The Innate Immune System in Venous Thrombosis


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Venous Thrombosis: A Serious and Common Problem

Venous thrombosis is a major cause of morbidity and mortality.

VTE Incidence ~300,000-2,000,000/year in the US.

Though common, relatively little is understood about the cellular and molecular events which initiate the acute phase of venous clot formation.
Risk Factors for Venous Thrombosis

Virchow’s Triad (1856)

- Endothelial Injury
- Venous Stasis
- Hypercoagulable state

Pregnancy, Malignancy, Surgery, Trauma, Age, Drugs, Obesity, Smoking, Hereditary Thrombophilias, Smoking, Inflammation.
Inflammation and VTE: Increased Risk of VTE in autoimmune disease.

VTE is a well-described complication of Crohn’s Disease and Ulcerative Colitis

Recent study of 13,756 patients with IBD, risk of VTE was increased compared to controls (HR 3.4, CI 2.7-4.3). The risk was amplified at time of disease flare (HR 8.4, CI 5.5-12.8). Lancet. 2010;375(9715):657.

Many studies have demonstrated a higher incidence of VTE in patients with other systemic autoimmune diseases.

Patients with connective tissue diseases had an increased incidence of VTE (IRR 2.3%) within 1 year post diagnosis compared to controls. Risks were highest with SLE and JRA. J Thromb Haemost. 2012 May;10(5):815-21
Inflammation and VTE: Risk of VTE and Acute Infection

Smeeth et al examined cases of first DVT (n= 7278) or first PE (n=3755) for association with URI or UTI in patients from the UK Health Improvement Network. Risk of VTE was increased proximal to both infections (IR= 2.1% and 1.9% for UTI and URI respectively). Lancet. 2006 Apr 1;367(9516):1075-9

Data from the Danish National Registry of Patients supported an increased risk of VTE in patients recently diagnosed with an infection in both community of hospital settings. Respiratory tract, urinary tract, skin, intra-abdominal and bacteremia were associated with at least two fold increased VTE risk. J Intern Med. 2012 Jun;271(6):608-18.

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<th>Inflammatory disease</th>
<th>Relative risk</th>
<th>Absolute risk</th>
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<tr>
<td>Infections NOS</td>
<td>1.7 – 2.5</td>
<td>n/a</td>
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1 Cases per 1,000 person-years

• VTEs are often associated with elevated levels acute phase reactants such as C reactive protein.

• Other inflammatory biomarkers have been associated with VTE including IL-6, IL-8, MCP-1, and TNF-α.
Early endothelial inflammation is associated with the release of Weibel-Palade bodies which contain vWF and P-selectin.

P-Selectin is required for venous clot formation in numerous animal models.

P-selectin mediates leukocyte rolling through interaction with P-selectin glycoprotein ligand 1

Clinical, epidemiologic, pathologic, and experimental evidence suggests a role for inflammation and innate immune cells early in venous thrombosis.

Endothelial injury is rarely demonstrable in VTE (except post-surgical).

What role does inflammation and the innate immune system play in the initiation and propagation of venous thrombosis in the absence of endothelial injury?
Stasis

Inflammation

Venous Thrombosis

Hypoxia
Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo

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Study Objectives

I) Develop a clinically relevant mouse model to establish venous thrombosis resulting from ‘low flow’ state in the absence of endothelial damage.

II) Use model to investigate role of innate immune response and inflammation early in thrombus formation.
Mice undergo ‘atraumatic’ laparotomy with placement of a permanent narrowing ligature on the IVC below left renal vein. Post-procedure, blood flow velocity is reduced ~80%.

Genetic mouse models were used in conjunction with intravital 2 photon microscopy to interrogate cellular and molecular events in early clot formation.
Within only 1 h of depressed venous blood flow, leukocytes started to roll along, adhere, or crawl on the venous endothelium (Fig. 3, A–C; and Video 3). In contrast, leukocytes barely interacted with the endothelium when venous blood flow was left unperturbed in sham-operated animals, excluding surgical preparation as trigger of the inflammatory response (unpublished data). Leukocyte accumulation increased significantly over time, and after 5–6 h leukocytes carpeted virtually the entire endothelial surface (Fig. 3, B and C), consistent with the ex vivo observations (Fig. 2, B–D). These findings clearly suggest that massive leukocyte accumulation precedes the development of DVT in response to restriction of venous blood flow.
Within only 1 h of depressed venous blood flow, leukocytes started to roll along, adhere, or crawl on the venous endothelium (Fig. 3, A–C; and Video 3). In contrast, leukocytes barely interacted with the endothelium when venous blood flow was left unperturbed in sham-operated animals, excluding surgical preparation as trigger of the inflammatory response (unpublished data). Leukocyte accumulation increased significantly over time, and after 5–6 h leukocytes carpeted virtually the entire endothelial surface (Fig. 3, B and C), consistent with the ex vivo observations (Fig. 2, B–D). These findings clearly suggest that massive leukocyte accumulation precedes the development of DVT in response to restriction of venous blood flow.

