Universitätsklinikum Hamburg-Eppendorf

Institut für Klinische Chemie und Laboratoriumsmedizin
Prof. Dr. Dr. Thomas Renné

Factor XII and Neutrophil Extracellular Traps in Thrombosis – Crosstalk between Coagulation and Inflammation

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
an der Medizinischen Fakultät der Universität Hamburg.

Vorgelegt von:

Marie Worm
aus Berlin

Hamburg 2020

Angenommen von der
Medizinischen Fakultät der Universität Hamburg am: 14.06.2021

Veröffentlicht mit Genehmigung der
Medizinischen Fakultät der Universität Hamburg.

Prüfungsausschuss, der*die Vorsitzende: Prof. Dr. Florian Langer

Prüfungsausschuss, zweite*r Gutachter*in: Prof. Dr. Dr. Thomas Renné

ABSTRACT

BACKGROUND: Thrombosis is a common disease in the western world. Coagulation factor XII (FXII) has been considered irrelevant for coagulation for a long time, but newer research shows its major role in many thrombotic diseases. This project aimed to characterize a deep vein thrombosis model in mice, to establish immunostainings for FXII and its potential activators, and to use those stainings to analyse the thrombosis model.
METHODS: Frozen and paraffin sections of murine deep vein thrombosis were stained with Haematoxylin and Eosin a Picrosirius red. A protocol for immunofluorescence was tested and enhanced, and stainings for neutrophil granulocytes, neutrophil extracellular traps (NETs), platelets, and FXII/FXIIa were established.
RESULTS: The results show that the thrombus size as well as the amount of red blood cells decreases over time, while the amount of collagen increases. The leucocyte density increases as well, and NETs show a maximum after 2 days. Platelets show a non-significant tendency to increase over time. FXII increases from 6 hours to 2 days after thrombus formation and stays on the same level afterwards. In double stainings, FXII shows an association with DNA and histones.
DISCUSSION/CONCLUSION: The results show the process of thrombus reorganization and resolution. NETs are found in later states of thrombosis and are likely to influence thrombus formation, stability, and reorganization with over all procoagulant properties. FXII initiates coagulation but probably also plays a role in thrombus organization in this setting. Neutrophil derived FXII could be an additional source of FXII.

ZUSAMMENFASSUNG

HINTERGRUND: Thrombosen sind eine häufige Erkrankung der westlichen Welt. Blutgerinnungsfaktor XII (FXII) wurde lange Zeit als irrelevant für die Blutgerinnung angesehen, aber neuere Forschung zeigt seine wichtige Rolle in vielen thrombotischen Erkrankungen. Dieses Project zielte darauf ab, ein tiefe Venenthrombosen-Modell in Mäusen zu charakterisieren, Immunfärbungen für FXII und seine potenziellen Aktivatoren zu etablieren und diese Färbungen zu nutzen, um das Thrombosen-Modell zu analysieren.
METHODEN: Gefrier- und Paraffinschnitte von tiefen Venenthrombosen aus Mäusen wurden mit Hämatoxylin und Eosin und Picrosirius rot angefärbt. Ein Protokoll für Immunfluoreszenz wurde getestet und verbessert und Färbungen für neutrophile Granulozyten, *neutrophil extracellular traps* (NETs), Thrombozyten und FXII/FXIIa wurden etabliert.
ERGEBNISSE: Die Ergebnisse zeigen, dass die Thrombusgröße sowie die Menge an Thrombozyten mit der Zeit abnehmen, während die Menge an Kollagen zunimmt. Die Leukozytendichte nimmt ebenfalls zu und NETs zeigen einen Höhepunkt nach zwei Tagen. Thrombozyten zeigen eine nicht signifikante Zunahmetendenz. FXII nimmt von 6 Stunden auf 2 Tage nach Thrombusentstehung zu und bleibt danach auf demselben Niveau. In Doppelfärbungen zeigt FXII eine Assoziation mit DNA und Histonen.
DIKUSSION/SCHLUSSFOLGERUND: Die Ergebnisse zeigen den Prozess der Thrombusneuorganisation und -auflösung. NETs finden sich in späteren Thrombusstadien und beeinflussen wahrscheinlich die Thrombusbildung, Stabilität und Reorganisation mit insgesamt prokoagulatorischen Eigenschaften. FXII initiiert die Blutgerinnung, aber spielt in diesem Rahmen vermutlich auch eine Rolle in der Thrombusorganisation. FXII aus Neutrophilen könnte eine zusätzliche Quelle für FXII sein.

TABLE OF CONTENTS

1 INTRODUCTION

1.1 Hemostasis and thrombosis

Blood is the universal transport and supply system of our body. It provides oxygen, nutrients, and hormones, carries carbon dioxide to the lungs, distributes heat and is a fundamental part of the immune system. The balance between blood flow and clotting must be maintained at all time. If a vessel is damaged, the blood needs to clot to stop bleeding and to seal wounds but if it clots inside an intact vessel it leads to the serious medical condition of thrombosis with its main complication thromboembolism (Barett, Barman et al. 2009).

Thrombosis appears in arterial and venous beds. Arterial thrombosis is mostly caused by arteriosclerotic plaques whereas the origins of venous thrombosis are more complex and divers. Known risk factors for venous thrombosis are surgery or trauma, malignancy, heart failure, inflammatory diseases and acute inflammation, varicose veins, immobilization, advanced age, adiposity, certain drugs (e.g. chemotherapy, Thalidomide, antipsychotics), congenital thrombophilia (e.g. Factor V Leiden, Protein S deficiency) and previous thromboembolic events (Kyrle and Eichinger 2005). Usually the genesis of a venous thrombosis is multifactorial. The classical Virchow's triad describes three conditions contributing to the development of a deep vein thrombus (DVT), namely endothelial injury, hemodynamic changes and hypercoagulability (Mammen 1992). Newer research emphasizes the role of inflammation in thrombotic diseases. It is an important risk factor for DVT and directly linked to intravascular coagulation, e.g. in sepsis (Saha, Humphries et al. 2011, Aksu, Donmez et al. 2012, Levi, van der Poll et al. 2012, Martinod and Wagner 2014).

Under healthy conditions the vessel endothelium produces anticoagulant and anti-inflammatory substances promoting a fluid state of the blood. It expresses prostacyclin, an effective platelet inhibitor and vessel dilator, thrombomodulin, a potent cofactor for thrombin mediated protein C activation and other anticoagulants e.g. heparin sulfate, tissue factor pathway inhibitor, and tissue plasminogen activator. Production of interleukin 10 (IL-10) inhibits leucocyte adhesion and activations (Mammen 1992, Wakefield, Myers et al. 2008). But if a vessel is damaged, blood is exposed to extracellular matrix proteins and endothelial cells are activated initiating platelet aggregation and coagulation which is an important physiological mechanism to stop bleeding.

A couple of mechanism are suggested how this balance can be disturbed and a thrombus is able to form. If the endothelium is damaged or irritated, e.g. by injury (surgery, trauma) or disturbed blood flow (e.g. varicose veins, immobilization) endothelial cells are activated. They release von Willebrand factor (vWF), tissue factor (TF), platelet-activating factor (PAF) and express P- and E-selectin on their surfaces. Platelet adherence and activation by vWF is crucial for thrombus formation. Activated platelets undergo a morphological change, adhere to the vessel wall and other platelets and release procoagulant substance from their granules, e.g. more vWF, coagulation factors, fibrinogen, P-selectin and inorganic polyphosphates (polyP). Together, the proteins released by the endothelium and platelets have the capability to activate more platelets, the extrinsic pathway of coagulation and leucocytes (Kyrle and Eichinger 2005, Brill, Fuchs et al. 2011, Fuchs, Brill et al. 2012). Moreover, polyP is able to activate coagulation factor XII (FXII) and therefore the intrinsic pathway of coagulation which is a powerful pathway leading to the formation of a blood clot (Muller, Mutch et al. 2009).

The importance of the individual factors contributing to DVT is unknown but it has been shown that activation of endothelial cells alone does not necessary lead to thrombosis (Thomas 1994). In addition, the endothelium can be completely intact in fresh thrombi suggestion other starting mechanism of DVT besides endothelial activation (Saha, Humphries et al. 2011).

Activated neutrophil granulocytes could provide a starting point for thrombosis. Neutrophils are recruited to an inflammatory site by a chemotactic gradient and activated by endothelial cells. They can undergo NETosis, a distinct form of cell death upon which they release large DNA structures referred to as neutrophil extracellular traps (NETs) (Fuchs, Abed et al. 2007). Nucleic acids are known to activate coagulation via the intrinsic pathway (Kannemeier, Shibamiya et al. 2007), NETs provide a scaffold for platelets and red blood cells (RBCs) and they contain histones that are able to activate platelets directly (Fuchs, Brill et al. 2012, Martinod and Wagner 2014). Vice versa, neutrophil-driven inflammation causes endothelial damage, thus activating endothelial cells, and further, neutrophils might be able to directly start the extrinsic pathway of coagulation by releasing TF (Saha, Humphries et al. 2011, Darbousset, Thomas et al. 2012). Taken together, activated neutrophils promote coagulation in many ways and are most likely an important part of thrombosis.

Red blood cells (RBCs) are one of the main components of a venous thrombus, especially in the early phase of DVT. The exact function of RBCs in a thrombus is unknown. They are passively trapped in the fibrin mesh of clotting blood and contribute essentially to the volume of a fresh thrombus, but they might also actively promote thrombosis. They interact with leukocytes and platelets by integrins expressed on their surfaces and they contain a large amount of iron which becomes oxidized if RBCs are destroyed. Oxidized iron can cause endothelial stress (Saha, Humphries et al. 2011, Aleman, Walton et al. 2014).

1.2 Coagulation factor XII

1.2.1 The contact system

The contact system is a cluster of proteins that locally assembles on negatively charged surfaces and contributes to inflammation and coagulation. It is initiated by the activation of coagulation factor XII (FXII, Hageman factor). The FXII zymogen is secreted mainly by the liver and circulates in plasma (Renne 2012). If FXII binds to negatively charged surfaces a small amount of activated FXII (FXIIa) is generated by autoactivation. The serine protease FXIIa

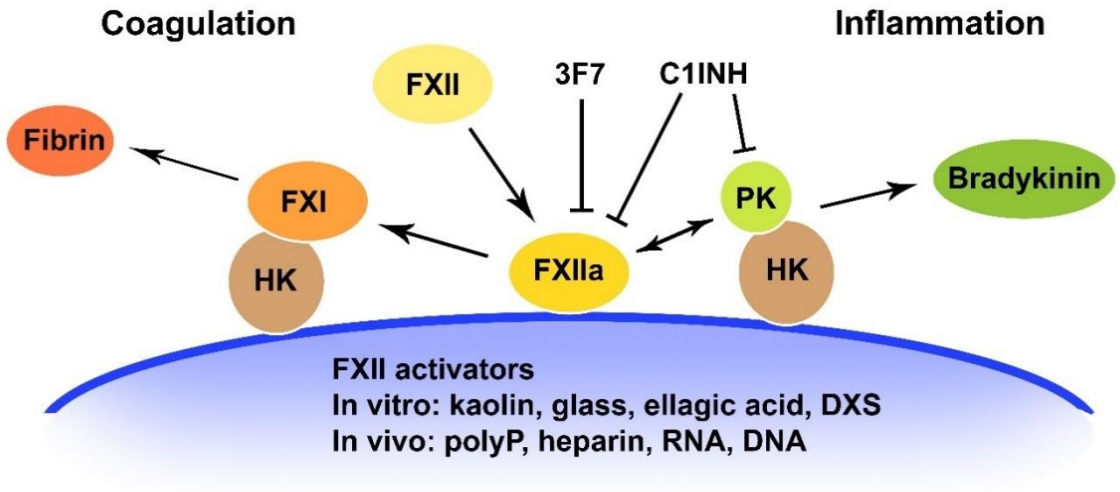


Figure 1: The contact system. Contact with negatively charged surfaces activates FXII and initiates procoagulant and proinflammatory reactions. Activated FXII starts the fibrin producing intrinsic pathway of coagulation by cleavage of FXI and the inflammatory kallikrein-kininogen system by PK mediated cleavage of HK. C1INH interferes both with FXIIa and PK while the antibody 3F7 specifically blocks FXIIa. DXS, dextran sulfate; FXII, factor XII; PK, plasma kallikrein; C1INH, C1 esterase inhibitor. Reference: Worm, Köhler et al. (2015).

cleaves plasma prekallikrein to plasma kallikrein (PK) which reciprocally activates more FXII, thus amplifying the signal. PK is not surface bound itself but recruited by high molecular weight kininogen (HK). When activated, PK releases the inflammatory mediator bradykinin (BK) from HK. BK is a powerful vessel dilator and increases vascular permeability (Shariat-Madar, Mahdi et al. 2002). Furthermore, it stimulates endothelial cells to release other inflammatory mediators such as prostaglandins and induces chemotaxis (Bjorkqvist, Jamsa et al. 2013). Another important substrate for FXIIa is coagulation factor XI (FXI). FXI is also surface bound via the protein HK and is cleaved by FXIIa to its active form FXIa. This starts the intrinsic pathway of coagulation leading to the formation of thrombin and to fibrin generation (Renne 2012).

The main regulatory player in the contact system is the serpin C1 esterase inhibitor (C1INH). This plasma protein is the most important endogenous inhibitor of FXIIa and PK and prevents overactivity of FXII (Maas and Renne 2012) (Figure 1).

1.2.2 FXII activation

FXII circulates in plasma as a single chain zymogen. Binding to negatively charged surfaces induces a conformational change and a self-proteolysis step at Arg_{353}-Val_{354} (auto-activation) occurs. A small amount of activated FXII is generated consisting of a heavy and a light chain connected by a disulfide-bond. This form is referred to as α-FXIIa and it cleaves both its substrates plasma prekallikrein and FXI. More α-FXIIa is reciprocally generated by plasma prekallikrein (PK). α-FXIIa remains surface bound via the heavy chain while the light chain contains the catalytic domain. Two additional cleavage steps at Arg_{334}-Asn_{335} and Arg_{343}-Leu_{344} release the light chain from the heavy chain resulting in β-FXIIa. The light chain retains its proteolytic activity towards PK but is unable to further cleave FXI (Bjorkqvist, Nickel et al. 2014, Labberton, Kenne et al. 2015).

In vitro activation of FXII is well characterized and many non-physiological materials are known to induce contact activation. The white clay material kaolin is commonly used in the diagnostic assay activated partial thromboplastin time (aPTT). The aPTT measures the time from contact activation to fibrin generation and is predominantly used to screen for coagulation deficiencies and monitor heparin therapy (Maas and Renne 2012). Other synthetic activators include glass, ellagic acid and metal ions such as Ni^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+} immobilized on phospholipid micelles (Mutch, Waters et al. 2012). Exposure of blood to medical devices, e.g. during hemodialysis or extracorporeal circulation with heart-lung machines, increases the risk of thrombosis. The polymer surfaces of common medical devices have the capability to activate FXII, thus an antithrombotic treatment is necessary (Svensson, Friberger et al. 1996). Furthermore, the glucan high molecular dextran sulfate (DXS) is mainly used in experimental settings. It activates FXII but exclusively triggers the bradykinin-pathway of the contact system without affecting coagulation (Bjorkqvist, Jamsa et al. 2013).

The in vivo triggering mechanisms of the contact system are not characterized as clearly. Extracellular RNA can activate FXII in plasma and DNA in the form of NETs promotes a procoagulant state possibly through FXII activation (Kannemeier, Shibamiya et al. 2007, Martinod and Wagner 2014). A very important in vivo activator of the contact system is inorganic polyphosphate (polyP) released from activated platelets. Not only does polyP contribute to thrombosis but it also provides a link between primary (cellular) and secondary (protein-mediated) hemostasis which remained a mystery for a long time (Muller, Mutch et al. 2009).

During vascular injury, blood is exposed to the subendothelial matrix. Collagen type I and laminin are among the most abundant proteins in the subendothelial basal membrane and they both have the potential to activate FXII (van der Meijden, Munnix et al. 2009, White-Adams, Berny et al. 2010). Also, arteriosclerotic plaque material initiates fibrin formation in a FXII-dependent manner similarly to collagen (Kuijpers, van der Meijden et al. 2014). In venous

thrombosis, various cell types shed microparticles (MPs) from their membrane such as platelets, leucocytes and endothelial cells. MPs possess a phosphatidylserine rich anionic membrane providing a surface for assembly of the contact system (Wakefield, Myers et al. 2008).

Alike the artificial FXII activator DXS, there are also some in vivo activators that selectively initiate the BK pathway without affecting coagulation. The highly sulfated polysaccharide heparin is released from IgE/antigen-complex activated mast cells and plays an essential role in allergic diseases. It induces vascular leakage and swelling via the contact system without activating the intrinsic pathway of coagulation (Oschatz, Maas et al. 2011). Misfolded protein aggregates, as found in patients with systemic amyloidosis, activate the contact system and trigger BK formation. Plasma levels of PK in these patients are elevated while FXIa remains normal (Maas, Govers-Riemslag et al. 2008).

In conclusion, the molecular mechanism of FXII activation requires surface binding and involves multiple cleavage steps. Many substances physiologically found in the body have the potential to activate FXII.

1.2.3 FXII in thrombosis

The FXII-driven contact system can initiate fibrin formation via the intrinsic pathway of coagulation. It has been observed that patients and animals with a FXII deficiency have a normal hemostatic capacity and do not suffer from excessive bleeding. Deficiencies of other coagulation factors such as Factor VIII or Factor IX, which are both activated by the extrinsic pathway of coagulation, cause severe, life threatening bleeding disorders. TF was believed to be largely, if not exclusively, responsible for fibrin formation in vivo for a long time (Ratnoff and Colopy 1955, Lammle, Wuillemin et al. 1991).

Newer research shows that FXII contributes essentially to thrombosis. FXII gene deficient mice are largely protected from experimental thrombosis while infusion with purified human FXII restores blood clotting (Renne, Pozgajova et al. 2005, Kleinschnitz, Stoll et al. 2006, Kenne and Renne 2014). The unique role of FXII for thrombosis while being dispensable for hemostasis offers the exiting idea of targeting FXII for safe anticoagulation. Currently used anticoagulants like heparin, vitamin K antagonists, or new oral anticoagulants such as Rivaroxaban successfully prevent thrombosis in many settings but they all increase the risk of bleeding as a dangerous side effect.

Prof. Renné' et al. developed the fully human recombinant antibody 3F7 which specifically blocks FXIIa and tested its anticoagulant properties. Mice injected with 3F7 were protected from $FeCl_3$-induced arterial thrombosis and in an arteriovenous shunt model in rabbits 3F7 inhibited shunt occlusion as efficiently as heparin. Another outstanding finding is that targeting FXIIa prevented occlusive blood clotting in an extracorporeal membrane oxygenation (ECMO) system adapted to rabbits. While the ECMO system occluded in less than 3 min in saline treated rabbits 3F7 injections protected the animals as effectively as heparin without increasing the bleeding risk (Larsson, Rayzman et al. 2014). Other groups confirmed these findings. Revenko et al. showed that mice treated with antisense oligonucleotides (ASOs) targeting FXII or plasma prekallikrein were protected from experimental arterial and venous thrombosis while the hemostatic capacity was normal (Revenko, Gao et al. 2011) and Matafonov et al. introduced another antibody targeting FXII that prevented thrombosis in an arteriovenous shunt system in baboons (Matafonov, Leung et al. 2014).

Taken together, these results show that FXII influences coagulation in vivo, as it is essential for thrombus formation, but it appears to be dispensable for hemostasis. This makes FXII an exciting novel target for safe anticoagulation.

1.2.4 Other roles of FXII

Hereditary angioedema is a rare inherited swelling disorder that is linked to FXII activation. It is characterized by episodes of severe acute swellings of skin and mucosa tissue which can be life threatening due to occlusion of the respiratory system (Zuraw 2008).The swelling is caused by excessive BK formation which induces vascular leakage and inflammation. Three types of hereditary angioedema (HAE) have been characterized. In HAE type I plasma levels of C1 esterase inhibitor (C1INH) are reduced, the main physiological inhibitor of FXII and PK. In HAE type II, C1INH plasma levels are normal but C1INH shows a reduced activity and in HAE type III a gain of function mutation in the F12 gene is found (Joseph, Tuscano et al. 2008). Current treatments for HAE patients include the infusion of C1INH, targeting the bradykinin receptor B_2 (B2R) or inhibiting PK (Cicardi, Banerji et al. 2010, Cicardi, Levy et al. 2010, Zuraw, Busse et al. 2010). Targeting FXII is a novel approach to treat HAE and could be especially efficient for HAE type III patients. Deficiency or pharmacologic inhibition of FXII reduced allergic responses in a murine anaphylaxis model (Sala-Cunill, Bjorkqvist et al. 2015).

The FXII zymogen serves as a growth factor as it contains epidermal growth factor-like domains and can induce proliferation of endothelial cells and angiogenesis (LaRusch, Mahdi et al. 2010). FXIIa plays a key role in thrombus formation via the intrinsic pathway of coagulation but it also interacts directly with fibrin. It can modulate the structure of a blood clot independently of thrombin generation. Areas with dense fibrin depositions colocalize with FXII in human carotid thrombi and *in vitro* FXII increases the fibrin density in a dose-dependent manner (Konings, Govers-Riemslag et al. 2011). FXIIa can also activate the fibrinolytic system contributing to thrombus resolution. It is able to directly convert plasminogen to plasmin, the main fibrinolytic enzyme, and reduces clot lysis time (Konings, Hoving et al. 2015). Therefore, the role of FXII in thrombosis is complex. It initiates coagulation and stabilizes the blood clot through direct interaction with fibrin, but it also contributes to fibrinolysis.

The contact system also interacts with the classical complement pathway, a part of the innate immune system. FXIIa is able to activate the C1r subcomponent of the complement protein C1 and C1INH also inhibits C1r and C1s besides FXII and PK (Kaplan and Ghebrehiwet 2010).

1.2.5 FXII histology in literature

Reference	Tissue	Antibody
(Jablonska, Markart et al. 2010)	Human Lung	Mouse anti-FXII (abcam)
(Konings, Govers-Riemslag et al. 2011)	Human carotid thrombi	Goat anti-human FXII (Affinity Biologicals)
(Kuijpers, van der Meijden et al. 2014)	human arteriosclerotic plaque	Mouse anti-FXII (US Patent Application 20090304685, D. Pritchard)
(Nickel, Ronquist et al. 2015)	human PC3 prostasome cancer cells	Recombinant human anti-FXIIa (Renné et al., 3F7)
(Nickel, Ronquist et al. 2015)	human PC3 prostasome cancer cells	Goat anit-FXII/FXIIa (Nordic MUbio)

Table 1: **Immunostainings for FXII in literature.** The online library PubMed (National Center for Biotechnology Information, U.S. National Library of Medicine) was searched for publications with histological depictions of FXII (http://www.ncbi.nlm.nih.gov/pubmed, 05.11.2015). The search terms "factor XII, fxii, and Hageman factor" were used and the figures of all listed publications were manually examined for images of FXII histology. The table shows the found references and the tissue and antibodies used.

The well-known project The Human Protein Atlas (HPA) is a publicly available database aiming to investigate the entire human proteome using well characterized antibodies (Uhlen, Fagerberg et al. 2015). The HPA website shows immunohistochemical stainings of FXII on paraffin sections of various healthy human tissue and cancer tissue with a polyclonal rabbit antibody (http://www.proteinatlas.org/ENSG00000131187-F12/tissue 07.11.2015) (Figure 2). A few authors show well established immunostainings for FXII, but currently (2015) there is no established protocol available for a FXII staining in murine tissue. The searched literature exclusively shows images of stainings performed on human tissue or human cell lines.

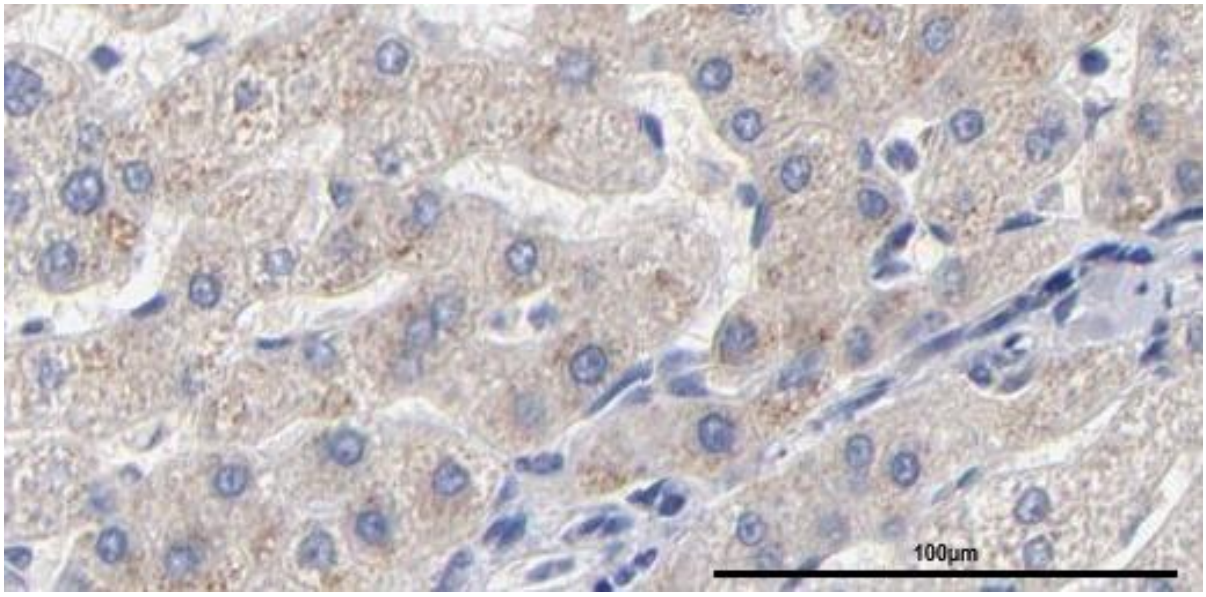


Figure 2: **Immunostaining for FXII by the HPA**. A paraffin section of a healthy human liver shows a positive staining (brown) in the hepatocytes.
Reference: http://www.proteinatlas.org/ENSG00000131187-F12/tissue/liver (07.11.2015)

1.3 Platelets

Platelets are small fragments of blood cells that derive from megakaryocytes in the red bone marrow and are continuously released into the blood stream. They do not contain a nucleus but many granules with procoagulant substances. If a vessel is injured platelets adhere and rapidly aggregate to form a plug that seals the wound. They are essential for hemostasis and also play a role in inflammation and thrombus formation.

If the vessel endothelium is damaged blood is exposed to the subendothelial matrix. Platelets adhere to collagen mainly mediated by vWF binding to the platelet receptor glycoprotein Ib-IX-V (GPIb-IX-V) but also by direct interaction with collagen. Binding of vWF to platelets is the major way of platelet recruitment in a thrombotic setting and platelets contain vWF in their granules (Bryckaert, Rosa et al. 2015). Consistently, vWF is largely associated with platelets in thrombi.

Activated platelets dramatically change their morphology. They spread and form dendrites driven by the submembranous complex of microtubules and actin. Cytoplasm-derived thromboxane A2 is secreted and the granules release procoagulant factors that can attract and activate more platelets. Alpha granules contain e.g. vWF, P-selectin, platelet factor 4, fibronectin, B-thromboglublin, fibrinogen and the coagulations factors V and XIII. Delta or dense granules contain ADP, calcium, serotonin and high amounts of inorganic polyP (Thon and Italiano 2012). Platelet-derived polyP is a negatively charged polymer with a chain length of 68-1000 phosphate residues. It has the capacity to activate the contact system as it has

been shown in vitro. If added to human or murine plasma, polyP induces FXII activation as efficiently as kaolin or dextran sulfate. Smaller synthetic polyP of a mean chain length ≤ 45 phosphate units does not trigger contact activation which emphasizes the importance of the surface size for assembly of the contact system. Animal experiments show that polyP induces BK formation in vivo as described by Prof. Renné's et al. PolyP initiated BK mediated vascular leakage in an edema model in wild type while $B2R^{-/-}$ and FXII gene deficient mice were protected. PolyP can also mediate in vivo activation of the intrinsic pathway of coagulation. Pulmonary thromboembolism was experimentally generated by intravenous infusion of polyP and led to lethal events in almost all wild type mice within 5 min. In contrast, FXII gene deficient mice were largely protected and most animals survived over 30 min (Muller, Mutch et al. 2009).

1.4 Neutrophil extracellular traps

1.4.1 Neutrophils

Neutrophil granulocytes are the most common type of immune cells and a crucial part of the innate immune system. They develop from myeloblasts in the red bone marrow and enter the blood stream which is tightly regulated by chemokines. During maturation, granules form in the neutrophil cytoplasm and the nucleus receives its characteristic lobed shape. Together with the smaller cell populations of eosinophil and basophil granulocytes adult neutrophils are referred to as polymorphonuclear cells (PMNs) on account of their lobed nuclei. Upon infection, the number of circulating neutrophils increases dramatically (Kumar, Abbas et al. 2014).

Neutrophils have a short life span of only 6-8 hours before they undergo a built-in cell death program. As they contain plenty of antimicrobial substances that are also toxic to host cells they need to be removed safely before becoming unstable. Most of the neutrophils undergo apoptosis which includes signaling for macrophages to phagocyte the dying cell and its remnants (Kumar, Abbas et al. 2014).

Usually PMNs are the first cell type at an infections site. They are attracted by chemokines and cytokines, become activated, and release their antimicrobial arsenal. Inflammatory signals such TNF-α, IL-1 and IL-17, or bacterial-derived lipopolysaccharides (LPS) activate endothelial cells and prompt them to express P- and E-selectins and several other intercellular adhesion molecules (ICAMs) (Borregaard 2010). Circulating neutrophils can recognize endothelial selectins with L-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) expressed on their surfaces. This interaction marks the starting point of neutrophil activation, tether the neutrophils to the vessel wall, and enable the characteristic rolling of the neutrophils along the endothelium. The cells slow down and move along the vessel wall while transitory bonds between selectins are formed and broken again. When PMNs reach a cell junction, β2-intergrins on the cells surfaces are able to bind to ICAMs on endothelial cells and arrest them (Sundd, Pospieszalska et al. 2011). They stop moving, start to spread, and mobilization of the antimicrobial arsenal begins. This enables the PMNs to migrate through the endothelium and leave the vessel. A chemotactic gradient leads them towards the inflammation site. Host- and microbial-derived chemoattractants bind to neutrophil receptors and Toll-like receptors (TLRs) can recognize pathogen-associated molecular patterns (PAMPs). A complex signaling cascade induces full activation of the neutrophils and enables them to perform their premier killing mechanisms of phagocytosis, degranulation, respiratory burst and the formation of neutrophil extracellular traps (NETs) (Amulic, Cazalet et al. 2012).

