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# Interactions between the innate immune and blood coagulation systems

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**Blood coagulation and inflammation are universal responses to infection and there is crosstalk between inflammation and coagulation that can either amplify or dampen the responses. Loss of appropriate interactions between these systems probably contributes to morbidity and mortality in infectious diseases. For instance, inflammatory cytokines and leukocyte elastase can downregulate natural anticoagulant proteins that help to maintain endothelial-cell integrity, control clotting, inhibit vasoactive peptides and dampen leukocyte infiltration into the vessel wall. This Review will summarize our current understanding of the mechanisms involved in the crosstalk between these two important systems.**

When infectious agents, regardless of their nature, initiate the innate immune response, it in turn triggers blood coagulation [1]. For instance, as viral infections, such as severe acute respiratory syndrome (SARS), progress into severe sepsis, markers of blood coagulation are increased markedly [2]. Blood coagulation components are not simply bystanders but can either amplify or inhibit the inflammatory response. Blood clotting can be initiated when inflammatory cytokines and endotoxin induce the *de novo* synthesis of tissue factor on leukocytes [3]. Exposure of tissue factor to the blood then triggers the coagulation cascade. Complement activation can lead to the formation of plasma membrane surfaces enriched in negatively charged phospholipids that amplify the coagulation reactions [4,5].

Opposing this coagulation cascade are the natural anticoagulant pathways. These pathways not only limit the coagulation response but also dampen the inflammatory response by minimizing leukocyte chemotaxis [6] and endothelial-cell interactions [7], and by suppressing apoptosis [8,9] and reducing cytokine expression [10–12]. However, the acute inflammatory response can lead to suppression of the natural anticoagulant pathways by consumption, proteolytic inactivation and downregulation of protein expression [13]. Loss of the coordinate control of the coagulation and inflammation pathways due to impaired function of the natural anticoagulant systems probably contributes to organ failure in severe sepsis.

The blood clotting process is extremely complex (the more biochemical details are reviewed in Refs [14,15]). Briefly, blood coagulation is initiated when tissue factor comes into contact with blood (Figure 1). Under normal circumstances, there is little tissue factor in the intravascular compartment. Most of the tissue factor is found constitutively expressed on extravascular cells, where it surrounds the vessels providing a mechanism to rapid seal breaches in vessel integrity [16]. Recent studies have indicated that there is a circulating form of tissue factor, probably localized on leukocyte-derived microparticles [17–19]. This microparticle-bound tissue factor might be concentrated on P-selectin on activated platelets in forming thrombi through interaction with its ligand, PSGL 1 (P-selectin glycoprotein ligand 1), from the activated leukocytes. Inflammatory stimuli can increase this response, both by stimulating tissue factor synthesis and promoting microparticle formation. Microparticles express high levels of negatively charged phospholipids on their surface [5], thus promoting both the tissue factor–factor VIIa-mediated factor X and IX activation, and enabling the propagation of coagulation by the factor Xa–factor Va activation of prothrombin and the factor IXa–factor VIIIa activation of factor X. Potent platelet agonists, such as thrombin in combination with collagen or complement C5b-9, can further augment the availability of negatively charged phospholipid membrane surfaces on cells, particularly platelets, thus enabling further amplification of the coagulation system [5,20]. Once initiated, the coagulation pathway has the potential to generate ~100 times more thrombin than is required to form a rapid, firm clot. Under most conditions, this is prevented by the natural anticoagulant mechanism. The major inhibitory mechanisms involve the protein C anticoagulant pathway, antithrombin–heparin, and the tissue factor pathway inhibitor (TFPI).

The protein C pathway is triggered when thrombin binds to thrombomodulin on the endothelial-cell surface. This complex, in concert with protein C bound to the endothelial-cell protein C receptor (EPCR), generates activated protein C. Once activated, protein C dissociates from the EPCR and can bind to protein S on membrane