Leukocytes were recruited to the IVC in large quantities already after 6 h of flow restriction (Fig. 2, B–D). Leukocytes adhered directly to the luminal aspect of the venous endothelium, whereas endothelial disruption was never detected (Fig. 2, B–D). Next, we performed intravital epifluorescence and two-photon microscopy (2P-IVM) to dissect the dynamics of leukocyte recruitment during DVT development in the IVC in response to...
Leukocytes are recruited early to endothelial surface

Fig 2: Leukocytes are recruited early to endothelial surface

A

B

C

D

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What are the kinetics of leukocyte recruitment by cell subset?

- Acradine orange (Leukocytes)
- LysM-eGFP (Neutrophils)
- CX3CR1-eGFP (Monocytes)

IVC Ligature

Imaging by 2 photon microscopy
Fig 3: Neutrophils and Monocytes are the main leukocyte subsets which accumulate early after DVT induction.
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Fig 4: Leukocyte Adhesion Depends on Endothelial P-Selectin

(A) RT-PCR of P-selectin in the IVC at baseline or 6 and 48 h after DVT induction (n=5 per group). Results are shown as mean ± standard deviation.

(B) RT-PCR of P-selectin in the IVC at baseline or 6 and 48 h after DVT induction (n=5) and CCL2 (n=7). Data are shown as mean ± SEM.

(C) Immunohistochemical stainings of the IVC in response to DVT induction. Firm cell adhesion is given as thrombus load in square millimeters of C57BL/6 mice 6 h after induction of DVT. Thrombus after 48 h in C57BL/6 and Innate immune cells and platelets initiate DVT | von Brühl et al. | Innate immune cells and platelets initiate DVT | von Brühl et al.

Leukocytes were stained with Acridine Orange and visualized by intravital video microscopy (arrowhead indicates aggregates; orange and visualized by intravital video microscopy (arrowhead indicates aggregates; arrows indicate single adherent cells). Bars, 50 µm. (D, Left) Representative in vivo images of adherent leukocytes in C57BL/6 mice 6 h after induction of DVT. (E, Left) Representative images of the excised IVC including the vWF. (F) Histological analysis of the IVC harvested IVC thrombi 48 h after DVT induction. Dots represent individual experiments; lines show the mean ± standard deviation. (C) Representative images of adherent leukocytes in C57BL/6 mice 6 h after induction of DVT.
Fig 4: Leukocyte Adhesion Depends on Endothelial P-Selectin
apart from acting as a potential source of myeloid TF, neutrophils deliver additional signals supporting venous thrombogenesis. Recently, activated neutrophils have been shown to release NETs, which consist of extracellular chromatin fibers with a backbone of histones (Brinkmann et al., 2004) and which may exert prothrombotic effects in vitro. Although release of NETs has been previously observed in a baboon model of venous thrombosis and in sepsis-induced microcirculatory thrombosis (Clark et al., 2007; Fuchs et al., 2010), the functional consequences of NETosis and their in vivo relevance for thrombogenesis in large veins remain undressed. Hence, we investigated here whether neutrophils might contribute to thrombus formation via release of NETs (Brinkmann et al., 2004). In fact, after 3 h of flow restriction, we found large amounts of intravascular extracellular DNA induced flow restriction in the IVC (Fig. 6A and not depicted).

To our surprise, neutropenic mice developed no or significantly smaller thrombi compared with isotype-treated controls within 48 h of flow restriction (Fig. 6B). This suggests that monocytes and monocyte-derived TF cannot fully compensate for a loss of neutrophils during DVT. Because TF expression by Ly6G+ neutrophils was weak compared with monocytes (Fig. 5E), it seemed likely that,

**Fig 5: Is Blood Cell TF Involved in DVT Formation?**

**Low-hTF**-No mouse TF (<1% human TF)

**HCV**-normal levels human TF

**A**

![Graph showing fibrin formation](image)

- **HCV**
- **low-hTF**

- **p < 0.001**

- **time after DVT induction**

- **fibrin formation (mean fluorescence intensity)**
Chimera expressing little TF on blood cells

