DR WOLFRAM RUF (Orcid ID : 0000-0002-6064-2166)

Article type : Review Article

Tissue Factor at the crossroad of coagulation and cell signaling

Short title: TF in coagulation and signaling

Hortensia Zelaya[*†], Andrea Rothmeier[‡], Wolfram Ruf [*‡$]

[*]Center for Thrombosis and Hemostasis, Johannes Gutenberg University Medical Center Mainz, Germany.

[†]National Scientific and Technical Research Council (CONICET) and National University of Tucumán, Tucumán, Argentina.

[‡]Department of Immunology and Microbiology, The Scripps Research Institute, La Jolla, CA, U.S.A.

[$]German Center for Cardiovascular Research (DZHK), Partnersite Rhein-Main

Correspondence:

Wolfram Ruf, MD

Center for Thrombosis and Hemostasis

Johannes Gutenberg University Medical Center

Langenbeckstr. 1

55131 Mainz

Germany

Tel: +49-6131-17-8222

Email: ruf@uni-mainz.de

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jth.14246

Summary

The tissue factor (TF) pathway plays a central role in hemostasis and thrombo-inflammatory diseases. Although structure-function relationships of the TF initiation complex are elucidated, new facets of the dynamic regulation of TF's activities on cells continue to emerge. Cellular pathways that render TF non-coagulant participate in signaling of distinct TF complexes with associated proteases through the protease-activated receptor (PAR) family of G-protein coupled receptors. Additional co-receptors, including the endothelial protein C receptor (EPCR) and integrins, confer signaling specificity by directing subcellular localization and trafficking. We here review how TF is switched between its role in coagulation and cell signaling through thiol-disulfide exchange reactions in the context of physiologically relevant lipid microdomains. Inflammatory mediators, including reactive oxygen species, activators of the inflammasome, and the complement cascade play pivotal roles in TF procoagulant activation on monocytes, macrophages and endothelial cells. We furthermore discuss how TF, intracellular ligands, co-receptors, and associated proteases are integrated in PAR-dependent cell signaling pathways controlling innate immunity, cancer, and metabolic inflammation. Knowledge of the precise interactions of TF in coagulation and cell signaling is important for understanding effects of new anticoagulants beyond thrombosis and identification of new applications of these drugs for potential additional therapeutic benefits.

Keywords: Endothelial protein C receptor; Hemostasis; Protein Disulfide-Isomerases; Proteinase-Activated Receptors; Thrombosis

Introduction

The isolation of tissue factor (TF) protein and the subsequent cloning of the coding sequence by three independent laboratories in 1987 revealed the evolutionary origin of TF as a member of the cytokine receptor family and set the stage for a rapid elucidation of the structure function relationships of the TF-FVIIa coagulation initiation complex. The interactions of macromolecular substrates FX and FIX have been mapped to some extent by mutagenesis and modeling [1] and structural information of prototypic inhibitor interactions with the TF-FVIIa complex [2, 3]. Despite this initial progress in understanding the structural basis for function of TF-initiated coagulation, novel posttranscriptional and posttranslational mechanisms have emerged that are crucial determinants for the distinct roles of TF in hemostasis and thrombosis. These pathways are also central for TF regulation, trafficking and localization in cells and therefore influence the pleiotropic functions of TF associated proteases in cell signaling involving the protease activated receptor (PAR) family of G-protein coupled receptors.

This review will focus on recent progress in three areas. First, we will discuss the role of TF expressed by vascular and extravascular cells in hemostasis and thrombosis. We will then address how inflammatory mediators and other stress signals feed into pathways that switch TF to a procoagulant form by thiol-disulfide exchange reactions in the context of physiologically relevant lipid microdomains. Lastly, we describe the progress made in our understanding of how TF, intracellular ligands, co-receptors, and associated proteases are integrated in PAR-dependent cell signaling pathways controlling innate immunity, cancer, and metabolic inflammation.

Cell type-specific roles of TF in thrombosis and hemostasis

TF is constitutively expressed in epithelia and perivascular barriers of brain, skin, lung, intestine and placenta. There is consensus that extravascular TF serves as a protective hemostatic envelope. In contrast, the function and cell-type specific expression of TF by intravascular cells,

including leukocyte populations, platelets and the endothelium, in pathophysiological processes remain incompletely understood. In addition, new insights into the biochemistry of TF-initiated coagulation and its regulation provide evidence for distinct pathways by which TF contributes to hemostasis versus thrombosis.

TF is traditionally viewed as the trigger, which provides limited amounts of thrombin for initiation of coagulation (see thin arrows in Figure 1A). Thrombin then activates the plasmatic cofactors V and VIII and enables coagulation amplification by FIXa generated by either TF-FVIIa, the FXII contact pathway or thrombin-activated FXIa (thick arrows in Figure 1A). These concepts were developed under static assay conditions in which thrombin rapidly accumulates and readily escapes dilution by blood flow and physiological neutralization by fibrinogen and the vessel wall. This led to an underappreciation of roles played by FXa in cofactor activation and its regulation.

Whole human blood and *in vivo* studies in rabbits with a tick-derived inhibitor specifically blocking FXa-mediated FV activation revealed a crucial role for FXa in generating an active prothrombinase complex [4] (thin arrows in Figure 1B). Several laboratories have also provided evidence that TF pathway inhibitor (TFPI) is the major physiological inhibitor that controls FXa-mediated activation of FV [5]. Whereas both splice isoforms, TFPIα and TFPIβ, inhibit TF-FVIIa, only TFPIα regulates prothrombinase formation by interaction of its positively charged carboxyl-terminus with an acidic FV B-domain exosite exposed by FXa cleavage or by increased alternative splicing in the East Texas bleeding disorder [6]. In addition, FV_{Leiden} is more resistant to control by TFPIα, contributing to the thrombosis risk in carriers of this prothrombotic mutation [7].

TFPIα inhibits FV activation in simulated reactions of TF-initiated coagulation, but, surprisingly, under the same conditions TFPIα did not interfere with FXa activation of the anti-hemophilic cofactor VIII [8]. The nascent product FXa can be trapped with a nematode inhibitor, NAPc2 [9]; in this complex FXa still activates FVIII, but not FV. Mutants of FVIIa capable of activating FX, but defective in releasing the newly formed product FXa, showed that the TF-FVIIa-FXa coagulation initiation complex directly activates both, cofactor VIII and enzyme FIX, leading to

thrombin generation in flowing blood (thick arrows in Figure 1B). Unlike the formation of an activate prothrombinase, this pathway was poorly inhibited by TFPIα and sufficient for fibrin formation in reconstituted whole blood under flow *ex vivo*. These experiments suggest that the hemostatic function of TF is triggered early and prior to amplified thrombin generation that requires undocking of FXa for generation of the prothrombinase. Imbalances in plasma exposure to active TF and circulating coagulation inhibitors, including TFPIα, thus favor direct prothrombinase generation by the TF pathway and intravascular thrombosis. This new concept of coagulation initiation by TF and the demonstrated distinct roles for TFPI in regulating TF initiation of the hemostatic FVIII pathway and the generation of prothrombotic prothrombinase raises important new questions on how anticoagulant drugs interfere with these distinct biochemical routes of the TF pathway.

Whereas epithelial cell-expressed TF has clearly been linked to the prevention of hemorrhage in a murine acute lung injury model [10] and arterial thrombosis following ferric chloride ($FeCl_3$) injury induced in mice involves vessel wall TF expressed by smooth muscle cells [11], the cellular sources of TF within the vascular compartment are diverse and TF prothrombotic activity is typically controlled by cellular activation, including for TF expressed by smooth muscle cells [12, 13]. Although TF expression by platelets has been discussed controversially, generation of platelet-like particles from *in vitro* differentiated human megakaryocytes demonstrates that platelets can carry both TF protein and mRNA in distinct subpopulations [14] and platelet activation can induce splicing of intron-retained TF mRNA for TF protein translation [15].

In mouse endothelial cells, increased reactive oxygen species (ROS) production following deficiency of the anti-oxidative protein paraoxonase-2 (PON2) causes an upregulation of TF activity. Coagulation abnormalities in *Pon*2-deficient mice are indicated by shortened clotting times *in vitro*, likely reflecting pre-activated coagulation factors, and are reversed by restoration of PON2 expression in endothelial cells [16]. Deficiency of sirtuin 3 (Sirt3), another anti-oxidative molecule inactivating superoxide dismutase 2 (SOD2), increases myeloid cell TF prothrombotic function in experimental murine thrombosis *in vivo* in the context of inflammation induced by endotoxin [17].