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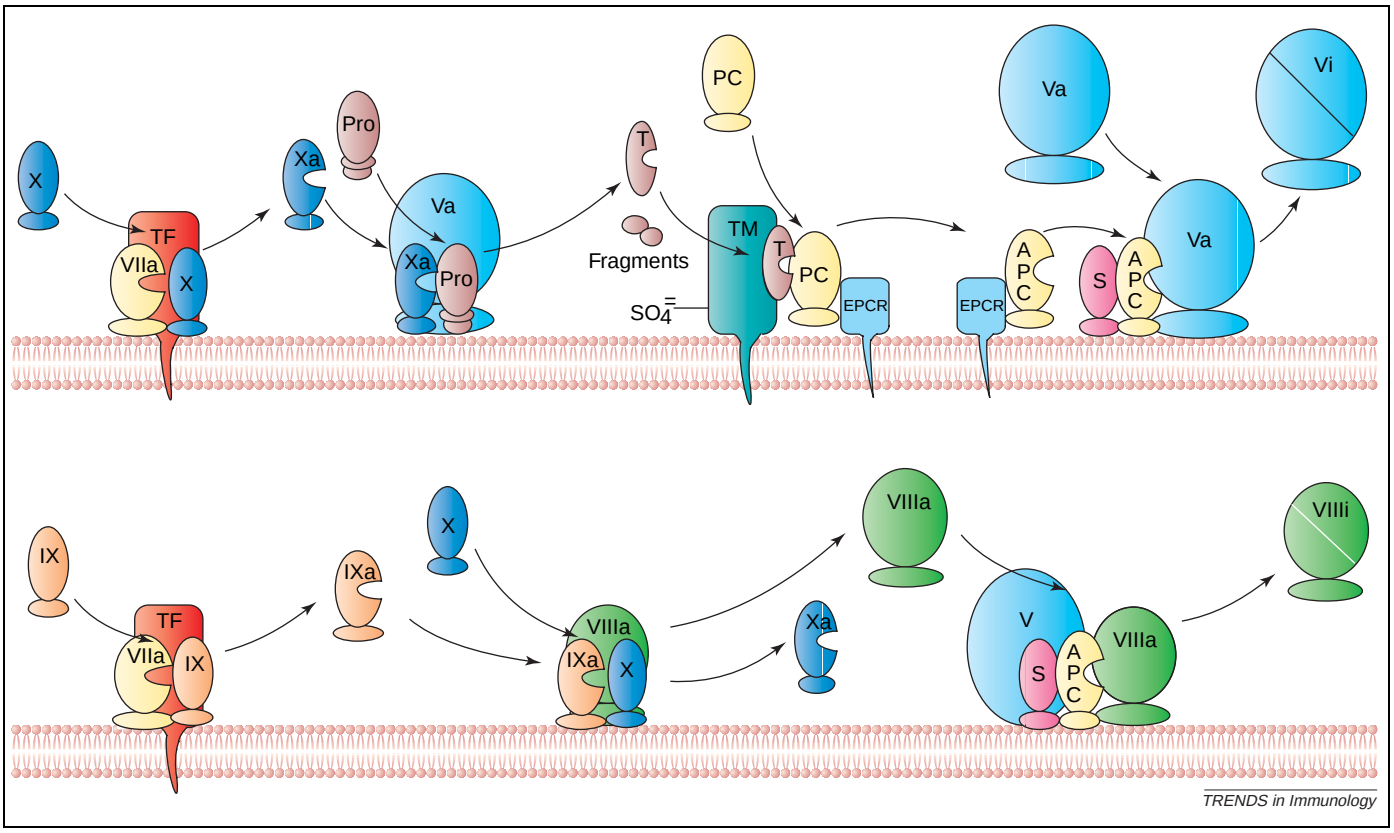


**Figure 1.** The function of membranes and cofactors in blood coagulation. The enzymes associate with cofactors on membrane surfaces. Factor VIIa associates with tissue factor (TF) to activate either factor X or factor IX. Factor IXa associates with factor VIIIa to activate factor X. Factor Va associates with factor Xa to activate prothrombin (Pro). Thrombin (T) associates with thrombomodulin (TM) to activate protein C (PC) and the activated protein C (APC) complexes with protein S (S) to inactivate factors Va and VIIIa, thereby blocking the coagulation cascade.

surfaces. The activated protein C–protein S complex then inactivates factors Va and VIIIa. Without these cofactors, factor Xa and factor IXa have much less than 1% of the capacity to activate the downstream zymogen. Practically, this means that inactivation of the cofactors shuts off the coagulation system completely. In addition, relative to thrombin in solution, thrombin bound to thrombomodulin is inhibited much more rapidly by antithrombin and protein C inhibitor [21].