Phagocytosis is the main strategy to eliminate pathogens and cell debris of our immune system. Phagocytic cells recognize particles or bacteria with a variety of receptors and actively internalize them together with a part of their cell membrane. A phagosome is formed within the cell, merged with other granules and eliminated. Neutrophils can either recognize pathogens directly by binding of PAMPs to pattern-recognition receptors (e.g. TLR) or mediated by opsonins such as immunoglobulins or complement proteins (Lee, Harrison et al. 2003).

Neutrophil granules contain plenty of antimicrobial substances and can be released as a defense against bacteria. The granules can be divided into three main classes. Azurophilic granules (peroxidase-positive or primary granules) contain myeloperoxidase (MPO), an enzyme that is essential for the respiratory burst, as well as defensins, lysozyme, bactericidal/permeability-increasing protein (PBI) and various serine proteases such as neutrophil elastase (NE), proteinase 3 (PR3) and cathepsin G. Specific granules (secondary granules) also contain antimicrobial substances such as NGAL, hCAP-18, lysozyme and the glycoprotein lactoferrin. The gelatinase granules (tertiary granules) mostly contain metalloproteases such as gelatinase and leukolysin. Activation of neutrophils leads to fusion of granules with the cell membrane or phagosomes and discharge of the granules content into extracellular space or phagosomes generating an antimicrobial milieu (Faurschou and Borregaard 2003).

Simultaneously to the mobilization of granules, neutrophils can produce reactive oxygen species (ROS) in a process called respiratory burst. It starts by assembly of the NADPH oxidase complex on cell or phagosomal membranes. The NADPH oxidase reduces molecular oxygen (O_2) to superoxide (O_2^-) which starts a cascade of highly reactive ROS production. Superoxide converts to hydrogen peroxide catalyzed by the superoxide dismutase and reacts with nitric oxide to peroxynitrite. Hydrogen peroxide is a substrate for MPO to produce hypochlorus acid. ROS are highly reactive molecules who modify and destroy microbial as well as host proteins. They are very effective to kill bacteria but they also cause inevitable collateral damage (Dahlgren and Karlsson 1999).

Additionally, to killing microbes, neutrophils communicate with most of the other types of immune cells. Tissue macrophages can attract neutrophils to sites of inflammation by releasing chemokines and vice versa activated neutrophils can recruit monocytes and enhance their phagocytic activity. Neutrophils are also able to activate dendritic cells (DCs), induce their maturation and they interact with natural killer cells (NKs) and lymphocytes (Amulic, Cazalet et al. 2012).

Thus, Neutrophils are specialized, powerful immune cells that are essential for defense against microbes and possess an arsenal of antimicrobial weapons.

1.4.2 Neutrophil extracellular traps

Neutrophil extracellular traps (NETs) are released by activated neutrophils if they undergo a distinct form of cell death referred to as NETosis. NETs are large extracellular DNA networks coated with histones and enzymes from neutrophil granules (Brinkmann, Reichard et al. 2004).

NETosis is a complicated process in which the chromatin is decondensed, the nucleus loses its lobed shape and its internal membranes, the cell is lysed, and a DNA/protein mesh is released into extracellular space.

Neutrophils can be stimulated to undergo NETosis by many pro-inflammatory substances, e.g. bacterial membranes, LPS or IL-8. Also, the artificial neutrophil activator phorbol myristate acetate (PMA) induces NETosis which is commonly used in research (Fuchs, Abed et al. 2007). After the neutrophils are activated, an extensive decondensation of chromatin is observed. Heterochromatin is converted to euchromatin and the nucleus starts to expand losing its characteristic lobed shape. Several mechanisms lead to DNA decondensation during NETosis. The enzyme peptidylarginine deiminase 4 (PAD4) citrullinates a large amount of histone H3 and H4 all over the nucleus. Positive charges are lost and therefore the bonds between DNA and histones loosens (Wang, Li et al. 2009). Neutrophil elastase (NE) is translocated to the nucleus and degrades histones, further releasing the DNA (Papayannopoulos, Metzler et al. 2010). Also, ROS production seems to play a role in DNA decondensation. NETosis is dependent on ROS produced by the NADPH oxidase complex and downstream products of the respiratory burst such as hydrogen peroxide. The neutrophil

activator PMA stimulates the NADPH oxidase and consistent with this, inhibition of the NADPH oxidase prevents NET formation (Fuchs, Abed et al. 2007).

The nucleus further expands until it fills most of the intracellular space. Internal nuclear membranes break down which is one characteristic of NETosis (Fuchs, Brill et al. 2012). The chromatin fuses with antimicrobial granular proteins such as additional neutrophil elastase (NE), myeloperoxidase (MPO), cathelin-related antimicrobial peptide (CRAMP) and cathepsin G (Brinkmann, Reichard et al. 2004, Papayannopoulos, Metzler et al. 2010). As the last step of NETosis, the neutrophils die by lysis of the cell membrane and the DNA web, mixed with

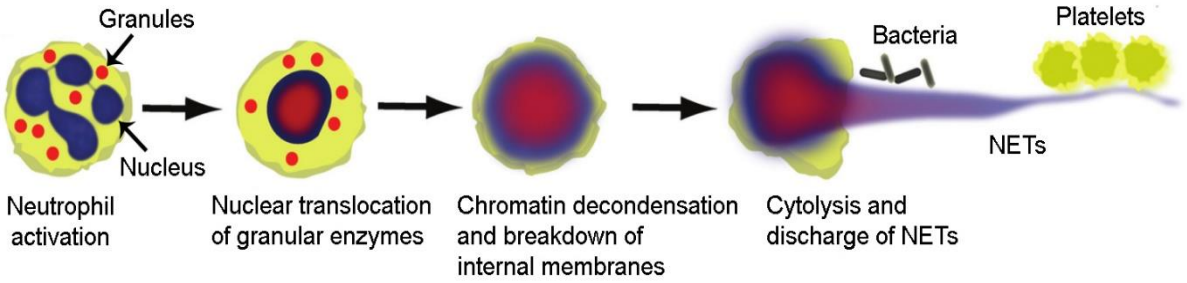


Figure 3: Scheme of NET formation and functions. The neutrophil is activated, granular enzymes such as NE translocate to the nucleus and the chromatin is decondensed. Internal membranes break down and the NET is released by cytolysis. NETs trap bacteria and provides a scaffold for platelet aggregation. Reference: Fuchs, Brill et al. (2012)

antimicrobial proteins, is released into extracellular space as a NET (Fuchs, Brill et al. 2012) (Figure 3).

NETosis differs clearly from apoptosis and necrosis and represents a third form of cell death. Apoptosis is characterized by induction of caspases and, in contrast to NETosis, chromatin is condensed and fragmented. During necrosis, the nucleus loses its structure and becomes a homogeneous mass, integrity of the cell membrane is lost, and proteins are released uncontrolled into extracellular space while NETs are never observed. Inconsistent to NETosis, in both apoptosis and necrosis the nuclear envelope remains intact until the cell is dead (Fuchs, Abed et al. 2007). Thus, NETosis is a distinct form of cell death that neutrophils use as a weapon against microbes.

NETs are able to efficiently trap bacteria, gram-positive species like Staphylococcus aureus as well as gram-negative like Salmonella typhimurium and kill them with the associated antimicrobial proteins. They help in preventing the spreading of an infection and also in eliminating the bacteria (Brinkmann, Reichard et al. 2004). NETs also have procoagulant properties. The large DNA network provides a scaffold for RBCs and platelets and supports their aggregation. Some of the histones associated with NETs can directly activate platelets. The DNA strands give a developing thrombus more stability as they form fibrillic structures similar to fibrin (Fuchs, Brill et al. 2012). Furthermore, NETs are large negatively charged structures that are likely to activate coagulation FXII as it has been shown for other nucleic acids and in vitro. This way NETs would directly promote the coagulation cascade leading to fibrin generation and not only adherence of the cellular thrombus components (Martinod and Wagner 2014). NETs are present in human and experimental murine DVT and they have a large impact in certain thrombotic diseases. $PAD4^{-/-}$ mice which are unable to perform NETosis are protected from experimental thrombosis in the inferior vena cava (Martinod, Demers et al. 2013) and a deficient NET-degradation is found in patients with acute thrombotic microangiopathy (Jimenez-Alcazar, Napirei et al. 2015).

1.5 Histological techniques

1.5.1 Cryosections vs. paraffin embedding

An important advantage of cryosectioning is an excellent preservation of antigens. The unfixed tissue is rapidly frozen conserving proteins in a near-native state (Bratthauer 2010). Detection of sensitive antigens is possible, and a wide range of antibodies can be used in immunostainings. Cryosectioning is also a fast and simple method to prepare tissue sections as it only requires a few materials and no time for tissue preparation e.g. fixation and dehydration, is needed. It is possible to acquire sections within minutes after collecting tissue samples making it a valuable technique not only for laboratories but also for fast intraoperative cancer diagnostics (Esbona, Li et al. 2012), (Bertz, Schmitz-Drager et al. 2012). A major disadvantage of cryosectioning is the fragility of cryoblocks. The original morphology can be lost due to cutting artifacts such as large gaps and cracks and the section thickness is limited to approximately 5 µm, depending on the tissue. Thinner sections tend to tear apart entirely and cannot be used but thicker sections give more background staining in immunofluorescence. Weaker specific signals of immunolabeled antigens can be masked entirely. Paraffin wax is harder and less brittle then Tissue-Tek® O.C.T.™ Compound used for cryoembedding and thinner sections can be achieved.

As a great advantage over cryosectioning, paraffin gives the tissue stability and support during sectioning and the tissue integrity is preserved a lot better. Especially with fragile tissue paraffin embedding produces fewer sectioning artifacts. A disadvantage of paraffin sectioning it the extensive tissue processing. Prior to embedding the organs are harshly fixed, dehydrated, and heated and the paraffin needs to be washed off from the sections before staining. Antigens might be lost or become undetectable by antibodies. Heat-mediated antigen retrieval is a valuable technique to partially counteract this problem (Shi, Taylor et al. 2013).

1.6 Project aims

Despite being dispensable for hemostasis, FXII is required for fibrin formation during thrombosis. However, the complex mechanisms of thrombus formation including FXII activation *in vivo* are not fully understood. I aimed to characterize the activation of the protease FXII in histological sections of murine deep vein thrombi using immunohistochemistry. A deeper understanding of the *in vivo* activation of FXII and its role in thrombus formation is important for a comprehensive knowledge of coagulation and could lead to new safer treatments and prevention strategies for thromboembolic and potentially inflammatory diseases.

Aim 1: To characterize the formation, reorganization, and resolution of murine deep vein thrombi using by paraffin sections stained with standard histological methods such as hematoxylin and eosin stain (H&E).

Aim 2: To characterize the timely and spatially distribution of potential sources of FXII activators, namely neutrophils, NETs, and platelets, using immunofluorescence techniques within thrombi.

Aim 3: To establish a specific method for the detection of FXII in murine tissue using immunofluorescence. The method should enable to detect the colocalization of FXII and its potential activators within murine deep vein thrombi.

2 MATERIALS AND METHODS

2.1 Murine DVT model

All experiments with living animals were performed by Miguel Jiménez-Alcázar, Laboratory of Molecular Inflammation, Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center Hamburg-Eppendorf. The tissue samples collected in his experiments were further used in this project.

In brief, 10-12 weeks old male wild type and genetically altered mice with a deletion of the F12 or the PAD4 gene (Pauer, Renne et al. 2004, Li, Li et al. 2010) with the C57BL/6J background were used in the experiments. Mice were subjected to anesthesia by isoflurane inhalation. After the plantar reflex could no longer be provoked, a laparotomy was performed by a midline incision following the Linea alba. The abdominal organs were exteriorized to acquire excess to the inferior vena cava (IVC). All visible branches of the IVC were bound with Prolene 6-0 (Ethicon, Scotland) thread. The aorta and the IVC were separated and the IVC was ligated right below the left renal vein with Prolene 6-0 thread. Next, the organs were arranged back into the abdominal cavity, the peritoneum and skin were sutured with Vicryl 6-0 and silk 6-0 (Ethicon, Scotland) thread, respectively. At the indicated time points of 6 hours, 2 days, 7 days, and 21 days after IVC ligation animals were again subjected to anesthesia by isoflurane inhalation and blood was obtained through retro-orbital bleeding. To collect the DVTs from below the IVC ligation the mice were sacrificed by cervical dislocation, then perfused with PBS and PFA and the DVTs were extracted along with surrounding vessel wall. The DVTs were placed in 4% neutral buffered PFA for 24 hours at 4°C for paraffin embedding or directly snap frozen for cryosectioning.

2.2 Tissue sectioning

Paraffin sectioning for this project was done by the Mouse Pathology Core Facility, University Medical Center Hamburg-Eppendorf. Briefly, fresh tissue was fixed in 2% PFA overnight, dehydrated through an alcohol series, washed in Xylene, infiltrated with molten paraffin and finally embedded in molten paraffin. Sections were cut with a standard microtome at 3 µm thickness.

For cryosectioning, fresh DVTs were washed 3 x 5 min in PBS and directly snap frozen in Tissue-Tek® O.C.T.™ Compound (Sakura) on a -60°C cooling plate. Sections were cut with a CryoStar™ NX70 Cryostat (Thermo-Scientific) according to the manufacturers manual at 5 µm thickness.

To encounter the difficulties of sectioning fresh frozen DVT, which are very fragile and brittle, a protocol to cryoprotect tissue and increase its stability was introduced later on in this project. It has been shown that submerging tissue samples in 30% sucrose solution in PBS prior to freezing can increase the quality of tissue sections in murine brain (Currle and Monuki 2007). For cryosectioning with sucrose treatment, fresh DVTs were fixed in 4% neutral buffered PFA for 1h and washed 3 x 5 min in PBS with rocking. The tissue was submerged to 30% (w/v) sucrose in PBS solution and incubated overnight at 4°C. First, the tissue floats on the surface but after a couple of hours' incubation time depending on the sample size, it sinks to the bottom. The DVTs were snap frozen in Tissue-Tek® O.C.T.™ Compound (Sakura) in an isopentane bath on dry ice (approx. -80%C). Cryoblocks were equilibrated to -20°C for 30 min and sectioned with a CryoStar™ NX70 Cryostat at 5 µm thickness.

For both sucrose treated and non-sucrose treated sections, the specimen was collected on slides (SuperFrost/Plus Objekträger, No. 2409/1, In vitro diagnostic, Glaswarenfabrik Karl Hecht KG) and dried 30 min at room temperature. Finished slides were stored at -20°C.

Subsequently, slides were thawed to room temperature in a sealed plastic bag for 30 min and stained.

2.3 Histological staining techniques

For Hematoxylin and Eosion staining, paraffin slides were deparaffinized in Xylene (Chemsolute® 12419, Th. Geyer) for one hour and rehydrated through a decreasing alcohol series (2 x 100% ethanol, 95% ethanol, 80% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, and 2 x aqua dest. For 5 min each). Slides were stained in Hematoxylin solution modified acc. to Gill III (Merck Millipore 105174) for 45 secs, rinsed in aqua dest. and differentiated with 0.3% acid alcohol (hydrochloric acid 0,3% in 70% ethanol) for 30 sec. The counterstain was done in Eosin Y (Sigma-Aldrich E5388) 0,25% working solution 30 sec. Slides were dehydrated through an increasing alcohol series (80% ethanol, 96% ethanol, 3 x 100% ethanol for 15 sec each), washed in Neo-Clear® (Merck Millipore 109843) for 15 sec and mounted with Neo-Mount® (Merck Millipore 109016).

For Picrosirius Red staining, slides were deparaffinized in Xylene for one hour, rehydrated through a decreasing alcohol series (2 x 100% ethanol, 95% ethanol, 80% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, and 2 x aqua dest. For 5 min each), stained in Picrosirius Red solution (0.5 g Direct Red 80, Sigma-Aldrich 365548, in 500 ml of saturated aqueous solution of picric acid) for one hour and washed 2 x 5 min in 0.1% acetic acid. Slides were rinsed with aqua dest. and counterstained with Hematoxylin solution modified acc. to Gill III for 45 secs. Slides were rinsed in auqa dest., dehydrated through an increasing alcohol series (80% ethanol, 96% ethanol, 3 x 100% ethanol for 15 sec each), washed in Neo-Clear® (Merck Millipore 109843) for 15 sec and mounted with Neo-Mount® (Merck Millipore 109016).

In H&E, nuclei show a basophilic staining in blue while most of the cytoplasmic components and fibrin are stained in acidophilic pink. RBCs are stained bright red. Alike H&E, nuclei are stained in blue with hematoxylin in the Picrosirius Red stain, while most of the other intracellular and extracellular components are stained in soft red or brown. Collagen shows a specific intensive dark red staining.

2.4 Immunofluorescence

2.4.1 Standard protocol for immunofluorescence

For cryosections, slides were thawed to room temperature for 30 min and fixed in 2% neutral buffered PFA for 10 min. Paraffin sections were deparaffinized in Xylene for 1 hour at room temperature and rehydrated through a decreasing alcohol series (2 x 100% ethanol, 95% ethanol, 80% ethanol, 70% ethanol, 50% ethanol, 30% ethanol, and 2 x aqua dest. For 5 min each).

A heat mediated antigen retrieval was performed for most stainings on paraffin sections, e.g. rabbit anti H3cit (abcam ab5103), mouse anti ssDNA (in house), rabbit anti FXIIa (Sigma-Aldrich SAB4500831). For the sodium citrate antigen retrieval buffer, stock solution A was prepared with 21,01 g citric acid and 1l aqua dest., stock solution B with 29,41 g sodium citrate and 1l aqua dest. The working solution of the buffer was prepared fresh each time with 4,5 ml stock solution A, 20,5 ml stock solution B and 250 ml aqua dest. Slides were placed in a cuvette filled with the working solution of the buffer which was placed in a jar filled with water. Slides were boiled for 8 min in a microwave and allowed to sit in the hot buffer for additional 15 min. Slides were washed 3 x 5 min in PBS.

Sections with and without antigen retrieval were circled with a delimiting pen (Dako Pen, Dako S200230-2). A blocking step to block unspecific protein binding was performed with either with 2% BSA (Sigma-Aldrich A3059) or ~1% gelatin from cold water fish skin (Sigma-Aldrich

G7765) in PBS + 0.1% Triton for 45 min at room temperature. Slides were washed 3 x 5 min in PBS.

Primary antibody was diluted according to the product information or at a dilution of 1:10 to 1:200 depending on the staining results in PBS + 0.05% Tween20 (PBST) and incubated overnight at 4°C. Slides were washed 3 x 5 min in PBST.

For a double immunofluorescent staining with primary antibodies raised in different host species (e.g. one mouse and one rabbit antibody), both antibodies were incubated simultaneously. Slides were washed 3 x 5 min in PBST.

A fluorophore labeled secondary antibody (Molecular Probes, Alexa Fluor® 488, 546 or 555 conjugated) specific for the fc-fragment of the primary antibody was diluted 1:500 in PBST and incubated one hour at room temperature. Slides were washed 3 x 5 min in PBST.

Nuclei were stained with DAPI (Invitrogen™ D1306) diluted 1:5000 in PBS for 2 min. Slides were washed 3 x 5 min in PBST and 1 x 5 min with aqua dest. and mounted with Vectashield® mounting medium (Vector Laboratories H-1400).

Detailed protocols, a complete list of all antibodies used and images of all performed immunostainings are included in the supplemental material.

2.4.2 Blocking buffers

Efficient blocking reduces unspecific antibody binding and improves the contrast of a specific immunostaining to the background. Three different blocking buffers were tested with PBS as a control. Cryosections were thawed to room temperature and fixed with 2% PFA for 10 min. Blocking buffers were added - Buffer A: 2% BSA in PBS + 0.1% Triton, Buffer B ~1% gelatin from cold water fish skin in PBS + 0.1% Triton, Buffer C 5% goat serum + 1% BSA in PBS + 0.1% Triton. Secondary antibody goat anti rabbit IgG Alexa Fluor® 546 conjugate (Molecular Probes A-11010) was incubated 1h at room temperature. Nuclei were stained with DAPI (Invitrogen™ D1306) diluted 1:5000 in PBS for 2 min. and slides were mounted with Vectashield® mounting medium (Vector Laboratories H-1400). No primary antibody was added as this experiment was done to evaluate background fluorescence depending on the blocking buffer.

2.4.3 Reducing autofluorescence

All tissue autofluorescence to a certain extend which can reduce the staining quality significantly. To test the effect of the lysochrome dye Sudan Black B as a background reducing agent, a normal immunofluorescent staining on paraffin sections with a primary antibody mouse anti ssDNA (in house, 2 µg/ml) and a secondary antibody goat anti mouse IgG Alexa Fluor® 488 conjugate (Molecular Probes, 10 µg/ml) was performed as described above. Prior to mounting, the slides were treated with a 0.1% Sudan Black B (Sigma-Aldrich 199664) solution in 70 % ethanol for 20 min.

2.4.4 Double indirect immunostaining with species equivalent antibodies

A lot of antibodies targeting FXII or potential FXII activators were raised in rabbits. A protocol for a double indirect immunostaining with species equivalent primary antibodies by Tornehave et al. (Tornehave, Hougaard et al. 2000) was tested and used for this project.

The first steps of the double staining followed the standard protocol for immunofluorescence as described above. Paraffin slides were deparaffinized in Xylene, rehydrated through an alcohol series and a heat mediated antigen retrieval in sodium citrate buffer was done in a microwave for 8 min. Sections were blocked with BSA or gelatin from cold water fish skin blocking buffer. The *first* primary antibody, e.g. rabbit anti vWF (Dako A0082, 2 µg/ml), was incubated over night at 4°C. The *first* secondary antibody goat anti rabbit IgG Alexa Fluor® 546 conjugate (10 µg/ml) was incubated 1h at room temperature.

To block binding of the *second* secondary antibody to the *first* primary antibody the slides were boiled in a microwave in sodium citrate buffer for 5 min as described for the antigen retrieval in the standard protocol.

The second immunostaining followed. The *second* primary antibody, e.g. rabbit anti Histone H3 (Abcam ab1794, 2 µg/ml), was incubated 1h at room temperature and subsequently the *second* secondary antibody goat anti rabbit IgG Alexa Fluor® 488 conjugate (10 µg/ml) was incubated 1h at room temperature. Nuclei were stained with DAPI (Invitrogen™ D1306) diluted 1:5000 in PBS for 2 min. Slides were washed 3 x 5 min in PBST and 1 x 5 min with aqua dest. and mounted with Vectashield® mounting medium (Vector Laboratories H-1400).

2.5 Primary antibodies included in this project

Target	Concentration	Marker for	Manufacturer (Product Number)
Lymphocyte antigen 6 complex locus G6D (Ly6G)	2 µg/ml	Neutrophils	Bio X Cell (BE0075-1)
Myeloperoxidase (MPO)	2 µg/ml	Neutrophils	Dako (A0398)
Neutrophil elastase (NE)	1 µg/ml	Neutrophils	abcam (ab68672)
cathelin-related antimicrobial peptide (CRAMP)	1 µg/ml	NETs	Innovagen (PA-CRPL-100)
Citrullinated histone 3 (H3cit)	2 µg/ml	NETs	abcam (ab5103)
Histone 3 (H3)	2 µg/ml	NETs/Histones/DNA	abcam (ab1791)
Histone complex 2ab	2 µg/ml	Histones/DNA	abcam (ab5103)
Single stranded DNA (ssDNA)	2 µg/ml	DNA	Sigma-Aldrich (MAB3034)
Von Willebrand Factor (vWF)	2 µg/ml	vWF/Platelets	Dako (A0082)
Cluster of differentiation 41 (CD41)	2 µg/ml	Platelets	Biozol (BLD-133901)
Fibrinogen	2 µg/ml	Fibrinogen	Bio-Rad (BSG-4440-8004)
Thrombin	2 µg/ml	Thrombins	Santa Cruz Biotechnology (sc-16972)
Macrophage receptor F4/80 (F4/80)	2 µg/ml	Macrophages	Bio-Rad (MCA497RT)
Active caspase-3 (Casp3)	5 µg/ml	Caspase 3	BD Biosciences (C92-605)
FXIIa	5 µg/ml	FXIIa	(Larsson, Rayzman et al. 2014) 3F7
FXIIa	2 µg/ml	FXIIa	Sigma-Aldrich (SAB4500831)
FXII/FXIIa	2 µg/ml	FXII/FXIIa	Sigma-Aldrich (HPA003825)

FXII	Dilution 1:500, 1:100	FXII	(Saito, Ishihara et al. 1985) P5-2-1
FXIIa	2 µg/ml	FXIIa	Excell Biotech Ltd. (FXII99-4)
FXII/FXIIa	2 µg/ml	FXII/FXIIa	Reference: Ravon D.M. et al. (F1)
FXII/FXIIa	Dilution 1:100	FXII/FXIIa	Nordic-MUbio (GAHu/FXII)
FXII	Dilution 1:500, 1:100	FXII	(Schwieder 2004) AB1-435
FXII/FXIIa	2 µg/ml	FXII/FXIIa	Affinity Biologicals (GAFXII-AP)

Table 2: Antibodies used in immunostainings. The Table shows all antibodies that were included in this project as primary antibodies for immunostainings. In the first row the targeted protein is named and in the third row the cell type or structure is it commonly found on.

2.6 Western blot

Western blots with murine wild type and $FXII^{-/-}$ plasma as well as healthy and FXII-deficient human plasma were performed with nine different antibodies targeting FXII. Purified FXII (haematologic technologies inc., human coagulation factor xii, HCXII-0155) was used as a control. Citrated mouse plasma was collected from the retro-orbital sinus, healthy human plasma was obtained from volunteer donors by the Institute of Transfusion Medicine, University Medical Center Hamburg-Eppendorf, FXII deficient plasma was from George King Bio-Medical (Product-No. 1200). Samples were diluted in Laemmli sample buffer (Bio-Rad 1610737), for reducing conditions β-mercaptoethanol (Carl-Roth 4227) was added. Samples were denatured at 95°C for 5 min in a Thermomixer® comfort (Eppendorf AG, Germany). 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE, SDS Sigma-Aldrich 71729, TEMED BioRad 161-0800, APS Serva Electrophoresis GmbH 13375) were precast. Samples were loaded and the gels were placed in an XCell SureLock® running chamber (Thermo Fisher Scientific) connected to a PowerEase® 500 power supply (Thermo Fisher Scientific). The chamber was filled with running buffer and the gels were run at 200V for one hour.

Next, the gels were placed inside a transfer cassette (Hoefer TE24) with a nitrocellulose membrane (BioRad 1620175) and fixed in a transfer chamber (Hoefer TE22). The chamber was filled with transfer buffer and proteins were transferred onto the membrane at 150 V for 2 hours. After transfer was complete, membranes were blocked with 0.5% fat-free milk powder in tris-buffered saline + 0.05% Tween20 for 1 hour at RT. Available antibodies targeting FXII were diluted according to the product information or a standard dilution of 1:1000 in 0.5% milk powder and incubated with the membranes for 1h at room temperature. Appropriate horseradish peroxidase conjugated secondary antibody (Invitrogen) was diluted 1:10.000 in 0.5% milk powder and incubated 1 hour at RT. Washing steps in between were done with 1x TBST. Blots were incubated with ECL™ Western Blotting Detection Kit (GE Healthcare) for 1 min at RT and exposed to ECL™ Hyperfilms™ (GE Healthcare). Films were developed with a Curix 60 (AGFA HealthCare).

2.7 Analyzing and quantifying images

Stainings were imaged with the ApoTome widefield imaging system from Carl Zeiss AG. The Zeiss software AxioVision Rel. 4.8 was used to acquire mosaic pictures and for pictures editing. For immunofluorescent stainings the background was set to black based on the isotype control and the contrast was enhanced orientated on the histogram of the grayscale image. Pictures were exported to .jpeg format and quantified with ImageJ 1.48v (Wayne Rasband, National Institutes of Health, USA). The thrombus was manually selected using the polygon selection tool and the area was determined with the measure area function. To quantify fluorescent stained area a threshold was set in grayscale images and covered areas were measured. A colored threshold in RGB images was used for H&E and Sirius Red stainings. Cells were automatically counted by selecting them with a threshold in a grayscale image and using the particle count function. Particles smaller than 50µm^2 where excluded. Manual cell counts were performed with the ImageJ cell counter plugin (Kurt De Vos, University of Sheffield, Academic Neurology, version 2010/12/07).

Statistical analyzes were performed with GraphPad Prism 5 (GraphPad Software, Inc.). t-test was used to compare two data sets to each other and one-way ANOVA to compare three or more data sets such as image quantifications of the four different time points of the DVT model.

3 RESULTS

3.1 Optimization of immunofluorescence techniques

3.1.1 Tissue sectioning method

For this project, paraffin- and cryoembedding was used to prepare tissue sections. DVTs are especially fragile and clotted blood is so brittle that a consistent quality of cryosections could not be achieved with a standard embedding protocol for the CryoStar™ NX70 Cryostat. The quality of paraffin sections was clearly superior over cryosections (Figure 4).