The previously underappreciated expression of TF by endothelial cells plays a major role in sickle cell disease (SCD) pathology. In this context, myeloid cell TF is responsible for coagulation activation in mouse models of SCD, but endothelial cell TF primarily contributes to PAR2 signaling-dependent vascular inflammation [18].

Genetic evidence supports the crucial role for myeloid cell-expressed TF in thrombosis [19, 20], but TF synthesized by monocytes or neutrophils likely contribute to distinct pathologies. In the localized laser-induced vessel injury in mice, endothelial cells release adenosine triphosphate (ATP) that triggers P2X1 receptors on neutrophils being recruited through interaction of lymphocyte function-associated antigen 1 (LFA-1) with intercellular adhesion molecule 1 (ICAM-1) to the injury site [21, 22]. TF is expressed by neutrophils and triggers thrombin and fibrin generation, as well as subsequent platelet recruitment and thrombus formation. Activation of neutrophils is required for TF exposure on the cell surface and neutrophil elastase enhances prothrombotic TF activity by degrading TFPI [23]. In this and other TF-dependent arterial thrombosis models [24-26], contact pathway FXII activation of FXI significantly contributes to thrombus stability and vascular occlusion.

Mouse neutrophil activation and the formation of neutrophil extracellular traps (NETs) contribute to thrombus stability [27], but initial TF-dependent fibrin formation in flow-restricted venous thrombosis is normal in NETosis-deficient mice [20]. In patients with sepsis, autophagy delivers TF to NETs released during activation, leading to exposure of prothrombotic TF [28]. Human neutrophils are also primed in atherosclerotic vessels to upregulate TF that can be subsequently exposed by neutrophils interacting with thrombin-stimulated platelets [29].

Eosinophils are among the neutrophil populations detected in thrombi from patients with in stent thrombosis [30] and epidemiological studies link the eosinophil activation marker, eosinophil cationic protein, to thrombotic events [31]. Human eosinophils carry TF in intracellular granules and stimulation with platelet activating factor (PAF) or granulocyte macrophage colony-stimulating factor (GMCSF) causes TF translocation to the membrane where it can initiate coagulation and supports trans-endothelial migration [32]. Coagulation activation by eosinophils not only requires TF,

but also the enzymatic modification of procoagulant phospholipid. Eosinophil activation induces surface exposure of 12/15-lipoxygenase-modified hydroxyeicosatetraenoic acid (HETE) phosphatidylethanolamines necessary for procoagulant activity, thrombin generation and thrombosis [31]. Phosphatidyl-ethanolamine (PE) is modified by the 12/15 lipoxygenase Alox15 expressed by eosinophils and Alox15 knock-out mice display reduced thrombosis. This and other studies provide evidence that immune cell-derived enzymatically oxidized HETE phospholipids play broader roles in prothrombotic and hemostatic reactions [33].

Myeloid cell TF activation in the context of inflammation

Whereas neutrophils typically translocate TF to the cell surface or to generated NETs, monocytes and macrophages express TF on the surface in an inactive or cryptic form that is converted to fully procoagulant TF by specific inflammatory agonist pathways. TF activation on monocytes or macrophages requires thiol-disulfide exchange and involves protein disulfide isomerase (PDI). Although PDI can be acutely released during vessel wall injury [34], *in vitro* studies demonstrate that PDI is detectable on the surface of intact cells [35] in complex with TF [36] and PDI on extracellular vesicles (EV) directly regulates TF activity [12].

The precise posttranslational modifications of cryptic TF may differ between vascular and extravascular cells, but the TF extracellular allosteric disulfide bond Cys^{186}-Cys^{209} can be S-nitrosylated, reduced or converted to mixed disulfides of TF Cys^{209} with thioredoxin (Trx) or glutathione[37]. When the allosteric disulfide is oxidized, affinity for FVIIa is high and recognition of macromolecular substrate FX is favored [36]. However, exposure of procoagulant phospholipid in the context of cellular activation is required, albeit insufficient alone, for the TF activity switch that involves thiol-disulfide exchange and PDI (Figure 2A). Moreover, agonists that activate TF frequently cause the release of EV with prothrombotic activities and thus couple local inflammatory reactions to remote propagation of thrombosis.

PDI has multiple targets during thrombus formation *in vivo* [38], but it plays a direct role in the function of prothrombotic EV released from myelo-monocytic and vessel wall cells activated by P2rX7 stimulation with the cell injury signal ATP [25]. ATP activation of P2rX7 is the central mechanism for cellular processing and release of interleukin (IL) 1β, which participates in a variety of inflammatory and cardiovascular diseases. In endotoxin-primed mouse macrophages, this pathway triggers endosomal ROS production required for Trx reductase-dependent inflammasome and caspase 1 activation [13]. Release of Trx from intracellular targets, including the inflammasome activator Trx-interacting protein (TXNIP), permits cytoskeletal changes and filopodia formation (Figure 2B). Reduced Trx is released into the extravascular space and responsible for reductive changes in cell surface proteins and required for TF incorporation into EV. Most important, inflammasome-generated caspase 1 promotes actin translocation across membranes and thereby enables the final severing of thrombo-inflammatory EV with a unique protein composition forming on filopodia.

Inhibition of glycosphingolipid-rich raft domains with the cholesterol chelator filipin prevents TF trafficking to filopodia and release onto EV [13]. *In vitro*, the major raft component sphingomyelin markedly suppresses TF activity in the presence of procoagulant phosphatidylserine (PS) [39], providing a supportive mechanism for TF encryption prior to cell activation. ATP stimulation rapidly increases PS levels on the outer membrane independent of Trx reductase [13], but TF activation requires the degradation of sphingomyelin by acidic sphingomyelinase which is activated independent of PDI [39]. Although PS is detectable after activation on macrophages and derived EV, the procoagulant activity of EV is most efficiently inhibited by duramycin, an antagonist of PE, and less so by lactadherin which specifically blocks PS [12]. PE is emerging as an important lipid in thrombosis because it also supports the prothrombotic activity of cancer cell-derived EV [40]. A challenge for future translational coagulation research will be the development of assay systems incorporating such lipid components that in cell-based and preclinical models emerged as relevant coagulation amplifiers.

The exposure of procoagulant phospholipids is essential for conversion of TF from a cryptic to a prothrombotic form. A molecular dynamic simulation of TF-FVIIa on a lipid surface revealed direct interactions between TF extracellular domain residues and the negatively charged headgroup of PS [41]. These TF residues are important for macromolecular substrate turnover [42], suggesting a mechanism by which TF structural changes in the carboxyl-terminus are influenced by a procoagulant lipid environment. However, PS exposure is typically not sufficient for TF activation on cells and require additional thiol-disulfide exchange mediated alterations in the TF protein.

As one example, macrophage TF surface availability is regulated by integrin α4β1 controlling the activity of ADP-ribosylation factor 6 (arf6) involved in integrin recycling [12]. Blockade of dynamin-dependent integrin internalization exposes PS and generates EV with TF and PS levels as seen following ATP stimulation; however, these EV lack PDI and have low prothrombotic activity in flowing blood [12]. A distinction between these two types of EV is the affinity of TF for its ligand FVIIa. PDI chaperone function enhances TF-FVIIa affinity and is blocked by an antibody to TF, 10H10 [43], which reduces EV TF-FVIIa affinity and markedly diminishes EV prothrombotic activity [12]. Similarly, monocyte TF is activated by antiphospholipid antibodies (aPLs) that expose PS independent of PDI, but TF activation nevertheless requires PDI oxidoreductase function and TF interactions that are blocked by anti-TF 10H10 [43].

Reduction of the TF allosteric disulfide by Trx is one elucidated mechanism that suppresses TF activity on monocytes. Inhibition of Trx is sufficient to increase TF activity in the context of PS exposure [44]. In addition, the complement cascade has emerged as an important activator of encrypted TF on monocytes *in vivo*. The polyclonal anti-thymocyte globulin (ATG) preparation is used for prevention and treatment of allograft rejection and graft-versus-host disease, but some patients develop low-grade intravascular coagulation during therapy. ATG rapidly induces procoagulant activity of preformed TF on the surface of monocytic cells dependent on Fc mediated complement activation *in vitro* [35]. TF conversion to an active form in this context requires PDI and thiol-disulfide exchange which is known to occur during complement activation (Figure 2A). Formation of the

membrane insertion complex C5b-7, but not the full assembly of the C5b-9 terminal complement complex is sufficient for PS exposure supporting TF activation.