The antithrombin–heparin mechanism neutralizes factor Xa, thrombin and factor IXa. It also inactivates factor VIIa but only when the factor VIIa is bound to tissue factor [22,23]. Antithrombin inhibition of the factor VIIa–tissue factor complex, factor IXa, factor Xa and thrombin are all thought to be accelerated by vascular heparin-like proteoglycans [24].

TFPI inactivates factor VIIa bound to tissue factor using a unique mechanism. The inhibitor has two functional inhibitory Kunitz domains. The second domain binds to and inhibits factor Xa, which, as a result of its ability to bind negatively charged membranes, concentrates the TFPI–factor Xa complex on the membrane surface, at which time the first Kunitz domain of the inhibitor reversibly neutralizes factor VIIa.

The physiological significance of these pathways is demonstrated by gene experiments in mice, which result in embryonic or neonatal lethality when any single pathway is disrupted [25–29]. In humans, complete deficiencies of protein C lead to neonatal microvascular thrombosis (purpura fulminans) and subsequent death unless treated [30]. Complete deficiencies of antithrombin and the TFPI in humans have not been described.

## Impact of acute inflammatory responses on natural anticoagulant mechanisms

Inflammation has multiple effects on the coagulation system (Figure 2). Antithrombin is consumed and/or inactivated in sepsis and other acute inflammatory injury. Antithrombin inhibitory activity decreases markedly during severe sepsis, often to $<50\%$ of normal levels [31]. Because the rates of inhibition of the target proteases are strongly dependent on the antithrombin concentration, this decrease in inhibitor concentration would contribute to increased stability of the coagulation enzymes and hence favor intravascular coagulation.

There is evidence that the vascular heparin-like molecules are reduced by inflammatory cytokines and neutrophil activation products [32]. Clinically, in severe sepsis, these heparin-like molecules can be downregulated or degraded [33], further diminishing the natural anticoagulant potential, especially when the antithrombin level has been reduced by consumption.

The protein C pathway appears to be especially sensitive to downregulation by inflammatory responses. Interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and endotoxin can downregulate thrombomodulin and the EPCR by inhibiting gene transcription [34,35], reducing the ability to generate activated protein C. Neutrophil

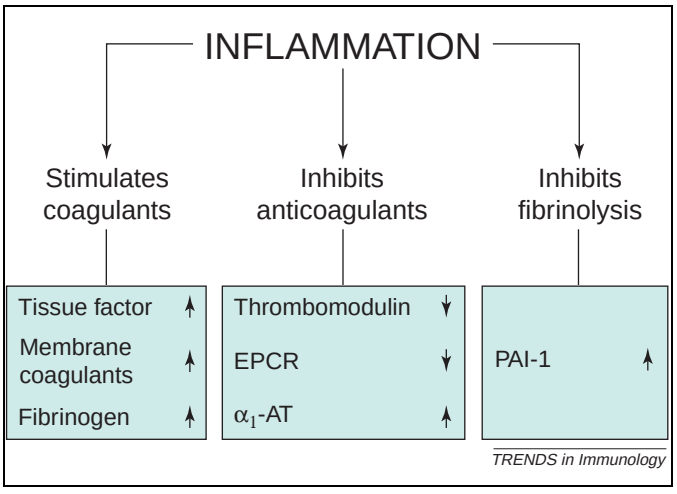


**Figure 2.** The impact of inflammatory mediators on the regulation of coagulation. Inflammatory mediators, such as TNF-α or endotoxin, can effect the changes indicated. An upward arrow indicates increases in levels and a downward arrow indicates decreases. Abbreviations: $\alpha_1$-AT, $\alpha_1$ antitrypsin; EPCR, endothelial-cell protein C receptor; PAI-1, plasminogen activator inhibitor-1; TNF-α, tumor necrosis factor-α.

elastase cleaves thrombomodulin from the endothelial-cell surface, generating a much less active form of thrombomodulin [36]. In septic patients, both the EPCR and thrombomodulin can be severely downregulated, as demonstrated both immunohistochemically [37] and by analysis of the ability of the patients to generate activated protein C [38]. In addition, protein C levels decrease dramatically in patients with severe sepsis. This is probably due to a combination of consumption and liver (the main site of protein C synthesis) dysfunction. The degree of protein C reduction correlates with a negative prognosis in septic patients [39].

