf-MEK-ERK pathway, resulting in ROS generation. ROS triggers the activation of MPO and NE, facilitating the translocation of MPO and NE into nucleus. NE promotes the decondensation of chromatin by degrading histones. In addition, the stimulation also increases the levels of intercellular calcium, might result in the activation of PAD4 that subsequently translocates into nucleus and citrullinates histones leading to chromatin decondensation. Thereafter, nucleus rounds up, nucleus envelope ruptures, cell membrane ruptures, and then eventually chromatin decorated with various granular proteins is released into extracellular space and cell is lysed. **(B)** The mechanism of vital NETosis. Vital NETosis is rapid and non-lysis. *S. aureus*-induced vital NET formation requires TLR2 and complement receptor and is independent of NOX-generated ROS. Activated PAD4 and NE induce chromatin decondensation, and then vesicles filled with NETs are generated. These vesicles are expelled from nucleus and released into extracellular space without the disruption of cell membrane. In vital NETosis, cell membrane integrity is maintained and the cell remains functional. NET, neutrophil extracellular trap; NETosis, a process of neutrophil extracellular trap formation; PMA, phorbol myristate acetate; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; MPO, myeloperoxidase; NE, neutrophil elastase; *S. aureus*, *Staphylococcus aureus*; PAD4, protein-arginine deaminase type 4; TLR2, toll-like receptor type 2.

DNA are delivered through the cytoplasm and released extracellularly (31) (**Figure 1B**). This rapid NETosis is also reported when neutrophils are stimulated by *Candida albicans* (33), *Leishmania* parasites (34), heparin (35), and LPS-stimulated platelets (36). Unlike in suicidal NETosis, neutrophils releasing NETs rapidly do not undergo cell lysis and remain functional, thus this process is termed as vital NETosis (37).

In addition to chromosomal DNA, mitochondrial DNA (mtDNA) has been reported as another source of NET DNA. Yousefi et al. demonstrated that neutrophils released mtDNA but not nuclear DNA in a ROS-dependent manner after priming of granulocyte/macrophage colony-stimulating factor (GM-CSF) and stimulation of LPS or complement factor 5a (C5a). In mtDNA, no nuclear proteins such as lamin B and nuclear matrix 45 (NP-45) were detected (38). Lood et al. demonstrated that ribonucleoprotein-containing immune complexes (RNP ICs), which are enriched in lupus, induced mobilization of mitochondria to the cell surface and release of mtDNA in a ROS-dependent manner (39). Van Dam et al. investigated the distinct pathways and characteristics of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV)-induced NETs and SLE-induced NETs. By quantifying the colocalization of TOMM20 and MitoSOX, the presence of mtDNA was confirmed in SLE-induced NETs but not in AAV-induced NETs (40).

Taken together, although pathways and mechanisms of NET formation are diverse and sophisticated upon different stimuli, they all come to an identical outcome: histone degradation, chromatin decondensation, and NETs released from neutrophils (41).

THE VON WILLEBRAND FACTOR AND ADAMTS13

The Glycoprotein von Willebrand Factor

Von Willebrand factor, a glycoprotein released by endothelial cells or stimulated platelets, is essential in hemostasis (42). Mature VWF monomer contains three modules: D (D¹-D3, D4), A (A1-A2-A3), and C (C1-C2-C3-C4-C5-C6-CK) (43). VWF monomers dimerize through the C-terminal cysteine knot (CK) domain in endoplasmic reticulum (ER). Dimers then assemble into multimers through D3 domain in Golgi (44). Interestingly, VWF multimers have been reported to self-associate forming branched structures under static and flow conditions (45–47). Notably, long VWF multimers, especially the UL-VWF exhibit prothrombotic activity (48). The activity of VWF is regulated by ADAMTS13 that specifically cleaves the peptide bond Tyr1605-Met1606 in A2 domain. VWF multimerization and ADAMTS13 proteolysis work together to

balance the size of VWF multimers, ensuring the proper adhesion, activation, and aggregation of platelets. At sites of vascular injury, the circulating “coiled” VWF concatemers are immobilized through pairs of interactions, such as A3 domain binding to collagen I (49) and C domains binding to fibrin network (50). Then, the flowing blood stretches these immobilized VWF concatemers into an elongated conformation (51). The length of VWF multimers significantly affects their responses to shear. A lower threshold of shear rate is required to extend long VWF multimers (52). A recent research suggested that tension, but not shear stress, regulates the affinity of VWF for platelet glycoprotein Ib α (GPIb α) (53).

A1A2A3 tridomain is essential for VWF function, as it contains binding sites for GPIb α on platelets (A1), collagen in subendothelial matrix (A1 and A3), and the peptide bond cleaved by ADAMTS13 (A2). The function of A1A2A3 is modulated by shear stress. The A1-GPIb α bond exhibits force-enhanced characteristics, which could be explained by catch-bond (54) or flex-bond mechanism (55). Liu et al. applied molecular dynamics (MD) simulations to further examine how a mutation regulates the affinity of A1 to GPIb α (56). It was demonstrated that the destabilization of the N-terminal arm of gain-of-function (GoF) A1 mutant and the increased movement of the α 2-helix could change the closed A1 conformation to a partially open state. The unfolding of the A2 domain is much easier than A1 and A3 domain, as the disulfide bond in A2 domain is located at the C-terminal end, which differs from the disulfide bond in A1 and A3 domain that links their C-terminal and N-terminal ends together (57). The unfolding forces for a single A2 domain range from 7 to 14 pN (58).

The function of A1A2A3 is also modulated by the interdomain interactions (59, 60). The binding of VWF A1 to GPIb α could be inhibited by its adjacent D'D3, A2, and A3 domains (8, 60, 61). The GPIb α binding site in A1 domain is exposed after the force-induced A2 domain unfolding (60). The half-life of A1-GPIb α bond is short, so it only can slow the rolling velocity of platelets (54). Subsequently, platelet receptor GPVI or β 1 integrins (α 2 β 1/ α 5 β 1) binds to respective ligands to form arrest adhesion (62). In addition, the integrin α IIb β 3 on platelet is activated through the “inside-out” and “outside-in” signaling, increasing the affinity to its ligands, such as VWF and fibrinogen, to contribute to platelet arrest adhesion and aggregation (63) (**Figure 2A**).