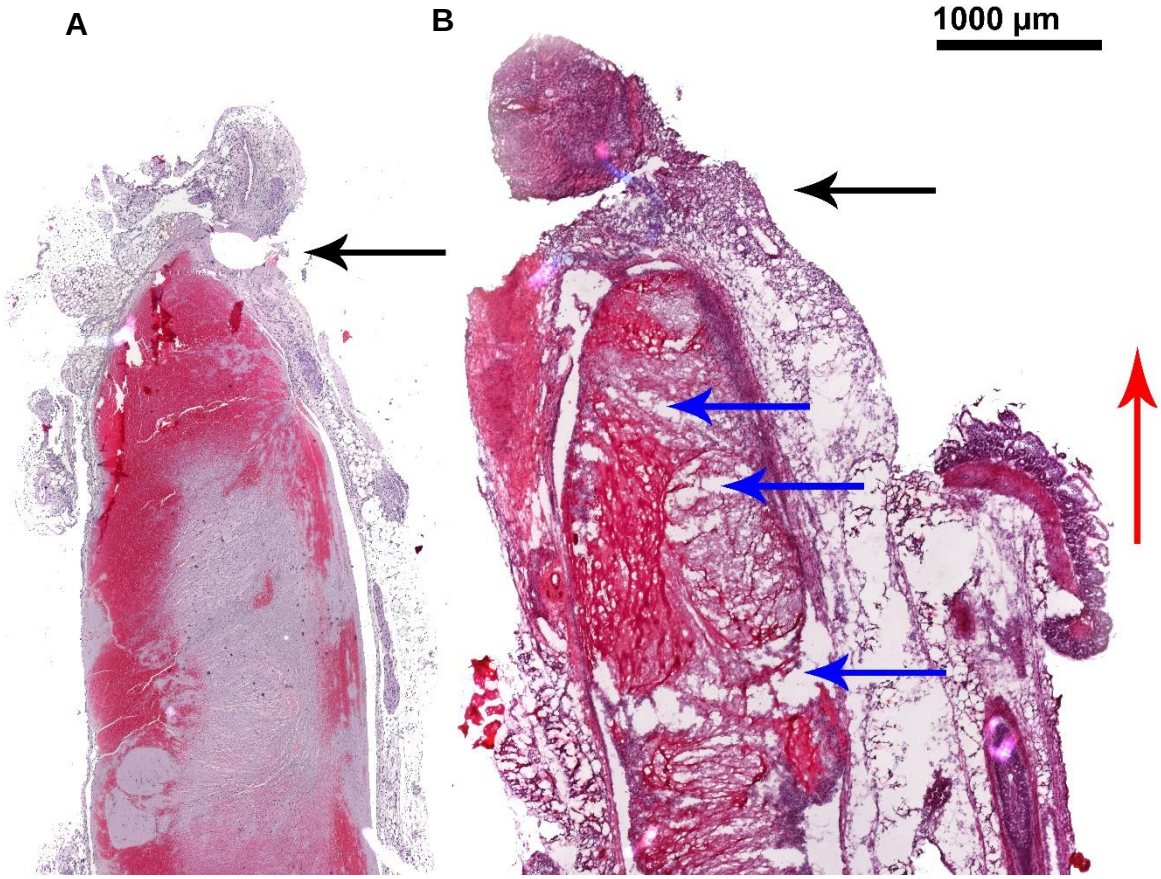


Figure 4: Paraffin-embeddings preserves the tissue morphology. A) Paraffin section, murine DVT 2 days after IVC ligation. B) Cryosection, murine DVT 7 days after IVC ligation. Hematoxylin-Eosin staining reveals larger gaps and cracks (blue arrows) in the cryosectioned tissue in comparison to paraffin-embedded tissue. Black arrows indicate the position of the ligation; red arrow indicates the flow direction of blood in the vessel.

3.1.2 Cryoprotecting tissue

Antigen preservation in cryosections is usually superior to paraffin sections in immunostainings, as it enables a wider range of antibodies binding to their targets. To enable a wider range of antibodies for immunostainings, a method to enhance the quality of cryosections was tested.

Fresh DVT were submerged to 30% (w/v) sucrose in PBS solution and incubated overnight at 4°C prior to freezing and sectioning. Ice crystal forming in a high percentage sucrose solution

are a lot smaller than in pure water, which makes the tissue less brittle. The quality of DVT sections with sucrose treatment is clearly superior over normal cryosections. There were visibly less cutting artifacts like gaps and cracks, the tissue didn't tear apart during sectioning and the overall integrity was preserved well. A stable quality of tissue sections could be achieved. In comparison to paraffin sections, the tissue tended to fold on the edges of the thrombus during sectioning. A DAPI staining of sucrose treated murine liver visualizes the better preservations of cells. In normal cryosections of the liver, most of the hepatocyte nuclei break and lose their characteristic round shape. In paraffin sections, they stay intact. With sucrose treatment, most of the nuclei remained stable and the overall morphology was comparable to paraffin (Figure 5).

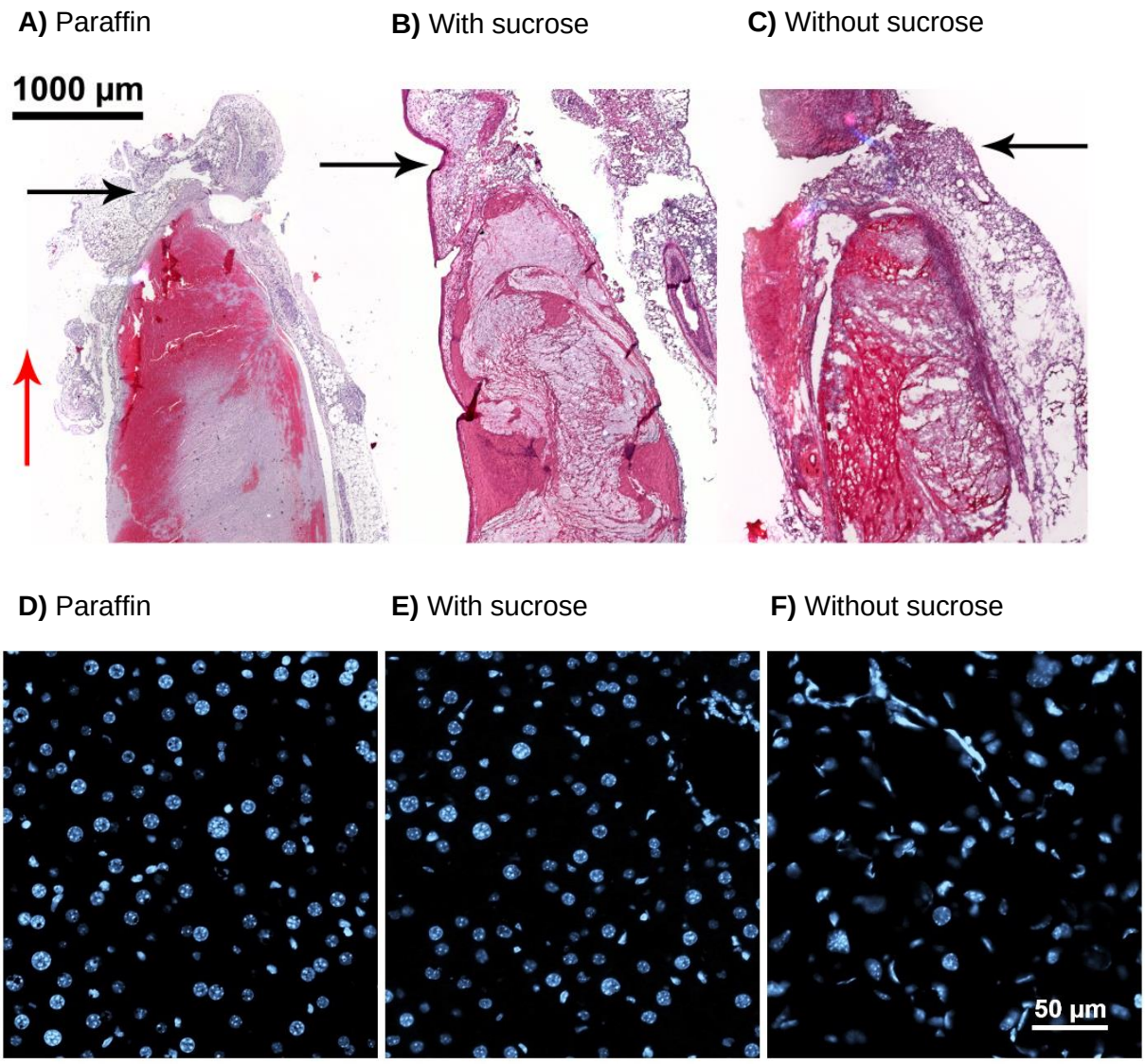


Figure 5: Cryoprotecting tissue with sucrose solution. Submerging tissue samples in 30% sucrose solution overnight enhances tissue stability during snap freezing and cryosectioning. A-C) H&E staining. A) Paraffin section, murine DVT 2 days after IVC ligation. B) Cryosection with sucrose treatment, murine DVT 2 days after IVC ligation. C) Cryosection without sucrose treatment, murine DVT 7 days after IVC ligation. D-F) Healthy murine liver stained with DAPI. D) Paraffin section. Most of the nuclei are intact. E) Cryosection with sucrose treatment. F) Cryosection without sucrose treatment. A lot of nuclei are deformed and lost their characteristic round shape.

3.1.3 Blocking buffers

Unspecific antibody binding and background staining are general issues in immunofluorescence, which can be reduces by the use of blocking buffers. Three different blocking buffers were tested containing BSA, gelatin from water fish skin or goat serum.

BSA and gelatin from cold water fish skin produced approx. the same amount of background fluorescence (mean brightness value 40,50 and 50,32) while goat serum blocking buffer was a lot brighter (mean brightness value 95,89, Figure 6). Both BSA and gelatin from cold water fish skin were used as blocking reagents for immunostainings. Especially all antibodies targeting FXII were tested with gelatin from cold water fish skin as BSA has a chance to contain bovine FXII. Gelatin from cold water fish skin does not consist of any mammalian proteins reducing the risk of unwanted cross-reactions.

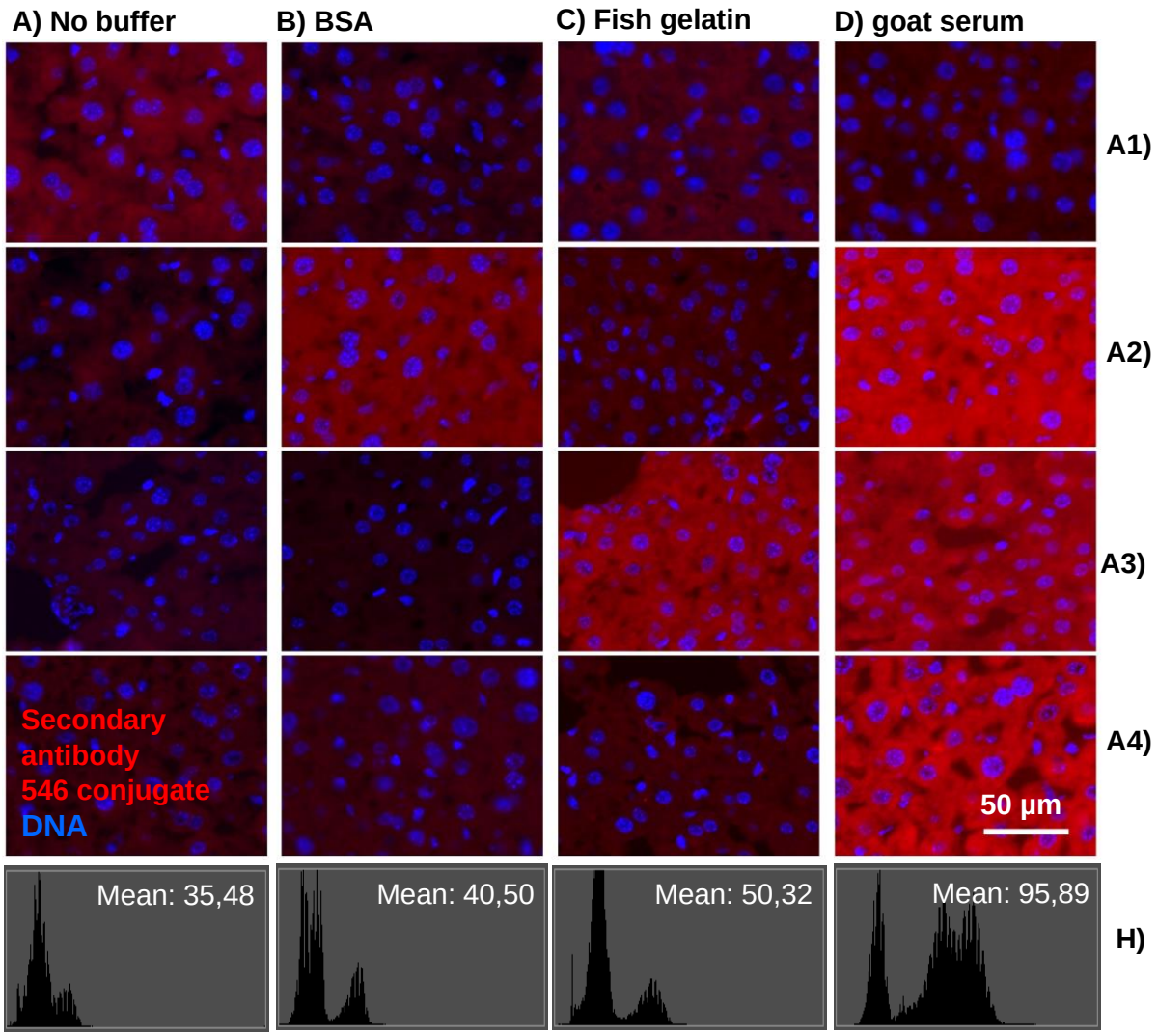


Figure 6: Unspecific antibody binding with different blocking buffers. Healthy murine liver (n = 4 animals), cryosections. Slides were incubated with blocking buffer for 30 min at RT, then secondary antibody goat anti-rabbit IgG Alexa Fluor® 546 conjugate was added and incubated for 1h at RT. A) PBS + 0.1 Triton. B) 2% BSA in PBS + 0.1 Triton. C) ~1% gelatin from cold water fish skin in PBS + 0.1 Triton. D) 5% goat serum + 1% BSA in PBS + 0.1 Triton. A1-4) Animal 1 to 4. H) Histograms of the red RGB channel of the combined images of all four animals with mean brightness values for each blocking buffer. Serum blocking buffer produces more unspecific staining than BSA or gelatin from cold water fish skin.

3.1.4 Reducing autofluorescence

Besides background staining, autofluorescence of tissue samples can cause background fluorescence in immunostainings. The contrast to specific signals is reduced and faint signals can be masked entirely. The lysochrome dye Sudan Black B binds unspecific to lipids and lipoproteins and is used to visualize lipids. It is non-fluorescent and it has been shown that it can reduce autofluorescence (Schnell, Staines et al. 1999).
An immunostaining with the antibody mouse anti ssDNA (Sigma-Aldrich MAP3034, 2 µg/ml) and a secondary antibody goat anti mouse IgG Alexa Fluor® 488 conjugate (Molecular Probes, 10 µg/ml) was performed on murine liver sections with treatment of 0.1% Sudan Black B (Sigma-Aldrich 199664) solution in 70 % ethanol for 20 min. prior to mounting. Liver is known to show a strong autofluorescence. Background fluorescence was clearly decreased while the specific immunostaining was unaffected (Figure 7). Subsequently, Sudan Black B was used in all immunostainings.

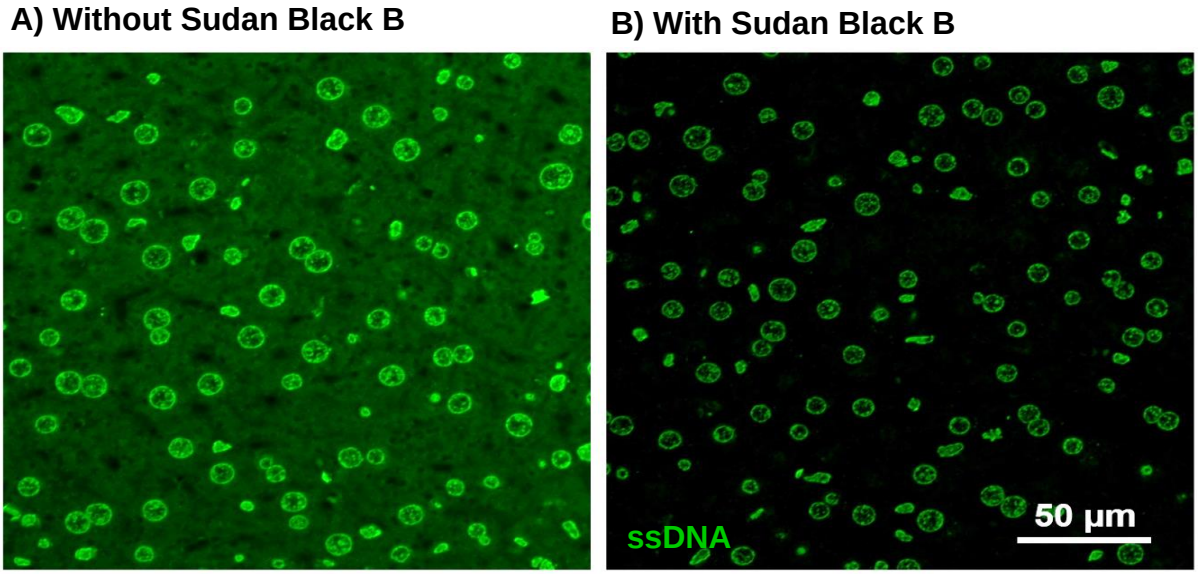


Figure 7: The lysochrome dye Sudan Black B reduces autofluorescence of liver. Immunostaining with an antibody against ssDNA on murine wild type liver. A) Without Sudan Black B treatment. B) With 0.1% Sudan Black B treatment. Slides were incubated with Sudan Black B after the normal immunostaining procedure. While the ssDNA staining is still bright, autofluorescence of hepatocytes is highly reduced.

3.1.5 Double indirect immunostaining with species equivalent antibodies

A double indirect immunofluorescent staining with the primary antibodies rabbit anti vWF (Dako A0082, 2 µg/ml) and rabbit anti Histone H3 (Abcam ab1794, 2 µg/ml) was done as described in methods to test the protocol for double stainings with species equivalent antibodies. The two antibodies rabbit anti vWF and rabbit anti Histone H3 show clearly different staining patterns. Performing the double staining with microwaving the slides in sodium citrate buffer to block binding of the *second* secondary antibody to the *first* primary antibody in between the two stainings gave two distinguishable signals. They matched with the signals observed in single stainings that were done simultaneously as a control. The microwave treatment successfully blocked free binding sites of the first primary antibody without eluting the antigen-antibody complex (Figure 8).

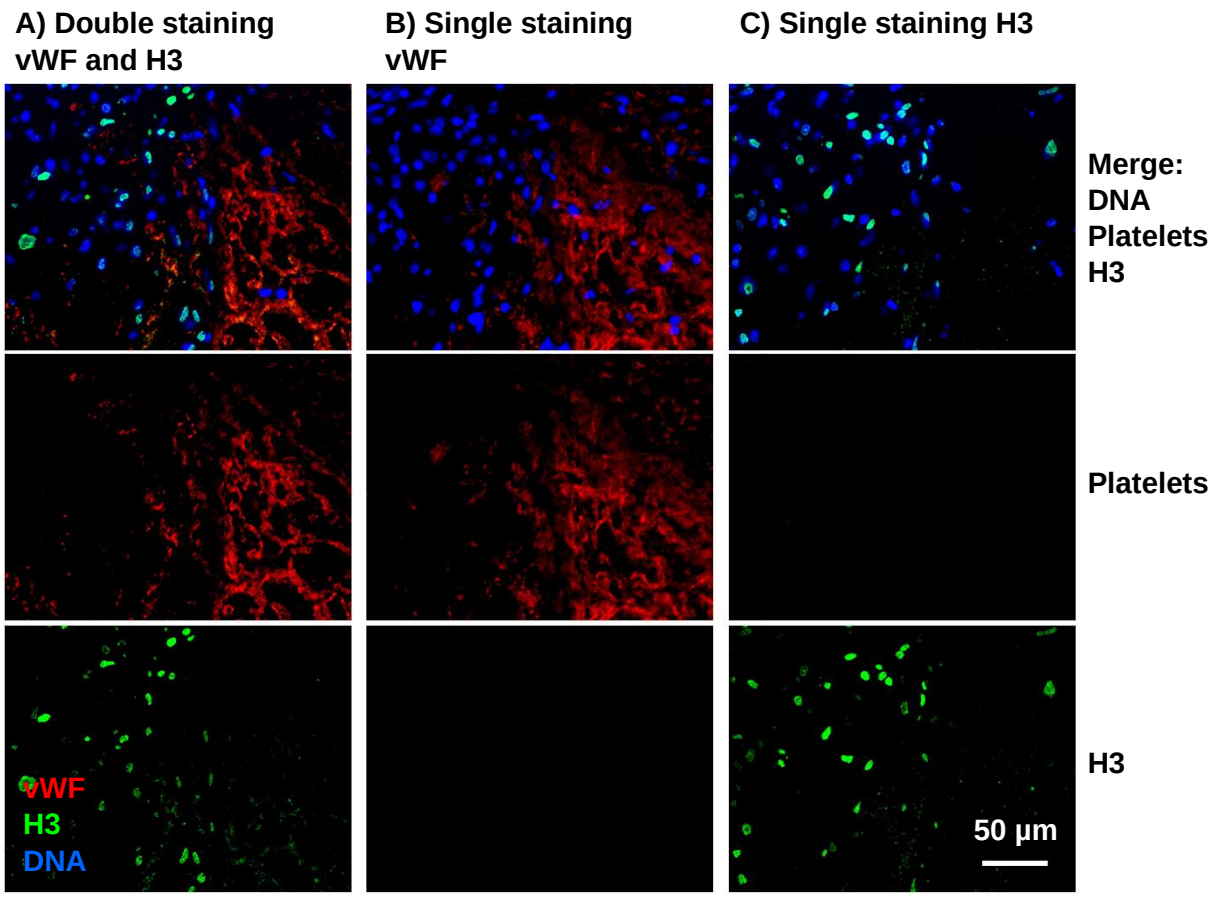


Figure 8: Double indirect immunofluorescent staining with two primary antibodies raised in rabbits. In this sequential staining approach microwave treatment after the first primary and first secondary antibody blocked free binding sites of the first primary antibody, allowing the second staining with an antibody raised in rabbit, but didn't elute the antigen-antibody-complex of the first staining. Paraffin sections of a Murine DVT 2 days after IVC ligation, wild type. A) Double staining with two polyclonal rabbit antibodies, anti-Von Willebrand factor (vWF) and anti-Histone H3. B) Single staining rabbit anti-vWF. C) Single staining rabbit anti-H3.

3.2 Immunofluorescence for FXII and its activators

3.2.1 Visualizing activators of FXII

To visualize FXII activation in vivo, immunostainings for NETs and platelets as its potential activators were established. This project aimed to characterize NETs and platelets in the DVT time course and establish a double staining with FXII as the next step.

NETs stimulate fibrin formation (Fuchs, Brill et al. 2012) and their properties make them a likely candidate for activating FXII in vivo. The large DNA fibers provide a negatively charged surface for assembly of the contact system and NETs are abundant in thrombosis. A specific marker for NETotic neutrophils and NETs is citrullinated Histone H3 (H3cit). To visualize NETs, immunostainings with the antibody rabbit anti H3cit (Abcam ab5103, 2 µg/ml) were performed according to the standard protocol described in methods with the secondary antibody goat anti rabbit IgG Alexa Fluor® 546 conjugate (Molecular Probes). As a control, the staining was done with protein arginine deiminase 4 (PAD4) gene deficient animals where no signal could be detected. PAD4 is an enzyme that citrullinates histones, which is an essential step in NETosis (Figure 9).

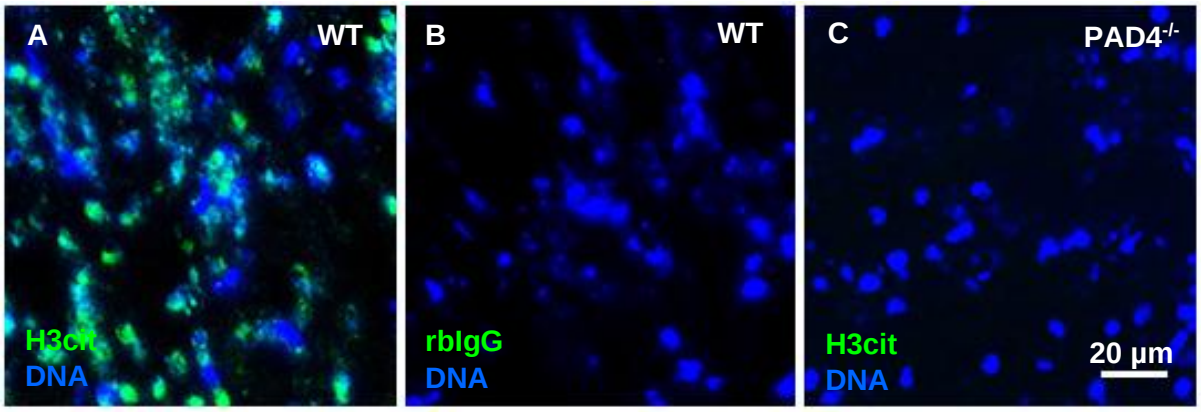


Figure 9: Visualizing NETs in thrombus. The antibody rabbit anti H3cit (Abcam ab5103, 2 µg/ml) shows a clear staining signal in an immunostaining. Tissue: murine DVT 2 days after IVC ligation, paraffin sections. A) Staining for H3cit on wild type. B) Isotype control. C) Staining for H3cit on PAD4$^{-/-}$.

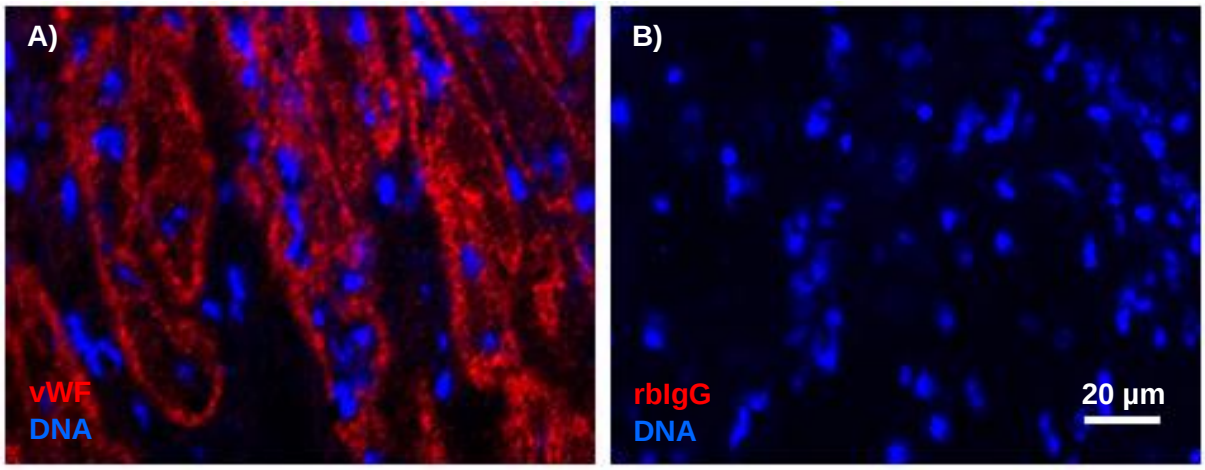


Figure 10: Visualizing platelets. The antibody rabbit anti VWF (Dako A0082, 2 µg/ml) is suitable for immunostainings for platelets. Tissue: murine DVT 2 days after IVC ligation, paraffin sections., wild type. A) Staining for vWF. B) Isotype control.

Platelets contain various procoagulant substances in their granules. Recently, it has been shown that PolyP from dense granules can activate FXII in vivo (Muller, Mutch et al. 2009) and therefore provide a link between primary and secondary hemostasis. Platelets, a prime source of PolyP during thrombosis, can be visualized in immunofluorescence. In this project, the antibody rabbit anti VWF (Dako A0082, 2 µg/ml) was used according to the protocol described in methods with goat anti rabbit IgG Alexa Fluor® 546 conjugate (Molecular Probes) as a secondary antibody (Figure 10). Platelets contain vWF in their granules and in a DVT setting vWF binds to its receptors on platelet surfaces and is highly associated with them (Bryckaert, Rosa et al. 2015). Therefore, vWF can be used as a marker for platelets in DVT.

Additional immunostainings for targets associated with DVT and inflammation were established, e.g. for neutrophils. The cells can be identified in a simple H&E staining due to their characteristic nucleus und neutrophilic cytoplasm, but it can be difficult to visualize them in immunofluorescence. Staining methods using antibodies are more specific and especially immunofluorescence is valuable to display multiple antigens simultaneously. Several antibodies were used in this project to visualize neutrophils. The antibodies rat anti Ly6G (Bio X Cell, BE0075-1) and rat anti Ly6G/Ly6C (BioLegend, 108401) both target a neutrophil surface protein. Further, rabbit anti MPO (Dako A0398) and rabbit anti NE (Abcam ab68672)

target granular proteases which are also associated with NETs. Besides H3cit, cathelicidin-related antimicrobial peptide (CRAMP) stained with rabbit anti CRAMP (Innovagen PA-CRPL-100) is a specific marker for NETs. Histones where visualized with the antibodies rabbit anti H3 (histone H3, Abcam ab1791) and mouse anti 2ab (histone complex H2A/H2B, (ab5103, Abcam). Extracellular DNA colocalized with MPO or NE is a strong indicator for NETs. DNA, in addition to DAPI, was visualized with the antibody mouse anti ssDNA (single stranded DNA, Sigma-Aldrich MAP3034). After a heat mediated antigen retrieval most of the DNA in the specimen is single stranded and therefore this antibody stained DNA ubiquitously.

Besides vWF, platelets where visualized with the antibody rat anti CD41 (Biozol BLD-133901). This staining only worked on cryosections where it showed a signal conformable to the vWF staining. The antibody sheep anti fibrinogen (Bio-Rad BSG-4440-8004) stained both fibrinogen and fibrin and the antibody goat anti thrombin (Santa Cruz Biotechnology sc-16972) prothrombin and thrombin. Macrophages infiltrate thrombus in a later state of a DVT than neutrophils. They were visualized with the antibody rat anti F4/80 (Bio-Rad MCA489RT) targeting a macrophage surface protein.

A complete list and images of all performed immunostainings is included in supplemental material.

3.2.2 Visualizing FXII

Antibodies targeting FXII/FXIIa tested in immunostainings					
Target	Host	Concentration/ Dilution	Manufacturer (Product Number)	Staining signal Paraffinsection	Staining signal Cryosection
FXIIa	Human	1:500	(Larsson, Rayzman et al. 2014) (3F7)	-	-
FXIIa	Rabbit	2 µg/ml	Sigma-Aldrich (SAB4500831)	Yes	yes
FXII/FXIIa	Rabbit	2 µg/ml	Sigma-Aldrich (HPA00835)	-	yes
FXII	Mouse	2 µg/ml	(Saito, Ishihara et al. 1985) (P5-2-1)	-	-
FXIIa	Sheep	2 µg/ml	Excell Biotech Ltd. (FXII99-4)	-	-
FXII/FXIIa	Mouse	2 µg/ml	Ravon D.M. et al. (F1)	-	-
FXII/FXIIa	Goat	2 µg/ml	Nordic-Mubio (GAHu/FXII)	-	-
FXII	Rabbit	1:500	(Schwieder 2004) (AB1-435)	-	Yes
FXII/FXIIa	Goat	2 µg/ml	Affinity Biologicals (GAFXII-AP)	-	-

Table 3: Staining FXII. Nine different antibodies targeting FXII/FXIIa were tested in immunostainings of murine wild type DVT. The antibody rabbit anti FXIIa (Sigma-Aldrich SAB4500831) produces a signal in immunostainings on paraffin sections. On cryosections, the three antibodies rabbit anti FXIIa (Sigma-Aldrich SAB4500831), rabbit anti FXII (Sigma-Aldrich HPA003825), and mouse anti murine FXII (Schwieder 2004), AB1-435) produced staining signals.