Once tight control of thrombin and other coagulation enzymes is lost, they can participate in promoting the inflammatory response (Figure 3). The enhanced inflammatory response enhances the cell-associated coagulation activities as described earlier. Therefore, anticoagulants that dampen the cellular inflammatory response also dampen the coagulant response [40].

### Impact of natural anticoagulant mechanisms on the inflammatory response and cellular apoptosis

The antithrombin–heparin pathway can modulate inflammatory responses not only by inhibiting coagulant enzyme-mediated cell signaling through the protease activated receptors but also by modulating cellular responses [12]. Antithrombin can downregulate the expression of CD11b/CD18 on leukocytes. Because factor X binding to this receptor augments factor X activation [41,42], the downregulation of this cellular factor X receptor decreases both leukocyte adhesion and coagulation. The addition of antithrombin to endothelial cells in culture increases prostacyclin formation [43] and decreases NF-κB signaling [10]. By modulating cellular responses to endotoxin, antithrombin appears to decrease both tissue factor and IL-6 expression in monocytes and endothelium [40]. Antithrombin binding to syndecan 4, a proteoglycan on neutrophils, inhibits chemokine-induced neutrophil migration [44]. Interestingly, heparin blocks this effect. In addition, administration of high levels of antithrombin to septic experimental animals and animals undergoing ischemia reperfusion injury reverses leukocyte recruitment [45] (Figure 4).

The protein C anticoagulant pathway has several components that reduce the inflammatory response. Thrombomodulin not only increases protein C activation but also prevents thrombin from activating protease-activated receptors (PARs) [46,47]. This is accomplished at least in part because the site on thrombin (anion binding exosite 1) responsible for binding PAR1 and thrombomodulin overlap (Figure 5). In addition, thrombin bound to thrombomodulin gains the ability to activate a plasma procarboxypeptidase R [also known as thrombin activatable fibrinolysis inhibitor (TAFI)] [48]. Recent studies have shown that his carboxypeptidase is a very potent inhibitor of the complement anaphylatoxin, C5a [49,50], and bradykinin [50]. By inhibiting these vasoactive