It has been demonstrated that VWF participates in leukocyte recruitment (65). The activated platelets on UL-VWF mediate leukocyte recruitment under static and flow conditions (65). Apart from its indirect role in leukocyte recruitment, VWF also directly regulates this process. The binding of D'D3 domain to P-selectin glycoprotein ligand 1 (PSGL-1) on leukocyte supports its rolling, and subsequently, VWF interacts with β ₂ integrin on leukocyte to promote its firm adhesion (66). In the presence of pathogens, VWF multimers also bind to NETs to form a network that can recruit both platelets and leukocytes and promote thrombosis (67). The size of such thrombi is efficiently controlled by ADAMTS13 and DNase I (68, 69). ADAMTS13 cleaves VWF multimers, while DNase I digests the DNA of NETs.

The Metalloprotease ADAMTS13

ADAMTS13 specifically cleaves VWF to convert the large multimers into small ones. Severe deficiency in ADAMTS13 results in thrombotic thrombocytopenic purpura (TTP), a fatal disorder characterized by systemic microvascular thrombosis (70). ADAMTS13 is a multi-domain protein including a metalloprotease domain (M), a disintegrin-like domain (D), a thrombospondin Type-1 domain (T), a cysteine-rich domain (C), a spacer domain (S), followed by seven additional thrombospondin Type-1 repeats (T2–8) and two CUB (complement c1r/c1s, sea urchin epidermal growth factor, and bone morphogenetic protein) domains (71, 72). Evidence shows that various domains of ADAMTS13 bind to VWF (73, 74). Notably, the lack of spacer domain results in a dramatic reduction in its proteolytic activity, suggesting spacer domain is vital (75, 76). Although the crystal structure of full length ADAMTS13 is not available to date, the crystal structure of its MDTCS provides critical insights into ADAMTS13 binding and cleavage (77, 78). Akiyama et al. firstly crystallized the DTCS structure in 2009 (79). Three exosites were reported on D, C, and S domain respectively. The cluster of four charged residues (Arg326, Glu327, His328, and Asp330) in the V-loop of D domain creates the exosite-1. De Groot et al. demonstrated that Arg349, Leu350, and Val352, close proximity to the cluster, interacted with unfolded VWF A2 (80), suggesting these residues function as a part of exosite-1. The V-loop in C domain creates another exosite (exosite-2). The cluster of His476/Ser477/Gln478 in the V-loop and the adjacent Arg488 play a pivotal role in VWF recognition. In addition, the U-loop and residues 494–496 flanking the V-loop may contribute to exosite-2. Exosite-3 comprises residues Tyr658, Arg659, Arg660, Tyr661, Tyr665, and two surrounding residues Arg568 and Phe692 in S domain. This exosite binds to the α 6 helix of VWF A2 and is critical in substrate recognition and proteolysis (81). A “molecular zipper” model has been proposed to depict the multi-step process of ADAMTS13 binding to VWF (82). In this model, the distal T and CUB domains firstly bind to a constitutively exposed binding site in VWF D4CK, the discrete exosites, starting from exosite-3, then sequentially bind to different segments of unfolded A2, bringing the M domain and the VWF scissile bond into proximity. The S1 and S1' subsites on the M domain engage with P1 Tyr1605 and P1' Met1606 of VWF to allow the proteolysis to occur (**Figure 2B**). Recently, Petri et al. crystallized a Fab-MDTCS structure. The authors proposed that the M domain exhibited a latent conformation, and the binding of exosite-1 to VWF allosterically activated the M domain to facilitate proteolysis (77).

The distal T-CUB domains of ADAMTS13 interact with its S domain, resulting in a “closed” conformation with low activity, as demonstrated by the electron microscopy (83) and small-angle X-ray techniques (84). This autoinhibition is relieved by the binding of VWF D4CK or monoclonal antibodies to the distal domains or mutating five residues in S domain (R568K/F592Y/R660K/Y661F/Y665F), resulting in an “open” conformation and increasing the proteolytic activity ~2.5-fold (**Figure 2B**). Such mutated ADAMTS13 is termed as GoF-ADAMTS13 (85).