In addition, TF on human and mouse monocytes is activated by complement-fixing aPLs. Whereas PDI and C3 are involved, as seen with ATG, the exposure of PS and TF activation are independent of C5 (Figure 2A). These aPL, which are not reactive with the β2-glycoprotein I cofactor, induce thrombosis independent of C5 and low-density lipoprotein receptor-related protein 8 (Lrp8) interacting with β2-glycoprotein I. In contrast, C3-deficiency prevents amplified venous thrombus formation in an inferior vena cava thrombosis model in mice, consistent with the *in vitro* properties of aPLs in TF activation [43]. Complement activation is also crucial in flow restricted venous thrombosis models in the mouse. Imaging of platelet and fibrin deposition has revealed distinct roles for C3 and C5 in this context [20]. Platelet activation is reduced in C3-deficient mice, but no defect is seen in C5-deficient mice, indicating direct activation of platelets by C3 products independent of terminal complement. On the contrary, in this model of myeloid cell TF-dependent thrombosis, PDI inhibitors as well as C3- and C5-deficiency attenuate fibrin deposition. In addition, C5-deficient mice display reduced PS exposure on leukocytes that are recruited efficiently to the flow restricted vessel wall. Thus, complement-coagulation crosstalk plays an important role in pathologies driven by monocyte TF expression.

TF-FVIIa and intracellular signaling

Apart from controlling coagulation, cellular mechanisms that render TF non-coagulant also support TF-FVIIa cell signaling. Coagulation proteases, including TF-FVIIa, TF-FVIIa-FXa, thrombin, and activated protein C (aPC) cleave the extracellular domains of PARs and elicit both heterotrimeric G protein and β-arrestin-coupled signaling [45]. Although all coagulation proteases can cleave PARs with variable efficiency, protease interactions with specific receptors, their subcellular localization in microdomains, PAR heterodimerization and alternative PAR cleavages result in biased agonism and sometimes opposing downstream cellular responses. Understanding the precise signaling pathways

elicited by the coagulation proteases is important for predicting potential therapeutic benefits of e.g. biased antagonists for PAR2 [46].

TF plays a central role in PAR signaling and forms two distinct signaling complexes (Figure 3). In the TF-FVIIa-Xa-endothelial protein C receptor (EPCR) complex, TF-FVIIa generated nascent product FXa cleaves PAR1 or PAR2 [47]. This signaling occurs at low concentrations of FVIIa (< 1 nM) and the TF molecule requires the presence of the Cys^{186}-Cys^{209} disulfide bound to have high affinity for FVIIa, full coagulant activity, and ternary TF-VIIa-Xa complex signaling [36]. In contrast, the binary TF-FVIIa complex cleaving PAR2 is formed by TF with low affinity for binding FVIIa requiring high FVIIa concentrations (1-20 nM) (Figure 3A). Reduction of the TF Cys^{186}-Cys^{209} allosteric disulfide bond decreases affinity for FVIIa without completely abolishing ligand binding, while fully preserving cell signaling when Cys^{186} is present, indicating a cell signaling role for this residue, potentially through mixed disulfide formation [36].

While aPC bound to EPCR is the prototypical PAR1 activator [48] and the FVIIa light chain cannot substitute for the same region in aPC in signaling [49], FVIIa in complex with EPCR also elicits TF-independent PAR1 signaling in endothelial cells [50]. In contrast, the binary TF-FVIIa complex activates PAR2 and has been linked to a variety of pathological conditions. In many cases, pathological TF-FVIIa signaling involves the short cytoplasmic domain of TF that in human TF is phosphorylated at Ser^{253} by protein kinase C (PKC) [51, 52] and at Ser^{258} by p38 [53] resulting in conformational changes influencing ligand binding [54, 55].

PAR2 cleavage and certain proximal signaling responses of TF-FVIIa do not require the TF cytoplasmic domain [56]. Instead, the TF cytoplasmic domain recruits adaptors for signaling complexes and protein trafficking, i.e. the regulatory subunit of phosphatidylinositol 3 kinase (PI3K) [57], the actin binding protein filamin [13, 58-60], and the prolyl-isomerase Pin1 [55, 61]. Interaction of the TF cytoplasmic domain with Pin1 not only influences TF protein half-life and incorporation into EV, but also Pin1-dependent transcriptional regulation of TF expression in human smooth muscle cells [55]. It is currently unclear whether Pin1 regulation involves PAR2 which is known to

constitutively signal independent of proteolytic cleavage [62]. In contrast, binding of the regulatory subunit of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) to the TF cytoplasmic domain is important for TF-FVIIa-PAR2 signaling that is connected to functions of cell adhesion receptors, particularly integrins of the β3 and β1 families [57].TF-FVIIa complex signaling through PAR2 has been implicated in cancer cell migration, invasion, proliferation and evasion from apoptotic cell death [56] and blocking the TF-integrin interaction with anti-TF antibody 10H10, which has minimal effects on coagulation, suppresses tumor growth [63]. Other TF-FVIIa inhibitors also prevent spontaneous tumor progression in syngeneic tumor models [64]. The interaction of TF with integrin is complex. Alternative splicing of TF creates a soluble isoform of TF without a transmembrane and cytoplasmic domain [65]. While alternatively spliced TF (asTF) does not support physiological roles of TF e.g. in embryonic development [66], it retains the ability to ligate integrins αvβ3 and α6β1 [67] that also interact with full-length TF [68]. Signaling of asTF is independent of FVIIa-PAR2 and promotes, through direct integrin ligation of endothelial cells, angiogenesis [67] and inflammation [69], or cancer cell survival [70].

The molecular details of TF-FVIIa complex formation with integrin and implications for pro-angiogenic and pro-migratory signaling have recently been further clarified. Immunoprecipitation with an antibody recognizing an active conformation of β1 integrin has shown that FVIIa induces TF complex formation with activated integrin through a specific binding site in the FVIIa protease domain remote from the catalytic cleft required for PAR2 cleavage [57]. This site overlaps with the macromolecular substrate binding site [1] and thereby precludes the activation of procoagulant substrates with a predicted impairment of TF-initiated coagulation. Signaling of the integrin-TF-FVIIa complex may therefore occur without interference from downstream thrombin signaling events.

TF-FVIIa-PAR2 signaling involves endosomal internalization of TF-FVIIa together with activated integrin dependent on the small GTPase arf6 (ADP-ribosylation factor 6), a regulator of integrin trafficking. Complex formation of TF-FVIIa with integrin is not required for PAR2 cleavage, but drives delayed mitogen activated kinase signaling and PI3 kinase activation required for the

expression of pro-angiogenic cytokines [57]. FVIIa catalytic activity and the TF cytoplasmic domain are dispensable for formation of the TF-FVIIa-integrin complex but required for PI3K-dependent IL8 induction and cell migration [57, 68]. PI3K plays a crucial role in TF-FVIIa enhanced cancer cell migration though its downstream target Protein kinase B (PKB, Akt). TF-FVIIa-PAR2 mediated Akt phosphorylation and inactivation of glycogen synthase kinase-3β (GSK-3β) results in β-catenin stabilization and gene transcriptional activation of promigratory and prometastatic genes [71].

In addition to integrins, TF-FVIIa proangiogenic signaling in non-cancerous keratinocytes depends on transactivation of epidermal growth factor receptor (EGFR) and proline rich tyrosine kinase 2 (PYK2) [72]. The TF-FVIIa complex also mediates transactivation of the insulin growth factor receptor (IGF-1R) preventing TRAIL (TNF-related apoptosis inducing ligand) dependent apoptosis in cancer cells [73]. The binary TF-FVIIa complex, but not the ternary TF-FVIIa-FXa or downstream coagulation proteases, induce cleavage of the ectodomains of tyrosine kinase receptors EphB2 and EphA2 which increases cell motility [74]. PAR2 signaling and, based on antibody blockade, TF-FVIIa-integrin interaction are not required for cleavage of a conserved arginine residue in the ligand-binding domain of Eph receptors. Extracellular cleavage of ephrins apparently does not involve downstream matrix metalloproteinases and is dependent primarily on serine protease activity. Additionally, TF-FVIIa has recently been shown to activate the transmembrane serine protease matriptase [75]. Although TF-FVIIa-PAR2 proangiogenic signaling is independent of matriptase [57], the role of matriptase in PAR2-independent TF-FVIIa triggered cell surface proteolysis requires further study. FVIIa mutant proteins defective in integrin complex formation and PAR2 cleavage will be useful tools to better understand the divergent roles of TF-FVIIa cell signaling pathways *in vitro* and *in vivo* [57, 76].