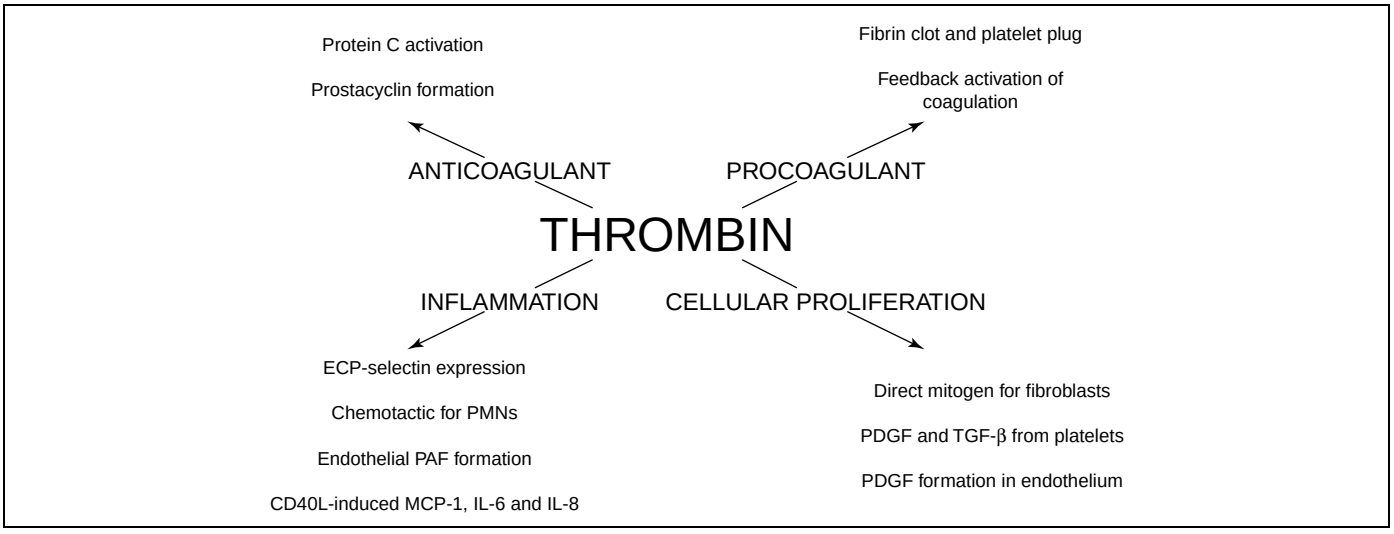


**Figure 3.** Thrombin is a multifunctional enzyme. Thrombin generates procoagulant, anticoagulant, inflammatory and mitogenic responses. These responses shift the hemostatic balance. Abbreviations: CD40L, CD40 ligand; EC, endothelial cell; IL-6, interleukin-6; MCP-1, macrophage chemotactic protein-1; PAF, platelet activating factor; PDGF, platelet-derived growth factor; PMNs, polymorphonucleocytes; TGF-β, transforming growth factor-β. Reproduced with permission from Ref. [77].

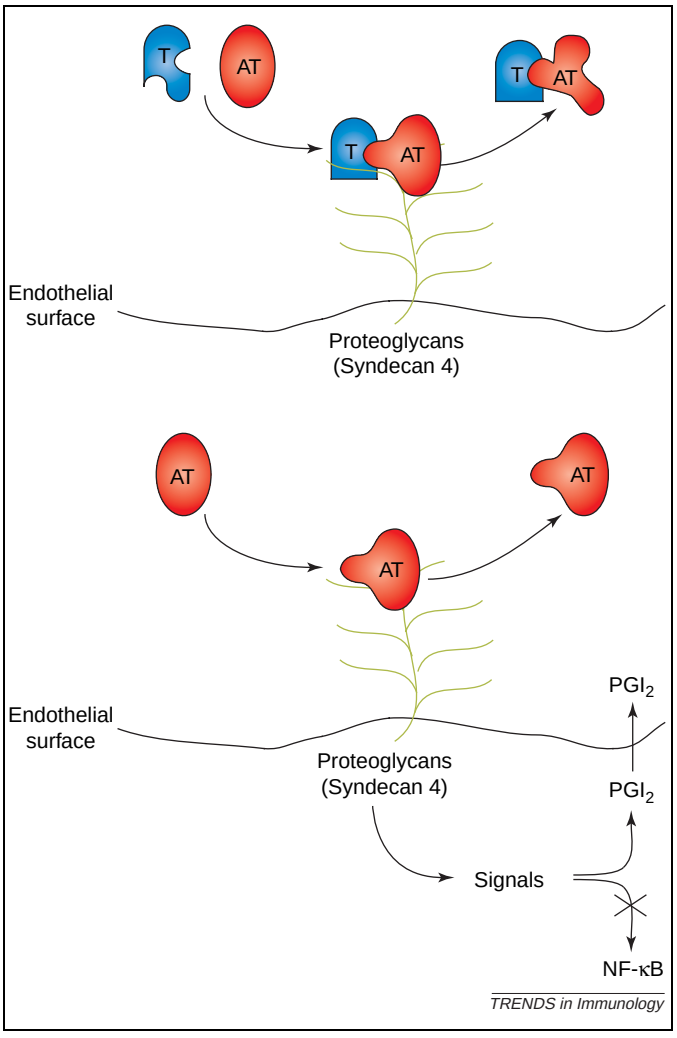


**Figure 4.** Antithrombin binds to heparin-like glycosaminoglycans, such as those on syndecan 4. Binding induces a conformational chance in antithrombin (AT). Thrombin (T) and some other coagulation enzymes react preferentially with the bound AT. In the case of T this requires T binding to the glycosaminoglycan (syndecan 4). Once T binds to the inhibitor, major conformational changes occur that lead to the release of the complex [78]. In the absence of T, AT binding to the glycosaminoglycan leads to cell signaling, increasing prostacyclin ($PGI_2$) formation and decreasing NF-κB activation.

substances, the generation of this carboxypeptidase, especially in the microcirculation [51], probably helps to prevent severe drops in blood pressure and microvascular injury, and subsequent edema, in animals with severe acute inflammatory injuries.