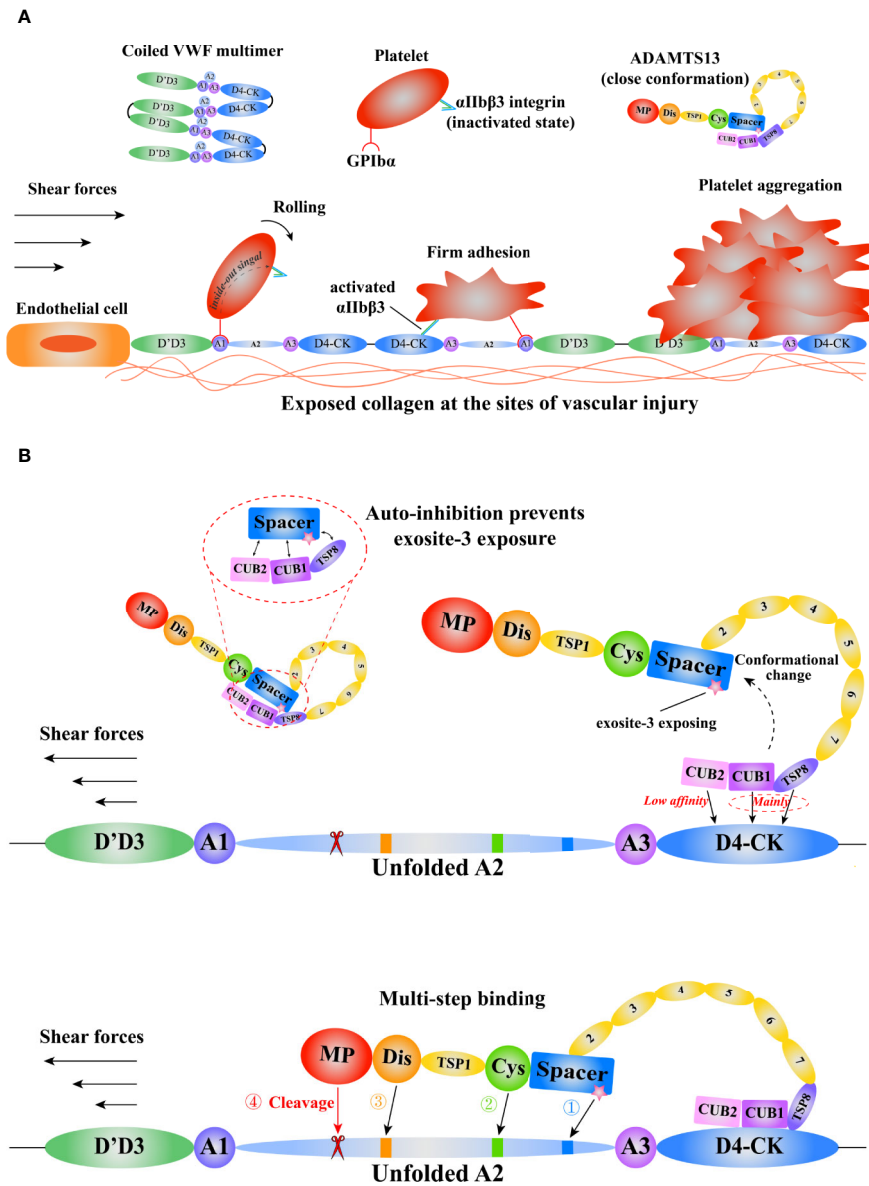


FIGURE 2 | A schematic diagram of the mechanisms of VWF and ADAMTS13 in thrombus formation. Adapted from Petri et al. (64). **(A)** The function of VWF in thrombosis. The coiled VWF multimers tether to the exposed subendothelial collagen at sites of vascular injury. Then, the globular VWF is gradually stretched into an elongated state under tension. Subsequently, the platelet receptor GPIIb/IIIa binds to the VWF A1 domain to induce the rolling of platelet, and activate the integrin αIIbβ3 that interacts with D4-CK domains resulting in firm adhesion, promoting platelet aggregation. **(B)** The model of VWF-mediated conformational change of ADAMTS13 and subsequent multi-step binding process. The circulating ADAMTS13 in a closed conformation prevents the exposure of the exosite-3 in Spacer domain. This auto-inhibition of ADAMTS13 is relieved mainly through the interactions between ADAMTS13 TSP8-CUB1 domains and VWF D4-CK domains. Then, the high shear forces and flexible TSP type 1 repeats (T1-T8) assist the “open” ADAMTS13 to get close to the unfolded VWF A2 domain. After a multi-step binding, the MP domain recognizes and cleaves the scissile bond Y¹⁶⁰⁵-M¹⁶⁰⁶ in A2 domain. VWF, von Willebrand Factor; GPIIb/IIIa, platelet glycoprotein IIb/IIIa; ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs, member 13; TSP, thrombospondin; CUB, complement C1r/C1s, Uegef (epidermal growth factor-related sea urchin protein), and Bmp1 (bone morphogenetic protein 1); MP, metalloproteinase.

By scanning WT- and GoF-ADAMTS13 molecules with atomic force microscopy (AFM), we proposed that ADAMTS13 might exist an additional “intermediate” conformation (86). However, further investigations are needed to reveal the physiological relevance of this conformation. South et al. reported that the GoF-ADAMTS13 and WT-MDTCS construct were able to

cleave human fibrinogen and suggested that ADAMTS13 in the “closed” conformation restricted its specificity to prevent off-target proteolysis (83, 87). Three flexible linker segments between T2/T3, T4/T5, and T8/CUB1 contribute to ADAMTS13 conformation modulation. The removal of these three flexible segments increases the recognition of the cryptic epitope in M

domain by the antibody 6A6, indicating that these three flexible segments modulate the binding of the distal domain to the proximal domain (88). South et al. proposed a model by integrating these findings to depict the conformational activation of ADAMTS13 by VWF (89).

Open ADAMTS13 conformation is found not only during acute acquired TTP (90), but also in patients in remission (91). Thus, open ADAMTS13 is proposed as a hallmark of acute acquired TTP and a novel biomarker to detect subclinical immune-mediated TTP in patients in remission as well. However, how the enhanced proteolytic activity of open ADAMTS13 is inhibited in acute acquired TTP patients remain elusive. It is possible that the exposure of cryptic epitopes in open ADAMTS13 may allow the binding of anti-spacer autoantibodies, thereby inhibiting ADAMTS13 function or inducing clearance of the resultant immune complexes. Designing new drugs to modulate ADAMTS13 conformation or prevent autoantibody binding might provide potential strategies for treating TTP.

Mechanical Force Modulates the Proteolysis of VWF by ADAMTS13

It has been well established that blood flow mediates the recognition and cleavage processes of VWF by ADAMTS13 (73). As mentioned above, high shear stress unfolds VWF A2 domain to expose the scissile bond Tyr1605-Met1606 for ADAMTS13 cleavage, down-regulating the activity of VWF multimers and preventing excessive platelet aggregation. By the use of single-molecule techniques, we and other groups have demonstrated that A2 unfolding is the prerequisite for ADAMTS13 cleavage (92–94). Recently, we reported, for the first time, that the interactions between VWF and ADAMTS13 were modulated by mechanical forces, exhibiting multiple bond characteristics: slip bonds, catch bonds, biphasic bonds, and triphasic bonds (73). These dynamic bonds might be critical, as such interaction can be mechanically strengthened or weakened by the formation of various dynamic bonds between different binding sites in circulation. We also proposed a novel computer strategy combining the steered molecular dynamics simulation and flexible docking techniques to investigate the binding of S domain to $\alpha 6$ helix of VWF A2 with various extensions. The data demonstrated a biphasic extension-regulated binding of $\alpha 6$ helix to S domain, suggesting that S domain prefers to bind partially extended $\alpha 6$ helix (81).