TF-PAR2 signaling in inflammatory diseases and cancer

Mice with a deletion of the TF cytoplasmic domain ($TF^{\Delta CT}$ mice) have helped to identify roles of TF-PAR2 signaling in physiology and pathology. $TF^{\Delta CT}$ mice display increased developmental and hypoxia-driven angiogenesis that is reversed by PAR2 deficiency or inhibitors of TF-FVIIa [77, 78]. While these and other studies [79, 80] indicate a regulatory role for the TF cytoplasmic domain, $TF^{\Delta CT}$ mice and $PAR2^{-/-}$ display similar pathology-protected phenotypes in other models [81]. Spontaneous breast cancer development is delayed in both strains and to a similar extent in double deficient mice, indicating that PAR2 and downstream TF cytoplasmic domain phosphorylation cooperate in cancer progression, consistent with human pathology data in breast cancer [82]. TF-FVIIa-PAR2 signaling has also been linked to human hepatocellular carcinoma (HCC) progression independent of downstream coagulation [83]. In addition, $TF^{\Delta CT}$ and PAR2-deficient mice were similarly protected from carbon tetrachloride (CCl_4) damage leading to hepatic fibrosis involving transforming growth factor (TGF) β and macrophages activation.

The TF cytoplasmic domain regulates TF trafficking in intestinal epithelial cells and gut microbiota promote N-glycosylation of TF and TF surface localization in enterocytes. The TF cytoplasmic domain and PAR1 signaling are both required for adaptive angiogenesis following colonization of the small intestine with microbiota [84]. While thrombin-PAR1 signaling triggered TF phosphorylation in enterocytes, it will be of interest for future studies to define which endogenous or microbiota-derived proteases can contribute to the regulation of intestinal homeostasis via PAR signaling.

Obesity is characterized by a hypercoagulable state and associated with increased risk for thrombosis [85]. In adipocytes, the TF cytoplasmic domain participates in TF-FVIIa induced suppression of Akt phosphorylation by insulin and thus impairs expression of negative regulators of weight gain. Accordingly, blocking TF-FVIIa interaction with an antibody in obese mice improved overall metabolism and energy expenditure [86]. TF is upregulated in the obese visceral adipose tissue and fibrin deposition is prominent surrounding the “crown-like” structure where macrophages

interact with stressed adipocytes. In mouse models, a genetically induced hyper-thrombotic state promotes obesity, but a mutant of fibrinogen devoid of interaction with the macrophage-expressed integrin αMβ2 (CD11b/CD18) is protected from diet-induced obesity with diminished systemic and adipose inflammation [87].

In addition, TF is expressed by adipose tissue macrophages and loss of TF-PAR2 signaling in hematopoietic cells prevents the development of adipose tissue inflammation and insulin resistance, key contributors to the development of type 2 diabetes and the metabolic syndrome [86]. In the liver, TF-PAR2 signaling drives the accumulation of $CD8^+$ T cells and promotes pathways of hepatic lipogenesis and gluconeogenesis that contribute to steatosis and hepatic insulin resistance [88]. Hepatic inflammation is also reduced in mice treated with the thrombin inhibitor dabigatran or defective macrophage fibrin interaction [87]. Thus, coagulation-induced fibrin deposition participates in inflammatory TF-PAR2 signaling in prevalent diseases of metabolic inflammation.

PAR2 signaling of TF-FVIIa generated nascent product FXa

As discussed above, initial studies showed that low concentrations of FVIIa elicited cell signaling only when FX was present [89] and that FXa associated with the TF-FVIIa complex efficiently cleaved PAR2 [90]. Because procoagulant and prothrombotic TF binds FVIIa with high affinity [12, 36], signaling of the nascent product FXa is integrated in cell surface TF-initiated coagulation (Figure 3B). This pathway may proceed without significant thrombin generation and thrombin-dependent PAR signaling, because TFPI controls the generation of active prothrombinase, as discussed above. In addition, TF-FVIIa-Xa ternary complex signaling is directly regulated by TFPI that suppresses PAR responses [91] and promotes lipid raft dependent recycling of the inhibited TF complex [92]. TF ternary complex signaling in endothelial, smooth muscle and cancer cells also requires EPCR, but not Gla-domain interactions of FVIIa with EPCR [47].

Although EPCR mediates endothelial protection in endotoxemia inflammation models [93], EPCR is crucial for signaling of the TF-FVIIa-FXa complex and the lipopolysaccharide (LPS) response of dendritic cells [94]. While PAR2 and toll like receptor (TLR) 4 form a heterodimer and PAR2 in general synergizes with TLR innate immune signaling [95, 96], the TF-FVIIa-FXa-EPCR complex selectively induces expression of the TLR3/4 signaling adaptor protein pellino-1, the transcription factor interferon (IFN) regulatory factor 8 and a set of IFN-regulated genes. This signaling response is absent in EPCR-, PAR2-, and TF-deficient cells following endotoxin stimulation *in vitro* and *in vivo* [94]. The induction of these genes downstream of TLR4 activation is blocked by anti-TF antibody inhibiting FVIIa binding or FXa inhibitors. Remarkably, stimulation with a direct PAR2 agonist restored the induction of this gene pattern when FXa was blocked, but not when the assembly of the TF-FVIIa complex was inhibited by anti-TF antibody. Thus, TF-FVIIa generation of FXa activating PAR2 and the formation of the TF-FVIIa-FXa-EPCR complex are an integral part of the TLR4 innate immune responses.

The cooperate roles of TF initiation complex signaling in innate immune cells is regulated by the anticoagulant PC pathway (Figure 3B). Thrombin-thrombomodulin activation of PC limits coagulation activation by inactivating the plasma coagulation cofactors FVa and FVIIIa. In addition, non-activated FV is a cofactor for aPC degradation of FVIIIa and this reaction requires the anticoagulant Protein S. This pathway is defective in homozygous FV_{Leiden} patients, but functional or even amplified in inflammatory conditions due to increased thrombin generation in heterozygous FV_{Leiden} carriers. The aPC-PS-FV, but not FVa or FV_{Leiden}, triad turned out to be a potent suppressor of inflammatory PAR2 signaling induced by the TF-VIIa-Xa-EPCR complex [97], identifying a novel regulation of the TF pathway signaling by the downstream anticoagulant pathway. Additional experiments showed that stabilizing the TF-FVIIa-FXa with a unique inhibitor or disabling aPC binding to EPCR prevents the regulation of the TF-dependent IFN response downstream of TLR4, indicating a mechanism of competition between aPC and FXa for recruiting EPCR to distinct signaling pathways. The regulation of TF signaling by aPC is also relevant for sepsis therapy. Treatment of septic mice

with a signaling selective aPC variant with greatly diminished anticoagulant function reduces mortality; this beneficial effect is lost in homozygous FV_{Leiden} mice. These experiments emphasize that both, pro- and anticoagulant, pathways are integrated into innate immune responses to microbial threats and potentially a variety of other, endogenous danger signals.

TF-dependent signaling in endothelial cells

Endothelial cell-expressed TF also participates in inflammation predominantly through cell signaling rather than coagulation activation. Although endothelial cell expression of TF has long been considered of little importance for pathological processes, the inflamed pulmonary vein endothelium in SCD expresses TF dependent on direct effects of hemolysis and leukocyte crosstalk [18]. The TF pathway is crucial for both coagulation abnormalities and chronic vascular inflammation in SCD, but endothelial cell-specific deletion of TF in a mouse model of SCD prevents selectively the upregulation of IL6, without changing markers of intravascular coagulation [18]. Blocking TF and anticoagulation with the thrombin inhibitor dabigatran or the FXa inhibitor rivaroxaban effectively reduces lung leukocyte infiltration. However, only FXa inhibition reduces IL6 levels, as also seen in anti-TF treated or PAR2 knock-out mice. While the participation of other components of the TF complex has not been analyzed, these data indicate a non-coagulant role for endothelial cell-expressed TF in vascular inflammation.

As mentioned above, endothelial cell TF is regulated by the anti-oxidative protein PON2, a cell associated protein with anti-atherogenic properties. *Pon2*-deficiency causes a posttranscriptional upregulation of endothelial cell TF activity and a proinflammatory state that is reversed by genetic restoration of PON2 in endothelial cells, anti-oxidant treatment, and antibody inhibition of TF [16]. Similar to SCD mice [18], *Pon2*-deficiency increases endothelial cell IL6 expression and CCL22 that is also regulated by ternary complex signaling in myeloid cells [94]. Loss of PON2 antioxidant function causes vascular inflammation and dysfunction that is reversed by anti-TF treatment. Knock-down of FVII synthesis in the liver and reduced circulating FVII levels increase

hemorrhaging following cerebral contusion injury in mice [98]. Infusion of recombinant FVIIa restores microvascular function, prevents endothelial cell apoptosis, and protects the brain. *In vitro* experiments with endothelial cells induced with inflammatory mediators to express TF showed that TF-FVIIa-FXa complex signaling through PAR2 suppresses p65 NF-κB phosphorylation independent of EPCR, but increases mitogen-activated protein (MAP) kinase signaling dependent on EPCR. While TF-FVIIa-FXa through downstream activation of matriptase and PAR2 is barrier protective in epithelial cells [75], *in vivo* experiments are necessary to better understand the effect of plasma FVII levels on brain endothelial protection. FVIIa is barrier protective through TF-independent, but EPCR-dependent PAR1 signaling [99], indicating overlapping functions of FVIIa with the endothelial protective aPC pathway. Mutant mouse models that are resistant to certain coagulation proteases [76, 94, 100] will be useful tools for future studies on the distinct roles of coagulation factors in vascular biology.