In addition to these functions, recent studies have shown that thrombomodulin has direct anti-inflammatory activity on the endothelium. Thrombomodulin is a multi-domain molecule. When the N-terminal lectin domain is deleted, protein C and carboxypeptidase R activation proceed normally. However, when this mutant is used to replace the normal thrombomodulin gene in mice, the mice recruit leukocytes much more avidly than wild-type mice in response to inflammatory stimuli. Infusion of the isolated lectin domain can reverse this response [52]. Cell culture studies demonstrated that this domain dampened the mitogen activated kinase and NF-κB responses in endothelium [51].

Activated protein C dampens NF-κB signaling in monocytes [53–55]. It also decreases inflammatory mediator-initiated generation of tissue factor on leukocytic cell lines [12,56,57] in an EPCR dependent fashion. In addition, activated protein C can inhibit tight neutrophil adhesion to the endothelium [6]. Activated protein C can also decrease endothelial-cell apoptosis [9]. This function is dependent on the EPCR and apparently involves activation of PAR1 [58]. In mouse models of stroke, activated protein C minimizes damage at least in part by inhibiting apoptosis through downregulation of P53 [59]. This inhibition of P53 expression was also dependent on EPCR and PAR1. How activating PAR1 generates anti-inflammatory activities remains an area of active investigation.

The recent determination of the EPCR structure suggests additional potential roles of the protein C pathway in regulating the immune response. EPCR shares considerable sequence identity to the MHC I–CD1 family of molecules [35]. The crystal structure of EPCR reveals that, like the CD1 family, EPCR has a tightly bound lipid, in this case phospholipid, located in a region virtually identical to the antigen-presenting groove in the CD1 family [60] (Figure 6). In CD1 proteins, glycolipids bind in this grove and can have a major role in the immune response to bacterial infection [61]. In addition, deficiency of CD1d in mice leads to autoimmune disease [62]. From the structural similarities, it is probable, but as yet unproven, that EPCR might have roles similar to those of CD1 molecules in the regulation of inflammation. Autoantibodies to EPCR and a correlation with autoimmune fetal loss have recently been demonstrated [63], consistent with its involvement in immune regulation.

## Platelet contributions to inflammatory responses

Platelets are also involved in the linking of inflammation and coagulation. Inflammatory mediators, such as IL-6, not only increase platelet production, but the platelets that are generated are more thrombogenic, demonstrating an increased sensitivity to platelet agonists, such as thrombin [64]. Platelets contain high concentrations of the proinflammatory mediator CD40 ligand. On platelet activation, CD40 ligand is released. This protein then induces tissue factor synthesis [65,66] and increases inflammatory cytokines, such as IL-6 and IL-8 [67,68].

## Conclusions

The clotting process involves molecules, such as the selectins, that were originally thought to be involved primarily in inflammation (Box 1). Natural anticoagulant proteins exhibit a spectrum of anti-inflammatory activities, including inhibiting NF-κB signaling, inhibiting complement C5a and bradykinin and preventing apoptosis. Severe acute inflammatory challenges can down-regulate the natural anticoagulants, thereby diminishing their ability to dampen the coagulant and inflammatory responses. When this happens, a progressive cycle can evolve in which the coagulation increases the inflammatory response, thereby increasing the coagulant response until severe vascular and organ injury occurs. Modulation of this complex series of interactive events promises to offer an attractive method for intervening clinically in diseases involving acute inflammatory