THE NET-VWF AXIS IN IMMUNOTHROMBOSIS PROVIDES NOVEL POTENTIAL THERAPEUTIC STRATEGIES

Mechanisms of NET-Induced Thrombosis

In recent years, NETs have been recognized as an important player in thrombosis (95, 96). NETs are found in thrombi from patients with venous or arterial thrombosis (96). The prothrombotic effect of NETs presents in triggering the

coagulation pathway and platelet aggregation. NETs provide a scaffold to recruit red blood cells, platelets and leukocytes, as well as to bind plasma proteins, such as VWF and fibronectin (67). This scaffold also supports fibrin deposition by binding with fibrinogen. In addition, two primary components of NET structure, DNA and histone, are involved in the coagulation pathways. Cell-free DNA derived from NETs mediates thrombin generation in FXII- or FXI-dependent pathway but not in tissue factor (TF) related pathway in patients with sepsis (64). Digestion or precipitation of DNA networks markedly diminishes their procoagulant effects. Histones induce thrombin generation in platelet rich plasma (PRP) by activating platelets *via* TLR2 and TLR4, which is driven by poloP, an activator in clotting cascade secreted by activated platelets, in a FXII-independent manner (97). In addition, histones, especially H4, directly interact with platelets and activate α IIb β 3 integrin on platelet surface inducing subsequent fibrinogen mediated platelet aggregation (98). Histones also induce platelet micro-aggregation in an α IIb β 3-independent fibrinogen-dependent manner. Histones cause severe tissue damage and thrombocytopenia, and induce death in mice, which could be prevented by treatment with heparin (98). Interestingly, intact NETs exhibit weaker procoagulant effect than individual DNA and histone (99). The serine proteases NE and CG promote tissue factor- and factor XII-dependent coagulation and thrombus growth by counteracting endogenous anticoagulants (100).

TF is a key initiator of the extrinsic coagulation cascade. TF activates FVII to form TF/FVIIa complex that subsequently activates FIX and FX mediating ensuing thrombin and fibrin generation (101). Neutrophils immediately adhere to endothelial cells through the interaction between leukocyte function antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) after laser-induced injury, and then express TF at the cell surface promoting fibrin generation and platelet aggregation *in vivo* (102), suggesting that neutrophils are a potential source of functional TF at the site of vascular injury. In addition, NETs also activate endothelial cells, inducing the elevated expression of vascular cell adhesion molecule-1 (VCAM-1), ICAM-1, and TF through interleukin-1 α (IL-1 α) and CG which could transform pro-IL-1 α to mature IL-1 α form (103). This NET-induced TF expression in endothelial cell surface accelerates plasma clotting *in vitro*. Elevated TF expression in neutrophils and the release of NETs decorated with TF have been found in patients with sepsis (104), AAV (105), ST-segment elevation acute myocardial infarction (STEMI) (106), SLE (107), and COVID-19 (108). In the courses of these diseases, neutrophils act as a pool of active TF. Neutrophilic TF is exposed by the formation of NETs triggered by activated platelets or stimuli in the pathological environment, which mediates localized thrombin generation and protease activated receptor-1 (PAR-1) signal-dependent platelet activation, promoting the formation of thrombus in these diseases. Neutrophils derived from healthy donors can form TF-bearing NETs stimulated by serum from these patients but not by PMA (103) or *Escherichia coli* (104), suggesting that elevated TF expression in neutrophils is mediated by special components in the pathological environment, such as cytokines in sepsis (104) and IgG in AAV (105).

The interaction between platelets and neutrophils plays a critical role in NET-induced thrombosis. Platelets interact with neutrophils to induce NETosis *via* the engagement of P-selectin/PSGL-1, the binding of neutrophil β_2 integrins with GPIIb/IIIa on platelet surface (109), and the release of soluble mediators, such as platelet factor 4 (PF4/CXCL4) (110) and high-mobility group box 1 (HMGB1) (111). HMGB1 enhances neutrophil recruitment and regulates NET formation (111, 112). Heterodimerization of CXCL4 and CCL5 enhances NET formation *via* integrin outside-in and G-protein-coupled receptor (GPCR) signaling (113). NETs in turn recruit and activate more platelets. By increasing the expression of platelet P-selectin (114) or activating TLR2 and TLR4 (97), histones induce platelet activation. Histones also associate with platelets to induce platelet aggregation *via* the stimulation of calcium influx and recruitment of plasma adhesion proteins (98). A previous study by Sandra Grässle et al. in 2014 revealed that the isolated DNA of NETs directly interacts with platelet released VWF (14).

NETs are prothrombotic and play a critical role in thrombosis. Extensive reviews are provided elsewhere (96, 115). We will discuss the interactions among NETs, VWF, and ADAMTS13 in mediating immunothrombosis in diseases below.

Mechanisms of NET-VWF Interaction and the Prothrombotic and Proinflammatory Effects

Pure DNA can directly bind to VWF A1 domain *via* electrostatic interactions (116), and this DNA-VWF interaction can be blocked by heparin (14). Notably, only activated VWF A1 domain can interact with isolated DNA and this interaction does not block the VWF cleavage by ADAMTS13. Preincubation of DNA significantly impairs the adhesion of platelets on the VWF coated surface under flow conditions, indicating that GPIIb/IIIa binding site in VWF A1 domain is blocked by DNA. However, this blockage can be eliminated under physiological conditions, in which erythrocytes are present, in a hematocrit-dependent manner. *S. aureus*- or PMA-stimulated neutrophils adhere to VWF coated surface in microfluidic experiments, but this adhesion is completely abolished by the preincubation of heparin or the treatment of DNase I (14), suggesting that neutrophils can be recruited to vessel wall *via* the VWF-DNA interaction *in vitro*, apart from the reported binding of VWF/PSGL-1 and VWF/ β_2 integrin (66). A recent study reported that three arginine residues, Arg1392, Arg1395, and Arg1399, in helix 4 of the VWF A1 domain were the main binding sites for double-strand DNA (dsDNA). VWF-dsDNA interaction depended on ionic strength and shear stress-mediated VWF activation, but not nucleotide length and sequence (117). In contrast to abundant dsDNA adhered to the WT-VWF coated surface under high shear stress, almost no dsDNA adhesion was observed on the VWF R1399A mutant coated surface under the same shear stress, indicating that Arg1399 residue in VWF A1 is essential to the DNA-VWF interaction (117). Furthermore, purified soluble histone can also bind to VWF by electrostatic forces and this binding does not influence VWF adhesive activity

(118). Neutrophil elastase, which contains positively charged side chain residues (119), is likely to bind to VWF *via* electrostatic interactions. Neutrophil elastases released from NETs still attached to the wall of liver vasculature after the treatment of DNase I in mice with methicillin-resistant *S. aureus* infection (15). Blocking VWF by antibody or cleaving VWF by ADAMTS13 did not affect neutrophil recruitment, but markedly prevented the accumulation of DNA fibers, decreased the amount of histone and elastase, and attenuated the tissue damage in liver sinusoid, supporting that the reduction of hepatic damage was due to the disruption of NET-VWF interaction (15). In a mouse model of hepatic ischemia-reperfusion injury, real-time intravital imaging demonstrated that leukocyte-vessel wall interactions in VWF knockout mice were significantly less than those in wild-type mice, supporting that VWF is a key mediator to recruit leukocytes to the sites of vascular damage (120). Less hepatic damage and better outcome were observed in VWF knockout mice or ADAMTS13 administered wild-type mice (120). In addition to endothelial cell-derived VWF, platelet-derived VWF is also associated with NETs. Carestia et al. reported that platelet-mediated NETs were significantly inhibited by blocking VWF released from activated platelets (110). They proposed that platelet-derived VWF might serve as a bridge that links activated platelets close to neutrophils, resulting in more platelet-mediated NET formation.