Conclusions and outlook

These studies have revealed new details of TF interactions contributing to the diverse TF functions in hemostasis, thrombosis and inflammation. TF is linked in its cellular regulation to intracellular adaptors and integrin trafficking pathways and thereby contributes to physiological control of cells. Inflammatory activation of cells can divert TF from these trafficking routes and cause pathologies, as illustrated for the generation of thrombo-inflammatory EV. Understanding these cellular and PAR signaling routes of TF will be essential for predicting effects of targeted selective anticoagulants and to devise new approaches that selectively interfere with pathological TF functions without perturbing hemostatic or protective signaling. This knowledge should be translated into improved diagnostic approaches required for the ultimate goal of individualized therapies in TF-dependent pathologies.

Acknowledgements

We thank our colleagues who participated in these studies. W. Ruf is supported by the National Heart Lung and Blood Institute (NHLBI), the Federal Ministry for Education and Research (BMBF) and the Alexander-von-Humboldt Foundation of Germany. H. Zelava received a Georg-Forster-Fellowship of the Alexander-von-Humboldt Foundation.

Disclosure of Conflict of Interests

W. Ruf participates in the Scientific Advisory Board of Iconic Therapeutics and the Board of Directors of MeruVasimmune LLC. In addition, he has a patent 'diagnostic assays for TF' pending. The other authors state that they have no conflict of interest.

References

1 Norledge B, Petrovan RJ, Ruf W, Olson A. The tissue factor/factor VIIa/factor Xa complex: a model built by docking and site-directed mutagenesis. *Proteins*. 2003; **53**: 640-8.
2 Huang M, Syed R, Stura EA, Stone MJ, Stefanko RS, Ruf W, Edgington TS, Wilson IA. The mechanism of an inhibitory antibody on TF-initiated blood coagulation revealed by the crystal structures of human tissue factor, Fab 5G9 and TF-5G9 complex. *J Mol Biol*. 1998; **275**: 873-94.
3 Teplyakov A, Obmolova G, Malia TJ, Wu B, Zhao Y, Taudte S, Anderson GM, Gilliland GL. Crystal structure of tissue factor in complex with antibody 10H10 reveals the signaling epitope. *Cell Signal*. 2017; **36**: 139-44.
4 Schuijt TJ, Bakhtiari K, Daffre S, Deponte K, Wielders SJ, Marquart JA, Hovius JW, van der Poll T, Fikrig E, Bunce MW, Camire RM, Nicolaes GA, Meijers JC, van 't Veer C. Factor Xa activation of factor V is of paramount importance in initiating the coagulation system: lessons from a tick salivary protein. *Circulation*. 2013; **128**: 254-66. 10.1161/CIRCULATIONAHA.113.003191.
5 Maroney SA, Mast AE. New insights into the biology of tissue factor pathway inhibitor. *J Thromb Haemost*. 2015; **13 Suppl 1**: S200-S7.
6 Vincent LM, Tran S, Livaja R, Bensend TA, Milewicz DM, Dahlback B. Coagulation factor V(A2440G) causes east Texas bleeding disorder via TFPIalpha. *J Clin Invest*. 2013; **123**: 3777-87.
7 Wood JP, Baumann Kreuziger LM, Ellery PER, Maroney SA, Mast AE. Reduced Prothrombinase Inhibition by Tissue Factor Pathway Inhibitor Contributes to the Factor V Leiden Hypercoagulable State. *Blood Adv*. 2017; **1**: 386-95.
8 Kamikubo Y, Mendolicchio GL, Zampolli A, Marchese P, Rothmeier AS, Orje JN, Gale AJ, Krishnaswamy S, Gruber A, Ostergaard H, Petersen LC, Ruf W, Ruggeri ZM. Selective factor VIII activation by the tissue factor-factor VIIa-factor Xa complex. *Blood*. 2017; **130**: 1661-70. 10.1182/blood-2017-02-767079.
9 Bergum PW, Cruikshank A, Maki S, Kelly CR, Ruf W, Vlasuk G. Role of zymogen and activated factor X as scaffolds for the inhibition of the blood coagulation factor VIIa-tissue factor complex by recombinant nematode anticoagulant protein c2. *J Biol Chem*. 2001; **276**: 10063-71.

10 Shaver CM, Grove BS, Putz ND, Clune JK, Lawson WE, Carnahan RH, Mackman N, Ware LB, Bastarache JA. Regulation of Alveolar Procoagulant Activity and Permeability in Direct Acute Lung Injury by Lung Epithelial Tissue Factor. *Am J Respir Cell Mol Biol*. 2015; **53**: 719-27.
11 Wang L, Miller C, Swarthout RF, Rao M, Mackman N, Taubman MB. Vascular smooth muscle-derived tissue factor is critical for arterial thrombosis after ferric chloride-induced injury. *Blood*. 2009; **113**: 705-13.
12 Rothmeier AS, Marchese P, Langer F, Kamikubo Y, Schaffner F, Cantor J, Ginsberg MH, Ruggeri ZM, Ruf W. Tissue Factor Prothrombotic Activity Is Regulated by Integrin-arf6 Trafficking. *Arterioscler Thromb Vasc Biol*. 2017; **37**: 1323-31. 10.1161/ATVBAHA.117.309315.
13 Rothmeier AS, Marchese P, Petrich BG, Furlan-Freguia C, Ginsberg MH, Ruggeri ZM, Ruf W. Caspase-1-mediated pathway promotes generation of thromboinflammatory microparticles. *J Clin Invest*. 2015; **125**: 1471-84.
14 Brambilla M, Facchinetti L, Canzano P, Rossetti L, Ferri N, Balduini A, Abbonante V, Boselli D, De Marco L, Di Minno MN, Toschi V, Corsini A, Tremoli E, Camera M. Human megakaryocytes confer tissue factor to a subset of shed platelets to stimulate thrombin generation. *Thromb Haemost*. 2015; **114**: 579-92. 10.1160/TH14-10-0830.
15 Schwertz H, Tolley ND, Foulks JM, Denis MM, Risenmay BW, Buerke M, Tilley RE, Rondina MT, Harris EM, Kraiss LW, Mackman N, Zimmerman GA, Weyrich AS. Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets. *J Exp Med*. 2006; **203**: 2433-40.
16 Ebert J, Wilgenbus P, Teiber JF, Jurk K, Schwierczek K, Dohrmann M, Xia N, Li H, Spiecker L, Ruf W, Horke S. Paraoxonase-2 regulates coagulation activation through endothelial tissue factor. *Blood*. 2018; **131**: 2161-72. 10.1182/blood-2017-09-807040.
17 Gaul DS, Weber J, Van Tits LJ, Sluka S, Pasterk L, Reiner MF, Calatayud N, Lohmann C, Klingenberg R, Pahla J, Vdovenko D, Tanner FC, Camici GG, Eriksson U, Auwerx J, Mach F, Windecker S, Rodondi N, TF LUS, Winnik S, Matter CM. Loss of Sirt3 accelerates arterial thrombosis by increasing formation of neutrophil extracellular traps and plasma tissue factor activity. *Cardiovasc Res*. 2018. 10.1093/cvr/cvy036.
18 Sparkenbaugh E, Pawlinski R. Prothrombotic aspects of sickle cell disease. *J Thromb Haemost*. 2017; **15**: 1307-16.
19 von Bruhl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, Khandoga A, Tirniceriu A, Coletti R, Kollnberger M, Byrne RA, Laitinen I, Walch A, Brill A, Pfeiler S, Manukyan D, Braun S, Lange P, Riegger J, Ware J, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med*. 2012; **209**: 819-35.
20 Subramaniam S, Jurk K, Hobohm L, Jackel S, Saffarzadeh M, Schwierczek K, Wenzel P, Langer F, Reinhardt C, Ruf W. Distinct contributions of complement factors to platelet activation and fibrin formation in venous thrombus development. *Blood*. 2017; **129**: 2291-302.
21 Darbousset R, Thomas GM, Mezouar S, Frere C, Bonier R, Mackman N, Renne T, Dignat-George F, Dubois C, Panicot-Dubois L. Tissue factor positive neutrophils bind to injured endothelial wall and initiate thrombus formation. *Blood*. 2012.
22 Darbousset R, Delierneux C, Mezouar S, Hego A, Lecut C, Guillaumat I, Riederer MA, Evans RJ, Dignat-George F, Panicot-Dubois L, Oury C, Dubois C. P2X1 expressed on polymorphonuclear neutrophils and platelets is required for thrombosis in mice. *Blood*. 2014; **124**: 2575-85.
23 Massberg S, Grahl L, von Bruehl ML, Manukyan D, Pfeiler S, Goosmann C, Brinkmann V, Lorenz M, Bidzhekov K, Khandagale AB, Konrad I, Kennerknecht E, Reges K, Holdenrieder S, Braun S, Reinhardt C, Spannagl M, Preissner KT, Engelmann B. Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. *Nat Med*. 2010; **16**: 887-96.
24 Kuijpers MJ, van der Meijden PE, Feijge MA, Mattheij NJ, May F, Govers-Riemslag J, Meijers JC, Heemskerk JW, Renne T, Cosemans JM. Factor XII regulates the pathological process of thrombus formation on ruptured plaques. *Arterioscler Thromb Vasc Biol*. 2014; **34**: 1674-80.