Nine different antibodies targeting FXII were tested in immunostainings on paraffin sections and cryosections of murine DVT 2 days after IVC ligation according to the protocols described in methods (Table 3).

On paraffin sections, the antibody rabbit anti FXIIa (Sigma SAB4500831) produced a staining signal while the isotype control was negative. The staining was performed with the standard protocol described in methods with a concentration of 2 µg/ml for the primary antibody and with goat anti rabbit IgG Alexa Fluor® 546, 532, or 488 conjugates (Molecular Probes, 10 µg/ml) as secondary antibodies. The signal was bright with a high contrast to the background, it had a clearly distinguishable pattern of large dots associated with nuclei stained with DAPI and it was reproducible (Figure 11, n = 13 experiments).

On cryosections, three antibodies produced a staining signal that did not appear in the isotype control: rabbit anti FXIIa (Sigma-Aldrich SAB4500831), rabbit anti FXII (Sigma-Aldrich HPA003825), and mouse anti murine FXII ((Schwieder 2004), AB1-435). The staining with the antibody rabbit anti FXIIa (Sigma-Aldrich SAB4500831) had the same pattern as on paraffin sections and it was bright with a strong contrast to the background. Stainings with the antibodies rabbit anti FXII (Sigma-Aldrich HPA003825) and mouse anti murine FXII ((Schwieder 2004), AB1-435) appeared remarkably similar, but both antibodies produced more background staining. The signal was localized around leucocytes within the thrombus and had a dotted pattern (Figure 11).

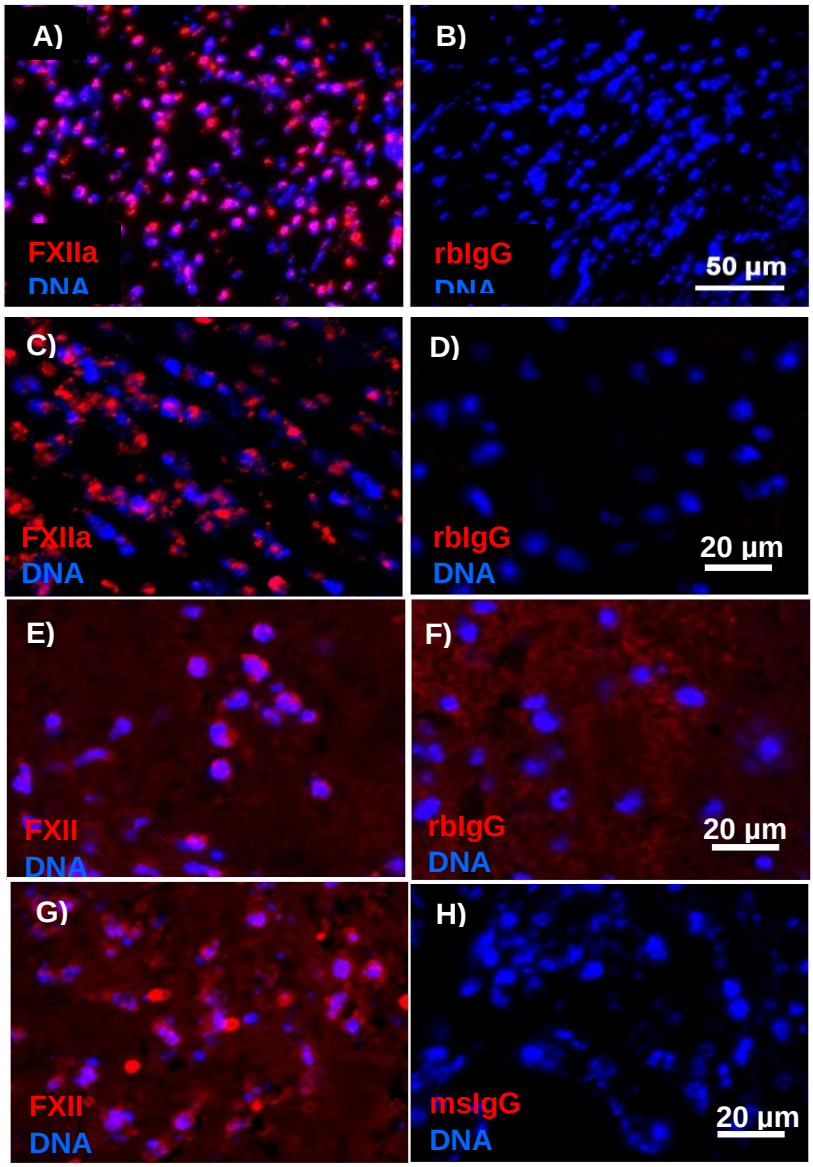
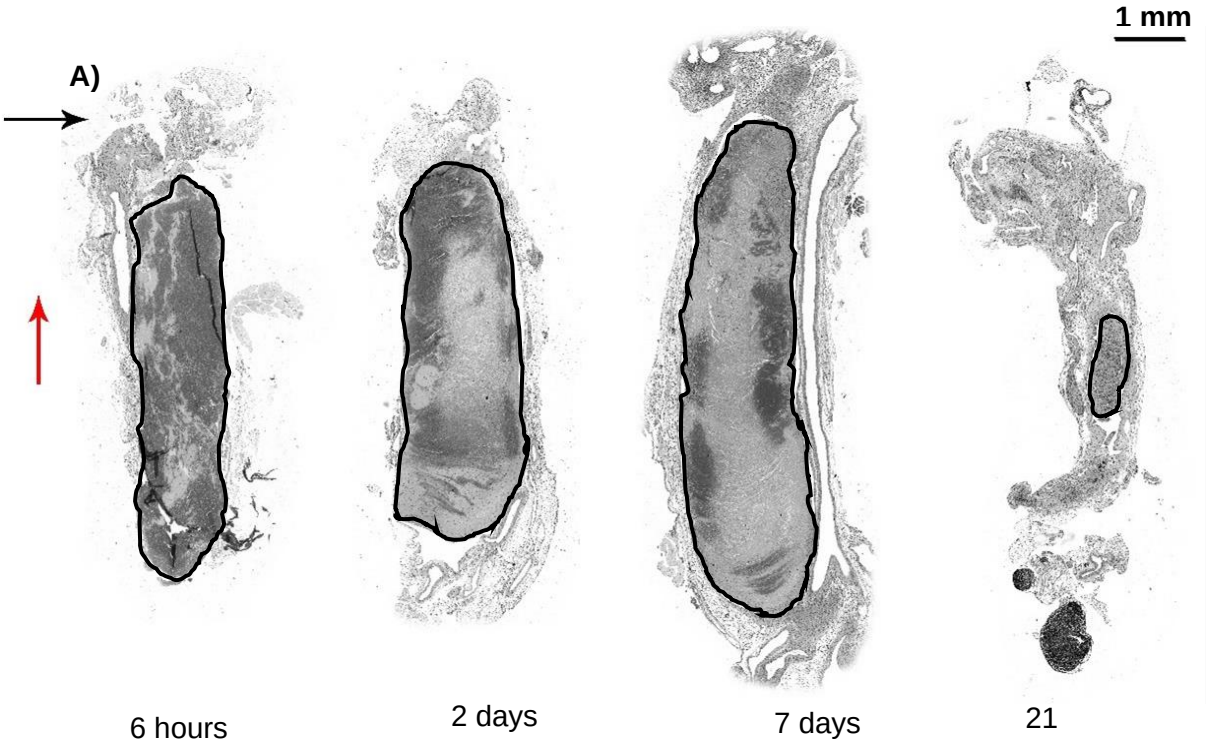


Figure 11: Staining for FXII/FXIIa.
Tissue: murine DVT 2 days after IVC ligation, WT.
A, B) Staining FXIIa on paraffin sections with the antibody rabbit anti FXIIa (Sigma-Aldrich SAB4500831).
C, D) Staining FXIIa on cryosections with the antibody rabbit anti FXIIa (Sigma-Aldrich SAB4500831).
E, F) Staining FXII on cryosections with the antibody rabbit anti FXII (Sigma-Aldrich HPA003825).
G, H) Staining FXII on cryosections with the antibody mouse anti murine FXII ((Schwieder 2004), AB1-435).

3.3 Thrombus characterization

Paraffin sections of DVTs from the different time points of the DVT time course model were used to characterize basic parameters like the thrombus size and its main components. Tissue sections were analyzed using histological techniques and image quantification. This approach gives a good overview of the development of the DVTs in the time course model.

The thrombus size decreases in the DVT time course. Comparing the thrombus size at the different time points shows a significant reduction from 2 days to 21 days (p = 0,0236). As a non-significant tendency, the size increases from 6 hours to 2 days where it reaches its maximum and decreases linearly from 2 days to 7 days and to 21 days (Figure 12).



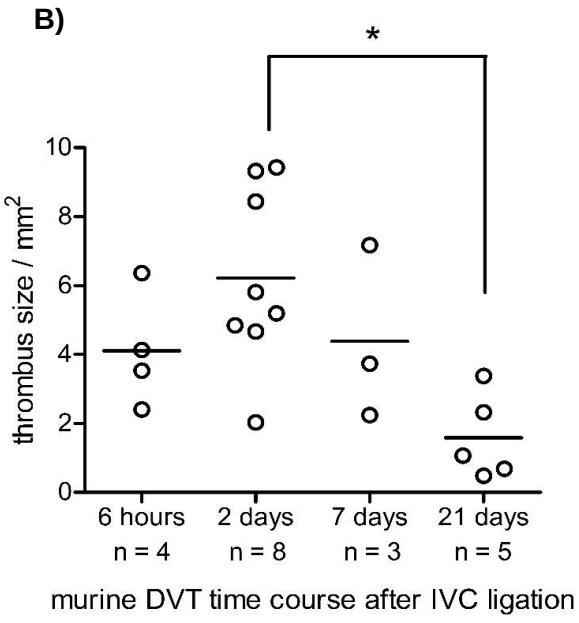


Figure 12: Thrombus size decreases over time. Sections of the murine wild type DVT time course were imaged and the size was measured with ImageJ.
A) representative example of DVTs in the time course. Black line marks the thrombus area, red arrow the directions of blood flow in the vessel and black arrow the position of the IVC ligation.
B) Quantitative analysis of the thrombus size over time with significant decrease from 2 to 21 days (p = 0,0236).

Also, the amount of red blood cells (RBCs) decreases in the DVT time course. RBCs are one of the main components of a fresh DVT. At the 6 hours' time point they filled over 60% of the thrombus size. The amount of RBCs decreases significantly from 6 hours to 21 days (p = 0,0026) with a tendency of a linear decrease by the 2 days' and 7 days' time points (Figure 13).

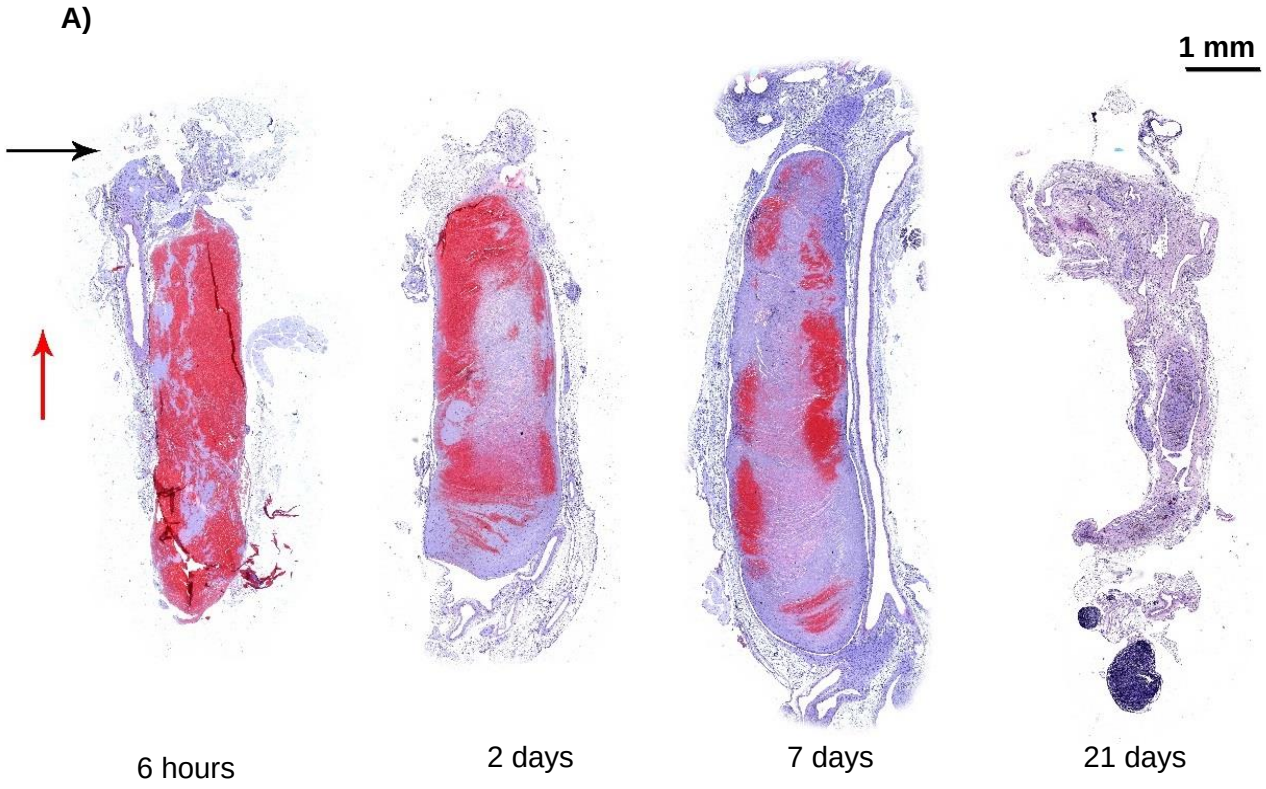


Figure 13: The amount of red blood cells decreases over time.
A) H&E staining of the murine DVT time course, representative images. RBCs are stained in bright red. The images visualize the decreasing amount of RBCs and also the reduction of thrombus size. Black arrow indicates the position of the ligation; red arrow indicates the flow direction of blood in the vessel.
B) Quantification of the H&E staining with ImageJ. The graph shows the percentage of bright red areas of the total thrombus in the murine DVT time course. Bright red areas decrease significantly from 6 hours' to 21 days' time point following a linear trend.

In contrast, collagen accumulates in the DVT time course. In the first 7 days, the amount of collagen is very low and stays at approximately the same level. At the 21 days' time point over 40% of the thrombus consist of collagen. The amount of collagen increases highly significant from 2 days to 21 days (p = 0,0025, Figure 14.

These results show that thrombosis is not a static event but undergoes a dramatic change over time. The thrombus is essentially rebuilt during the complex process of thrombus development and resolution.

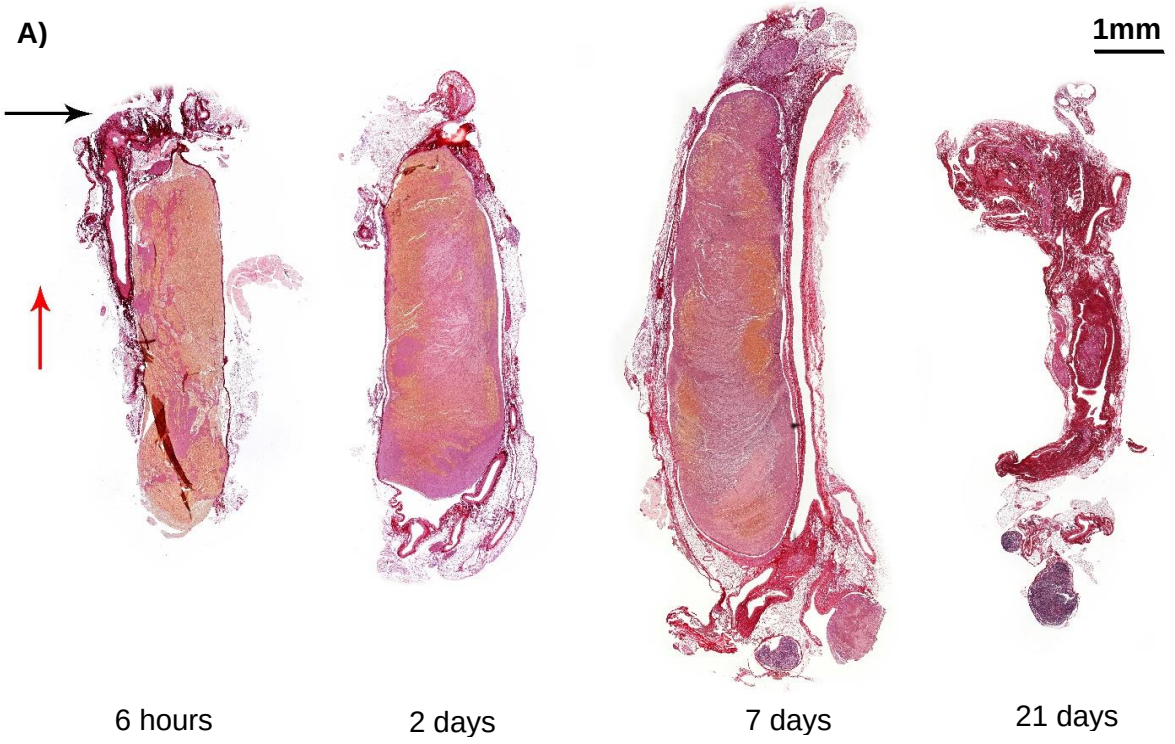


Figure 14: Collagen accumulates in the DVT time course.

A) Sirius Red staining of the murine wild type DVT time course. The images visualize the increasing amount of collagen stained in dark red within the thrombus. Collagen appears in the vessel wall remnants at all time points and in the thrombus after 21 days. Black arrow indicates the position of the ligation; red arrow indicates the flow direction of blood in the vessel.

B) Quantification of the Sirius Red staining. The dark red areas were measured with ImageJ. The graph shows the percentage of dark red areas of the total thrombus size in the murine DVT time course. Dark red areas increase significantly from the 2 days' to the 21 days' time point.

3.4 NETs in the DVT time course

NETs have an important impact on DVT in mouse models and appear in human thromboembolic diseases (Martinod and Wagner 2014). In experimental thrombosis NETs provide a scaffold for RBCs and platelets similar to vWF and fibrin and they interact with endothelial cells (Brill, Fuchs et al. 2012).

Leucocytes with neutrophils as their most common cell type migrate into the thrombus and influence thrombus remodeling. The leucocyte density increases in the DVT time course. To quantify the leucocyte density sections of a total of 20 murine wild type DVT were stained with DAPI. Nuclei within the thrombus were counted using the ImageJ particle count function and set in ratio with the thrombus size to receive the leucocyte density. The leucocyte density increases significantly from 6 hours to 21 days ($p = 0,0030$) and from 2 days to 21 days ($p = 0,0437$). It must be considered that especially at the 21 days' time point other cells with nuclei, such as fibroblasts and macrophages, are found within the thrombus which cannot be differentiated from leucocytes by this method (Figure 15).

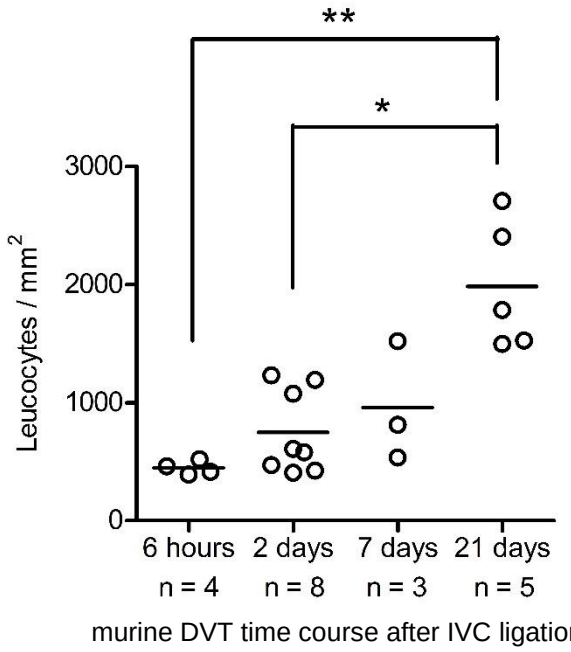
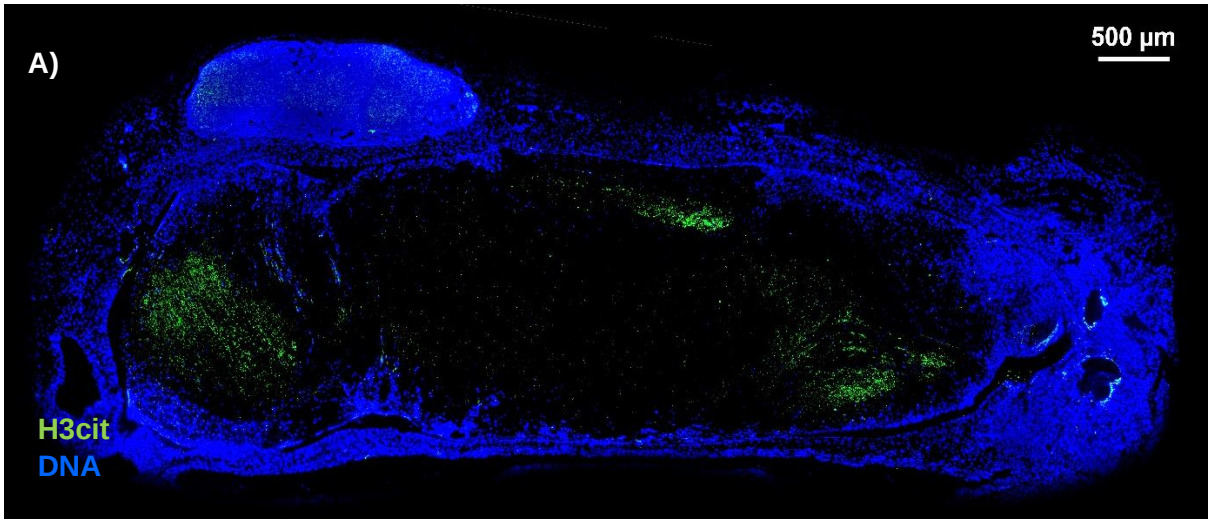
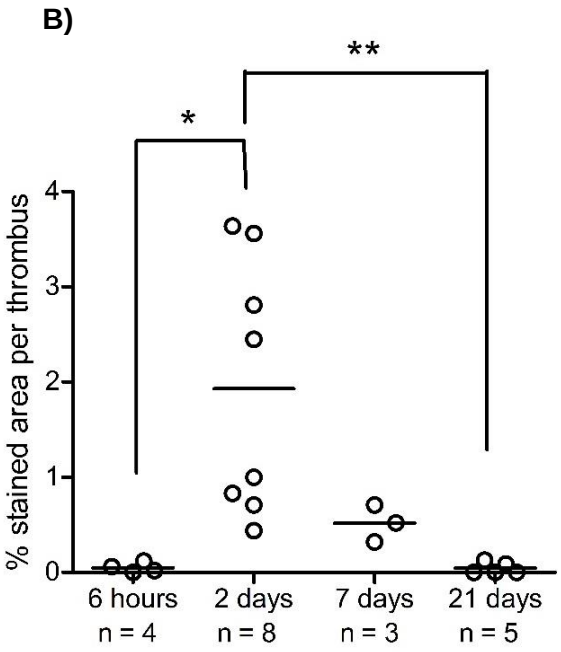


Figure 15: Leucocyte density. The graph shows the amount of leucocytes per mm^2 in sections of a murine DVT. Sections were stained with DAPI and the amount of stained nuclei within the thrombus was calculated with ImageJ 1.48v particle counter. There is a significant increase of leucocytes from 6 hours to 21 days and 2 days to 21 days. Leucocytes migrate into the thrombus and influence its development and remodeling.

NETs show a maximum at the 2 days' time point of the DVT time course. 6 hours after IVC ligation only sporadic NETs can be detected within thrombi. NETs increase significantly from 6 hours to the 2 days' time point ($p = 0,0164$) and then decrease again by 21 days ($p = 0,0038$, Figure 16). In conclusion, only very few NETs are present in the initial state of thrombus formation but a lot more after 2 days.



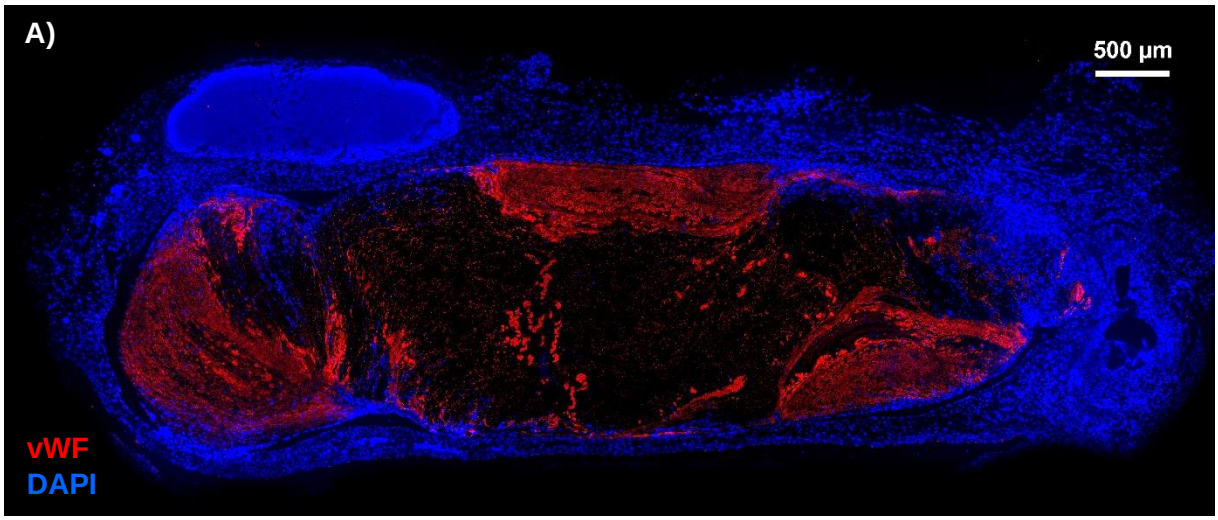


murine DVT time course after IVC ligation

Figure 16. NETs in the DVT time course.
NETs show a maximum at the 2 days' time point of the DVT time course.
A) Representative immunostaining for H3cit as a marker for NETs. Murine DVT 7 days after IVC ligation.
B) Quantification of the H3cit staining of the murine DVT time course with ImageJ. The graph shows the percentage of stained areas of the total thrombus. The amount of NETs increases from 6 hours to 2 days and then decreases again to 21 days.

3.5 vWF in the DVT time course

Platelets are crucial for both hemostasis and thrombosis and recent findings have shown that platelet-derived PolyP activates FXII initiating the intrinsic pathway of coagulation (Muller, Mutch et al. 2009). The mean platelet amount increased slightly from every time point to the next in the murine DVT time course but only shows a tendency of platelet accumulation. It has to be considered, that the increasing amount of platelets could also be an effect of decreasing thrombus size while the total number of platelets remains stable (Figure 17).



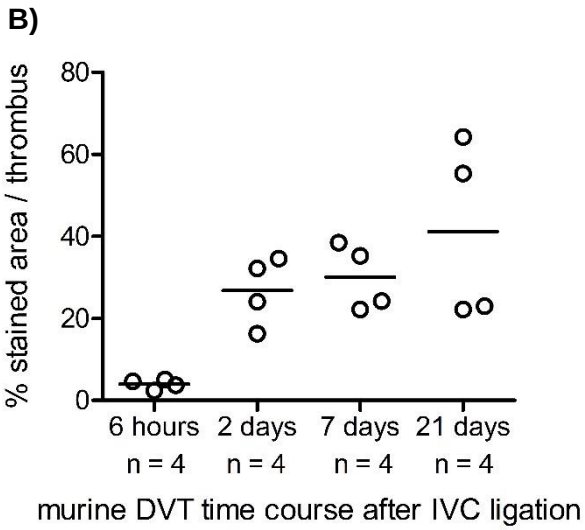


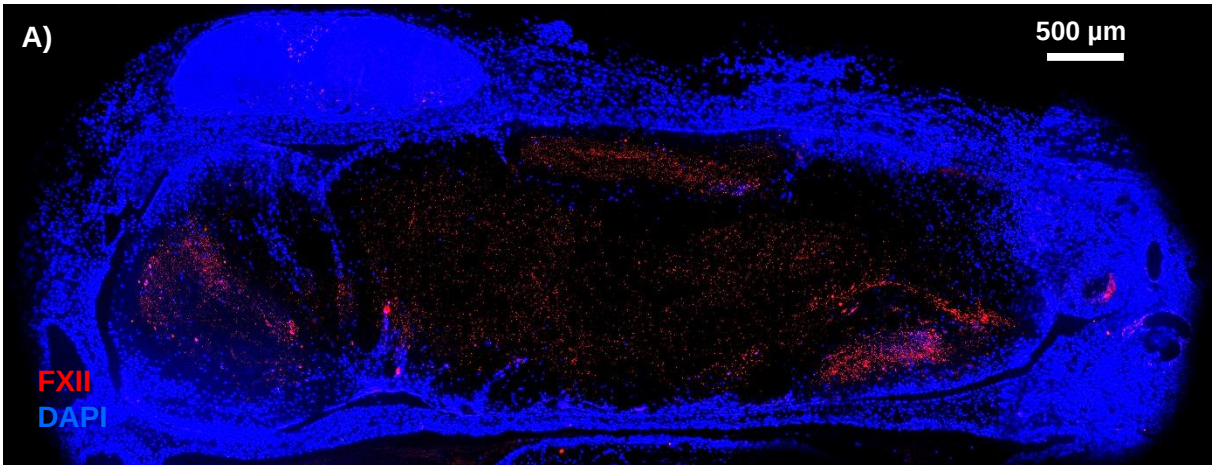
Figure 17: The amount of platelets increases over time in the DVT time course.
A) Representative immunostaining for vWF as a marker for platelets. Murine DVT 7 days after IVC ligation.
B) Quantification of the vWF staining of the DVT time course with ImageJ. The graph shows a non-significant tendency of platelet accumulation.

3.6 FXII in the DVT time course

FXII is crucial for the formation of a DVT and influences its devolvement in many ways. It accumulates after 2 days in the DVT time course. Immunostainings with the antibody rabbit anti FXIIa (Sigma-Aldrich SAB4500831, 2 µg/ml) were performed on paraffin sections of a total of 20 murine wild type DVT of the time course. At the 6 hours' time point, only little of the thrombus is positive for FXII but the amount increases significantly to the two days' time point ($p = 0{,}0202$) and then remains at approximately the same level. The first two time points show a similar kinetics to NETs but in contrast to NETs FXII levels do not decrease (Figure 18).