Previous studies have demonstrated that both VWF and NETs have proinflammatory effect (121, 122). In this point of view, the NET-VWF network, which attaches NET structure and recruits more leukocytes to stimulated endothelium, is likely not only to facilitate leukocyte infiltration into the surrounding tissue but also to amplify the proinflammatory effects of NETs. Thus, it is conceivable that VWF released from endothelial cells and platelets interacts with NETs to promote the progression of thrombosis and inflammation.

NETs Mediating VWF and ADAMTS13 Activities May Form a Vicious Circle to Aggravate the Phenotype of Thrombotic Microangiopathies

NET contents mediate the activities of both VWF and ADAMTS13. Reduced ADAMTS13 activity concomitant with increased plasma VWF levels has been observed in thrombotic microangiopathies (TMAs) associated with systemic inflammation, such as in severe sepsis, sepsis-induced disseminated intravascular coagulation (DIC), malignancy, and autoimmune diseases (123). The mechanism is not fully understood, but activated neutrophils and NETosis may contribute to the deficiency of ADAMTS13. During systemic inflammation, activated neutrophils or NETosis release various cytokines, proteases, peptides, and ROS, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). Several inflammatory cytokines have been found to regulate the release and cleavage of UL-VWF under flow conditions (124). Interleukin 8, tumor necrosis factor α (TNF- α), and IL-6/IL-6 receptor complex stimulate the release of UL-VWF multimers, leading to VWF-platelet string formation on endothelial cell-

cultured surface under flow. Notably, IL-6 inhibits the cleavage of UL-VWF by ADAMTS13 under flow but not in static conditions. In patients with sepsis-induced DIC, low molecular weight forms of ADAMTS13 are detected in plasma, indicating that ADAMTS13 deficiency may be due to the proteolysis of proteases in plasma apart from the impairment of biosynthesis (125). Neutrophil elastase and plasmin inactivate ADAMTS13 *via* proteolysis *in vitro* (125, 126) but not in the presence of human plasma under static conditions (127). Similarly, leukocyte proteases released from activated neutrophils or NETosis, including NE, PR3, CG, and MMP-9, cleave plasma-derived VWF at or near the ADAMTS13 cleavage site in a dose-dependent manner under shear stress *in vitro* (128). MPO catalyzes the generation of HOCl from H₂O₂ and Cl⁻ leading to oxidant tissue injury (129). Chen et al. reported that HOCl oxidized Met1606 at the ADAMTS13 cleavage site in VWF A2 domain, converting methionine to methionine sulfoxide, resulting in the oxidation of both VWF A2 peptide and plasma VWF multimers (130). These oxidized substrates were more difficult to be cleaved by ADAMTS13, indicating that this oxidative modification markedly impairs ADAMTS13 cleavage (130). Interestingly, HOCl generated by the MPO-H₂O₂-Cl⁻ system also oxidized methionine in ADAMTS13 and significantly inactivated ADAMTS13 to cleave VWF A2 peptide and plasma VWF multimers under denatured conditions (127). Further analysis by using LC-MS/MS showed that total seven methionine in MDTCS domains of ADAMTS13 were oxidized, among which Met249, Met331, and Met496 were critical for ADAMTS13 activity, and the extent of oxidation of each methionine was positively correlated with the reduction of ADAMTS13 activity. Notably, the oxidized ADAMTS13 could be further oxidized by HOCl with high concentration or activated neutrophils in the presence of 0.5% human plasma, resulting in marked activity loss. Even in the presence of 90% human plasma, >30% of Met249 and Met496 in ADAMTS13 were oxidized. These findings suggested that oxidation of ADAMTS13 by HOCl and subsequent proteolytic function impairment may occur in physiological conditions. Taken together, both VWF substrate and ADAMTS13 can be oxidized by MPO-H₂O₂-Cl⁻ system released from activated neutrophils or NETosis, leading to decreased ADAMTS13 activity and increased plasma VWF levels, eventually resulting in excessive platelet aggregation and occlusion at sites of vascular injury.

Human neutrophil peptides (HNPs), also known as α -defensins, play an important role in innate immune system (131). HNPs, which not only have proinflammatory effects but also enhance coagulation by activating platelets (132) and inhibiting fibrinolysis (133), have been observed abundantly in human atherosclerotic arteries (134) and increase in hyperlipidemia patients with coronary heart disease (135). HNPs released from activated neutrophils and NETosis also modulate the activities of both VWF and ADAMTS13. HNPs, mainly HNP1, HNP2, and HNP3, have antimicrobial properties and play an important role in innate immune response (136). Pillai et al. reported that HNPs dramatically inhibited the cleavage

of FRET-VWF73 substrates and VWF multimers by ADAMTS13 *in vitro* (131). It might be due to HNPs and spacer domain of ADAMTS13 having the same RRY motif. HNPs competitively bound VWF A2 and blocked the interaction between ADAMTS13 and VWF, thereby impairing ADAMTS13 cleavage. Increased plasma levels of HNPs were observed in patients with acute TTP, implying the association between inflammation and ADAMTS13 deficiency in acute TTP (131). In patients with traumatic brain injury, high plasma levels of HNPs and VWF, concomitant with decreased plasma ADAMTS13 activity, associate with increased mortality (137). Surprisingly, HNPs also exhibit antithrombotic effects. HNP1 inhibits the adhesion and aggregation of murine platelets on the collagen-coated surface or on TNF- α activated endothelial cells under arterial shear stress in the absence of ADAMTS13 (138). Under arterial flow, VWF multimers are activated by high shear stress and expose the free cysteine thiols that facilitate VWF-VWF lateral association by forming new covalent disulfide bonds (139), leading to the formation of larger and thicker VWF multimer networks and the recruitment of more blood cells. HNP1 inhibits the formation of UL-VWF strings *via* the direct binding of its free cysteine thiols to free cysteine thiols in VWF (138).