**Fig 5: Is Blood Cell TF Involved in DVT Formation?**

- **Bone Marrow**
  - Low-hTF
  - HCV

- **Images**
  - (A) Immunofluorescence imaging on vG-stained serial sections after 1–6 h of flow restriction by intravital microscopy. Representative in vivo images are shown on the right. Bar, 100 µm.
  - (B) Thrombus load was assessed on vG-stained serial sections after 48 h in control mice (LysM^ Cre^/ox/ox) and HCV mice (LysM^ Cre^/ox/ox). Data are shown as mean ± SEM.
  - (C) Thrombus weight (at 48 h) in control mice (LysM^ Cre^/ox/ox) and HCV mice (LysM^ Cre^/ox/ox). Data are shown as mean ± SEM.
  - (D) Adherent leukocytes at 48 h in control mice (LysM^ Cre^/ox/ox) and HCV mice (LysM^ Cre^/ox/ox). Data are shown as mean ± SEM.
  - (E) Immunohistochemical detection of cell TF (red) on Ly6G-positive (green) and myeloid cells (blue). LysM^ Cre^/ox/ox (HCV) mice (top) and LysM^ Cre^-TF^ flox/flox (low-hTF) mice (bottom).
Are Neutrophil Extracellular Traps (NETs) involved in DVT induction?

Neutrophil NET formation is a process by which Neutrophils actively extrude DNA associated complexes which include granular proteins and anti-bacterial molecules.

NETs serve a putative innate anti-microbial immune function

NET formation has also been implicated in pathologic conditions such as autoimmune disease, TRALI, and VTE.
ogen surrounded the NETs, we analyzed in more detail of NETs during DVT development (Fig. 6 F).

Intravital 2-PIVM revealed that extracellular DNA originates from Ly6G+ neutrophils (Fig. 6 D and Video 7)

Because platelets and a dense network of extracellular DNA originating from MPO, NE, and histones (H2A-H2B-DNA, H3) in neutrophils (Fig. 6 D and Video 6).

NETs were decorated with TF and protein disulphide isomerase, an enzyme implicated in the activation of blood cell–prothrombotic surface (not depicted; Reinhardt et al., 2008).

Hence, we next examined the functional relevance of NETs whether NETs significantly suppressed DVT growth (Fig. 6, G and H).

Figure 6. NETs in DVT induction.

(A) Leukocyte accumulation in vivo at 6 h of thrombosis. Arrowheads: extracellular DNA; arrows: neutrophil. Bar, 50 µm.

(B) Thrombus weight (left) in the IVC was determined in WT injected with normal saline i.v. and HU mice. Shown is a representative of each group. Quanti- tations of NETs at 48 h in the enoxaparin-treated animals (n = 6 per group). Data are shown as mean ± SEM. Data were obtained from 3 experiments of intravital microscopy. Ly6G-positive neutrophils (green, FITC anti-Ly6G antibody) attached to the ves- sel wall (blue) release Sytox orange–positive nuclei as shown (right) as mean ± SEM. Dots represent individual experiments; lines show the mean.

(C) Visualization of NETs in vivo by 2-photon fluorescence microscopy. Ly6G-positive neutrophils (green, Sytox Green) and aggregated neutrophils; arrows: single, adhering to the vessel wall; arrowheads: NETs. Bar, 100 µm.

(D) Visualization of NETs in vivo by 2-photon microscopy. Ly6G–treated WT mice (n = 3 per group). Data are shown as mean ± SEM.

(E) Localization of NETs stained with Hoechst after DNase1 treatment with anti-Ly6G and isotype control antibody (not depicted; Reinhardt et al., 2008). Arrows, nuclei; arrowheads, NETs. Bar, 50 µm.

(F) Measurement of NETs in the IVC. Bars, 50 µm.

The quantitative analysis of NETs stained with Hoechst is also shown (also see Video 7).

(H) After injection of DNase1, thrombi stained with Hoechst after DNase1/actinase and DNA restriction. Dots represent measured NETs in thrombi after DNase1 treatment. Arrowhead: NETs. Bar, 10 µm.

Arrows, nuclei; arrowheads, NET structures inside the IVC 4 h after induction of thrombosis stained with Hoechst after DNase1/actinase and DNA restriction. Dots represent measured NETs in thrombi after DNase1 treatment. Arrowhead: NETs. Bar, 10 µm.
Innate immune cells and platelets initiate DVT | von Brühl et al.

Whether NETs concentrate prothrombotic factors on their surfaces. In fact, the NETs were decorated with TF and protein disulfide isomerase, an enzyme implicated in the activation of blood cell–derived TF, supporting the concept that they might act as a prothrombotic surface (not depicted; Reinhardt et al., 2008).

Hence, we next examined the functional relevance of NETs for DVT formation and treated mice with DNase1. Administration of DNase1 reduced NETs and at the same time significantly suppressed DVT growth (Fig. 6, G and H).

Intravital 2-PIVM revealed that extracellular DNA originates from Ly6G+ neutrophils (Fig. 6 D and Video 7). Extracellular DNA was located in close proximity to neutrophils and stained positive for the neutrophil granule proteins MPO, neutrophil elastase (NE), and the histone proteins H2A-H2B and H3, confirming that these structures were NETs (Fig. 6 E and not depicted). NETs were virtually absent in neutropenic mice, indicating that neutrophils are the major source during DVT development (Fig. 6 F).

