Novel insights into the mechanisms of venous thrombosis

By

Holly Payne

A thesis submitted to the University of Birmingham for the degree of DOCTOR OF PHILOSOPHY

Institute of Cardiovascular Sciences

College of Medical and Dental Sciences

University of Birmingham

July 2017

Abstract

Venous thrombosis is a major health concern, with an annual incidence of ~1 per 1000 adults (Cushman 2007). This includes deep vein thrombosis (DVT) and pulmonary embolism (PE), the fatal consequence of a clot detaching and moving to the lungs, together these diseases are termed venous thromboembolism (VTE). Current treatment options for VTE are often associated with serious side effects and bleeding complications, highlighting the need for more effective prophylaxis. This study therefore aimed to identify new targets to treat DVT, which would not have the associated negative side effects.

This study shows the platelet receptor CLEC-2 (C-type lectin receptor 2) plays an important role in DVT, probably through interaction with podoplanin in the IVC wall, and that lack of CLEC-2 is protective in this disease.

We show other immune cells may also play a role in DVT, and demonstrate that mast cell deficiency is protective in vivo. Furthermore, we suggest that the mast cell constituent responsible for the prothrombotic phenotype is likely to be histamine. Preliminary data also suggests that T-cells may have a protective role in DVT, and that thrombin may be important for the release of neutrophil extracellular traps (NETs) from neutrophils inside a growing thrombus.

Publications arising from this thesis

Payne H, Ponomaryov T, Watson SP, Brill A (2017) Mice with a deficiency in CLEC-2 are protected against deep vein thrombosis. *Blood.* April 6;129(14):2013-2020

Moreau T, Evans AL, Vasquez L, Tijssen MR, Yan Y, Trotter MW, Howard D, Colzani M, Arumugam M, Wu WH, Dalby A, Lampela R, Bouet G, Hobbs CM, Pask DC, **Payne H**, Ponomaryov T, Brill A, Soranzo N, Ouwehand WH, Pedersen RA & Ghevaert C. (2016). Large-scale production of megakaryocytes from human pluripotent stem cells by chemically defined forward programming. *Nature Communications.* 7 Apr. 7:11208

Ponomaryov T, **Payne H**, Fabritz L, Wagner DD, Brill A (2017) Mast cell granular contents are crucial for deep vein thrombosis in mice. *Circulation Research.121(8):941-950*

Payne H, Brill A. (2017) Stenosis of the inferior vena cava as a murine model of deep vein thrombosis. *JoVE.*

Book chapter

Arman M, **Payne H**, Ponomaryov T & Brill A.(2015) Role of Platelets in inflammation. *The Non-Thrombotic Role of Platelets in health and Disease. Intech.* Chapter 3

Acknowledgments

Firstly I would like to thank my supervisor Alex Brill for allowing me to complete this PhD and for all of the help, support and guidance over the past 3-4 years. Also for sharing his expert knowledge, and particularly having patience with teaching me in-vivo techniques. Thanks also to the University of Birmingham who have funded my PhD and allowed me to conduct my research, and the Biomedical Services Unit for all of their support with animal work.

I would also like to thank Steve Watson my second supervisor, for his expert advice and continued support over the duration of my PhD. A special thank you to Steve for supporting and helping me transition to a part-time course, in order to train as a member of the GB hockey team in the lead up to the Rio Olympic games. I will be forever grateful that I was able to continue with my PhD whilst training. Special thanks also to Tanya Ponomaryov for all the help and advice as well as the valuable discussions we have had over the past few years.

I would like to give a huge thank you to all of the members of the Platelet group in Birmingham, for everyone's kindness and support, and also making the lab a great place to work over the years. Particularly to all of my fellow PhD students who have not only helped with work problems, sharing ideas and reagents, but also for making it a fun environment in and out of the lab. Also, I would like to say thank you to everyone I have collaborated with during my PhD.

Outside of the lab I would like to say a huge thank you to all of my family and friends who have been there throughout, and have allowed me to escape from PhD work when needed. Special thanks to all my friends who have provided an outlet from both work and hockey. But most of all a huge thanks you to my parents who have always been so supportive over the years, I definitely would not have been able to do it without you.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

LIST OF TABLES

CHAPTER 1

CHAPTER 2

ABBREVIATIONS

ADP	Adenosine diphosphate
BMDMC	Bone marrow-derived mast cells
BSA	Bovine serum albumin
CLEC-2	C-type lectin-like receptor 2
DVT	Deep vein thrombosis
ELISA	Enzyme-linked immunosorbent assay
FcεRI	Immunoglobulin E receptor I
FcRγ-chain	Fc receptor common γ-chain
GPCRs	G protein-coupled receptors
GPVI	Glycoprotein VI
ICAM-1	Intercellular Adhesion Molecule 1
ITAM	Immunoreceptor tyrosine-based activation
IVC	inferior vena cava
LAT	linker for activation of T-cells
LPS	Lipopolysaccharide
MC	Mast cells
NETs	Neutrophil extracellular traps
PARs	Protease-activated receptors
PE	Pulmonary embolism
PF4	platelet factor 4
PRP	platelet rich plasma
RA	Rheumatoid arthritis
RBCs	Red blood cells

SCF	Stem Cell Factor
sP-selectin	Soluble P-selectin
TNF-α	Tumor Necrosis Factor α
tPA	Tissue Plasminogen Activator
TxA_2	Thromboxane A2
VTE	venous thromboembolism
vWF	von Willebrand Factor
WPB	Weibel-Palade Body
WT	wild type

Chapter 1
General Introduction

1.1 Blood composition

Blood has four major components; plasma, which makes up about 55% of the mixture, the other 45% being red blood cells, white blood cells and platelets. Plasma, the liquid component of the blood is important for transporting blood cells throughout the body (American Society of Hematology). It also has a crucial role in maintaining the body's fluid balance and transports a complex mixture of solutes including clotting proteins, waste products, and antibodies. Blood has a number of important functions including the transport of oxygen and nutrients to the lungs and tissues, the transport of immune cells to fight infection, as well as forming clots to prevent excessive blood loss.

1.1.1 Erythrocytes

Erythrocytes or red blood cells (RBCs) are the most abundant cells in the blood making up ~40% of the volume. Their production is controlled by erythropoietin, a hormone produced in the kidneys. They originate as immature cells in the bone marrow and go through 8 stages of differentiation before being released into the blood stream at stage 7 of this process. They begin as pluripotent haematopoietic stem cells, before differentiating into common myeloid progenitor cells and have then become committed to their developmental pathway. Early erythroblasts form and this is where ribosome synthesis occurs. These cells further differentiate into late erythroblasts and normoblasts, where haemoglobin begins to accumulate. Finally the cell's nucleus is ejected and they are released into the blood stream as reticulocytes. In the blood stream these reticulocytes mature into full-blown erythrocytes.

They are characterized by their biconcave disc shape as they have no nucleus, and are ~7um in diameter. This shape allows RBCs to be more flexible and fit through the capillaries of the body. Red cells contain haemoglobin, a molecule that binds to oxygen in the lungs allowing it to be transported around the body to metabolizing tissues; it also binds to carbon dioxide and returns this to the lungs to be exhaled.

1.1.2 Granulocytes

Granulocytes are types of white blood cells, and immune cells, which as their name suggests, contain small granules with enzymes that are released upon infection or during allergic reactions. Granulocyte populations are made of neutrophils, basophils and eosinophils.

Eosinophils and basophils are the least common of the granulocytes, and represent only ~1-5% and 1% of the circulating white blood cells respectively. Basophils contain both heparin and histamine and are important inflammatory cells in response to allergic reactions. Eosinophils may also act as antigen presenting cells allowing them to activate the adaptive immune system, although this role is still under debate (Rothenberg 2006).

Neutrophils are the most abundant type of white blood cells. They are characterized by their classical lobed nucleus and are and essential part of the innate immune system. They respond rapidly to inflammation and also act as phagocytic cells. More recently a novel mechanism for neutrophils has been proposed which may potentiate thrombosis. They are able to form neutrophil extracellular traps (NETs), which are extracellular chromatin fibers bound to histones and granular antimicrobial proteins. Neutrophil death through

liberation of NETs is termed NETosis. The concept of 'immunothrombosis' has recently emerged and it is now known that during thrombus formation in vessels, neutrophils contribute to generating an intravascular scaffold, which may function both to support thrombus growth and to trap/destroy pathogens (Brill et al 2012; Brinkmann et al 2004; Engelmann et al 2013).

The signaling pathways that lead to NETosis have been investigated and certain mechanisms have been put forward, however they are still largely unknown and it has not yet been possible to visualize this process happening in real time. NETs are released through a cell death pathway, which is dependent on the production of Reactive Oxygen Species (ROS). ROS production causes delobulation of the nucleus and break down of the nuclear membrane. This allows the chromatin to mix with the granular proteins before the plasma membrane finally ruptures (Brinkmann et al 2009). Neutrophils express high nuclear levels of the enzyme peptidylarginine

deiminase 4 (PAD4) and this catalyzes citrullination of histones (replaces positively charged arginine for uncharged citrulline). It is hypercitrullination of histones by PAD4 that mediates chromatin decondensation and further NET formation (Wang et al 2012). It was also shown by Wang et al that inhibition of PAD4 decreased the formation of NETs, and so histone citrullination is used as a marker of NET formation. Figure 1.1 below shows the current signaling pathways believed to lead to NETosis.

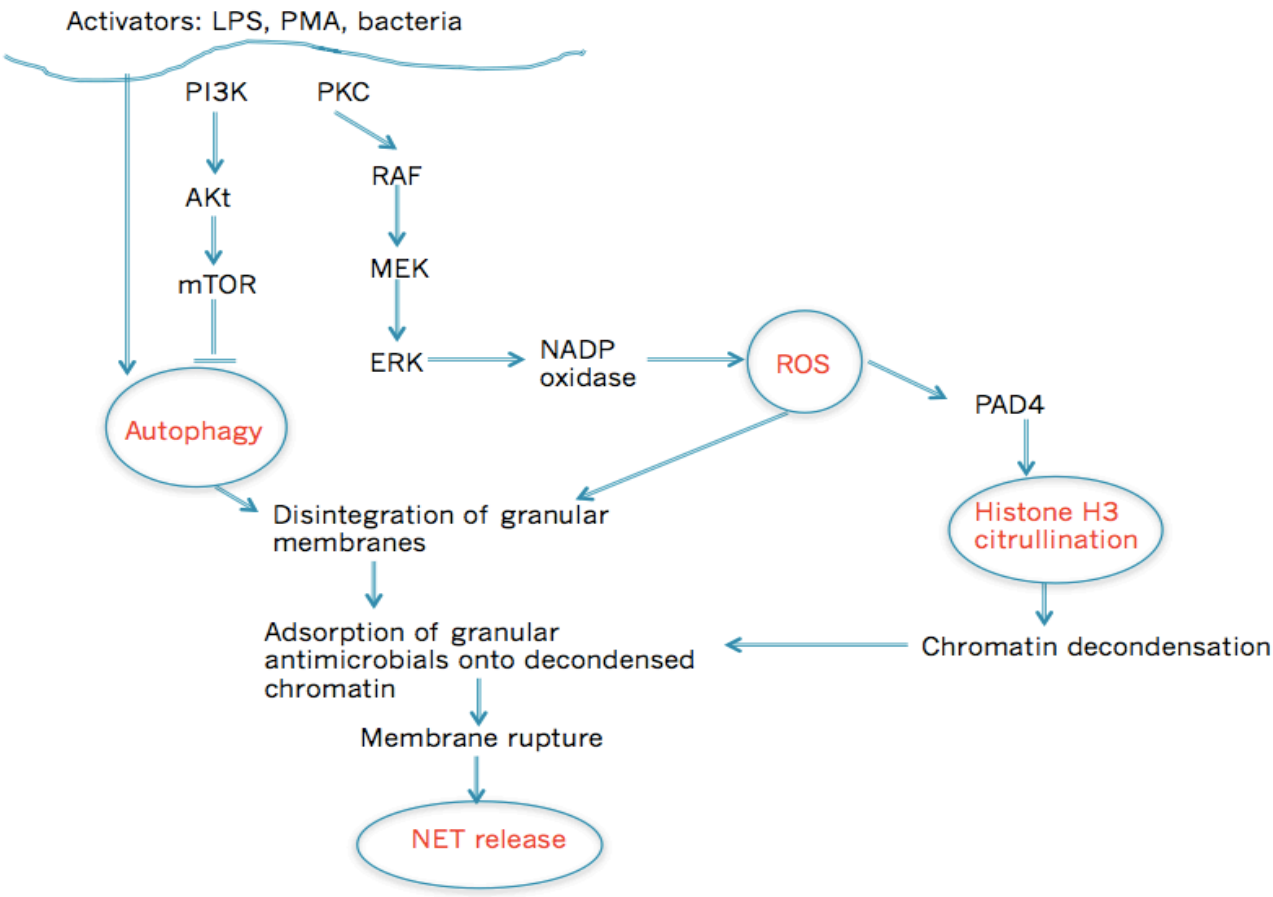


Figure 1.1 – NETosis signaling pathway

Upon neutrophil activation, the RAF-MEK-ERK pathway is necessary to lead to ROS production, this leads to disintegration of the granular membranes and activates the enzyme peptidylarginine deiminase 4 to citrullinate histone H3, further leading to chromatin decondensation. Following membrane rupture neutrophil extracellular traps (NETs) are then released.

PI3K: Phosphoinositide 3-Kinase, AKT: Protein Kinase B, mTOR: The mammalian Target of Rapamycin, PKC: Protein Kinase C, RAF: Rapidly Accelerated Fibrosarcoma, MEK: Mitogen-activated protein kinase, ERK: Extracellular signal-regulated kinases.

There has been controversy over whether the release of NETs is an active process in order to act a host defense mechanism, or if it is simply just cell rupture in response to trauma or infection. Yipp and Kubes (2013) describe both of these types or cell death as distinct pathways, named either vital or suicidal NETosis.

Vital NETosis (see figure 1.2 below) allows neutrophils to maintain their conventional host defense functions. This form of cell death occurs following direct microbial exposure, NETs are then released by vesicles or nuclear budding which allows the outer neutrophil membrane to remain intact, allowing them to carry out their normal function. Gram-positive bacteria activate neutrophils to release NETs by binding to complement receptor 3 (CL3) and TLR2. Whereas gram-negative bacteria induce NET release by activation of TLR4 on platelets followed by neutrophil-platelet interaction via CD11a.

In contrast, suicidal NETosis occurs through activation of PKC and the RAF-MEK-ERK pathway (see figure 1.1). Elastase is then translocated into the nucleus by NADPH from cytosolic granules, where it breaks down the chromatin. Myloperoxidase (MPO) is then also involved in chromatin decondensation and is needed for nuclear envelope breakdown. After NETs have formed inside the cell the outer membrane entirely ruptures and the NET is released so the cell no longer remains functional.

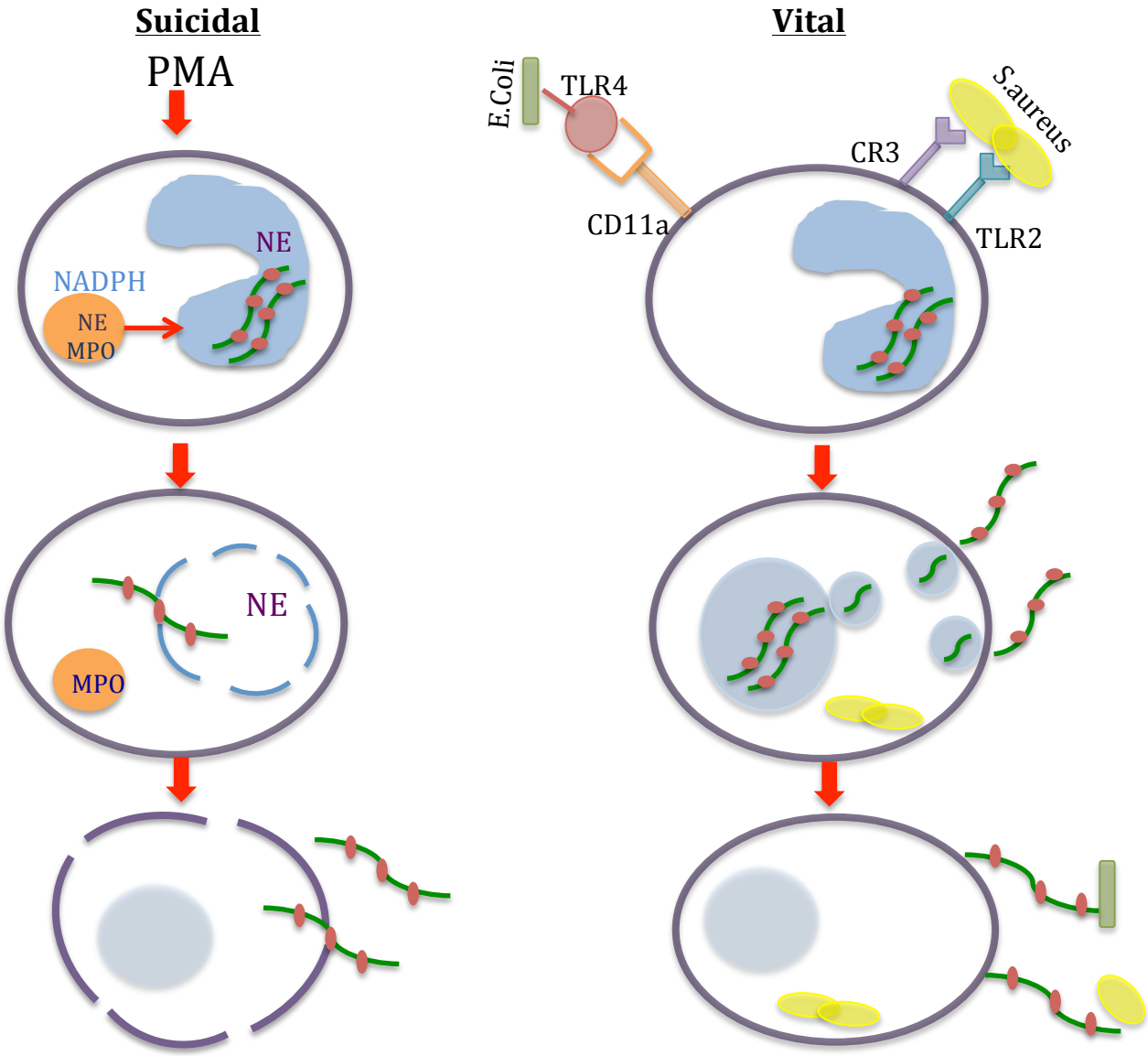


Figure 1.2 – Vital and suicidal NETosis pathways

During suicidal NETosis NADPH translocates elastase into the nuclease where it breaks down chromatin. Myeloperoxidase (MPO) is then involved in nuclear envelope breakdown and finally the entire membrane ruptures to release NETs leaving the cell nonfunctional. In Vital NETosis, direct bacterial exposure leads to signaling via both TLR2 and CR3 (gram positive bacteria), or via TLR4 on platelets followed by CD11a on neutrophils (gram negative bacteria). NETs are then released via nuclear budding and vesicles, leaving the cell intact and functional.

Recent evidence suggests that NETs may also promote thrombosis. Fuchs et al (2010) showed that blood perfusion over NETs leads to platelet adhesion, aggregation and activation. Destruction of the NET structures by DNase prevented platelet accumulation. NETs are likely to play a role in DVT. In a murine model of DVT, it has been shown that extracellular chromatin (most probably NETs) is a structure in a venous thrombus, and that both this scaffold and the histones attached to it contribute to DVT in mice (Brill et al 2012; Von Bruhl et al 2012).

1.1.3 Lymphocytes

There are 3 types of lymphocytes; natural killer cells (NK cells), T-cells and B-cells.

T and B cells are part of the acquired or 'adaptive' immune system, and have a large range of receptors that are able to recognize and mount an immune response to specific pathogens. This adaptive response also creates an immunological memory, meaning if the same pathogen is encountered again, memory cells are able to respond quicker, providing long lasting immunity.

T- progenitor cells originate in the bone marrow; from there they migrate to the thymus (thymocytes) to mature into T-cells. There are two types of T-cells; cytotoxic T-cells which express CD8+, and helper T-cells that express CD4+.

All cells express major histocompatibility complex (MHC) class I receptors. MHC class I receptors are used to present the foreign antigen from infected cells to the specific CD8+ T cell receptors. Upon recognition of the antigen peptide, the T-cell is activated and destroys the infected cell.

T-helper cells are able to produce cytokines to direct the immune response and act as a cross talk between the innate and acquired immune system. T- helper cells recognize specific Antigens presented by antigen presenting cells (phagocytes, dendritic cells and macrophages) via MHC class II receptors. The T-cells also express a CD28 receptor, which simultaneously binds to CD80 or CD86 on the antigen-presenting cell. This leads to activation of the CD4+ T-cell and causes differentiation into an effector cell (see figure 1.3).

These effector CD4+ cells via their CD40L are able to bind to B-cell CD40 receptors, activating them into either plasma cells or memory cells. B cells are produced in the bone marrow but mature in the spleen. Once activated, they differentiate into plasma cells, which are able to produce a range of antibodies. Recognizing antigens directly through their own B-cell receptor can also activate B- cells.

Innate immune responses are rapid, and non-specific to particular pathogens in the way the adaptive response, but instead provide a generic response that is not long lasting. The innate leukocytes include mast cells, eosinophils and basophils, and the innate phagocytic cells include neutrophils, macrophages and dendritic cells.

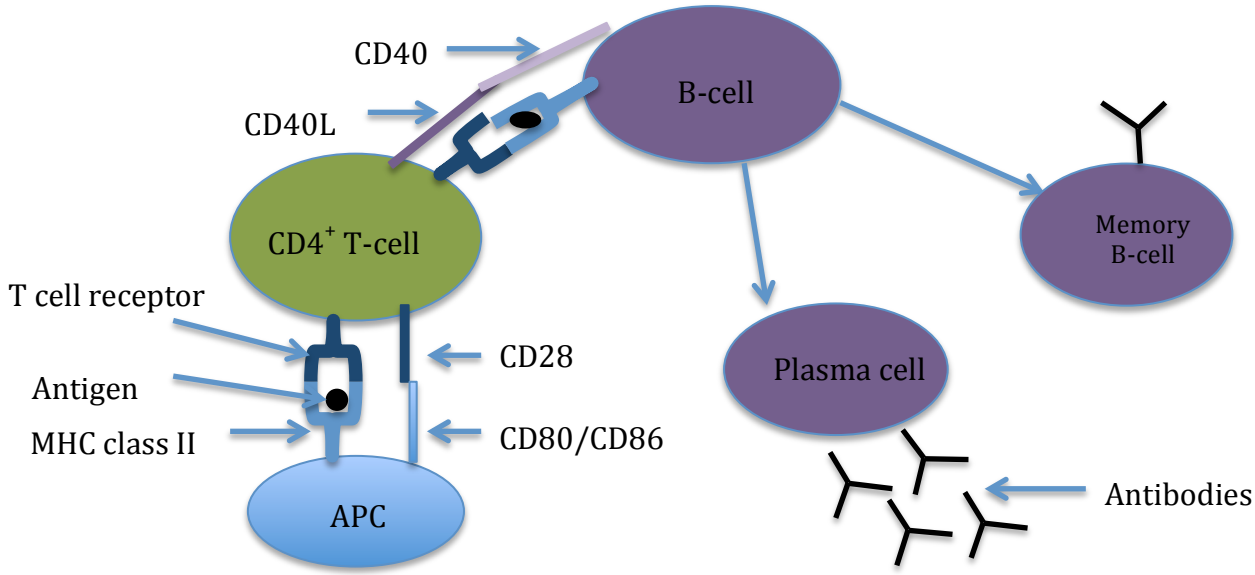


Figure 1.3 –T-helper cell and B-cell signaling

T-helper (CD4+) cells recognize antigens presented by MHC class II receptors. They also have CD28 receptors that bind to the antigen presenting cells (APC) via CD80 or D86 on the APC. This leads to activation of CD4+ effector cells, which via their CD40L and can bind to B-cell CD40, activating them to become either plasma cells or memory cells.

1.1.4 Mast cells

Mast cells originate from the haematopoetic progenitor in bone marrow and also from a bifunctional basophil-MC lineage progenitor in spleen. They are unique in that they enter the circulation as early progenitor cells rather than end stage cells (Gurish et al 2012). Mast cells express a specific set of antigens including FcεRI (a receptor for immunoglobulin E) and Kit (a receptor for stem cell factor). They are the cells most frequently associated with allergy and anaphylaxis, however mast cells are extremely heterogeneous cells, they are constitutively

present in most tissues, and their function will depend on the location in which they are found.

A unique characteristic of Mast cells is they contain granules. These granules contain potent anticoagulants such as heparin and tissue plasminogen activator (TPA), endothelial activators such as histamine and TNF-α, as well as a number of other enzymes (tryptases and chymases) (Moon et al 2014).

Mast cells have the ability to secrete their granule contents and release this vast array of mediators with a variety of biological functions. Their role in allergic inflammation (a risk factor for deep vein thrombosis) has been well documented (Majoor et al 2013). Mast cells can be activated by a number of different ways, and can rapidly release mediators from their storage granules to induce inflammation. Allergens are able to crosslink with immunoglobulin E (IgE) receptors on mast cells, causing them to degranulate. This response can also be bought about through microbial pathogens and pattern recognition receptors for pathogen-associated molecular patterns (PAMPs), or through receptors for damage-associated molecular patterns (DAMPs) as a result of physical injury.

One of the most well studied mediators released from mast cell granules is histamine. Mast cells release histamine when the body encounters a toxic substance, in response to an allergen or when they detect injury. Mast cells do not circulate in the blood, but are released from the tissues in which they reside to secrete histamine (Amin 2012), this acts on blood vessels at the site of damage causing them to dilate allowing for increased blood flow to the area in need.

Mast cells are involved in a number of diseases, including chronic inflammatory diseases such as asthma, as well as IgE –dependent allergic reactions. In these cases it is the ongoing mast cell activation and increased spontaneous release of

histamine that is detrimental. In other cases however, mast cells have been shown to have a protective role, for example in a mouse model of acute septic peritonitis (Echtenacher et al 1996).

Mast cells have been shown to be implicated in arterial diseases such as atherosclerosis and abdominal aortic aneurysms (AAA). It was shown that the mast cell constituents responsible for increasing the incidence of abdominal aortic aneurysms were chymases and tryptases (Wang et al 2012). Lack of mast cells was shown to be protective in AAA, and mice lacking mast cells showed a reduced degradation of media elastin and also decreased macrophages and T-cells at damaged sites. Bankl et al (1999) demonstrated that there was an association between mast cells and DVT. This group reported that mast cell distribution was increased in veins of the lower limb in patients with DVT compared to in non-thrombosed limbs.

1.2 Physiological role of platelets

Platelets are small (~1-3 μm in diameter) anucleated cells derived from megakaryocytes in the bone marrow. In healthy individuals, normal platelet count can range from 150,000 – 450,000 per μL. Millions of new platelets are produced each day with the average life span of circulating platelets being 5-9 days. Their role is to maintain hemostasis and they are recruited to sites of vessel damage to prevent bleeding. Platelets circulate in the vascular system in a resting sate, and do not interact with the endothelium unless it becomes damaged, in this case they respond rapidly to form a haemostatic plug. Platelets have a number of surface receptors, mediating interactions with other cells and

proteins at sites of damage. Receptors are also necessary to activate platelets, and play a role in platelet recruitment, adhesion and aggregation. Some of the important receptors on the platelet membrane are GPIb-IX-V, GPVI, αIIbβ3 and CLEC-2 (Figure 1.4 below).

Platelets also contain a number of signaling proteins and granule contents. In healthy individuals platelets play an important role in forming vascular plugs at sites of injury in blood vessels. However platelet activation at sites of diseased endothelium can lead to a number of thrombotic disorders such as myocardial infarction, stroke, and atherosclerosis (Lindemann et al 2001, Nieswandt et al 2005). More recently they have also been shown to be important in maintaining vascular integrity (Gros et al 2014) and inflammation (Massberg 2013). It has been reported that platelets can actually safeguard developing lymphatics and vessels at the site of leukocyte infiltration in inflamed organs and in tumors (Ho-Tin-Noe et al 2011).

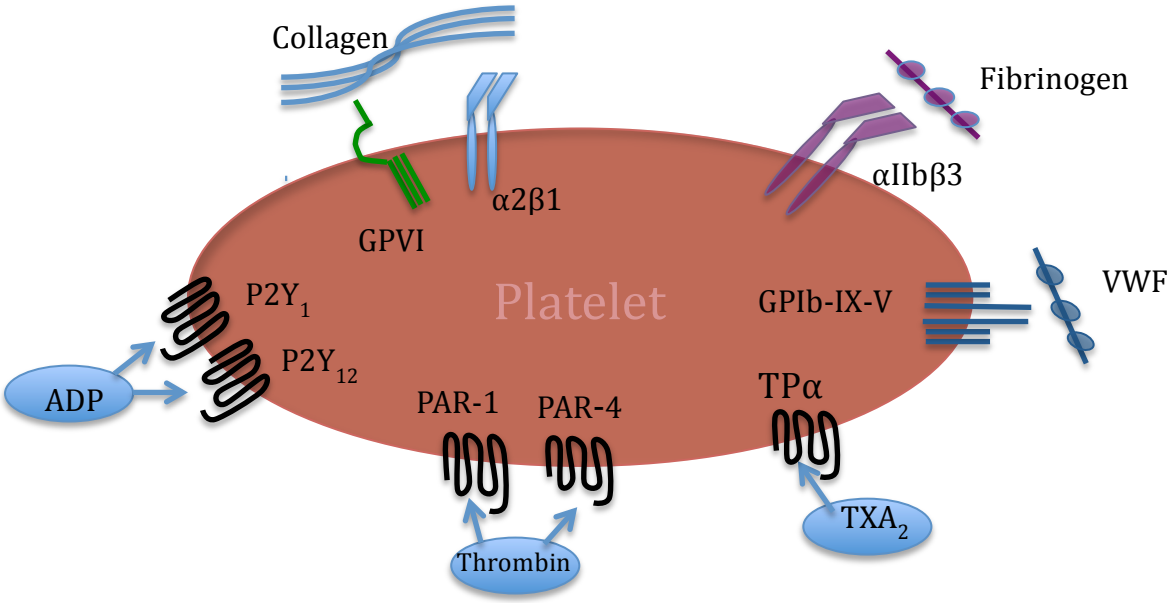


Figure 1.4 – major platelet receptors interacting with their specific ligands

Shows many of the major platelet receptors and which ligands they interact with to cause platelet activation and activation of further signaling pathways.

1.2.1 Platelet production

There is a constant need for circulating platelets to remain at a level of around 150-450x10^9 platelets per litre, therefore new cells are produced as they are removed from the circulation. Platelets arise from haematopoietic stem cells in the bone marrow, before common myeloid progenitor cells then commit to the immature megakaryocyte lineage (Patel et al 2005). Megakaryocytes then mature and differentiate into pro-platelets, which finally release platelets into the circulation (Kaushansky 2005). Thrombopoietin plays a critical role in this megakaryocyte differentiation.

1.2.2 Platelet anatomy

Platelets have a limited ability to synthesize their own proteins, as they are anucleated. They are able however to uptake plasma proteins such as fibrinogen. It has been reported however that despite being anucleated, both megakaryocytes and primary platelets possess a functional spliceosome. Signal dependent splicing is a novel role of platelets, which is highly complex despite the lack of nucleus (Denis et al 2005).

Platelets also possess an actin cytoskeleton and a microtubule network, allowing the cell to undergo marked structural changes during platelet spreading (Calaminus et al 2008). They also contain secretory vesicles in the cytoplasm; α-granules, dense granules and lysosomes. Table 1.1 below shows the contents of the α-granules and dense granules and their functions. Individuals with either a low granular number or an inability to release the granule contents can suffer from mild to severe bleeding disorders such as grey platelet syndrome (Deppermann et al 2013)

Table 1.1- major contents of platelet α- and dense granules

The main contents of platelet α- and dense granules and their functions.

α-granules			Dense granules		
Content	**Function**	**Role**	**Content**	**Function**	**Role**
-VWF -Fibrinogen	Adhesion proteins	Thrombus formation and platelet aggregation	Poly-p	Activation of factor XII	Mediators involved in platelet aggregation and thrombus formation
-VEGF -EGF -PDGF	Growth factors	Angiogenesis and wound repair	Serotonin ADP ATP Ca2+	Feedback mediators	
- P-selectin	Membrane protein	Leukocyte binding			
-PF4 RANTES	Chemokines	Leukocyte recruitment			

1.3 Platelet signaling

Platelets have a number of receptors, which signal in different ways, leading to Ca^{2+} mobilization and secretion of granule contents. A number of the platelet receptors work via a feedback mechanism, either positive feedback or inhibitory, and this can lead to platelet activation and is crucial for their function. The receptors involved in this are discussed below.

A distinction has been made between two types of signaling; inside-out and outside-in signaling. Whilst 'inside-out' signaling activates the ligand binding function of the integrins, 'outside-in' signaling leads to cellular responses as a result of ligand binding to the integrin (Shen 2012). For example inside-out signaling is initiated by the binding of an agonist to a plasma membrane receptor, this could determine if αIIbβ3 is able to find Von Willebrand factor (vWF) or fibrinogen, which would allow platelet aggregation to occur as these act as bridging molecules between receptors on adjacent platelets (Shattil 1998). Outside-in signaling refers to post-ligand binding events, in platelets these are regulated by αIIbβ3 and responsible for functions such as granule secretion and platelet spreading (Shattil 1998).

1.3.1 - G protein-coupled receptors (GPCRs)

G protein coupled receptors (GPCRs) are a large family of membrane receptors, which bind to a variety of external signals. They are sometimes known as 'seven-transmembrane receptors' as they have seven domains that span the membrane. They are able to enhance platelet activation via binding to a number of agonists released from platelet granules and from damaged endothelial cells, leading to a positive feedback mechanism. Such as the GPCRs $P2Y_1$ and $P2Y_{12}$, which bind to ADP released from granules and damaged endothelial cells, and along with other receptors enhance secretion and platelet aggregation (Dawood et al 2007).

A subfamily of GPCRs are the protease-activated receptors (PARs), unlike other GPCRs these are not activated via ligand binding, but instead by cleavage of part of their extracellular domain. Serine proteases e.g. thrombin activate PARs by cleaving the amino terminus of the receptor, exposing the N-terminus which acts

as a 'tethered ligand'. There are 4 known PARs, PAR 1 and PAR 4 are the most important in human platelets, and these are activated by thrombin (Kahn et al 1999) whilst PAR 3 and 4 are important in mouse platelets. The Thromboxane A_2 (TXA2) prostanoid receptor (TP) is another typical GPCR, which has 7 transmembrane segments. The effects of Thromboxane A_2 are mediated by binding to this TP receptor. TXA2 is produced by activated platelets, and stimulates platelet aggregation by increasing the expression of GPIIb/IIIa on the platelet membrane, as well as vasoconstriction (Smyth EM 2010).

1.3.2- Integrins

Integrins are a family of hetrodimeric transmembrane receptors, which act as bidirectional signaling molecules. Platelets express both β1 and β3 integrins, which are present in a low-affinity state in the membrane, and shift to a high-affinity state during platelet activation (Nieswand et al 2009). A highly expressed important receptor on the platelet surface is αIIbβ3, which mediates platelet aggregation. αIIbβ3 mediates platelet aggregation by binding to plasma fibrinogen and also vWF. Binding of these ligands to the receptor results in 'outside- in signaling' and allows platelet aggregation to proceed as previously discussed.

1.3.3 – ITAM containing receptors

Immunoreceptor tyrosine-based activation motifs (ITAMs) are a conserved sequence of amino acids, Yxx(L/I)x6-12Yxx(L/I) which are important for signal transduction in platelet and a number of other immune cell receptors, as well as Fc receptors; FcγRIIA and FcγRI (Daeron M 1997). ITAMs are typically phosphorylated by Src family kinases, which enable recruitment and activation

of Syk family kinases (Moicsai et al 2010). The important platelet receptors that signal through ITAM receptors are described below.

1.4 Major Platelet receptors involved in thrombosis

1.4.1 GPVI

GPVI Is the major collagen receptor expressed only on platelets and megakaryocytes. It belongs to the Ig superfamily of surface receptors and has two Ig domains. There are around 3700-9300 GPVI receptors on the platelet surface (Burkhart et al 2012). Association of GPVI with the FcRγ chain is required for its expression. GPVI in its monomeric form has a low affinity for collagen but dimerization increases the affinity. GPVI becomes activated upon phosphorylation of the ITAM region in the FcRγ chain by Src and SyK family kinases, which ultimately leads to activation of PLCγ2 (Watson et al 2010). Src family kinases (SFKs) Fyn and Lyn associate with a poly-proline region of the GPVI cytoplasmic tail through their SH3 domains. This phosphorylation of the FcRγ chain provides a docking site for SH2 domain of SyK. This binding further leads to a cascade of signaling events through adaptor proteins such as the linker for activation of T-cells (LAT). Phosphorylation of LAT allows binding of adaptor proteins Grb2 and Gads and also phospholipase C (PLC)γ2. PLCγ2 is then activated by recruitment of SLP-76 and Ca^{2+} is mobilized (Nieswandt, Watson 2003).

The role of collagen-dependent platelet adhesion via GPVI in arterial thrombosis remains controversial. Using the ferric chloride injury model to induce thrombus formation, some groups have reported that GPVI is crucial for platelet

recruitment to the injured arterial wall. In contrast to wild-type animals, GPVI knock-out mice do not show arterial occlusion (Massberg 2003; Dubois 2006). The authors conclude that sub-endothelial collagens are the major trigger for arterial thrombosis and that GPVI is important for recruitment of platelets to the vessel wall. In contrast, others have shown that GPVI plays a less significant role. Using the same model to induce arterial thrombosis, Eckly et al (2011) showed that deficiency in both GPVI or β1 integrin's did not suppress thrombus formation. The role that GPVI may play in venous thrombosis is unknown.

However it was recently shown in an in vivo ferric chloride model that whilst GPVI was not important for thrombus initiation, it was important for complete occlusion (Alshehri et al 2015), with GPVI deficient mice showing delayed occlusion times. The authors show that GPIV is a receptor for fibrin, and it is this interaction that is important in forming an occlusive thrombus. They show that platelets are able to spread on fibrin, and that the ectodomin of GPVI can bind both monomeric and polymerized fibrin. It is suggested that this interaction is important for thrombus growth and stability, and may be why GPVI deficient mice show increased embolization in the $FeCL_3$ model.

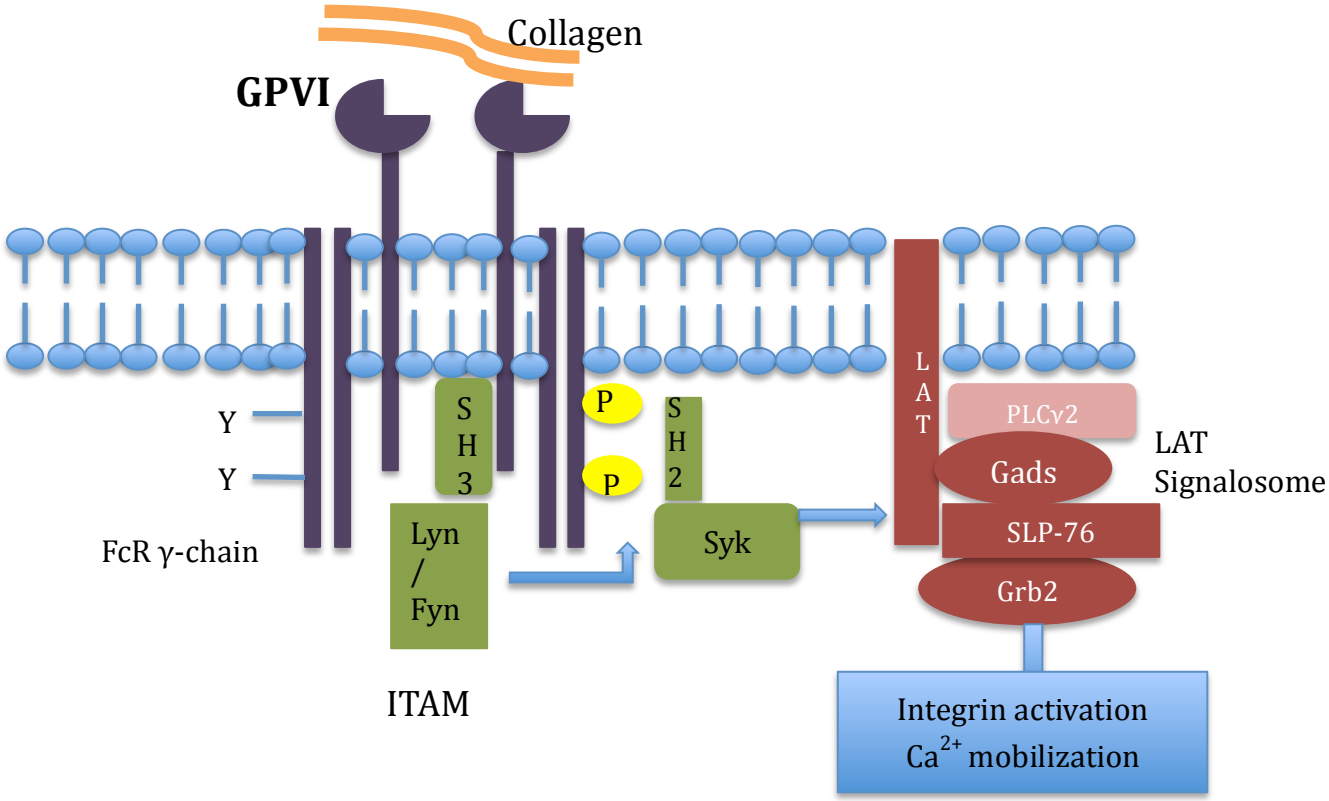


Figure 1.5 – Schematic of GPVI signaling pathway

The signaling cascade that occurs after GPVI activation by collagen, involving the FcRγ-chain, the formation of the LAT signalosome and finally culminating in integrin activation and Ca^{2+} mobilization.