During NET formation, PAD4, which converts positively charged arginine residues in histones to neutral citrulline residues (140), is essential for chromatin decondensation, and subsequently released with NETs. Recently, Sorvillo et al. demonstrated that PAD4 citrullinated plasma ADAMTS13 on specific arginine residues, resulting in a dramatic reduction of ADAMTS13 enzymatic activity and thus leading to increased VWF-platelet string formation in mesenteric venules of mice (141). PAD4 also decreased the time to vessel occlusion and markedly reduced thrombus embolization in murine mesenteric venules underwent ferric chloride-induced injury. Citrullinated ADAMTS13 was detected in plasma from healthy donors, septic patients and donors with other comorbidities, suggesting ADAMTS13 is citrullinated *in vivo* (141).

DNA-histone complexes released from NETosis or other forms of cell death are also associated with acute TMAs. In patients with acute TTP, elevated levels of DNA-histone complexes and MPO are detected compared to those in healthy donors, and these increased levels are inversely correlated with platelet counts, implying that DNA-histone complexes and MPO may contribute to thrombopenia (142). Zheng et al. successfully established novel ADAMTS13^{-/-} zebrafish lines that exhibited spontaneous but mild feature of TTP (143). They found that lysine-rich histone induced a more severe and persistent TTP phenotype and higher mortality rate in ADAMTS13^{-/-} than in wild-type zebrafish, indicating that histone might trigger and aggravate TTP in individuals with severe ADAMTS13 deficiency. Increased plasma levels of VWF antigen were also observed in ADAMTS13^{-/-} zebrafish after treatment with histone. Both spontaneous and histone-induced TTP phenotypes were effectively prevented in ADAMTS13^{-/-} *via* VWF gene knock-out, suggesting that VWF might be a potential therapeutic target in inflammation-induced acute TTP phenotype (143).

Taken together, contents released from activated neutrophils and NETosis, on the one hand, inhibit ADAMTS13 activity by oxidation, citrullination, proteolysis or competitively binding to VWF A2 substrate, resulting in the increased concentration of VWF antigen, promoting the formation of UL-VWF multimers and their prothrombotic effects at sites of vascular injury. On the other hand, like ADAMTS13, several proteases cleave VWF, exhibiting their antithrombotic effects. However, whether these antithrombotic effects work *in vivo* is uncertain. These findings shed light on the relationship between inflammation and ADAMTS13 deficiency in TMAs. The interaction between NETs and VWF retains abundant NET components and activated neutrophils on damaged vessel wall. More granulocyte contents are in close proximity to VWF and VWF-bound ADAMTS13, and exert their prothrombotic effects to promote the development of thrombosis. This NET-VWF interaction may lead a vicious cycle: NET component-mediated deficiency of ADAMTS13 promotes the formation of UL-VWF multimers which in turn recruit and attach more activated neutrophils and NET components to vessel wall, aggravating the symptom of inflammation and thrombosis (Figure 3). Therefore, targeting NET-VWF axis by the administration of recombinant human ADAMTS13 and/or DNase I, maybe a potential therapeutic strategy for TMAs and other systemic inflammatory diseases.

NET-VWF Axis Is a Potential Therapeutic Target for Acute Ischemic Stroke

NETs and VWF play a critical role in the pathophysiology of acute ischemic stroke (AIS) (144, 145). AIS is caused by occlusion and stenosis of internal carotid artery (ICA) or vertebral artery, which obstruct cerebral blood flow and eventually result in brain damage. AIS causes massive death worldwide and only two treatments are approved by the FDA to date (1): mechanical removal of thrombus *via* endovascular thrombectomy, and (2) pharmacological thrombolysis by using t-PA that promotes degradation of fibrin in thrombus by activating endogenous plasminogen (146). However, endovascular treatment is not always available and t-PA therapy is limited by the narrow therapeutic time window (4.5 h after stroke onset). It has been proposed that NETs contained in thrombi may be one of the reasons for treatment failure. Abundant neutrophils and NETs are observed in almost all thrombi retrieved from patients with ischemic stroke (147). Removing NETs by DNase I or impeding NET formation by Cl-amidine, a PAD4 inhibitor, significantly inhibited arterial thrombosis in ischemic mouse brain and ameliorated stroke outcome, such as decreasing the infarct volume and maintaining the basal blood flow level (146). *In vitro* experiments showed that addition of extracellular DNA and histones to fibrin increased the thickness, stiffness and