Further, FXII shows a colocalization with leucocytes in DVT. The single staining of FXII with DAPI shows an association of the FXII signal with nuclei. It shows a pattern of dots localized around nuclei within the thrombus, which are mostly leucocytes in this setting, whereas the vessel walls show no staining (Figure 19).

Next, a double immunostaining for FXIIa and H3 was performed on wild type and on protein arginine deiminase 4 (PAD4) gene deficient animals ($PAD4^{-/-}$). The experiment was designed to further investigate the colocalization of FXII and nuclei and to see if there is a difference when no NETs are present. A double immunostaining for FXIIa and H3 was done on paraffin section of murine wild type and $PAD4^{-/-}$ DVT two days after IVC ligation. The antibodies rabbit anti H3 (Abcam ab1791) and rabbit anti FXIIa (Sigma-Aldrich SAB4500831) were used both at a concentration of 2 µg/ml according to the protocol for double immunostainings with two



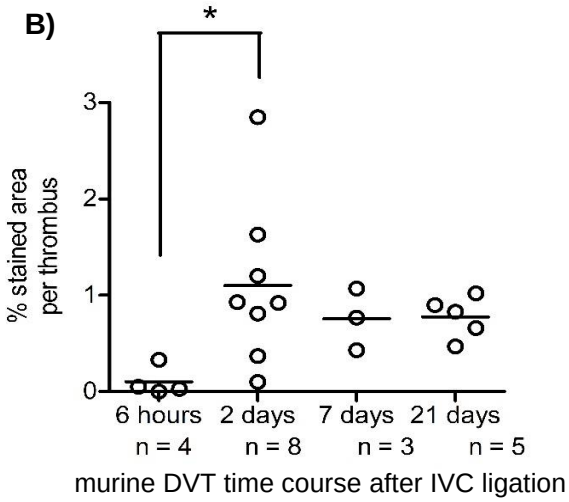


Figure 18: FXII accumulates at the 2 days' time point in the DVT time course.
A) Representative immunostaining for FXII, murine DVT 7 days after IVC ligation.
B) Quantification of the FXII staining of the DVT time course with ImageJ. The amount of FXII is very low at the 6 hours' time point and increases to 2 days.

primaries raised in the same host species as described in methods. Hitherto, homologous areas of the thrombi were selected, and stained particles were counted with ImageJ 1.48v.

The results show no significant difference in the FXIIa particle count between wild type and PAD4$^{-/-}$. Whether NETs are present are not, does not seem to influence the overall amount of FXIIa in a thrombus in this setting. Next, particles positive for FXIIa and for DNA as well as lone FXIIa particles were counted. This showed that almost all FXIIa particles were also

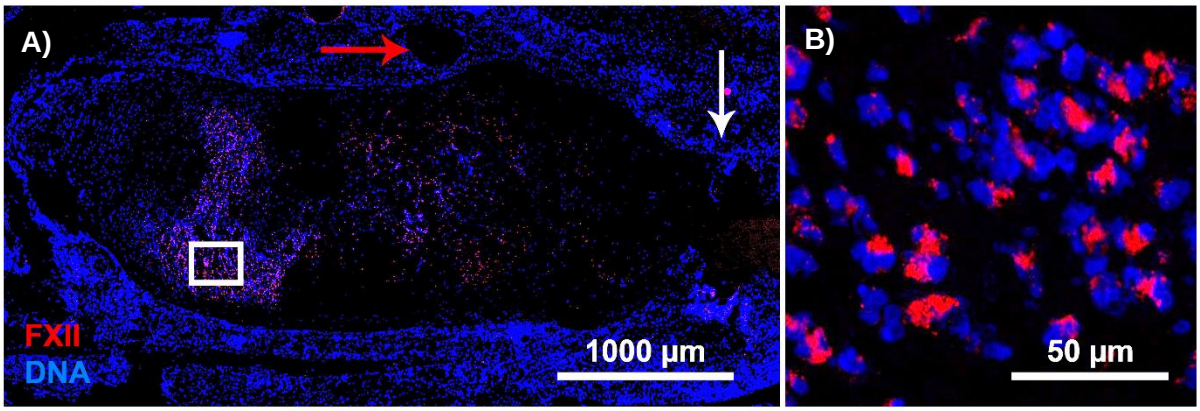


Figure 19: FXII colocalizes with leukocytes in DVT. Immunostaining with the antibody rabbit anti FXIIa (Sigma-Aldrich SAB4500831) shows an association of FXII with leukocytes in the DVT mouse model. The images show a murine DVT 2 days after IVC ligation, wild type. A) Overview. White arrow indicates the position of the ligation; red arrow indicates the flow direction of blood in the vessel. B) 20-times magnification of the marked square.

positive for DNA (p = 0,0179) which emphasizes the association of FXIIa and DNA in this setting. Again, there was no significant difference between wild type and PAD4$^{-/-}$. Particles positive for FXIIa and H3 were counted as well. Most particles FXIIa particles also showed a H3 staining (p = 0,0136), and again wild type and PAD4$^{-/-}$ showed no significant difference (Figure 20).

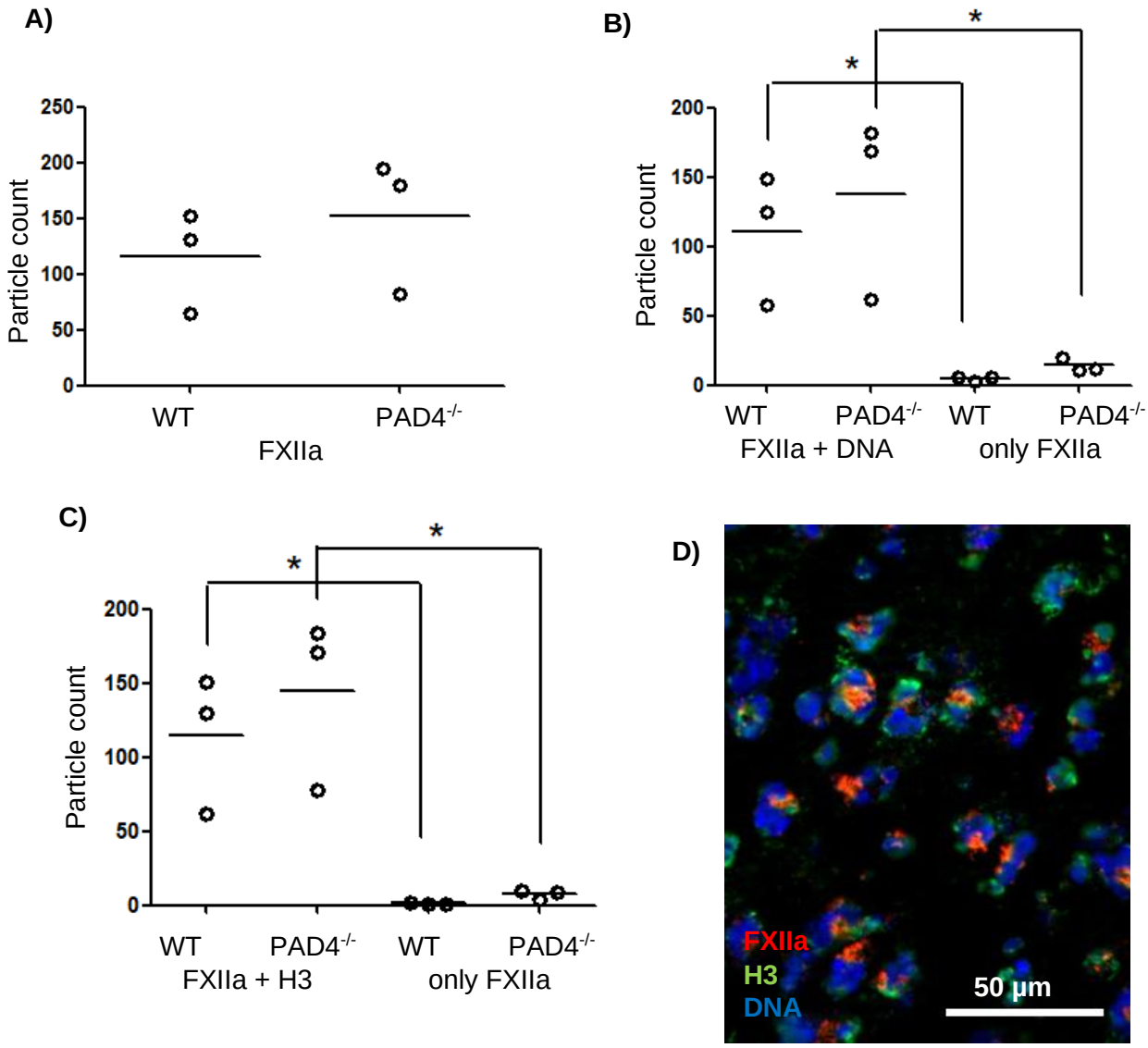


Figure 20: FXIIa is associated with leucocytes in wild type and PAD4$^{-/-}$. A double immunostaining with the antibodies rabbit anti H3 (Abcam ab1791) and rabbit anti FXIIa (Sigma-Aldrich SAB4500831) was performed on three wild type and three PAD4$^{-/-}$ DVT 2 days after IVC ligation. The graphs show the count of stained particle in homologous thrombus areas (y-axis) for different signals (x-axis). A) The particle count of all particles positive for FXIIa shows no difference between wild type and PAD4$^{-/-}$. B, C) x-axis shows particles that were positive for FXIIa and DNA (B) or particles positive for FXIIa and histone H3 (C), and particles that were exclusively positive for FXIIa, both in wild type and PAD4$^{-/-}$Almost all FXIIa is associated with DNA and histone H3. Again, there is no significant difference in wild type and PAD4$^{-/-}$. D) Representative image of the immunostaining.

4 DISCUSSION

4.1 Thrombus development

The development and resolution of a venous thrombus is a complicated process which involves many cells and proteins. There are many risk factors for a thrombotic event, but the exact mechanisms are unknown. Endothelial damage or neutrophil activation can initiate thrombosis but those are not always found.

The development of a thrombus can be classified into three distinct states. The first state is called unorganized. The newly formed thrombus mainly consists of red blood cells (RBCs) and platelets. The organizing state is characterized by infiltration of inflammatory cells, in the beginning mainly neutrophils. Fibroblasts migrate into the edges of the thrombus and collagen deposition and neovascularization starts. In the third state, the organized state, the thrombus has a fibrotic appearance marked by collagen rich, fibrous connective tissue and hemosiderin-laden macrophages (Seidman and Mitchell , Savchenko, Martinod et al. 2014). The three states also mirror the process of thrombus resolution which is closely related to wound healing. It is characterized by a perithrombotic inflammation, neovascularization, and fibrosis which occurs mainly during the organizing state of thrombosis. Although inflammation in general is prothrombotic it seems to be also required for thrombus resolution, especially monocytes/macrophages seem to play and essential role in neovascularization and fibrin clearance. Neutrophils and macrophages express proteases such as matrix metalloproteases that degrade the fibrin matrix and both cell types are phagocytes. Together with platelets they release pro-angiogenic factors such as basic fibroblast growth factor (bFGF), IL-8 and vascular endothelial growth factor VEGF. Neovascularization is essential to restore blood flow through the vein (Modarai, Burnand et al. 2005, Henke and Wakefield 2009).

Northeast et al. found that a fresh thrombus grows until it reaches its maximum size after two days and starts to shrink again (Northeast, Soo et al. 1995). This is also what was observed in this project (Figure 11). The 2 days' time point might be a hallmark in thrombus development in which most of the thrombus is in an organizing state and resolution starts. Fibrinolysis probably overtakes fibrin deposition and a significant number of leucocytes already infiltrated the thrombus.

RBCs are one of the main components of a thrombus in an early state. Their function is not known exactly but over time the iron in the hemoglobin is oxidized by ROS which leads to cell lysis and causes stress on the endothelium (Aleman, Walton et al. 2014). In this project a decreasing amount of RBCs was observed which fits with the concept that they passively increase the volume of a DVT in the beginning and then die due to cell lysis. The kinetics of RBCs in the time course show that they are abundant in a freshly formed thrombus, but no new RBCs are recruited later and their amount decreases (Figure 12). They are important for thrombus formation, although it is unknown if they play an active role in it.

In the organizing and organized state of thrombosis tissue fibrosis can be observed. This is also observed in wound healing, in which it causes the formation of scars. In thrombosis, the vessel wall is remodeled and thickened which can lead to post-thrombotic syndrome, a disease characterized by vein valve failure and persistent venous obstruction. A rapid thrombus resolution is essential to prevent post-thrombotic syndrome (Diaz, Wrobleski et al. 2015). An ongoing fibrosis in the DVT especially in the late state of thrombosis was also observed in this project. Collagen is one of the main components of fibrous connective tissue and characterizing its deposition with the Picrosirius Red staining showed its accumulation in the DVT time course. 6 hours after IVC ligation there was barely any collagen within the thrombus but at the 21 days' time point where all of the thrombus is in the organized state only a small fibrotic plug with collagen as one of its main components remained of the thrombus (Figure 13).

The changes in thrombus size, the decreasing amount of RBCs and collagen accumulation all characterize the complex process of thrombus formation, development and resolution. The fresh thrombus is rich in RBCs and grows until the 2 days' time point then the organizing state and the resolution starts. The thrombus shrinks, RBCs are eliminated, and fibrosis progresses.

4.2 NET kinetics

Neutrophils play a dual role in thrombosis. Activated neutrophils can promote thrombosis by releasing NETs and through direct interaction with endothelial cells and coagulation factors but on the other hand they can also promote fibrinolysis and play a role in neovascularization.

Neutrophils start migrating into the thrombus soon after its formation. An increasing number of leucocytes in the DVT time course has been observed in this project. 6 hours after IVC ligation there were already approximately 500 leucocytes/mm^2 thrombus area and the density increased up to the 21 days' time point to approximately 2000 leucocytes/mm^2 thrombus area (Figure 14).

The initial neutrophil recruitment is mainly mediated by P-selectin which is expressed on endothelial cells and platelets. The neutrophils release procoagulant NETs which provide a scaffold for RBCs and platelets and give the thrombus more stability (Fuchs, Brill et al. 2012). DNases are required to degrade the NETs and it has been shown that blood clots containing DNA and histones are more resistant to fibrinolysis (Savchenko, Martinod et al. 2014). In this project, only very few NETs were found within the thrombi 6 hours after IVC ligation. They reached a maximum at the 2 days' time point and then decreased again (Figure 15). Therefore, NETs are mainly present in the organizing state of thrombosis which is also what Savchenko et al. found in human thrombi (Savchenko, Martinod et al. 2014). NETs show a similar kinetics to the thrombus size in the DVT time course with a strong peak at 2 days (Figure 15). The thrombus grows until the second day were already a lot of NETs are present, thus, NETs are likely to promote thrombus formation, stability, and/or reorganization. This is also supported by the fact that $PAD4^{-/-}$ mice are partially protected from experimental thrombus formation (Martinod, Demers et al. 2013). NETs are quite common in the organizing state of thrombosis and they influence thrombus resolution which starts in this phase of thrombus development.

NETs show procoagulant properties, but neutrophils can also promote fibrinolysis and neovascularization. Neutrophil elastase stimulates matrix metalloproteases which are essential for the degradation of the thrombus matrix, and they express pro-angiogenic factors (Henke and Wakefield 2009). Therefore, neutrophils likely play a role in thrombus resolution, but their impact seems to be inferior compared to macrophages. Overall, the procoagulant properties of neutrophils predominate their effects on thrombus resolution. Activated neutrophils create an inflammatory milieu which is generally procoagulant, they interact with endothelial cells and promote thrombus formation by releasing NETs. The additional thrombus stability given by NETs slows down thrombus resolution and also the inflammatory mediators released by neutrophils increase fibrosis. NETs are frequently found in human thrombotic diseases and furthermore, inhibiting P-selectin, which prevents neutrophil activation accelerates thrombus resolution and diminishes fibrosis of the vessel wall (Myers, Henke et al. 2002). Fibrin deposition is decreased, and less post-thrombotic syndromes occur (Diaz, Wrobleski et al. 2015).

Targeting neutrophils or NETs offers new strategies to treat venous thrombosis or thromboembolism. In combination with other drug classes, inhibiting NETosis offers a new approach to prevent thromboembolic events and especially posthrombotic syndrome. DNases to degrade NETs combined with tPA which is the current standard in the treatment of fresh, occlusive thrombosis could lead to a faster way of therapeutic thrombolysis (Brill, Fuchs et al. 2012, Kim, Nam et al. 2015).

4.3 FXII staining

With the improvements in tissue freezing and reducing background immunofluorescence, four different antibodies targeting FXII were successfully used in immunostainings. The most reliable staining was achieved with the antibody rabbit anti FXIIa (Sigma-Aldrich SAB4500831). It's labelled as targeting FXIIa specifically but it likely also binds FXII as the staining patter was the same as with the other antibodies and, apart from the fully human recombinant antibody 3F7 (Larsson, Rayzman et al. 2014), no antibody has been reliably tested for its specificity for FXIIa.

Staining the DVT time course for FXII showed increasing levels of FXII to the two days' time point and a stable kinetic afterwards. FXII activation is generally considered to initiate thrombosis formation (Bjorkqvist, Nickel et al. 2014) and in this project there was FXII found in the early state of thrombosis, but in this particular setting of complete occlusion of the inferior vena cava it might play a more important role in later states of thrombosis as it's quantity increased. It's role in the later states of thrombosis is yet unknown, but it is likely linked with leucocytes as the double stainings showed a close association with them. This was very consistent with all stainings. There was no difference between the FXIIa staining of wild type and PAD4-/- mice, so the connection between FXII and leucocytes/DNA in this case seems to be independent of NET formations or at least not restricted to NETs. The observed increase of FXII levels could also come from neutrophil derived FXII. It has recently been reported that neutrophils are able to produce FXII, that has distinct functions from circulating hepatic derived FXII, e.g. contributes to neutrophil trafficking at sites of inflammation (Stavrou, Fang et al. 2018).

5 CONCLUSION AND OUTLOOK

The development of a DVT is a complicated process which involves many cell types and plasma proteins, and which is connected to inflammation.

Aim 1 of this project was to characterize a murine DVT time course with standard histological staining techniques. I showed that the size of DVT and the amount of red blood cells decrease over time while collagen accumulates. This represents the reorganization process of a DVT.

Aim 2 was to characterize the timely and spatially distribution of potential sources of FXII activators. I showed that leucocytes migrate into a newly formed thrombus and release NETs, which are most frequently found in the organizing state of thrombus. Both NETs and Platelets are found in a fresh formed thrombus but are more common later, which might indicate that also FXII is activated in later states. However, it remains unclear how NETs exactly contribute to thrombosis in the DVT model used in this project. In the experiments conducted with $PAD4^{-/-}$ mice no obvious difference to wild type was observed, but the experiments where primarily designed to investigate a connection between FXII and NETs. An open question for further research could be to analyze the $PAD4^{-/-}$ mouse model in the DVT time course in greater detail.

Aim 3 was to establish a specific method for the detection of FXII in murine tissue using immunofluorescence to enable colocalizations of FXII and its potential activators. I established a staining protocol for FXII/FXIIa and showed, that FXIIa was most common in the organizing state of thrombosis. Further, I showed that FXII is associated with nuclei in this setting. The exact function of FXIIa in the later states of thrombosis and its role in neutrophil activities is unclear and a continuative question that awaits further research.

6 REFERENCES

Aksu, K., A. Donmez and G. Keser (2012). "Inflammation-induced thrombosis: mechanisms, disease associations and management." Curr Pharm Des **18**(11): 1478-1493.

Aleman, M. M., B. L. Walton, J. R. Byrnes and A. S. Wolberg (2014). "Fibrinogen and red blood cells in venous thrombosis." Thromb Res **133 Suppl 1**: S38-40.

Amulic, B., C. Cazalet, G. L. Hayes, K. D. Metzler and A. Zychlinsky (2012). "Neutrophil function: from mechanisms to disease." Annu Rev Immunol **30**: 459-489.

Barett, K. E., S. M. Barman, S. Boitano and H. Brooks (2009). Ganong's Review of Medical Physiology, 23rd Edition, McGraw-Hill Medical.

Bertz, S., B. J. Schmitz-Drager, C. Protzel and A. Hartmann (2012). "[Intraoperative frozen section diagnosis of the genitourinary tract]." Pathologe **33**(5): 441-449.

Bjorkqvist, J., A. Jamsa and T. Renne (2013). "Plasma kallikrein: the bradykinin-producing enzyme." Thromb Haemost **110**(3): 399-407.

Bjorkqvist, J., K. F. Nickel, E. Stavrou and T. Renne (2014). "In vivo activation and functions of the protease factor XII." Thromb Haemost **112**(5): 868-875.

Borregaard, N. (2010). "Neutrophils, from marrow to microbes." Immunity **33**(5): 657-670.

Bratthauer, G. L. (2010). "Preparation of frozen sections for analysis." Methods Mol Biol **588**: 67-73.

Brill, A., T. A. Fuchs, A. K. Chauhan, J. J. Yang, S. F. De Meyer, M. Kollnberger, T. W. Wakefield, B. Lammle, S. Massberg and D. D. Wagner (2011). "von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models." Blood **117**(4): 1400-1407.

Brill, A., T. A. Fuchs, A. S. Savchenko, G. M. Thomas, K. Martinod, S. F. De Meyer, A. A. Bhandari and D. D. Wagner (2012). "Neutrophil extracellular traps promote deep vein thrombosis in mice." J Thromb Haemost **10**(1): 136-144.

Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch and A. Zychlinsky (2004). "Neutrophil extracellular traps kill bacteria." Science **303**(5663): 1532-1535.

Bryckaert, M., J. P. Rosa, C. V. Denis and P. J. Lenting (2015). "Of von Willebrand factor and platelets." Cell Mol Life Sci **72**(2): 307-326.

Cicardi, M., A. Banerji, F. Bracho, A. Malbran, B. Rosenkranz, M. Riedl, K. Bork, W. Lumry, W. Aberer, H. Bier, M. Bas, J. Greve, T. K. Hoffmann, H. Farkas, A. Reshef, B. Ritchie, W. Yang, J. Grabbe, S. Kivity, W. Kreuz, R. J. Levy, T. Luger, K. Obtulowicz, P. Schmid-Grendelmeier, C. Bull, B. Sitkauskiene, W. B. Smith, E. Toubi, S. Werner, S. Anne, J. Bjorkander, L. Bouillet, E. Cillari, D. Hurewitz, K. W. Jacobson, C. H. Katelaris, M. Maurer, H. Merk, J. A. Bernstein, C. Feighery, B. Floccard, G. Gleich, J. Hebert, M. Kaatz, P. Keith, C. H. Kirkpatrick, D. Langton, L. Martin, C. Pichler, D. Resnick, D. Wombolt, D. S. Fernandez Romero, A. Zanichelli, F. Arcoleo, J. Knolle, I. Kravec, L. Dong, J. Zimmermann, K. Rosen and W. T. Fan (2010). "Icatibant, a new bradykinin-receptor antagonist, in hereditary angioedema." N Engl J Med **363**(6): 532-541.

Cicardi, M., R. J. Levy, D. L. McNeil, H. H. Li, A. L. Sheffer, M. Campion, P. T. Horn and W. E. Pullman (2010). "Ecallantide for the treatment of acute attacks in hereditary angioedema." N Engl J Med **363**(6): 523-531.

Currle, D. S. and E. S. Monuki (2007). "Flash freezing and cryosectioning E12.5 mouse brain." J Vis Exp(4): 198.

Dahlgren, C. and A. Karlsson (1999). "Respiratory burst in human neutrophils." J Immunol Methods **232**(1-2): 3-14.

Darbousset, R., G. M. Thomas, S. Mezouar, C. Frere, R. Bonier, N. Mackman, T. Renne, F. Dignat-George, C. Dubois and L. Panicot-Dubois (2012). "Tissue factor-positive neutrophils bind to injured endothelial wall and initiate thrombus formation." Blood **120**(10): 2133-2143.

Diaz, J. A., S. K. Wrobleski, C. M. Alvarado, A. E. Hawley, N. K. Doornbos, P. A. Lester, S. E. Lowe, J. E. Gabriel, K. J. Roelofs, P. K. Henke, R. G. Schaub, T. W. Wakefield and D. D. Myers, Jr. (2015). "P-selectin inhibition therapeutically promotes thrombus resolution and prevents vein wall fibrosis better than enoxaparin and an inhibitor to von Willebrand factor." Arterioscler Thromb Vasc Biol **35**(4): 829-837.

Esbona, K., Z. Li and L. G. Wilke (2012). "Intraoperative imprint cytology and frozen section pathology for margin assessment in breast conservation surgery: a systematic review." Ann Surg Oncol **19**(10): 3236-3245.

Faurschou, M. and N. Borregaard (2003). "Neutrophil granules and secretory vesicles in inflammation." Microbes Infect **5**(14): 1317-1327.

Fuchs, T. A., U. Abed, C. Goosmann, R. Hurwitz, I. Schulze, V. Wahn, Y. Weinrauch, V. Brinkmann and A. Zychlinsky (2007). "Novel cell death program leads to neutrophil extracellular traps." J Cell Biol **176**(2): 231-241.

Fuchs, T. A., A. Brill and D. D. Wagner (2012). "Neutrophil extracellular trap (NET) impact on deep vein thrombosis." Arterioscler Thromb Vasc Biol **32**(8): 1777-1783.

Henke, P. K. and T. Wakefield (2009). "Thrombus resolution and vein wall injury: dependence on chemokines and leukocytes." Thromb Res **123 Suppl 4**: S72-78.

Jablonska, E., P. Markart, D. Zakrzewicz, K. T. Preissner and M. Wygrecka (2010). "Transforming growth factor-beta1 induces expression of human coagulation factor XII via Smad3 and JNK signaling pathways in human lung fibroblasts." J Biol Chem **285**(15): 11638-11651.

Jimenez-Alcazar, M., M. Napirei, R. Panda, E. C. Kohler, J. A. Kremer Hovinga, H. G. Mannherz, S. Peine, T. Renne, B. Lammle and T. A. Fuchs (2015). "Impaired DNase1-mediated degradation of neutrophil extracellular traps is associated with acute thrombotic microangiopathies." J Thromb Haemost **13**(5): 732-742.

Joseph, K., T. B. Tuscano and A. P. Kaplan (2008). "Studies of the mechanisms of bradykinin generation in hereditary angioedema plasma." Ann Allergy Asthma Immunol **101**(3): 279-286.

Kannemeier, C., A. Shibamiya, F. Nakazawa, H. Trusheim, C. Ruppert, P. Markart, Y. Song, E. Tzima, E. Kennerknecht, M. Niepmann, M. L. von Bruehl, D. Sedding, S. Massberg, A. Gunther, B. Engelmann and K. T. Preissner (2007). "Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation." Proc Natl Acad Sci U S A **104**(15): 6388-6393.

Kaplan, A. P. and B. Ghebrehiwet (2010). "The plasma bradykinin-forming pathways and its interrelationships with complement." Mol Immunol **47**(13): 2161-2169.

Kenne, E. and T. Renne (2014). "Factor XII: a drug target for safe interference with thrombosis and inflammation." Drug Discov Today **19**(9): 1459-1464.

Kim, Y. D., H. S. Nam, S. H. Kim, E. Y. Kim, D. Song, I. Kwon, S. H. Yang, K. Lee, J. Yoo, H. S. Lee and J. H. Heo (2015). "Time-Dependent Thrombus Resolution After Tissue-Type Plasminogen Activator in Patients With Stroke and Mice." Stroke **46**(7): 1877-1882.

Kleinschnitz, C., G. Stoll, M. Bendszus, K. Schuh, H. U. Pauer, P. Burfeind, C. Renne, D. Gailani, B. Nieswandt and T. Renne (2006). "Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis." J Exp Med **203**(3): 513-518.

Konings, J., J. W. Govers-Riemslag, H. Philippou, N. J. Mutch, J. I. Borissoff, P. Allan, S. Mohan, G. Tans, H. Ten Cate and R. A. Ariens (2011). "Factor XIIa regulates the structure of the fibrin clot independently of thrombin generation through direct interaction with fibrin." Blood **118**(14): 3942-3951.

Konings, J., L. R. Hoving, R. S. Ariens, E. L. Hethershaw, M. Ninivaggi, L. J. Hardy, B. de Laat, H. Ten Cate, H. Philippou and J. W. Govers-Riemslag (2015). "The role of activated coagulation factor XII in overall clot stability and fibrinolysis." Thromb Res **136**(2): 474-480.

Kuijpers, M. J., P. E. van der Meijden, M. A. Feijge, N. J. Mattheij, F. May, J. Govers-Riemslag, J. C. Meijers, J. W. Heemskerk, T. Renne and J. M. Cosemans (2014). "Factor XII regulates the pathological process of thrombus formation on ruptured plaques." Arterioscler Thromb Vasc Biol **34**(8): 1674-1680.

Kumar, V., A. K. Abbas and J. C. Aster (2014). Robbins & Cotran Pathologic Basis of Disease. Oxford, Elsevier Ltd.

Kyrle, P. A. and S. Eichinger (2005). "Deep vein thrombosis." Lancet **365**(9465): 1163-1174.

Labberton, L., E. Kenne and T. Renne (2015). "New agents for thromboprotection. A role for factor XII and XIIa inhibition." Hamostaseologie **35**(2).

Lammle, B., W. A. Wuillemin, I. Huber, M. Krauskopf, C. Zurcher, R. Pflugshaupt and M. Furlan (1991). "Thromboembolism and bleeding tendency in congenital factor XII deficiency--a study on 74 subjects from 14 Swiss families." Thromb Haemost **65**(2): 117-121.

Larsson, M., V. Rayzman, M. W. Nolte, K. F. Nickel, J. Bjorkqvist, A. Jamsa, M. P. Hardy, M. Fries, S. Schmidbauer, P. Hedenqvist, M. Broome, I. Pragst, G. Dickneite, M. J. Wilson, A. D. Nash, C. Panousis and T. Renne (2014). "A factor XIIa inhibitory antibody provides thromboprotection in extracorporeal circulation without increasing bleeding risk." Sci Transl Med **6**(222): 222ra217.

LaRusch, G. A., F. Mahdi, Z. Shariat-Madar, G. Adams, R. G. Sitrin, W. M. Zhang, K. R. McCrae and A. H. Schmaier (2010). "Factor XII stimulates ERK1/2 and Akt through uPAR, integrins, and the EGFR to initiate angiogenesis." Blood **115**(24): 5111-5120.

Lee, W. L., R. E. Harrison and S. Grinstein (2003). "Phagocytosis by neutrophils." Microbes Infect **5**(14): 1299-1306.

Levi, M., T. van der Poll and M. Schultz (2012). "Infection and inflammation as risk factors for thrombosis and atherosclerosis." Semin Thromb Hemost **38**(5): 506-514.