25 Furlan-Freguia C, Marchese P, Gruber A, Ruggeri ZM, Ruf W. P2X7 receptor signaling contributes to tissue factor-dependent thrombosis in mice. *J Clin Invest*. 2011; **121**: 2932-44.
26 Renne T, Pozgajova M, Gruner S, Schuh K, Pauer HU, Burfeind P, Gailani D, Nieswandt B. Defective thrombus formation in mice lacking coagulation factor XII. *J Exp Med*. 2005; **202**: 271-81.
27 Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD, Jr., Wrobleski SK, Wakefield TW, Hartwig JH, Wagner DD. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci U S A*. 2010; **107**: 15880-5.
28 Kambas K, Mitroulis I, Apostolidou E, Girod A, Chrysanthopoulou A, Pneumatikos I, Skendros P, Kourtzelis I, Koffa M, Kotsianidis I, Ritis K. Autophagy mediates the delivery of thrombogenic tissue factor to neutrophil extracellular traps in human sepsis. *PLoS ONE*. 2012; **7**: e45427.
29 Stakos DA, Kambas K, Konstantinidis T, Mitroulis I, Apostolidou E, Arelaki S, Tsironidou V, Giatromanolaki A, Skendros P, Konstantinides S, Ritis K. Expression of functional tissue factor by neutrophil extracellular traps in culprit artery of acute myocardial infarction. *Eur Heart J*. 2015; **36**: 1405-14.
30 Riegger J, Byrne RA, Joner M, Chandraratne S, Gershlick AH, Ten Berg JM, Adriaenssens T, Guagliumi G, Godschalk TC, Neumann FJ, Trenk D, Feldman LJ, Steg PG, Desmet W, Alfonso F, Goodall AH, Wojdyla R, Dudek D, Philippi V, Opinaldo S, et al. Histopathological evaluation of thrombus in patients presenting with stent thrombosis. A multicenter European study: a report of the prevention of late stent thrombosis by an interdisciplinary global European effort consortium. *Eur Heart J*. 2016; **37**: 1538-49. 10.1093/eurheartj/ehv419.
31 Uderhardt S, Ackermann JA, Fillep T, Hammond VJ, Willeit J, Santer P, Mayr M, Biburger M, Miller M, Zellner KR, Stark K, Zarbock A, Rossaint J, Schubert I, Mielenz D, Dietel B, Raaz-Schrauder D, Ay C, Gremmel T, Thaler J, et al. Enzymatic lipid oxidation by eosinophils propagates coagulation, hemostasis, and thrombotic disease. *J Exp Med*. 2017; **214**: 2121-38.
32 Moosbauer C, Morgenstern E, Cuvelier SL, Manukyan D, Bidzhekov K, Albrecht S, Lohse P, Patel KD, Engelmann B. Eosinophils are a major intravascular location for tissue factor storage and exposure. *Blood*. 2007; **109**: 995-1002.
33 Slatter DA, Percy CL, Allen-Redpath K, Gajsiewicz JM, Brooks NJ, Clayton A, Tyrrell VJ, Rosas M, Lauder SN, Watson A, Dul M, Garcia-Diaz Y, Aldrovandi M, Heurich M, Hall J, Morrissey JH, Lacroix-Desmazes S, Delignat S, Jenkins PV, Collins PW, O'Donnell VB. Enzymatically oxidized phospholipids restore thrombin generation in coagulation factor deficiencies. *JCI Insight*. 2018; **3**. 10.1172/jci.insight.98459.
34 Reinhardt C, von Bruhl ML, Manukyan D, Grahl L, Lorenz M, Altmann B, Dlugai S, Hess S, Konrad I, Orschiedt L, Mackman N, Ruddock L, Massberg S, Engelmann B. Protein disulfide isomerase acts as an injury response signal that enhances fibrin generation via tissue factor activation. *J Clin Invest*. 2008; **118**: 1110-22.
35 Langer F, Spath B, Fischer C, Stolz M, Ayuk FA, Kroger N, Bokemeyer C, Ruf W. Rapid activation of monocyte tissue factor by antithymocyte globulin is dependent on complement and protein disulfide isomerase. *Blood*. 2013; **121**: 2324-35.
36 Ahamed J, Versteeg HH, Kerver M, Chen VM, Mueller BM, Hogg PJ, Ruf W. Disulfide isomerization switches tissue factor from coagulation to cell signaling. *Proc Natl Acad Sci USA*. 2006; **103**: 13932-7.
37 Langer F, Ruf W. Synergies of phosphatidylserine and protein disulfide isomerase in tissue factor activation. *Thromb Haemost*. 2014; **111**: 590-7.
38 Stopa JD, Neuberg D, Puligandla M, Furie B, Flaumenhaft R, Zwicker JI. Protein disulfide isomerase inhibition blocks thrombin generation in humans by interfering with platelet factor V activation. *JCI Insight*. 2017; **2**: e89373.
39 Wang J, Pendurthi UR, Rao LVM. Sphingomyelin encrypts tissue factor: ATP-induced activation of A-SMase leads to tissue factor decryption and microvesicle shedding. *Blood Adv*. 2017; **1**: 849-62.