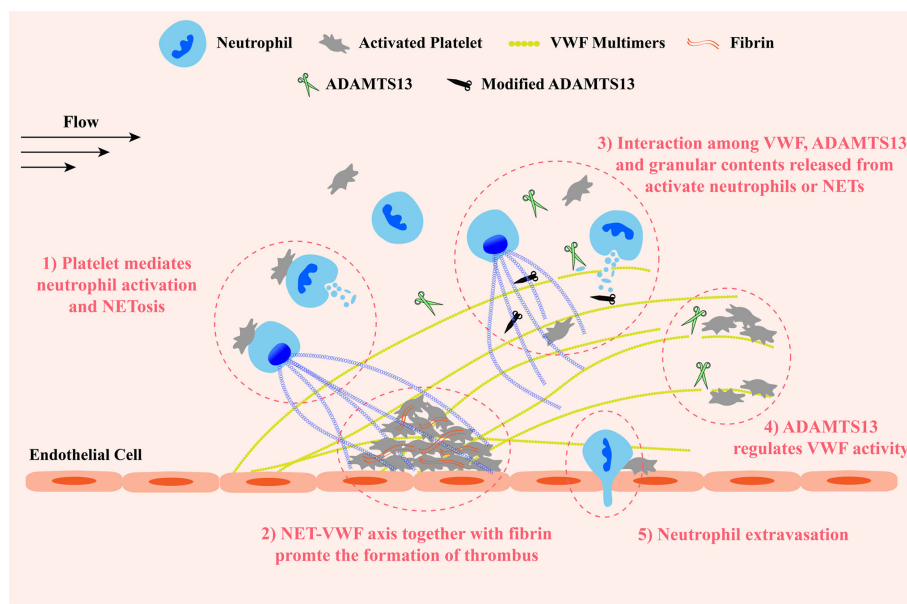


FIGURE 3 | A schematic diagram of the VWF-NET axis in thrombus formation and tissue damage at sites of vascular injury. Upon damage, VWF multimers are released from endothelial cells and capture circulating platelets to injury sites. VWF multimers recruit blood cells including neutrophils and erythrocytes. ADAMTS13 cleaves excessive VWF multimers to regulate their activity preventing the growth of thrombus. 1) The interaction among neutrophils, platelets, and VWF forms a vicious cycle to promote the development of thrombosis and inflammation: platelets mediate neutrophil activation and NETosis, then NETs and granular proteins released from activated neutrophils are retained to the vessel wall by directly interacting with VWF and exert prothrombotic and proinflammatory effects, inducing the release of VWF multimers and promoting platelet adhesion in turn. 2) Both NETs and VWF modify fibrin networks enhancing their procoagulant activity and the resistance of fibrinolysis providing insight into potential therapeutic targets of rt-PA resistance thrombi. 3) Granular components released from activated neutrophils and NETosis not only inactivate ADAMTS13 *via* chemical modification or competitive combination but also cleave VWF. 4) ADAMTS13 binds and cleaves VWF multimers to prevent excessive platelet aggregation. 5) Neutrophils extravagates into the intima with the help of VWF-platelet complex. VWF, von Willebrand Factor; ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs, member 13.

stability of the fibrin network, making the clot harder to be dissolved by t-PA, but the treatment with both DNase I and t-PA showed significant clot lysis (148). These findings were further supported by Ducroux et al. (149). They found that NET content in thrombi from AIS patients was positively associated with endovascular procedure length and device number of passes, and targeting NETs by DNase I accelerated t-PA-induced thrombolysis in *ex vivo* assay. These results suggested that NETs alter the mechanical properties of thrombus, increasing the resistance of thrombus to endovascular treatment and recombinant t-PA (rt-PA) therapy (149). Recent clinical analysis also supported that high numbers of NETs enhanced the compactness and stability of stroke thrombi, making the vessels harder to be recanalized and resulting in worse clinical outcome (150).

It has been reported that VWF is incorporated into fibrin network *via* covalently crosslinking by factor X IIIa (151) or in a thrombin-dependent manner (50). Thrombi from ischemic stroke patients contain on average $20.3 \pm 10.1\%$ of VWF (152), and VWF (especially endothelial cell-derived VWF) is proved to mediate ischemic stroke by promoting postischemic thromboinflammation in a brain ischemic/reperfusion injury model (153). Infusion of recombination human ADAMTS13 markedly dissolved the t-PA resistant thrombi in a dose-dependent manner, resulting in reduced cerebral infarct sizes in a mice model with $FeCl_3$ -induced middle cerebral artery occlusion (152). AIS patients with low ADAMTS13 activity may have poor early neurological improvement after rt-PA therapy, implying that ADAMTS13 favors rt-PA to perform its thrombolytic effect (154). A recent study further analyzed the components of the thrombi from ischemic stroke patients at the molecular and cellular levels (12). In sections of stroke thrombi, leukocytes and extracellular DNA mainly presented in platelet-rich regions and the boundary areas between platelet-rich and red blood cell-rich regions (12). Interestingly, in addition to abundant leukocytes and extracellular DNA, the interaction between dense thin fibrin and VWF was observed and platelets were filled within these fibrin-VWF structures in platelet-rich regions (12). VWF serves as a mediator to bring NETs and fibrin into close proximity, facilitating NETs to modify fibrin structure and to perform its procoagulant effect. rt-PA is the only thrombolytic drug approved by the FDA for ischemic stroke, but it works only for less than half of patients (12, 152). The reason for the rt-PA resistance is not fully understood, but it is believed that excessive NETs presented in thrombus are the critical factor. Targeting the NET-VWF axis seem to be a promising and effective intravenous thrombolysis therapy than existing rt-PA treatments in rt-PA-resistant thrombi, as Staessens et al. have discussed (12).

The reason for excessive NET formation in AIS thrombus is unclear, however, it is reasonable to speculate that platelets play an important role in uncontrolled NETosis and the following rt-PA resistance in stroke thrombus. Denorme et al. had discussed that VWF-GPIIb α is a thrombo-inflammatory axis in ischemic stroke (155). Endothelial cell-bound VWF activated by hydrodynamic force mediates initial platelet adhesion *via* the binding of its A1 domain to platelet GPIIb α to allow subsequent

platelet activation and aggregation. In contrast to its direct binding to endothelial cell *via* P-selectin/PSGL-1 interaction under venous low shear stress, leukocytes are recruited to endothelial surface under arterial high shear stress by the ideal substrate, VWF-platelet complex (65). This complex can also alter vascular permeability and thus promote leukocyte extravasation (156, 157). Breaking the interaction between VWF and GPIIb α markedly reduced brain injury and improve functional outcome in murine stroke model (155). Similarly, NET formation mediated by platelet TLR4 promoted the growth of arterial thrombus in an ischemic stroke mouse model (146). DNase I responded thrombi from patients with AIS had denser platelet distribution than those DNase I nonresponded ones. Thrombi with higher platelet density showed more weight loss after DNase I treatment. These results implied that the amount of NETs is associated with the density of platelets in stroke thrombi. Moreover, a recent study reported that interactions between NETs and activated platelets played an important role in the hypercoagulability of stroke patients with ICA occlusion (158). Plasma from carotid lesion site in stroke patients not only induced platelet activation in a thrombin-dependent manner, but also exposed phosphatidylserine (PS, a critical catalyst in the coagulation cascade) on the surfaces of platelets, platelet-derived microparticles (PMPs) and neutrophils. Activated platelets mediated the formation of NETs decorated with PS. These PS-bearing NETs were observed in the carotid thrombus and provided a platform to bind PMPs and coagulation factors, including prothrombin, fibrinogen, and Factor X, and thus promoted thrombin and fibrin formation (158).