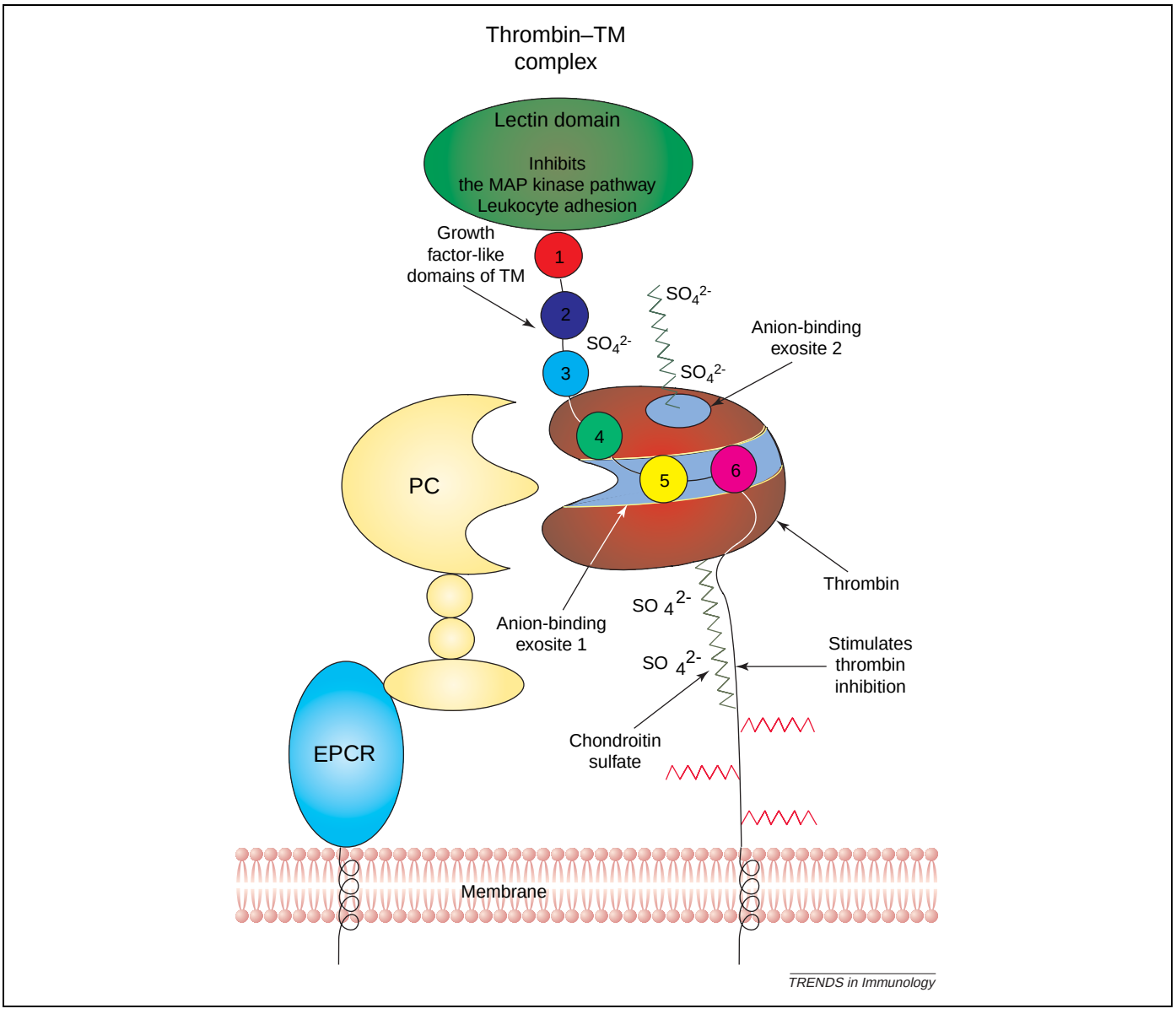


**Figure 5.** Thrombin binding to TM involves anion-binding exosite 1 on thrombin (shown as a strip through the middle of thrombin) and EGF domains 4 to 6 on TM. A chondroitin sulfate moiety on TM increases the affinity for thrombin but is not required for function. This chondroitin sulfate interacts with anion-binding exosite 2 on thrombin, a second, basic area near the heparin-binding site. The lectin domain of TM inhibits leukocyte adhesion and the MAP kinase pathway. Other functions of TM require the presence of different EGF domains. Domains 1–6 stimulate fibroblast growth; domains 3–6 are required for TAFI activation; domains 4–6 are required for PC activation; and thrombin-clotting activity is blocked by domains 5–6. The activation of PC to APC by the thrombin–TM complex is enhanced by binding of PC to EPCR through its gla domain [60]. Abbreviations: APC, activated protein C; EGF, epidermal growth factor; EPCR, endothelial-cell protein C receptor; MAP, mitogen-activated protein; PC, protein C; TAFI, thrombin activatable fibrinolysis inhibitor; TM, thrombomodulin.

challenges, such as severe sepsis. The recent successful demonstration that recombinant activated protein C can reduce the 28-day all cause mortality in patients with severe sepsis [69] provides support for this proposal.