Because platelets and a dense network of fibrin surrounded the NETs, we analyzed in more detail...

Fig 6. NETs in DVT induction.
degradation of NETs in vitro (Fuchs et al., 2010), reduced the formation of NETs during DVT in vivo (Fig. 6), an effect which might add to the antithrombotic actions of the drug.

Together, these findings indicate that NETs are not innocent bystanders but rather contribute to neutrophil-driven coagulation during DVT propagation. Heparin, which has a high affinity for histones (Pal et al., 1983) and fosters...

Fig 7. Platelets and Leukocytes Interact to Support DVT Formation
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revealed that most platelets adhered either directly to the intact endothelium or were attached to adherent leukocytes (Fig. 7 C and Videos 8–10). Platelets and leukocytes formed heterotypic aggregates (Fig. 7, B–D). Considering that only 10% of the circulating platelets are labeled in our Platelets support DVT formation.

In addition to leukocytes, platelets are recruited during DVT formation (Fig. 7 A). Intravital microscopy indicated that platelet adhesion occurred within 2 h of \( \Delta P \) and increased over time (not depicted and Fig. 7 B). After 6 h, 2P-IVM

Figure 8. Platelets induce NET formation, which triggers FXIIa-dependent thrombus propagation. (A) Freshly isolated human neutrophils were incubated with platelet supernatant. Cells were stained with a primary antibody directed against DNA–histone complexes and DAPI and visualized by confocal microscopy. Incubation with DNase1 where indicated. Bars, 50 µm. Arrowheads, cell nucleus; arrows, NET. (B) The total number of NETs (left) and NETs per leukocyte (right) was quantified on cross sections of thrombi 48 h after \( \Delta P \) in IL4-R/Iba1 mice and WT animals (\( n = 3 \) per group). Data are shown as mean ± SEM. (C) Analysis of thrombus formation (milligrams) in the IVC of C57BL/6 (\( n = 9 \)), FXII−/− mice (\( n = 7 \)), and FXI−/− mice (\( n = 7 \)) 48 h after \( \Delta P \). Dots represent individual experiments; lines show the mean of each group. (D) Quantification of fibrin density as percent of fibrin-covered area in the IVC thrombus (\( n = 4 \) per group). Data are shown as mean ± SEM. (E) The effects of co-incubation of activated platelets (P) and neutrophils (N) on FXII activation in vitro. NETosis was inhibited by an antibody directed against the H2A–H2B–DNA complex. Data are shown as mean ± SEM. (F) Confocal visualization of FXII on NETs, released from isolated human neutrophils. Arrow, FXII bound to Sytox Green+ NETs. Bars, 10 µm.
Since Factor XII can be activated by negatively charged surfaces, do NETs activate factor XII in DVT formation?
Fig 7. NETs serve as Factor XII scaffold and facilitate Factor XII activation.
Study provides novel insight into how restricted venous flow can initiate an inflammatory response during DVT induction.

- Flow restriction upregulates several chemokines in vessel wall. Leukocytes are recruited early, predominately neutrophils.

- Innate immune cells play an active role in thrombus formation both by the delivery of TF and NETs. Platelets enhance this process (in the absence of endothelial disruption).

- Data suggests NET formation forms a prothrombotic scaffold for activation of factor XII.
Stasis

Inflammation

Venous Thrombosis
Proposed Model of Innate Immune Cell Involvement in Venous Thrombosis

A. Hypoxia, ULVWF, and DNA, respectively. Monocytes/macrophages (MØ) release an additional source of DNase and generate plasmin and promote fibrin formation, and exacerbate platelet and endothelial activation.

B. Activated platelets and neutrophils (RBC) adhesion, promote fibrin formation, and exacerbate platelet and endothelial activation.

C. Tissue factor (TF)–containing microparticles that enhance thrombin generation in the growing thrombus.

Figure 1. Proposed Model of Innate Immune Cell Involvement in Venous Thrombosis.
Neutrophils appear to be important for early venous thrombosis formation. What is the contribution of immune cell subsets to thrombolysis?

Are the mechanisms described in this study clinically relevant to human patients with VTE?

Do these mechanisms apply to different clinical scenarios such as clots that form in neutropenic patients?

Are any of the mechanisms of clot initiation/propagation described in the study hyperactive in patients with unidentified thrombophilias?
Questions for Further Investigation

Do these results suggest novel ways to intervene in VTE?

• DNAse?

• Neutrophil Elastase (NE) and peptidylarginine deiminase 4 (PAD4)?

• RAF-MEK-ERK pathway? (GW5074, U0126)
Questions?
Additional References:


J Thromb Haemost. 2012 May;10(5):815-21

Lancet. 2006 Apr 1;367(9516):1075-9


J Cell Biol. 2010;191:677–691