Taken together, both NETs and VWF can not only recruit platelets and leukocytes, but also promote clot coagulation and leukocytes infiltration. It is tempting to speculate that NET-VWF interaction provides a larger and more stable network structure for the recruitment of blood cells and the binding of coagulation factors at sites of vascular injury under flow, facilitating the interactions among the components, such as platelets/leukocytes/NETs and fibrin/VWF/NETs, and thus promoting the development of thrombosis and inflammation, aggravating vessel and organ damage (Figure 3). Therefore, targeting the NET-VWF axis by DNase I and/or ADAMTS13 is a potential therapeutic strategy for AIS.

NET-VWF Axis Is a Potential Therapeutic Target in COVID-19

Recently, COVID-19, which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (159), is a global health care challenge, with rapid spread and high mortality (160). COVID-19 is characterized by acute respiratory distress syndrome (ARDS), in which, acute respiratory failure, endothelial injury, immunothrombosis, and imbalance between coagulation and inflammation have been reported (161, 162). Zuo et al. reported that high levels of NETs were found in sera from patients with COVID-19, and these sera could trigger the formation of NETs from neutrophils collected from healthy volunteers *in vitro* (160). A further study proved that SARS-CoV-2 could directly trigger NET

formation *via* an ACE2-serine protease TMPRSS2 dependent pathway and induce lung epithelial cell death *in vitro* (163). Nicolai et al. demonstrated that in COVID-19 patients, inflammatory microvascular thrombi containing NETs were present in lung, kidney, and heart (164). Middleton et al. also demonstrated that elevated levels of PF4 and RANTES, which were demonstrated to trigger NETosis, were detected in plasma from COVID-19 patients and COVID-19 plasma-induced NET formation was inhibited by neonatal NET-Inhibitory Factor (nNIF), indicating that NETs contributed to immunothrombosis in COVID-19 patients (165). In addition, thrombocytopenia, hyperactive coagulation, pulmonary damage, acute cardiac and kidney injuries were observed in patients with severe COVID-19 (166–168). Barnes et al. observed extensive neutrophil infiltration in pulmonary capillaries from the autopsy specimen of a COVID-19 patient and proposed that therapies targeting NETs might alleviate the severity of COVID-19 (167). NET formation, which results in the production of inflammatory cytokines, might contribute to cytokine storm and worse outcome of COVID-19 (167, 169). COVID-19 and NET-associated diseases share common manifestations, it thus is reasonable to propose that NETs may be a novel therapeutic target for COVID-19 (167). Dornase alfa, the recombinant human DNase I, is FDA-approved for cystic fibrosis treatment (170). It is also used off-label as mucolytic in other diseases, such as ARDS, and may be beneficial in the context of COVID-19 (171). Nine such clinical trials are registered (NCT04432987, NCT04387786, NCT04409925, NCT04359654, NCT04445285, NCT04402944, NCT04355364, NCT04402970, and NCT04459325), including two trials are in phase 3 (NCT04355364 and NCT04402970) and one trial is completed (NCT04459325).

Along with NETs, VWF and ADAMTS13 are also reported to be involved in COVID-19. Ladikou et al. observed high levels of VWF and coagulation factor VIII that released from injured endothelial cells could contribute to hypercoagulability and the elevated rate of venous thromboembolism (VTE) in COVID-19 patients, suggesting that VWF could be used for the hierarchy of endothelial damage and thrombotic risk (172). By assessing the markers of endothelial cell and platelet activation, Goshua et al. demonstrated that mortality of COVID-19 patients was correlated with VWF antigen and soluble thrombomodulin (173). In addition, the reduction of ADAMTS13 was observed in COVID-19 patients and proposed to use to predict mortality (174). However, normal ADAMTS13 activity was also observed in COVID-19 inpatients (175).

Although the involvement of NETs and VWF in endothelial damage and COVID-19 has been elucidated, no direct evidence yet demonstrates their interactions contributing to COVID-19 progression. Further studies are necessary to shed new light on the interactions and the mechanisms of NET-VWF in COVID-19, and provide the potential therapeutic strategies.

CONCLUSIONS

The field of NETosis is in an exciting phase. Most of the literature mainly focuses on which stimuli can induce NET formation and

which proteins can inhibit the process. The well-defined singling pathway that is distinct from other cell death pathways remains elusive. In addition, the initiation of NETosis is still an open question: what determines NETosis to occur in response to the stimulation that also triggers phagocytosis and degranulation. Recently, Xie et al. identified eight neutrophil populations by profiling >25,000 differentiating and mature mouse neutrophils using single-cell RNA sequencing (176). Further investigations are needed to reveal whether different neutrophil populations possess different capabilities to generate NETs in response to stimuli. New findings would allow us to better define the physiological significance of NETosis.

Over the past decade, abundant elegant animal and human studies have demonstrated that NETs contribute to thrombus formation and propagation in arterial, venous, and cancer-associated thrombosis. The interactions between NETs and VWF form a larger and thicker net-work accelerating the immunothrombosis in diseases. Combinational usage of DNase I and ADAMTS13 is therefore a potential therapeutic strategy. Several studies have investigated the bindings of VWF A1 to NET DNA and histone. Further studies are needed to provide more insights into the basis of the molecular structure of these bindings. The new findings might facilitate the designs of new drugs selectively against the NET-VWF binding, which might be crucial for the patients with acquired TTP. Notably, the conformation and function of VWF are regulated by the blood flow. Whether and how the blood flow alters the binding between NETs and VWF is an interesting question and less explored. Apart from the NET-VWF interaction, we extensively review the direct or indirect inactivation of ADAMTS13 by granulocyte contents released from activated neutrophils or NETosis. It is interesting to investigate whether the modifications of ADAMTS13 by NETs modulate its conformation and thus the enzymatic activity.

The interplay among NET, VWF and ADAMTS13 might form a vicious cycle, resulting in reduced ADAMTS13 activity and subsequently elevated plasma levels of VWF, which is positively correlated with severity and mortality in TMAs, AIS, and COVID-19. Targeting the NET-VWF axis may pave a new road to therapeutic strategies for immunothrombosis in diseases.

AUTHOR CONTRIBUTIONS

JL and JY designed the paper. JL, JY, ZW, QL, JH, TH, and WL wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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