Li, P., M. Li, M. R. Lindberg, M. J. Kennett, N. Xiong and Y. Wang (2010). "PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps." J Exp Med **207**(9): 1853-1862.

Maas, C., J. W. Govers-Riemslag, B. Bouma, B. Schiks, B. P. Hazenberg, H. M. Lokhorst, P. Hammarstrom, H. ten Cate, P. G. de Groot, B. N. Bouma and M. F. Gebbink (2008). "Misfolded proteins activate factor XII in humans, leading to kallikrein formation without initiating coagulation." J Clin Invest **118**(9): 3208-3218.

Maas, C. and T. Renne (2012). "Regulatory mechanisms of the plasma contact system." Thromb Res **129 Suppl 2**: S73-76.

Mammen, E. F. (1992). "Pathogenesis of venous thrombosis." Chest **102**(6 Suppl): 640s-644s.

Martinod, K., M. Demers, T. A. Fuchs, S. L. Wong, A. Brill, M. Gallant, J. Hu, Y. Wang and D. D. Wagner (2013). "Neutrophil histone modification by peptidylarginine deiminase 4 is critical for deep vein thrombosis in mice." Proc Natl Acad Sci U S A **110**(21): 8674-8679.

Martinod, K. and D. D. Wagner (2014). "Thrombosis: tangled up in NETs." Blood **123**(18): 2768-2776.

Matafonov, A., P. Y. Leung, A. E. Gailani, S. L. Grach, C. Puy, Q. Cheng, M. F. Sun, O. J. McCarty, E. I. Tucker, H. Kataoka, T. Renne, J. H. Morrissey, A. Gruber and D. Gailani (2014). "Factor XII inhibition reduces thrombus formation in a primate thrombosis model." Blood **123**(11): 1739-1746.

Modarai, B., K. G. Burnand, J. Humphries, M. Waltham and A. Smith (2005). "The role of neovascularisation in the resolution of venous thrombus." Thromb Haemost **93**(5): 801-809.

Muller, F., N. J. Mutch, W. A. Schenk, S. A. Smith, L. Esterl, H. M. Spronk, S. Schmidbauer, W. A. Gahl, J. H. Morrissey and T. Renne (2009). "Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo." Cell **139**(6): 1143-1156.

Mutch, N. J., E. K. Waters and J. H. Morrissey (2012). "Immobilized transition metal ions stimulate contact activation and drive factor XII-mediated coagulation." J Thromb Haemost **10**(10): 2108-2115.

Myers, D. D., Jr., P. K. Henke, S. K. Wrobleski, A. E. Hawley, D. M. Farris, A. M. Chapman, B. S. Knipp, P. Thanaporn, R. G. Schaub, L. J. Greenfield and T. W. Wakefield (2002). "P-selectin inhibition enhances thrombus resolution and decreases vein wall fibrosis in a rat model." J Vasc Surg **36**(5): 928-938.

Nickel, K. F., G. Ronquist, F. Langer, L. Labberton, T. A. Fuchs, C. Bokemeyer, G. Sauter, M. Graefen, N. Mackman, E. X. Stavrou, G. Ronquist and T. Renne (2015). "The polyphosphate-factor XII pathway drives coagulation in prostate cancer-associated thrombosis." Blood **126**(11): 1379-1389.

Northeast, A. D., K. S. Soo, L. G. Bobrow, P. J. Gaffney and K. G. Burnand (1995). "The tissue plasminogen activator and urokinase response in vivo during natural resolution of venous thrombus." J Vasc Surg **22**(5): 573-579.

Oschatz, C., C. Maas, B. Lecher, T. Jansen, J. Bjorkqvist, T. Tradler, R. Sedlmeier, P. Burfeind, S. Cichon, S. Hammerschmidt, W. Muller-Esterl, W. A. Wuillemin, G. Nilsson and T. Renne (2011). "Mast cells increase vascular permeability by heparin-initiated bradykinin formation in vivo." Immunity **34**(2): 258-268.

Papayannopoulos, V., K. D. Metzler, A. Hakkim and A. Zychlinsky (2010). "Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps." J Cell Biol **191**(3): 677-691.

Pauer, H. U., T. Renne, B. Hemmerlein, T. Legler, S. Fritzlar, I. Adham, W. Muller-Esterl, G. Emons, U. Sancken, W. Engel and P. Burfeind (2004). "Targeted deletion of murine coagulation factor XII gene-a model for contact phase activation in vivo." Thromb Haemost **92**(3): 503-508.

Ratnoff, O. D. and J. E. Colopy (1955). "A familial hemorrhagic trait associated with a deficiency of a clot-promoting fraction of plasma." J Clin Invest **34**(4): 602-613.

Renne, T. (2012). "The procoagulant and proinflammatory plasma contact system." Semin Immunopathol **34**(1): 31-41.

Renne, T., M. Pozgajova, S. Gruner, K. Schuh, H. U. Pauer, P. Burfeind, D. Gailani and B. Nieswandt (2005). "Defective thrombus formation in mice lacking coagulation factor XII." J Exp Med **202**(2): 271-281.

Revenko, A. S., D. Gao, J. R. Crosby, G. Bhattacharjee, C. Zhao, C. May, D. Gailani, B. P. Monia and A. R. MacLeod (2011). "Selective depletion of plasma prekallikrein or coagulation factor XII inhibits thrombosis in mice without increased risk of bleeding." Blood **118**(19): 5302-5311.

Saha, P., J. Humphries, B. Modarai, K. Mattock, M. Waltham, C. E. Evans, A. Ahmad, A. S. Patel, S. Premaratne, O. T. Lyons and A. Smith (2011). "Leukocytes and the natural history of deep vein thrombosis: current concepts and future directions." Arterioscler Thromb Vasc Biol **31**(3): 506-512.

Saito, H., T. Ishihara, H. Suzuki and T. Watanabe (1985). "Production and characterization of a murine monoclonal antibody against a heavy chain of Hageman factor (factor XII)." blood **1985 65: 1263-1268**.

Sala-Cunill, A., J. Bjorkqvist, R. Senter, M. Guilarte, V. Cardona, M. Labrador, K. F. Nickel, L. Butler, O. Luengo, P. Kumar, L. Labberton, A. Long, A. Di Gennaro, E. Kenne, A. Jamsa, T. Krieger, H. Schluter, T. Fuchs, S. Flohr, U. Hassiepen, F. Cumin, K. McCrae, C. Maas, E. Stavrou and T. Renne (2015). "Plasma contact system activation drives anaphylaxis in severe mast cell-mediated allergic reactions." J Allergy Clin Immunol **135**(4): 1031-1043.e1036.

Savchenko, A. S., K. Martinod, M. A. Seidman, S. L. Wong, J. I. Borissoff, G. Piazza, P. Libby, S. Z. Goldhaber, R. N. Mitchell and D. D. Wagner (2014). "Neutrophil extracellular traps form predominantly during the organizing stage of human venous thromboembolism development." J Thromb Haemost **12**(6): 860-870.

Schnell, S. A., W. A. Staines and M. W. Wessendorf (1999). "Reduction of lipofuscin-like autofluorescence in fluorescently labeled tissue." J Histochem Cytochem **47**(6): 719-730.

Schwieder, M. (2004). "Aktivierungsmechanismen des Blutgerinnungsfaktors XII auf Oberflächen."

Seidman, M. A. and R. N. Mitchell "Surgical Pathology of Small- and Medium-Sized Vessels." Surgical Pathology Clinics **5**(2): 435-451.

Shariat-Madar, Z., F. Mahdi and A. H. Schmaier (2002). "Assembly and activation of the plasma kallikrein/kinin system: a new interpretation." Int Immunopharmacol **2**(13-14): 1841-1849.

Shi, S. R., C. R. Taylor, C. B. Fowler and J. T. Mason (2013). "Complete solubilization of formalin-fixed, paraffin-embedded tissue may improve proteomic studies." Proteomics Clin Appl **7**(3-4): 264-272.

Sundd, P., M. K. Pospieszalska, L. S. Cheung, K. Konstantopoulos and K. Ley (2011). "Biomechanics of leukocyte rolling." Biorheology **48**(1): 1-35.

Svensson, M., P. Friberger, O. Lundstrom and B. Stegmayr (1996). "Activation of FXII during haemodialysis." Scand J Clin Lab Invest **56**(7): 649-652.

Thomas, D. (1994). "Venous thrombogenesis." Br Med Bull **50**(4): 803-812.

Thon, J. N. and J. E. Italiano (2012). "Platelets: production, morphology and ultrastructure." Handb Exp Pharmacol(210): 3-22.

Tornehave, D., D. M. Hougaard and L. Larsson (2000). "Microwaving for double indirect immunofluorescence with primary antibodies from the same species and for staining of mouse tissues with mouse monoclonal antibodies." Histochem Cell Biol **113**(1): 19-23.

Uhlen, M., L. Fagerberg, B. M. Hallstrom, C. Lindskog, P. Oksvold, A. Mardinoglu, A. Sivertsson, C. Kampf, E. Sjostedt, A. Asplund, I. Olsson, K. Edlund, E. Lundberg, S. Navani, C. A. Szigyarto, J. Odeberg, D. Djureinovic, J. O. Takanen, S. Hober, T. Alm, P. H. Edqvist, H. Berling, H. Tegel, J. Mulder, J. Rockberg, P. Nilsson, J. M. Schwenk, M. Hamsten, K. von Feilitzen, M. Forsberg, L. Persson, F. Johansson, M. Zwahlen, G. von Heijne, J. Nielsen and F. Ponten (2015). "Proteomics. Tissue-based map of the human proteome." Science **347**(6220): 1260419.

van der Meijden, P. E., I. C. Munnix, J. M. Auger, J. W. Govers-Riemslag, J. M. Cosemans, M. J. Kuijpers, H. M. Spronk, S. P. Watson, T. Renne and J. W. Heemskerk (2009). "Dual role of collagen in factor XII-dependent thrombus formation." Blood **114**(4): 881-890.

Wakefield, T. W., D. D. Myers and P. K. Henke (2008). "Mechanisms of venous thrombosis and resolution." Arterioscler Thromb Vasc Biol **28**(3): 387-391.

Wang, Y., M. Li, S. Stadler, S. Correll, P. Li, D. Wang, R. Hayama, L. Leonelli, H. Han, S. A. Grigoryev, C. D. Allis and S. A. Coonrod (2009). "Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation." J Cell Biol **184**(2): 205-213.

White-Adams, T. C., M. A. Berny, I. A. Patel, E. I. Tucker, D. Gailani, A. Gruber and O. J. McCarty (2010). "Laminin promotes coagulation and thrombus formation in a factor XII-dependent manner." J Thromb Haemost **8**(6): 1295-1301.

Worm, M., E. C. Köhler, R. Panda, A. Long, L. M. Butler, E. X. Stavrou, K. F. Nickel, T. A. Fuchs and T. Renné (2015). "The factor XIIa blocking antibody 3F7: a safe anticoagulant with anti-inflammatory activities." Annals of Translational Medicine **3**(17).

Zuraw, B. L. (2008). "Clinical practice. Hereditary angioedema." N Engl J Med **359**(10): 1027-1036.

Zuraw, B. L., P. J. Busse, M. White, J. Jacobs, W. Lumry, J. Baker, T. Craig, J. A. Grant, D. Hurewitz, L. Bielory, W. E. Cartwright, M. Koleilat, W. Ryan, O. Schaefer, M. Manning, P. Patel, J. A. Bernstein, R. A. Friedman, R. Wilkinson, D. Tanner, G. Kohler, G. Gunther, R. Levy, J. McClellan, J. Redhead, D. Guss, E. Heyman, B. A. Blumenstein, I. Kalfus and M. M. Frank (2010). "Nanofiltered C1 inhibitor concentrate for treatment of hereditary angioedema." N Engl J Med **363**(6): 513-522.

7 SUPPLEMENTAL MATERIAL

7.1 Protocols

7.1.1 Cryoembedding with sucrose treatment

Sacrificing the animal and perfusion

1. Prepare the surgery and make sure everything needed is within reach.
2. Initiate deep anaesthesia and wait until reflexes can't be triggered any more.
3. Bleed the animal through the venous plexus behind the eye using a glass capillary. Catch the blood in an Eppendorf tube with coagulation activators (e.g. kaolin)
4. Kill the animal with cervical dislocation.
5. Make a cut from caudal to cranial to open the abdomen and the chest. Expose the heart.
6. Perfuse the animal with 1x PBS. Liver should change colour from deep red to soft pink.
7. Perfuse with 4% PFA.
8. Remove the desired organs and transfer them to 4% PFA. Fix for 1-2 h.

Cryoprotection

1. Wash tissue 3x 5 min in 1x PBS with rocking.
2. Transfer tissue to a 30% sucrose solution (w/v) in 1x PBS. Tissue will not sink.
    a. 150g in 500ml 1x PBS. Mix until completely dissolved. Store at 4°C.
3. Place the tissue in the fridge at 4°C overnight or until the tissue sank completely (approx. 6h).

Embedding and freezing

1. Label cryomolds.
2. Fill a Styrofoam box with dry ice and place a steel or hard plastic box in it.
3. Place some dry ice pellets in the box and slowly add isopentane. Perform this step under a fume hood.
4. Fill the bottom of the cryomold with OCT.
5. Transfer the tissue to the cryomold and orient it the desired way. Chilled OCT is more viscose and helps to hold the tissue in place.
6. Fill the cryomold completely with OCT. Try to minimize bubbles.
7. Immerse the cryomold into the isopentane and wait until the OCT is completely frozen.
8. Frozen tissue should be stored at -80°C, wrapped with plastic foil and placed in plastic bag to prevent drying.
9. Section and stain as soon as possible.

Sectioning

1. Equilibrate cryoblocks to -20°C for at least 30 min prior to sectioning.
2. Make the desired amount of sections (see NX70 Cryostar Thermoscientific manual).
3. Air dry cryosections at RT for 30 min.
4. Store slides at -80°C. Put the box in a plastic bag with silica gel.
5. Before using the slides let them thaw in another plastic bag with silica gel for at least 30 min at RT.

7.1.2 Immunofluorescent staining on cryosections

Sample preparation

1. Thaw frozen slides in a sealed plastic bag to room temperature
2. Mark sections with hydrophobic pen (e.g. Dako pen)

Fixation

1. Incubate the slides in 2% PFA for 15 min at room temperature
2. Wash the slides 3x 5min in 1x PBS

Antigen Retrieval, if necessary

1. Prepare Antigen-Retrieval Buffer as follows:
2. 4.5 ml solution A + 20.5 ml solution B + Aqua dest. ad 250 ml
    a. Solution A: 0,1 M citric acid (21,01 g citric acid per 1 l dH2O)
    b. Solution B: 0,1 M sodium citrate (29,41 g sodium citrate per 1 l dH2O)
    c. Stock Solutions can be stored at 4°C2.
3. Place a cuvette with the Antigen-Retrieval Buffer in a glass dish filled with water
4. Place the slide in the cuvette and boil up in a microwave oven (takes approx. 10 min)
5. Boil the slides 5x 2 min
6. Take cuvette out of the dish and cool down to room temperature (approx.. 20-30 min)
7. Rinse slides with 1x PBS
8. Wash 3x 5 min in 1x PBS

Blocking

1. Prepare Blocking Buffer: 1x PBS, 1% cold water fish gelatin, 0.1% Triton X-100 (store at -20°C).
2. Incubate the slides in Blocking Buffer for 45min at room temperature
3. Rinse with 1x PBS
4. Wash 2x 5min in 1x PBS

Primary Antibody

1. Dilute primary Antibody and Isotype Control in 1x PBS + 0.05% Tween-20 according to the product information, final concentration e.g. 2 µg/ml
2. Incubate over night at 4°C in a wet chamber
3. Rinse with 1x PBS + 0.05% Tween-20
4. Wash 3x 5min in 1x PBS + 0.05% Tween-20

Secondary Antibody

1. Dilute secondary Antibody in 1x PBS + 0.05% Tween-20: Final Concentration e.g 10 µg/ml.
2. Incubate 60 min at room temperature protected from light
3. Rinse with 1x PBS + 0.05% Tween-20
4. Wash 3x 5min in 1x PBS + 0.05% Tween-20

DNA Staining

1. Dilute DAPI 1:5000 in 1x PBS
2. Incubate slides 2min with diluted DAPI
3. Rinse with 1x PBS
4. Wash 2x 5 min in 1x PBS and 1x 5min in Aqua dest.

Autofluorescence reduction

1. Prepare Sudan Black B solution: 0.1% Sudan Black B in 70% ETOH. Can be stored at room temperature.
2. Incubate slides 20 min.
3. Wash 3x 5 min in 1x PBS and 1x 5min in Aqua dest.

Cover Sections

1. Cover sections with Vectashield and Cover slip
2. Store stained slides protected from light. Image as soon as possible.

7.1.3 Immunofluorescent staining on paraffin sections

Deparaffinisation

1. 1h Xylene
2. 2x 5 min 100% ETOH
3. 5 min 95% ETOH
4. 5 min 80% ETOH
5. 5 min 70% ETOH
6. 5 min 50% ETOH
7. 5 min 30% ETOH
8. 5 min Aqua dest.

Antigen Retrieval

1. Prepare Antigen-Retrieval Buffer as follows:
2. 4.5 ml solution A + 20.5 ml solution B + Aqua dest. ad 250 ml
    a. Solution A: 0,1 M citric acid (21,01 g citric acid per 1 l dH2O)
    b. Solution B: 0,1 M sodium citrate (29,41 g sodium citrate per 1 l dH2O)
    c. Stock Solutions can be stored at 4°C
3. Place a cuvette with the Antigen-Retrieval Buffer in a glass dish filled with water
4. Place the slide in the cuvette and boil up 8 min in a microwave oven
5. Take cuvette out of the dish and cool down 15 min
6. Mark the sections with a hydrophobic pen (e.g. Dako pen)
7. Wash 3x 5 min in 1x PBS

Blocking

1. Prepare Blocking Buffer: 1x PBS, 1% cold water fish gelatin, 0.1% Triton X-100 (store at -20°C).
2. Incubate the slides in Blocking Buffer for 45min at room temperature
3. Rinse with 1x PBS
4. Wash 2x 5min in 1x PBS

Primary Antibody

5. Dilute primary Antibody and Isotype Control in 1x PBS + 0.05% Tween-20 according to the product information, final concentration e.g. 2 µg/ml
6. Incubate over night at 4°C in a wet chamber
7. Rinse with 1x PBS + 0.05% Tween-20
8. Wash 3x 5min in 1x PBS + 0.05% Tween-20

Secondary Antibody

5. Dilute secondary Antibody in 1x PBS + 0.05% Tween-20: Final Concentration e.g 10 µg/ml.

6. Incubate 60 min at room temperature protected from light
7. Rinse with 1x PBS + 0.05% Tween-20
8. Wash 3x 5min in 1x PBS + 0.05% Tween-20

DNA Staining

5. Dilute DAPI 1:5000 in 1x PBS
6. Incubate slides 2min with diluted DAPI
7. Rinse with 1x PBS
8. Wash 2x 5 min in 1x PBS and 1x 5min in Aqua dest.

Autofluorescence reduction

4. Prepare Sudan Black B solution: 0.1% Sudan Black B in 70% ETOH. Can be stored at room temperature.
5. Incubate slides 20 min.
6. Wash 3x 5 min in 1x PBS and 1x 5min in Aqua dest.

Cover Sections

3. Cover sections with Vectashield and Cover slip
4. Store stained slides protected from light. Image as soon as possible.

7.1.4 Double immunofluorescent staining on paraffin sections with two primary antibodies raised in the same species

Deparaffinization

1. 1h Xylene
2. 2x 5 min 100% ETOH
3. 5 min 95% ETOH
4. 5 min 80% ETOH
5. 5 min 70% ETOH
6. 5 min 50% ETOH
7. 5 min 30% ETOH
8. 5 min Aqua dest.

Antigen Retrieval

1. Prepare Antigen-Retrieval Buffer (citrate buffer) as follows:
   4.5 ml solution A + 20.5 ml solution B + Aqua dest. ad 250 ml
   a. Solution A: 0,1 M citric acid (21,01 g citric acid per 1 l dH2O)
   b. Solution B: 0,1 M sodium citrate (29,41 g sodium citrate per 1 l dH2O)
   c. Stock Solutions can be stored at 4°C
2. Place a cuvette with the Antigen-Retrieval Buffer in a glass dish filled with water
3. Place the slide in the cuvette and boil up 8 min in a microwave
4. Take cuvette out of the dish and cool down 15 min
5. Mark the sections with a hydrophobic pen (e.g. Dako pen)
6. Wash 3x 5 min in 1x PBS

Blocking

1. Prepare Blocking Buffer: 1x PBS, 1% cold water fish gelatin, 0.1% Triton X-100 (store at -20°C).
2. Incubate the slides in Blocking Buffer for 45min at room temperature

3. Rinse with 1x PBS
4. Wash 2x 5min in 1x PBS

First primary Antibody

1. Dilute first primary Antibody and Isotype Control in 1x PBS + 0.05% Tween-20 according to the product information, final concentration e.g. 2 µg/ml
2. Incubate over night at 4°C in a wet chamber
3. Rinse with 1x PBS + 0.05% Tween-20
4. Wash 3x 5min in 1x PBS + 0.05% Tween-20

First secondary Antibody

1. Dilute first secondary Antibody in 1x PBS + 0.05% Tween-20: Final Concentration e.g 10 µg/ml.
2. Incubate 60 min at room temperature protected from light
3. Rinse with 1x PBS + 0.05% Tween-20
4. Wash 3x 5min in 1x PBS + 0.05% Tween-20

Microwave treatment

1. Place a cuvette with the Antigen-Retrieval Buffer in a glass dish filled with water
2. Place the slide in the cuvette and boil up 5 min in a microwave
3. Take cuvette out of the dish and cool down 20 min
4. Mark the sections with a hydrophobic pen (e.g. Dako pen)
5. Wash 3x 5 min in 1x PBS

Second primary Antibody

1. Dilute second primary Antibody and Isotype Control in 1x PBS + 0.05% Tween-20 according to the product information, final concentration e.g. 2 µg/ml
2. Incubate over night at 4°C in a wet chamber
3. Rinse with 1x PBS + 0.05% Tween-20
4. Wash 3x 5min in 1x PBS + 0.05% Tween-20

Second secondary Antibody

5. Dilute second secondary Antibody in 1x PBS + 0.05% Tween-20: Final Concentration e.g 10 µg/ml.
6. Incubate 60 min at room temperature protected from light
7. Rinse with 1x PBS + 0.05% Tween-20
8. Wash 3x 5min in 1x PBS + 0.05% Tween-20

DNA Staining

9. Dilute DAPI 1:5000 in 1x PBS
10. Incubate slides 2min with diluted DAPI
11. Rinse with 1x PBS
12. Wash 2x 5 min in 1x PBS and 1x 5min in Aqua dest.

Autofluorescence reduction

7. Prepare Sudan Black B solution: 0.1% Sudan Black B in 70% ETOH. Can be stored at room temperature.
8. Incubate slides 20 min.
9. Wash 3x 5 min in 1x PBS and 1x 5min in Aqua dest.

Cover Sections

5. Cover sections with Vectashield and Cover slip
6. Store stained slides protected from light. Image as soon as possible.

7.1.5 Western blot

Preparation of Running buffer, Transfer buffer, samples and gel.

1. Running buffer: Add 50 ml 20x MES SDS Running Buffer to 950 ml water to prepare 1x Running buffer. For a small gel, 500ml is enough.
2. Transfer Buffer 4L: 800 ml Methanol + 80 ml CAPS fill up to 4L with deionized water. CAPS buffer: 0,5 M pH11; 1L total volume (110,6g to 1L deionized water)
3. Sample preparation: dilute 3 µl of sample in 5 µl Laemmli 4x sample Buffer and 1,5 µl Dithiothreitol and fill up with aqua dest. to 20 µl. Add 1 µl β-mercaptoethanol for reducing conditions. Heat samples at 95°C for 5 min.
4. Gel: Precast 12% SDS-PAGE gels.

Electrophoresis and Removing Gel

1. Prepare the Chamber: Remove the gel cassette and rinse it with deionized water. Insert the XCELL Sure Assembly in its unlocked position. Place the gel cassette on each side and lock by moving the tension lever to the locked position.
2. Fill each chamber (upper and lower chamber) with the Running buffer.
    a. Ensure that the upper buffer chambers are not leaking.
3. Load 10µl sample into each well of the gel. Always add the gel after filling up the chamber with buffer.
4. Run gel at 200V constant; 120mA; 25 W for 1 hour.
5. Remove the gel: After running is complete, separate the gel cassette with the gel knife. Carefully separate the gel from the plates with hands. Cut out the stacking gel with the wells and the bottom part of the gel.

Blotting

1. Put the gel in a dish, containing 1x Transfer buffer.
2. Soak 3 pieces of Blotting filter paper, sponges and blotting membrane in the Transfer buffer.
3. Place 1 piece of the pre-soaked blotting filter paper on the anode plate (black) on a sponge of a semi-dry blotting apparatus.
4. Place the gel on the top of the filter paper and the membrane on top of the gel. Add a filter paper on top. Roll with a roller to remove air bubbles.
5. Fill the blotting apparatus with 1x Transfer buffer and put the cassette in the tank
6. Transfer at constant 400mAor (100V constant) for at least two hour or overnight in the cold room.

Blocking

1. Prepare the blocking solution by weighing 2.5g Milk powder and adding 50 ml TBST.
2. After complete transfer put the membrane in blocking Solution for at least 1 hour.

Primary antibody

1. Take 500ml of blocking solution in two 2ml tubes and make it to 2 ml with TBST.
2. Dilute the ab 1791 Total Histone 1/400 = 5µl Ab to 2ml.

3. Add the antibody dilution to the membrane, seal the membrane in a plastic bag and incubate overnight on an overhead shaker at 4°C.

Secondary antibody

1. Wash the membrane 4x in TBST for 15min. at room temperature on shaker in dishes.
2. Dilute the second antibody (anti rabbit: HRP DiaNova) 1:20.000 (1 µl antibody in 1ml blocking solution+ 49ml TBST).
3. Incubate 1h at room temperature on a shaker.
4. Wash the membrane 4x in TBST for 15 min in dishes /RT on a shaker.

Developing

5. Incubate with ECL Reagent for 1 min.
6. Put the membrane in a cassette for developing.
7. Expose with ECL Hyperfilms and develop in Curix 60 (AGFA HealthCare).