Of interest, antithrombin [70] and TFPI both failed in large randomized clinical trials [71], despite, similar to activated protein C, having proven efficacious in non-human primate models of severe sepsis [72,73]. Several possibilities could explain the failure of the natural anticoagulants TFPI and antithrombin in the trials. First, in the clinical situation, bleeding complications limit dosage to a greater extent than in the animal studies. For instance, the anticoagulant doses in the baboon sepsis model were much higher than in the human trials [74]. Therefore, the doses of the anticoagulants might not have been adequate. In the antithrombin trial, heparin was used in many of the patients [70]. As noted earlier, heparin blocks several cellular effects of antithrombin. Although there was no overall effect on survival with antithromin, the patients with heparin alone or antithrombin alone tended to have a better survival rate than the patients who received both [70].

Alternatively, the trial result differences lie in some different functions among the natural anticoagulants. Antithrombin and activated protein C share several anti-inflammatory functions *in vitro* and in animal models. Few studies have explored differences between the natural anticoagulants with respect to the cellular responses and particular vascular beds that are impacted when an ongoing inflammatory challenge is in place at the

**Box 1. Questions for future research**

The exact signaling mechanisms responsible for the protective effects of natural anticoagulants need to be characterized more fully. At the preclinical level, differences in the protective effects of natural anticoagulants need to be explored more fully, particularly in situations where disease has already progressed, as would almost always be the case in patients. Future research will also need to address the impact of common disease processes, such as diabetes, on both the innate immune response and the ability of natural anticoagulant pathways to modulate the innate immune response. Combinations of microarray and proteomics approaches might aid in these studies.

In addition, although our understanding of the regulation of blood coagulation in systems with artificial membrane surfaces is relatively sophisticated, cellular control of coagulation is still an emerging area. For instance, activated protein C is a potent anticoagulant involving liposomes. By contrast, it is a relatively poor anticoagulant on platelet surfaces. In model systems of blood coagulation, in which platelets are present over endothelium, it appears that activated protein C anticoagulant functions occur on the endothelial cell rather than the platelet surface [76]. The nature of the membrane surface responsible for these differences remains to be elucidated.

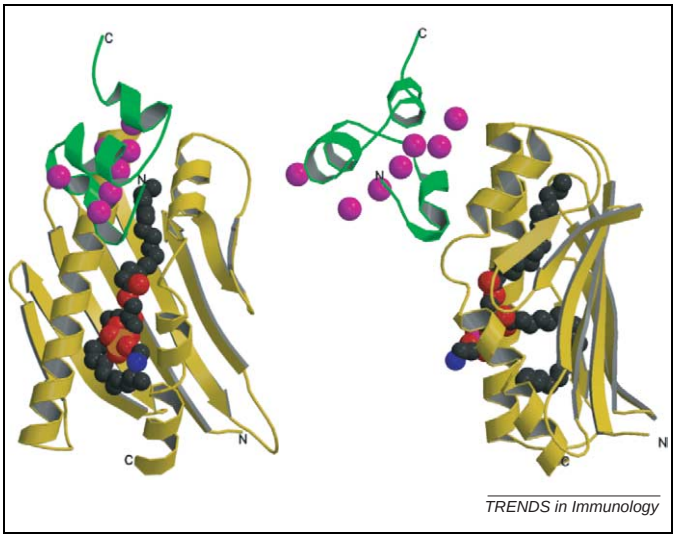


**Figure 6.** The EPCR molecule with a portion of the PC Gla domain and a lipid molecule. In the EPCR (yellow ribbon), two α-helices and an eight-stranded β-sheet create a groove that is filled with phospholipid (the space filling blue balls in the center). Binding of $Ca^{2+}$ ions (magenta spheres) to the PC Gla domain (green ribbon) exposes the N-terminal 'omega' loop, which in the absence of EPCR interacts with the phospholipid surfaces on the membrane. There do not appear to be direct interactions between the PC Gla domain and the lipid molecule located in the groove of the EPCR. The model of the complex consists of residues 7–177 of the rsEPCR and the first 33 residues of the PC Gla domain. Abbreviations: EPCR, endothelial-cell protein C receptor; PC, protein C.

time of inhibitor administration. Furthermore, in the patient populations, many co-morbidities might decrease efficacy of one of the inhibitors selectively. Co-morbidities, such as atherosclerosis, diabetes and hypertension, can exacerbate the innate inflammatory response by a variety of mechanisms, including downregulation of the natural anticoagulants [75].

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