40 Stark K, Schubert I, Joshi U, Kilani B, Hoseinpour P, Thakur M, Grunauer P, Pfeiler S, Schmidergall T, Stockhausen S, Baumer M, Chandraratne S, von Bruhl ML, Lorenz M, Coletti R, Reese S, Laitinen I, Wormann SM, Algul H, Bruns CJ, et al. Distinct Pathogenesis of Pancreatic Cancer Microvesicle-Associated Venous Thrombosis Identifies New Antithrombotic Targets In Vivo. *Arterioscler Thromb Vasc Biol*. 2018; **38**: 772-86. 10.1161/ATVBAHA.117.310262.
41 Ohkubo YZ, Morrissey JH, Tajkhorshid E. Dynamical view of membrane binding and complex formation of human factor VIIa and tissue factor. *J Thromb Haemost*. 2010; **8**: 1044-53. 10.1111/j.1538-7836.2010.03826.x.
42 Ruf W, Miles DJ, Rehemtulla A, Edgington TS. Tissue factor residues 157-167 are required for efficient proteolytic activation of factor X and factor VII. *J Biol Chem*. 1992; **267**: 22206-10.
43 Muller-Calleja N, Ritter S, Hollerbach A, Falter T, Lackner KJ, Ruf W. Complement C5 but not C3 is expendable for tissue factor activation by cofactor-independent antiphospholipid antibodies. *Blood Adv*. 2018; **2**: 979-86. 10.1182/bloodadvances.2018017095.
44 Ansari SA, Pendurthi UR, Rao LVM. The lipid peroxidation product 4-hydroxy-2-nonenal induces tissue factor decryption via ROS generation and the thioredoxin system. *Blood Adv*. 2017; **1**: 2399-413. 10.1182/bloodadvances.2017010132.
45 Hamilton JR, Trejo J. Challenges and Opportunities in Protease-Activated Receptor Drug Development. *Annu Rev Pharmacol Toxicol*. 2017; **57**: 349-73. 10.1146/annurev-pharmtox-011613-140016.
46 Cheng RKY, Fiez-Vandal C, Schlenker O, Edman K, Aggeler B, Brown DG, Brown GA, Cooke RM, Dumelin CE, Dore AS, Geschwindner S, Grebner C, Hermansson NO, Jazayeri A, Johansson P, Leong L, Prihandoko R, Rappas M, Soutter H, Snijder A, et al. Structural insight into allosteric modulation of protease-activated receptor 2. *Nature*. 2017; **545**: 112-5.
47 Disse J, Petersen HH, Larsen KS, Persson E, Esmon N, Esmon CT, Teyton L, Petersen LC, Ruf W. The Endothelial Protein C Receptor Supports Tissue Factor Ternary Coagulation Initiation Complex Signaling through Protease-activated Receptors. *J Biol Chem*. 2011; **286**: 5756-67.
48 Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. *Science*. 2002; **296**: 1880-2.
49 Gleeson EM, McDonnell CJ, Soule EE, Willis FO, Rushe H, Rehill A, Smith OP, O'Donnell JS, Preston RJS. A novel protein C-factor VII chimera provides new insights into the structural requirements for cytoprotective protease-activated receptor 1 signaling. *J Thromb Haemost*. 2017; **15**: 2198-207.
50 Mohan Rao LV, Esmon CT, Pendurthi UR. Endothelial cell protein C receptor: a multiliganded and multifunctional receptor. *Blood*. 2014; **124**: 1553-62. 10.1182/blood-2014-05-578328.
51 Dorfleutner A, Ruf W. Regulation of tissue factor cytoplasmic domain phosphorylation by palmitoylation. *Blood*. 2003; **102**: 3998-4005.
52 Ahamed J, Ruf W. Protease-activated receptor 2-dependent phosphorylation of the tissue factor cytoplasmic domain. *J Biol Chem*. 2004; **279**: 23038-44.
53 Ettelaie C, Elkeeb AM, Maraveyas A, Collier ME. p38alpha phosphorylates serine 258 within the cytoplasmic domain of tissue factor and prevents its incorporation into cell-derived microparticles. *Biochim Biophys Acta*. 2013; **1833**: 613-21.
54 Sen M, Herzik M, Craft JWJ, Creath AL, Agrawal S, Ruf W, Legge GB. Spectroscopic characterization of successive phosphorylation of the tissue factor cytoplasmic region. *Open Spectrosc J*. 2009; **3**: 58-65.
55 Kurakula K, Koenis DS, Herzik MA, Jr., Liu Y, Craft JW, Jr., van Loenen PB, Vos M, Tran MK, Versteeg HH, Goumans MTH, Ruf W, de Vries CJM, Sen M. Structural and cellular mechanisms of peptidyl-prolyl isomerase Pin1-mediated enhancement of Tissue Factor gene expression, protein half-life, and pro-coagulant activity. *Haematologica*. 2018. 10.3324/haematol.2017.183087.
56 Versteeg HH, Ruf W. Emerging insights in tissue factor-dependent signaling events. *Semin Thromb Hemost*. 2006; **32**: 24-32.

57 Rothmeier AS, Liu E, Chakrabarty S, Disse J, Mueller BM, Ostergaard H, Ruf W. Identification of the integrin-binding site on coagulation factor VIIa required for proangiogenic PAR2 signaling. *Blood*. 2018; **131**: 674-85. 10.1182/blood-2017-02-768218.
58 Koizume S, Ito S, Yoshioka Y, Kanayama T, Nakamura Y, Yoshihara M, Yamada R, Ochiya T, Ruf W, Miyagi E, Hirahara F, Miyagi Y. High-level secretion of tissue factor-rich extracellular vesicles from ovarian cancer cells mediated by filamin-A and protease-activated receptors. *Thromb Haemost*. 2015; **115**.
59 Collier ME, Maraveyas A, Ettelaie C. Filamin-A is required for the incorporation of tissue factor into cell-derived microvesicles. *Thromb Haemost*. 2014; **111**: 647-55.
60 Ott I, Fischer EG, Miyagi Y, Mueller BM, Ruf W. A role for tissue factor in cell adhesion and migration mediated by interaction with actin binding protein 280. *J Cell Biol*. 1998; **140**: 1241-53.
61 Ettelaie C, Collier MEW, Featherby S, Greenman J, Maraveyas A. Peptidyl-prolyl isomerase 1 (Pin1) preserves the phosphorylation state of tissue factor and prolongs its release within microvesicles. *Biochim Biophys Acta*. 2018; **1865**: 12-24. 10.1016/j.bbamcr.2017.09.016.
62 Sevigny LM, Zhang P, Bohm A, Lazarides K, Perides G, Covic L, Kuliopulos A. Interdicting protease-activated receptor-2-driven inflammation with cell-penetrating pepducins. *Proc Natl Acad Sci U S A*. 2011; **108**: 8491-6.
63 Versteeg HH, Schaffner F, Kerver M, Petersen HH, Ahamed J, Felding-Habermann B, Takada Y, Mueller BM, Ruf W. Inhibition of tissue factor signaling suppresses tumor growth. *Blood*. 2008; **111**: 190-9.
64 Zhao J, Aguilar G, Palencia S, Newton E, Abo A. rNAPc2 inhibits colorectal cancer in mice through tissue factor. *Clin Cancer Res*. 2009; **15**: 208-16.
65 Bogdanov VY, Balasubramanian V, Hathcock J, Vele O, Lieb M, Nemerson Y. Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nat Med*. 2003; **9**: 458-62.
66 Sluka SH, Akhmedov A, Vogel J, Unruh D, Bogdanov VY, Camici GG, Luscher TF, Ruf W, Tanner FC. Alternatively spliced tissue factor is not sufficient for embryonic development. *PLoS ONE*. 2014; **9**: e97793-.
67 van den Berg YW, van den Hengel LG, Myers HR, Ayachi O, Jordanova E, Ruf W, Spek CA, Reitsma PH, Bogdanov VY, Versteeg HH. Alternatively spliced tissue factor induces angiogenesis through integrin ligation. *Proc Natl Acad Sci U S A*. 2009; **106**: 19497-502.
68 Dorfleutner A, Hintermann E, Tarui T, Takada Y, Ruf W. Crosstalk of integrin $\alpha 3\beta 1$ and tissue factor in cell migration. *Mol Biol Cell*. 2004; **15**: 4416-25.
69 Srinivasan R, Ozhegov E, van den Berg YW, Aronow BJ, Franco RS, Palascak MB, Fallon JT, Ruf W, Versteeg HH, Bogdanov VY. Splice Variants of Tissue Factor Promote Monocyte-Endothelial Interactions by Triggering the Expression of Cell Adhesion Molecules via Integrin-Mediated Signaling. *J Thromb Haemost*. 2011; **9**: 2087-96.
70 Kocaturk B, van den Berg YW, Tieken C, Mieog JS, de Kruijf EM, Engels CC, van der Ent MA, Kuppen PJ, van de Velde CJ, Ruf W, Reitsma PH, Osanto S, Liefers GJ, Bogdanov VY, Versteeg HH. Alternatively spliced Tissue Factor promotes breast cancer growth in a B1 integrin-dependent manner. *Proceedings of the National Academy of Sciences*. 2013; **110**: 11517-22.
71 Roy A, Ansari SA, Das K, Prasad R, Bhattacharya A, Mallik S, Mukherjee A, Sen P. Coagulation factor VIIa-mediated protease-activated receptor 2 activation leads to beta-catenin accumulation via the AKT/GSK3beta pathway and contributes to breast cancer progression. *J Biol Chem*. 2017.
72 Wiiger MT, Prydz H. The epidermal growth factor receptor (EGFR) and proline rich tyrosine kinase 2 (PYK2) are involved in tissue factor dependent factor VIIa signalling in HaCaT cells. *Thromb Haemost*. 2004; **92**: 13-22.
73 Aberg M, Eriksson O, Mokhtari D, Siegbahn A. Tissue factor/factor VIIa induces cell survival and gene transcription by transactivation of the insulin-like growth factor 1 receptor. *Thromb Haemost*. 2014; **111**: 748-60.