1.4.2 – CLEC-2

CLEC-2 is a C-type lectin receptor, and type II membrane protein that is expressed on platelets and megakaryocytes. It was initially discovered as a receptor to the platelet activating snake venom rhodocytine (Suzuki-Inoue et al 2006). It signals in a similar way to GPVI but in contrast only has a single YxxL in its cytoplasmic tail, so is known as hemITAM (Watson et al 2010). CLEC-2 mediates platelet activation through SyK and Src kinases. A novel mechanism was proposed whereby SyK is activated through cross-linking of two phosphorylated CLEC-2 receptors, 2 SyK SH2 domains bind to the phosphorylated YxxL in the cytoplasmic tail of CLEC-2, which are present as dimers on the platelet surface (Hughes 2010).

The only known ligand to physiologically interact with CLEC-2 is podoplanin (Herzog et al 2003), a transmembrane protein involved in lymphatic vessel formation as well as tumor metastasis. Podoplanin is not only expressed on lymphatic endothelial cells, but also on kidney podocytes and type 1 lung alveolar cells (Ozaki et al 2007). Although podoplanin is not expressed in platelets or blood endothelial cells, some groups have suggested that CLEC-2 is an essential receptor in hemostasis and thrombosis.

May et al (2009) showed *in vivo* that CLEC-2 deficient platelets can adhere normally under flow but are unable to aggregate properly. Similar findings were reported by Ozaki et al (2010), who suggest that CLEC-2 is required for the 'platelet piling up' process, which eventually leads to stable thrombus formation. They demonstrate that single cell adhesion is normal on a collagen surface, but thrombus formation is severely impaired. Both groups show that arterial

thrombus formation was prevented in CLEC-2-deficient mice using the ferric chloride injury model.

May et al (2009) also report that the lack of CLEC-2 leads to increased tail bleeding times, although others have not confirmed this (Ozaki 2010). Any evidence that CLEC-2 plays a role also in venous thrombosis was lacking.

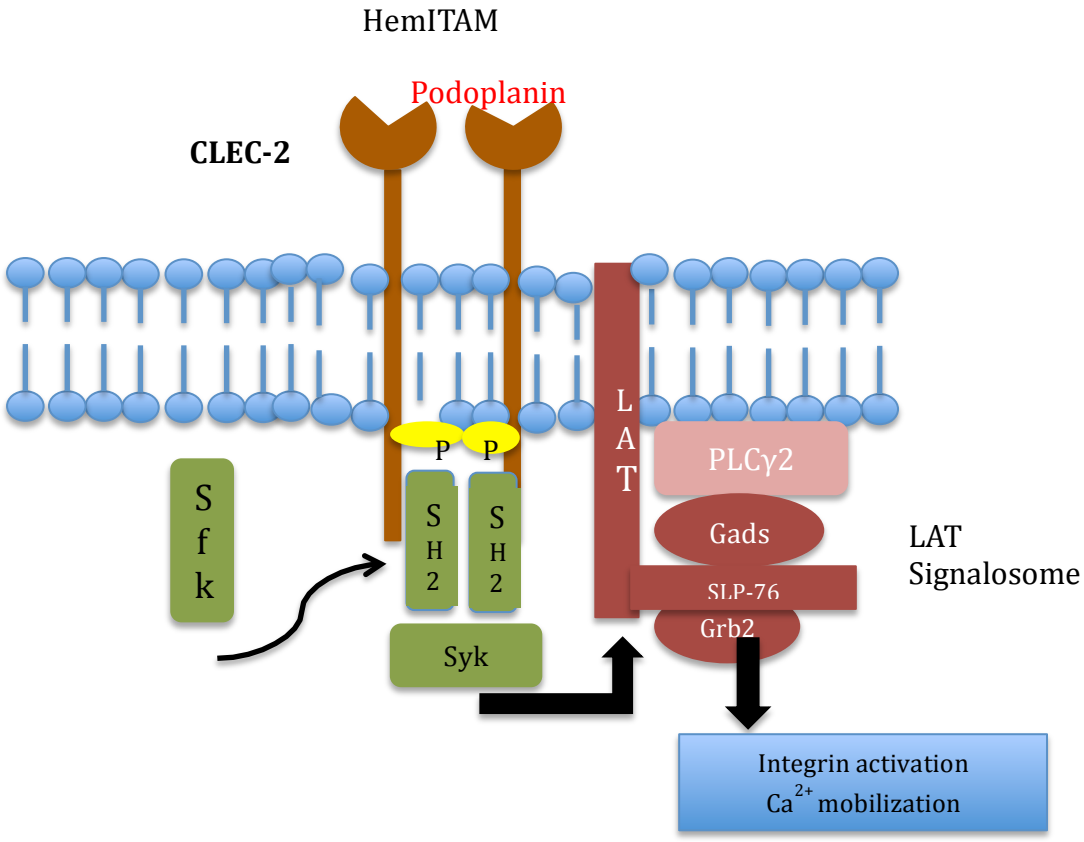


Figure 1.6 – Schematic of the CLEC-2 signaling pathway

Upon binding to podoplanin, CLEC-2 becomes activated. This leads to phosphorylation of the HemITAM by SyK and Src family kinases (sfk), which further leads to downstream signaling and formation of the LAT signalosome. Finally these signaling events lead to integrin activation and Ca^{2+} mobilization.

1.4.3- GPIbα.

This receptor is part of the GPIb-IX-V complex, which also includes GPIbβ, GPIX and GPV.

GPIbα is the main receptor for VWF along with integrin αIIbβ3. Whilst this integrin requires inside out signaling to become activated, GPIbα is constitutively active on the platelet surface (Bergmeier et al 2008). The N-terminal domain of GPIbα is the major ligand-binding region; it is able to bind VWF, thrombin, P-selectin and coagulation factors (Savage et al 1998). Removing the extracellular domain of GPIbα abolishes the ability of platelets to adhere to sites of arterial damage (Cosemans et al 2013).

It has been suggested by Bergmeier et al (2006) that GPIbα is more important than its main ligand VWF, in arterial thrombosis. This group demonstrated that mice deficient for VWF were still able to form thrombi (although unlike wild type, VWF KO mice thrombi never occlude the vessel). In contrast, in GPIbα deficient mice, platelet-vessel wall interaction was virtually absent and thrombi did not form at all. These findings suggest that GPIbα is crucial for the initial platelet adhesion to the vessel wall in arterial thrombosis. This group also reported that platelets lacking αIIbβ3, the main integrin for platelet aggregation, despite not being able to form thrombi on their own, can incorporate into growing WT thrombi efficiently. This implies that, at least under arterial flow, GPIbα is more important for recruitment and adhesion of platelets than αIIbβ3.

The group of Smyth et al (2001) however reported no occlusion in the carotid artery of αIIbβ3 deficient mice using the ferric chloride model (in wild-type animals vessels occlude). They also showed that injection of tissue factor could overcome αIIbβ3 deficiency in initiating systemic intravascular

thrombosis, suggesting that a combined therapy of both anticoagulants and anti-αIIbβ3 agents may be efficacious.

1.4.4- αIIbβ3

This is the major integrin found on the platelet surface. It is involved in platelet aggregation and therefore hemostasis and thrombosis. Its major ligands are fibrinogen, VWF and fibronectin and the activation of this integrin is mediated through 'inside-out signaling' (see 1.3). The cytoplasmic tails of αIIb and β3 as well as the transmembrane regions are in contact, keeping the integrin in its 'inactive' state. When this complex collapses by the binding of cytoplasmic tail binding partners (e.g. talin) the integrin becomes active (Plow et al 2007).

Platelet agonists regulate αIIbβ3 function. Platelets require activation to enable αIIbβ3 to bind fibrinogen and VWF and cause platelet aggregation (Bennett 2005). Fibrinogen binding to platelets via αIIbβ3 is important for platelet aggregation. Fibrinogen and VWF are important for bringing adjacent activated platelets together (platelet cohesion) and can also promote stable aggregation under high shear. Platelet surface αIIbβ3 can also bind soluble fibrinogen, which is implicated in further platelet recruitment to a growing thrombus (Jackson 2003).

1.5 - Platelet activation & coagulation cascade

When the endothelium becomes damaged, collagen is exposed and platelets are able to bind to it through their collagen specific glycoprotein $\alpha_2\beta_1$ surface receptors. This adhesion is mediated by von Willebrand Factor (vWF) released from the activated endothelium, and from platelets. vWF forms a crosslink

between the collagen fibrils and the GPIb-GPIX-GPV complex. In addition vWF also binds to and stabilizes coagulation factor VIII.

Binding of platelet GPVI to collagen then triggers a signaling cascade that activates platelet integrins. Activated platelets also release the contents of their stored granules including ADP, vWF, platelet activating factor (PAF), and thromboxane A2 (TXA2) this in turn activates more platelets.

Platelets themselves are also important in the activation of the coagulation cascade, where they provide an efficient surface for the assembly of the enzyme complexes of the coagulation system.

Whilst the 'classical' view of the coagulation cascade was that is divided into the intrinsic and extrinsic pathways, we now know that these are overlapping processes rather than alternative pathways.

In the intrinsic pathway, all clotting factors are already present in the blood, starting with conversion of FXII to FXIIa following the secretion of platelet polyanions. The extrinsic pathway is initiated following vessel damage and exposure of tissue factor (TF) (Ajjan et al 2005). Once TF is exposed, this is able to bind to factors VII/VIIa, and in the presence of calcium ions, forms a complex that further activates the 'cascade', converting FX to FXa, FIX to FIXa and FVIIa itself. This finally culminates in the formation of thrombin at the damaged vessel wall, which further activates platelets; followed by conversion of fibrinogen to fibrin that stabilizes the formed clot (Cimmino G et al 2016).

In resting states, phosphatidylserine (PS) is found in the inner platelet membrane. Following platelet activation by either exposure to collagen, sheer stress or thrombin, this phosphatidylserine moves to the outer surface of the

plasma membrane. This leads to increased activation of prothrombin and factor X, as well as the appearance of high affinity binding sites for factors Va and VIIIa (Ahmadd et al 1992). The transbilayer movement of PS in the platelet membrane is accompanied by the generation of phosphatidylserine-rich microvesicles. Platelet derived microvesicles are localized to the site of vessel damage, and incorporated into the growing thrombus (Merten et al 1999). The presence of PS at the phospholipid membrane facilitates the binding of FVIIa, FXIa, FXa and prothrombin (PT), this is due to an electrostatic charge between positively charged ϒ- carboxyglutamic acid (GLA) domains in the clotting proteins and the PS on the membrane (Owens et al 2011).

As well as tissue factor exposure following vessel wall injury, tissue factor bearing microparticles can also bind to platelets. A bridge is formed through the interaction of P-selectin on the platelet surface and P-selectin glycoprotein Ligand-1 (PSGL-1). These PSGL-1 and TF rich microparticles are then able to transfer tissue factor to the platelet membrane (Falati et al 2003).

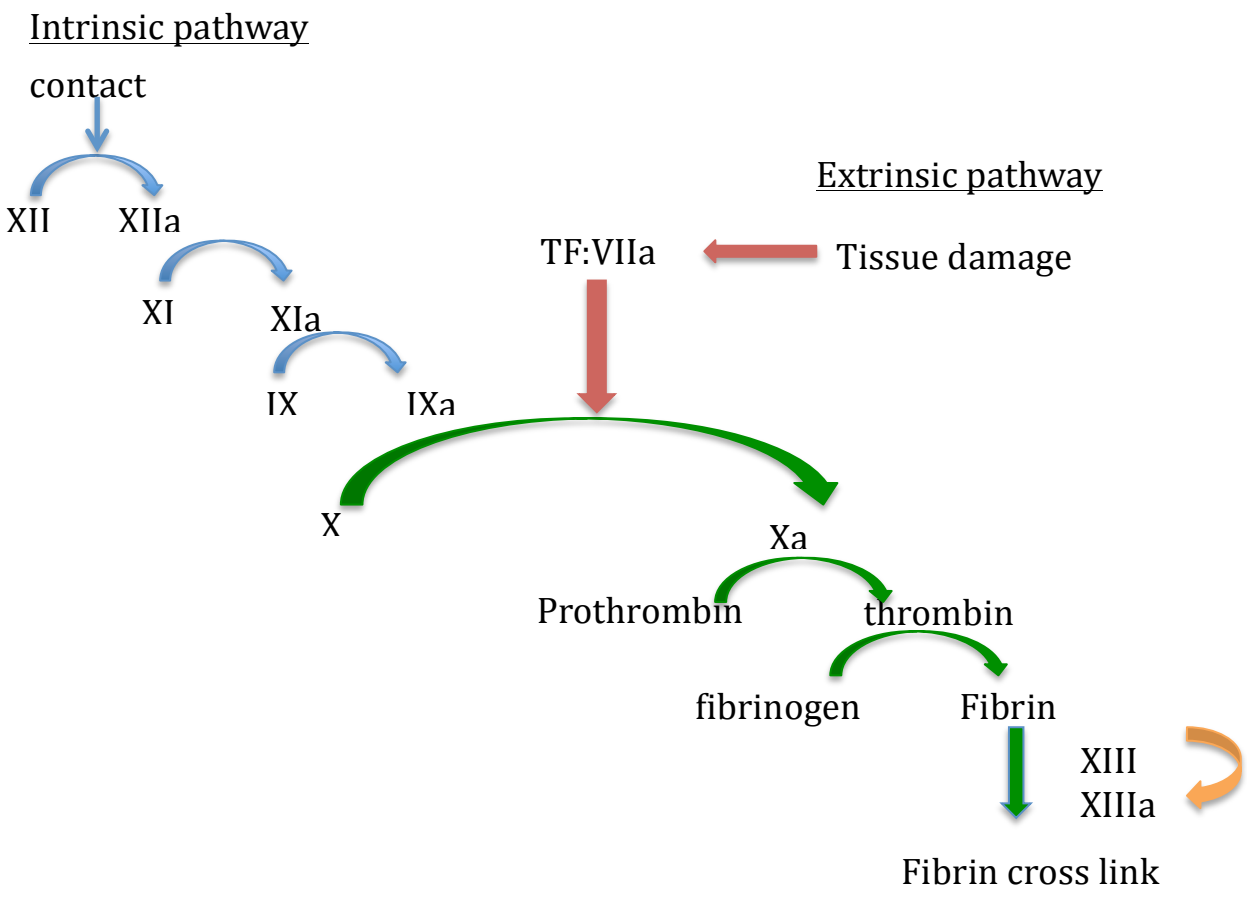


Figure 1.7 – Intrinsic and extrinsic coagulation cascades

The coagulation cascade of both the contact activation pathway (intrinsic pathway) and the tissue factor pathway (extrinsic) both involve a series of downstream cleavage steps by serine proteases. Both pathways fundamentally culminate in the production of fibrin, which stabilizes the clot.

In order to prevent platelet activation on an intact healthy endothelium, inhibitory mechanisms are in place. In healthy vessels, the endothelium expresses nitric oxide synthase (NOS) and cyclo-oxgenase (COX-1), which produces Nitric Oxide (NO) and prostacyclin (PGI2) respectively (Mitchell et al 2007). The actions of NO and PGI2 are synergistic in inhibiting platelet activation. Nitric Oxide produced by NOS activates Guanosine cyclase, which in turn results in the formation of cyclic guanosine monophosphate (cGMP), which then inhibits platelet activation (Feil at al 2003). Release of PGI2 from the endothelium also inhibits platelet activation; it interacts with platelet G protein-coupled PGI_2 receptor coupled to Gαs, causing accumulation and activation of cAMP (Raslan & Naseem 2015). It is through these secondary messengers; cAMP and cGMP, that platelet inhibition occurs, activating protein kinase A (PKA) and protein kinase G (PKG) respectively leading to further phosphorylation of targeted platelet proteins.

1.6 Role of platelets in inflammation

Inflammation is a complex set of responses produced by the innate immune system in response to a variety of pathological stimuli, such as microbes or damage-associated molecular patterns (DAMPs). Systemic inflammation can however occur as a result of an infection, leading to sepsis, or in response to a substantial trauma or chronic disease. The classical symptoms of inflammation are redness, swelling, heat, pain and compromised function.

The inflammatory response involves multiple mechanisms but an important part is the recruitment of immune cells e.g. neutrophils and monocytes by the vessel wall (Thomas et al 2015). Platelets also play an important role in regulating

inflammation, and are not only needed for hemostasis and thrombosis but have also been described as inflammatory cells (Gros et al 2014; Jenne & Kubes 2015). Platelets contain a number of inflammatory mediators, some of which they synthesize de novo, others that they store and secrete from granules (α-granules, dense granules and lysosomes). The release of these substances upon platelet activation allows leukocytes to be recruited to the site of injury (Shi 2011). Table 1.2 below lists some of these important platelet-derived inflammatory mediators.

Table 1.2. Inflammatory mediators synthesized by and stored in platelets

Location	Molecule	Family
α- granules	PF4/ CXCL4	Chemokine
α- granules	β-thromboglobulin(CXCL7/NAP-2)	Chemokine
α- granules	RANTES (CCL5)	Chemokine
α- granules	CD40L	Cytokine
α- granules	PDGF	Growth factor
α- granules	TGF-β	Growth factor
α- granules	TNF-α	Cytokine
α- granules	IL-1α	Cytokine
α- granules	CXCL1	Cytokine
α- granules	CXCL5	Cytokine
α- granules	CXCL12	Cytokine
α- granules	CCL3	Chemokine
α- granules	CCL7	Chemokine
α- granules	CXCL7	Chemokine
α- granules	CCL17	Chemokine
α- granules	CXCL8	Chemokine
Dense granules	ATP	Nucleotide
Dense granules	Serotonin	Monoamine
Dense granules	Polyphosphates	Phosphates
Dense granules	Glutamate	Amino Acid
Synthesized	IL-1β	Cytokine
Synthesized	Thromboxane A2	Eicosanoid

Platelet α-granules contain many large proteins, the most abundant protein secreted by activated platelets is Platelet Factor 4 (PF4), which accounts for

~25% of α-granules content (Lambert et al 2009). It has many functions such as acting as a chemoattractant for monocytes, it also accelerates atherogenesis by causing vascular inflammation and promoting retention of lipoproteins in the vascular wall. PF4 prevents LDL interacting fully with its receptor and therefore results in its retention on the cell surface (Gawaz et al 2005).

Platelets also synthesize as well as store biologically active proteins; pro-IL-1β is synthesized as a result of thrombin activation. In vivo IL-1β is shown to accumulate in thrombi formed in the ferric chloride model. IL-1β from platelets causes up-regulation of endothelial adhesion receptors and release of proinflammatory IL-6 and IL-8 from endothelial cells (Lindemann et al 2001).

Platelet dense granules also contain inflammatory mediators such as Polyphosphate (polyP). PolyP has been shown to have both procoagulant and proinflammatory functions (Muller et al 2009). Upon platelet activation its release activates the FX11- driven contact activation system, which in turn casues release of bradykinin (and inflammatory mediator) and subsequent accumulation of neutrophils and increased vascular permeability.

All of these platelet inflammatory mediators, which are stored and released, may contribute to multiple inflammatory diseases such as rheumatoid arthritis and atherosclerosis, demonstrating the importance of platelets in inflammation.

1.6.2 Platelet crosstalk with endothelium and leukocytes

Under physiological conditions (non-inflammatory) platelets and leukocytes do not bind to each other, and endothelial cells produce platelet inhibitors e.g. prostacyclin to limit platelet/endothelium interaction (Arman et al 2015).

In patients with inflammatory diseases for example sepsis or cerebrovascular ischemia, activated platelets can be found in the blood (Stokes & Granger 2012). Once activated, these platelets not only pose a greater risk of excessive thrombosis in these patients but they also stimulate the release of Weibel-Palade body constituents leading to increased recruitment of leukocytes to the vessel wall (May et al 2008).

Platelet interaction with the endothelium leads to deposition of proinflammatory cytokines at the vessel wall such as RANTES, as well as accumulation of monocytes. Platelet binding to the activated endothelium is mediated through P-selectin, CD40L and glycoproteins Ibα, αIIb and GPVI (Burger 2003).

Platelets and leukocytes can activate each other in a bi-directional fashion. P-selectin on activated platelets binds to PSGL-1 on leukocytes, which subsequently leads to activation of integrin's on the leukocyte membrane (Mac-1 and LFA-1) (Ghasemzadeh 2013). Mac-1 is able to bind either directly to GP1bα on platelets, or bind α11bβ3 through fibrinogen. Platelet mediated activation of Mac-1 on leukocytes activates coagulation Factor X and therefore thrombin generation, demonstrating platelet-leukocyte interaction triggers the coagulation cascade.

1.7 – Arterial thrombus formation

Multiple signaling events lead to platelet activation and adhesion, culminating finally in thrombus formation. At damaged sites in vessels collagen in the sub-endothelial matrix is exposed, for example following the rupture of atherosclerotic plaques. Platelet binding to this collagen leads to further platelet recruitment. The processes that lead to thrombus formation are described in detail below and shown in figure 6:

1. Tethering – The initiation of thrombus formation is highly influenced by the shear rate of blood flow. At low shear (0-1000s^{-1}) such as in large veins and venules platelet aggregation is primarily mediated by crosslinking of fibrinogen with αIIbβ3. At higher shear rate (1000-10000s^{-1}) such as in arterioles this association is not strong enough to maintain platelet tethering to the vessel wall. Instead, under high sheer VWF coated on collagen fibers is most important by interacting with GPIb-IX-V receptors on platelets (Maxwell et al 2007). This allows initial platelet capture or 'tethering' to the damaged endothelium. This initial capture has a fast on-off rate of association and dissociation between the receptor and ligand. A stable adhesion is therefore not formed, and platelets roll along the endothelium in the direction of flow (Offermanns 2006).

2. Stable adhesion - Platelets become activated during their interaction with thrombogenic surfaces. The tethering process to vWF allows GPVI, the major collagen receptor to come into contact with the exposed collagen fibers in the vessel wall. This leads to integrin activation, and integrins αIIbβ3 and α2β1 form additional adhesion bonds with VWF and collagen respectively, promoting cell arrest and stable adhesion.

3. Spreading- Once platelets have been activated this stimulates reorganization of the actin cytoskeleton, and extension of filopodia and lamellipodia. This leads to the transformation of the biconcave disks to fully spread platelets with an increased surface area. This larger surface area provides a further more stable adhesion as the area to attach to exposed collagen and VWF is increased (Aslan 2012).

4.Thrombus growth – Once platelet integrin's are activated this leads to secretion of α and dense granule contents such as ADP and TxA2. These feedback messengers lead to further activation and aggregation of platelets (Watson, Harrison 2007). Either the intrinsic or extrinsic coagulation pathways achieve the consolidation of a growing thrombus. This is by tissue factor expressed on the endothelial surface, or by the factor XII pathway driven by collagen and platelet polyphosphates (Cosemans et al 2013). Tissue-factor rich microparticles are also incorporated into the growing thrombus via a P-selectin –PSGL-1 dependent mechanism. The platelets also provide a pro-coagulant surface for thrombin generation. This thrombin generation converts fibrinogen into fibrin that stabilizes and consolidates the thrombus. The fibrin network can further trap RBCs and leukocytes leading to effective vessel occlusion.

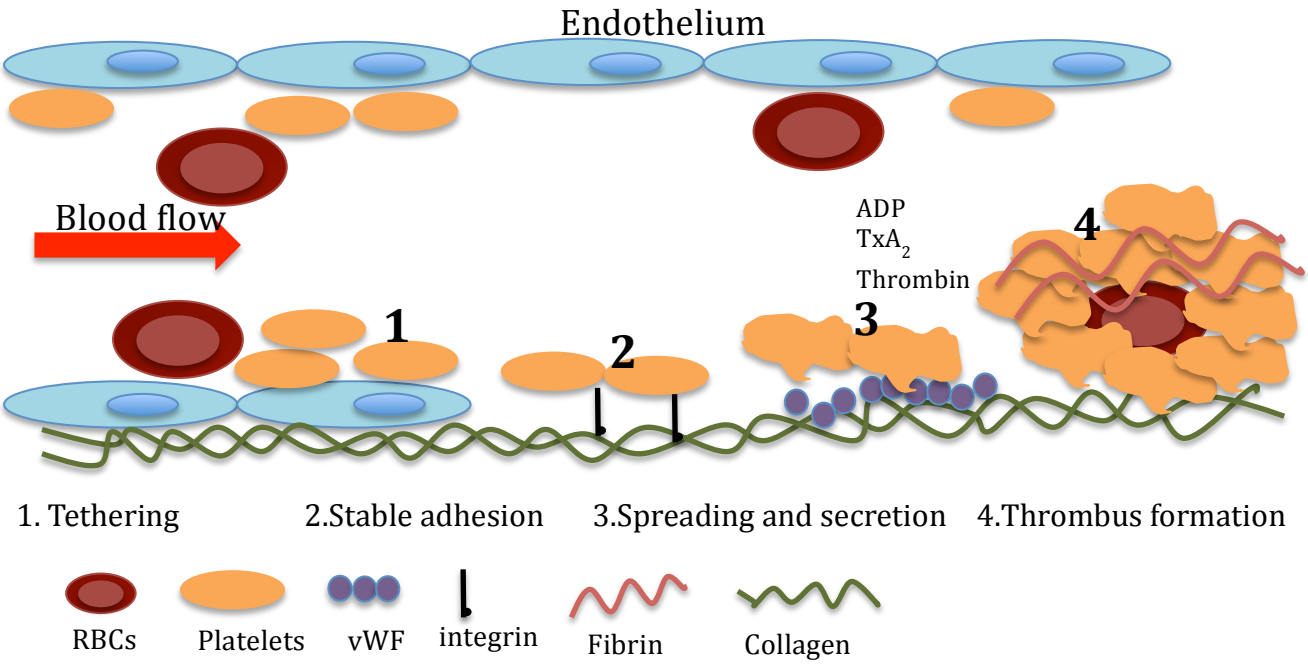


Figure 1. 8 – Events leading to arterial thrombus formation

1) The blood flow transports platelets to the damaged vessel wall, where they tether via weak interactions between vWF and GPIb-IX-V. 2) Tethering allows for closer proximity and interactions between GPVI and the exposed collagen, this leads to integrin activation. 3) Platelets spread and begin to secrete their granule contents 4) ADP and TxA_2 recruit more platelets to the growing thrombus. The thrombus becomes more stable via fibrin generation.

1.7.2 Arterial vs. Venous thrombosis

Thrombosis is closely linked to haemostasis, which is a beneficial protective response leading to bleeding arrest after vessel injury. Physiological and pathological clotting processes share the same coagulation pathways and both involve activation of platelets. Thrombosis is a huge economic burden, with the estimated cost of management being £640 million a year. Thrombosis can be broadly divided into two categories, arterial and venous.

Mechanisms of arterial thrombosis have been extensively studied. Arterial thrombosis is predominantly triggered by atherosclerotic plaque rupture or injury to the vessel wall. Endothelial denudation and exposure of subendothelial matrix proteins, such as collagen and VWF, is a prerequisite and central mechanism of thrombosis in arteries. This triggers platelet recruitment to the exposed sub-endothelium via a number of surface receptors. Arterial thrombosis underlies very serious conditions such as myocardial infarction and ischemic stroke, a leading cause of death in the UK. The mechanisms of arterial thrombus formation are described previously in the chapter in detail (1.6).

In contrast, venous thrombosis is usually initiated in the absence of endothelial denudation but instead is caused by blood stasis and hypercoagulability. In general, venous thrombi grow slower than arterial, and consist of RBC's and fibrin with incorporation of platelets. Mechanisms leading to venous thrombosis initiation and propagation remain incompletely understood.

1.8 Deep vein thrombosis (DVT)

1.8.1 DVT epidemiology

Deep vein thrombosis is the formation of a thrombus within a deep vein, predominantly the legs. Pulmonary embolisms (PE) can form when clots in the lower limbs detach and move to the lungs, this is usually life threatening. Together deep vein thrombosis and pulmonary embolism form the serious disease, venous thromboembolism (VT). The incidence of venous thrombosis is approximately 1 per 1000 adults annually, with the incidence being slightly higher in men than women. In all cases of VT approximately 70% of them manifest in DVT, with the remaining 30% involving PE, with or without DVT (Cushman 2007).

Venous thrombosis is a severe disease, with the major outcomes including death, post-thrombotic syndrome, recurrence and other bleeding complications. It has been reported that ~6% of people with DVT and ~10% of those with PE die within the first month of an episode, whilst the mortality rate of those with PE is estimated to be ~30% (Cushman 2007).

Venous thrombosis is a disease associated with ageing, with cases rising rapidly after 45 years old, with cases even higher in those over 80 years. In the elderly mortality tends to be higher with an increased incidence of PE compared to just DVT, and elderly people also have several other diseases and these may interact to predispose this population to thrombosis. There also seems to be differences in incidence of venous thrombosis among different ethnic groups. Lower incidences are reported in the US, Asian and Hispanic populations compared to white populations, with African-Americans reportedly having a 25% higher rate of the disease (Tsai et al 2002).

1.8.2 Risk factors for DVT

There are many risk factors for venous thrombosis, and many of them may interact, including endogenous patient characteristics as well as other environmental factors. These risk factors include old age, pregnancy, obesity, cancer and genetic factors affecting coagulation. As well as other external factors including surgery, trauma, whilst both hospitalization and long haul flights can lead to prolonged periods of venous stasis. Symptoms of DVT can include swelling in the affected area of the lower leg, pain in the leg often like a cramping, or it may occur without any obvious symptoms. For PE however, symptoms can include chest pains, shortness of breath, and dizziness.

Currently advice given to try and prevent this disease is to keep as active as possible and not to have a sedentary life style. It is suggested that you should avoid sitting for long periods for example in bed post-surgery or long haul flights, and should aim to move your lower limbs as much as possible. Other lifestyle factors such as quitting smoking and diet changes to prevent obesity may go some way to prevent this disease.

1.8.3 Current treatment options for DVT and disadvantages

Current treatment options for people with the disease primarily aim to prevent the clot from growing, or prevent the clot detaching causing a pulmonary embolism. Secondly, the aim is then to try and prevent recurrence. Anticoagulants such as heparin or warfarin, which interact with clotting factors are often used as a treatment option, these decrease the bloods ability to clot, and can prevent clots getting bigger. Warfarin is a vitamin K antagonist, and produces its anticoagulant effect by interfering with the vitamin K cycle, which

ultimately prevents prothrombin becoming carboxylated, and interacting with the phospholipid membrane. These medications however are often associated with serious side effects, especially if not taken exactly as instructed. Other treatment options for more serious cases of DVT or PE can include giving patients tissue plasminogen activators (TPA) to break up blood clots. This is however only given in extremely serious life threatening cases, as they can cause very severe bleeding complications. In order to prevent post-thrombotic syndrome compression stockings are often given to patients who have had DVT, alternatively inferior vena cava filters can sometimes be fitted it patients who need to stop anticoagulant treatment. These filters trap large fragments of blood clots and prevent them detaching and causing a pulmonary embolism (NHS).

This highlights that currently treatments either aim to prevent clots getting bigger or prevent the chance of PE occurring, but there still isn't any good treatments options that tackle DVT without potentially serious bleeding complications as a side effect. Considering the high incidence of this disease and the increasing ageing population, better drug targets need to be identified that won't have these associated side effects.

1.8.4 Mechanisms of DVT

Virchow's triad predicts that there are three main causes of venous thrombosis; stasis, changes in blood coaguability or changes in the vessel wall. Whilst chronic low levels of inflammation impact on arterial thrombosis, this has little impact on venous thrombosis, however acute inflammation can increase venous thrombosis (Esmon 2009). Venous thrombosis is initiated without any visible injury to the vessel wall (expect for thrombosis associated with surgery). Venous thrombosis is believed to be initiated in the venous valvular sinus, it is in these

venous valve pockets that stasis occurs; this is also associated with increased hypoxia, which provides a hypercoagulable microenvironment (Aird 2007). Hypoxia could further lead to up-regulation of tissue factor on the endothelium as well increasing P-selectin expression, which further recruits leukocytes and tissue factor containing micro particles, both of which are important for thrombus formation (Myers et al 2003).

It has previously been shown using the IVC stenosis model, where blood flow is partially restricted, that wild type mice form venous thrombi comparable to human deep vein thrombi. Both thrombi contain a red part rich in RBC's and fibrin (Zahn lines), and a white part rich in platelets, suggesting they have similar mechanisms of thrombus formation. It was shown by Brill et al (2011) that von Willebrand factor expressed on the endothelium interacts with platelets, and plays and important role in the development of DVT. Mice deficient in VWF were protected from thrombosis in both the IVC stasis and stenosis models. They show that flow restriction by IVC ligation results in endothelial activation, and VWF release from Weibel-Palade bodies (WPB). Platelets and leukocytes are then recruited and bind to VWF and P-selectin, respectively, on the activated endothelium. Thus, endothelial activation and liberation of WPB is pivotal for DVT initiation.

1.8.5 Potential roles of immune cells in DVT

It has been shown that T-regulatory cells (T-regs) may have a role in changing the functional status of the endothelium. This makes T-regs important players in ischemic stroke. There is a controversy over whether these immune cells promote stroke or play a protective role. Some studies have demonstrated that

adoptive transfer of T-regs into Rag1-/- mice (deficient in lymphocytes) increased the susceptibility for brain ischemia in a model of middle cerebral artery occlusion (MCAO) (Kleinschnitz et al 2013). In contrast, others have shown that infarct size is significantly enlarged when T-regs are depleted (Liesz et al 2009). T-regs adhere to the vascular endothelium and it is therefore possible that they activate or downregulate it; I have therefore looked at if T-regs have a role in DVT.

Other immune cells may also be involved in DVT pathogenesis, e.g., mast cells. It has been shown that mast cells accumulate in the vicinity of thrombosed veins in humans (Valent et al 2002). Mast cells are extravascular and found in close proximity to capillaries and post-capillary venules. They secrete a number of factors such as histamine, a WPB secretagogue, as well as heparin (an anticoagulant) and tissue-type plasminogen activator (tPA), which promote thrombolysis. Thus, mast cells can potentially be implicated in thrombosis and I investigated the role mast cells play in DVT using mast cell deficient mice in the IVC stenosis model.

1.9 - Aims of thesis

The mechanisms leading to venous thrombosis initiation have not been fully elucidated. The role of major platelet receptors CLEC-2 and GPVI have been extensively studied in arterial thrombosis but not in venous thrombosis. These platelet ITAM receptors participate in the maintenance of vascular integrity and endothelial 'well-being' which we know is critical in DVT initiation, therefore I aimed to understand their roles. I also aimed to investigate the role other immune cells may play in venous thrombosis. Overall, the major aim was to identify new targets to prevent DVT, which are not involved in normal hemostasis and therefore will not lead to bleeding complications as a side effect. The specific aims were:

1. To investigate the role of CLEC-2 and GPVI in DVT, and compare this to their role in arterial thrombosis
2. To investigate the role of mast cells in DVT
3. To investigate the role of T-cells in DVT
4. To study the signaling pathways involved in NETs, and the role they play in thrombosis

Chapter 2
Materials and methods

2.1 Materials

2.1.1 Antibodies and Reagents

Details of all antibodies and reagents used throughout this thesis are listed below in the table.

Table 2.1 – Antibodies and reagents

Antibodies	Source
Anti-CD3	Abcam (Cambridge, UK)
Anti-GR1	BD Bioscience (San Diego, USA)
Anti-CD45	Abcam (Cambridge, UK)
Anti-CD41	Emfret (Germany)
Syrian hamster anti-mouse PDPN	eBioscience (Hatfield, UK)
Mouse anti-tubulin	Sigma (Poole, UK)
Anti-Histone H3 Ab	Abcam (Cambridge, UK)
Rabbit anti-human VWF	Dako (Denmark)
FceR1-PE	eBioscience clone MAR-1 (Hatfield, UK)
CD117 (C-Kit)-APC	eBioscience clone 2B8 (Hatfield, UK)
Mouse anti-DNP-IgE	Sigma (Poole, UK)
DNP-HAS	Sigma (Poole, UK)
Other Reagents	**Source**
Compound 48/80	Sigma (Poole, UK)
Sodium cromoglycate	Sigma (Poole, UK)
Ketotifen	Sigma (Poole, UK)
Prostacyclin (PGI_2)	Caymen chemicals (Cambridge, UK)
Histamine	Sigma (Poole, UK)
PMA	Abcam (Cambridge, UK)
LPS (from Escherichia Coli)	Sigma (Poole, UK)
Ionomycin	Abcam (Cambridge, UK)
Glucose Oxidase Thrombin	Sigma (Poole, UK) Sigma (Poole, UK)

2.1.2 Transgenic mice

All animal experiments were performed with ethical approval from Animal Welfare Ethical Review Body and the UK Home Office, under project licenses 70/8286 and 40/3745.

GPVI-/-, platelet specific CLEC-2 deficient (PF4-cre colony) and CLEC-$2^{fl/fl}$ PF4cre - GPVI $^{-/-}$ (double deficient) mice were used in the laser injury model. Platelet

factor 4 (PF4) is a megakaryocyte-platelet lineage specific marker, and the CLEC-2 gene is deleted in this particular cell lineage (Tiedt et al 2007). Double deficient mice were created by crossing GPVI constitutive KO's, with CLEC-2 floxed mice further generating cre^{+} and cre^{-} litters.

For DVT experiments investigating the role of CLEC-2, all mice were on a C57BL/6 background. For conditional deletion on CLEC-2 expression from platelets, 4 week old $Clec1b^{fl/fl}$ x $Rosa26^{+ERT2cre}$ and their $Clec1b^{fl/fl}$ x $Rosa26^{+/+}$ control littermates were fed with a tamoxifen diet TAM 400 (400mg tamoxifen in citrate form per Kg of diet, Harlan, UK) for 2 weeks, followed by 4 weeks of regular diet.

To investigate the role of podoplanin and CLEC-2 in DVT, mice were used with podoplanin deleted in either cells of hematopoietic or endothelial origin. Vav1-cre $pdpn^{fl/fl}$ (hematopoietic cell-specific knockout) and Tie2-Cre $pdpn^{fl/fl}$ (knocks out podoplanin in cells of endothelial origin) were crossed with $pdpn^{fl/fl}$ mice with $Vav1^{cre+}$ and $Tie2^{cre+}$ mice respectively (Jackson Lab, strain 008863). Podocin-cre $pdpn^{fl/fl}$ mice were created in house by crossing $pdpn^{fl/fl}$ and $podocin^{cre+}$ mice.

For mast cell experiments, mast cell deficient mice $Kit^{w\text{-}sh}$ and $Kit^{w\text{-}v}$ on a C57Bl/6 background were purchased from the Jackson Lab. These mice strains have different mutations in the C-Kit receptor, leading to the absence of mast cells. C57BL/6 mice were purchased from Charles River used for experiments with topical application of histamine and compound 48-80.

For all experiments investigating the role of T-cells in DVT both nude mice (absent T-cells) and Rag1-/- mice (deficient in B and T lymphocytes) were used.

C57Bl/6 mice from Charles River were used for injection of anti-CD25 antibody (to deplete T-regulatory cells).

2.1.3 Adoptive transfer of mast cells into $Kit^{w\text{-}sh}$ mice

Ten millions of in vitro differentiated MCs were injected into 3-4 weeks old $Kit^{W\text{-}sh}$ mice through the tail vein. In 8 – 10 weeks, mice were subjected to 48 h IVC stenosis.

2.1.4 Mast cell depletion in mice

Mice were injected i.p every 12 hours for 4 days with the mast cell degranulator; compound 48/80 dissolved in sterile saline. Mice were injected with 200µl of the solution according to the following protocol: day 1- 0.6mg/kg, day 2- 1.0mg/kg, day 3- 1.2mg/kg and day 4, 2.4mg/kg. This method results in peritoneal mast cell depletion of ~84% (Carvalho M et al 2005). DVT surgery (IVC stenosis) was performed 24 h after the mice received their last injection.

2.1.5 Mast cell membrane stabilization in vivo

Sodium cromoglycate and Ketotifen inhibit mast cell degranulation by acting as a mast cell membrane stabilizers. Mice were injected intraperitoneally (i.p) with cromolyn (100mg/kg body weight), or i.p Ketotifen (25mg/kg body weight) at two time points; 24 h and 30 min prior to DVT surgery (IVC stenosis).

2.1.6 Treatment of mice with anti-podoplanin antibody

For deep vein thrombosis experiments (chapter 3) WT littermates were injected with an anti-podoplanin antibody (Syrian hamster anti-mouse, clone 8.1.1) twice: at 24 h and 30 min before surgery (100μg/mouse at 24 h and 50μg/mouse respectively).

For intravital microscopy experiments, WT littermates received one injection of the antibody (100μg/mouse) immediately before surgery. Control mice received the same dose of IgG. At the doses stated about, it has been shown that this antibody blocks podoplanin in mice for at least 48 hrs.

2.2 Blood collection

2.2.1 human blood collection

Ethical approval for blood donation from healthy volunteers was granted by Birmingham University Internal Ethical Review (ERN_11-0175). Healthy volunteers who were drug free consented to the collection of venous blood. Blood was drawn into sodium citrate (4% citrate 1:9 ratio) or into EDTA.

2.2.2 Mouse Blood collection

In order to isolate platelets for transfusion into clec1bfl/fl PF4cre mice (chapter 3) blood was collected from the retro-orbital plexus of WT syngeneic C57Bl/6 mice, and stabilized in sodium citrate.

2.3 Mouse platelet preparation

Blood collected in sodium citrate was centrifuged at 100 g for 5 minutes to obtain the platelet rich plasma (PRP) this was performed in a microcentrifuge (ThermoScientific, Paisley, UK). The red blood cells (RBCs) were sedimented by centrifugation of the PRP along with the top layer of erythrocytes at 100 g for 3.5 minutes. The supernatant PRP was incubated with prostaglandin I_2 (2 μg/ml, 5 min, 37^{o}c) and centrifuged at 100 g for 5 minutes (Vargas et al 1982). After removing the supernatant the platelet pellet was then resuspended in Modified Tyrode's buffer (134mM NaCl, 2.9mM KCl, 0.34mM Na_2HPO4. $12H_20$, 12mM $NaHCO_3$, 20mM HEPES, 1mM $MgCl_2$, 5mM glucose, pH 7.35). Platelets from a number of mice were pooled together in buffer and infused into the recipient donor via injection into the tail vein.

2.4 Culture of murine bone marrow-derived mast cells (BMDMCs)

Femur and tibia bones were taken from mice, and these were flushed using a syringe containing RPMI to remove the bone marrow cells. The bone marrow cells were collected, centrifuged and resuspended in RPMI containing; 10% foetal bovine serum (FBS), 100U/ml penicillin, o.1 mg/ml streptomycin, 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1mM nonessential aminoacids and 1 mM MEM amino acids (all purchased from Sigma) in the presence of 5ng/ml recombinant mouse IL_3 (Peprotech). Cells were maintained for 4 weeks with continuous enrichment for non-adherent fractions of mast cell precursors. After this time mast cells constituted over 90% of the population, and this was assessed by FACS staining using antibodies against mouse FceR1-PE (eBioscience, clone MAR-1) and CD117(c-Kit)-APC (eBioscience, clone 2B8).

Mast cell functionality was checked by stimulating them to degranulate by sensitizing them overnight with 100 ng/ml mouse anti-DNP-IgE(sigma), they were then washed and stimulated with 100 ng/ml DNP-HAS (sigma) for 1 h (fast release) or 6 h (slow release). To monitor the mediators released β-hexosaminidase activity was measured in the mast cell releasates.

2.5 Neutrophil isolation from mouse bone marrow

The femur bones were taken from mice, and using a 25 gauge needle were flushed out using 0.01% EDTA to collect the marrow. The cell suspension was then passed through a 70μm filter, and the cells were then spun at 1500 rpm for 5 minutes. The supernatant was discarded and the cells resuspended in 1ml EDTA. Ice-cold water was then added (4ml) to lyse red blood cells, after 30 seconds isotonicity was restored by adding 4 X PBS (2ml). The cells were spun again at 1500 rpm for 5 minutes, the supernatant discarded and the remaining cell pellet resuspended in 2ml EDTA. A percoll gradient was then set up at bands of 72%, 64%, and 52% percoll, with a 2ml layer of the cell suspension on top. The gradient was then spun at 1500 g for 30 minutes to separate out the neutrophils. The neutrophils are separated out as a band at the interface between 72% and 64% percoll. The band containing the neutrophils was removed and washed to remove any percoll by spinning at 1500rpm using PBS. The wash step was repeated twice, and the neutrophil cell pellet resuspended in 1ml PBS.

2.6 Isolation of neutrophils from human blood

Human blood was collected in either EDTA or heparin (10U/ml) as an anticoagulant. A layer of 6 ml Histopaque 1119 (Sigma) was added to a falcon tube, followed by a layer of 5 ml whole blood on top of the Histopaque. This was then spun at 800 g for 20 minutes, and separates out into 4 bands. The top two bands are discarded and the lower 'redish' band containing granulocytes and some erythrocytes were transferred to a separate tube. The cells were washed in PBS and centrifuged for 10 minutes at 300 g. A 100% percoll solution was then made up, and using 1x PBS a gradient of 85%, 80%, 75%, 70% and 65% percoll was the prepared by layering 2 ml of each solution on top of each other in decreasing order. After spinning and resuspending in PBS, 2 ml of the cell solution was layered on top of the percoll gradient. This was then centrifuged at 800 g for 20 minutes. The top layer and most of the 65% layer was then discarded and the remaining layers up to the 85% interphase were collected into a few falcon tube. The cells were washed in PBS and centrifuged at 300 g for 10 minutes. The supernatant was then removed and the cell pellet resuspended in PBS.

2.7 Animal experimentation – in vivo models

2.7.1 Laser injury model of the cremaster muscle

Male mice were anesthetized using tribromethanol (240 mg/kg). The mice were placed in the supine position; the carotid artery was isolated and cannulated. A tracheostomy tube is also inserted into the trachea to help the mouse to breath.

The mouse's testicle was then exteriorized and the connective tissue surrounding it was carefully removed with blunt forceps. Once an incision has then been made along the midline of the testicle the cremaster can then be carefully spread and pinned down onto a circular stage. The microvasculature can then be visualized on the microscope connected to a camera.

The cremaster preparation was constantly kept wet throughout the procedure using a warm bicarbonate-buffered physiological salt solution (PSS) prepared in ultrapure H_20.

The mouse was injected via the cannulated carotid artery with an antibody to fluorescently label platelets (Emfret analytics) at a dose of 0.1ug/g. Injury is produced in both arteries and veins by targeting the built in laser to 'shoot' at the vessel wall, thrombus formation was then visualized.

2.7.2 Intravital microscopy of mesenteric vessels (ferric chloride model)

Mice were anesthetized with midazolam and hypnorm (200 µl hypnorm, 200 µl midazolam, 400 µl water, given at a dose of 10 µl/g body weight) and an incision was made along the abdomen. The mesentery was exteriorized and carefully spread out flat so both arteries and veins could be visualized. A paper strip was saturated in a 10% ferric chloride solution and placed across the vessels for 5 minutes. After this time point the strip was removed and observed down the microscope for either 40 min (maximum cut off time) or until the vessel had occluded. Time taken for the vessel to occlude was recorded. The mice were injected retro-orbitally with a platelet-labeling antibody (1µl per gram body weight) to visualize thrombus formation and Rhodamine 6G to visualize rolling leukocytes.

2.7.3 Flow restriction in the inferior vena cava (IVC): a model of DVT in mice

The established flow restriction model of DVT has been previously described (Brill et al 2011). Mice were anesthetized using isoflurane, placed in a supine position, and attached to a mask with a constant flow of isoflurane-oxygen mixture. An incision was made along the midline of the abdomen and the guts exteriorized. Saline was applied to the guts regularly throughout the experiment to prevent drying out. The Inferior vena cava (IVC) was gently separated from the aorta. Any side branches were closed using a 7.0 polypropylene suture. To achieve stenosis (partial flow restriction) the IVC was ligated over a 30-gauge needle or 'spacer'. The suture was tied over the needle and then the needle was removed, this achieves ~90% closure of the vessel lumen but doesn't cause endothelial injury. The peritoneum was then closed using a 6.0 suture, and the skin was stapled back together. After 48 hours the mice were culled and the thrombi, if they developed in the IVC, were taken for analysis.

2.7.4 Investigating the role of mast cells using the IVC stenosis model

For mast cell experiments (chapter 4) the mast cell degranulator compound 48/80, or histamine was topically applied after stenosis. The IVC was partially closed as described above, and for these experiments a piece of watmann paper was soaked in a solution of compound 48/80(1 mg/ml), histamine (100 mg/ml) or saline as a control. The strip was placed on the IVC at the place of stenosis for 10 minutes. After this, the paper was removed and the peritoneum and skin were then closed as described above.

2.7.5 Stasis model of DVT

In order to achieve complete stasis of the IVC, back branches and side branches were all fully closed. Back branches were cauterized, and this achieved 100% closure of the vessel lumen. The rodent stasis model of DVT has been described before elsewhere (Henke at al 2001). Thrombus development was then checked in these mice 24 h post-surgery.

2.7.6 Intravital microscopy of the IVC

After mice had undergone IVC stenosis as described above (2.7.3) they were allowed to recover consciousness. 6 hours after surgery, they were anesthetized using tribomethanol, and syngenic washed platelets (2.5% of the total number of circulating platelets) labeled with calcein AM were injected intravenously. The mouse was placed in a supine position, the IVC exposed and a round coverslip was placed on top of the IVC. Fluorescent platelets were visualized 1-2 mm below (in caudal direction) the ligation site using 3i VIVO-SDC confocal system, with Yokogawa CSU-10 and Photometrics Evolve EMCCD camera, on an Olympus BX61WI upright microscope. The air objective x10 was used, and focus was adjusted to the upper focal plane. Flow was recorded for at least 1-minute and Fiji/ImageJ was used to quantify areas of adhered platelets. Median-based intensity projection from 10 randomly chosen consecutive acquired images (corresponding to 1 s) was employed to generate a single projected image. After automatic set of threshold, total area of adhered platelets was measured by a Fiji incorporated function 'analyze particles' using image J software (NIH, USA).

2.8 Immunohistochemistry

DVT thrombi, and also thrombi still in the intact IVC were collected from mice and fixed in formalin. The tissue was frozen in OCT blocks, and cryosections 10μm thick were cut and mounted onto polylysine adhesion slides (ThermoScientific Paisley, UK) for staining and stored at -80^{o}c.

Fluorescent staining of thrombi cyrosections

For staining for podoplanin (chapter 3) slides were removed from -80^{o}c and immersed in ice-cold acetone for 10 minutes. After leaving them to air dry for 15 minutes, they were blocked with 1% BSA, 5% goat serum in 0.1% Tween for 1h at room temperature (RT) without permeabilization. They were stained with the primary antibody: hamster anti-mouse PDPN (eBioscience), rabbit anti-human VWF (DAKO) overnight at 4oC. After washing, Alexa-Fluor labeled secondary antibodies (molecular probes) were added for 45 min at RT. Nuclei were also stained using ToPro3.

Staining for T-cells and neutrophils in thrombi (chapter 5) was performed in a similar way described above. But slides were permeabilized using 0.3% Triton in blocking buffer (3% BSA in PBS), washed and incubated in blocking buffer for 1 h at RT. Primary antibodies anti-CD3 and anti-Gr-1 were used to stain for T-cells and neutrophils respectively, whilst anti-CD45 and anti-CD41 were used to stain for leukocytes and platelets (Overnight at 4^{o}c). The next day secondary antibodies were added and ToPro3 was used to stain nuclei.

After staining images were taken on the Leica confocal microscope. Scanning of the whole thrombus was performed using Axio Scan.Z1 (Zeiss) and the images were analyzed using Fiji/imageJ.

2.9 Western blotting

Thrombi were taken from mice following DVT surgery (IVC stenosis), the IVCs were also taken separately from control and operated mice, and these were all lysed. 50 μg of total protein was loaded per lane and separated in SDS-PAGE. Protein gels were blotted to PVC membranes in Turbo Blot Transfer system. They were blocked with 3% BSA and developed using the primary antibodies: hamster anti-mouse PDPN (eBioscience, clone 8.1.1) and mouse anti-tubulin (Sigma). Densitometry of bands was performed and results were normalized to the density of the corresponding tubulin band.

2.10 Measuring soluble P-selectin

The content of soluble P-selectin (sP-selectin) in plasma samples (chapter 3) was measured using an ELISA kit (R&D systems) in accordance to manufacturer's instructions.

2.11 Activating neutrophils for NET formation

A 24 –well cell culture plate was prepared by placing a 13mm round glass cover slip into each well, or wells were coated in Poly-lysine (Sigma). $2x10^5$ neutrophils in RPMI were seeded into each well and allowed to settle in an incubator at 37°C for 1 h. In order to activate the neutrophils to produce neutrophil extracellular traps (NETs) they were treated with the following; PMA (100 nM), LPS (100 μg/ml), ionomycin (20 μM) or glucose oxidase (10 mU/ml) and were incubated at 37°C for 3 h. After 3 h incubation, the cells were stained with DNA dye DAPI, fixed in 2% formalin and NET formation was analyzed using

a fluorescent microscope. In some wells, cells were also stained for citrullinated histone H3 using an anti-Histone H3 antibody (Abcam)

2.11.1 Quantitative analysis of NET formation

The extracellular chromatin was stained with DAPI and the area covered by NETs was quantified by the ImageJ software. The result was expressed as a percentage of the area covered by NETs to the total area of each view field.

2.12 Statistics

The sample size estimation was performed using G*Power 3.1.9.2 (Faul et al 2007) Based on our current experience with the model at the University of Birmingham, thrombi prevalence in control mice after 48 h IVC stenosis varies between 60 – 100%. Therefore, we assumed the expected difference (effect size) between experimental groups to be at least 0.8, error probability of 0.1 and the desired power value of 0.80. The calculated sample size for DVT experiments was 15 animals per group. In cases when statistical significance was achieved we used lower numbers of animals.

Non-parametric results (e.g., length and weight of thrombi) were compared by the Mann-Whitney test. Parametric data (e.g., area covered by adhered platelets) were compared using two-tailed unpaired Student's t-test or (in case of multiple comparisons) ANOVA. Thrombosis prevalence was compared using a contingency table followed by the Fisher's exact square method. Difference between experimental groups was considered significant at $p<0.05$

Chapter 3

The role of GPVI and CLEC-2 in deep vein thrombosis

3.1 Introduction

Platelets are critical in hemostasis, and upon vascular injury are the first cells arriving at the site of damage. They are able to adhere to the vascular wall via exposed subendothelial proteins such as collagen, and after capture of VWF they become activated and develop a preliminary clot, sealing the damaged area in the vessel wall.

Platelets become activated by the binding of various stimulatory ligands such as collagen and ADP, which triggers a signaling cascade, ultimately leading to a conformational change in αIIβb3 (known as inside out signaling). This leads to platelet aggregation and formation of the primary clot. Thrombin then acts to converts fibrinogen into fibrin; this polymerized fibrin along with platelets forms a hemostatic plug. Clot retraction, or 'shrinking' then usually occurs over a period of days as a result of fibrin capturing coagulation factors from platelets.

One such mechanism of platelet activation is implemented through receptors containing an ITAM or structurally similar (hem)ITAM domains. Murine platelets express the receptors GPVI and CLEC-2, which are ITAM and (hem)ITAM bearing receptors respectively. In contrast human platelet also have an additional ITAM-containing protein FcϒRIIA (Bergmeier 2006). GPVI binds collagen and fibrin, whereas CLEC-2 is a receptor for podoplanin. Until now, podoplanin expression has been detected in various cell types, such as lymphatic endothelium and tumor cells, but not in the blood vasculature (Suzuki-Inoue 2010; Leyre 2013), with the exception of recent findings by Hitchcock et al (2015).

The physiological role of podoplanin (PDPN) that has been most well researched is its interaction with CLEC-2 on platelets leading to their aggregation and

activation (Astarita 2012). Podoplanin is also known to play a critical role in development of organs such as the heart and lungs, as well as the lymphatic system, demonstrated in studies using podoplanin-deficient mice. Much of the understanding of podoplanin and its mechanisms has come from studying tumor cells, which upregulate PDPN expression during epithelial-mesenchymal transition, this up-regulation is linked to increased metastasis (Astarita 2012).

We already know that podoplanin plays a critical role in development. It is expressed in the developing mouse embryo on day E9, but over time is restricted to certain cells such as alveolar type 1 and lymphoid cells by adult stage, and is critical in the development of these organs (Astarita 2012). Deficiency in podoplanin in mice embryos is lethal, and they often die in utero, or ones that survive die within a few days after birth (Mahtab et al 2008). Recent critical findings were that podoplanin and CLEC-2 drive cerebrovascular patterning, and are important in vascular integrity maintenance during development (Lowe et al 2014). Lowe et al found that podoplanin expression on the neuro-epithelium (epithelium of the neural tube) was important in interacting with CLEC-2 on platelets. This interaction mediated platelet adhesion, aggregation and secretion, which ultimately guided the maturation of the developing vasculature, supported vascular integrity and prevented hemorrhage. They showed that constitutive deletion of CLEC-2 or podoplanin led to a similar pattern of hemorrhaging from E11.5, this was associated with defective angiogenesis of cerebral blood vessels.

Both CLEC-2 and GPVI receptors play a role in platelet activation and arterial thrombosis. Platelet activation by diesel exhaust particles has been shown to be dependent on both receptors (Alshehri 2015).

CLEC-2 has been reported to play a role in ferric chloride- and photochemically induced thrombosis in mesenteric arterioles (May 2009; Suzuki-Inoue 2010). May et al showed that whilst CLEC-2 deficient platelets displayed normal adhesion under flow, subsequent aggregate formation was dramatically defective in both vivo and in vitro. They report that CLEC-2 deficient mice have increased tail-bleeding times, and increased protection from occlusive arterial thrombus formation. Demonstrating an essential role of CLEC-2 in hemostasis and thrombosis. Similar findings were reported by Ozaki et al (2010). They suggest that CLEC-2 is required for the 'platelet piling up' process, which eventually leads to stable thrombus formation. They demonstrate that single cell adhesion appeared to be normal on a collagen surface, but thrombus formation was severely impaired. Both groups show that arterial thrombus formation was prevented in CLEC-2 knock out mice, with Nieswandt et al (2009) also reporting this leads to increased tail bleeding times, although this increase in bleeding time is unlikely as many others have not confirmed this (Watson 2010, Bender 2013). However, any evidence that CLEC-2 may also play a role in venous thrombosis has been lacking.

Massberg et al. used a ferric chloride injury model to demonstrate that GPVI-deficient mice have longer time to occlusion with some mice showing no occlusion at all (Massberg 2003). Bender et al. (2013) and Alshehri (2015) reported impaired thrombus stability after ferric chloride application in mice after GPVI-depletion or in GPVI-deficient mice.

Recently, it was found that GPVI can bind fibrin and mediate via this mechanism various platelet activation events (Mammadova-Bach 2015; Alshehri 2015). Kalia and co-authors reported severely impaired thrombus development in FcRg-deficient mice that don't express GPVI (Kalia 2008). In contrast, others have shown that GPVI plays a less significant role. Eckly et al (2011) using the same laser injury model to induce arterial thrombosis, showed that deficiency in both GPVI and β1 integrin's did not suppress thrombus formation. The role that GPVI may play in venous thrombosis had not previously been reported.

The ITAM and (hem)ITAM receptors seem to play only minimal role in normal hemostasis. One report demonstrated that CLEC-2 deficiency prolonged tail-bleeding time (May 2009), whereas others found an insignificant trend or no increased blood loss (Suzuki-Inoue 2010; Moroi, Watson; Hughes 2010; Bender 2013). Lack of GPVI did not result in impaired hemostasis in one study (Lockyer et al 2006) and led to a moderately prolonged bleeding time in another one (Nieswandt 2001). However, deficiency in both CLEC-2 and GPVI, results in severely impaired both hemostasis and thrombosis (Bender 2013).

In addition to their hemostatic roles, platelets play a role in a number of non-hemostatic processes. Platelets have been shown to maintain vascular integrity and prevent hemorrhage under inflammatory conditions (Goerge 2008; Gros et al 2014; Lee et al 2016). Recent studies demonstrated the critical role of platelet (hem)ITAM receptors in this process (Boulaftali 2013). They demonstrated that inhibition of GPVI, or deficiency in CLEC-2, partially reduced the ability of platelets to maintain vascular integrity in both LPS induced inflammation in the lung, and immune-complex induced inflammation in the skin. Hillgruber et al

found that neutrophil diapedesis across the endothelial barrier was the primary cause of hemorrhage under thrombocytopenic conditions. GPVI and CLEC-2 are critical in prevention of this inflammatory induced hemorrhage. Thus platelet ITAM and hem(ITAM) receptors modulate the functional state of the endothelium in inflammation.

Deep vein thrombosis (DVT) and pulmonary embolism, the fatal consequence of clots detaching and moving to the lungs are designated together as venous thromboembolism (VTE). This disease poses a disastrous health problem, with around 1 per 1000 adults developing the disease annually (Cushman 2007). The prevalence of VTE has not significantly decreased over the past 2.5 decades (Heit 2008; Heit 2006) despite new prophylactic methods, suggesting new therapeutic targets are needed for this disease.

Although the mechanisms of venous thrombosis are not completely understood, we know it occurs without endothelial denudation (Sevitte 1974). In contrast to arterial thrombosis, which is based on platelet accumulation at the site of ruptured atherosclerotic plaques. One of the main causes of DVT, with the exception of cancer or trauma related DVT, is stagnant blood flow in the venous valves, a component of the Virchow's triad (Esmon 2009). As a result of flow distortion, local hypoxia may develop and result in an even more pro-thrombotic environment. At static conditions, blood which pools inside valvular pockets has been shown to become rapidly hypoxic, with pO2 returning back to normal levels when flow is restored and blood exchange in pockets become quicker again (Hamer et al 1981). We know that hypoxia can induce the release of Weibel-Palade bodies (WPB), which are the storage granules of endothelial cells, containing P-selectin and VWF, implemented in inflammation (Pinsky et al

1996). Brill et al (2011) recently showed that the release of WPB's and cell recruitment to the vessel wall are critical in a murine model of DVT.

Thus, DVT develops in a similar way to sterile inflammation, with initiation depending on the recruitment of immune cells and platelets to the vessel wall. Because of this, DVT can be described as a thromboinflammatory disease or 'immunothrombosis' (Engelmann & Massberg 2013), as venous thrombosis is closely linked with inflammatory mechanisms.

We therefore hypothesized that the platelet receptors responsible for regulating the functional properties of the vessel wall during inflammation could also play a role in DVT.

In this chapter, we used both the laser injury model and the well-established model of IVC stenosis (which mimics the flow distortion in venous valves), to investigate the roles of GPVI and CLEC-2 in venous and arterial thrombosis in a comparative aspect. The main aims of this chapter are (i) to investigate if the absence of GPVI affects thrombosis (ii) to investigate the role of CLEC-2 in thrombosis, and (iii) to determine if podoplanin expression in the vessel wall correlates with the degree of thrombosis.

3.2 Results

3.2.1 CLEC-2 plays a critical role in DVT formation in mice

To investigate the role of CLEC-2 in venous thrombosis (DVT) we used the well-established model of IVC stenosis, described in methods 2.7.3. 48 hours after mice underwent DVT surgery (stenosis application), mice with an inducible loss of CLEC-2 (methods) were protected from developing DVT in 100% of cases, compared to 60% of the mice in the littermate control group, which did produce a thrombus ($P < 0.03$ figure 3.1 A). The frequency of thrombosis in the control group was similar to what is normally seen in C57BL6 mice using this same model, where it can range from 60 to 100%. No phenotype was observed in the stasis model (data not shown). In order to test if this role was specific to CLEC-2 found of platelets, we used the same model on platelet-specific knock out mice `and their littermate controls. In the control group, 81% of mice produced a thrombus post stenosis, compared to 38% in the knockout mice ($p<0.05$, figure 3.1B). We then aimed to investigate if this response could be reversed. We transfused the knockout mice with WT platelets (to around 50% of circulating platelets) and thrombosis was restored in these mice (as shown in figure 3.1B), further supporting the role of platelet specific CLEC-2 in DVT.

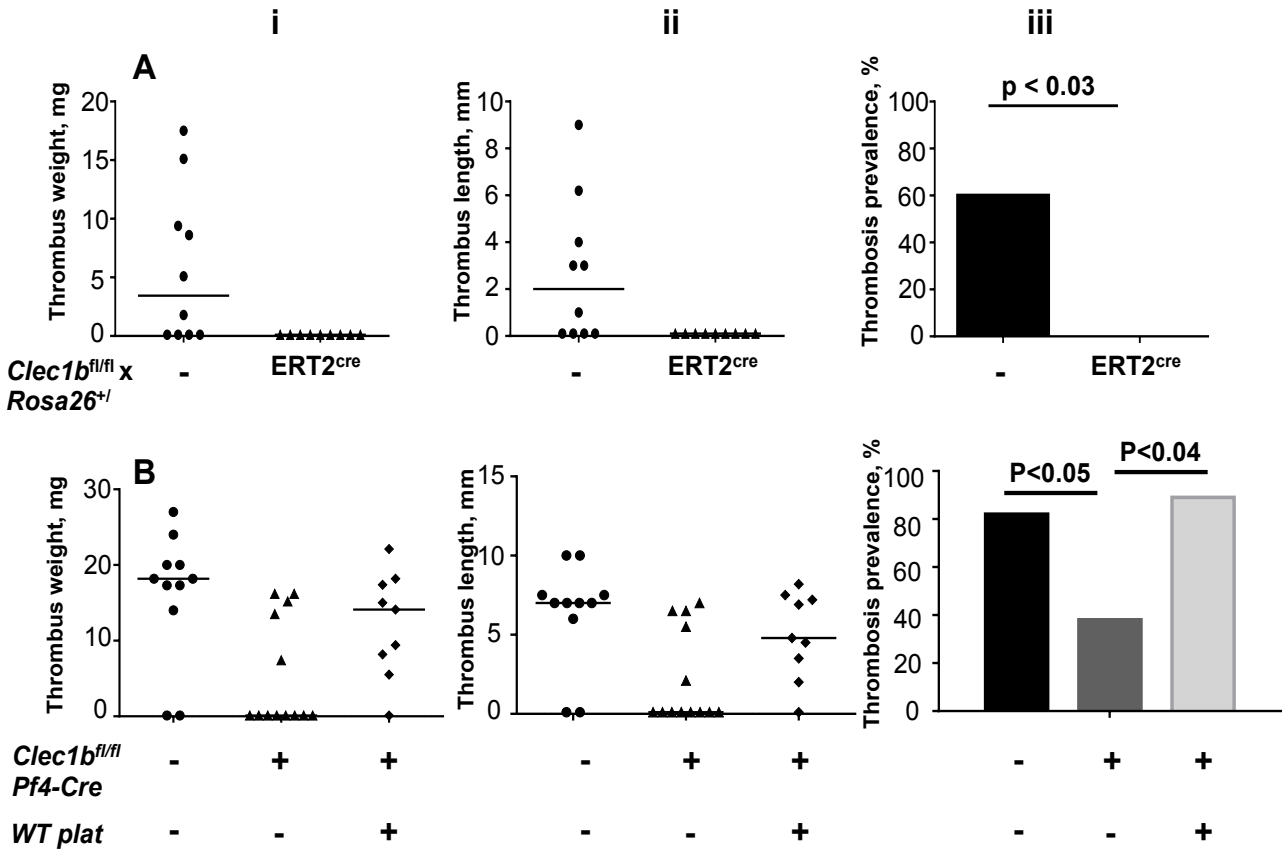


Figure 3.1 - CLEC-2 exacerbates DVT

Panels i, ii and iii show thrombus weight, thrombus length and thrombosis prevalence respectively. Each dot represents one mouse, with the horizontal line representing the median A) Thrombus prevalence after 48 hour IVC stenosis application in clec1b$^{fl/fl}$ xRosa26$^{+/creERT2}$ (n=10) and Clec1b$^{fl/fl}$ littermates (n=9). B) Thrombus prevalence 48 hours after IVC stenosis in Clec1b$^{fl/fl}$ PF4-Cre mice (n=13) that received a transfusion of WT platelets (8 x 10^8) and control littermates (n=11). Of importance is the restoration of thrombus after transfusion of WT platelets.

(Performed in collaboration with A.Brill.)

3.2.2 GPVI is not critical for DVT formation in mice

To investigate if GPVI also played a critical role in venous thrombosis similarly to CLEC-2, we again used the IVC stenosis model to replicate DVT in GPVI KO mice. After 48 hours stenosis, 30% of GPVI deficient mice produced a thrombus compared to 69% of control mice (p = 0.07, figure 3.2). Whilst this data suggests that GPVI may play some role, it does not appear to be significant in leading to DVT.

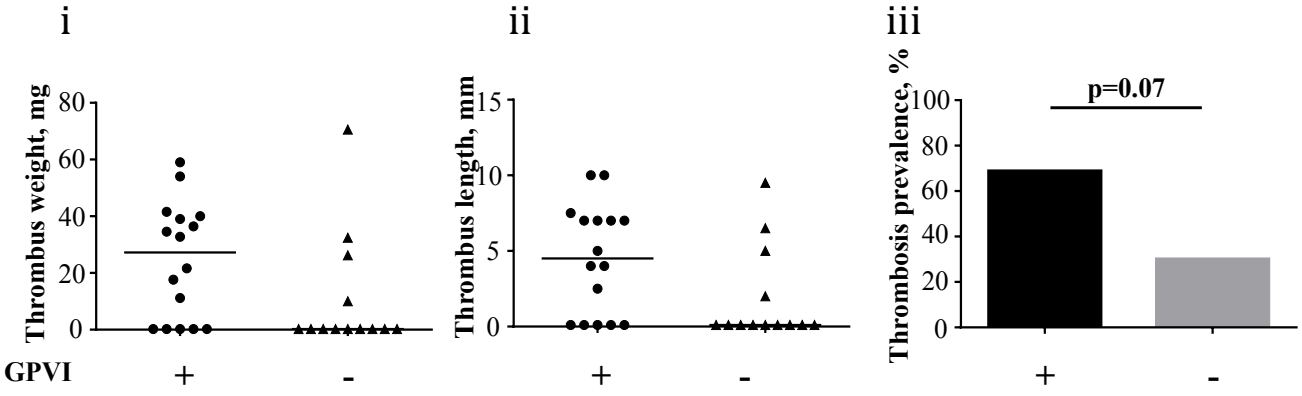


Figure 3.2 – GPVI is not critical in DVT formation

Graph i) shows thrombus weight, ii) thrombus length and iii) thrombosis prevalence. Each dot represents one mouse. Thrombus prevalence in GPVI deficient mice (n= 13) was not statistically significantly different from control mice (n=16) (p=0.07 iii). *(Performed in collaboration with A.Brill)*

3.2.3 Mice without CLEC-2 have a reduced recruitment of platelets to the vessel wall

It had previously been reported that endothelial activation, secretion of Weibel-Palade bodies and the resulting accumulation of platelets and immune cells at the vessel wall are critical events in the development of DVT (Brill et al 2011, Von Bruhl et al 2012). We therefore used intravital microscopy to assess platelet recruitment to the vessel wall in CLEC-2 deficient and WT mice. Mice underwent stenosis of the IVC as previously described, 6h post stenosis application mice were then opened and intravital microscopy was performed (methods 2.7.5). In WT controls, a large proportion of platelets were adhered to the vessel wall and remained fixed during the period of observation (supplementary videos can be observed on request). The average area covered by adhered platelets was 7.8 ± 0.7% of the view field (Figure 3.3 A). In contrast, in CLEC-2 deficient mice most platelets rapidly flowed away in the direction of the blood flow and did not firmly attach to the IVC wall. The average area covered by adhered platelets in mice lacking CLEC-2 was 1.3 ± 0.4% (Figure 3B, $P < 0.003$). This implies that the release of platelet-recruiting molecules from the endothelium is strongly reduced in the absence of CLEC-2.

We then further investigated if podoplanin, the ligand for CLEC-2, was involved in this process. A specific neutralizing antibody was used to inhibit podoplanin. In these mice, a similar phenotype was observed to that in the CLEC-2 deficient mice. The area covered by adhered platelets in IgG controls was 6.5 ± 1.3%, compared to an area of 2.6 ± 0.4% after antibody administration; this was significant at $P< 0.03$ (Figure 3.3 C & D). Together these results suggest that both

CLEC-2 and podoplanin expression are required for platelet recruitment to the vessel wall, potentially through direct binding.

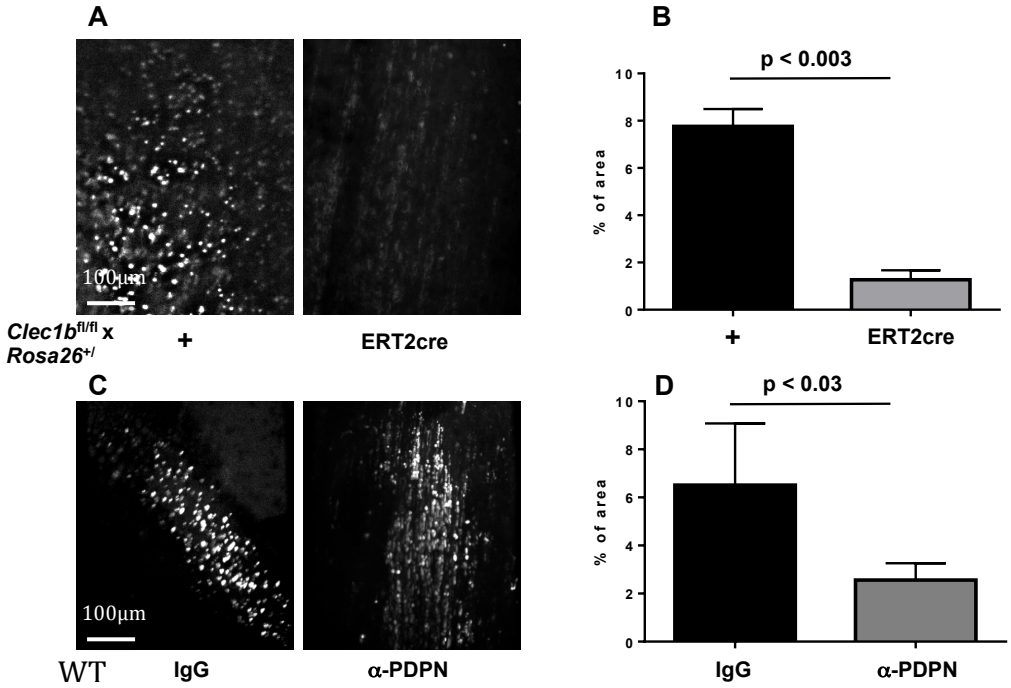


Figure 3.3- Stenosis-induced platelet recruitment is reduced in the absence of CLEC-2 and inhibition of podoplanin

Clec1b$^{fl/fl}$xRosa26$^{+/creERT2}$ and their Clec1b$^{fl/fl}$ littermates underwent IVC stenosis for 6h. Intravital microscopy was then used to visualize the deposition of infused fluorescently labeled syngeneic platelets on the IVC wall. A) Representative image of adhered platelets (in white) B) The percent of area covered by immobilized platelets. This was significantly different at $p<0.003$ (n=4, data are presented as mean ± SEM).

C) WT mice administered an podoplanin neutralizing antibody (100µg/mouse, I.V) or an IgG control prior to surgery. D) Shows the percent of area covered by recruited platelets (n= 4 for both group) this was significant at $p< 0.03$.

(Performed in collaboration with A.Brill)

3.2.4 Podoplanin is expressed in the IVC vessel wall

As both CLEC-2 and podoplanin deficient mice produced a similar phenotype in terms of platelet recruitment to the vessel wall during stenosis, we wanted to investigate this role that they may both play in venous thrombosis further. We tested to see if podoplanin was expressed in the IVC wall. Immunostaining showed that there was a low, but definative level of podoplanin expression in the vessel wall of sham-operated IVC's (Figure 3.4 Ai). Podoplanin expression was substantially increased in IVCs after thrombosis development, shown by the staining in Fig 3.4 ii. Podoplanin was expressed exclusively under the endothelium of the vessel (abluminal side) but not in the thrombus itself. The endothelium of the vessel wall was distinguished by positive staining for PECAM-1 (CD31) (Figure 3.4 Ci) and the thrombus was observed by staining for VWF (Figure 3.4 Cii). During DVT, platelets can penetrate the vessel wall and they may then come into contact with sub endothelial matrix components, potentially podoplanin (Figure 3.4 D).

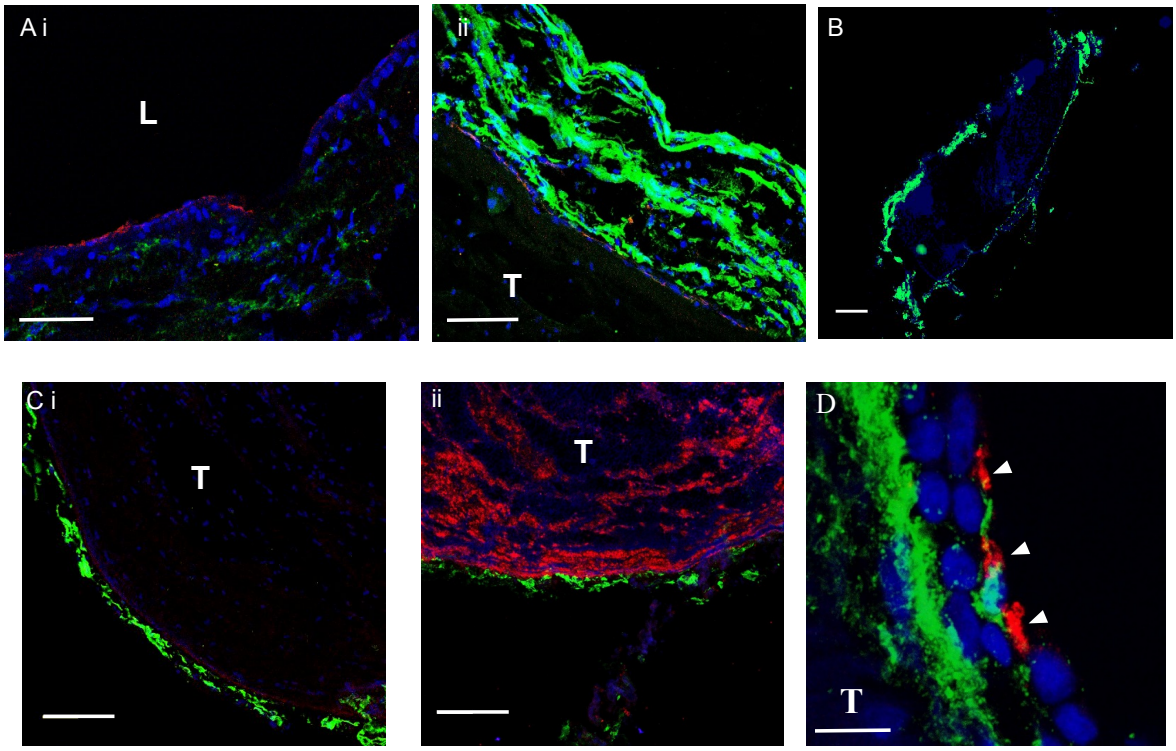


Figure 3.4 - IVC wall expresses podoplanin

Ai) shows the low level of staining for podoplanin (green) in a sham-operated IVC, compared to in **Aii)** where staining for podoplanin is much higher in a thrombosed IVC. 'L' designates lumen, 'T' designates thrombus. **B)** Shows the whole thrombus after 48 h of stenosis inside the IVC

Ci & Cii) Show that podoplnain expression is localized to the abluminal side of the endothelium and not in the thrombus itself, by staining for PECAM-1 (red) and VWF respectively (red).

D) Shows staining of IVC with a thrombus after 48 h stenosis. Arrowheads show where platelets area able to penetrate the vessel wall and localize in the vicinity to podoplanin.

A, scale bar is 50µm; B, Scale bar is 500µm; C scale bar is 100µm, D scale bar is 10µm. Representative images are taken n = 3 with 10-15 images from each IVC.

(*Performed in collaboration with T.Ponomaryov)*

3.2.5 Podoplanin expression in the IVC wall increases with thrombosis

To confirm the presence of podoplanin in the vascular wall further, we performed western blotting (methods 2.9). Podoplanin was not found in 4 out of the 5 thrombi tested as shown in Figure 3.5A, with only very small trace amounts found in the fifth thrombus, but this can probably be attributed to the fact remnants of the vessel wall may have contaminated the sample. Podoplanin was detected in the intact IVC (sham-operated) that had not undergone stenosis, suggesting it is constitutively expressed. This is shown in Fig 3.5 B in the three left lanes. After 48 h stenosis, leading to thrombosis, this resulted in an increased podoplanin expression in the IVC wall compared to non-thrombosed IVC's. This was measured by band densitometry and normalized corresponding to the control (tubulin) shown in Figure 3.5 B. Podoplanin expression increased from 3.6 ± 1.3 arbitrary units in the sham operated IVC, to 33.4 ± 8.3 arbitrary units in the IVC with a thrombus post stenosis.

Furthermore, we also measured podoplanin expression in the vessel wall of IVC's that underwent stenosis for the same time period, but did not result in thrombus formation. We found that podoplanin expression demonstrated an insignificant trend to increase in the intact IVC, but to a lesser extent that in those IVCs that resulted in thrombosis after stenosis. Podoplanin expression in the stenosed IVC without a thrombus increased to 14.3 ± 4.7 a.u compared to the intact IVC ($p=0.053$ Figure 3.5 C). Therefore, podoplanin expression in the IVC wall does increase as a result of flow restriction, but the magnitude of this increase corresponds to the event of thrombosis.

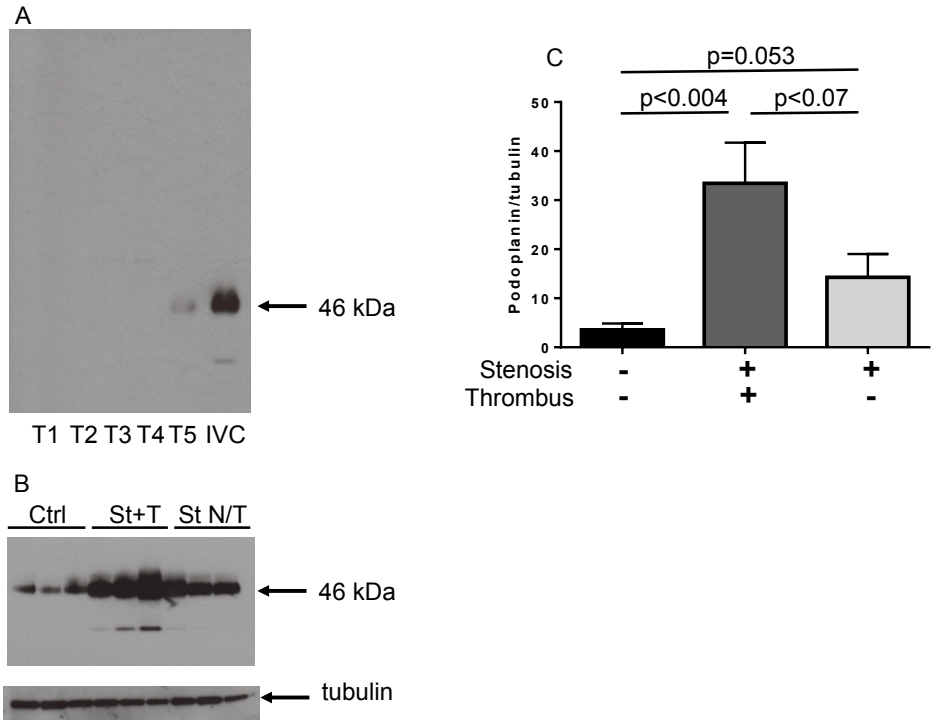


Figure 3.5 – Podoplanin expression in the IVC wall increases with thrombosis

A) Western blot of 5 thrombi compared to 1 IVC after 48 h stenosis. Note no podoplanin expression in 4 out of 5 thrombi, with only trace amounts detected in thrombi 5. Compared to the intact IVC where it is constitutively expressed.

B) Lanes 1-3 shows intact IVC, lanes 4-6 shows IVCs after stenosis with thrombi (st+T), lanes 7-9 show IVC after stenosis without thrombi (St N/T). This was measured by band densitometry against the tubulin loading control.

C) Shows that IVC's post stenosis have significantly higher expression of podoplanin in their vessel walls compared to control IVC's, with there being a more significant increase if stenosis has resulted in thrombosis ($p<0.004$). Data in bar graph represent mean ± SD, n = 6 for each group.

3.2.6 Testing of anti-podoplanin antibody

To test the specificity of the anti-podoplanin antibody, and therefore to allow us to be confident that the results described below (3.2.7) were due to podoplanin deletion, staining was performed. Specificity of the antibody was proven by a high amount of staining in the kidneys of WT mice (Podoplanin staining in green). No staining in mice with tissue-specific podoplanin deficiency (figure 3.6).

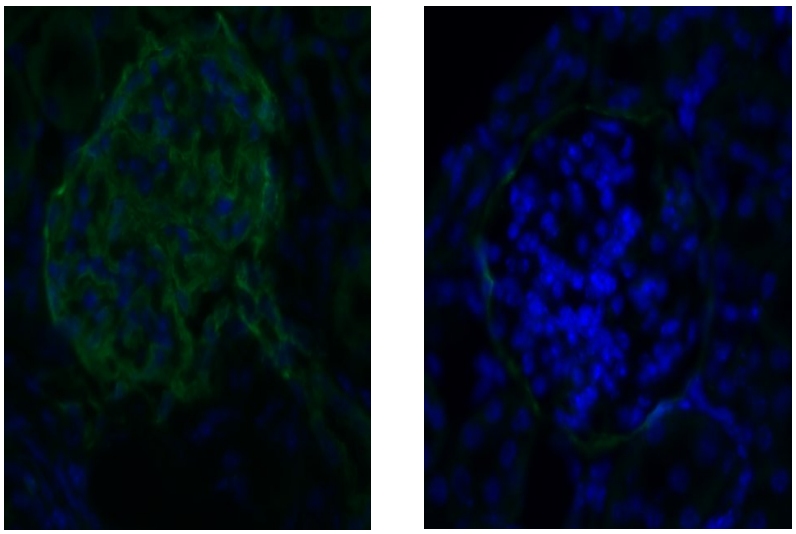


Figure 3.6 Testing of the anti-podoplanin antibody

Shows positive staining for podoplanin (green) in the kidneys of WT mice but no staining in the tissue specific podoplanin deficient mice. (*Performed in collaboration with T.Ponomaryov)*

3.2.7 Anti-podoplanin antibody decreases size of thrombi in murine DVT

In order to determine the role podoplanin plays in DVT we treated mice with anti-podoplanin blocking antibody (clone 8.1.1) as described in methods 2.1.6. There was no significant difference in thrombosis prevalence between IgG treated littermate control mice and antibody-treated mice, at 82% and 90% respectively (shown in figure 3.6 iii). As no significant difference was found in thrombosis prevalence we could then compare thrombus size using a non-parametric Mann-Whitney test. Mice treated with the podoplanin-blocking antibody had significantly lower weight, and length of thrombi compared to IgG treated mice ($p<0.02$ shown in figure 3.6 i & ii). This result suggests that podoplanin is also involved in DVT, affecting thrombus propagation rather than thrombus initiation.

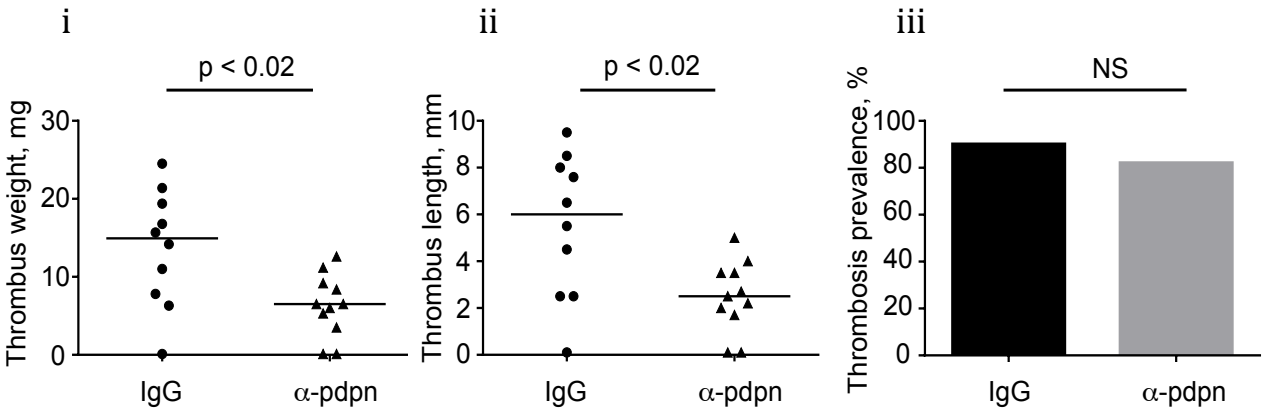


Figure 3.7 – lack of podoplanin results in decreased thrombus size in murine DVT

i & ii) Show thrombus weight and thrombus length respectively in mice following IVC stenosis. Both show that mice that received an anti-podoplanin

blocking antibody (clone 8.1.1) had a decreased thrombus size and weight compared to mice that received and IgG control ($p < 0.02$). Lines in the dot plots represent median. N=10 (IgG), N=11 (antibody)

iii) Shows that there was no significant difference in thrombosis prevalence.

(Performed in collaboration with A.Brill)

3.2.8 - Deletion of podoplanin in hematopoietic or endothelial cells does not prevent DVT

We have shown that podoplanin is expressed in the IVC wall and that this is up-regulated during DVT, with increased expression correlating to the degree of thrombosis. The source of this podoplanin is however yet to be identified. We next aimed to check whether the source of this podoplanin could be hematopoietic or endothelial cells. We therefore used mice with podoplanin specifically knocked out on these cells. $Vav1^{cre+}pdpn^{fl/fl}$ is a hematopoietic cell-specific knockout, while $Tie2^{Cre+}pdpn^{fl/fl}$ mice have podoplanin knocked out in cells of endothelial origin (mice described in methods 2.1.2). Both strains showed no phenotype in the DVT stenosis model, with thrombosis prevalence 48 h after IVC stenosis application, being comparable to normal WTs (Figure 3.8.1). Furthermore, mice lacking podoplanin in these cells still retained podoplanin expression in the vessel wall, as shown in Figure 3.8.2 Suggesting that these cells are not the source. The ubiquitous podoplanin staining in the IVCs of these mice also showed a 'layer-like' pattern and rules out lymphatic vessels and lymph nodes as a potential source.

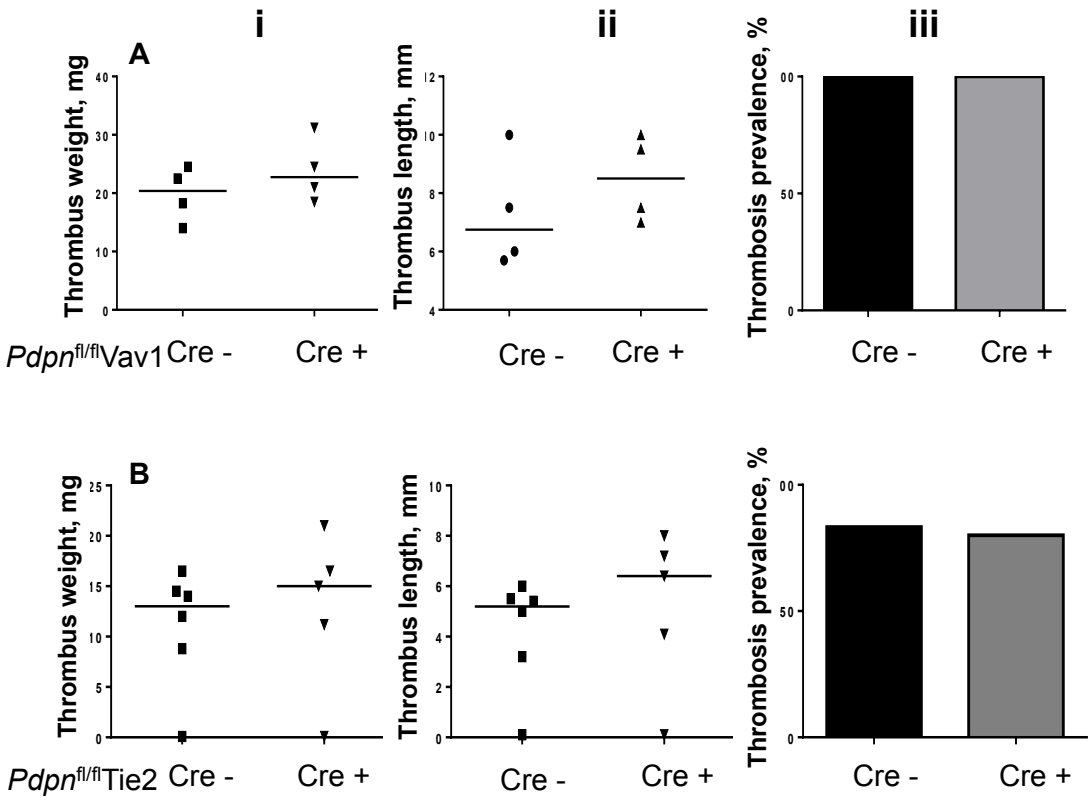


Figure 3.8.1 - DVT is not prevented after podoplanin deletion in hematopoietic or endothelial cells

Colum i) shows thrombus weight, ii) thrombus length and iii) thrombosis prevalence. A) Shows Vav1^{cre+}pdpn$^{fl/fl}$ mice with podoplanin deleted in hematopoietic cells compared to control group (n=4 for both) B) shows Tie2^{Cre+}pdpn$^{fl/fl}$ with podoplanin deleted in endothelial cells compared to control group (cre- n=6, cre+ n=5). Of note, neither knockout showed a phenotype after 48 h stenosis. *(Performed in collaboration with A.Brill)*

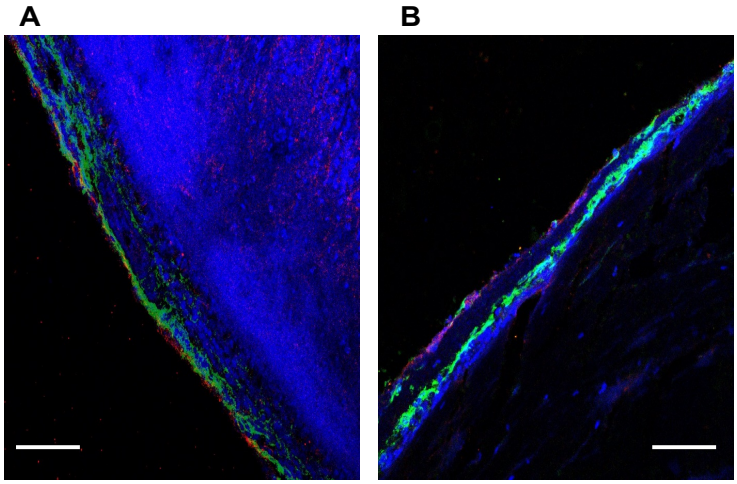


Figure 3.8.2 - Deletion of podoplanin in hematopoietic or endothelial cells does not prevent its expression in the IVC

IVCs with thrombi after 48 h stenosis were stained for PECAM-1 (red) and podoplanin (green). Representative images taken from N=3. Nuclei are in blue. Representative staining's from A, *pdpn*$^{fl/fl}$Vav1^{cre}+ and B, *pdpn*$^{fl/fl}$Tie2^{cre+} shows podoplanin staining (Green) in the vessel walls of mice lacking podoplanin in hematopoietic cells, and endothelial cells. *(Performed in collaboration with T.Ponomaryov)*

3.2.9 Lack of GPVI but not CLEC-2 impairs thrombus formation after laser injury

To study whether the differential roles of CLEC-2 and GPVI in DVT are specific to venous thrombosis or may be expanded to other endothelial activation-triggered thrombosis models, we next used the model of laser stimulation of arterioles in the cremaster muscle. Despite different thrombus composition (platelets constitute major component of a thrombus after laser injury (Celi 2002),

whereas in DVT, thrombi largely consist of red blood cells and fibrin), in both models, endothelial activation rather than denudation and injury, initiates thrombus development (Furie 2015). Different patterns of thrombus development are depicted in Figure 3.9 (graphs on the right side) as a function of integral fluorescence intensity of time. In control mice, despite some natural variability in size, thrombi developed within 1-2 min and were always 3-dimentional (Figure 3.9). Similar thrombi were observed in mice lacking CLEC-2. Animals, deficient for GPVI, demonstrated a phenotype, when thrombi started to develop but were unstable and constantly embolized. The resulting thrombus remained two-dimensional with a layer of platelets adhered to the site of injury but no thrombus propagation was observed. Combined deficiency of both receptors gave a phenotype similar to deficiency of GPVI only (movies available on request). Thus, absence of CLEC-2 is dispensable for laser-induced thrombosis, whereas GPVI is required for thrombus stability in this model.

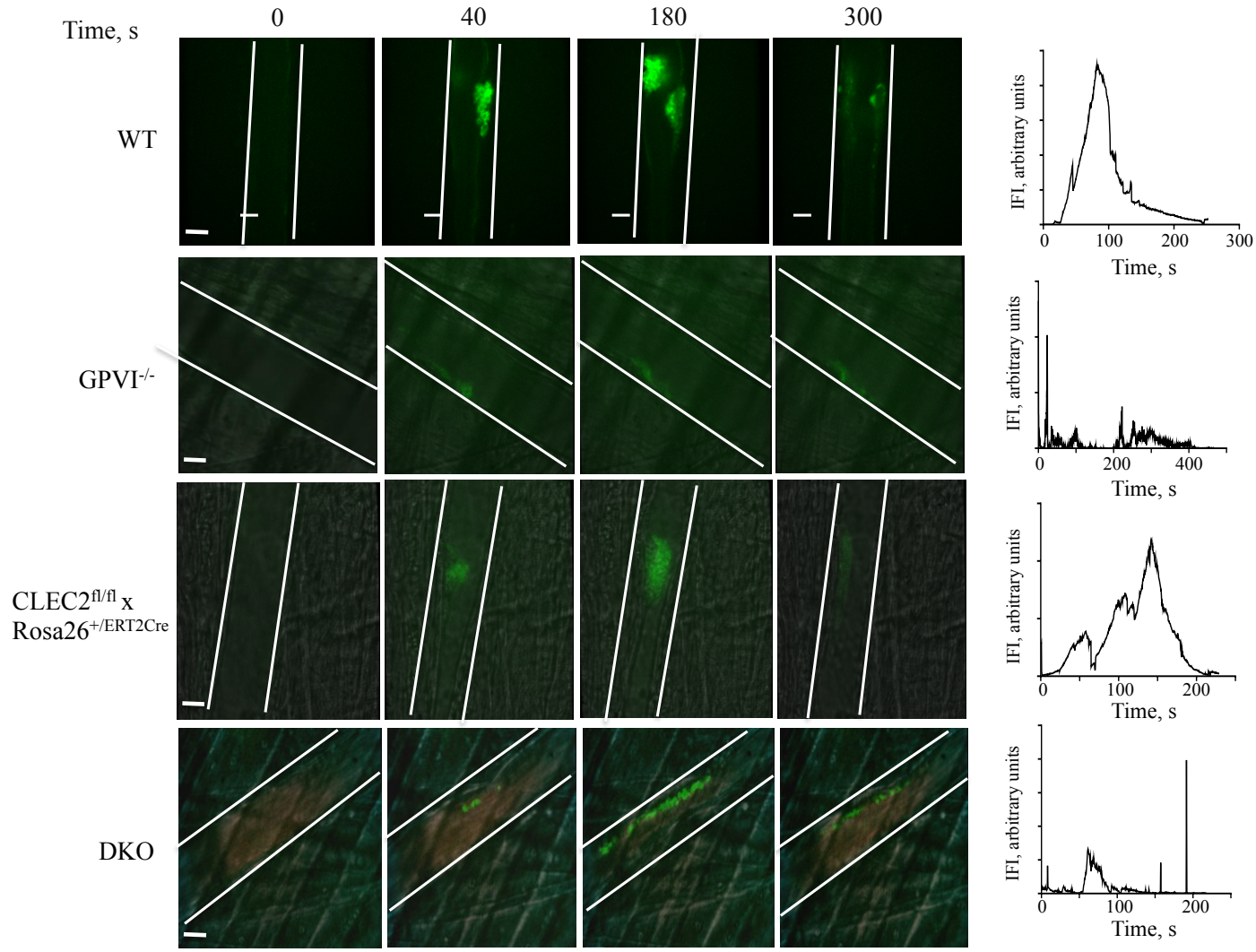


Figure 3.9 – lack of GPVI, but not CLEC-2 impairs thrombus formation in mice following laser injury

Fluorescent platelet-labeling antibody (green) was infused intravenously and thrombosis was induced by laser impulse in cremaster muscle arterioles of mice deficient for CLEC-2, GPVI, both receptors or WT controls. Thrombus development was recorded; integral fluorescence intensity was calculated and presented as a function of time (graphs). Representative images demonstrate thrombi at different time points (above). DKO stands for double knockouts. White lines delineate the vessel. Note the difference in the pattern of thrombus development between WT/CLEC-2-deficient and GPVI/double-deficient mice.

3.3 Discussion

This study demonstrates the differential roles that ITAM receptors CLEC-2 and GPVI play in thrombosis. We show that mice deficient in GPVI had only an insignificant trend towards decreased DVT prevalence. Whereas mice lacking CLEC-2 were protected against DVT, whilst this resulted in complete protection in CLEC-2 knockouts, those mice with a specific platelet CLEC-2 knocked out showed a partial, but a significant protection from DVT development.

This dramatic effect of CLEC-2 deficiency is unlikely to be related to impaired platelet-platelet interactions because laser-induced thrombosis in these mice was intact. Laser-initiated thrombosis absolutely depends on platelet aggregation because platelet accumulation can be prevented by a GPIIb-IIIa inhibitor eptifibatide (Atkinson et al 2010).

Neutrophils have also been suggested to express CLEC-2 in some studies, however others have reported this is not the case, and remains controversial (Kerrigan et al 2009; Lowe et al 2015). Our results showing that platelet-specific knockout mice have reduced thrombosis prevalence, suggests that platelet CLEC-2 plays a role in DVT initiation.

As can be seen in the results, the median thrombus size in the WT littermates of the Clec1b$^{fl/fl}$ x Rosa26$^{+/-}$ and the Clec1b$^{fl/fl}$ Pf4 $^{cre+}$ varies slightly. The reason for this is unclear but could be attributed to a number of factors such as treatment with tamoxifen, or that the mice carry different transgenes.

It may seem reasonable to hypothesize that because the platelet specific knockout mice present with blood-lymphatic mixing, that this could somehow contribute to the fact they show reduced thrombosis. However, we show that thrombosis is restored in the Clec1b$^{fl/fl}$ Pf4 $^{cre+}$ mice following transfusion of WT

platelets; despite the fact they still show blood-lymphatic mixing. Furthermore, the Clec1b$^{fl/fl}$ x Rosa26$^{+/-}$ inducible knockouts show an even greater phenotype than the platelet specific knockouts, and these mice do not have the defect in blood-lymphatic mixing.

It had been previously shown that a shift in the state of the endothelium (one of the factors in Virchow's triad) to a proinflammatory state, and the consequential release of WPB and recruitment of platelets and leukocytes to the vessel wall is a crucial step in the formation of deep vein thrombosis (Brill et al 2011). Meaning that the mechanisms of DVT initiation and development are similar to those in sterile inflammation.

We know that platelets are predominately recruited to the vessel wall in DVT as a result of VWF secretion by WPB's. A deficiency in CLEC-2 prevented this platelet accumulation at the IVC wall after stenosis, at the stage preceding thrombus development (shown in figure 3.3A). This observation suggests that the endothelium may remain quiescent under the conditions normally triggering thrombosis in the presence of CLEC-2 i.e. endothelial activation is suppressed. This suggests that platelets, in a CLEC-2 – dependent fashion, either directly interact with the vessel wall modifying its functional properties, or secrete and agent mediating this effect. It is likely that this direct interaction between CLEC-2 and the vessel wall is crucial for platelet recruitment and thrombosis initiation.

This direct interaction between CLEC-2 and the vessel wall may occur not only at the endothelial surface, but also at sites of endothelial junctions. As platelets penetrate the vessel wall during thrombosis, they might also interact with cell types other than the endothelium.

Stenosis of the IVC results in the formation of a 'bulb' where the vessel has been compressed, in which there will be a low flow rate with highly distorted flow. Blood pressure is also increased in this region. This could cause local hypoxia, and potentially up regulate podoplanin expression in the vessel wall, as has been demonstrated previously in other tissues (Kolenda et al 2011). These factors described above, along with local hypoxia, could result in the loosening of endothelial cell-cell junctions allowing platelet CLEC-2 to interact with podoplanin, or another possible ligand. Podoplanin up-regulation may not only be a cause of thrombosis, but it could also occur as a result of thrombus formation, while it is possible that they both processes work in tandem and in fact form a positive feedback loop.

This anti-thrombotic phenotype we observed in the stenosis model, was not seen when we repeated the same method using the complete stasis model. This could be attributed to the fact that thrombosis in the stasis model is a result of tissue factor driven blood coagulation (Day et al 2005), and the change in the pro-inflammatory state of the endothelium, which appears to be so crucial after partial occlusion, is less important in this model.

It has been reported that CLEC-2 and GPVI play a role in maintaining vascular integrity under inflammatory conditions (Goerge et al 2008). And it was shown that platelets lacking these ITAM receptors were unable to prevent hemorrhage (Bergmeier 2006). Therefore ITAM receptors, in particular CLEC-2, are able to modify the properties of the endothelium, which has a central role in DVT development. Although the mechanism, through which platelet (hem)ITAM receptors implement this function, is still unclear, it might be involved in the

decreased endothelial ability to recruit cells and protection against DVT in CLEC-2-deficient mice. A similar niche role of CLEC-2, which is again independent of GPVI, has been shown in Salmonella infection-induced thrombosis (Hitchcock et al 2015). They demonstrate a link between infection, inflammation and thrombosis, following bacterial infection in the liver, podoplanin expressing monocyte-lineage cells accumulate. As a result of bacteremia and inflammation, vascular endothelial cells become damaged, exposing platelets to these podoplanin-expressing cells, leading to a thrombotic response (Hitchcock 2015).

Given that DVT may be considered a thromboinflammatory disease, the prothrombotic effect we have shown for CLEC-2 may be mediated by neutrophils. It has been shown that flow restriction in the IVC, which results from stenosis, can lead to increased expression of CCL2 and CXCL1, trafficking agents which attract and activate neutrophils (Von Bruhl et al 2012). It has recently been discovered that platelets protect the vessel wall from inflammatory bleeding, part of the mechanism for this includes the prevention of deleterious neutrophil diapedesis (Goerge et al 2008). It has also been demonstrated that CLEC-2 and podoplanin are able to secure the vessel wall in high endothelial venules during lymphocyte transmigration in lymph nodes (Herzog et al 2013). Platelets are therefore known to support leukocyte recruitment to the vascular wall through various mechanisms, such as releasing chemoattractants or acting as a bridge between activated endothelium and leukocytes. Platelets potentiate the recruitment of leukocytes to the vessel wall during early stages of DVT development.

Leukocytes not only modulate physiological properties of the vascular wall, but they also play a role in the pathogenesis of venous thrombosis through the release of Neutrophil Extracellular Traps (NETs) (Brill 2012, Fuchs et al 2012). NETs act as a scaffold to stabilize the thrombus and provide a surface for the activation of coagulation factors (Massberg 2017). The decreased attachment of platelets at the IVC wall following stenosis in CLEC-2 deficient mice could impair leukocyte recruitment, and could be a factor contributing to protection against DVT in these mice.

As previously stated, DVT can be considered a thromboinflammatory disease. Other studies have supported the role of CLEC-2 in thromboinflammatory settings. It was shown that after infection with salmonella, a high level of thrombosis develops in the liver of control mice, but markedly decreased in CLEC-2 deficient mice (Hitchcock et al 2015). Thrombosis induced by salmonella was dependent entirely on CLEC-2 and similarly in our experiments, GPVI did not contribute to DVT with mice lacking this receptor only showing an insignificant trend towards decreased DVT prevalence.

These results align with our results, and assume that CLEC-2 plays an important role in venous thrombosis under septic or aseptic inflammatory conditions, and GPVI plays a less important role.

Currently, the only known natural ligand for CLEC-2 is podoplanin, but this has not previously been shown to be expressed in the vascular wall. It is already known podoplanin is expressed in the lymphatic endothelium, kidney podocytes and type 1 alveolar lung cells (Suzuki et al 2010). Recently, it was shown to be up

regulated in the tissues next to the blood vasculature after salmonella infection (Hitchcock et al 2015).

Our results demonstrate that podoplanin is expressed in the IVC wall, staining shows that it is located in the vicinity immediately below the endothelium, which is similar to the salmonella-induced pattern. We show that podoplanin is constitutively expressed in the intact IVC vessel wall, whilst it is dramatically up regulated following IVC stenosis. Furthermore, podoplanin expression was further increased if stenosis of the IVC resulted in thrombosis, and was much higher than in those IVCs post stenosis where thrombi did not develop. It is not clear whether increased podoplanin is a cause or consequence of thrombosis, although this result indirectly suggests that podoplanin in the IVC wall is related to DVT.

We can speculate that platelets accumulate at the IVC vessel wall as a result of their exposure to podoplanin, which would in turn result in further platelet recruitment and activation, leading to further increased podoplanin expression.

We performed further experiments to verify a causal relationship between podoplanin expression in the vessel wall and DVT, using an anti-podoplanin inhibitory antibody. While it is not clear how to antibody penetrates the endothelial monolayer, to get to podoplanin found on the abluminal side of the endothelium we can make a suggestion. It is plausible that increased blood pressure allows the vessel wall to stretch, which in turn allows endothelial gaps to widen allowing the antibody to make contact with the podoplanin in the vessel wall.

Administration of the anti-podoplanin antibody resulted in smaller size of thrombi, while thrombosis prevalence remained the same as in control mice. The

fact that IgG control mice and the mice administered the antibody had comparable thrombosis prevalence, implies that podoplanin is implicated in thrombus propagation rather than its initiation. Because absence of CLEC-2 and absence of podoplanin produce different (though both antithrombotic) phenotypes, we cannot say categorically that they work together as a receptor-ligand couple in this process.

Other mechanisms cannot be excluded. For example, it is possible that CLEC-2 binds to another unidentified ligand, not podoplanin. And that platelets still interact will the vessel wall via this alternative mechanism when podoplanin is neutralized with the antibody, but platelet activation is impaired which results in reduced thrombus growth. Alternatively, we cannot exclude the possibility that the antibody may not 100% block podoplanin in vivo.

The question still remains as to what is the source of podoplanin in the IVC vessel wall. We have however been able to rule out a number of potential options. We have shown that it is unlikely to be from hematopoietic or endothelial cell origin, since mice with podoplanin knocked out specifically on these cells still retained podoplanin expression in the vessel wall (figure 3.8.2) and DVT formation in these mice was normal.

Another potential source of podoplanin in the vessel wall could be from fibroblastic cells. It has previously been shown that fibroblastic reticular cells surrounding high endothelial venules highly expressed podoplanin (Herzog et al Nature 2013). It has also been shown to be expressed on myofibroblasts of the prostate and in cancer-associated fibroblasts (Schacht et al 2005).

Whilst a less likely option, as the venous wall only contains small amounts, vascular smooth muscle cells (VSMC) cannot be ruled out as a potential contributor to venous thrombosis. In addition to podoplanin, VSMCs express another potential CLEC-2 ligand, S100A13 (Inoue et al 2015). This ligand becomes highly expressed of VSMC that are exposed to oxidative stress. This could potentially happen in the IVC during stenosis as a result of the local hypoxia.

Our results show that lack of another ITAM receptor, GPVI did not lead to protection against DVT. However GPVI deficient mice showed poor thrombus stability in the laser injury model (figure 9), which is consistent with other group's findings, where GPVI has been shown to play a role in a photochemical injury model (Marjoram et al 2014). Interestingly, mice lacking both ITAM receptors, GPVI and CLEC-2, have shown a sever defect in both thrombosis and hemostasis (Nieswandt 2009), which indicates that combined inhibition of both receptors is not a beneficial anti-thrombotic strategy.

Several groups have not observed this same anti-thrombotic phenotype in GPVI deficient mice. The group of Dubois et al (2006) did not report impaired thrombus development in the laser injury model. This discrepancy could however be attributed to the fact that we used different mouse strains. Dubois et al used FcR-γ null mice, which lack platelet surface GPVI, whereas we used GPVI knockouts, and these receptors have previously been shown to produce different results in various models. For example lack of GPVI affects carotid artery occlusion, but absence of FcRγ does not (Marjoram et al 2014). Whilst another

group reported a strong antithrombotic phenotype in FcRγ- deficient mice (Kalia et al 2008)

It has recently been identified that GPVI not only binds to collagen, but also to fibrinogen, leading to platelet activation (Alshehri et al 2015, Mammadova-Bach et al 2015). This has implications in ferric chloride induced thrombosis in vivo. Without GPVI-fibrinogen binding, platelet αIIbβ3 cannot be fully activated, and cannot efficiently bind to fibrinogen. This prevents firm platelet-platelet interactions and therefore thrombus growth. This reduced platelet cohesion could explain the unstable thrombus formation we see in our model (figure 3.9) and the partial protection we see against DVT in the GPVI-deficient mice.

In conclusion, we have answered the main aims of this chapter. We have shown that both CLEC-2 and podoplanin have a significant impact in DVT. Complete deficiency in CLEC-2 protects mice totally from DVT, with mice lacking CLEC-2 only on platelets showing a smaller, but still significant protection from DVT. GPVI deficiency showed only a trend towards DVT protection but this was not significant. The IVC wall expresses podoplanin and this is important in venous thrombosis. Although further studies are required to ascertain to what extent podoplanin mediates the prothrombotic effect of CLEC-2. As CLEC-2 deficient mice only show a minor impairment in normal hemostasis, this could be a potential target for DVT treatment in the future.

Chapter 4

The role of Mast cells in the development of deep vein thrombosis

4.1 Introduction

Deep vein thrombosis (DVT), and its associated complication pulmonary embolism (PE), as previously discussed in chapter 3, is a serious health problem in the western world. With DVT being responsible for over 100,000 deaths in the US annually, as reported by the American Surgeon General (centers for disease control and prevention), and affecting 1 in every 1,000 people in the UK (NHS). Despite this disease being a major heath concern, the mechanisms leading to venous thrombosis have still not been fully elucidated, and the reported cases are not declining annually. In fact, even after development of efficient current treatment options, patients are still presenting with post thrombotic syndrome and chronic thromboembolic pulmonary hypertension from DVT and PE respectively (Ashrani et al 2009). This usually requires prolonged periods of anticoagulation therapy and general poor quality of life, not to mention the additional cost and medical attention as a result. Therefore there is a great need for a better understanding of the mechanisms of DVT and alternative treatment options as current anticoagulant treatments such as Warfarin and Clexane are efficient, but associated with bleeding complications. The paradigm of our approach is to identify new targets to tackle DVT through a 'backdoor approach' this is not involved in normal hemostasis. One such target is the immune system.

As described in chapter 1, we know that thrombosis in veins develops in a different manner to that in arteries. In veins thrombi develop without any sign of endothelial denudation, whereas thrombus formation in arteries occurs as a result of exposure of the subendothelial adhesion proteins after atherosclerotic plaque rupture. In venous valves, blood flow can become stagnant and stasis, a

component of Virchow's triad (Bovill et al 2011), can lead to local hypoxia, and prolonged period of blood exchange in valves increases thrombogenicity (Hamer et al 1981).

A common cause of this 'blood pooling' in valves and stagnant flow is a result of prolonged periods of inactivity such as during long-haul flights, or post-surgery when patients are in bed-ridden positions with limited mobility.

It has previously been demonstrated (refer to chapter 3) that an inflammatory component plays a role in DVT initiation. We know that local hypoxia caused as a result of stagnancy in the venous valves, may lead to release of P-selectin, and WVF from WPB's. This in turn leads to the recruitment of platelets and leukocytes. While we know the importance of platelets in thrombosis initiation, as they provide a surface for clotting factors, other cells may also play a role. Neutrophils have been shown to support DVT propagation by producing NETs (Brill et al 2012; Von Bruhl et al 2012). We sought to investigate if mast cells, which have previously been associated with other cardiovascular diseases, are implicated in DVT.

Mast cells (MC) are part of the innate immune system. Their development is different to other myeloid-derived cells, in that mast cells originate from the hematopoietic progenitor in the bone marrow, but they leave and enter the circulation as early progenitors rather than end-stage cells (Gurish et al 2012). Once mast cells have moved to the tissues and matured, they can be distinguished as they express a specific set of antigens; the receptor FcεRI which has a high affinity for immunoglobulin E (IgE), and Kit (stem cell factor receptor).

Mast cells do not circulate permanently in the blood but reside in the connective tissue (Amin 2012). Mast cells are constitutively present in most tissues, there are different subsets including peritoneal, mucosal and connective tissues mast cells. They are functionally heterogeneous cells depending on which tissue they mature in. A specific subset of mast cells which highly express FcεRI II and MHC II can act as antigen presenting cells (Theoharies et al 2012). Although their most well documented role is as effector cells in allergic diseases, they have a number of other roles such as in homeostatic responses and immunoregulation (Moon et al 2014). Allergic inflammation has been shown to be a risk factor for DVT and PE (Lippi et al 2016). Mast cells are located in the vicinity of blood vessels as well being observed at the site of human DVT (Bankl et al 1999).

Another distinctive feature of mast cells is that they contain granules. They have the ability to secrete their granular contents and release a variety of important mediators with a diverse range of biological activities. These include Amines; (histamine and polyamines), proteoglycans, proteases (tryptases and chymases), lysosomal enzymes and cytokines (Moon et al 2014).

It is known that mast cells play a role in allergic inflammation; one of the most well studied mediators released from MC granules involved in this process is histamine. Histamine is released from mast cells when they detect injury, or when the body encounters a toxic substance (Amin 2012). Histamine acts on the nearby blood vessels at the site of toxicity or damage to cause blood vessels to dilate, allowing more blood to flow to the infected area. Histamine also acts upon endothelial cell WPB's causing them to exocytose and release VWF (Conte 2015).

Asthma is considered to be a chronic inflammatory disease, and this is associated with ongoing mast cell activation. In the bronchoalveolar lavage of asthmatics it has been shown to contain an increase in spontaneous release of histamine from mast cells, compared to that of non-asthmatics (Amin 2012). While mast cells play a detrimental role in IgE-dependent allergic reactions, they may also have a protective role in other diseases. Some reports have shown mast cells to be protective in bacterial infections, for example in a mouse model of acute septic peritonitis (Echtenacher et al 1996). Using the caecum ligation and puncture (CLP) method, mice that were deficient in mast cells showed an increased mortality compared to mice with mast cells. If these deficient mice were then reconstituted with cultured mast cells this protected them from the lethal effects of CLP. Demonstrating a protective role of mast cells in bacterial peritonitis (Echtenacher at al 1996). Another group also showed that mast cells had a protective role against enterobacterial infection, they show mast cells exerted this effect by releasing inflammatory cytokines and recruiting neutrophils, and this recognition of enterobacteria was mediated TLR4 on mast cells (Supajatura et al 2001).

Mast cells have also been shown to play an important role in the pathogenesis of other diseases such as atherosclerosis and abdominal aortic aneurysm. Abdominal aortic aneurysm (AAA) is an inflammatory arterial disease. The number of medial and adventitial MC's (particularly those that have degranulated) is significantly increased in atherosclerotic aortas compared to

non- diseased aortas. This number is further increased in AAA (Swedenborg et al 2011).

The role of mast cells in AAA has been well studied using various mouse models, and it was shown that Kit $^{W\text{-}sh/W\text{-}sh}$, which lack MC's were protected from AAA. Lack of mast cells in these mice was associated with a significant reduction in media elastin degradation and adventitial angiogenesis, along with a reduction in macrophages and T-cells at lesion sites (Swedenborg et al 2011).

This phenotype could be reversed, by adoptive transfer of exogenous mast cells from WT mice (Wang et al 2012). Furthermore if mice were treated with compound 40/80, which stimulates mast cell degranulation, abdominal aortic aneurysms were larger compared to mice treated with a vehicle, and also showed an increase in elastin degradation and lesion inflammation. All of these effects could be partially suppressed by administering mice with the mast cell stabilizing agent, cromolyn (Wang et al 2012).

Further studies were performed to elucidate which components of the mast cell granules were responsible for this. Chymase and tryptase are mast cell specific proteases and account for over 25% of the total cellular proteins (Schwartz et al 1987) they are commonly used to detect mast cells.

Pharmacological inhibition of chymase or tryptase decreased AAA formation, along with reducing other associated pathologies (Wang et al 2012), demonstrating the important role these mast cell constituents play in AAA development. This study demonstrated the importance of mast cells, at least in an arterial inflammatory setting.

An association has previously been made between mast cells and DVT. Bankl et al (1999) performed a study comparing the number and distribution of mast cells in the deep veins of the lower limb in patients with and without DVT. Giemsa staining and immunohistochemistry, using antibodies against mast cell granule constituents (e.g. Tryptase and chymase) showed an increase in number of mast cells in DVT compared to nonthrombosed limb veins (Bankl et al 1999). Overall we know that mast cells contain both anticoagulants (heparin, tPA) and pro-inflammatory mediators (histamine, TNF-α) and therefore could potentially have either a pro-thrombotic or anti-thrombotic phenotype in DVT. In this chapter we aimed to investigate this.

4.2 Results

4.2.1 Mast cell deficient mice are protected against DVT

To investigate if mast cells play a role in DVT, we performed IVC stenosis (as described methods 2.7.3) to $Kit^{W\text{-}sh}$ mice that lack mast cells vs. their WT littermates and looked for thrombosis prevalence at two different time points; 24 hours and 48 hours after stenosis application. Whilst in the WT group 75% of the mice produced a thrombus after 24 h stenosis, and 90% produced a thrombus after 48 h, none of the mast cell deficient mice developed a thrombus at either time point (Figure 4.2 a,b). To further confirm this phenotype, we performed the same experiment with a different strain of Kit mutant mice $Kit^{W\text{-}v}$ and their littermate controls. As can be seen in figure 4.2C, the same phenotype was observed, after 48 h stenosis, 89% of control mice produced a thrombus compared to none of the mutant mice.

Because the Kit mutation could potentially mediate other abnormalities apart from lack of mast cells, such as impaired germ cell development, altered blood count, or abnormal vascular permeability, we wanted to rule these out as potential causes or mechanisms for the affected thrombosis phenotype observed. We therefore cultured murine bone marrow-derived mast cells, and performed adoptive transfer of these in vitro-differentiated MCs into $Kit^{W\text{-}sh}$ mice to restore their pool of mast cells, and see if the phenotype could be reversed. We cultured murine bone marrow-derived mast cells (BMDMC) using methods that have previously been described in the literature (methods 2.4). The in vitro differentiated cells were assessed by FACS staining for c-Kit and FcεR and its was shown they were around > 90% mature after about 4 weeks. The functionality of

these mast cells was confirmed by their ability to release β-hexaminidase upon stimulation (figure 4.1).

In vitro differentiated mast cells (10 X 10^6) were injected into the tail vein of 3-4 week old $Kit^{W\text{-}sh}$ mice. 8-10 weeks after the adoptive transfer these mice were subjected to 48 h IVC stenosis. As can be seen in figure 4.2b, 67% of the mice that received these mast cells produced a thrombus. This is significantly higher than the $Kit^{W\text{-}sh}$ mice without the adoptive transfer, and it is also comparable to thrombosis prevalence in WT controls. This confirms that it is the lack of mast cells that protects mice against DVT.

4.2.2 – Evaluating the purity and functionality of in-vitro differentiated mast cells

We performed further experiments to make sure that the mast cells we had cultured, and were going to adoptively transfer into the Kit-$^{W\text{-}sh}$ mice were functional. In order to assess if the mast cells were mature we performed FACS staining (figure 4.1 a). Staining with antibodies against mouse FcεR1 and CD117, showed that more than 90% of the mast cells expressed both receptors, and were therefore classed as mature. The mast cell functionality was tested, by testing their ability to secrete their granule contents when stimulated.

In order to assess the efficiency of the mast cells to release the mediators, β-hexaminidase activity was measured in the supernatants (figure 4.1 b). The two batches of mast cells, which were used for the adoptive transfer experiments, are shown below (figure 4.1a - MC#3 and MC#4).

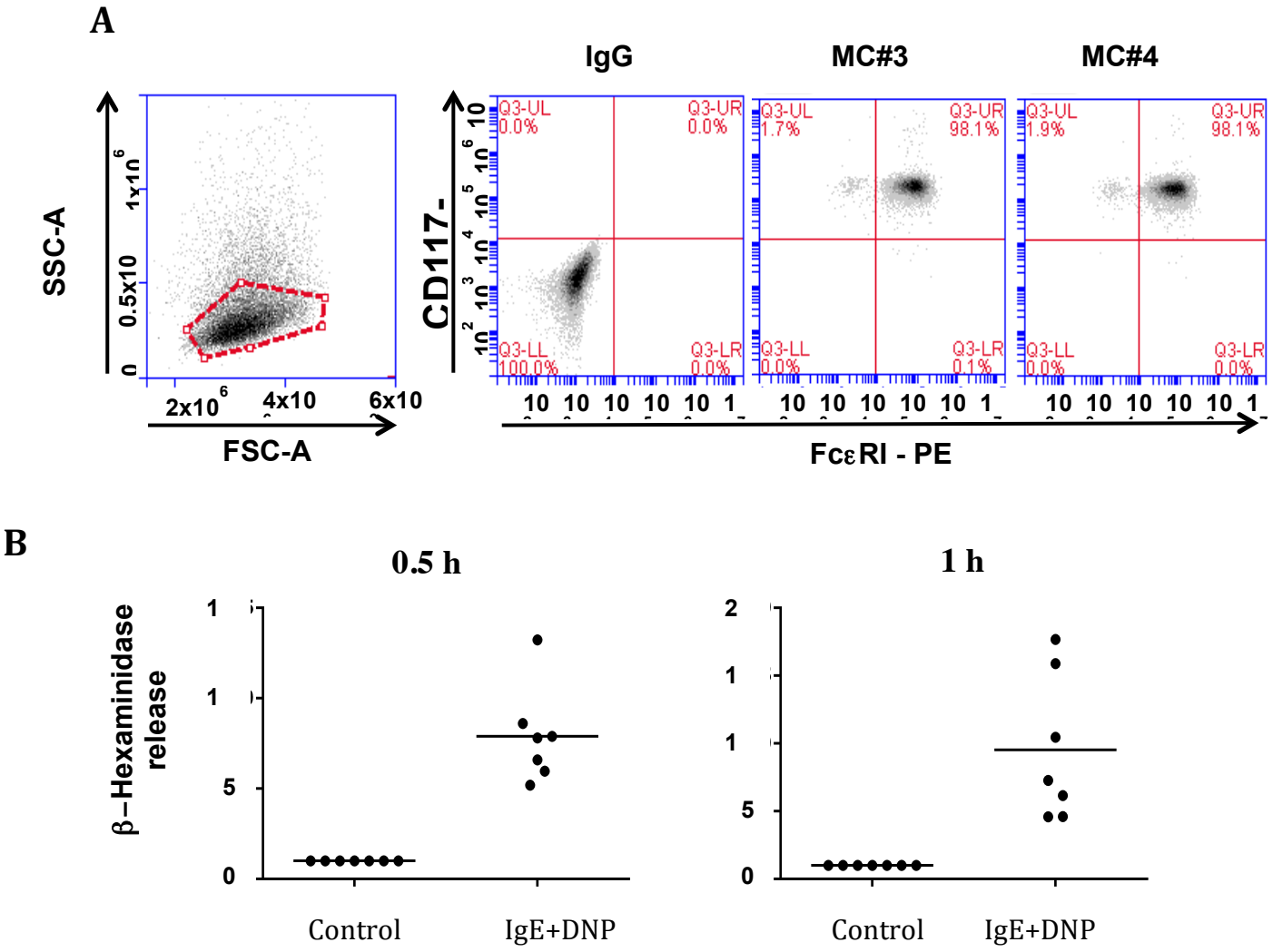


Figure 4.1 - Evaluation of purity and functionality of the in vitro differentiated Mast cells

(A) To assess maturity of mast cells FACS staining was performed for FCεRI and CD117. Over 90% of cells expressed both receptors. Two MC batches used in the adoptive transfer experiments are presented (MC#3 and MC#4) (**B**) β-hexaminidase activity was measured in the mast cell supernatant to assess the cells functionality and ability to secrete their granular contents. *(Performed in collaboration with T.Ponomaryov)*

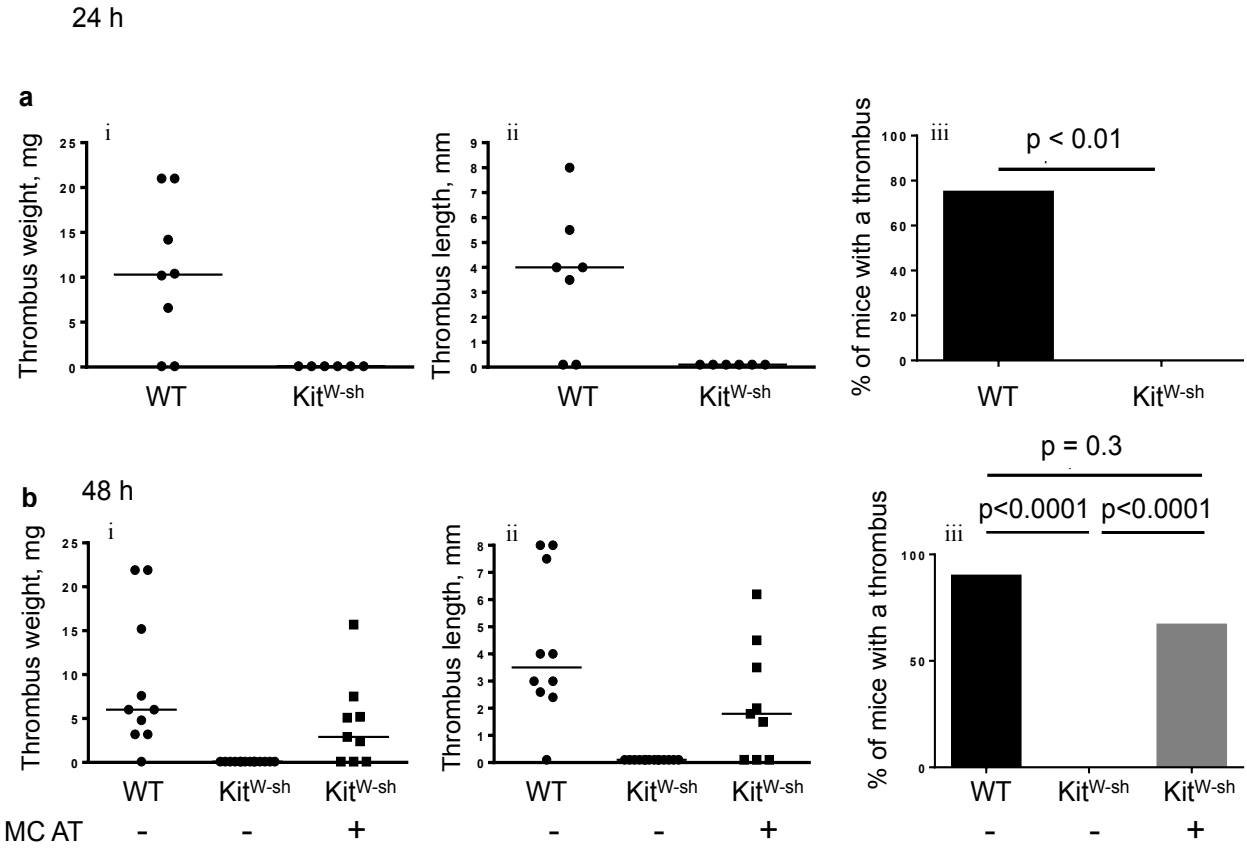


Figure 4.2 - Kit$^{W\text{-}sh}$ and Kit$^{W\text{-}v}$ mice are protected against DVT, and mast cell repopulation restores thrombosis

Stenosis of the IVC was performed in Kit$^{W\text{-}sh}$ mice (lacking mast cells) and their WT littermates. Thrombosis prevalence was measured at either (**a**) 24 h after stenosis or (**b**) 48 h after stenosis. Another group of Kit$^{W\text{-}sh}$ mice underwent adoptive transfer (AT) of 10 x10^6 in vitro-differentiated mast cells, 8-10 weeks prior to stenosis. Thrombus weight, length and prevalence were measured.

(**a**) WT n= 8, Kit$^{W\text{-}sh}$ n= 6; (**b**) WT n= 10, Kit$^{W\text{-}sh}$ n= 14, Kit$^{W\text{-}sh}$ following adoptive transfer n= 9. Note that mice without mast cells were protected against DVT and thrombosis was restored after adoptive transfer of mast cells.

(Performed in collaboration with A.Brill)

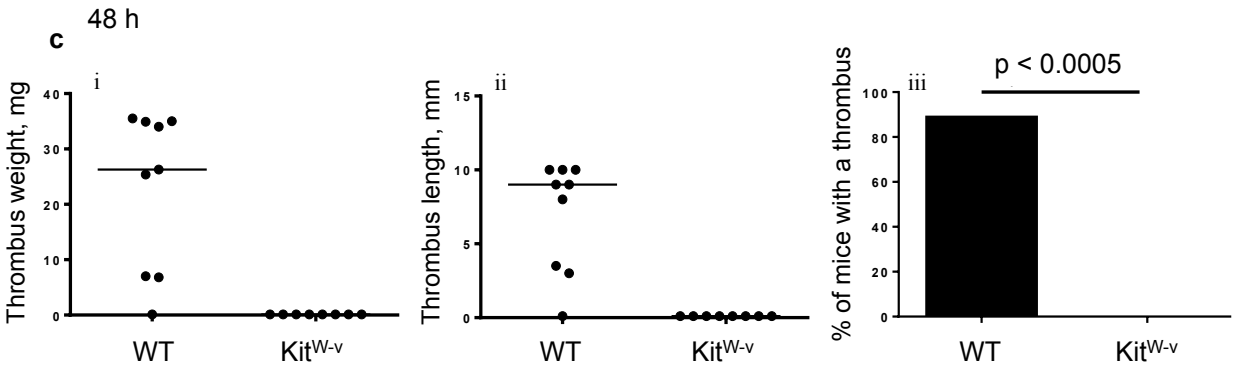


Figure 4.2 continued - Kit$^{W\text{-}sh}$ and Kit$^{W\text{-}v}$ mice are protected against DVT, and mast cell repopulation restores thrombosis

(**c**) IVC stenosis was performed in another strain of Kit mice; Kit$^{W\text{-}v}$.

Kit$^{W\text{-}v}$ mice n=8, WT littermates n=9. Thrombus weight, length and prevalence were measured. Again this strain of mast cell deficient mice was protected against DVT. *(Performed in collaboration with A.Brill)*

4.2.3 Objectively assessing the presence or absence of thrombi in WT and Kit$^{W\text{-}sh}$ mice

In order to provide further evidence with an objective verification of the presence of thrombi in either WT or Kit$^{W\text{-}sh}$ mice, we used ultrasound Doppler (figure 4.3).

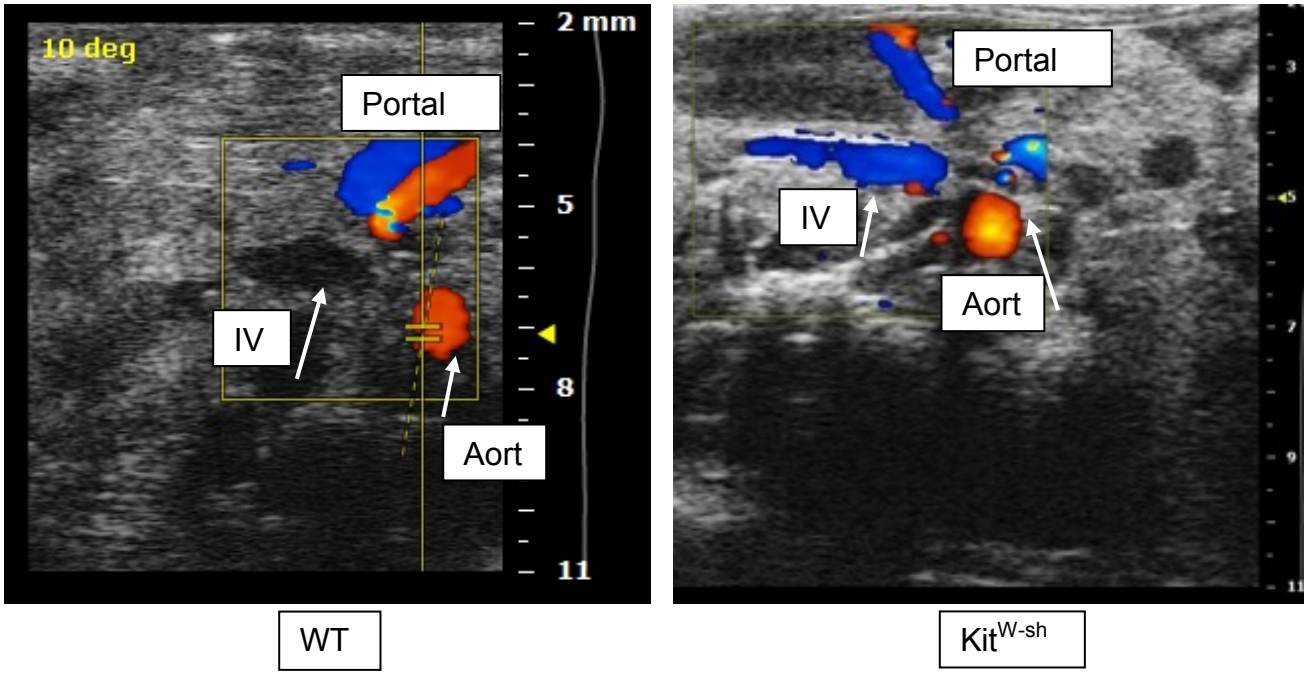


Figure 4.3 Presence of thrombi in WT but not in Kit$^{W\text{-}sh}$ mice detected by ultrasound Doppler

Ultrasound Doppler was performed on WT and Kit$^{W\text{-}sh}$ mice (n=4 mice per group) 48 h after IVC stenosis. No blood flow was detected in the IVC of WT mice, suggesting the presence of a thrombus. In Kit$^{W\text{-}sh}$ mice blood flow could be detected, showing that a thrombus did not form in the IVCs of these mice. *(Performed in collaboration with A.Brill & L.Fabritz)*

4.2.4 Mast cells are present in the IVC wall, and there number decreases with thrombosis

In order to assess if mast cells are present in the IVC wall where we believe them to be implicated in thrombosis, we performed staining with Toluidine blue dye. IVCs from WT mice were frozen, sectioned and stained with toluidine blue, which specifically stains mast cell granules. As can be seen in figure 4.4 mast cells were present in the IVC wall, specifically in the tunica media and adventitia. We compared the number of mast cells present in the IVC wall 48 h after stenosis in mice that did and did not produce a thrombus, as well as in WT unchallenged IVCs. The number of mast cells in the IVC wall just below the area of ligation, 48 h after stenosis, decreased in those mice that produced a thrombus compared to intact IVCs. The number of mast cells present in stenosed IVCs with a thrombus was 12.1 ± 1.0, compared to 25.6 ± 2.1 in intact untreated IVCs ($P < 0.0001$). Interestingly, in IVCs that have undergone stenosis, but have not resulted in thrombus formation, contain a similar number of mast cells to intact IVCs. Together these results suggest that during deep vein thrombus formation, mast cells may either migrate out of the IVC wall, or they degranulate, which is why toluidine blue staining is lost. The fact that mast cell numbers remain similar in stenosed IVCs without a thrombus, and intact IVCs, and decrease in those with a thrombus suggests that degranulation of the mast cells is associated with thrombosis.

A

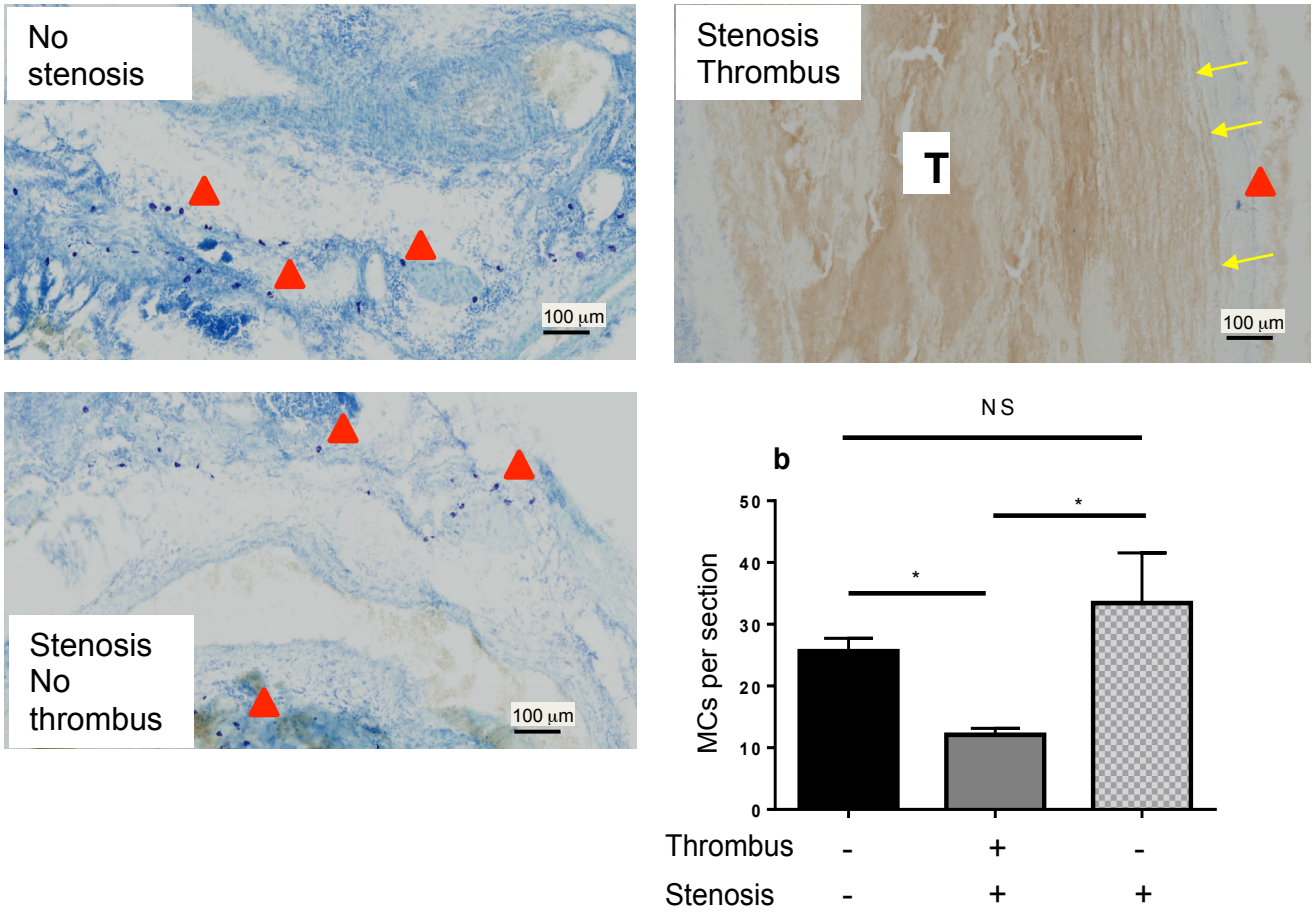


Figure 4.4 Granule-containing MCs are present in the IVC wall and decrease in number with thrombosis.

(**a**) IVC was excised from unchallenged mice (upper left) or mice after 48 h stenosis with (upper right) or without (upper right) a thrombus. Mast cells were stained with toluidine blue. Red arrowheads depict MCs, T designates thrombus. Yellow arrows show boarder between thrombus and the vessel wall.

(**b**) Black bar (intact IVC) n = 11, grey bar (IVC after stenosis with a thrombus) n = 11, chequered bar (IVC after stenosis with no thrombus) n = 7. The bars show the number of mast cells counted in each transverse section.

4.2.5 Mast cell granule depletion prevents DVT

Having observed that the that absence of mast cells is protective from DVT, we next wanted to investigate if pharmacological depletion of mast cells granules gave the same result.

Mice were given an i.p. Injection of the mast cell degranulator compound 48/80 in sterile saline consecutively for 4 days prior to surgery (methods 2.1.4). The mice were then subjected to IVC stenosis 24 h after the last injection of the compound. This treatment regime should have depleted mast cells by ~84%.

IVC stenosis was performed for 48 h as described in methods 2.7.3, thrombosis prevalence, thrombus length and weight were then measured. None of the mice administered the compound 48/80 developed a thrombus, compared to 86% of the control mice that received and injection of sterile saline (figure 4.5). This result phenocopied what we previously saw in mast cell deficient mice, suggesting that functional mast cells and their granules are critical in venous thrombosis.

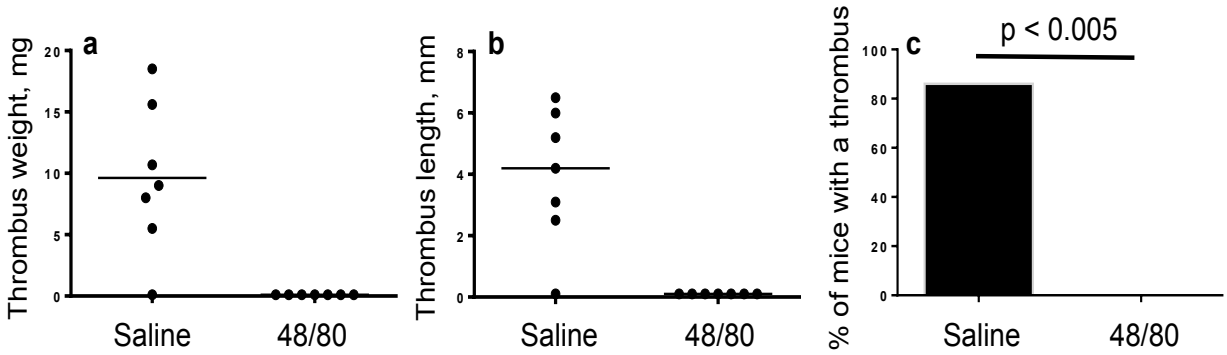


Figure 4.5- Mast cell granule depletion prevents DVT.

Mice either received consecutive daily injections of sterile saline, or the mast cell degranulator compound 48/80 (n= 7 in both groups). After 48 h stenosis thrombus weight (a), length (b) and thrombosis prevalence (c) were measured. 86% of control mice produced a thrombus compared to none of the 48/80 treated mice. *(Performed in collaboration with A.Brill)*

4.2.6 Absence of mast cells results in suppressed endothelial activation

As we already know, secretion of WPB constituents and endothelial activation are crucial in the initiation of DVT (Brill et al 2011). We next aimed to investigate if mast cells have a role in regulating the activation of the endothelium. We firstly measured the plasma levels of VWF in the blood of WT and $Kit^{W\text{-}sh}$ mice as VWF is released from WPBs upon endothelial activation. As can be seen below in figure 4.6 a, plasma levels of VWF were higher in unchallenged WT compared to mast cell deficient mice, suggesting an association between circulating levels of VWF and the presence or absence of mast cells in the absence of challenge, and the role of mast cells in WPB regulation.

As we also know that cell recruitment is a prerequisite for DVT development, and that stenosis of the IVC promotes this recruitment. We sought to investigate if this cell recruitment process is modified in the absence of mast cells.

Mice were subjected to IVC stenosis for 6 h, and mice that had not already produced thrombi at this time point were used in the further experiments. These mice were then injected with washed platelets labeled with calcein AM, and intravital microscopy of the IVC was performed (as described in methods 2.7.5). As can be seen in figure 4.6 b & c, after stenosis in WT mice multiple platelets stay adhered to the IVC vessel wall, compared to in $Kit^{w\text{-}sh}$ mice, where platelets did not stay attached or make contact with the endothelium, but instead were carried away in the direction of the blood flow. Only single platelets were adhered to the vessel wall (videos available upon request).

This was then quantified using 10 randomly chosen consecutive images (corresponding to 1s recording) and the total area of adhered platelets was measured (methods 2.7.5). Figure 4.6 c) shows that in the WT mice, the total area covered by adhered platelets was 6.9 ± 0.73%. Compared to 2.4 ± 0.54% in $Kit^{W\text{-}sh}$ mice ($p<0.003$). This result could suggest that mast cells are involved in endothelial activation critical for DVT initiation.

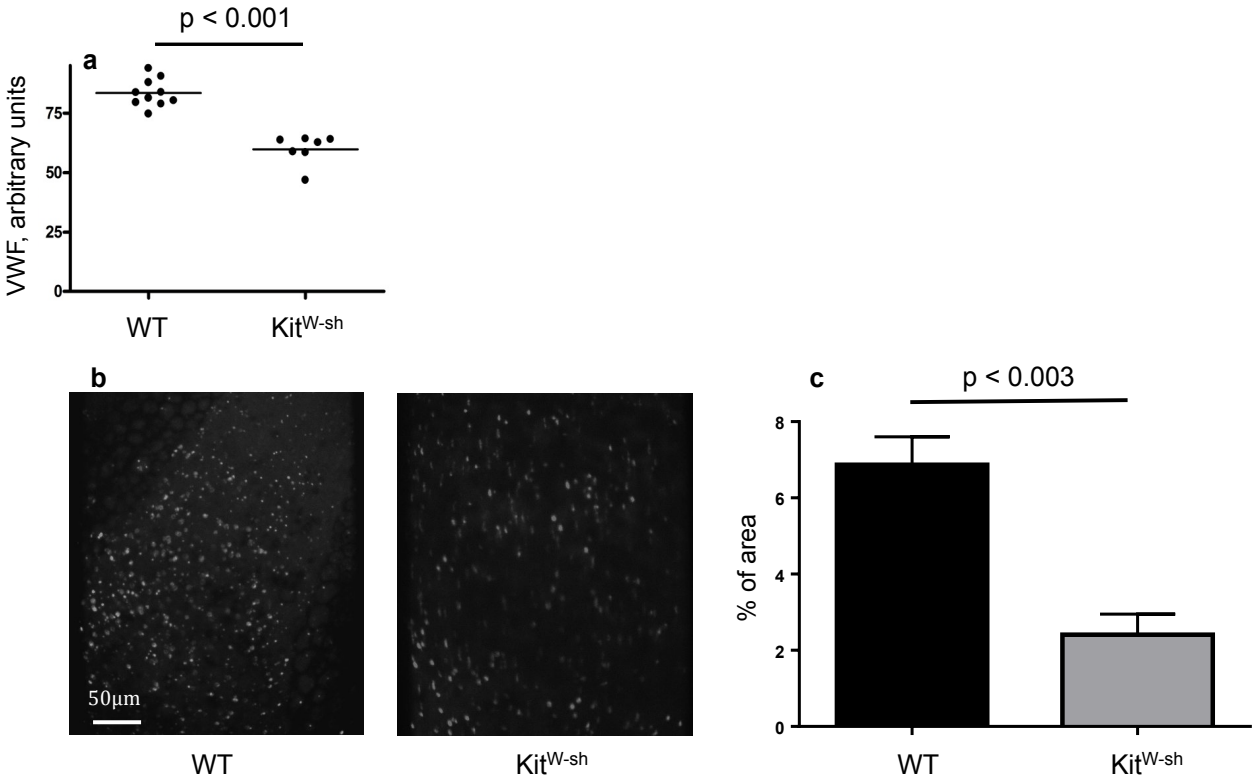


Figure 4.6 - Mast cells regulate plasma levels of VWF and platelet recruitment to the stenosed IVC wall.

(a) Plasma levels of VWF were measured in the blood from both WT and Kit$^{W\text{-}sh}$ mice (n= 7-10). (b) Representative images of 10 consecutive frames of intravital microscopy of the stenosed IVC (6 h after stenosis application with no thrombus), showing platelets (fluorescently labelled) adhered to the vessel wall. (c) The percentage area covered by platelets in WT and Kit$^{W\text{-}sh}$ mice (n=4 for both groups) 6 h after stenosis application.

4.2.7 Mast cell involvement in activation of the endothelium

To verify if the mast cells are involved in the endothelial activation, we performed further in vitro studies. In vitro differentiated mast cells were stimulated with IgE, followed by DNP, and mast cell releasate was then added to HUVEC cells for 20 h. ICAM-1 expression was measured by FACS, as ICAM-1 expression is involved in leukocyte accumulation near the vessel wall. As can be seen in figure 4.7, mast cell releasate was able to stimulate expression of ICAM-1 on HUVECS at the level comparable with the reference endothelial activator, cytokine TNF-α. We also measured the levels of soluble P-selectin, a biomarker of venous thrombosis (Antonopoulos 2014, Ramacciotti 2011) in the blood samples of both WT and $Kit^{W\text{-}sh}$ mice, after stenosis, or in unchallenged mice.

Blood was taken from the retroorbital plexus of both WT and $Kit^{W\text{-}sh}$ mice, (n= 5 to 9) prior to surgery and then 6 h post stenosis. Figure 4.8 shows stenosis induced an increase in soluble P-selectin levels in WT mice, but not it $Kit^{W\text{-}sh}$ mice, whereas basal levels of sP-selectin were similar. Together these results suggest that a deficiency in mast cells may cause a lower level of cell recruitment to the vessel wall during DVT initiation, and a reason for this could be impairment in the release of adhesive substances from the endothelium.

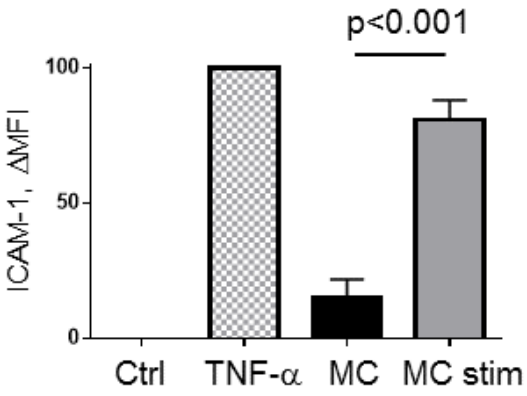


Figure 4.7 - MC releasate induces ICAM-1 expression on HUVEC in vitro

The releasate of mast cells after simulating them with IgE followed by DNP, was added to HUVECs for 20 h. induction of ICAM-1 expression by 10ng/ml TNF-α was taken as a 100% . *(Performed in collaboration with T.Ponomaryov)*

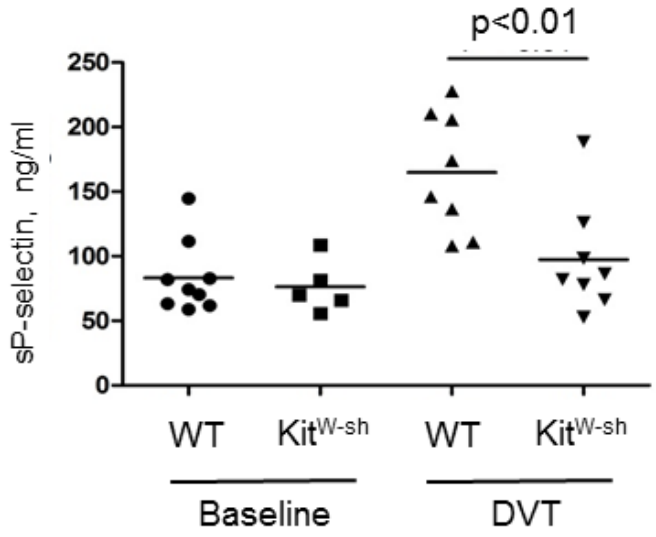


Figure 4.8- Plasma sP-selectin levels are elevated during DVT in WT mice but not MC-deficient mice.

Blood samples were taken from both WT and $Kit^{W\text{-}sh}$ mice prior to surgery and 6 h post stenosis (n= 5 to 9). Plasma sP-selectin levels were measured using a commercial ELISA kit.

4.2.8 Inhibiting mast cell degranulation protects against DVT

Mast cell biological functions, such as in allergic diseases, are implemented mainly due to release of their granule components. Drugs which are already commercially available, such as those to treat allergies, work primarily through stabilizing the mast cell membrane and preventing their degranulation. We next aimed to investigate if this approach could be a potential way to prevent DVT.

We used two different mast cell membrane stabilizers, sodium cromoglycate (Cromolyn), and Ketotifen, both of which inhibit mast cell degranulation in mice (Ramos 2010, Finn 2013).

WT mice received an injection of either membrane stabilizer, or sterile saline, 30 minutes prior to surgery and 24 h after. IVC stenosis was performed for 48 h and then thrombosis prevalence, weight and length of thrombi were measured.

Cromolyn was administered to mice at a dose of 100mg/kg body weight, whilst the other group received an injection of sterile saline (n=16 for both groups). As can be seen in figure 4.9 a-c below, thrombosis prevalence fell from 71% in saline treated mice, to 31% in those that received Cromolyn ($p<0.04$).

The same experiment was performed using Ketotifen, at a dose of 25mg/kg body weight. 7 mice received ketotifen, and 10 mice received sterile saline. Figure 4.9 d-f shows that thrombosis prevalence fell from 80% in saline treated mice to 14% in those that received ketotifen ($p<0.02$). These results suggest that mast cell membrane stabilisers could potentially be a promising anti-thrombotic treatment and prevent DVT.

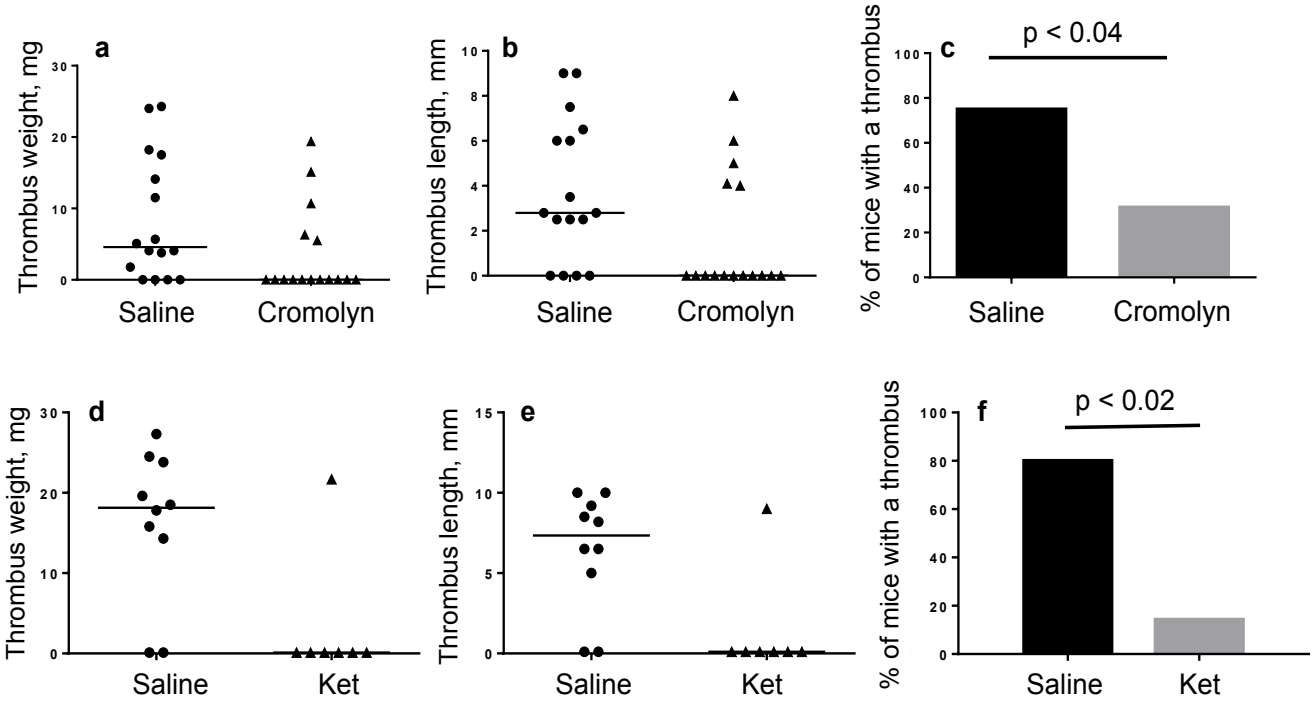


Figure 4.9 - Inhibition of MC degranulation by membrane stabilization protects against DVT.

(a-c) WT mice were administered either sterile saline or cromolyn (100mg/kg) at 24 h and 30 min prior to IVC stenosis. (n=16 for both groups).

(d-f) WT mice were administered either sterile saline or Ketotifen (25mg/kg). Saline n= 10, Ketotifen n=7. Thrombus formation was checked in all groups 48 h after stenosis. Thrombus weight, length and thrombosis prevalence are shown, with the horizontal bar representing the median.

(Performed in collaboration with A.Brill)

4.2.9 Topical application of either compound 48/80 or histamine increases thrombosis in WT mice, and induces DVT in $Kit^{W\text{-}sh}$ mice.

In order in investigate further how mast cells and their granule contents play a role in the development of DVT, we performed topical application of the secretogogues, compound 48/80, or histamine, onto the IVCs of mice during stenosis.

After stenosis application a strip of Whatman paper was soaked in a solution of either compound 48/80 (1mg/ml), histamine (100mg/ml) or saline. The paper strip was placed on the IVC for 10 minutes immediately after stenosis induction. 6 h post surgery the mice were opened to investigate the development of thrombi.

WT mice were used for topical application of compound 48/80, histamine or saline. $Kit^{W\text{-}sh}$ mice were used for topical application of histamine or saline only.

Figure 4.10 shows that in WT control mice, thrombosis prevalence was 25% after IVC stenosis. When WT mice were treated with compound 48/80- thrombosis prevalence increased to 80% ($p<0.03$), whilst when treated with histamine, mice produced a thrombus in 87.5% of cases ($p<0.02$ Vs. saline).

None of the $Kit^{W\text{-}sh}$ treated with saline produced a thrombus, but when these mice received topical application of histamine, thrombosis prevalence dramatically increased to 100%. Demonstration that histamine can induce DVT in mast cell deficient mice, mimicking thus the effect of mast cells, suggesting the effect is probably mediated by histamine release.

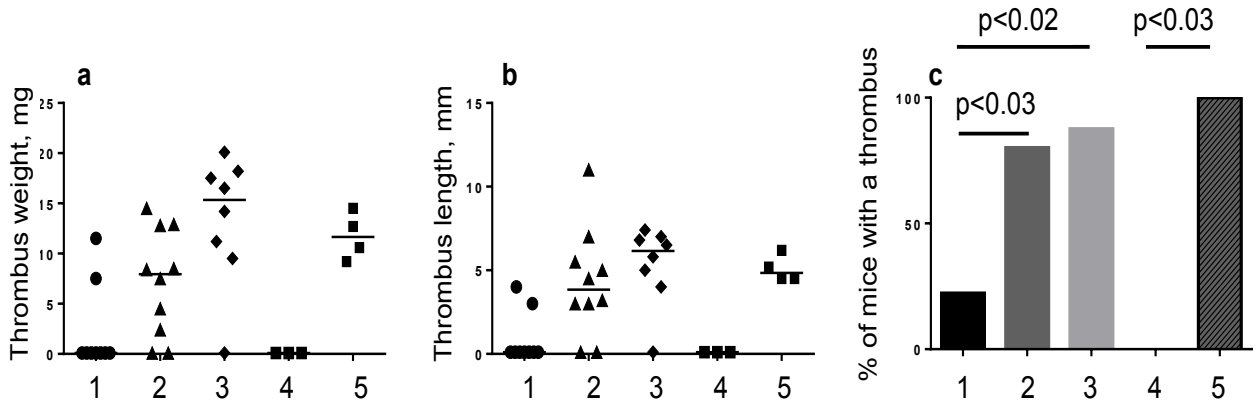


Figure 4.10 -Topical application of compound 48-80 or histamine potentiates thrombosis in WT and histamine induces DVT in Kit$^{W\text{-}sh}$ mice.

The groups shown above; 1) WT + saline, 2) WT + 48-80, 3) WT + Histamine, 4) Kit$^{W\text{-}sh}$ + saline, 5) KitW^{-sh} + Histamine.

WT and Kit$^{W\text{-}sh}$ mice underwent IVC stenosis for 6 hours. Immediately after stenosis application a strip of paper soaked in either compound 48/80 (1mg/ml), histamine (100mg/ml) or saline was applied to the IVCs of mice for 10 min. After 6 h thrombus formation, weight and length were tested.

(Performed in collaboration with A.Brill)

4.2.10 No difference is seen in arterial thrombosis or normal hemostasis between WT and $Kit^{W\text{-}sh}$ mice

We wanted to investigate if the prothrombotic effect of mast cells is specific to DVT or if they also exacerbate arterial thrombosis. We used the ferric chloride model of thrombosis, which induces thrombus formation as a result of denudation of the endothelium. As can be seen in figure 4.11a, no difference was observed between WT and $Kit^{W\text{-}sh}$ mice in time to occlusion in the ferric chloride model. This further suggests that the endothelium is required for mast cells to exert their prothrombotic activity, and that the effect of mast cells is specific to venous thrombosis.

In order to rule out any other factors, which could be causing the anti-thrombotic phenotype in $Kit^{W\text{-}sh}$ mice, we then compared normal hemostasis parameters in WT and $Kit^{W\text{-}sh}$ mice. Tail bleeding times were taken by cutting the tail at 3mm, and monitoring the time until bleeding arrest. No difference was seen in tail bleeding times between WT and $Kit^{W\text{-}sh}$ mice (figure 4.11 b). We also performed coagulation tests, looking at Prothrombin time (PT) and activated partial thromboplastin time (aPTT). In both of these coagulation tests, no difference was seen between WT and $Kit^{W\text{-}sh}$ mice.

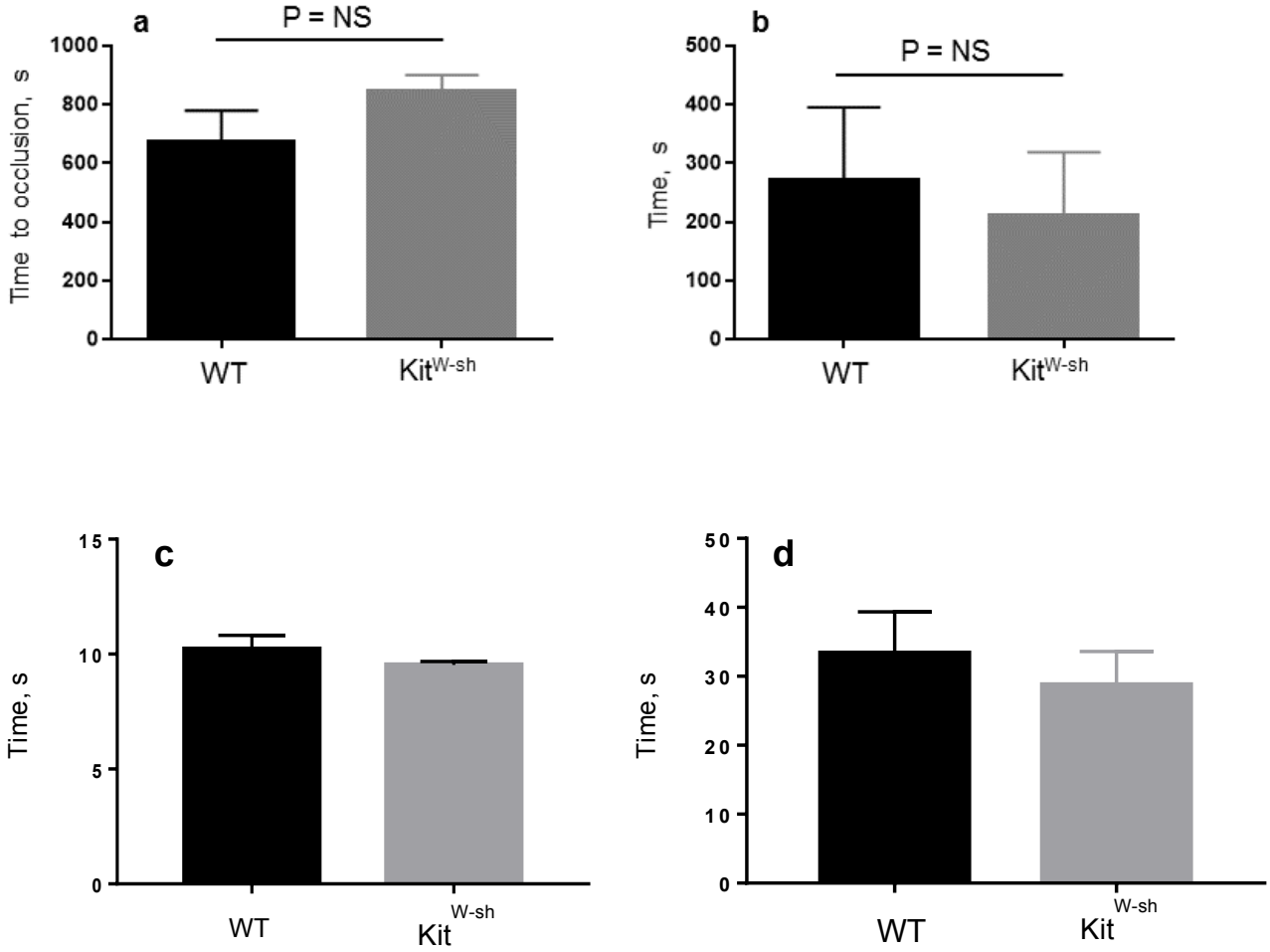


Figure 4.11 - No difference in normal hemostasis and arterial thrombosis between WT and Kit$^{W\text{-}sh}$ mice.

a) Fluorescent platelets were infused into WT and Kit$^{W\text{-}sh}$ mice (n= 5) and a strip soaked in 10% ferric chloride was applied to their mesentery for 5 mins. The time to vessel occlusion was recorded. b) The tip of the tail was cut at 3mm in both WT and Kit$^{W\text{-}sh}$ mice and time to bleeding arrest was measured (n= 6 to 8). C) PT and d) aPTT test were performed using a coagulometer (n= 5).

(Performed in collaboration with A.Brill)

4.3 Discussion

Current options for DVT prophylaxis are associated with bleeding complications, because they mostly target clotting factors, which are essential components of both pathological thrombosis and normal hemostasis. Therefore the existing drugs used to prevent DVT also impact on normal hemostasis and bleeding arrest. Throughout this chapter we have demonstrated that mast cells play a role in DVT, and are involved in DVT initiation. Also we have shown that a potential treatment option for venous thrombosis could be to inhibit mast cells, which are not directly involved in hemostasis, and therefore this is unlikely to result in bleeding complications as a side effect.

Venous thrombosis occurs as a result of blood pooling, and flow distortion. These lead to local hypoxia, endothelial activation, subsequent release of WPB contents, and recruitment of immune cells and platelets, which is essential in thrombosis initiation (Von Bruhl et al 2012). P-selectin deficient mice, in which immune cells cannot be recruited, are protected from DVT. Therefore mechanisms of DVT appear not only to involve blood coagulation, but also have parallels to sterile inflammation. DVT can therefore be considered an immunothrombosis (Engelmann et al 2013) as inflammatory components are involved in its formation.

Mast cells were initially considered as good candidates for a potential role in DVT, but weather this role was to be protective or prothrombotic was to be investigated. This is because mast cell granules contain some inhibitors of coagulation such as heparin and tPA, and may therefore inhibit thrombosis, but also contain pro-inflammatory mediators such as histamine and TNF-α, and

given that inflammatory components play a role in DVT mast cells could potentially be prothrombotic.

In this chapter we have shown that two strains of mast cell deficient mice; $Kit^{W\text{-}sh}$ and $Kit^{W\text{-}v}$ are completely protected against DVT, using the IVC stenosis model. This phenotype was striking, with 0% of mice producing a thrombus in either strain. This result suggests that of mast cells exacerbate venous thrombosis, and the mast cells contain a pro-thrombotic components/s, whose role in thrombus formation outweighs that of the other anti-coagulant components such a heparin.

A potential reason why mast cell prothrombotic components play a more important role in DVT that the anti-thrombotic components, could be due to their location in the vessel wall. As mast cells are found in the tissues surrounding the IVC, they have the ability to secrete their granules contents to the abluminal side of the endothelium. Here the inflammatory mediators may activate the endothelium, but the anticoagulant components (heparin and tPA) cannot actually reach the blood steam to mediate their effect.

However we cannot categorically state this is the only process leading to DVT, as a small number of WT mice do not produce a thrombus in the stenosis model. Therefore mast cells may not be prothrombotic in some cases, and other processes may be involved. It should also be noted these results have been obtained in mouse models, and further translational studies are needed in patients to verify their validity in humans.

Mast cell deficient mice we used in these experiments, carrying mutations in the c-Kit receptor (CD117) are known to have other abnormalities. We wanted to rule out any of these other abnormalities as potential causes of the antithrombotic phenotype shown in the Kit mutant mice, and prove it is indeed the lack of mast cells that specifically cause this effect.

It has been reported that $Kit^{W\text{-}sh}$ mice also have abnormal vascular permeability, and that $Kit^{W\text{-}v}$ mice have macrocytic anemia, both stains of mast cell deficient mice may also have other not yet known defects as a result of the Kit mutation.

To rule these possibilities out, we performed adoptive transfer of in vitro differentiated mast cells into the $Kit^{W\text{-}sh}$ mice; it has been previously shown that this approach restores tissue pools of mast cells within 2-3 months (Wolters et al 2005). Adoptive transfer restored thrombosis in mast cell deficient mice, further supporting the fact that lack of mast cells is responsible for the anti-thrombotic phenotype, not any other defects caused by the c-Kit mutation. Although it is not known if engraftment of transferred mast cells is the same as the normal mast cell distribution pattern, this would be a likely explanation however if the phenotype was not restored following mast cell infusion.

We knew that mast cells were found near blood vessels (Metcalfe et al 1997), but we wanted to further investigate if they are found in the IVC. Staining with toluidine blue showed that mast cells were located the IVC wall, close to the abluminal part of the endothelium. Staining showed that there were fewer granule containing mast cells in ICVs after stenosis where a thrombus had formed. Whereas, if a thrombus did not form, there was no significant change in mast cell number between WT and IVCs which underwent stenosis. This result

suggests that there is a link between functional mast cells and DVT formation, and the fact that less mast cells are found in IVCs where a thrombus has formed, suggests their released granule contents might be linked to DVT. We cannot be sure however if mast cells become fully degranulated which is why they do not stain with toluidine blue, or if they migrate out of the IVC wall. However disappearance of mast cells in thrombosed IVCs does suggest that their released cargo triggers DVT.

We wanted to next investigate if mast cells could be targeted pharmacologically, which would probably be more clinically relevant, as well as showing this phenotype in mice with genetic mutations. Many drugs are already on the market which inhibit mast cell degranulation such as anti-histamines, which are used in allergies. We firstly used a mast cell secretagogue 48-80 and administered it over a period of 4 days to mice in order to degranulate mast cells. This appeared to be protective in DVT, and none of the mice administered 40-80 developed a thrombus.

We then performed a similar experiment, using the mast cell membrane stabilizers sodium cromoglycate and Ketotifen. This produces a similar effect but instead of depleting the granules contents, prevents the mast cell secreting their contents. Both membrane stabilizers also protected mice from DVT development. When used as a mast cell stabilizers these compounds do not have an anti-platelet effect as shown by Platelet Function Analyzer-100, therefore we know this effect is not attributed to platelet inhibition. The anti-thrombotic effect seen after chronic administration of 40-80 could be attributed to the down

regulation of coagulation factor VII (Chu et al 2000), and therefore it may also inhibit the coagulation cascade, as well as deplete mast cell granules.

We further explored this by investigating if topical application of the mast cells secretagogue, compound 48-80 could potentiate thrombosis. A dramatic increase in thrombosis prevalence was seen in WT mice treated with this compound compared to controls. This suggested it is a mast cell granule constituent responsible for this phenotype. A likely candidate was histamine, seeing as is a small molecule and can stimulate the endothelium causing WPB to be released. We therefore performed local topical application of histamine to the IVCs of WT and Kit mice. Histamine potentiated thrombosis in the WT mice, and was able to induce DVT in mast cell deficient mice. These results suggest that histamine is the likely candidate in mast cells that is responsible for the pro-thrombotic effect.

We also performed intravital microscopy to investigate how mast cells may effect platelet recruitment to the vessel wall post stenosis. In mast cell deficient mice only single platelets firmly adhered to the vessel wall with the majority of them being carried away in the direction of the blood flow. We know platelet recruitment is vital for DVT formation, and that this process is dependent on WPBs releasing WVF. We measured plasma levels of WVF and show that these are reduced in $Kit^{W\text{-}sh}$ mice compared to WTs. Therefore mast cells somehow maintain VWF secretion, and probably contribute to DVT by activating the endothelium to release WPBs.

We further show in in-vitro studies that mast cell releasate was able to stimulate expression of ICAM-1 on HUVECS. ICAM-1 is an adhesion receptor for leukocytes.

Mast cells are therefore able to support DVT by up regulating endothelial cells and causing WBPs to secrete WVF and P-selectin.

Plasma soluble P-selectin is known to be a biomarker of DVT as it promotes DVT in mice (Antonopoulos et al 2014; Ramacciotti et al 2011). We show that mast cells are able to regulate sP-selectin in the plasma, as while IVC stenosis caused an increased in plasma sP-selectin levels in WT mice, this increase was not observed in $Kit^{W\text{-}sh}$ mice lacking mast cells. The increase in plasma sP-selectin in WT mice following stenosis supports the similarities of DVT in humans and the animal model. The elevated plasma sP-selectin could originate from either platelets or the endothelium, but regardless of the origin, this result shows it is also regulated by mast cells.

In conclusion, throughout this chapter we have provided evidence that mast cells are important regulators of DVT. And blocking mast cells can be protective against venous thrombosis. The pro-thrombotic effect mediated by mast cells is most likely to be through release of histamine, although we cannot rule out other mast cell granule components. Because temporarily blocking mast cell is unlikely to have any bleeding side effects, as $Kit^{W\text{-}sh}$ mice show normal bleeding times, mast cells are potentially a good target for preventing DVT in humans.

Chapter 5

The role of T-cells in venous thrombosis

5.1 Introduction

It is now commonly known that during the formation of deep vein thrombosis, inflammatory cells accumulate in the vessel wall and in the thrombus, and it is not only platelets and RBCs that are important in this thrombus formation (Diaz 2011). Inflammation and thrombosis are now considered to be intimately linked; in fact DVT can be considered as an immunothrombotic disease (Saha et al 2011).

Many treatments for DVT are concerned with preventing thrombus growth or extension such as anticoagulants, but few have an effect on existing thrombi which are able to resolve naturally, a process in which immune cells are believed to play a critical role (Mondarai et al 2005).

Treatments that speed up the thrombus resolution process, for example mechanical removal or thrombolysis, are associated with bleeding complications and increased risk of mortality. Treatment options, which could accelerate the natural thrombus resolution process, would be very attractive, as these would be associated with less complicated side effects (Patterson et al 2010).

A number of immune cells are implicated in inflammatory diseases, and some have been shown to play important roles in diseases such as atherosclerosis and DVT. One such important immune cell is the T-lymphocyte, or T-cell.

T-cells are a type of white blood cells that play a critical role in the immune response. There are different subsets of T-cells; helper, regulatory or cytotoxic cells. Cytotoxic T-cells or 'killer' cells are activated by various cytokines and bind to, and destroy infected cells. T-helper cells secrete cytokines, and these chemical messengers stimulate B-cells to differentiate into antibody producing plasma

cells. Regulatory T-cells (Tregs) play a critical role in suppressing an excessive immune response and maintaining self-tolerance. A deficiency in T-reg cells can cause a number of allergies or autoimmune diseases. There are different subpopulations of Treg cells including natural Tregs, which are most well studied and express the surface markers CD4, CD25 and Foxp3. These natural Treg cells develop in the thymus and have a predetermined function to be specialist suppressive cells. Other types such as inducible Tregs are more adaptive, and acquire their function after coming into contact with specific antigens (Xu et al 2013).

There have been reports that Tregs play a protective role in a number of diseases including myocardial infarction, atherosclerosis and in ischemia/reperfusion injury in liver and kidney. This protective role has been attributed to the ability of Tregs to relieve an excessive inflammatory response (Xu et al 2013).

A number of studies have shown T-cells to be implicated in arterial diseases. For example atherosclerosis (Mor et al 2007), however there is still controversy over whether T-cells play a protective or deleterious role.

Because atherosclerosis is considered to be an inflammatory disease, T-cells appeared to be good candidates for potentially regulating this inflammation. This is because they influence plaque development by secreting mediators, and they are regulated by both soluble and membrane bound molecules from many cells at the disease site (Robertson et al 2006).

It was shown by Robertson et al (2006) that the predominant T cell subset found in atherosclerotic lesions in apoE-/- and LDLR-/- mice were CD4+ T-cells. In these mice it was shown that a deficiency in adaptive immunity led to a decrease atherosclerosis. If immune deficient scid/scid mice were reconstituted with

CD4+ cells, this accelerated atherosclerosis, demonstrating the important role they play in the disease.

Further work supported this theory, using anti-CD4 antibodies to deplete these cells in C57BL6 mice; this reduced fatty streak development, with the mice on an atherogenic diet (Huber et al 2001). Authors suggest that T-cell activation is important for the early progression of the disease, but not for its initiation. T-cells play a vital role in the balancing act between if an atherosclerotic lesion will develop into a stable plaque, or if an immune reaction could lead to rupture of the plaque and eventually end in stroke.

It has been shown that T-regulatory cells (Tregs) may have a role in changing the functional status of the endothelium. The role that they play in ischemic stroke is still very controversial. Two theories have been put forward, one that they have a protective role against ischemic injury by modulating the inflammatory response. And conversely, that they could potentially be exacerbating the insult by causing micro vascular dysfunction (Xu et al 2013).

Liesz et al have shown that Tregs might have a protective role in post-stroke injury by preventing secondary infarct growth. They used 2 different mouse model approaches to show this. The model of middle cerebral artery occlusion (MCAO) was used in mice that had been administered anti-CD25 antibody two days previously, which specifically depletes Tregs. Mice deficient in Tregs showed significantly enlarged infarct size, and significantly worse neuronal function. They further verified this finding by performing adoptive cell transfer of Tregs into Rag2-/- mice that lack lymphocytes. Mice that received Tregs had significantly smaller infarcts following MCAO than those mice that only received

CD4+ T-cells, demonstrating that lack of Tregs is probably associated with worse outcome. Furthermore other studies have supported this protective role, Li et al (2012) showed that post stroke, adoptive transfer on Tregs within 24 h could dramatically reduce ischemic stroke volume, and showed prolonged beneficial effects.

In contrast to these findings, Kleinschnitz et al (2013) show that Tregs may have a negative role, and could in fact exacerbate ischemic-reperfusion damage 24h after stoke. They used DEREG mice, which had depleted Tregs to show that following 60 mins MCAO, there was in fact a decrease in infarct volume and an improvement in neurological function. Similarly to Liez et al (2009), they also performed adoptive transfer studies into Rag2-/- mice. However Kleinschnitz et al report that mice that received adoptively transferred Tregs, had increased infarct size.

While the controversy remains over if Tregs have a positive or negative impact on post-stroke outcome, some studies have contradicted both and have actually found them to have neither effect (Stubbe et al 2013). Ren et al (2011) also used the DEREG mouse model, and following 60 min MCAO showed no significant difference in infarct size between mice with or without Tregs.

Tregs adhere to the vascular endothelium and it is therefore possible that they activate or down regulate it, as well the role of T-cells in arterial thrombotic disorders, others have previously reported that T-cells may play a role in regulating post-thrombotic vein wall inflammation, and resolution of deep vein thrombosis in vivo (Luther 2016).

Siefert et al (2014) studied the role of T-cells in DVT resolution. They used the stasis model to replicate DVT in T cell depleted mice, achieved using an anti-CD3 antibody. Thrombus resolution in both control and T-cell depleted animals was measured by thrombus and vein wall weight. Mice that were depleted of T-cells during thrombus resolution (day 0-12) resulted in a 35% increase in thrombi size by day 12 compared to controls. This highlights that T-cell depletion impairs thrombus resolution. Thrombi were also analyzed, and those from mice in which T-cells had been depleted, showed decreased levels of several important mediators of thrombus resolution such as plasminogen activator inhibitor-1 (PAI-1) and urokinase plasminogen activator (uPA). Their results suggest that T-cells are important in regulating the expression of these mediators upon recruitment to the resolving thrombus and that T-cells are important players in the process of resolution.

Others have also reported this role in thrombus resolution. It is known that both CD4+ and CD8+ T-cells infiltrate the thrombus and vein wall upon DVT initiation, Luther et al (2016) suggest that in the vein wall the cells most predominantly recruited are effector-memory T-cells. And these cells are important in thrombus resolution by also recruiting neutrophils and monocytes to the thrombus. They show that depletion of effector-memory T-cells delayed thrombus resolution.

While previous studies have shown that T-cells play a role in the pathogenesis of many arterial thrombotic disorders, and that they are also important in the resolution of DVT, we wanted to further investigate if they played a role in the initiation of DVT.

T cells are not the only immune cells suggested to have a role in vascular diseases. B-cells have also been shown to play a role for example in atherosclerosis. B cells can be divided into two subsets; B1 and B2 cells. It has been suggested that B1 cells are atheroprotective, and they exert this protection via the production of natural IgM antibodies (Tsiantoulas et al 2015). Whilst in contrast it is suggested that B2 cells are proatherogenic. For certain autoimmune disorders such as rheumatoid arthritis, depletion of B cells has become a valuable treatment option.

5.2 Results

5.2.1 – Nude mice appear to have a prothrombotic phenotype in DVT

In order to investigate if T-cells play a role in deep vein thrombosis, we again used the IVC stenosis model as previously described in methods (2.7.3) on Foxn1nu, or 'nude' mice. Nude mice have a mutation in the FOXN1 gene, and subsequently have an absent thymus, and therefore are unable to produce T-lymphocytes. IVC stenosis was performed for two different time points, for 48 h and for 6 h. 48 h after stenosis, 70% of nude mice (with absent T-cells) produced a thrombus, and 60% of nude mice produced a thrombus after 6 hours (figure 5.1). However only 40% of WT mice produced a thrombus after 48 h, and only 10% of WT mice after 6 h. Thrombosis prevalence in the WT mice were however lower than would be expected, and lower than described in the literature (Brill et al 2011). This discrepancy could be attributed to different sources of WT mice (Jackson Lab Vs. Taconic of Charles River), or differences in their diet.

Although a preliminary result, there seemed to be an obvious phenotype in the nude mice (figure 5.1), with thrombosis prevalence being significantly higher in those mice without T-cells, compared to WTs at both time points. This could suggest that T-cells offer a protective role in DVT development.

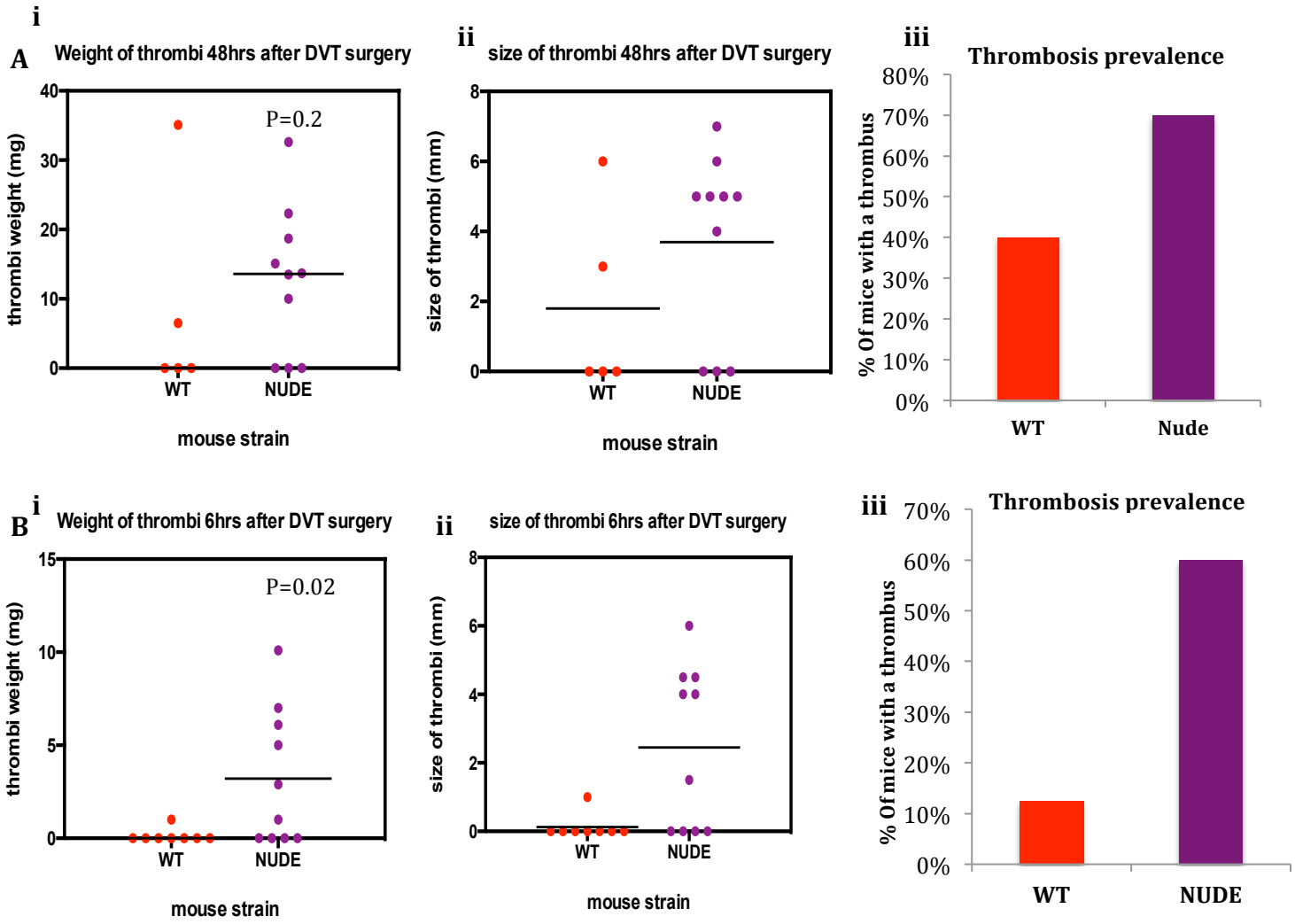


Figure 5.1 – Nude mice have a prothrombotic phenotype in DVT

(**a**) Following 48 h IVC stenosis, 70% of nude mice (with absent T-cells) produced a thrombus compared to 40% of WT mice this not significantly different (p = 0.2). Thrombus weight and length are shown in Ai & Aii with Aiii showing thrombosis prevalence (**b**) The same procedure was repeated for 6 h, and thrombosis prevalence was 60% in nude mice, compared to 10% in WTs (p=0.02). Each dot represents one mouse (48 h stenosis WT n= 5, Nude n= 10, 6h stenosis WT n= 8, Nude n=10). Statistical analysis was performed using a contingency table, followed by a Fisher's exact test. *(Performed in collaboration with A.Brill)*

5.2.2- T-regulatory cells do not appear to be protective in DVT

As nude mice have an absent thymus, they are depleted of all T-cells. We decided to do further studies but investigating the role more specifically of one subset of T-cells; the T-regulatory cells (Tregs). Tregs have already been reported to play a role in a number of diseases including atherosclerosis and ischemia reperfusion injury in liver and kidney (Xu et al 2013). As previously stated this protective role was attributed to the ability of Tregs to relieve an excessive inflammatory response. As DVT has inflammatory components, we investigated the role these T-regulatory cells may play.

C57BL6 mice were injected with an anti-CD25 antibody 48 hours prior to IVC stenosis. T-regulatory cells constitutively express IL-2R alpha chain (CD25) on their surface therefore the anti-CD25 antibody depletes the T-reg population (Noris et al 2007). The control group was injected with an IgG control antibody. Thrombi were then removed and measured 6h post stenosis.

In contrast to the nude mice, those mice that received an anti-CD25 Ab did not have a pro-thrombotic phenotype. 40% of mice that received the anti-CD25 antibody produced a thrombus, compared to 20% of the IgG control mice. This was not significantly different (0.32), and suggests that Tregs do not play a protective role in DVT. However thrombosis prevalence was measured after 6 h, compared to in the nude mice that were measured after 48 h stenosis. Also only 20% of control mice produced a thrombus, which as previously stated is lower than we would expect, possibly due to differences in the source of mice. Another consideration is that we did not achieve 100% depletion of Tregs with the anti-CD25 Antibody depletion.

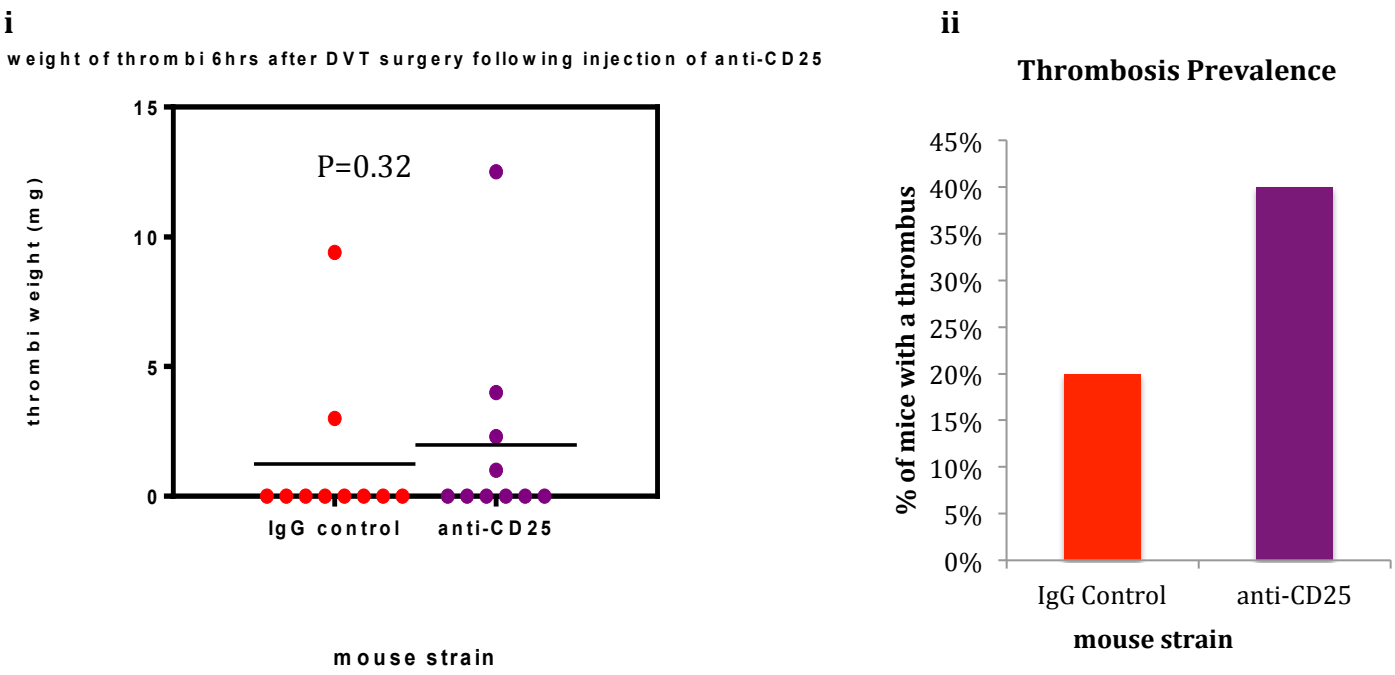


Figure 5.2 – No phenotype was observed after injection of C57BL6 mice with anti-CD25 Ab.

C57BL6 mice received an injection of either IgG control or anti-CD25 antibody 48 hours prior to IVC stenosis. Thrombosis prevalence and thrombi weight are shown above 6 h after stenosis. Each dot represents one mouse, for both groups n=10. ii) Shows Thrombosis prevalence was 20% in the IgG control, and 40% in anti-CD25 group. There was no significant difference in deep vein thrombosis prevalence between those mice that had T-regulatory cells and those that did not (p=0.32). Statistical analysis was performed using a contingency table, followed The Fisher's exact test. *(Performed in collaboration with A.Brill)*

5.2.3 – Rag1-/- mice are not prothrombotic in DVT

Rag-1 is a V(D)J recombination activation gene, which plays a role in the rearrangement and recombination of immunoglobulin and T-cell receptor genes. Rag1-/- mice are unable to perform this recombination and have no mature B- or T – lymphocytes (Mombaerts et al 1992). These mice have not been reported to have any neuroanatomical or behavioral abnormalities, despite RAG-1 expression being found in the central nervous system. As previously discussed both B- cells and T-cells have been reported to play both protective and detrimental roles in various disease development.

As these mice lack all T and B-lymphocytes, we hypothesized they may have a similar prothrombotic phenotype to the one observed in nude mice. As can be seen below in figure 5.2, this was not the case, and only 30% of Rag1-/- mice produced a thrombus compared to 50% of WT mice following 48 hours IVC stenosis. This suggests that T and B-lymphocytes may not offer a protective role in DVT as we previously thought.

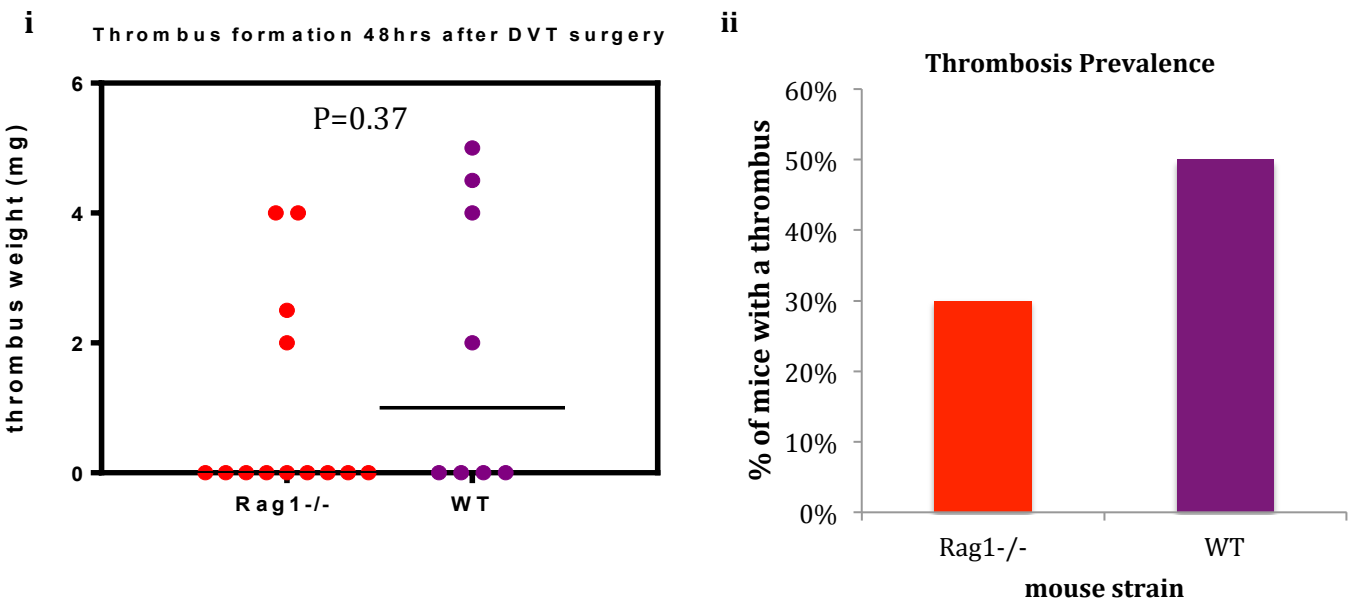


Figure 5.3 - Rag1-/- mice (no mature B or T lymphocytes) are not prothrombotic

Both Rag1-/- and WT mice underwent stenosis of the IVC and after 48 hours thrombus prevalence and weight was measured. 30% of Rag1-/- mice produced a thrombus, compared to 50% of WT mice; this was not a significant difference (p=0.37). Each dot represents one mouse (Rag1-/- mice n= 13, WT mice n= 8). Statistical analysis was performed using a contingency table, followed by the Fisher's exact test. *(Performed in collaboration with A.Brill)*

5.2.4 T-cells are present in DVT thrombi of C57BL6 mice partially co-localized with neutrophils

As we hypothesized that T-cells may be protective in deep vein thrombosis, or at least play some role, we looked at if where they are localized in the thrombus. Figure 5.4 shows staining of a cross section of thrombi from a WT mouse after 48 h IVC stenosis. CD3 is a T-cell marker and these are shown in green, Gr1 is a neutrophil marker and these are shown as red in figure 5.4. It appears that T-cells may partially co-localize with neutrophils. It has been suggested that neutrophils may bind to the activated endothelium prior to platelets during thrombus formation, at least in the artery, and this may be a critical step (Darbousset et al 2012). It is also known that neutrophils are important in the clotting cascade as they release extracellular traps (NETs) and bind to factor XIII. It is possible that NETs may also recruit T-cells in the same way as they recruit platelets and RBCs (Fuchs et al 2010). And it is unknown whether the interaction of T-cells with neutrophils could affect NETosis.

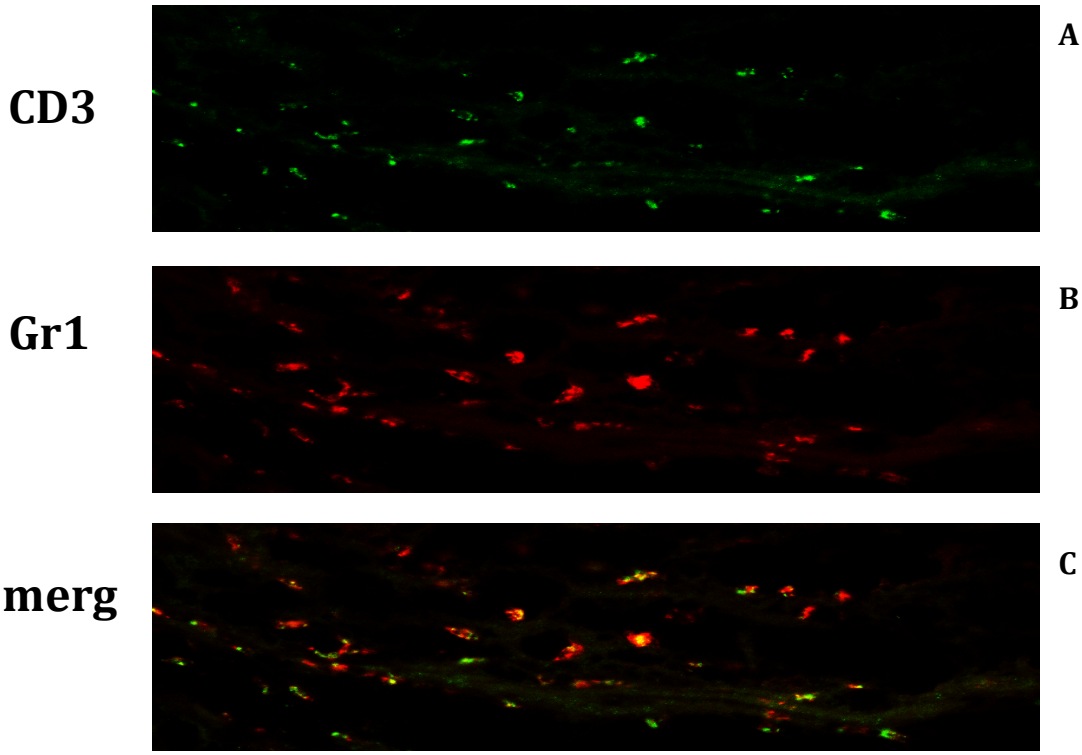


Figure 5.4- T-cells are present in DVT thrombi and partially co-localize with neutrophils

Staining of a thrombus 48 h post IVC stenosis in a WT mouse. Representative image taken from N=3. **A**) Shows staining of T-cells (Green), CD3 is a T-cell marker **B**) shows staining of neutrophils (Red), Gr1 is neutrophil marker. **C**) The merged staining of both CD3 and Gr1 reveals that neutrophils and T-cells may co-localize within the thrombus during DVT.

5.3- Discussion

Studies have previously shown that inflammatory cells accumulate in both the vein wall and in the thrombus during the progression of venous thrombotic disorders (Saha et al 2011). Further research has confirmed that inflammation and thrombus are intimately linked, this is now well known. However the exact contribution of each immune cell to venous thrombosis is still controversial, and the mechanisms are not fully understood.

We know that activation of the endothelium is a crucial step in the formation of DVT; this activation leads to up-regulation of tissue factor (TF), which is critical in thrombogenesis (Chu 2011). The activation of the endothelium by immune cells is therefore probably an important inflammatory process resulting in thrombosis.

As previously discussed, T-cells, specifically T-regulatory cells, have been suggested to play a role in a number of cardiovascular disorders such as myocardial infarction and atherosclerosis. This protective role has been attributed to the ability of Tregs to relieve an excessive inflammatory response. There is still some controversy however over whether these cells play a protective or deleterious role in the disease pathogenesis.

We therefore wanted to investigate, using a number of mouse models, if T-cells may play a role in deep vein thrombosis, which has an inflammatory component. We showed that Foxn1nu 'nude mice', which have and absent thymus and an inability to produce T-lymphocytes, have a prothrombotic phenotype in DVT (results, figure 5.1). We performed IVC stenosis for two time point's 6h and 48h in both nude and WT mice. There was a significant difference between

thrombosis prevalence in nude and WT mice after 6 h IVC stenosis (60% thrombosis prevalence), but although there was a trend this was not significantly different after 48h (70% thrombosis prevalence). Although this is preliminary data, it does suggest that T-cells may offer a protective role in DVT, and lack of T-cells is detrimental. This could be due to their ability to reduce an excessive inflammatory response, and therefore reduce the number of platelets and further inflammatory mediators to the site. However in this experiment, thrombosis prevalence in WT mice was much lower than would be expected, and lower than is reported in the literature. We show WTs to have 40% and 10% thrombosis prevalence after 48 h and 6 h respectively, and we would usually expect to see ~60%. This difference could however be attributed to different sources of WT mice, or differences in their diet. Further studies are needed to confirm this phenotype, and these studies should use a greater number of WT mice, and they should be littermate controls.

As nude mice have an absent thymus they do not have the ability to produce any T-cells at all. We wanted to investigate further which specific subset of T-cells were responsible for the phenotype seen in the nude mice, and therefore deceiver which T-cells may be protective in DVT. T-regulatory cells were considered to be a good candidate as they have already been shown to play a role in a number of cardiovascular diseases that have an inflammatory component.

To do this we used an anti-CD25 antibody that specifically depletes T-regulatory cells (Tregs), as they constitutively express IL-2R alpha chain (D25) on their surface. C57BL6 mice received an injection of the antibody 48 h prior to IVC

stenosis. In contrast to nude mice, only 40% of mice lacking Tregs produced a thrombus, and 20% of WT mice (Results, figure 5.2). Again, as previously stated, for unknown reasons thrombosis prevalence in the WT mice was much lower than would usually be expected. Also we measured thrombosis prevalence and thrombus weight 6 h after IVC stenosis, but not after 48 h. In order to further study if Tregs may have a role in DVT, we would repeat the experiment and perform IVC stenosis for two time points, both 6 h and 48 hrs. Another factor that we cannot rule out is that the anti-CD25 antibody did not achieve 100% depletion of Tregs. The other possibility is that T-regulatory cells are not the subset of cells that offer the protection seen in the nude mice.

We next decided to use Rag1-/- mice to see if these have a phenotype similar to that of the nude mice. Rag1-/- mice have no T-cells or B-cells, as they are not able to perform VDJ recombination, the process where T and B-cells randomly assemble different gene segments in order to generate their unique antigen receptors. B-cells have also previously been implicated in a number of inflammatory diseases, specifically in atherosclerosis, but whether they are atheroprotective or proatherogenic is still under debate. We suspected that the absence of both B and T- cells in combination may give a prothrombotic phenotype. As can been in results, figure 5.3, this was not the case. Only 30% of Rag1-/- mice produced a thrombus, compared to 50% of the WT mice. Thrombosis prevalence in the WT mice was around what we would normally expect. This result suggested that T-cells and B-cells, or at least in combination do not exert a protective role in DVT, as mice lacking both of these immune cells still show lower levels of thrombosis than in controls.

If T-cells are implicated in DVT, as the data from the nude mice suggests, we wanted to know whereabouts, if at all, they are located in the thrombi following IVC stenosis, and if they possibly interact with any other immune cells.

Because recent findings suggest neutrophils may also be important in promoting DVT through their release of their extracellular chromatin, forming NETs (Brill et al 2012). We hypothesized that these NETs may be important in recruiting or capturing T-cells in the same way they recruit platelets and RBCs. Figure 5.4 C, revealed that when staining for CD3 (a T-cell marker) and Gr1 (a neutrophil marker) that these cells did in fact co-localize within the thrombus. This could suggest because of their close proximity, that they do interact in some way. And potentially this may not be so T-cells promote DVT, but they could be involved in thrombus resolution.

As our results are very preliminary, and it is still not clear if T-cells may exert a protective role in deep vein thrombosis, further work is needed to repeat these experiments and increase the number of mice used in all groups. We would also aim to get around 60% thrombosis prevalence in WT mice as is suggested in the literature, and use littermate controls for all studies.

Chapter 6

Neutrophil extracellular traps (NETs)

6.1 – Introduction

Neutrophils are considered primarily to be an innate immune cell, with a primary function to act as a phagocytic cell against foreign bacteria. More recently however, their function has been shown to be multifaceted, playing a role in coagulation and many other processes (Kimball 2016).

It was first shown by Brinkman et al (2004) that in response to lipopolysaccharide (LPS), neutrophils release their granule chromatin and proteins; these extracellular fibers are able to bind both gram-positive and gram-negative bacteria. These extracellular chromatin fibers, bound to histones and granular antimicrobial proteins, are termed neutrophil extracellular traps (NETs), and the process by which this occurs is NETosis.

Whilst it is known that NETs play a key role in the form of an innate immune response, by capturing and killing bacteria, ensuring they cannot spread and providing a high local concentration of antimicrobial agents, they have also recently been suggested to potentiate thrombosis. During thrombus development, neutrophils contribute to the formation of an intravascular scaffold, where its function is likely to be both to support thrombus growth and to capture and destroy pathogens (Engelmann et al 2013).

NETs have not only been implicated in venous thrombosis, but also have a role in sepsis, trauma and cancer-related thrombosis (Kimball et al 2016). Although they have a role in a variety of diseases, it was reported that NETs have a common role in all of them, that they increased ischemia and the chance of further injury through micro or macrovascular thrombosis.

Whilst the exact mechanisms of NETosis are still largely unknown, it is known that it is a distinct form of cell death, different to necrosis and apoptosis. The cell

death pathway that leads to NET production is dependent on ROS production (see fig 1 chapter 1.1). ROS causes break down of the nuclear membrane, and delobulation of the nucleus. The chromatin is then able to mix with granular proteins before eventually the plasma membrane ruptures and the contents are released. ROS production also promotes peptidylarginine Deminase (PAD4) to citrillinate histones.

Neutrophils express high levels of the enzyme PAD4, and this is used as a marker of NET formation. It is this enzyme that allows citrullination, the replacement of positively charged arginine for uncharged citrulline. Wang et al (2009) show that it is hypercitrullination of histones by PAD4 that mediates chromatin decondensation and further NET formation. They also show that inhibition of PAD4 decreased NET formation.

One of the earliest studies that showed NETs have an important role in thrombosis was conducted by Fuchs et al (2010). He showed that NETs caused platelet adhesion, activation and aggregation, whilst treatment with DNAase or the anticoagulant heparin, prevented thrombosis by dismantling this NET scaffold. They showed NETs not only recruited red blood cells, they also promoted fibrin deposition.

Further work by Brill et al (2012) showed that NETs may actually promote deep vein thrombosis (DVT) and NETs form an important structural part of a venous thrombus. The IVC stenosis mouse model was used in these experiments, and the authors demonstrated that following IVC stenosis, mice had increased plasma levels of extracellular DNA. Also thrombi that formed in these mice were taken for immunohistochemical staining, which revealed that neutrophils (which had Gr-1 positive staining) were found in both the red part and the white part of the

thrombus. Furthermore, citrullinated histone H3 (CitH3), a marker of NETs, was found in the red part of the thrombus, associated with Gr-1 staining.

If mice were administered DNAase, this was protective after both 6 h and 48 h stenosis. In contrast if mice received an infusion of calf thymus histones, this increased DVT following stenosis, likely due to the increased levels of plasma VWF this induced. They were therefore able to show that this extracellular chromatin, probably originating from neutrophils, was a crucial structural part of the thrombus and both this scaffold, and histones appear to contribute to DVT.

Recent studies have shown that NETs may collaborate with platelets to promote intravascular coagulation in mouse models of sepsis (McDonald et al 2017). During sepsis NETs are released, and as well as having a positive effect in contributing to host defense, they may also contribute to tissue damage and are believed to contribute to coagulation. McDonald et al showed that NETs were actually critical in the development of sepsis- induced intravascular coagulation, regardless of the type of bacterial infection (gram positive or negative). If NETs were inhibited during sepsis, this reduced organ damage, increased microvascular perfusion and reduced intravascular coagulation. They also performed in-vivo imaging of mouse models of sepsis, and demonstrated that within the NETs, there was increased platelet aggregation, thrombin activation and fibrin clot formation.

Many studies have recently shown that NETs could also have an important role in non-infectious sterile inflammation. Following NETosis intracellular proteins are exposed to the extracellular space, this could potentially lead to the presentation of auto-antigens to the host immune system (Jorch & Kubes 2017).

They have been shown to play a role in several non-infectious autoimmune, and autoinflammatory diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and Psoriasis. Rheumatoid arthritis (RA) is an autoimmune disease, which is characterized by chronic synovial inflammation at the joints, which can result in both bone and cartilage injury. RA patients have an increased level of NET producing neutrophils in the synovial fluid of their synovial cavities; circulating neutrophils of these patients are also more easily simulated to undergo NETosis in comparison to healthy individuals, and have increased amounts of ROS (Delgado-Rizo et al 2017). It is thought that NETs act as a source of autoantigens in these patients, when neutrophils release NETs along with their granular contents, cytoplasmic self-antigens are also released into the joints (Khandpur et al 2013).

Similarly, people with systemic lupus erythematosus (SLE) have increased levels of anti-ribonucleo-protein and anti-DNA antibodies in their serum, and the increased in these antibodies is believed to be due to NETs. In patients with Lupus, not only do their NETs contain higher levels of pathogenic autoantigens compared to healthy controls, leading to increased tissue damage, they also have impaired NET degradation so they are more persistent (Jorch & Kubes 2017).

The exact mechanisms of NET formation are still largely not understood, or what causes NETs to form in thrombosis. We already know thrombin is critical in thrombosis, and it has also previously been shown to stimulate chemotaxis of neutrophils. Jenkins et al (1995) showed that thrombin triggered directional movement of neutrophils at low concentrations, while at higher concentrations cell movement was inhibited. Treatment of neutrophils with thrombin receptor

activating peptide (TRAP), failed to stimulate actin polymerization, believed to be required for neutrophil movement in chemotaxis. This indicated that another receptor, not PAR1 probably exists for thrombin induced neutrophil chemotaxis. It was then later shown by another group that neutrophils stimulated with thrombin appear to have a dramatic reduction in the number of cytoplasmic granules, as well as numerous empty vesicles in their cytoplasm (Stuardo et al 2004). These studies therefore showed that thrombin is having an effect on neutrophils, but through what mechanisms is not known, it may be through a PAR independent mechanism. We then wanted to ask the question, if thrombin could induce NET formation in thrombosis.

As well as understanding the cause of NETosis in thrombosis, we wanted to further investigate the exact mechanism behind this. Previous studies have shown that the cytoskeleton may be important in NET release (Neeli et al 2009). Usually any large changes in cell morphology involve the cytoskeleton. The actin cytoskeleton is already known to be involved in several functions of neutrophils such as phagocytosis, cell attachment and migration, whilst microtubules play an important role in transporting granules. As NETosis involves a change in cell morphology and release of granule contents, it seemed reasonable to predict that both the actin and tubulin networks may play a role in this, but at which stage is unclear.

Neeli et al tested this hypothesis by incubating neutrophils with either nocodazole, a drug that interferes with tubulin polymerization, or cytochalasin D, which interferes with actin filament polymerization, prior to treatment with LPS. They measured both levels of NET release, and of histone H3 deimination.

Around 30% of neutrophils treated with just LPS produced NETs. However, following treatment with nocodozole prior to LPS, most neutrophils had a similar appearance to control cells, with only 5% of the cells showing NET release. These cells also showed a reduced level of histone H3 deminination. Similarly, treatment of neutrophils with cytochalasin D also resulted in most of the neutrophils maintaining their naïve morphology, and only 9% of them produced NETs.

Although the results are striking, it is not clear exactly how the cytoskeleton plays a role in this NET release. One suggestion is the actin filaments may play a role in allowing the rupture of the plasma membrane and deployment of NETs. Actin filaments could act to push the chromatin through a breach in the plasma membrane. This hypothesis seems plausible since those neutrophils treated with cytochalasin D (which disrupts actin polymerization) prior to LPS, showed chromatin was able to mix with the cytoplasmic contents following nuclear envelope breakdown, but the plasma membrane still remained a barrier to NET release.

In this chapter I aimed to uncover the signaling pathways that may lead to NET formation, based on what is already known, we decided to target specific stages in the NETosis pathway with inhibitors to see if these played an important role, and if we could inhibit NETosis happening. Figure 1 below shows at which points in the pathway we were trying to inhibit NETosis. We also wanted to investigate what causes NETosis during thrombosis, specifically in DVT, a sterile inflammatory environment.

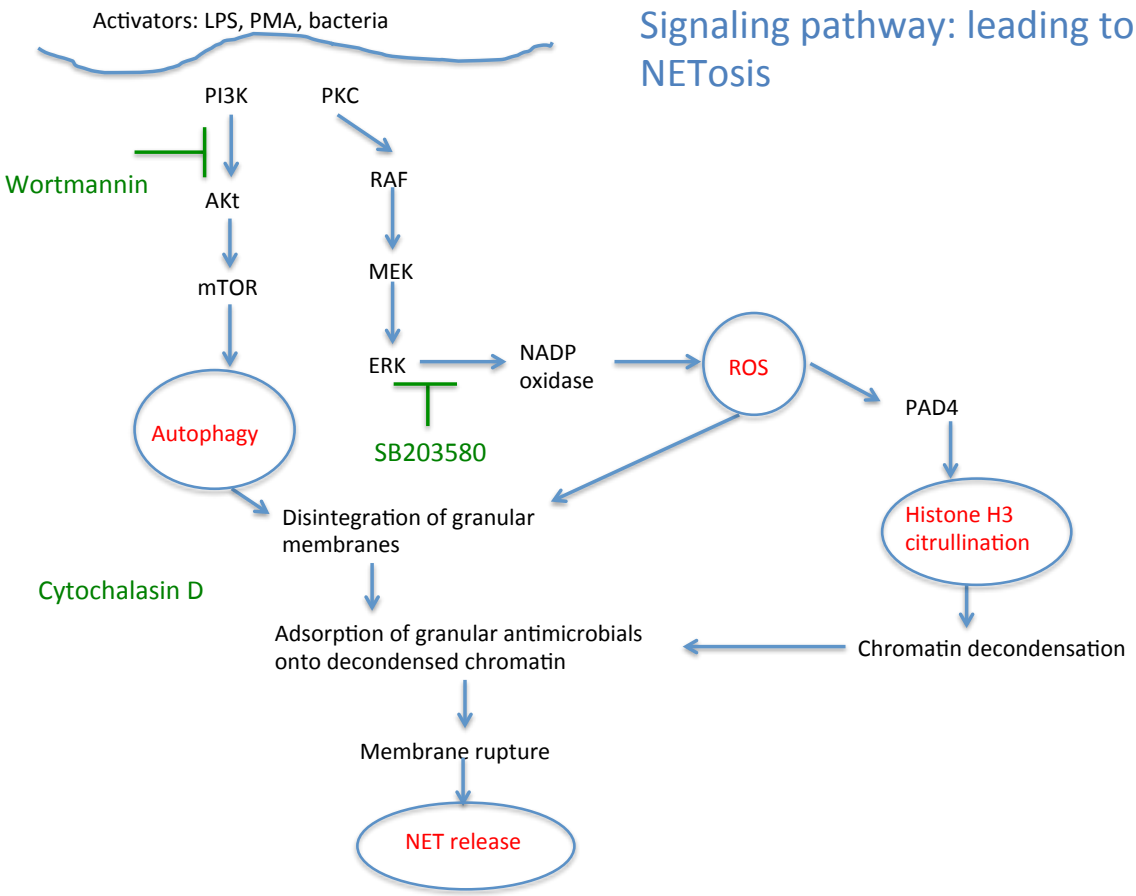


Figure 6.1 - Inhibiting different stages of the NETosis pathway

We targeted different stages in the NETosis pathway using various inhibitors to see if we could prevent neutrophils producing NETs, using Wortmannin, SB203580 and cytochalsin D.

PAD4:Peptidylarginine deiminase 4, PI3K: Phosphoinositide 3-Kinase, AKT: Protein Kinase B, mTOR: The mammalian Target of Rapamycin, PKC: Protein Kinase C, RAF: Rapidly Accelerated Fibrosarcoma, MEK: Mitogen-activated protein kinase, ERK: Extracellular signal-regulated kinases.

6.2 Results

6.2.1 NET formation by murine neutrophils

Neutrophils were isolated from the bone marrow of mice as described in methods 2.5 by using a percoll gradient. We wanted to make sure we could replicate the well-known protocol for neutrophil activation, and we tested a range of common activators to see if they produced NETs in murine neutrophils in our hands. A 24 well cell culture plate was prepared by putting a 13mm round glass cover slip into each well, 2 X 10^5 neutrophils were then seeded into each well in 500µl RPMI, and incubated at 37^{o}C for 1h. After this time, neutrophils were activated with 100nM PMA, 100µg/ml LPS or 20µM Ionomycin for 3h. Cells were then fixed and stained with DAPI, the DNA dye so NETS can be visualized. As can be seen below in figure 6.1, NETs were produced by murine neutrophils using all of the activators.

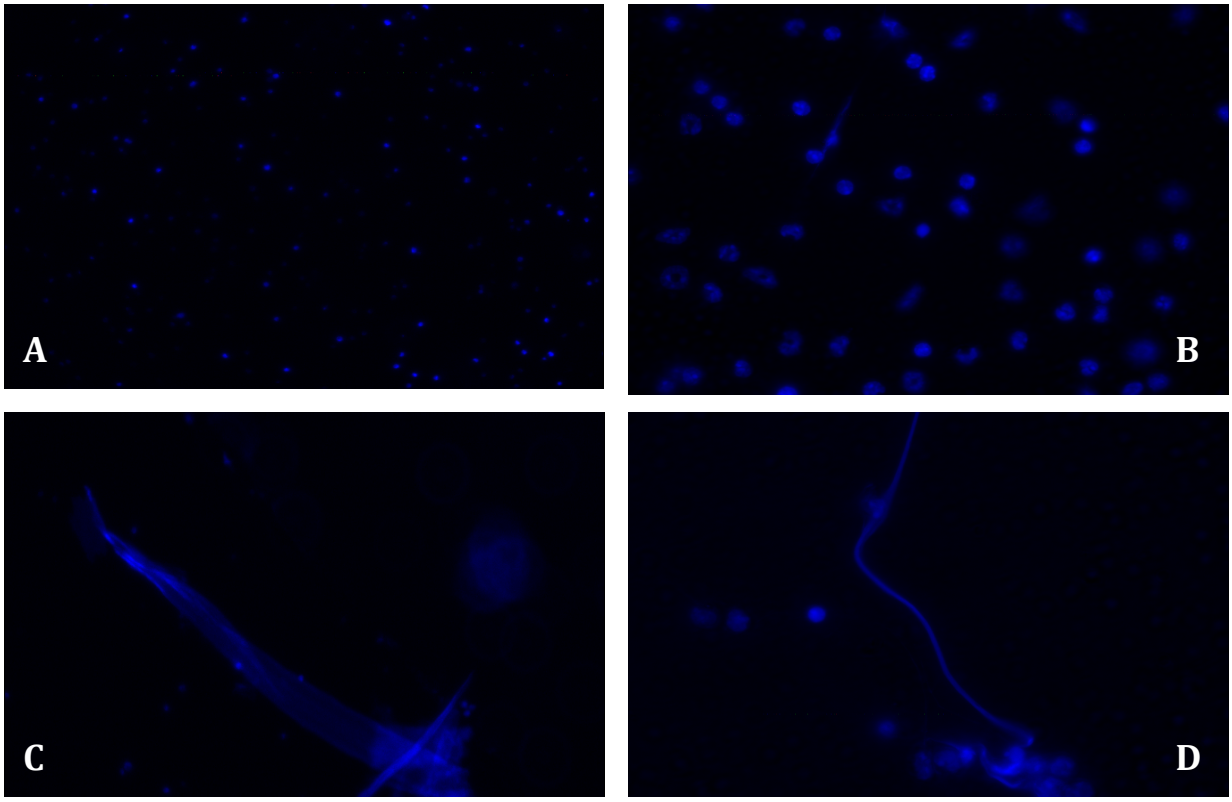
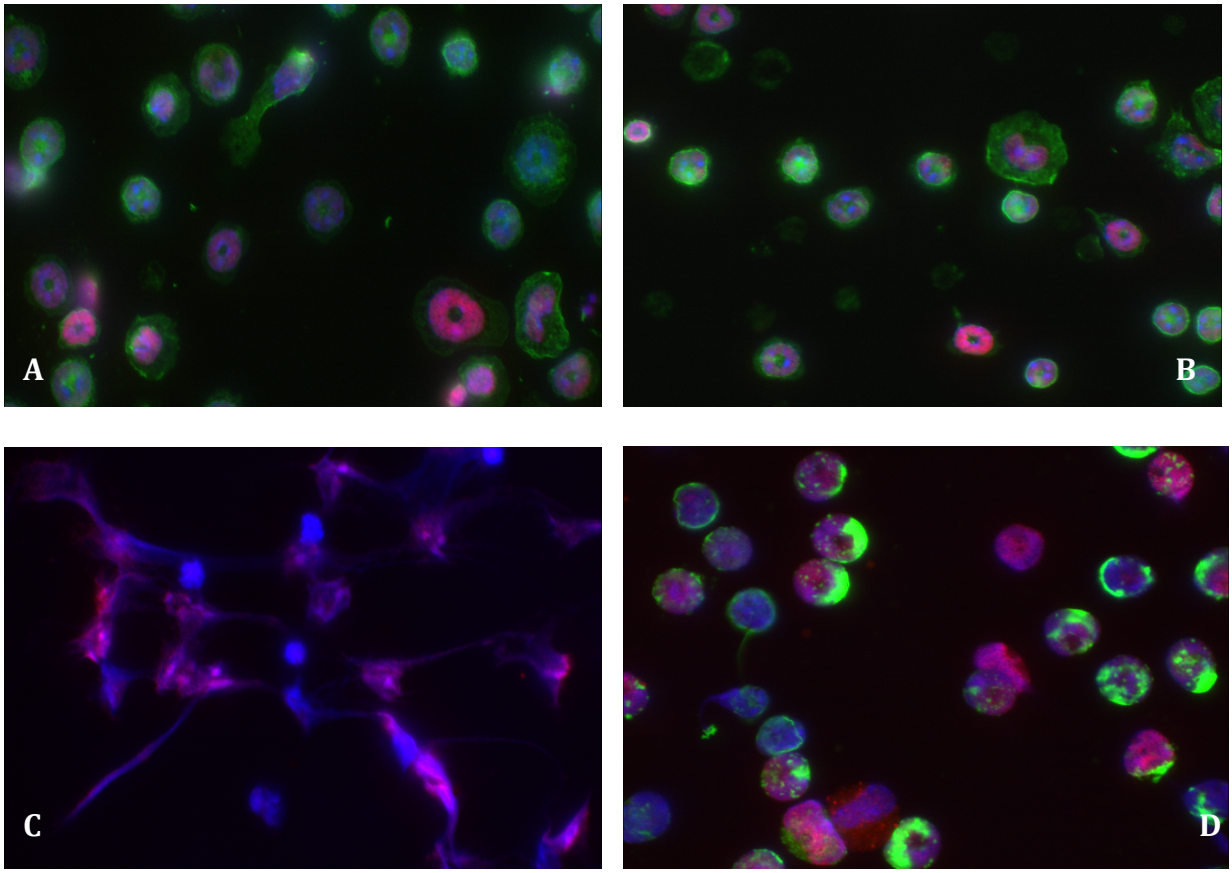


Figure 6.2 – Murine neutrophils activated to produce NETs

Neutrophils were stimulated with various activators for 3h, and stained with the DNA dye DAPI to visualize NET formation. 2 X 10^5 neutrophils were seeded per well. A) Control un-stimulated neutrophils, B) Neutrophils activated with 100nm PMA, C) Neutrophils activated with 100 μg/ml LPS D) Neutrophils activated with 20 μM Ionomycin

6.2.2 Immunostaining for citrullinated histone H3 in murine neutrophils

Citrullination of histone H3, the process whereby positively charged arginine is replaced for uncharged citrulline is a marker of NET formation. The enzyme PAD4 is responsible for this process, and it is known that hypercitrullination by PAD4 mediates chromatin decondensation and NET formation. As Cit-H3 staining is now commonly used as a method to detect NETs, we performed this immunostaining using an anti-Histone H3 antibody. Figure 6.3 below shows in the control untreated neutrophils (A&B), no NETs were produced, clear lobulated neutrophils can be seen (purple), and Phalloidin (green) staining also shows the actin cytoskeleton is evenly distributed. Whilst In those neutrophils activated with 20μm Ionomycin (Figure 6.3 C & D), NETs can be clearly seen by the Cit-H3 and DNA staining, interestingly figure D shows the distribution of the actin cytoskeleton (green) to the edge of the cell, possibly before it is used to deploy the NETs.



= Citrullinated histone H3

= DNA

= Polymerized actin cytoskeleton

Figure 6.3- Immunostaining for citrullinated histone H3 in murine neutrophils

A & B) un-stimulated neutrophils. C & D) neutrophils stimulated with 20μM Ionomycin. Immunostaining was performed using DAPI (DNA stain), anti-Histone H3 antibody (pink) and Phalloidin (green) to stain the actin cytoskeleton.

6.2.3 Activating human neutrophils to produce NETs

We found that working with murine neutrophils was inconsistent and with neutrophils in the control group often being activated, probably as a result of the isolation process. We therefore decided to isolate and stimulate neutrophils from human whole blood, as this is common practice with a more standardized protocol. Neutrophils were isolated as described in methods 2.6. They were then activated with 5μm Ionomycin, 10μg/ml LPS or 100nm PMA. The extracellular chromatin was stained using DAPI dye. As can be seen in figure 6.4 below, NETs formed in human neutrophils using all of the activators. Unexpectedly however, few NETs could also be seen in the control (figure A below), neutrophils may have been activated during the isolation procedure. However following quantification, NETs were produced to a much greater extent if treated with activators, the greatest NET production being seen following addition of 5μM Ionomycin (figure D). Quantification was performed by taking 4 representative images per treatment condition; the percentage of NET coverage per area of view field was then calculated.

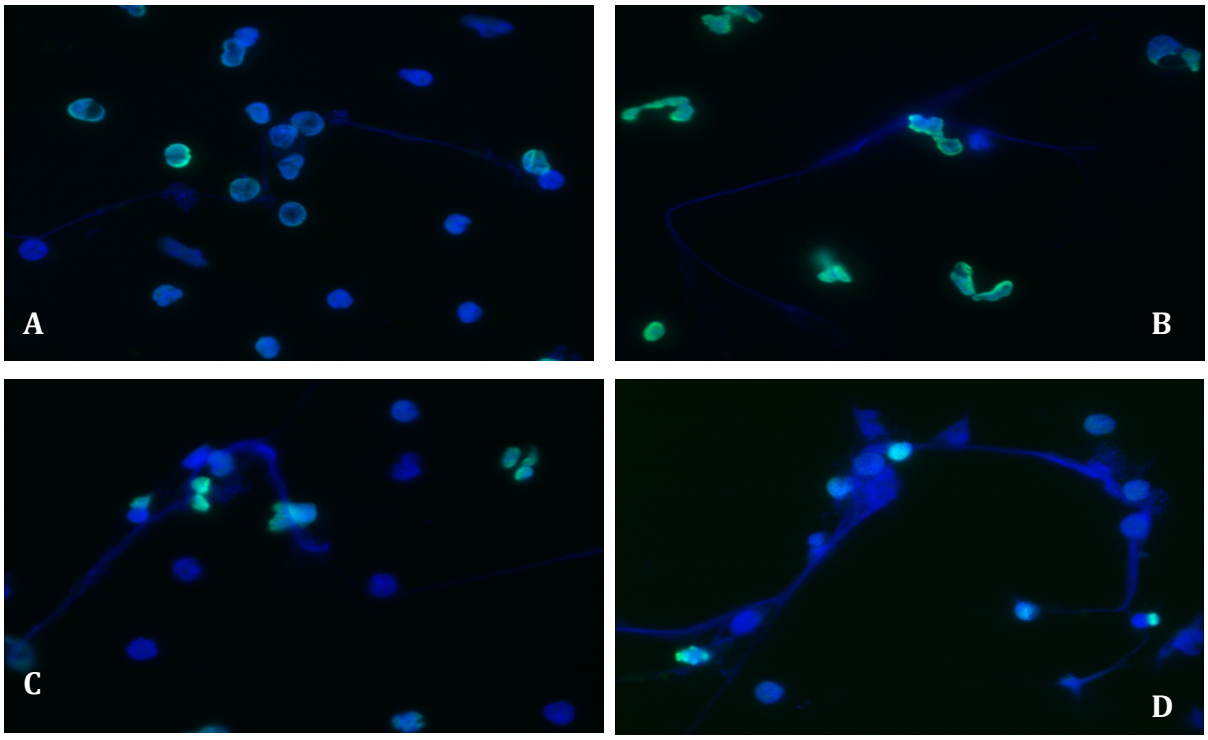


Figure 6.4 – Activation of human neutrophils

Neutrophils were stimulated with various activators for 3h, and stained with the DNA dye DAPI to visualize NET formation. 2 X 10^5 neutrophils were seeded per well. A) Control un-stimulated neutrophils. B) Neutrophils activated with 100nM PMA. C) Neutrophils activated with 10µg/ml. LPS D) Neutrophils activated with 5µM Ionomycin.

6.2.4 Targeting different stages of the NETosis pathway to prevent NET formation

As NETs have been found to be implicated in a number of diseases, including thrombosis, we wanted to investigate the signaling pathways which leads to their formation, and to see if we could target specific parts of the pathway to inhibit NETosis. We used either PMA (100nM) or Ionomycin (2µM) as the activators for NETs, as I had previously seen high levels of NETosis in human neutrophils at these concentrations. In addition to these activator's, 1h later

neutrophils were incubated with one of the following inhibitors: Wortmannin (10μM), Cytochalasin D (10μM) or SB203580 (10μm). Figure 6.1 above demonstrates at which stages in the NETosis pathway these inhibitors were targeting. As it has been previously suggested that the actin cytoskeleton may play a role in the deployment of NETs (Neeli et al 2009), we wanted to see if we could replicate this, using cytochalasin D the actin polymerization inhibitor. This may work to prevent rupture of the plasma membrane, and therefore prevent NET release.

We also chose to test Wortmannin, which is a PI3K inhibitor. One of the possible routes, which may lead to NET release, is autophagy, a regulated form of cell death. Wortmannin inhibits PI3K, and therefore interrupts the PI3K-Akt-mTOR pathway that culminates in autophagy. The other pathway we wanted to investigate was the raf-MEK-ERK pathway, it has been suggested that it is important for generation of ROS, and that this pathway induces the expression of antiapoptotic proteins, which inhibit apoptosis to allow for NETosis (Hakkim et al 2011). We therefore used the P38 MAPKinase inhibitor (or ERK inhibitor), SB203580 to see if inhibiting MAPKinase had an impact on NET release.

Figure 6.5 below shows representative images of all conditions, both activators with and without the 3 inhibitors. We saw no obvious difference in the amount of NETs produced between any of the neutrophils that received an inhibitor, or those which received an activator only. We therefore concluded that it was very unlikely that these parts of the pathway would be good therapeutic targets for preventing NETosis in thrombosis, and that other signaling pathways may be more important. We therefore need to do further work to establish at what stage

we could inhibit NETosis, one future question would be to investigate the role of Src an Syk Kinases, which are found upstream of PKC.

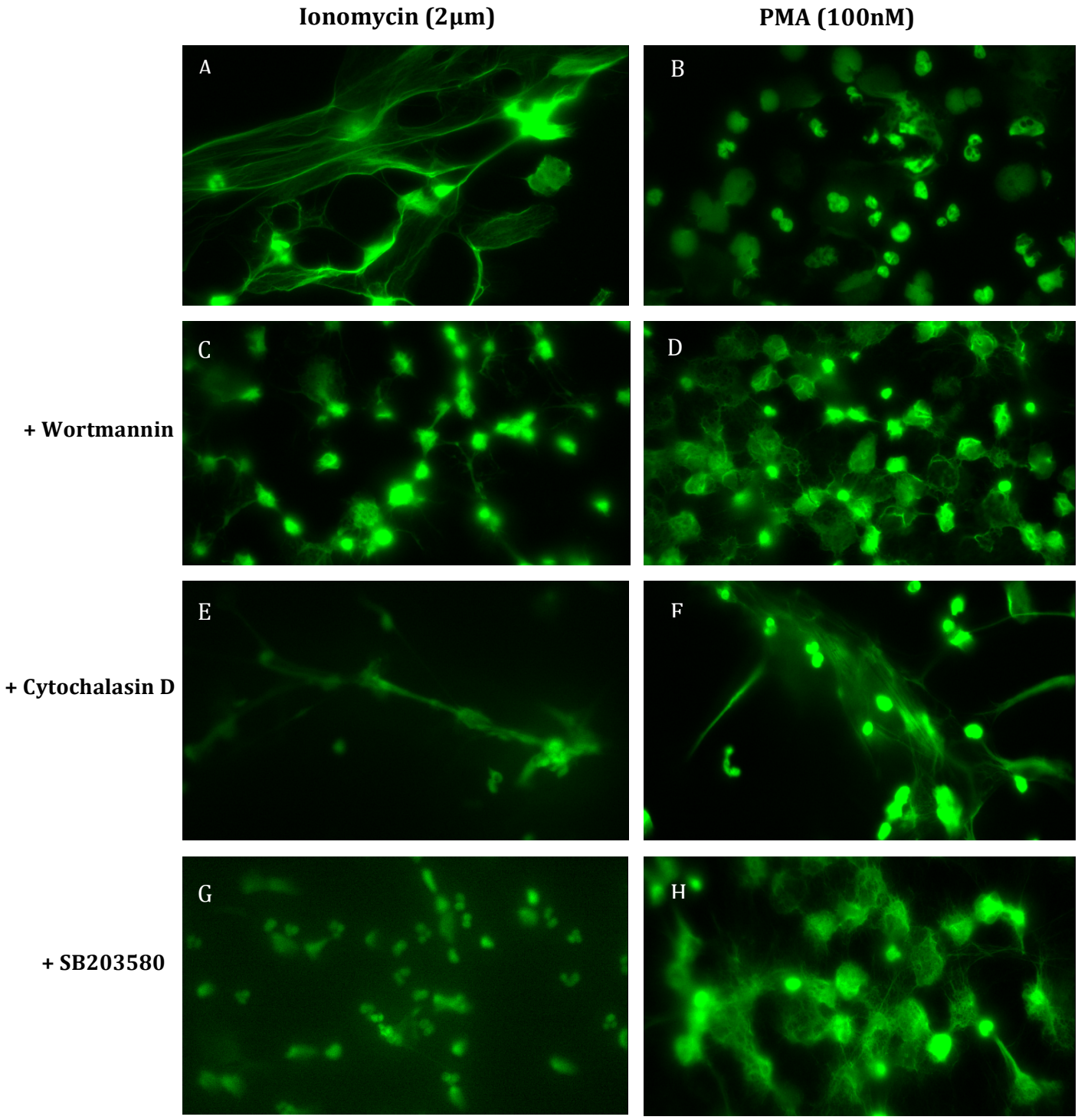


Figure 6.5 NET formation using a range of inhibitors

2 X 10^5 neutrophils were seeded per well and activated with either Ionomycin (2μM) or PMA (100nM). Various inhibitors were added to each group to try and prevent NETosis; wortmannin (10μM), cytochalasin D (10μM) or SB203580 (10μM). No difference in NETosis was found between the inhibitors and controls.

6.2.5 The role of thrombin in NETosis

We know that NETs have been reported to play a role in thrombosis. It was shown by Brill et al (2012) using the mouse IVC stenosis model, that NETs may actually promote deep vein thrombosis, and they form an important structural part of the thrombus. We wanted to investigate what promotes this NET release in DVT, a sterile inflammatory environment. The obvious target to investigate was thrombin, as we already know this is critical in thrombosis. Thrombin has also previously been shown to trigger directional movement of neutrophils (Jenkins 1995), although the receptor through which thrombin works was not identified. It has also been shown to lead to a dramatic reduction in the number of cytoplasmic granules in neutrophils (Studardo et al 2004). We wanted to investigate if thrombin is the candidate for inducing NETs in DVT.

Neutrophils were seeded into a 24 well plate, with 3 wells for each treatment condition. 3 wells contained un-stimulated neutrophils, then 3 contained of each of the activators; PMA (100nM) or Ionomycin (2μM) to act as the controls. 3 different concentrations of thrombin were then tested; 0.1 units/ml thrombin 1unit/ml and 5units/ml. This experiment was repeated 5 times, with the average area of NETs per view field, per condition being calculated. Figure 6.6A below shows representative images of neutrophils treated with each of the concentrations of thrombin, compared to the control untreated neutrophils.

Figure 6.6B shows the results of all 5 experiments combined, and the graph represents the % area of view field covered by NETs. A significant difference was found between both 0.1 unit/ml and 1unit/ml thrombin and the control using an ANOVA, followed by Dunnetts multiple comparisons test, but no significant difference was found between 5units/ml thrombin and the control. Although preliminary data, these results could suggest that thrombin could be responsible for the induction of NET formation during thrombosis.

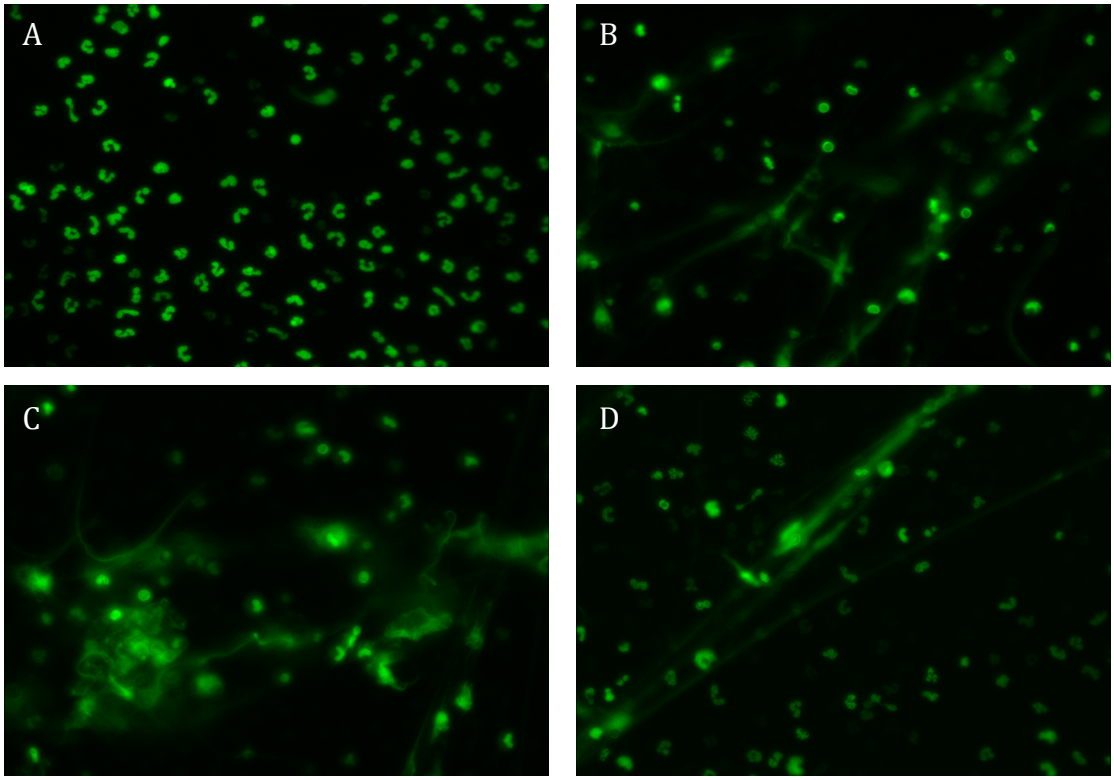


Figure 6.6A - Representative images of neutrophils treated with different concentrations of thrombin.

A) Control– untreated neutrophils, B) neutrophils treated with 0.1 units/ml thrombin, C) neutrophils treated with 1unit/ml thrombin, D) neutrophils treated with 5 units/ml thrombin

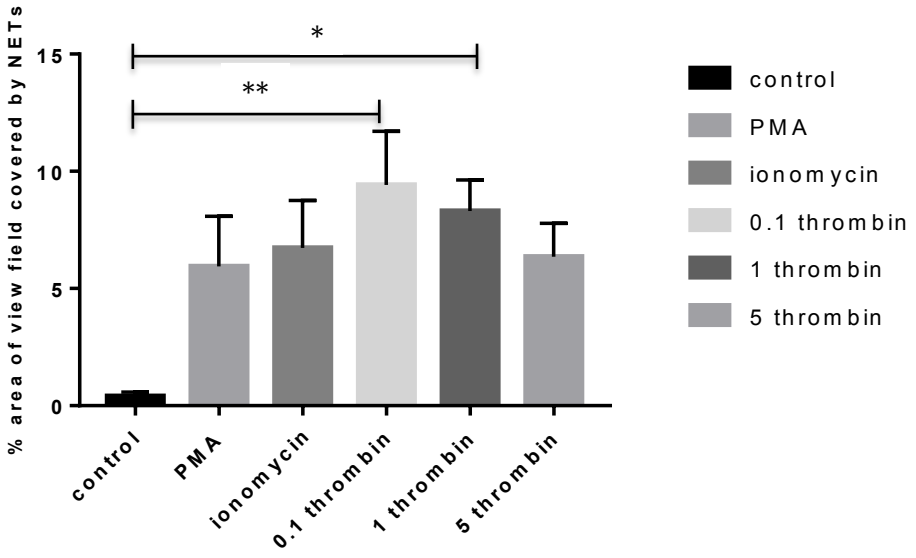


Figure 6.6 B – The average % area of view field covered by NETs following addition of different concentrations of thrombin to neutrophils

2 X 10^5 neutrophils were seeded into each well of a 24 well plate, with 3 wells per treatment condition. The graph shows the average % area of view field covered by NETs taken from 5 experiments, N= 15 per condition. Neutrophils were either not activated, or treated by 100nM PMA, 2µm Ionomycin, or one of 3 concentrations of thrombin; 0.1units/ml, 1unit/ml or 5units/ml. ** denotes P=0.0047, * denotes P=0.0143. Only 0.1 units/ml and 1unit/ml thrombin are significantly different compared to control, others groups are non-significant. Bars show mean with ± SEM.

6.3 Discussion

Studies have recently shown that neutrophils play multifaceted roles in a variety of illnesses, and are no longer considered to just be phagocytic cells, important for protecting the host against bacterial infections (Kimball 2016). We know that neutrophils produce NETs, and these have a variety of roles, not only during infectious diseases to trap bacteria, but also in sterile inflammatory settings such as that found in thrombosis.

Work by Fuchs et al showed that NETs were capable of promoting platelet adhesion, aggregation and activation, whilst it was later shown that they are involved in deep vein thrombosis, forming an important structural part of the thrombus (Brill et al 2012). We therefore wanted to further explore the role of NETs in thrombosis, and to understand the mechanisms of NETosis in a thrombotic environment.

We investigated various stages of the NETosis pathway to try and find a good target for preventing NET formation. We were unable to see any difference in NET formation using Wortmannin, Cytochalasin D or SB203580 as inhibitors. It is unlikely that autophagy is the main cause of NET release, as wortmannin inhibits PI3K, which in turn would prevent the PI3K-Akt-mTOR pathway culminating in autophagy. It is more likely that an alternative form of cell death is responsible for NET release. We also found no inhibition of NET formation using SB203580, a MAPKinase inhibitor. We hypothesized that this inhibitor may lead to decreased ROS production, and subsequently decreased NET release, as we know ROS is important for activating PAD4, and for disintegration of the granular membrane. However this was not the case, if we were to continue this

study further we would look at the role of kinases upstream of PKC, for example investigating if Src and Syk play a role in NET release.

We also wanted to investigate the effects of cytochalasin D on NET release; this interferes the actin polymerization and had previously been reported to inhibit NET formation after activation with LPS in a study by Neeli et al (2009). We were not however able to see this same inhibition, and NET release was similar in the control and cytochalasin D treated neutrophils. We did however use different activators to the ones used in this study, PMA and Ionomycin. In the future we would repeat this study testing all of the activators prior to inhibition, and at a range of concentrations. Although the actin cytoskeleton may be important for NET release, and aid in the deployment of the extracellular chromatin, it may not be the primary cause of NET release and other pathways may be more important.

The next question we wanted to investigate was what causes NET release in a thrombotic environment, specifically in DVT. NETs have been found in the thrombi formed in the deep veins, and we know that this is a sterile inflammatory environment; therefore the cause of this NET release was not due to bacteria or other infectious agents. The obvious candidate was thrombin. Thrombin has a diverse range of biological roles, and acts as both a pro-coagulant and a pro-inflammatory mediator (Hongbao et al 2008). It plays a crucial in thrombosis, and it also plays a role during inflammation in acting as a neutrophil chemoattractant (Esmon 2000). Thrombin induces neutrophils to move towards its chemical gradient (the site of injury for example) to carry out their function. It is possible that thrombin also activates the neutrophils to produce NETs. We tested a range of concentrations of thrombin; 0.1 units/ml, 1

unit/ml and 5 units/ml. We found that thrombin did induce NET production at all concentrations, at levels comparable to that of PMA and Ionomycin, or in the case of 1 unit/ml thrombin, an even higher level of NET production was measured compared to the other activators (see figure 6.6B). When compared to the control group, a significant difference was found using and ANOVA between the treatment conditions and 0.1 and 1 unit/ml thrombin. There was an insignificant difference however between 5units/ml thrombin and the control. These results are promising, and suggest that thrombin may be the candidate that induces NETosis in a thrombotic environment. Further work is needed to confirm this finding.

If we were to continue with these experiments further, we would aim to increase the N numbers, these results show the average of 4 experiments, and we would want to try and take the average of at least 10 experiments. Quantification of NETs can be problematic; applicability of measuring fluorescent intensity is questionable because the intensity of NETs and intact nuclei is insufficiently different to ensure reliable discrimination. Therefore, we developed quantification based on manual delineation of NETs and currently express the area covered by NETs as a percentage of the total area of each view field. We were able to do this by randomly selecting 3 different areas per well, per treatment condition. The random nature of our selection process may mean quantification is not entirely accurate and the only way to overcome this is to increase the N numbers.

Another problem we encountered was getting a low level of NET production in the control, which is unusual; this could explain the non-significant result when comparing PMA and ionomycin against the control, which are usually used as

standards. This low level of NET production was probably due to neutrophils being activated during the isolation process. We would need to optimize the neutrophil isolation protocol in order to get better quality isolation, with less contamination of RBC's and other factors, which could potentially activate neutrophils.

Chapter 7

General Discussion

7.1 Summary of Results

Platelets play a critical role in thrombosis, and many of the major platelet receptors that play important roles in arterial thrombosis have already been studied, but less so in venous thrombosis. The overall aim of this thesis was to identify new targets to prevent deep vein thrombosis, which will not lead to bleeding complications as a side effect.

In this thesis I wanted to (i) investigate the role of CLEC-2 and GPVI in venous thrombosis and compare this to their roles in arterial thrombosis. (ii) To investigate if other immune cells, namely mast cells and T-cells play a role in DVT (iii) to study the signaling pathways leading to NETosis, and the cause of NETosis in thrombosis.

7.1.1 The role of GPVI and CLEC-2 in DVT

I have assessed the role of both GPVI and CLEC-2 in DVT using in vivo models, the well-established IVC stenosis model to replicate DVT, and the laser injury model. Whilst I found GPVI deficiency resulted in only an insignificant trend towards decreased DVT prevalence in the stenosis model, CLEC-2 deficiency resulted in protection against DVT. I also show that podoplanin, the ligand for CLEC-2 is constitutively expressed in the IVC wall, and this expression is up regulated following IVC stenosis. This podoplanin expression was then further increased if IVC stenosis resulted in thrombus formation. Further experiments to investigate the relationship between podoplanin expression in the vessel wall and DVT were performed.

I then showed in the in-vivo IVC stenosis model, that mice administered with an anti-podoplanin antibody, still formed thrombi with a prevalence similar to that found in control mice, however thrombus size was decreased in mice without

podoplanin. This implied that podoplanin is most likely involved in thrombus propagation, rather than thrombus initiation. Although both CLEC-2 and podoplanin appear to be important in DVT, we cannot categorically state that they work together as a receptor-ligand couple. Further investigation is needed to ascertain the exact source of podoplanin in the IVC.

I did however rule out a few potential sources of podoplanin in the IVC. I show it is unlikely to be from endothelial or hematopoietic cells, as mice with podoplanin knocked out on these cells maintained podoplanin expression in the IVC.

I was also able to demonstrate that the dramatic phenotype seen in the CLEC-2 knock out mice using the IVC stenosis model, was not due impaired platelet-platelet interactions, because thrombus formation was normal in these mice using the laser injury model. GPVI deficient mice however showed an interesting phenotype in the laser injury model, thrombi in these mice started to form but embolized, not allowing for thrombus propagation, this result conflicts with those published by Dubois et al 2006. However mice used by Dubois et al in their experiment were FcRγ-deficient mice, not GPVI knock-out mice, this could be a reason for the discrepancy shown.

Although further studies are required to investigate if podoplanin mediates the prothrombotic effect CLEC-2, and if so where the source of this podoplanin is, we can conclude that complete deficiency of CLEC-2 protects mice from DVT, and therefore is an attractive therapeutic target considering these mice have minimal impairments in hemostasis.

7.1.2 The role of mast cells in DVT

Mechanisms of DVT involve not only blood coagulation, but also aspects of sterile inflammation. We know recruitment of both immune cells and platelets are important for DVT and we wanted to investigate the role mast cells may have. Interestingly mast cells contain both pro-inflammatory mediators in their granules e.g. Histamine, as well as inhibitors of coagulation such as heparin.

In this thesis we report a novel role of mast cells in DVT, we show that two strains of mast cell deficient mice were completely protected against DVT in the IVC stenosis model. This suggested to us that mast cells contain a pro-thrombotic component, which overrides the anti-thrombotic abilities of other mast cell constituents.

We then performed further experiments to confirm that it was indeed lack of mast cells causing this anti-thrombotic phenotype, and not from other defects that may be caused by the c-KIT mutation in $KIT^{W\text{-}sh}$ mice. We demonstrate that adoptive transfer of in vitro differentiated mast cells into mast cell deficient $KIT^{W\text{-}sh}$ mice restored thrombosis following IVC stenosis.

To support our results further we performed more experiments instead targeting mast cells pharmacologically. Compound 48-80 exhausted the intracellular pool of mediators of mast cells, and Sodium cromoglycate and ketotifen are mast cell membrane stabilizers, which prevent the liberation of the granule contents. Both of these approaches protected mice from DVT, supporting the assumption the mast cell contents play a role in DVT. As we hypothesized that histamine might be the likely candidate responsible for this phenotype, we performed In-Vivo experiments using topical application of histamine to the IVC. This was able to induced DVT in mast cell deficient mice. Together my results

show that mast cells (probably working through histamine, although other constituents may be involved) may be a good therapeutic target for DVT prevention in humans.

7.1.3 Other immune cells in DVT: T-cells and neutrophils

We know that DVT is now considered to be an immunothrombotic disorder, and that various inflammatory cells accumulate in the thrombus and the vessel wall during DVT. In this thesis we explored further the novel role of T-cells and the more commonly studied role of NETs in DVT.

I show using the in vivo IVC stenosis model that nude mice lacking all T-cells have a prothrombotic phenotype in DVT. Suggesting that T-cells may offer a protective role in DVT and that lack of T-cells is detrimental. We hypothesized that this results was probably due to T-cells ability to reduce and excessive inflammatory response, and therefore reducing the number of platelets and other inflammatory mediators recruited to the site.

Because nude mice have no T lymphocytes at all, we wanted to perform further studies to investigate which specific subset of T-cells were responsible for the protective role observed. We used anti-CD25 to deplete T-regulatory cells and performed IVC stenosis to these mice. We show that T-regulatory cells are probably not the subset of cells responsible for protection from DVT since a non-significant prothrombotic phenotype was observed in these mice. We also show that Rag1-/- mice that lack both B and T-cells are not protected against DVT and we did not see the same phenotype we observed in the nude mice.

Overall whilst we suggest that nude mice are prothrombotic, and that T-cells may exert a protective role in DVT, further work is needed to confirm these

findings. Especially as thrombosis prevalence in our controls for this experiment were not at the usual levels we would expect.

I also wanted to investigate where in the thrombus T-cells may be located and we hypothesized that NETs which are responsible for capturing platelets and RBCs in the thrombus, may also be responsible for capturing T-cells.
I show in this study that neutrophils and T-cells do in fact co-localize in the thrombus. Although this is preliminary data, it may suggest that NETs and T-cells could interact in DVT, and this could be to play a role in thrombus resolution.

Previous studies had already reported a role of NETs in thrombosis (Brill et al 2012). They had already shown that NETs form an important structural part of the thrombus in DVT, I wanted to investigate what is the cause of this NET release in thrombosis, a sterile inflammatory environment. The obvious candidate for us to investigate was thrombin, after addition of a range of concentrations of thrombin, we show that it can induce NET release and both 0.1 and 1 unit/ml thrombin resulted in a significant increase in NET coverage compared to control untreated NETs. Although these are very preliminary experiments, we show promising signs that thrombin can induce NET formation and therefore may be a likely candidate for the cause of NETosis during DVT.
I also wanted to investigate which stages of the NETosis pathway could potentially be targeted in order to prevent NET formation. I performed various in vitro experiments using a range of inhibitors; Wortmannin, which inhibits PI3K, cytochalasin D that inhibits actin polymerization and SB203580 which is a MAP kinase inhibitor. I did not observe a decrease in NET formation comparing

any or the inhibitors to the control, I therefore concluded from this experiment that other stages of the pathway were probably more important for NET release and further work is needed to both optimize the study to ensure control samples have minimal neutrophil activation, and to identify other potential stages in the pathway to target.

7.2 Future directions

Throughout this thesis I have identified new targets, including both CLEC-2 and mast cells, which we show to be involved in DVT. In murine models, mice lacking either CLEC-2 or mast cells are protected against DVT and therefore we suggest these are prothrombotic. We have also demonstrated that T-cells may have a protective role in DVT. However all of these results are only the beginning on a long chain of studies that now need to be performed. Target Validation should now take place to see if these identified targets produce the same results in a clinically relevant setting in humans.

Although our results appear to be striking in mice, translational studies need be performed in humans; this should begin with clinical trials. These trials should be randomized double-blind trials, for example to test if drugs preventing mast cell degranulation correlate with a positive outcome in DVT, patients should randomly be assigned either a placebo or mast cell membrane stabilizer, with both the patient and clinician not knowing which they are receiving.

Other factors need to be considered in these translational studies such as the stage of the disease. The outcomes of our studies in mice are all at the first stage of DVT onset, studies need to be performed in humans to see if these targets/cells still have the same effect in patients who are experiencing

reoccurrence of the disease, or in patients who also have pulmonary embolisms for example, the biggest major complication with DVT. Other parameters should also be measured in the same way we measured in mice, for example investigating if plasma levels of histamine are increased in patients with DVT.

There are some inherent limitations of our studies, for example in all experiments we perform IVC stenosis using young, healthy 8-10 week old mice. Seeing as the prevalence of DVT is highest among an ageing population, who potentially also have many other diseases which may be contributing to their outcome, there is an argument that our model is not representative of the biggest target population. We therefore need to question if this surgery could be performed on aged mice, which questionably would be more representative of the population in which DVT occurs most frequently. A strength of out study in comparison to others however is that we did use both male and female mice, whereas most other studies use mainly male mice, we can therefore infer that our observations are the same across both genders.

Another limitation of this study could be the IVC stenosis model itself. Whilst it is widely used and accepted, stenosis only mimics blood flow distortion, which we do know is important in venous thrombosis, however other factors may be at play in humans which this mouse model cannot replicate.

7.3 Final conclusions

The overall findings of this thesis show I have identified a novel role of both CLEC-2 and mast cells in DVT. I show that CLEC-2 deficiency is protective in DVT, and that CLEC-2 may interact with its ligand podoplanin found in the IVC wall to exert its prothrombotic phenotype. I have shown a novel role of mast cells in

DVT, with mast cell deficiency being protective. I also suggest that the mast cell constituent responsible for the prothrombotic phenotype in DVT is most likely to be histamine. Whilst only preliminary results, I also show that T-cells may have a protective role in DVT and that thrombin may be important for the release of NETs by neutrophils in the thrombus.

These are all potentially very good targets for preventing or treating DVT in humans seeing as they all have limited bleeding complications as a side effect.

REFERENCES

Ahmad SS, Rawala-Sheikh R, Walsh PN.1992. Components and assembly of the factor X activating complex. Semin Thromb Hemost 18: 311–323

Aird WC. 2007. Vascular bed-specific thrombosis.*J Thromb Haemost.;283-91*

Ajjan R and Grant PJ. 2006. Coagulation and atherothrombotic disease. Atherosclerosis 186: 240–259

Alshehri OM, Hughes CE et al 2015. Fibrin activates GPVI in human and mouse platelets. *Blood*; 126(13):1601-1608

American Surgeon General CDC – centers for disease control and prevention Amin K .2012. The role of mast cells in allergic inflammation. *Respiratory Medicine;* 106(1):9-14

Antonopoulos CN, Sfyroeras GS, Kakisis JD, Moulakakis KG and Liapis CD .2014. The role of soluble P selectin in the diagnosis of venous thromboembolism. *Thromb Res*;133:17-24.

Arman M, Payne H, Ponomaryov T & Brill A. 2015. 'Role of platelets in inflammation' ISBN 978-953-51-2208-1

Ashrani AA and Heit JA. 2009. Incidence and cost burden of post-thrombotic syndrome. *J Thromb Thrombolysis*;28:465-76.

Aslan JE, Itakura A, Gertz JM & McCarty OJ. 2012. Platelet shape change and spreading. *Methods Mol Bol*;788:91-100

Astarita JL, Acton SE, Turley SJ. 2012.Podoplanin: emerging functions in development, the immune system, and cancer. *Front Immunol*;3:283.

Atkinson et al .2010. Laser-induced endothelial cell activation supports fibrin formation. *Blood;* 116(22):4675-4683

Bankl HC, Grossschmidt K, Pikula B, Bankl H, Lechner K and Valent P.1999 Mast cells are augmented in deep vein thrombosis and express a profibrinolytic phenotype. *Hum Pathol*;30:188-94.

Bender M, May F, Lorenz V, et al. 2013. Combined in vivo depletion of glycoprotein VI and C-type lectin-like receptor 2 severely compromises hemostasis and abrogates arterial thrombosis in mice. *Arterioscler Thromb Vasc Biol*;33(5):926-934.

Bennet J.S 2005 Structure and function of the platelet integrin αIIbβ3. *J Clin Invest;*115(2): 3363-3369

Bergmeier W et al. 2008. Glycoprotein Ibα and von Willebrand factor in primary platelet adhesion and thrombus formation: Lessons from mutant mice. *Thromb Haemost*; 99:264-270

Bergmeier W et al.2006. The role of platelet adhesion receptor GPIbα far exceeds that of its main ligand, Von Willebrand factor, in arterial thrombosis. *PNAS;* Vol.103:16900-19805

Boulaftali Y, Hess PR, Getz TM, et al. 2013.Platelet ITAM signaling is critical for vascular integrity in inflammation. *J Clin Invest*;123(2):908-916.

Bovill EG and van der Vliet A.2011. Venous valvular stasis-associated hypoxia and thrombosis: what is the link?1.*Annu Rev Physiol*;73:527-45.

Brill A, Fuchs TA, Chauhan AK, et al. 2011 von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. *Blood*;117(4):1400-1407.

Brill A, Fuchs TA, Chauhan AK, Yang JJ, De Meyer SF, Kollnberger M, Wakefield TW, Lammle B, Massberg S and Wagner DD.2011. von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. *Blood*;117:1400-7.

Brill A, Fuchs TA, Savchenko AS, Thomas GM, Martinod K, De Meyer SF, Bhandari AA and Wagner DD.2012. Neutrophil extracellular traps promote deep vein thrombosis in mice. *J Thromb Haemost* ;10:136-44.

Brill et al 2012. Neutrophil extracellular traps promote deep vein thrombosis. *JTH*;10(1):136-144

Brinkmann V et al .2004. Neutrophil Extracellular Traps kill bacteria. *Science*; 303(5663): 1532-1535.

Brinkmann V, Reichard U, Goosmann et al 2004. Neutrophil Extracellular traps kills bacteria.*Science*; Vol 303;5663: 1532-1535

Brinkmann V, Zychlinsky A. 2012. Neutrophil extracellular traps: is immunity the second function of chromatin? *J Cell Biol;* 198: 773–783.

Burger PC, Wagner DD. 2003. Platelet P-selectin facilitates atherosclerotic lesion development. *Blood*; 101(7):2661-2666.

Burkhart JM, Vaudel et al 2012. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*;120(15):73-82

Calaminus SD, Thomas S, Mccarty Oj et al. 2008. Identification of a novel, actin-rich structure, the actin nodule, in the early stages of platelet spreading. *J Thromb Haemost;* (6)**:**1944-52.

Carvalho M, Benjamin C et al. 2005. Effect of mast cells depletion on the failure of neutrophil migration during sepsis. *Eur J Pharmacol*.525:161-169

Chu AJ 2011. Tissue factor, Blood coagulation, and beyond:an overview.*International Journal of Inflammation*; Volume 2011:367284

Chu AJ, Wang ZG and Fox MJ. III. 2000. Instantaneous inhibition by compound 48/80 of tissue factor-initiated extrinsic coagulation is mediated by the downregulation of factor VII activation. *Arch Biochem Biophys*;377:357-65.

Cimmino G et al 2017. The Two faces of thrombosis: Coagulation cascade and platelet aggregation. Are platelets the main therapeutic target?. J Thromb Cir. 3:(1) 117

Conte IL et al. 2015. Is there more than one way to unpack a weibel-Palade body? *Blood*; 126(18):2165-2167

Cosemans JMEM, Angelioolo-Scherrer A, Matthejj NJA, Heemskerk JWM. 2013. The effects of arterial flow on platelet activation,thrombus growth , and stabilization. *Cardiovas Res*;99(2):342-352

Cushman M 2007 Epidemiology and Risk factors for venous thrombosis. *Semin Hematol*; 44(2):62-69

Daeron M. Fc receptor biology. *Annu rev Immunol*, 15, 203-34.

Darbousset, R., Thomas, G. M., Mezouar, S., Frère, C., Bonier, R., Mackman, N., et al. 2012. Tissue factor-positive neutrophils bind to injure endothelial wall and initiate thrombus formation. *Blood;* 120, 2133–2143

Dawood BB, Wilde J & Watson SP. 2007. Reference curves for aggregation and ATP secretion to aid diagnose of platelet-based bleeding disorders: effect of inhibition of ADP and thromboxane A(2) pathways. *Platelets;* (18):329-45.

Day SM, Reeve JL, Pedersen B, et al.2005 Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall. *Blood*;105(1):192-198.

Delgado-Rizo et al 2017. Neutrophil Extracellular Traps and its implications in inflammation. *Front.Immunol.* 8:81

Denis MM et al 2005. Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets. *Cell*;122(3):379-91

Depperman C, Cherpokova D, Nurden P et al. 2013. Gray platelet syndrome and defective thrombo-inflammation in Nbeal2-deficient mice. *J Clin Invest*.

Diaz JA. 2011. Inflammation and Acute Venous Thrombosis. *US Oncology & Hematology*;7(1):68-71

Dubois C et al. 2006. Glycoprotein VI-dependent and –independent pathways of thrombus formation in vivo. *Blood*.107:3902-3906.

Echtenacher B, Mannel DN & Hultner L .1996. Critical protective role of mast cells in a model of acture septic peritonitis. *Nature;* 381.6577

Eckly A et al. 2011. Mechanisms underlying $FeCl_3$- induced arterial thrombosis. *J Thromb Haemost;*(9):779-89

Engelmann B & Massberg S. 2013. Thrombosis as an intravascular effector of innate immunity. *Nature Reviews Immunol*;(13): 34-35

Esmon C.T .2003. Inflammation and thrombosis. *J Thromb Haemost*; (1):1343-8
Feil R, Lohmann SM et al 2003. Cyclic GMP-dependent protein kinases and the cardiovascular system: insights from genetically modified mice. *Cir Res*. 93. 907-16

Falati S, Liu Q, Gross P.et al. 2003. Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. J Exp Med 197: 1585–1598

Finn DF & Walsh JJ. 2013. Twenty-first century mast cell stabilizers.*Br J Pharmacol;* 170(1):23-37

Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD, Jr, Wrobleski SK, Wakefield TW, Hartwig JH, Wagner DD .2010. Extracellular DNA traps promote thrombosis. *PNAS*; 107: 15880-15885

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

Furie B & Furie BC 2012. Thrombus formation in vivo. *J.Cin.Invest*;115(12):3355-3362

Gawaz M,Langer H, May AE. 2005.Platelets in inflammation and atherogenesis. *J Clin Invest*;115(12):3378-3384.

Ghasemzadeh M, Hosseini E. 2013. Platelet-leukocyte crosstalk: Linking proinflammatory responses to procoagulant state. *Thromb Res*;131(3):191-197.

Goerge T, Ho-Tin-Noe B, Carbo C, et al. 2008.Inflammation induces hemorrhage in thrombocytopenia. *Blood*;111(10):4958-4964.

Gros A, Ollivier V, Ho-Tin-Noe B. 2014. Platelets in inflammation: regulation of leukocyte activities and vascular repair. *Front Immunol*;5:678.

Gurish MF and Austen KF. 2012. Developmental origin and functional specialization of mast cell subsets. *Immunity*;37:25-33.

Hakkim A, Fuchs TA et al. 2011. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nature Chemical Biology*. Vol 7.

Hamer JD, Malone PC and Silver IA. 1981. The PO2 in venous valve pockets: its possible bearing on thrombogenesis. *Br J Surg*;68:166-70.

Heit J et al. 2006. Trends in Indicdence of deep vein thrombosis and pulmonary embolism: a 35-year population-based study. *Blood*;108:430a.

Heit JA et al. 1999. Predictors of survival after deep vein thrombosis and pulmonary embolism: a population-based, cohort study. *Arch Intern Med*. 159(5):445-53

Heit JA. 2008. The epidemiology of venous thromboembolism in the community. *Arterioscler Thromb Vasc Biol* ;28(3):370-372.

Henke PK, Wakefield TW, Kadell AM, et al. 2001. Interleukin-8 administration enhances venous thrombosis resolution in a rat model. *J Surg Res*.;99(1):84-91.

Herzog BH, Fu J, Wilson SJ, et al. 2013.Podoplanin maintains high endothelial venule integrity by interacting with platelet CLEC-2. *Nature*;502(7469):105-109.

Hillgruber C, Poppelmann B, Weishaupt C, et al. 2015. Blocking neutrophil diapedesis prevents hemorrhage during thrombocytopenia. *J Exp Med*;212(8):1255-1266.

Hitchcock JR, Cook CN, Bobat S, et al.2015. Inflammation drives thrombosis after Salmonella infection via CLEC-2 on platelets. *J Clin Invest*;125(12):4429-4446.

Ho-Tin-Noe B et al .2011. How platelets safeguard vascular integrity. J Thromb Haemost.1:56-65

Hongbao MA et al 2008. Thrombin. *Nature and Science*. 6(2)

Huber SA, Sakkinen P, David C, et al. 2001. T-helper-cell phenotype regulates atherosclerosis in mice under conditions of mild hypercholesterolemia. *Circulation*;103:2610–16

Hughes CE, Pollitt AY, Mori J et al. 2010. CLEC-2 activates Syk through dimerization. *Blood,* 115**,** 2947-2955.

Inoue O, Hokamura K, Shirai T, et al. 2015. Vascular Smooth Muscle Cells Stimulate Platelets and Facilitate Thrombus Formation through Platelet CLEC-2: Implications in Atherothrombosis. *PLoS One*;10(9):e0139357.

Jackson S.P et al .2003. Integrin αIIbβ3- dependent calcium signals regulate platelet-Fibrinogen interactions under flow. *J Biol Chem*; 278:34812-34822

Jenkins AL, Howells GL, Scott E.1995. The response to thrombin of human neutrophils: evidence for two novel receptors. *Journal of cell science*;108:3059-3066

Jorch SK & Kubes P. 2017 An emerging role for neutrophil extracellular traps I noninfectious disease. *Nature medicine*; 23:3

Kahn ML et al. 1999 Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J Clin Invest; 103(6):879-887

Kalia N et al. 2008. Critical role of FcR gamma-chain, LAT, PLCgamma2 and thrombin in arteriolar thrombus formation upon mild, laser-induced endothelial injury in vivo. *Microcirculation*;15: 325–35.

Kaushansky K. 2005. The molecular mechanisms that control thrombopoiesis. *The Journal of Clinical Investigation;* 115**,** 3339-3347.

Kerrigan AM, Dennehy KM, Mourao-Sa D, et al. 2009. CLEC-2 is a phagocytic activation receptor expressed on murine peripheral blood neutrophils. *J Immunol*;182(7):4150-4157.

Khandpur R et al. 2013 NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med*; 5:178

Kimball AS, Obi AT, Diaz JA and Henke PK. 2006. The emerging role of NETs in venous thrombosis and immunothrombosis. *Frontiers in immunology*; Vol 7;236

Kleinschnitz C, Kraft P, Dreykluft A, et al. Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature. *Blood*. 2013;121(4):679–691.

Kolenda J, Jensen SS, Aaberg-Jessen C, et al. 2011. Effects of hypoxia on expression of a panel of stem cell and chemoresistance markers in glioblastoma-derived spheroids. *J Neurooncol*;103(1):43-58.

Lambert MP, Rauova L,Bailey M et al 2007. Platelet factor 4 is a negative autocrine in vivo regulator of megakaryopoiesis: clinical and therapeutic implications. *Blood*;110(4):1153-1160.

Lee RH, Bergmeier W. 2016. Platelet immunoreceptor tyrosine-based activation motif (ITAM) and hemITAM signaling and vascular integrity in inflammation and development. *J Thromb Haemost*;14(4):645-654.

Li P, Gan Y, Sun BL, et al. 2012. Adoptive regulatory T-cell therapy protects against cerebral ischemia. *Annals of Neurology*;74(3):458-71

Liesz A, Suri-Payer E, Veltkamp C, et al. 2009. Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nature Medicine*; 15(2):192–199

Lindemann S, Tolley ND, Dixon DA et al. 2001. Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. *J Cell Biol*; 154(3):485-490.

Lippi G and Favaloro EJ. 2016. Allergy and Venous Thromboembolism: A Casual or Causative Association. *Semin Thromb Hemos* ;42:63-8.

Lockyer S, Okuyama K, Begum S, et al. 2006.GPVI-deficient mice lack collagen responses and are pro- tected against experimentally induced pulmonary thromboembolism. Thromb Res;118:371-380

Lowe KL, Navarro-Nunez L, Benezech C, et al. 2015 The expression of mouse CLEC-2 on leucocyte subsets varies according to their anatomical location and inflammatory state. *Eur J Immunol*;45(9):2484-2493.

Luther N et al 2016. Innate effector-memory T-cell activation regulates post-thrombotic vein wall inflammation and thrombus resolution. *Circulation research;*119(12): 1286-1295

Mahtab, E. A. F. et al .2008. Cardiac malformations and myocardial abnormalities in podoplanin knockout mouse embryos: correlation with abnormal epicardial development. *Dev. Dyn;* 237, 847–857.

Majoor CJ, Kamphuisen PW, Zwinderman AH, et al.2013. Risk of deep vein thrombosis and pulmonary embolism in asthma. Eur Respir J; 42: 655–661.

Mammadova-Bach E et al. 2015. Platelet glycoprotein VI binds to polymerized fibrin and promotes thrombin generation. *Blood;* 126**,** 683-91.

Marjoram et al. 2014. α2β1 Integrin, GPVI Receptor, and Common FcRγ Chain on Mouse Platelets Mediate Distinct Responses to Collagen in Models of Thrombosis; *PLos One*. 9(11)

Massberg S et al.2004. Soluble glycoprotein VI dimer inhibits platelet adhesion and aggregation to the injured vessel wall in vivo. *FASEB J*;18:397-399

Massberg S. et al.2003. A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. *J.Exp.Med; Vol* 197(1): 41-49

Maxwell MJ, Westein E, Nesbitt W.S et al. 2007. Identification of a 2-stage platelet aggregation process mediating shear-dependent thrombus formation. Blood; 109.2

May AE, Seizer P, Gawaz M. 2008. Platelets: inflammatory firebugs of vascular walls. *Arterioscler Throm Vasc Biol*.3. 5-10

May F, Hagedorn I, Pleines I, et al. 2009. CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis. *Blood*;114(16):3464-3472.

McDonld B et al 2017 Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice. *Blood*; 09-741298

Merten M, Pakala R, Thiagarajan P, Benedict CR. 1999. Platelet microparticles promote platelet interaction with subendothelial matrix in a glycoprotein IIb/IIIa-dependent mechanism. Circulation 99: 2577–2582

Metcalfe DD, Baram D and Mekori YA. Mast cells. *Physiol Rev*; 1997;77:1033-79.

Mitchell JA et al 2007. Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium. *Exp Physiol*.93.1.14-147

Modarai B, Burnand KG, Humphries J, Waltham M, Smith A. 2005. The role of neovascularisation in the resolution of venous thrombus. *Thromb Haemost.*;93:801–809.

Mocsai A, Ruland J & Tybulewicz VLJ. 2010. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nature reviews*. *Immunology*. 10,387-402

Mombaerts P et al 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*;68(5):869-77

Moon TC, Befus AD & Kulka M. 2014. Mast cell mediators: Their differential release and the secretory pathways involved*. Front immunol*;5:569

Mor A, Planer D et al. 2007. Role of naturally occurring CD4+ CD25+ regulatory T cells in experimental atherosclerosis. *Arterioscler Thromb Vasc Biol*;27:893–900

Moroi M 1989. A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J Clin Invest.*;84:1440–1445.

Muller F,Mutch NJ, Schenk WA, et al 2009. Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. *Cell*; 139(6):1143-1156.

Myers DD, Hawley AE, Farris DM, Wrobleski SK, Thanaporn P, Schaub RG, Wagner DD, Kumar A, Wakefield TW .2003. P-selectin and leukocyte microparticles are associated with venous thrombogenesis. *J Vasc Surg; 38(5):1075-89.*

Neeli I, Dwivedi N, Khan S and Radic M. 2009. Regulation of extracellular chromatin release from neutrophils. *J Innate Immun;*1:194-201

Nieswandt B & Waston SP. 2003. Platelet –collagen interaction: is GPVI the central receptor? *Blood*;102(2):449-61

Nieswandt B et al. 2001. Glycoprotein VI but not a2β1 integrin is essential for platelet interaction with collagen. *The EMBO journ;* 20(9):2120-2130

Nieswandt B, Aktas B et al 2005. Platelets in atherothrombosis: lessons from mouse models. *JTH*; Vol 3 (8):1725-1736

Nieswandt B, Varga-Szabo, Elvers M. 2009. Integrins in platelet activation. *JTH* 9;1538-7836

Noris M et al. 2007. Regulatory T cells and T cell Depletion: Role of immunosuppressive Drugs. JASN.Vol 18 (3): 1007-1018

Owens AP, Mackman N, Weber C & Mause S.2011. Microparticles in hemostasis and thrombosis. Circulation Research. 108:1284-1297.

Ozaki Y et al 2007 Involvement of the snake toxin receptor CLEC-2 in podoplanin-mediated platelet activation by cancer cells. *J Biol Chem*;282:3165-72

Ozaki Y. et al. 2010. Essential in vivo roles of the c-type lectin receptor CLEC-2. *J.Biol. Chem;* 285: 24494-24507

Patel SR., Hartwig JH& Italiano JR. 2005. The biogenesis of platelets from megakaryocyte proplatelets. *J Clin Invest;* 115**,** 3348-54.

Patterson BO, Hinchliffe R, Loftus IM, Thompson MM, Holt PJ. 2010. Indications for catheter-directed thrombolysis in the management of acute proximal deep venous thrombosis. A*rterioscler Thromb Vasc Biol*;30:669–674.

Pinsky DJ et al. 1996. Hypoxia-induced exocytosis of endothelial cell Weibel-Palade bodies. A mechanism for rapid neutrophil recruitment after cardiac preservation. *J Clin Invest* ;97(2):493-500.

Plow E.F et al (2007) Platelet integrin αIIbβ3: activation mechanisms. *J. Thromb. Haemost*; **5**:1345-52

Ramacciotti E, Blackburn S, Hawley AE, Vandy F et al.2011. Evaluation of soluble P-selectin as a marker for the diagnosis of deep venous thrombosis. *Clin Appl Thromb Hemost* ;17:425-31.

Ramos L, Pena G, Cai B, Deitch EA and Ulloa L. 2010. Mast cell stabilization improves survival by preventing apoptosis in sepsis. *J Immunol*;185:709-16.

Raslan Z & Naseem KM 2015. Compartmentalization of cAMP-dependent signaling in blood *platelets*: The role of lipid rafts and actin polymerization. *Platelets*.26.349-57

Ren X, Akiyoshi K, Vandenbark AA, Hurn PD, Offner H. 2011. CD4+FoxP3+ regulatory T-cells in cerebral ischemic stroke. *Metabolic Brain Disease* ;26(1):87–90.

Robertson, AK, Hansson, GK. 2006. T cells in atherogenesis: for better or for worse?. *Arterioscler Thromb Vasc Biol*. ;26:2421–2432

Rothenberg ME & Hohan SP. 2006. The Eosinophil. *Annual Review of immunology*. Vol.24:147-174

Saha P, Humphries J, Modarai B et al. Leukocytes and the natural history of deep vein thrombosis: current concepts and future directions. *ATVB*.2011;31(3):506-512

Schacht V, Dadras SS, Johnson LA, Jackson DG, Hong YK, Detmar M. 2005. Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumors. *Am J Pathol* ;166(3):913-921.

Sevitt S. 1974. The structure and growth of valve-pocket thrombi in femoral veins. *J Clin Pathol* ;27(7):517-528.

Shattil SJ et al 1998. Integrin signaling: The platelet paradigm. Blood. Vol 91(8);2645-2657

Shen B, Delaney MK & Du X .2012. Inside-out, outside-in, and inside-outside in: G protein signaling in integrin-mediated cell adhesion, spreading and retraction. *Curr Opin Cell Biol*;(5);600-606

Shi G,Morrell CN. 2011.Platelets as initiators and mediators of inflammation at the vessel wall. *Thromb Res*;127(5):387-390.

Siefert S, Gabe J, Chabasse C et al 2014. The role of T cells in resolution of Deep venous thrombosis in vivo. Journal of vascular surgery. Vol 60 (3);817-818

Smyth EM 2010. Thromboxane and the thromboxane receptor in cardiovascular disease. Clin Lipidol. 5(2):209-219

Smyth. SS et al .2001. Variable protection of B3-intergrin-deficicnt mice from thrombosis initiated by different mechanisms. *Blood*; 98:1055-1062

Stokes KY & granger DN 2012. Platelets: a critical link between inflammation and microvascular dysfunction. J Physiol. 590:1023-1034

Stuardo M, Gonzalez CB, Nualaert F et al 2004. Stimulated human neutrophils form biologically active kinin peptides from high and low molecular weight kininogens. *Journal of Leukocyte Biology*; 75(4):631-40

Stubbe T, Ebner F, Richter D, et al.2013. Regulatory T cells accumulate and proliferate in the ischemic hemisphere for up to 30 days after MCAO. *Journal of Cerebral Blood Flow and Metabolism*;33(1):37–47.

Supajatura V et al.2001. Protective roles of mast cells against enterobacterial infection are mediated by Toll-like receptor 4.*J Immunol*.15;167(4):2250-6

Suzuki-Inoue K, Fuller GL, García A, Eble JA et al.2006. A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. *Blood;* 107(2):542-9.

Suzuki-Inoue K, Inoue O, Ding G, et al. 2010. Essential in vivo roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. *J Biol Chem*;285(32):24494-24507.

Savage B et al 1998. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell*; 4;94(5):657-66

Swedenborg J, Mayranpaa MI, Kovanen PT. 2011. Mast cells: Important players in the orchestrated pathogenesis of Abdominal Aortc Aneurysms. *ATVB*; 31:734-740

Thomas MR et al 2015. The role of platelets in inflammation. *Thromb Haemost*.31;114(3):449-58

Theoharides TC et al 2012. Mast cells and inflammation. *Mol.Basis of Disease*;Vol.1822(1):21-33

Tsai AW, Cushman M, Rosamond WD et al. 2002. Cardiovascular risk factors and venous thromboembolism incidence: the longitudinal investigation of thromboembolism etiology. *Arch Intern Me.; 162(10):1182-9*

Tsiantoulas D, Sage AP, Mallat Z, Binder CJ. 2015. Targeting B cells in Atherosclerosis. ATVB;35:296-302.

Vargas JR et al 1982. The use of prostacyclin in the separation from plasma and washing of human platelets. *Prostaglandins*.23.929-945

Valent P, Baghestanian M, Bankl HC et al. 2002. New aspects in thrombosis research: possible role of mast cells as profibrinolytic and antithrombotic cells. *Thromb Haemost;* 87(5):786-90

Von Bruhl et al 2012. Monocytes, neutrophils and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. JEM.209(4); 819

von Bruhl ML, Stark K, Steinhart A, Chandraratne S, et al. 2012. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med*;209:819-35.

Wang Y, Li M, Stadler S, Correll S et al. 2009 Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J Cell Biol;* 184: 205–213.

Wang YI & Shi GP.2012. Mast cell chymase and Tryptase in Abdominal Aortic Aneurysm formation. *Trends Cardiovasc Med*; 22(6):150-155

Watson SP & HarrisonP. 2007. The Vascular Function of Platelets. *Postgraduate Haematology.* Blackwell Publishing Ltd.

Watson SP, Herbert JMJ & Pollitt AY. 2010. GPVI and CLEC-2 in hemostasis. JTH;8:1457-1467

Wolters PJ, Mallen-St Clair J, Lewis CC, Villalta SA, Baluk P, Erle DJ and Caughey GH.2005. Tissue-selective mast cell reconstitution and differential lung gene expression in mast cell-deficient Kit(W-sh)/Kit(W-sh) sash mice. *Clin Exp Allergy*;35:82-8.

Xu X, Li M & Jiang Y. 2013. The paradox role of Regulatory T cells in ischemic stroke. *The Scientific World Journal;* Vol 2013;8

Yipp BG, Kubes P. 2013. NETosis: how vital is it?. *Blood*;122:2784-2794