7.2 Antibodies used in and images of immunostainings

Primary antibodies used in immunofluorescent stainings

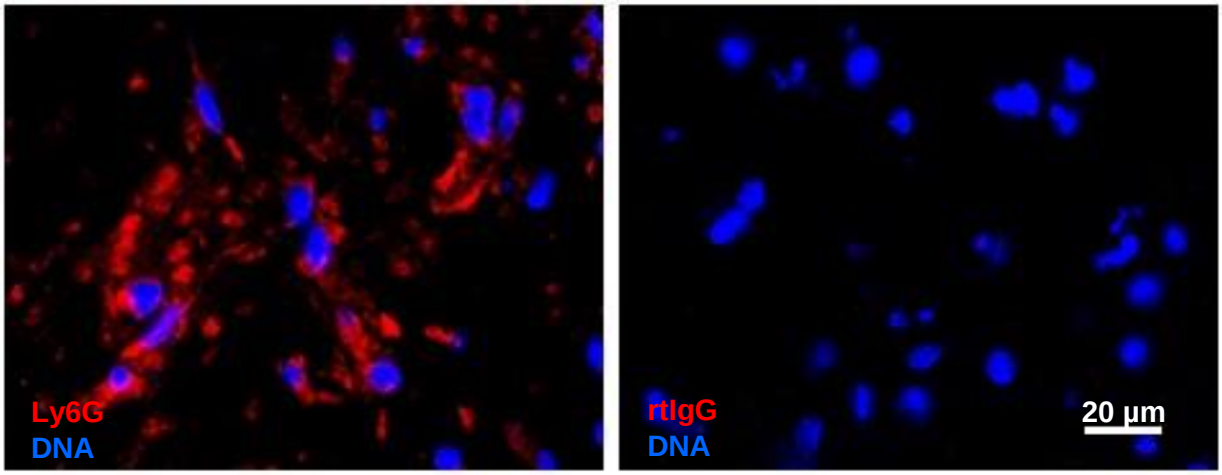
Target	Ly6G	Ly6G/Ly6C	MPO	NE
Marker for:	Neutrophils	Neutrophils	Neutrophils	Neutrophils
Manufacturer (Product Number)	Bio X Cell (BE0075-1)	BioLegend (108401)	Dako (A0398)	abcam (ab68672)
Host	rat	rat	rabbit	rabbit
Clonality	monoclonal	monoclonal	polyclonal	polyclonal
Embedding	paraffin	paraffin	paraffin	paraffin
Antigen retrieval	Heat mediated	Heat mediated	Heat mediated	Heat mediated
1st antibody concentration	2 µg/ml	2 µg/ml	2 µg/ml	1 µg/ml
2nd antibody concentration	10 µg/ml	10 µg/ml	10 µg/ml	10 µg/ml

Target	CRAMP	H3cit	H3	2ab
Marker for:	NETs	NETs	NETs	Histones/DNA
Manufacturer (Product Number)	Innovagen (PA-CRPL-100)	abcam (ab5103)	abcam (ab1791)	in house
Host	rabbit	rabbit	rabbit	mouse
Clonality	polyclonal	polyclonal	polyclonal	**?**
Embedding	paraffin	paraffin	paraffin	paraffin
Antigen retrieval	Heat mediated	Heat mediated	Heat mediated	Heat mediated
1st antibody concentration	1 µg/ml	2 µg/ml	2 µg/ml	2 µg/ml
2nd antibody concentration	10 µg/ml	10 µg/ml	10 µg/ml	10 µg/ml

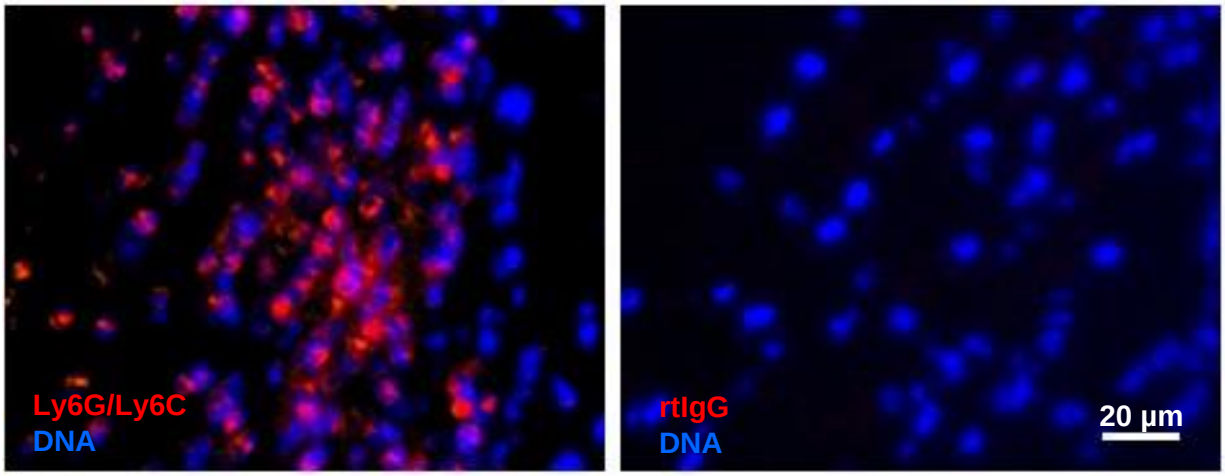
Primary antibodies used in immunofluorescent stainings

Target	ssDNA	vWF	CD41	Fibrin
Marker for:	DNA	Platelets	Platelets	Fibrin
Manufacturer (Product Number)	in house	Dako (A0082)	Biozol (BLD-133901)	in house (59D8)
Host	mouse	rabbit	rat	mouse
Clonality	?	polyclonal	monoclonal	monoclonal
Embedding	paraffin	paraffin	OCT	paraffin
Antigen retrieval	Heat mediated	Heat mediated	-	Heat mediated
1st antibody concentration	2 µg/ml	2 µg/ml	2 µg/ml	2 µg/ml
2nd antibody concentration	10 µg/ml	10 µg/ml	10 µg/ml	10 µg/ml

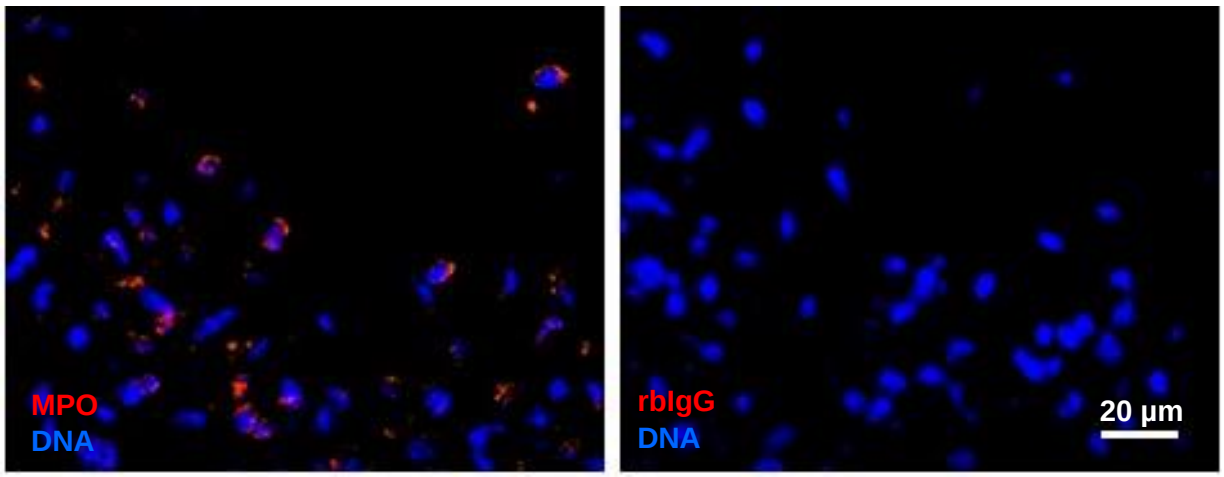
Target	Fibrinogen	Thrombin	F4/80	Active Caspase-3
Marker for:	Fibrinogen/ Fibrin	Thrombin/ Prothrombin	Macrophages	Caspase-3
Manufacturer (Product Number)	Bio-Rad (BSG-4440-8004)	Santa Cruz Biotechnology (sc-16972)	Bio-Rad (MCA497RT)	BD Biosciences (C92-605)
Host	sheep	goat	rat	rabbit
Clonality	polyclonal	polyclonal	monoclonal	monoclonal
Embedding	paraffin	paraffin	paraffin	paraffin
Antigen retrieval	Heat mediated	Heat mediated	Heat mediated	Heat mediated
1st antibody concentration	2 µg/ml	2 µg/ml	2 µg/ml	5 µg/ml
2nd antibody concentration	10 µg/ml	10 µg/ml	10 µg/ml	10 µg/ml



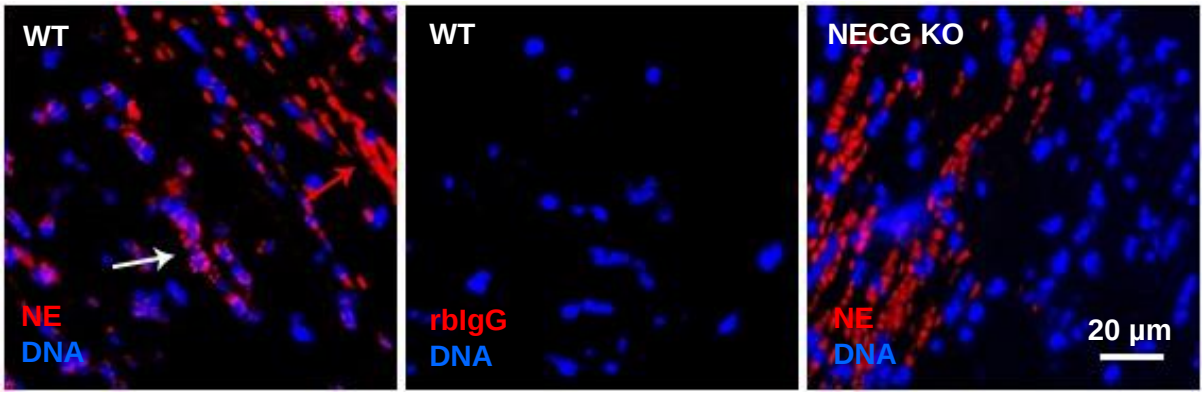
Tissue: paraffin section, murine DVT 7 days after IVC ligation, WT
Antibody: rt-a-Ly6G (Bio X Cell BE0075-1)



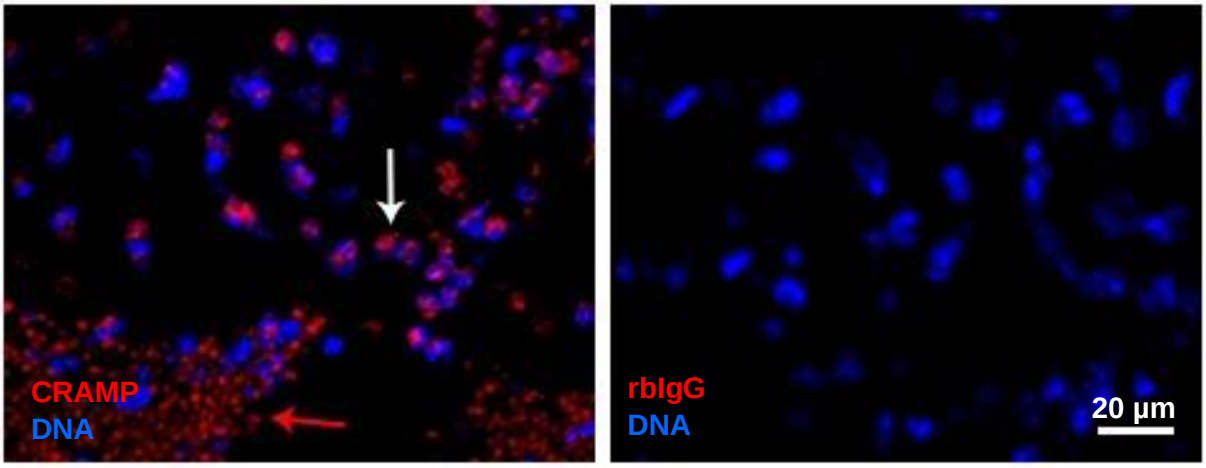
Tissue: paraffin section, murine DVT 2 days after IVC ligation, PAD4 KO
Antibody: rt-a-Ly6G/Ly6C (BioLegend 108401)



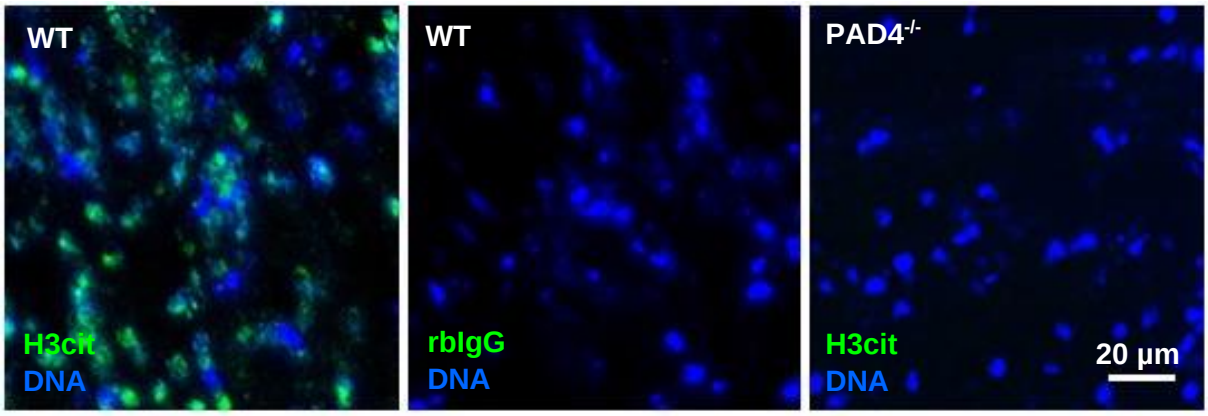
Tissue: paraffin section, murine DVT 2 days after IVC ligation, WT
Antibody: rb-a-MPO (Dako A0398)



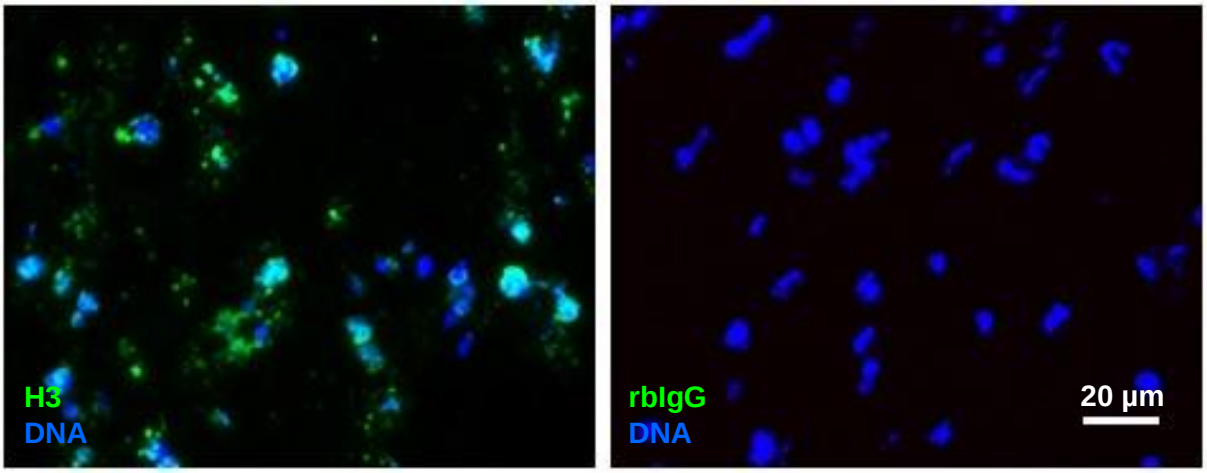
Tissue: paraffin section, murine DVT 2 days after IVC ligation, WT
Antibody: rb-a-NE (Neutrophil Elastase, abcam ab68672)
Red arrow: red blood cells. White arrow: NE.



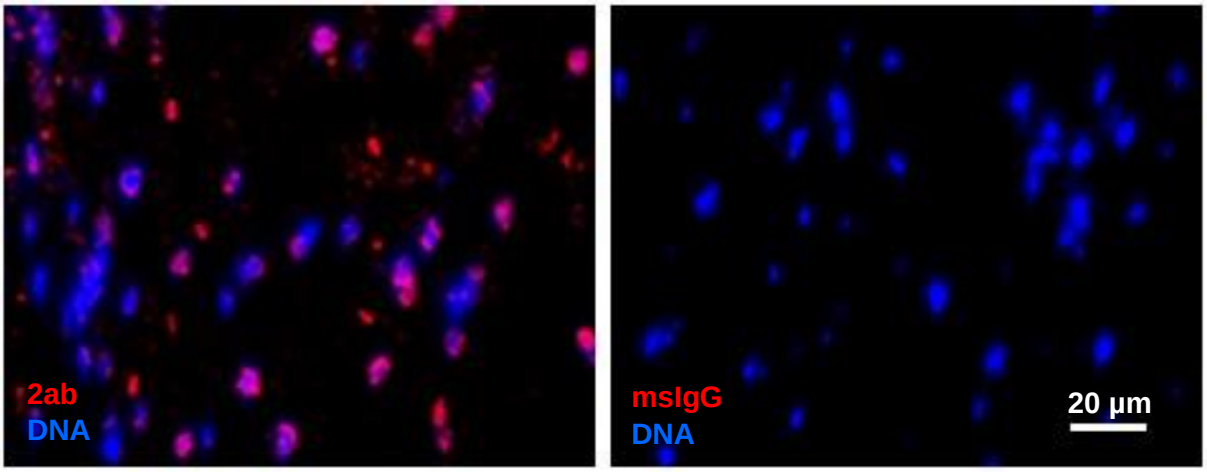
Tissue: paraffin section, murine DVT 2 days after IVC ligation, WT
Antibody: rb-a-CRAMP (Innovagen PA-CRPL-100)
Red arrow: red blood cells. White arrow: CRAMP.



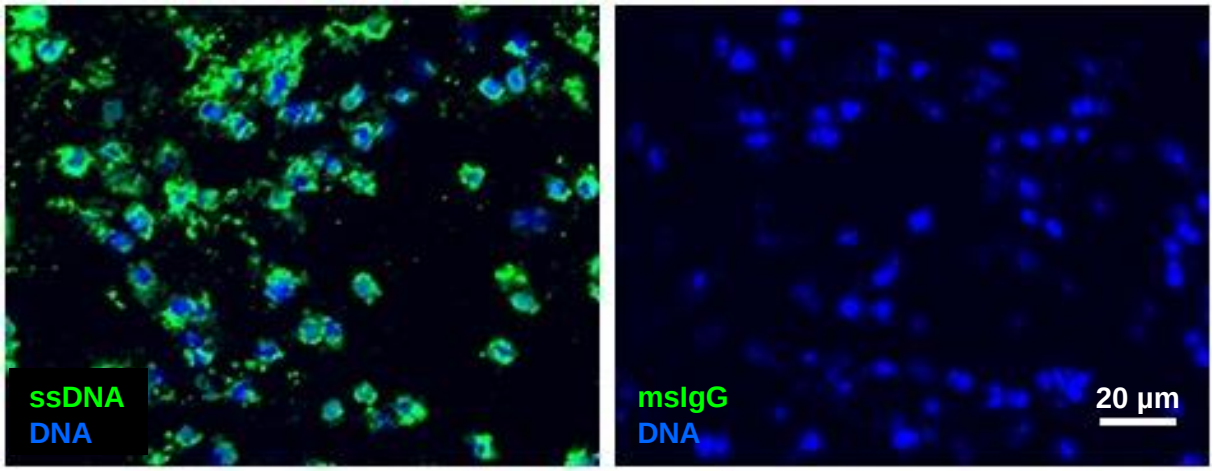
Tissue: paraffin section, murine DVT 2 days after IVC ligation, WT
Antibody: rb-a-H3cit (abcam ab5103)



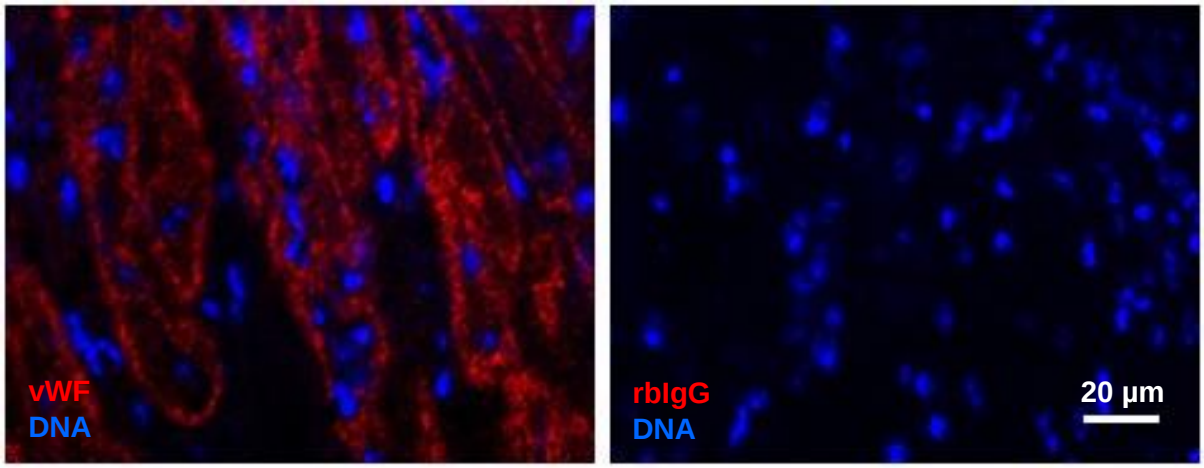
Tissue: paraffin section, murine DVT 2 days after IVC ligation, WT
Antibody: rb-a-H3 (abcam ab1791)



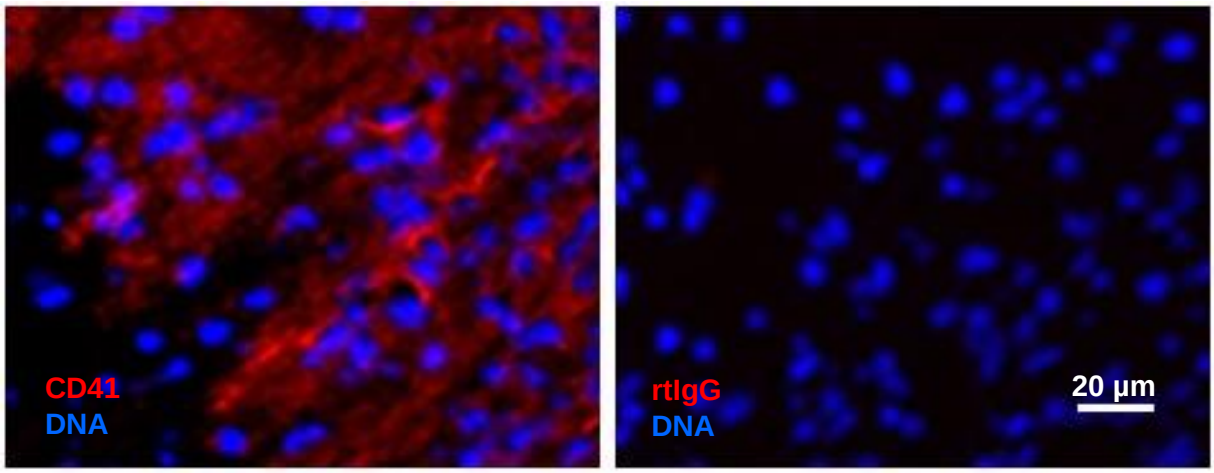
Tissue: paraffin section, murine DVT 2 days after IVC ligation, PAD4 KO
Antibody: ms-a-2ab (in house)



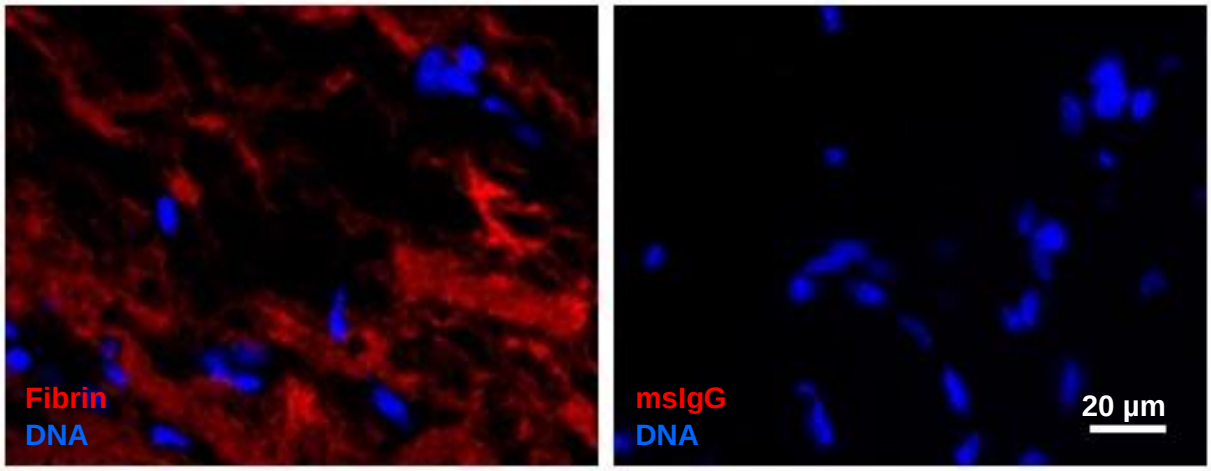
Tissue: murine DVT 2 days after IVC ligation, PAD4 KO
Antibody: ms-a-ssDNA (in house)



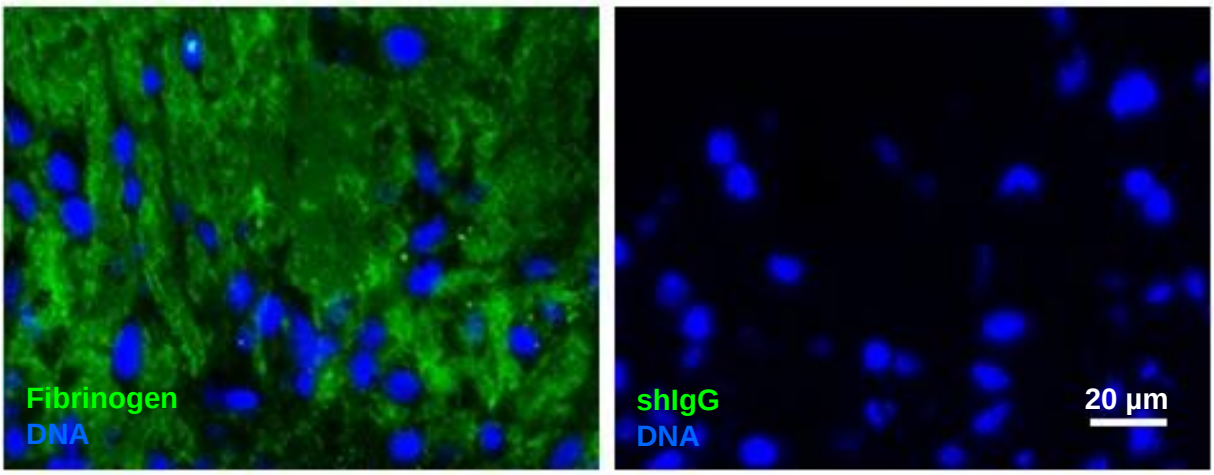
Tissue: paraffin section, murine DVT 2 days after IVC ligation, WT
Antibody: rb-a-vWF (Dako A0082)



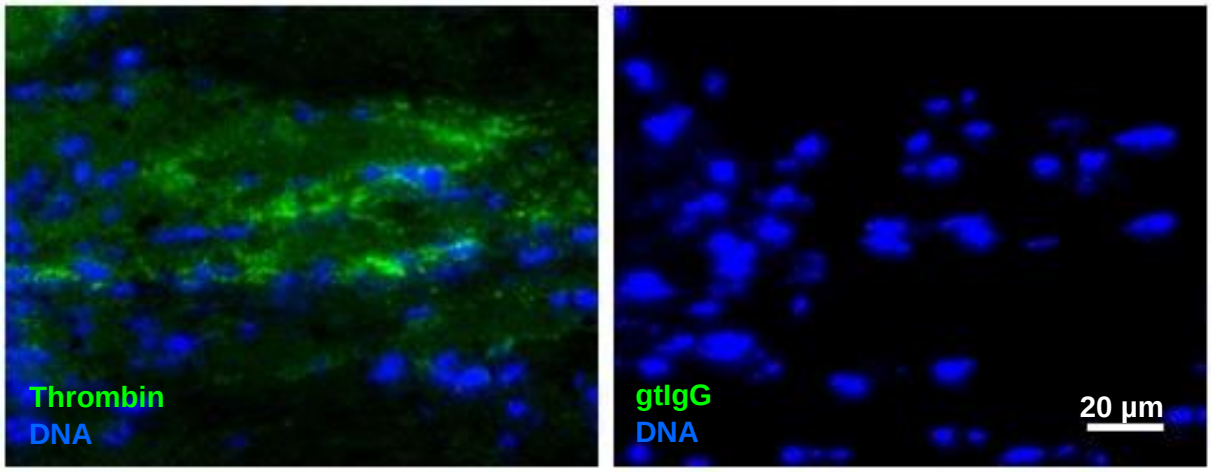
Tissue: cryosection, murine DVT 2 days after IVC ligation, PAD4 KO,
Antibody: rt-a-CD41 (Biozol BLD-133901)



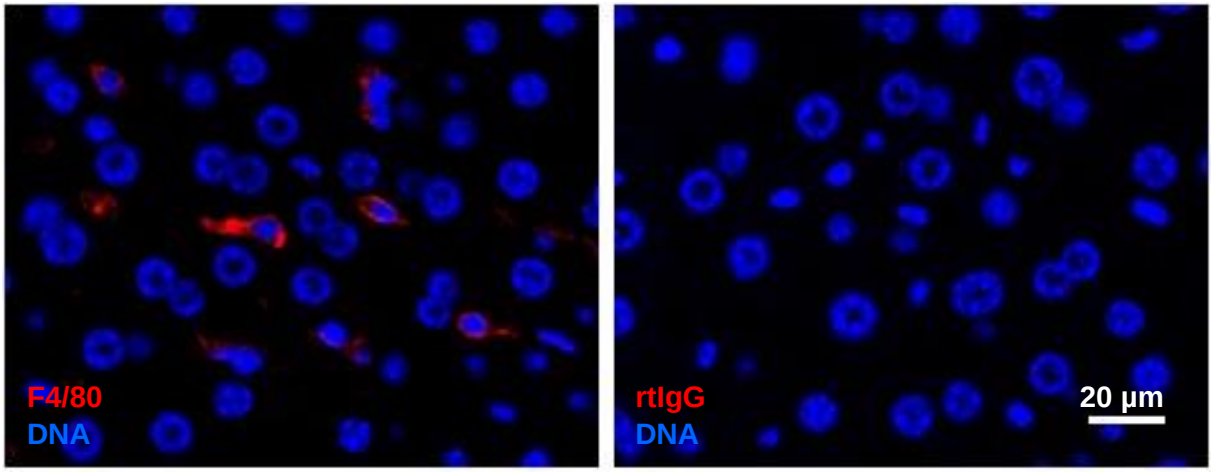
Tissue: paraffin section, DVT 7 days after IVC ligation, WT
Antibody: ms-a-Fibrin (in house)
Staining worked approx. half of the times, weak signal.