74 Eriksson O, Ramstrom M, Hornaeus K, Bergquist J, Mokhtari D, Siegbahn A. The Eph tyrosine kinase receptors EphB2 and EphA2 are novel proteolytic substrates of tissue factor/coagulation factor VIIa. *J Biol Chem*. 2014; **289**: 32379-91.
75 Le Gall SM, Szabo R, Lee M, Kirchhofer D, Craik CS, Bugge TH, Camerer E. Matriptase activation connects tissue factor-dependent coagulation initiation to epithelial proteolysis and signaling. *Blood*. 2016; **127**: 3260-9.
76 Larsen KS, Ostergaard H, Olsen OH, Bjelke JR, Ruf W, Petersen LC. Engineering of substrate selectivity for tissue factor-factor VIIa complex signaling through protease activated receptor 2. *J Biol Chem*. 2010; **285**: 19959-66.
77 Belting M, Dorrell MI, Sandgren S, Aguilar E, Ahamed J, Dorfleutner A, Carmeliet P, Mueller BM, Friedlander M, Ruf W. Regulation of angiogenesis by tissue factor cytoplasmic domain signaling. *Nature Med*. 2004; **10**: 502-9.
78 Uusitalo-Jarvinen H, Kurokawa T, Mueller BM, Andrade-Gordon P, Friedlander M, Ruf W. Role of Protease Activated Receptor 1 and 2 Signaling in Hypoxia-Induced Angiogenesis. *Arterioscler Thromb Vasc Biol*. 2007; **27**: 1456-62.
79 Apostolopoulos J, Hickey MJ, Sharma L, Davenport P, Moussa L, James WG, Gregory JL, Kitching AR, Li M, Tipping PG. The cytoplasmic domain of tissue factor in macrophages augments cutaneous delayed-type hypersensitivity. *J Leukoc Biol*. 2008; **83**: 902-11.
80 Ahamed J, Niessen F, Kurokawa T, Lee YK, Bhattacharjee G, Morrissey JH, Ruf W. Regulation of macrophage procoagulant responses by the tissue factor cytoplasmic domain in endotoxemia. *Blood*. 2007; **109**: 5251-9.
81 Schaffner F, Versteeg HH, Schillert A, Yokota N, Petersen LC, Mueller BM, Ruf W. Cooperation of tissue factor cytoplasmic domain and PAR2 signaling in breast cancer development. *Blood*. 2010; **116**: 6106-13.
82 Ryden L, Grabau D, Schaffner F, Jonsson PE, Ruf W, Belting M. Evidence for tissue factor phosphorylation and its correlation with protease activated receptor expression and the prognosis of primary breast cancer. *Int J Cancer*. 2010; **126**: 2330-40.
83 Chen KD, Wang CC, Tsai MC, Wu CH, Yang HJ, Chen LY, Nakano T, Goto S, Huang KT, Hu TH, Chen CL, Lin CC. Interconnections between autophagy and the coagulation cascade in hepatocellular carcinoma. *Cell Death Dis*. 2014; **5**: e1244.
84 Reinhardt C, Bergantall M, Greiner TU, Schaffner F, Ostergren-Lunden G, Petersen LC, Ruf W, Backhed F. Tissue factor and PAR1 promote microbiota-induced intestinal vascular remodelling. *Nature*. 2012; **483**: 627-31.
85 Samad F, Ruf W. Inflammation, Obesity and Thrombosis. *Blood*. 2013; **122**: 3415-22.
86 Badeanlou L, Furlan-Freguia C, Yang G, Ruf W, Samad F. Tissue factor-PAR2 signaling promotes diet-induced obesity and adipose inflammation. *Nat Med*. 2011; **17**: 1490-7.
87 Kopec AK, Abrahams SR, Thornton S, Palumbo JS, Mullins ES, Divanovic S, Weiler H, Owens AP, III, Mackman N, Goss A, van RJ, Luyendyk JP, Flick MJ. Thrombin promotes diet-induced obesity through fibrin-driven inflammation. *J Clin Invest*. 2017; **127**: 3152-66.
88 Wang J, Chakrabarty S, Bui Q, Ruf W, Samad F. Hematopoietic Tissue Factor-protease activated receptor 2 signaling promotes hepatic inflammation and contributes to pathways of gluconeogenesis and steatosis in obese mice. *American Journal of Pathology*. 2015; **185**: 524-35.
89 Camerer E, Huang W, Coughlin SR. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc Natl Acad Sci USA*. 2000; **97**: 5255-60.
90 Riewald M, Ruf W. Mechanistic coupling of protease signaling and initiation of coagulation by tissue factor. *Proc Natl Acad Sci USA*. 2001; **98**: 7742-7.
91 Ahamed J, Belting M, Ruf W. Regulation of tissue factor-induced signaling by endogenous and recombinant tissue factor pathway inhibitor 1. *Blood*. 2005; **105**: 2384-91.
92 Ott I, Miyagi Y, Miyazaki K, Heeb MJ, Mueller BM, Rao LVM, Ruf W. Reversible regulation of tissue factor-induced coagulation by glycosyl phosphatidylinositol-anchored tissue factor pathway inhibitor. *Arterioscler Thromb Vasc Biol*. 2000; **20**: 874-82.

93 Li W, Zheng X, Gu J, Hunter J, Ferrell GL, Lupu F, Esmon NL, Esmon CT. Overexpressing endothelial cell protein C receptor alters the hemostatic balance and protects mice from endotoxin. *J Thromb Haemost*. 2005; **3**: 1351-9.
94 Liang HP, Kerschen EJ, Hernandez I, Basu S, Zogg M, Botros F, Jia S, Hessner MJ, Griffin JH, Ruf W, Weiler H. EPCR-dependent PAR2 activation by the blood coagulation initiation complex regulates LPS-triggered interferon responses in mice. *Blood*. 2015; **125**: 2845-54.
95 Rallabhandi P, Nhu QM, Toshchakov VY, Piao W, Medvedev AE, Hollenberg MD, Fasano A, Vogel SN. Analysis of proteinase-activated receptor 2 and TLR4 signal transduction: a novel paradigm for receptor cooperativity. *J Biol Chem*. 2008; **283**: 24314-25.
96 Antoniak S, Mackman N. Multiple roles of the coagulation protease cascade during virus infection. *Blood*. 2014; **123**: 2605-13.
97 Liang HP, Kerschen EJ, Basu S, Hernandez I, Zogg M, Jia S, Hessner MJ, Toso R, Rezaie AR, Fernandez JA, Camire RM, Ruf W, Griffin JH, Weiler H. Coagulation factor V mediates inhibition of tissue factor signaling by activated protein C in mice. *Blood*. 2015; **126**: 2415-23.
98 Yuan Q, Zhang D, Wu S, Yu J, Yu L, Sun Y, Du Z, Li Z, Zhou L, Wu X, Hu J. FVIIa prevents the progressive hemorrhaging of a brain contusion by protecting microvessels via formation of the TF-FVIIa-FXa complex. *Neuroscience*. 2017; **348**: 114-25. 10.1016/j.neuroscience.2017.02.020.
99 Sundaram J, Keshava S, Gopalakrishnan R, Esmon CT, Pendurthi UR, Rao LV. Factor VIIa binding to endothelial cell protein C receptor protects vascular barrier integrity in vivo. *J Thromb Haemost*. 2014; **12**: 690-700.
100 Sinha RK, Wang Y, Zhao Z, Xu X, Burnier L, Gupta N, Fernandez JA, Martin G, Kupriyanov S, Mosnier LO, Zlokovic BV, Griffin JH. PAR1 biased signaling is required for activated protein C in vivo benefits in sepsis and stroke. *Blood*. 2018; **131**: 1163-71. 10.1182/blood-2017-10-810895.

Figure legends

Figure 1: Alternative models of coagulation initiation. (A) In the traditional view, TF generates small amount of thrombin that amplifies its own production through cofactor FVIII and FV activation. **(B)** In the new coagulation scheme, the nascent product FXa formed by TF-FVIIa directly activates both, cofactor VIII and enzyme FIX, prior to inhibition by TFPI and independent of thrombin feedback. FVa generation requires FXa undocking from TF-FVIIa, thus exposing free FXa to inhibitory control by TFPI.

Figure 2: Pathways of TF activation and EV generation. (A) TF modifications of the allosteric Cys^{186}-Cys^{209} disulfide bond by S-Nitrosylation (SNO), glutathionation (GSH) or thioredoxin (Trx) are reversed by thiol-disulfide exchange reactions and PDI during complement C3 activation. In this context, complement fixing antithymocyte globulin (ATG) required C5b-C7 insertion for PS exposure, whereas aPLs upregulate PS independent of complement. **(B)** ATP-triggered activation of the P2X7 receptor is a prothrombotic pathway that activates TF on vessel wall cells and macrophages and recruits TF from raft domains to EV through activation of the inflammasome and caspase 1.

Figure 3: Schematic representation of TF signaling complexes. (A) TF-FVIIa complex signaling via PAR2 involves integrin-complex formation and endosomal internalization dependent on arf6. **(B)** The TF-FVIIa-Xa-EPCR complex directly activates PAR2 and is controlled by TFPI and the anticoagulant aPC-PS-FV complex competing for EPCR occupancy. Release of FXa is crucial for coagulation initiation and thrombin generation. Excessive intravascular thrombin generation may furthermore interact with direct TF signaling by eliciting PAR1/PAR2 heterodimer signaling.