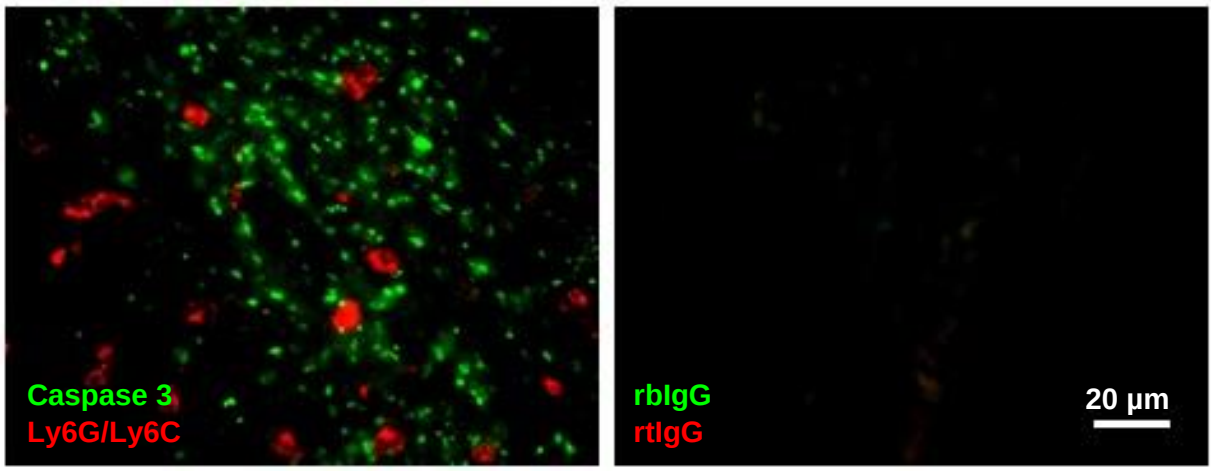
Tissue: paraffin section, murine DVT 7 days after IVC ligation, WT
Antibody: sh-a-Fibrinogen (Bio-Rad BSG-4440-8004)



Tissue: paraffin section, murine DVT 2 days after IVC ligation, WT
Antibody: gt-a-Thrombin (Santa Cruz Biotechnology sc-16972)



Tissue: paraffin section, murine Liver, WT
Antibody: rt-a-F4/80 (Bio-Rad MCA497RT9)

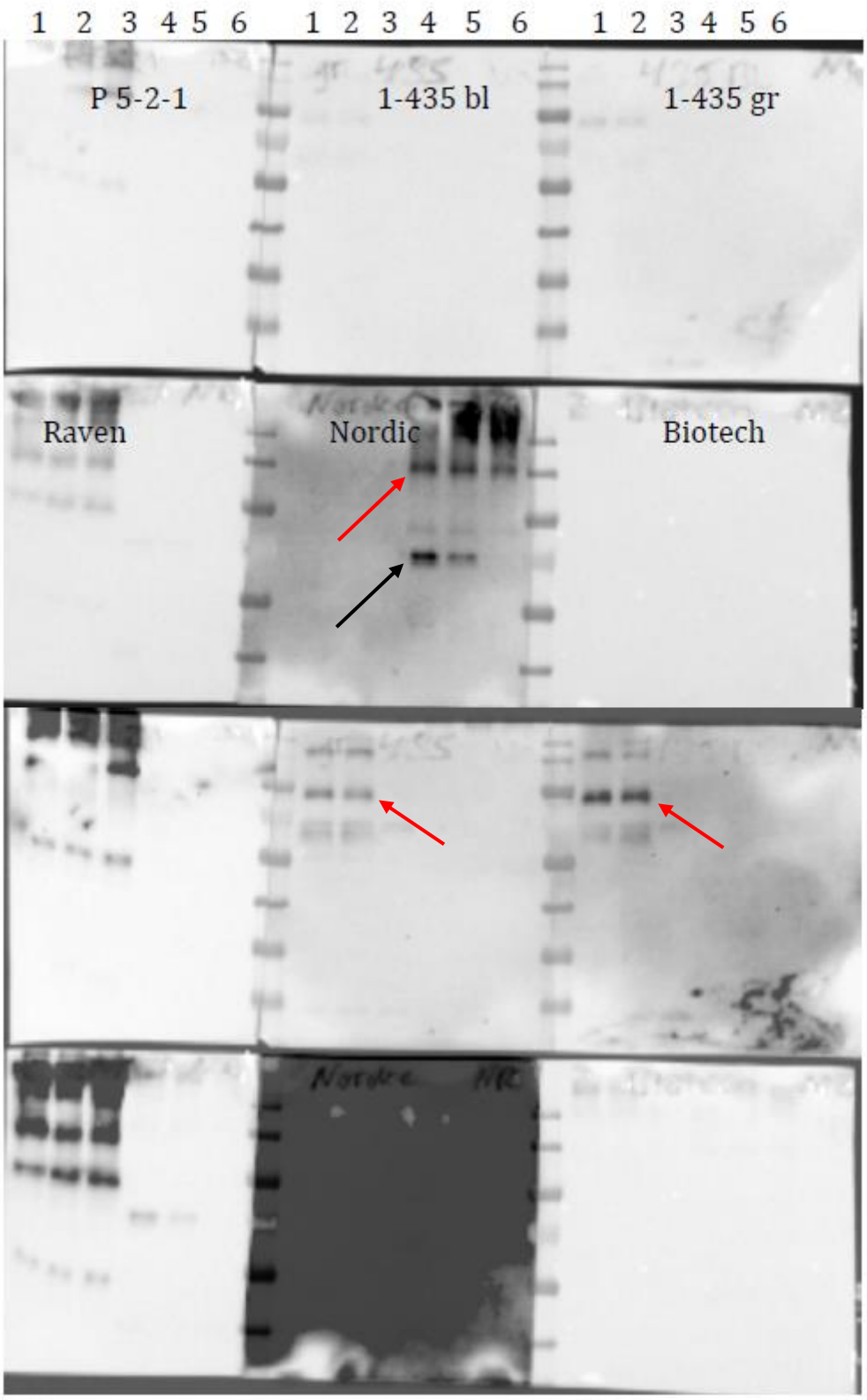


Tissue: paraffin section, murine DVT 2 days after IVC ligation, WT
Antibody: rb-a-Caps3 (Beckton-Dickinson C92-605), rt-a-Ly6G/Ly6C (BioLegend 108401)

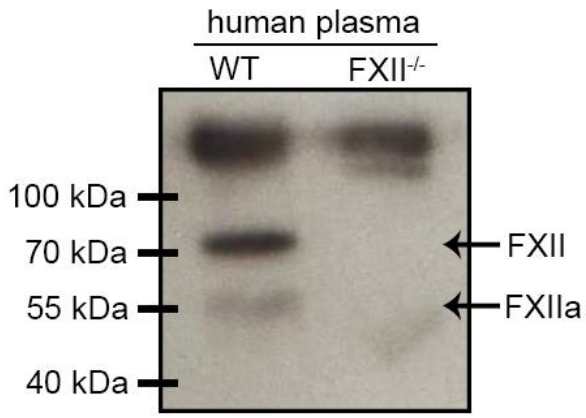
7.3 Western blots for FXII

Antibodies targeting FXII

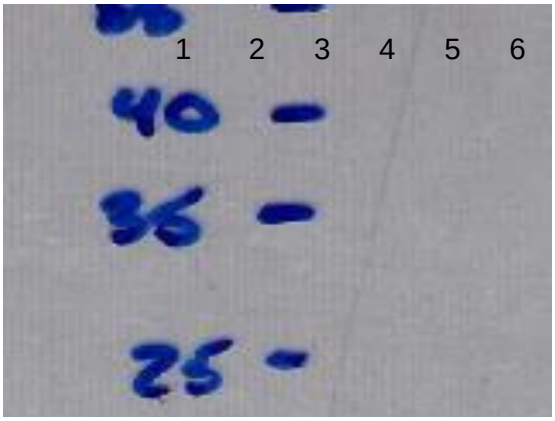
Target	Host	Manufacturer (Product Numer)
FXIIa	human	In house (3F7)
FXIIa	rabbit	Sigma-Aldrich (SAB4500831)
FXII/FXIIa	rabbit	Sigma-Aldrich (HPA003825)
FXII	mouse	In house (P5-2-1)
FXIIa	sheep	Excell Biotech Ltd. (FXII99-4)
FXII/FXIIa	mouse	Ravon D.M. et al. (F1)
FXII/FXIIa	goat	Nordic-Mubio GAHu/FXII)
FXII	rabbit	In house (AB1-435)
FXII/FXIIa	goat	Affinity Biologicals (GAFXII-AP)



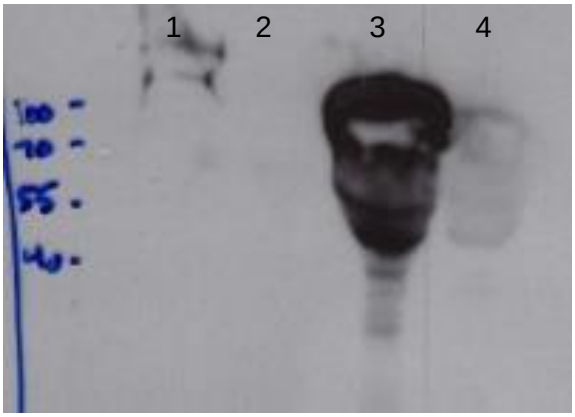
Western blot for FXII with plasma, non-reducing conditions
Markers: 170, 130, 100, 70, 55, 40, 25 kDa
1: mouse WT, 2: mouse WT + DXS, 3: mouse FXII$^{-/-}$
4: human, human + DXS, FXII def. human
Upper half: 11 sec exposure, lower half: 100 sec. exposure
Red arrows: FXII bands, black arrow: FXIIa band



Western blot for FXII with human plasma, non-reducing conditions
Antibody: Sigma-Aldrich (SAB4500831)



Western blot for FXII with human plasma, non-reducing conditions
Antibody: Sigma-Aldrich (HPA003825)
1: purified FXII
2: normal plasma
3: FXII def. plasma
4: plasma + kaolin
5: plasma + polyP
6: purified FXII



Western blot for FXII with plasma, reducing conditions
Antibody: Affinity Biologicals (GAFXII-AP)
1: human
2: human FXII def.
3: murine WT
4: murine FXII-/-

8 PUBLICATION

The Factor XIIa blocking antibody 3F7: a safe anticoagulant with anti-inflammatory activities

Marie Worm[1], Elodie C. Köhler[1], Rachita Panda[1], Andy Long[1], Lynn M. Butler[1,2], Evi X. Stavrou[3], Katrin F. Nickel[1,2], Tobias Fuchs[1,2] & Thomas Renné[1,2]

From the [1]Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [2]Division of Clinical Chemistry, Department of Molecular Medicine and Surgery and Center of Molecular Medicine, Karolinska Institutet and University Hospital, Stockholm, Sweden. [3]Division of Hematology and Oncology, Department of Medicine, Case Western Reserve University, Cleveland, Ohio; Department of Medicine, Louis Stokes Veterans Administration Hospital, Cleveland, Ohio, USA

Correspondence:
Thomas Renné, M.D. Ph.D.
Clinical Chemistry, Department of Molecular Medicine and Surgery
Karolinska Institutet
Karolinska University Hospital Solna (L1:00)
SE-171 76 Stockholm, Sweden
and
Institute of Clinical Chemistry and Laboratory Medicine (O26)
University Medical Center Hamburg-Eppendorf
Martinistrasse 51
D-20246 Hamburg, Germany
Tel.: +46-8-517-73390; +49-40-7410 58984
Fax: +46-310376; +49-40-7410 57576
Email: thomas@renne.net

Published in: Annals of Translational Medicine 2015;3(17):247
Submitted Aug 31, 2015. Accepted for publication Sep 06, 2015.
doi: 10.3978/j.issn.2305-5839.2015.09.07

Abstract
The plasma protein factor XII (FXII) is the initiating protease of the procoagulant and proinflammatory contact system. Factor XII activates both the bradykinin producing kallikrein-kinin system and the intrinsic pathway of coagulation. Contact with negatively charged surfaces induces auto-activation of zymogen FXII that results in activated FXII (FXIIa). Various *in vivo* activators of FXII have been identified including heparin, misfolded protein aggregates, nucleic acids and polyphosphate. Murine models have established a central role of FXII in arterial and venous thromboembolic diseases. Despite the central function of FXII in pathologic thrombosis, its deficiency does not impair hemostasis in animals or humans. The selective role of FXIIa in thrombosis, but not hemostasis, offers an exciting novel strategy for safe anticoagulation based on interference with FXIIa. We have generated the recombinant fully human FXIIa-blocking antibody 3F7, which abolished FXIIa enzymatic activity and prevented thrombosis in a cardiopulmonary bypass system in large animals, in the absence of increased therapy-associated bleeding. Furthermore, 3F7 also interfered with bradykinin-driven edema in the severe swelling disorder hereditary angioedema type III. Taken together, targeting FXIIa with 3F7 appears to be a promising approach to treat edema disorders and thrombosis.

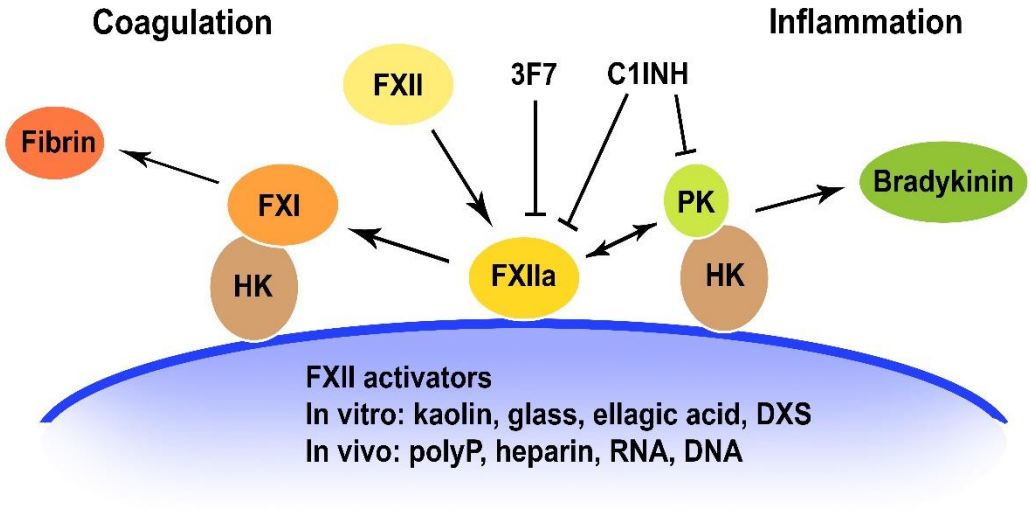


Figure 1. Contact with negatively charged surfaces activates FXII and initiates procoagulant and proinflammatory reactions. Activated FXII starts the fibrin producing intrinsic pathway of coagulation by cleavage of FXI and the inflammatory kallikrein-kinin system by PK mediated cleavage of HK. C1INH interferes both with FXIIa and PK while 3F7 specifically blocks FXIIa only.

The contact system
The contact system is a cascade of five plasma proteins with procoagulant and proinflammatory activities. It consists of the serine proteases factor XII (FXII, Hageman factor), factor XI (FXI), plasma kallikrein (PK), the non-enzymatic cofactor high molecular weight kininogen (HK) and C1 esterase inhibitor (C1INH). Activated FXII initiates the contact system that drives the intrinsic pathway of coagulation and the inflammatory kallikrein-kinin system leading to fibrin and bradykinin (BK) formation, respectively [1, 2]. FXII zymogen is primarily released by hepatocytes and circulates in plasma as a single chain protein [3]. FXII binding to negatively charged surfaces induces a conformational change. Surface bound FXII activates itself by limited proteolysis of the single peptide-bond Arg_{353}-Val_{354} (auto-activation), leading to activated FXII (FXIIa), a two chain serine protease. A single disulfide bond in each FXIIa molecule connects the heavy chain to the light chain. FXIIa cleaves its substrate FXI to generate activated FXI (FXIa) and plasma prekallikrein to PK. PK reciprocally activates FXII,

leading to amplification of the process [4]. Two further cleavage steps commencing at residues 334 and 343 release the light chain from the heavy chain that remains surface bound [5]. The contact system proteins locally assemble on surfaces of various cardiovascular cells. While FXII binds directly to surfaces, HK is required to bind FXI and PK [6, 7]. C1INH is the principle endogenous inhibitor of FXIIa and PK and regulates the enzymatic activity of the contact system proteases [8] (Figure 1).

Factor XII in coagulation

Fibrin production by the classic coagulation cascade can be initiated by two distinct mechanisms involving vessel wall (extrinsic) or blood borne (intrinsic) factors. Both pathways lead to the formation of thrombin, a protease that converts fibrinogen to insoluble fibrin. The extrinsic pathway is initiated when tissue factor (TF) is exposed at sites of vascular injury and binds to circulating coagulation factor VII (FVII) [9]. In contrast, the intrinsic pathway is initiated by contact mediated activation of FXII and subsequent cleavage of FXI [10].

A commonly used diagnostic coagulation test, the activated partial thromboplastin time (aPTT), is based on FXII-mediated contact activation. It is used to monitor coagulation in patients treated with heparin, it is prolonged in the presence of lupus anticoagulant and in inherited or acquired deficiencies in contact system proteins. Despite its high relevance *in vitro*, FXII was considered to have no function for coagulation *in vivo*. In contrast to all other coagulation proteases, deficiency in FXII in humans does not lead to any bleeding disorder, despite a marked prolongation of the aPTT [11, 12]. Studies on FXII deficient ($FXII^{-/-}$) mice revealed an essential role of FXII in thrombosis. $FXII^{-/-}$ mice were largely protected from vessel occlusive thrombus formation in venous and arterial vascular beds in response to chemical or mechanical endothelial injury [13] I would add ref 14 & 15 and skip the next sentence; it reflects venous and arterial thrombosis[13], [14] [15]. Like their human counterparts $FXII^{-/-}$ mice have a normal hemostatic capacity but a prolonged aPTT [16]. Substitution of $FXII^{-/-}$ mice with human FXII normalized the prolonged aPTT and restored defective thrombus formation [13], indicating that FXII operates similarly in mice and humans. In addition to triggering fibrin production, direct binding of FXIIa to fibrin(ogen) modulates the fibrin clot structure leading to increased fiber density and reduced fibrin meshwork pore size [17].

FXII in hereditary angioedema

The rare inherited swelling disorder hereditary angioedema (HAE) is caused by excessive BK signalling [8]. HAE is characterized by acute swelling attacks involving the skin, the oropharyngeal, laryngeal or gastrointestinal mucosa. Swelling events can be life-threatening due to development of laryngeal edema and airway compromise[18]. The classic HAE types 1 and 2 (HAE I and HAE II) are caused by deficiencies of C1INH, the major plasma inhibitor of FXIIa and PK. HAE I and HAE II result due to deficiency in or functional loss of C1INH and hence, these HAE types can be treated by infusion of functional C1INH [8].

A third variant of HAE exists and despite the fact that patients suffer from similar symptoms as HAE types I and II, normal plasma levels of a fully functional C1INH are found in HAE type III patients [19]. HAE III is associated with a single missense mutation in the *F12* gene leading to an amino acid exchange, Thr309Lys [20]. It was subsequently shown that Thr309 could also be mutated to an Arginine [21]. The underlying mechanism of HAE III has been enigmatic for over a decade; recently defective glycosylation in the two mutated FXII forms was identified. Contact activation is largely increased in mutated FXII variants, leading to excess BK formation in patient plasma. Both activated mast cell and contact-triggered edema was largely increased in $FXII^{-/-}$ mice infused with recombinant FXII_Thr309Lys or FXII_Thr309Arg and a novel transgenic mouse with inducible expression of human FXII_Thr309Lys. Together, these data show that loss of glycosylation leads to an increased contact-induced activation of Thr309-mutated FXII that triggers excessive BK formation. Of note, aPTT and thrombus formation were normal in HAE III patient plasma and HAE III mouse models [22].

FXII activators

Binding to negatively charged surfaces activates zymogen FXII. Various *in vitro* activators of FXII have been identified such as the white clay material kaolin that is used in diagnostic assays [4]. Other non-physiologic activators include glass or ellagic acid and synthetic phospholipid micelles decorated with bivalent cations [23]. Moreover, exposure of blood to polymer surfaces exposed in medical devices can lead to FXII activation. Clinical procedures such as hemodialysis and extracorporeal circulation that expose blood to large non-biologic surfaces trigger FXIIa generation and thus, are associated with a high thrombotic risk requiring anticoagulant treatment [24]. The synthetic polysaccharide high molecular weight dextran sulphate (DXS) is commonly used in experimental settings to activate FXII. It exclusively activates the kallikrein-kinin system without interfering with coagulation [2]. Similarly to DXS, the mast cell-derived polysaccharide heparin has the ability to trigger contact system activation. IgE/antigen activated mast cells release heparin in allergic disease animal models leading to BK formation without causing increased coagulation [25]. Consistently, activated mast cell-driven BK formation via the contact system critically contributes to anaphylaxis and possibly other allergic diseases[26]. Insoluble misfolded aggregated proteins selectively activate the kallikrein-kinin system without triggering coagulation [27] and BK formed by this mechanism contributes to inflammatory reactions seen in Alzheimer disease [28]. Extracellular RNA and DNA have been identified as natural occurring FXII activators that trigger coagulation and cleavage of the phosphoester bonds in the polymer backbone; use of RNAse provided thromboprotection in a murine thrombosis model [29]. Activated neutrophils can undergo a cell death program that leads to the formation of neutrophil extracellular traps (NETs) consisting of nuclear DNA with histones and microbicidal proteins. This process is called NETosis and constitutes a host defence mechanism against microbial pathogens which become trapped in the NETs[30]. The ability of large DNA clusters to activate FXII [31] has been implicated in both macrovascular [32] and microvascular thrombosis [33]. Activated platelets release the inorganic polymer polyphosphate that is stored in platelet dense granules [34]. Synthetic platelet size polyphosphate triggers coagulation in an FXII-dependent manner *in vitro* [35]. Human plasma experiments, thrombosis and edema models in mice revealed that polyphosphate activated FXII *in vivo* and interference with the polyphosphate-FXII pathway interfered with thrombosis and vascular leakage [36]. Supporting the critical role of polyphosphate in FXII activation, inherited deficiency in the polymer delays clotting in platelet rich plasma and thrombosis in Hermansky-Pudlak syndrome patients [37] and inositol hexakisphosphate kinase 1 deficient mice [38], respectively. The polyphosphate-FXII pathway operates independently of TF-driven (extrinsic) coagulation [39]. Recent studies using genetically modified mice and patient plasma have shown a critical role of the polyphosphate-FXII pathway in prostate cancer associated thrombosis. Polyphosphates on cancer cells and derived microparticles potently activate FXII and drive venous thrombosis in *vivo* [40]. Consistently, platelet derived microparticle-driven clotting is critically dependent on FXII suggesting that microparticles from platelets also expose polyphosphate on their surface [41].

Targeting FXII

The fact that FXII is essential for thrombosis but dispensable for hemostasis offers the first safe strategy for anticoagulation. We produced the fully human recombinant antibody 3F7 that specifically binds to the catalytic domain of FXIIa with high affinity and completely inhibits its protease activity. Antibody 3F7 blocks FXIIa-mediated clotting in human plasma and thrombosis in mouse models. We adapted an extracorporeal membrane oxygenation (ECMO) system, used for infant therapy, and applied it to a rabbit model. 3F7 provided thromboprotection as efficiently as heparin however, in sharp contrast to heparin, 3F7 did not increase bleeding [42]. Furthermore, targeting FXIIa with 3F7 interfered with edema in a humanized HAE III mouse model and BK-formation in HAEIII patient plasma [22]. Currently,

treatments for acute edema attacks include infusion of C1INH [43], inhibition of kallikrein with ecallantide (DX-88) [44] and targeting the bradykinin receptor B2R (icatibant) [45]. Inhibition of FXIIa with 3F7 could represent a novel therapeutic strategy both in the prophylaxis and acute edema attack stages of HAE. [22]. Clinical applications of 3F7 require further investigation and will present new possibilities for a novel safe drug to potently interfere with thrombosis and inflammation.

ACKNOWLEDGEMENTS

MW is supported by a stipend of the German Research Society provided to the IRTH of the SFB877. This work was supported in part by grants from Hjärt Lungfonden (20140741), Stockholms läns landsting (ALF, 20140464), Vetenskapsrådet (K2013-65X-21462-04-5), German Research Society (SFB877, TP A11 and SFB841, TP B8), and a European Research Council grant (ERC-StG-2012-311575_F-12) to TR.

References

1. Renne, T., *The procoagulant and proinflammatory plasma contact system.* Semin Immunopathol, 2012. **34**(1): p. 31-41.
2. Renne, T., et al., *In vivo roles of factor XII.* Blood, 2012. **120**(22): p. 4296-303.
3. Stavrou, E. and A.H. Schmaier, *Factor XII: what does it contribute to our understanding of the physiology and pathophysiology of hemostasis & thrombosis.* Thromb Res, 2010. **125**(3): p. 210-5.
4. Maas, C. and T. Renne, *Regulatory mechanisms of the plasma contact system.* Thromb Res, 2012. **129 Suppl 2**: p. S73-6.
5. Kenne, E. and T. Renne, *Factor XII: a drug target for safe interference with thrombosis and inflammation.* Drug Discov Today, 2014.
6. Renne, T., et al., *Characterization of the H-kininogen-binding site on factor XI: a comparison of factor XI and plasma prekallikrein.* J Biol Chem, 2002. **277**(7): p. 4892-9.
7. Renne, T., et al., *Mapping of the discontinuous H-kininogen binding site of plasma prekallikrein. Evidence for a critical role of apple domain-2.* J Biol Chem, 1999. **274**(36): p. 25777-84.
8. Bjorkqvist, J., A. Sala-Cunill, and T. Renne, *Hereditary angioedema: a bradykinin-mediated swelling disorder.* Thromb Haemost, 2013. **109**(3): p. 368-74.
9. Mackman, N., *Tissue-specific hemostasis: role of tissue factor.* J Thromb Haemost, 2008. **6**(2): p. 303-5.
10. Renne, T., et al., *Factor XI deficiency in animal models.* J Thromb Haemost, 2009. **7 Suppl 1**: p. 79-83.
11. Lammle, B., et al., *Thrombo-embolism and bleeding tendency in congenital factor XIi deficiency: A study of 74 subjects from 14 Swiss families.* Thromb. Haemost., 1991. **65**: p. 117-121.
12. Muller, F., D. Gailani, and T. Renne, *Factor XI and XII as antithrombotic targets.* Curr Opin Hematol, 2011. **18**(5): p. 349-55.
13. Renne, T., et al., *Defective thrombus formation in mice lacking coagulation factor XII.* J Exp Med, 2005. **202**(2): p. 271-81.
14. Stavrou, E.X., et al., *Reduced thrombosis in Klkb1-/- mice is mediated by increased Mas receptor, prostacyclin, Sirt1, and KLF4 and decreased tissue factor.* Blood, 2015. **125**(4): p. 710-9.
15. Kleinschnitz, C., et al., *Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis.* J Exp Med, 2006. **203**(3): p. 513-8.
16. Pauer, H.U., et al., *Targeted deletion of murine coagulation factor XII gene-a model for contact phase activation in vivo.* Thromb Haemost, 2004. **92**(3): p. 503-8.

17. Konings, J., et al., *Factor XIIa regulates the structure of the fibrin clot independently of thrombin generation through direct interaction with fibrin.* Blood, 2011. **118**(14): p. 3942-51.
18. Tse, K. and B.L. Zuraw, *Recognizing and managing hereditary angioedema.* Cleve Clin J Med, 2013. **80**(5): p. 297-308.
19. Bork, K., et al., *Hereditary angioedema with normal C1-inhibitor activity in women.* Lancet, 2000. **356**(9225): p. 213-7.
20. Cichon, S., et al., *Increased activity of coagulation factor XII (Hageman factor) causes hereditary angioedema type III.* Am J Hum Genet, 2006. **79**(6): p. 1098-104.
21. Dewald, G. and K. Bork, *Missense mutations in the coagulation factor XII (Hageman factor) gene in hereditary angioedema with normal C1 inhibitor.* Biochem Biophys Res Commun, 2006. **343**(4): p. 1286-9.
22. Bjorkqvist, J., et al., *Defective glycosylation of coagulation factor XII underlies hereditary angioedema type III.* J Clin Invest, 2015. **125**(8): p. 3132-46.
23. Mutch, N.J., E.K. Waters, and J.H. Morrissey, *Immobilized transition metal ions stimulate contact activation and drive factor XII-mediated coagulation.* J Thromb Haemost, 2012. **10**(10): p. 2108-15.
24. Svensson, M., et al., *Activation of FXII during haemodialysis.* Scand J Clin Lab Invest, 1996. **56**(7): p. 649-52.
25. Oschatz, C., et al., *Mast cells increase vascular permeability by heparin-initiated bradykinin formation in vivo.* Immunity, 2011. **34**(2): p. 258-68.
26. Sala-Cunill, A., et al., *Plasma contact system activation drives anaphylaxis in severe mast cell-mediated allergic reactions.* J Allergy Clin Immunol, 2015. **135**(4): p. 1031-43 e6.
27. Maas, C., et al., *Misfolded proteins activate factor XII in humans, leading to kallikrein formation without initiating coagulation.* J Clin Invest, 2008. **118**(9): p. 3208-18.
28. Zamolodchikov, D., et al., *Activation of the factor XII-driven contact system in Alzheimer's disease patient and mouse model plasma.* Proc Natl Acad Sci U S A, 2015. **112**(13): p. 4068-73.
29. Kannemeier, C., et al., *Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation.* Proc Natl Acad Sci U S A, 2007. **104**(15): p. 6388-93.
30. Fuchs, T.A., et al., *Novel cell death program leads to neutrophil extracellular traps.* J Cell Biol, 2007. **176**(2): p. 231-41.
31. Oehmcke, S., M. Morgelin, and H. Herwald, *Activation of the human contact system on neutrophil extracellular traps.* J Innate Immun, 2009. **1**(3): p. 225-30.
32. Fuchs, T.A., et al., *Extracellular DNA traps promote thrombosis.* Proc Natl Acad Sci U S A, 2010. **107**(36): p. 15880-5.
33. Jimenez-Alcazar, M., et al., *Impaired DNase1-mediated degradation of neutrophil extracellular traps is associated with acute thrombotic microangiopathies.* J Thromb Haemost, 2015. **13**(5): p. 732-42.
34. Ruiz, F.A., et al., *Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes.* J Biol Chem, 2004. **279**(43): p. 44250-7.
35. Smith, S.A., et al., *Polyphosphate modulates blood coagulation and fibrinolysis.* Proc Natl Acad Sci U S A, 2006. **103**(4): p. 903-8.
36. Muller, F., et al., *Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo.* Cell, 2009. **139**(6): p. 1143-56.
37. Muller, F. and T. Renne, *Platelet polyphosphates: the nexus of primary and secondary hemostasis.* Scand J Clin Lab Invest, 2011. **71**(2): p. 82-6.
38. Ghosh, S., et al., *Inositol hexakisphosphate kinase 1 maintains hemostasis in mice by regulating platelet polyphosphate levels.* Blood, 2013. **122**(8): p. 1478-86.
39. Nickel, K.F., et al., *Time-dependent degradation and tissue factor addition mask the ability of platelet polyphosphates in activating factor XII-mediated coagulation.* Blood, 2013. **122**(23): p. 3847-9.
40. Nickel, K.F., et al., *The polyphosphate-factor XII pathway drives coagulation in prostate cancer-associated thrombosis.* Blood, 2015. pii: blood-2015-01-622811, in press

41. Kilinc, E., et al., *Factor XII Activation is Essential to Sustain the Procoagulant Effects of Particulate Matter.* J Thromb Haemost, 2011. **9**(7): p. 1359-67.
42. Larsson, M., et al., *A factor XIIa inhibitory antibody provides thromboprotection in extracorporeal circulation without increasing bleeding risk.* Sci Transl Med, 2014. **6**(222): p. 222ra17.
43. Zuraw, B.L., et al., *Nanofiltered C1 inhibitor concentrate for treatment of hereditary angioedema.* N Engl J Med, 2010. **363**(6): p. 513-22.
44. Cicardi, M., et al., *Ecallantide for the treatment of acute attacks in hereditary angioedema.* N Engl J Med, 2010. **363**(6): p. 523-31.
45. Cicardi, M., et al., *Icatibant, a new bradykinin-receptor antagonist, in hereditary angioedema.* N Engl J Med, 2010. **363**(6): p. 532-41.

9 ACKNOWLEDGMENTS

This project was partially funded by a stipend of the integrated research training group of the SFB877 – Proteolysis as a Regulatory Event in Pathophysiology – Christian-Albrechts-Universität zu Kiel.

To my supervising professor Thomas Renné: Thank you for the idea for this project, for helpful discussions, for your patience, and support.

To my supervisor Tobias Fuchs: Thank you for leading me through this project, for giving me advice but also freedom to develop my own ideas, for always answering even the most basic questions and for your patience and support when something didn't work as planned.

To my fellow lab members Andy Long, Rachita Panda, Miguel Jiménez-Alcazar, Luke Hopf, Elodie Köhler, Kristin Klätschke, Josephine Bitterling and Andrea Knoke: Thank you for teaching me all the basics of lab work, for helping and supporting me without asking for anything in return, for always standing together, for being a great team, for some really funny evenings with lots to laugh about and for supporting me in some very difficult times. I wouldn't have finished this project without you.

To Christine Desel and all the other members of the SFB877: Thank you for your support, some very interesting seminars and talks and the chance to present and discuss my project to a group.

To the UKE microscopy imaging facility and mouse pathology facility: Thank you for providing the equipment and materials needed for this project and for helpful advice on imaging and immunostaining.

To my family and friends: Thank you for enduring my endless babbling about immunofluorescence and microscopes, for always being there for me and encouraging me.

To Michael Link: Thank you for your love, for your kindness and forgiveness which gave me strength and courage.

10 CURRICULUM VITAE

Der Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.

11 EIDESSTATTLICHE VERSICHERUNG

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Datum: Unterschrift: