Aus dem Centrum für Thrombose und Hämostase

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The role of T-lymphocyte-derived prothrombin in $CD8^+$ T-cell proliferation and survival

Die Rolle von aus T-Lymphozyten stammendem Prothrombin bei der Proliferation und dem Überleben von $CD8^+$ T-Zellen

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List of Abbreviations

ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
APC/PC	Activated Protein C/Protein C
BBB	Blood-brain barrier
BMC-DI	Bone marrow chimeric D-Insight mice
BTB	Blood-testis barrier
C3, C5	Complement factor 3, Complement factor 5
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CNS	Central nervous system
CYP	Cytochromes P450
DC	Dendritic cell
DI	Division index
DIC	Disseminated intravascular coagulation
DiI	Fraction diluted
EC	Endothelial cell
EI	Expansion index
EPCR	Endothelial cell protein C receptor
FACS	Fluorescence-activated cell sorting
FII/FIIa	Prothrombin/thrombin
FI-XIII	Coagulation factors I to XIII
Fluc	Firefly luciferase
FVL	Factor V Leiden mutation
GGCX	Gamma-glutamyl carboxylase enzyme
GPCR	G-protein coupled receptor
GPIb-IX, GPIIb/IIIa	Glycoprotein Ib-IX, Glycoprotein IIb/IIIa
HIT	Heparin-induced thrombocytopenia
INR	international normalized ratio
LCMV	Lymphocytic choriomeningitis mammarenavirus

LPS	Lipopolysaccharides
MACS	Magnetic-activated cell sorting
MCP-1	Macrophage chemoattractant protein-1
MGP	Matrix Gla protein
MHCI/II	Major histocompatibility complex I/II
MP	Microparticle
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NK	Natural killer cell
NOAC	Novel oral anticoagulant
PAMP	Pathogen-associated molecular pattern
PAR	Protease-activated receptor
PF	Precursor frequency
PI	Proliferation index
PKC	Protein kinase C
PRR	Pattern recognition receptors
RI	Replication index
TAFI	thrombin-activatable fibrinolysis inhibitor
TBA	Tail bleeding assay
TBT	Tail bleeding time
TCR	T-cell receptor
TF	Tissue factor
TGF-β	Transforming growth factor-beta
TIL	Tumor-infiltrating lymphocytes
TNFα	Tumor necrosis factor-alpha
uc/cVKD factor	Uncarboxylated/carboxylated Vitamin K dependant factor
VKA	Vitamin K antagonist
VKORC1	Vitamin K epoxide reductase enzyme-1
vWF	von Willebrandt factor

Zusammenfassung

Thrombin (FIIa) ist eine multifunktionelle Serinprotease, die durch die Aktivierung vom Prothrombin gebildet wird. Thrombin spielt eine zentrale Rolle bei der primären und sekundären Hämostase, indem es die Thrombozyten aktiviert. Die Aktivierung der Thrombozyten führt zur Plättchenaggregation und das Fibrinogen wird in Fibrinmonomeren umgewandelt. Neben seinen weitreichenden Funktionen bei der Hämostase und der Bildung von Blutgerinnseln, spielt das Thrombin in verschiedensten Abläufen im Körper wie Angiogenese, Gewebereparatur, Embryonalentwicklung und Krebsentstehung eine Rolle, die durch PARs vermittelt werden. In den letzten Jahren haben viele Studien gezeigt, dass das Thrombin ein wichtiges regulatorisches Protein ist, das die Entzündung und Blutstillung verbindet.

Frühere Ergebnisse aus unserem Labor haben gezeigt, dass das Thrombin nicht nur in der Leber exprimiert wird, sondern auch aus extrahepatischen Quellen wie die T-Zellen stammt. Die Hochregulierung des aus den T-Zellen stammenden Thrombins während einer akuten Entzündung deutet auf seine unerkannten Aufgaben in physiologischen und pathologischen Prozessen im Körper hin. In dieser Studie haben wir die möglichen Auswirkungen des von $CD8^+$ T-Zellen stammenden Thrombins auf die Proliferation und das Überleben von $CD8^+$ T-Zellen untersucht. Wir haben dafür serumfreien Zellkulturen aus primären $CD8^+$ T-Zellen benutzt, die aus der Milz von Mäusen gewonnen wurden. CFSE-markierte $CD8^+$ T-Zellen wurden fünf Tage lang beobachtet, und ihre Vermehrungs- und Überlebensraten wurden mittels Durchflusszytometrie analysiert. Wir haben FII mit genetischen und pharmazeutischen Ansätzen ins Visier genommen. Die systematische und in-vitro-Behandlung von $CD8^+$ T-Zellen aus den Wildtyp-Mäusen mit Phenprocoumon führte zu einer signifikanten Verringerung der Überlebens- und Vermehrungsrate. Die Zugabe vom Thrombin (FII) nach der Phenprocoumon-Behandlung rettete die T-Zellen und stellte ihre Vermehrungs- und Überlebensfähigkeit wieder her, während die Prothrombin-Zugabe keine signifikanten Änderungen auslösen konnte. Die Behandlung mit dem direkten Thrombin-Inhibitor Argatroban verringerte das Überleben und die Proliferation von $CD8^+$ T-Zellen. Die genetische Ablation von FII in T-Zellen führte zu einer deutlich geringeren Proliferation von $CD8^+$ T-Zellen aus transgenen CD4Cre-Mäusen, hatte aber keinen Einfluss auf die Überlebensrate. Diese Ergebnisse deuten darauf hin, dass das aus T-Zellen stammende Thrombin die Vermehrung und das Überleben von T-Zellen beeinflusst.

T-Zellen können als potenzielle Thrombin-Träger fungieren, und FII, das von T-Zellen stammt, kann die Immunantwort durch die T-Zell-Vermehrung und Transport in bestimmten Geweben regulieren, in denen normalerweise leberproduziertes Thrombin ausgeschlossen ist. Die Entdeckung der Effekte von aus T-Zellen stammendem FII auf die Vermehrung und das Überleben von T-Zellen ist wichtig, um seine bisher unbekannte Rolle in den extrahepatischen Kompartimenten bei akuten und chronischen Entzündungskrankheiten aufzudecken.

1. Introduction

1.1 Hemostasis

Hemostasis is the physiological mechanism that ceases bleeding from blood vessels by forming a plug and helps preserve vascular integrity following an injury (1,2). Healthy vessels have anticoagulant properties to prevent blood from clotting under normal conditions. However, upon endothelial damage, a great number of procoagulant molecules get exposed and interact with components of the circulating blood. Consequently, a platelet plug is formed, allowing the maintenance of normal circulation in non-injured parts of the body (1). Hemostasis is a complex and tightly regulated mechanism. Therefore shifting the equilibrium of the hemostasis toward a more pro- or anticoagulant state may result in excessive bleeding or thrombosis, which can be life-threatening (1).

Hemostasis consists of two main processes: primary and secondary hemostasis. Primary hemostasis comprises 3 phases, including platelet adhesion, platelet aggregation, and platelet activation. As a result of primary hemostasis, the injury is sealed with a loose primary clot (3). As the primary clot is not stable enough, an insoluble fibrin mesh must be formed at the injury site, supporting the primary clot. During the secondary hemostasis, a fibrin mesh is generated by the coagulation cascade. Primary and secondary hemostasis are closely linked together and happen simultaneously (1,4).

1.1.1 Primary Hemostasis

Following an injury of the vessel endothelium, collagen and other tissue proteins such as von-Willebrand-factors (vWF) get exposed. Von-Willebrand-factors are released from injured endothelial cells and secreted from α-granules of the platelets during the activation step (see details below). They act as “injury detectors” that bind to subendothelial collagen, other matrix proteins like fibronectin and laminin, and von-Willebrand-receptors (vWR=GPIb-IX) that are localized on platelets. Platelets adhere to collagen fibers of the subendothelial matrix via von-Willebrand-factors (vWF) at the site of the endothelial lesion. This results in the adhesion of circulating platelets at the site of the endothelial injury. During the activation step, platelets secrete various mediators such as serotonin and ADP from their δ-granules and produce thromboxane A_2, which leads to platelet aggregation and vasoconstriction via G-protein coupled receptors (GPCRs) (5). Activated platelets also produce thrombin on their surfaces, which acts through a

subgroup of GPCRs called PARs (proteinase-activated receptors) and ensures continuous activation and platelet recruitment. Platelets change their discoidal flat shape and adopt a spherical form with pseudopodia (3,6). The shape change allows the interlocking of the platelets and the inside-out activation of the fibrinogen receptor (GPIIb/IIIa) on the platelet membrane (5). Fibrinogen secreted by the platelets binds to GPIIb/IIIa receptors on the membrane and crosslinks the platelets with each other, creating the primary clot.

1.1.2 Secondary Hemostasis

Secondary hemostasis is defined as the process in which fibrinogen is converted to fibrin, resulting in a red stable clot. Several components, such as clotting factors (FI-XIII), phospholipids, and Calcium (Ca^{+2}) ions are involved in secondary hemostasis. It is a highly complex process; however, two models can provide an explanation of how fibrin is generated in vivo (Figure 1).

According to the historical “cascade” model, different plasma proteins get activated in a sequence. This model consists of two pathways: intrinsic and extrinsic pathways, which converge on the common pathway. The intrinsic pathway begins with the FXII activation through collagen, high molecular weight kininogen, and kallikrein. Activated FXII (FXIIa) and thrombin (FIIa) cleave FXI. FXIa activates FIX, and at the same time, FVIII is activated via thrombin. FVIIIa, FIXa, Ca^{+2} ions, and phospholipids build an enzyme complex, which is called the intrinsic tenase. On the other hand, the extrinsic pathway begins with the tissue factor (TF=FIII) and FVII. Once the endothelium of a vessel is disrupted, TF originating from the subendothelial tissue is exposed and it cleaves the FVII to FVIIa. FVIIa, TF, Ca^{+2} ions, and phospholipids build the extrinsic-tenase complex. The formation of both tenases marks the beginning of the common pathway. These enzyme complexes are called “tenase” because they convert FX to the active form FXa. FXa forms the prothrombinase complex with its cofactor FV, phospholipids, and Ca^{+2} ions. The prothrombinase complex cleaves prothrombin (FII) to thrombin (FIIa), which cleaves fibrinogen into stable fibrin fibers and also activates FXIII. Lastly, FXIIIa covalently crosslinks the fibrin monomers to build the final insoluble fibrin clot (3,7,8) (details in Figure 1A).

Although the cascade model is a practical model for understanding the coagulation process and how involved soluble plasma proteins might interact with each other, it comes short of explaining how the blood clots in vivo, including the presence of cellular

components or how the deficiency of some factors like FVIII or FIX can cause excessive bleeding disorders, while FXI or FXII deficiency does not. Taking this into account, Monroe et al. (2006) proposed a new cell-based coagulation model, which comprises initiation, amplification, and propagation (Figure 1B). In the initiation phase, TF and FVIIa build the extrinsic tenase complex and activate the factors FIX and FX. FXa cleaves prothrombin and converts it into a small amount of thrombin (FII). Thrombin activates platelets and cofactors FV and FVIII in the amplification phase. Thrombin also converts FXI into FXIa, which functions as a booster of FIXa production. Shortly, thrombin creates a suitable environment for massive thrombin generation. Activated cofactors (FVa and FVIIIa) help the accumulation of tenase and prothrombinase complexes during propagation. These complexes convert FX to FXa on the platelet surface. Finally, high amounts of prothrombin are converted to thrombin via FXa in a short time, which is also called the “burst of thrombin” (7,8). Thrombin cleaves fibrinogen to fibrin and FXIII crosslinks them to a stable, insoluble clot.

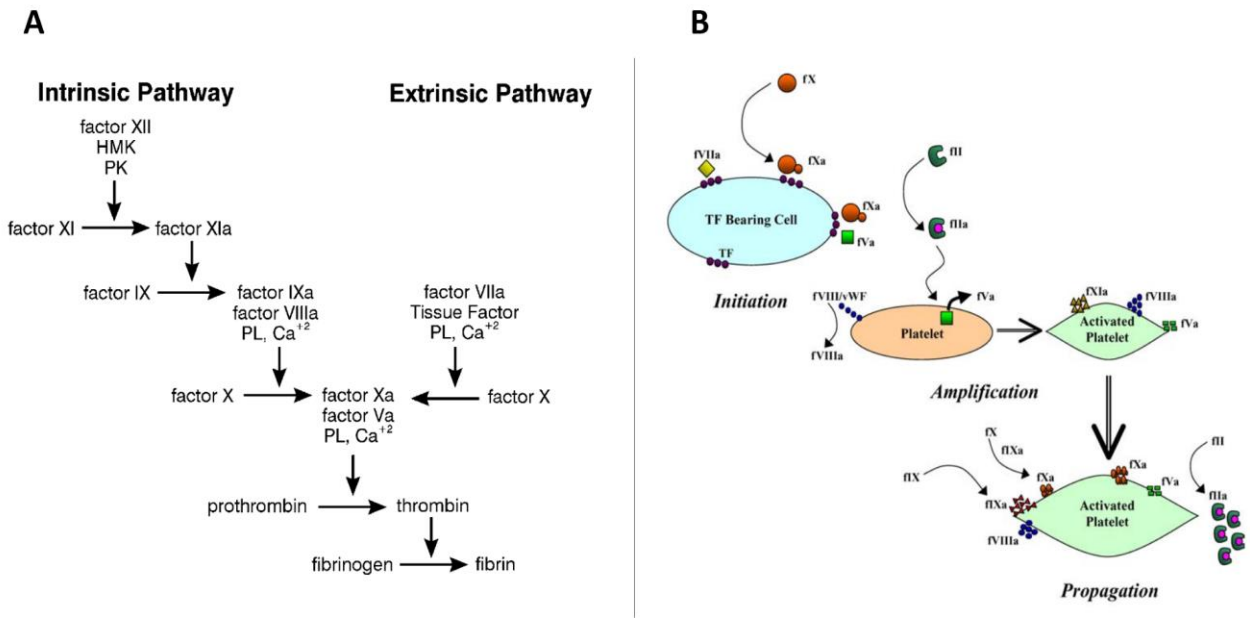

Figure 1: Different models of secondary hemostasis.

Figure 1A shows the cascade model, which was proposed in the 1960s by McFarlene, Davie, and Ratnoff. The figure was modified from Monroe et al. (9). This model provides an overview of how coagulation works and is suited for understanding in vitro tests. In **Figure 1B**, the new cell-based model according to Monroe et al. is presented (9). The figure was modified from Becker et al. (10). It comprises three steps: initiation, amplification, and propagation. This model offers a better understanding of how coagulation might work in vivo and how it comes to bleeding disorders in the absence of some clotting factors. For detailed explanations of both models, see the text above (1.1.2).

1.2 Thrombin: The key regulator of hemostasis

Thrombin is a 36 kD, Na^+ activated allosteric serine-protease from the chymotrypsin family. It has two polypeptide chains; A and B chains, which are bound with a disulfide bond. B-chain has 3 additional intramolecular disulfide bonds (11,12). Thrombin has 2 exosites. Exosite I is the binding epitope for fibrinogen, fibrin, thrombomodulin, and the thrombin receptors PAR1 and PAR3. Exosite II is located on the other side of the enzyme and is the binding site for heparin, other glucosaminoglycans, the platelet receptor GpIb, and some autoantibodies (13).

Thrombin is a fundamental enzyme for primary and secondary hemostasis, where it can play opposing roles. The allosteric activation of thrombin by binding of Na^+ ions gives thrombin a highly procoagulant feature; resulting in the cleavage of fibrinogen, FV, FVIII, and FXI. Prothrombotic functions and thrombin signaling through PAR1, PAR3, and PAR4 are also intensified in the Na^+ presence. The activation of clotting factors and cleavage of fibrinogen by thrombin facilitate the formation of a stable blood clot in the injury sites. Thrombin also contributes to stabilizing the clot by activating TAFI, an anti-fibrinolytic enzyme (12,14). Conversely, thrombin also has anticoagulant roles. Allosteric binding of thrombin to the thrombomodulin receptor on the endothelial cells impedes fibrin formation and PAR activation. Additionally, it boosts the activation of protein C, which inactivates both cofactors FVa and FVIIIa, therefore suppressing the coagulation cascade. Antithrombin, a plasma serpin, is also a substrate of thrombin. Upon binding, thrombin cleaves a ‘bait’ loop and becomes irreversibly attached to the antithrombin. This results in the clearance of thrombin in plasma, thus downregulation of the clot formation. The presence of glycosaminoglycans like heparin can accelerate this process even more (15). Other than hemostatic functions, thrombin has multiple pleiotropic effects, which are mediated through PARs (see 1.7) (11).

1.3 Thrombin formation

The road to the active enzyme thrombin (FIIa) starts with prothrombin (FII), an inactive zymogen precursor of active thrombin (16). The F2 gene, encoding FII, is localized at chromosome 11p11-q12 (17). After the translation, a prepropeptide with 622 residues corresponding to protein with 70 kD is generated. The prepropeptide evolves into the mature prothrombin with 579 residues (18,19). Prothrombin contains 5 domains: propeptide, Gla domain, kringle-1 and kringle-2 domains, and serine protease domain

(20). Prothrombin has to be gamma-carboxylated after its production for optimal protein function. Gamma-carboxylated prothrombin then gets activated through the prothrombinase complex (FVa, FXa, Ca^{+2}, phospholipids), and finally, thrombin is formed. Thrombin is capable of cleaving fibrinogen to fibrin during clot formation, executing anticoagulant effects, and participating in various cell signaling events.

1.3.1 Gamma-carboxylation of prothrombin and Vitamin-K cycle

Prothrombin is a vitamin K-dependent protein along with coagulation factors FVII, FIX, FX, proteins C and S, matrix Gla protein (MGP), and osteocalcin. In the post-translational phase, the first ten glutamic acid residues of prothrombin (Glu) are converted into gamma-carboxyglutamic acid (Gla) through carboxylase activity. Gla is critical for membrane binding due to its metal-binding properties. It binds to Ca^{+2} ions, leading to the exposure of the phospholipid-binding site, and enables optimal anchoring to the cell membrane (21,22).

Gla has important biological roles and is found in vertebrates and invertebrates. Nearly all mammalian tissues have carboxylase activity (22). The gamma-glutamyl carboxylase enzyme (GGCX) catalyzes the conversion of Glu residues to Gla residues with the help of the cofactor vitamin K. Dietary intake of vitamin K is limited; therefore, it needs to be recycled (23). During the carboxylation of the Glu residue, reduced vitamin K (hydroquinone) is converted into vitamin K-2,3-epoxide. Vitamin K-2,3 epoxide is reduced back to hydroquinone form via the VKORC1 enzyme in 2 steps. In the first step of the reduction, vitamin K quinone is formed, which is then reduced into vitamin-K hydroquinone in the second step. Vitamin K-cycle ends with the formation of the active vitamin K-hydroquinone as shown in Figure 2 (23,24).

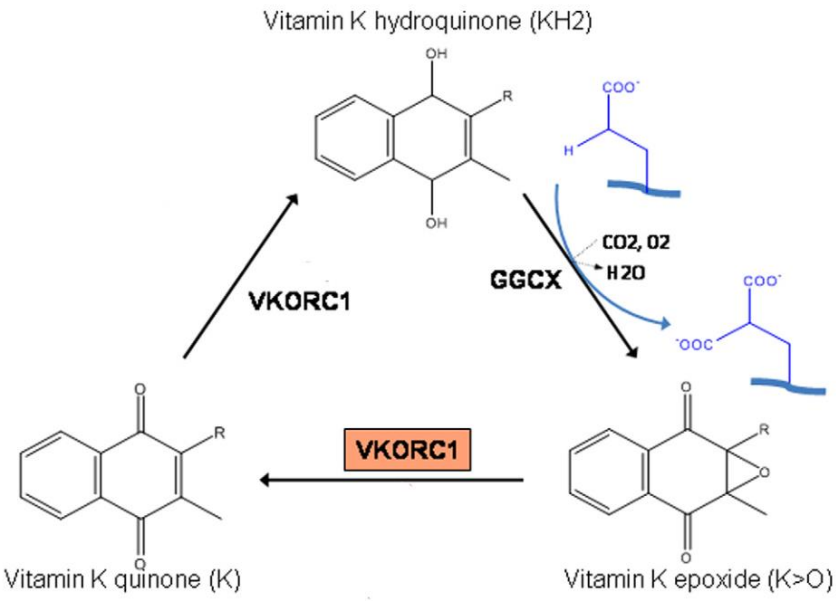

Figure 2: Vitamin K cycle and the gamma-carboxylation of the vitamin K-dependent proteins.

The vitamin K cycle is depicted in the figure modified from Hodroge et al. 2011 (24). Blue molecules represent vitamin K-dependent proteins like prothrombin, which are then carboxylated by the GGCX enzyme.

1.3.2 Prothrombin activation

Thrombin formation is a result of the prothrombin activation through the prothrombinase complex (see 1.1.2 for details of clot formation). The prothrombinase complex cleaves prothrombin at R271 and R320, which are located in the kringle-2 and serine-protease domains. As a result, Gla domains are cleaved along with the kringle domains, and active serine-protease thrombin is released. There are two pathways for prothrombin activation to thrombin (Figure 3). The prethrombin-2 pathway is initiated through the cleavage at R271 by the prothrombinase complex, which leads to the formation of prethrombin-2. Prethrombin-2 is an inactive intermediate, and once it is cleaved at the R320 site, active serine-protease thrombin is formed (20,25,26). Under normal physiological conditions, prothrombin is activated along the prethrombin-2 pathway (16). The second pathway is the meizothrombin pathway. The prothrombinase complex cleaves prothrombin at the R320 site, forming the active intermediate meizothrombin. The meizothrombin is then cleaved at R271, and thrombin is formed (20,25,26). The prothrombin activation via the meizothrombin pathway occurs at synthetic phospholipid and non-platelet surfaces (16).

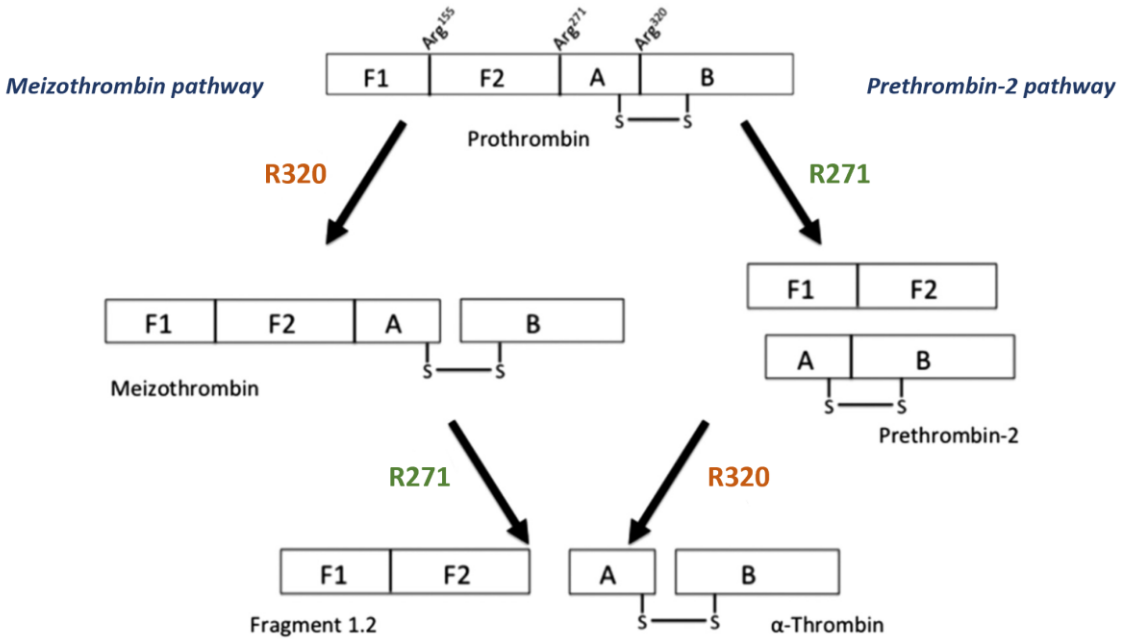

Figure 3: Prothrombin activation pathways.

Prothrombin activation is depicted in the figure modified from Haynes et al. 2012 (26). Fragment 1 (F1) contains Gla and kringle-1 domains, and Fragment 2 (F2) consists of the kringle-2 domain. The meizothrombin pathway is depicted on the left side, and the prethrombin-2 pathway is shown on the right side. The cleavage at R271 leads to the removal of fragments 1 and 2, while the cleavage at R320 forms a light and heavy chain that are held together through a disulfide bond. At the end of both pathways, fragments 1 and 2 are cleaved, and the alpha-thrombin (the active serine-protease) is formed (26).

1.4 Anticoagulants

Anticoagulants are substances that are used for the prevention and treatment of thrombosis. While they have been one of the most prescribed drugs in the last decades, there are several types of anticoagulants, each with different mechanisms of action. The first substance discovered with anticoagulant characteristics was coumarin in 1941 when a mysterious hemorrhagic cattle disease (also named spoiled sweet clover disease) emerged. Researchers were able to identify dicumarol, a vitamin-K antagonist as the responsible substance, which since then revolutionized antithrombotic therapy (27,28). In the last decades, novel oral anticoagulants (NOACs) with specific targets have been introduced. They are expanding the spectrum of anticoagulant therapies and offer many advantages.

1.4.1 Vitamin K Antagonists

Vitamin K antagonists (VKAs) have been used in antithrombotic therapies for more than six decades. They prevent the recycling of vitamin K by inhibiting the vitamin K

epoxide reductase enzyme (VKORC). (Vitamin K cycle, see 1.3.1) VKORC enzyme is responsible for the reduction of oxidized vitamin K to vitamin K hydroquinone. Inhibition of the vitamin K regeneration results in an indirect inactivation of the vitamin K-dependent coagulant factors like prothrombin (FII), FVII, FIX, FX; natural anticoagulant proteins C, and S; and the other vitamin K-dependent proteins like osteocalcin, matrix Gla protein, periostin, and Gas6 (29). VKAs are administrated orally and require up to 5 days until the coagulation factors are inactivated and the antithrombotic effects set in (30).

There are many derivates of vitamin K antagonists. The most clinically used derivates of coumarins (4-hydroxycoumarins) are warfarin, acenocoumarol, and phenprocoumon (Figure 4) (31). Warfarin is the most used coumarin in the USA, while acenocoumarol and phenprocoumon are the most prescribed coumarin derivates in Europe. VKAs are mostly prescribed for the prevention and treatment of venous embolisms, stroke risk reduction, prosthetic heart valves, and pulmonary embolisms. As the VKA effects vary intra- and interindividually, there is a need for constant monitoring via INR and prothrombin time measurements (32).

Each coumarin derivate has different half-lives varying between 6 to 60 hours. Vitamin K antagonists are metabolized with CYP-enzymes. CYP2C9 is the main catalyst of hydroxylation during coumarin metabolism. CYP3A4 is the additional catalyst along with the CYP2C9 in phenprocoumon metabolism. Thus, phenprocoumon proves itself to be an effective drug even in poor CYP2C9 metabolizers, as its pharmacokinetics are not affected by various CYP2C9 polymorphisms (32).

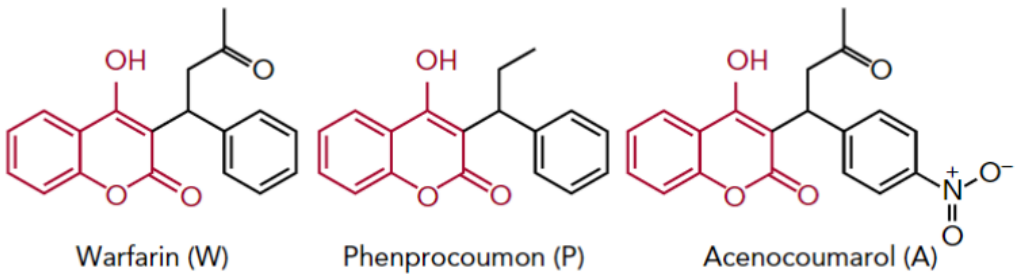

Figure 4: Molecule structures of vitamin K antagonists.

Modified from Chen et al. 2018 (31).

Vitamin K antagonists have proven themselves to be important agents for anticoagulation. Recent research has shown that the treatment with vitamin K antagonists might also have effects beyond anticoagulation. Verma et al. showed that patients treated with VKAs have moderately lower leukocytes, lymphocytes, and monocytes compared to control groups who did not receive VKA treatment (29). Stolz et al. demonstrated in a comparative study that warfarin and rivaroxaban (FXa-Inhibitor; see Table 1) were both able to reduce inflammatory lesions in the spinal cord by autoimmune encephalitis in mice. They concluded that warfarin and rivaroxaban had a mild preventive effect on local inflammation (33). These findings suggest that VKAs have possible anti-inflammatory effects.

1.4.2 New direct-acting anticoagulants

In the past decades, although heparins and vitamin K antagonists have been widely used in antithrombotic therapies, their limitations led to the identification of new target-specific drugs in the last twenty years (34). Some of these limitations were that VKAs have a narrow therapeutic window, and many possible drug interactions and heparins can cause a life-threatening low platelet syndrome named HIT (heparin-induced thrombocytopenia). New direct-acting anticoagulants that do not require constant monitoring can be used in patients with HIT history, they are as effective as VKAs and heparins and they pose a lower risk for bleeding (34,35). They have short elimination half-lives, which makes them safer and better controllable compared to VKAs and heparins. Some of their indications are non-valvular atrial fibrillation, deep vein thrombosis, and pulmonary embolism (36). These direct agents are able to inhibit active factors FIIa and FXa reversibly. The inhibition of factor Xa results in the cessation of thrombin (FIIa) generation, whereas FIIa inhibition stops the conversion of fibrinogen to fibrin monomers (37,38). After the first direct-acting anticoagulant argatroban was approved in 2000 in the USA, many other new anticoagulants followed in the next years. Currently, there are many anticoagulants that can be administered orally or parenterally (for an overview, see Table 1).

	Factor-IIa Inhibitors	*Factor-Xa inhibitors*
Oral	*Ximelagatran**	Rivaroxaban
	Dabigatran	Apixaban
		Edoxaban
		Betrixaban
Parenteral	Argatroban	
	Bivalirudin	-
	*Lepirudin**	
	*Desirudin**	

Table 1: Overview of direct-acting FIIa and FXa inhibitors.

Many direct-acting new anticoagulants can be applied orally or parenterally. In this table, the substances are shown according to their application form and which factor they inhibit. The asterisks represent the drugs that are not in use. Ximelagatran is not in use since 2004 because of its liver-toxic side effects. Bivalirudin, lepirudin, and desirudin are recombinant hirudins. Desirudin is not approved in Germany, and lepirudin production stopped in 2012. Betrixaban has been in use in the USA since 2017 but has not been approved in the EU. To this date, there is no known parenteral factor Xa inhibitors in the market.

The factor IIa inhibitor argatroban is a small molecule, synthetical L-Arginine derivate, that is approved for HIT prophylaxis and treatment. It has an elimination half-life of 45 minutes and is metabolized via CYP3A4/5 enzymes in the liver. It binds selectively and specifically to the catalytic site of thrombin and is effective against free, clot-, and fibrin-bound thrombin (37). Given that thrombin also plays a central role in non-hemostatic processes like inflammation, its inhibition via argatroban also has anti-inflammatory effects. Hoppensteadt et al. showed that argatroban downregulates CD40L, which is mainly secreted from activated $CD4^+$ and $CD8^+$ T-cells. CD40L regulates inflammation by affecting T-cell-mediated effector functions, and it leads to the expansion of $CD8^+$ cells (39,40). Shavit et al. have shown that direct thrombin inhibitors downregulate inflammation in the brain (41). Recently, in the global COVID-19 pandemic, the direct thrombin inhibitor argatroban was considered for antithrombotic and anti-inflammatory therapy in severe cases of patients who do not respond to heparins (41,42). Although future studies are needed to prove beneficial effects, the direct-acting inhibitors are not only helpful for thrombosis prevention but are also potential candidates in the treatment of critical inflammatory diseases (43).

1.5 Innate and adaptive immunity

People live in an environment with microorganisms, which can evoke various diseases, if not eliminated. For this, there is need for a host defense system. There are two systems for the host defense: innate and adaptive immunity. Both have humoral and cellular components that make it possible to react to the microorganisms rapidly and also allow them to recognize the antigens of the microorganisms and build a long-time immunity. Thus, these systems need to work together in a balanced way for optimal protection against bacteria, viruses, parasites, and many more microorganisms.

1.5.1 Innate immunity

Innate immunity can be perceived as the first-line defense system, which acts rapidly and, in an antigen-unspecific way to eradicate entering microorganisms. Innate immunity is an ancient system that is shared with other vertebrates, and its components can be transferred from mother to child. It detects microorganisms, keeps the balance between pro-and anti-inflammatory mechanisms, and interacts with the adaptive immune system. The recognition system work through PRRs (pattern recognition receptors), which are present over the generations and modified by natural selection with each generation. PRRs recognize PAMPs (LPS, dsRNA, membrane components, microbial DNA), which are characteristic of pathogens. Innate immunity has humoral and cellular components. Among humoral components are acute-phase proteins like CRP, lysozyme, interferons, and the complement system. Cellular components are monocytes, which turn into macrophages once they are in the infection site, neutrophils, basophilic and eosinophilic granulocytes, DCs, NKs, mast cells, platelets, and innate lymphoid cells. Leukocytes extravasate via adhesion molecules and proinflammatory mediators and cross the vessel walls (diapedesis). Diapedesis and chemotaxis (movement with chemical stimuli) are mediated by chemokines like IL-8 (neutrophil attractant) and MCP-1 (macrophage chemoattractant protein-1). Some mediators such as C3b mark the cells and antigens to facilitate phagocytosis, which is called opsonization. As soon as the leukocytes reach the invasion sites, they start phagocytosis and eliminate the invader microorganisms (3,44).

1.5.2 Adaptive immunity

Adaptive immunity is a more sophisticated system that functions in an antigen-specific way. It builds a long-time memory of the invading microorganisms and provides long-lasting protection. Its function is to recognize and destroy invading pathogens like the innate immune system. However, contrary to innate immune response, it needs days to weeks in order to specialize its components against the microorganisms. It has humoral and cellular components. The humoral components of the adaptive immune system are the antibodies secreted from the B-cells, and the cellular components are T- and B-lymphocytes. They can be differentiated with their CD (cluster of differentiation) markers on their surfaces. T-cells have $CD4^+$ and $CD8^+$ subsets, and B-cells carry CD19 (44).

During T-cell maturation, T-cell precursors migrate to the bone marrow or thymus and develop T-cell markers. In that stage, T-cells are in a double negative condition, $CD4^-$/$CD8^-$. After that, surface markers CD4 and CD8 start to form, along with the TCR. At that stage, the double-positive $CD4^+$/$CD8^+$ T-cells, which recognize MHC molecules and do not recognize the body's own antigens, survive. As the last step, T-cells that recognize MHCI better lose the CD4 positivity, whereas the ones that recognize MHCII lose the CD8 positivity. Then, they migrate to the peripheral lymphatic organs like the spleen, lymph nodes, Peyer´s patches, mucosal tissues, and bronchus-associated lymphatic tissues (3,45,46). TCRs of T-cells recognize MHC molecules on the APC (antigen-presenting cells) like macrophages, dendritic cells, and B-cells and differentiate into different T-cell subtypes. $CD4^+$ T-cells are T-helper cells, which activate macrophages (Th_1) and B-cells (Th_2). $CD8^+$ T-cells are cytotoxic cells. They recognize MHCI molecules of the infected cells or the tumor cells and secrete perforins and mediators to induce apoptosis (3,44,45).

1.6 Thrombin in non-hemostasis

Thrombin is known for being the key regulator in hemostasis. However, thrombin is not only a crucial serine-protease in coagulation, but it also has multiple pleiotropic effects, including actions on inflammatory cells that are mediated through PAR activation and signaling (see 1.7) (11,47).

Thrombin is a potent platelet activator. These effects are mediated through PARs, and it leads to shape changes, platelet aggregation, secretion of mediators such as ADP,

thromboxane A_2 from platelet granules, and chemokine secretion (48). During injury, thrombin also regulates vasodilatation and vasoconstriction processes depending on the cells on which it is acting and can alter the barrier function and the permeability of the vessels (47,49). Thrombin mediates the expression of P-selectin on endothelium and platelets, advancing the rolling and interactions of the granulocytes, monocytes, and lymphocytes with endothelial cells (ECs) at the injury site (50). It also mobilizes CD40L from the platelet surface, which promotes TF synthesis on macrophages and stimulates ECs to secrete chemokines and adhesion molecules, leading to the generation of recruitment and extravasation signals for leukocytes (50,51). Thus, thrombin is a key regulator linking inflammation and hemostasis.

Thrombin can activate proinflammatory pathways by inducing mast cell degranulation and the production of cytokines such as TNFα, IL-1β, IL-6, IL-8, and MCP-1 (48,50,52,53). The link between coagulation and inflammation is bidirectional; therefore, secreted proinflammatory cytokines can promote coagulation reciprocally. Thrombin is also capable of activating C3 and C5 of the complement system independent of the other pathways, leading to the activation and lysis of the target cells (54). Thrombin has a mitogenic effect on fibroblasts, endothelial cells, mesenchymal cells, and lymphocytes (48,55,56). Many immune cells such as natural killer cells, monocytes, $CD8^+$ and $CD4^+$ T-cells, and $\gamma\delta$ T-cells express the thrombin receptor PAR. These cells are crucial for antimicrobial and antitumor immunity; thus, thrombin affects innate and adaptive immunity (57). Recently, it was shown that thrombin can activate pro-IL-1α directly into IL-1α. Pro-IL-1α is found on macrophages, dendritic cells, and platelets. IL-1 is known to promote immune cell recruitment and adaptive immunity by T-helper cell differentiation and effector T-cell proliferation (58).

Thrombin's extensive functions are currently being heavily investigated in various research disciplines (59–63) with the aim of better understanding the underlying mechanisms of its broad actions in various systems. To this end, knock-out mouse models targeting prothrombin have been generated, which resulted in near complete pre-or neonatal mortality of the modified mice (64,65). This suggests that (pro)thrombin is instrumental in embryonic development, wherein a minimum of 5-10% prothrombin activity is needed for the development of normal embryos (66). This represents a significant experimental hurdle for further investigations.

1.7 PARs

Protease-activated receptors are G-protein coupled receptors that participate in transmembrane signaling upon proteolytic cleavage. There are four members of the PAR family in the mammalian genome: PAR1, PAR2, PAR3, and PAR4. Vu et al. characterized PAR1 in 1991, and since then, other PAR receptor discoveries followed (67,68). PAR1, PAR2, and PAR3 are clustered in chromosome 5q13 and PAR4 is found at 19p12 (69,70). PARs are located on various cells, such as endothelial cells, platelets, fibroblasts, smooth muscle cells, leukocytes, T-cells, and tumor cells (57,71,72). Thus, they play crucial roles in immunity, inflammation, wound repair, pain, cell proliferation, coagulation, embryologic development, and tumor growth. The expression profiles of different cell types may vary between species (68).

PARs can be activated in various ways. PARs carry their ligands, which are demasked upon cleavage of the receptor. The proteases cleave the N-terminal exodomain of the PARs, creating a new N-terminus. The new N-terminus serves as a tethered ligand, which binds to the PAR intramolecularly and initiates the transmembrane signaling (60,71). Activation through this mechanism is called the canonical pathway. PARs can also be activated by synthetic peptide activation, where peptides similar to tethered ligands are administrated. The signaling pathways not only vary between tissues and cells but also depend on the receptor ligands. PARs have different cleavage sites for different proteases. Therefore, activation of the same receptors may induce different signaling pathways and responses, which is called “biased signaling”. An example of biased signaling is the active protein C (APC), which cleaves PAR1 in the presence of EPCR at a distinct site than the canonical site, initiating anti-inflammatory and anti-apoptotic pathways.

PARs can dimerize to form homo- or heterodimers. These dimers can mediate distinct signaling pathways than mono-PARs. PAR3 bound to PAR1 can enhance the interaction between PAR1 and thrombin, resulting in increased endothelial permeability (73). The PAR signaling is initiated after PAR couples to G-proteins ($G_{12/13}$, G_q, $G_{i/z}$) (74). The PAR activation and signaling via thrombin can lead to 2224 various intracellular signaling patterns, which makes it complicated to investigate these pathways (75). An example of PAR signaling (PAR1) and the resulting cellular changes are depicted in Figure 5.

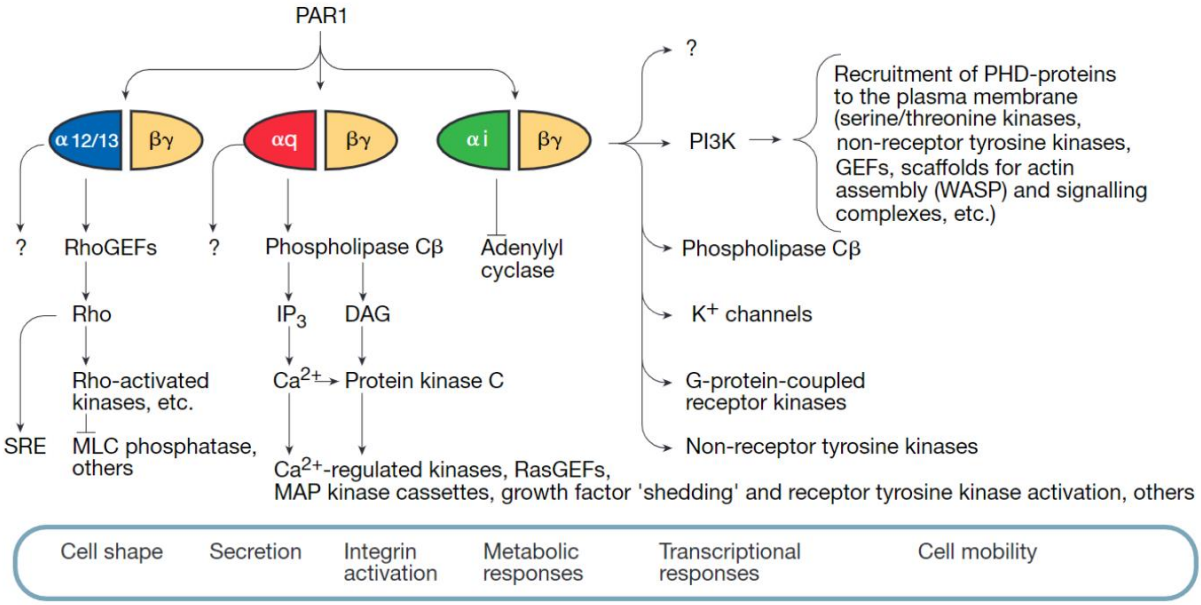

Figure 5: PAR1 signaling pathways.

The figure modified from Coughlin et al. (47) depicts the PAR1 signaling. The activation of $G\alpha_{12/13}$ results in cell shape changes in platelets and enhances permeability and cell migration in endothelial cells via Rho protein activation. The other subunit $G\alpha_q$ leads to calcium mobilization, which activates the pathways for secretion, aggregation, and integrin activation of platelets and posttranslational modifications in mesenchymal and endothelial cells. $G\alpha_I$ improves platelet responses. $G\beta\gamma$ activation supplies the membrane with protein binding sites (47).

Each proteinase-activated receptor is activated through various substrates. PAR1 is activated through thrombin, FXa, APC, trypsin, and granzyme A; PAR2 is activated through trypsin, tryptase, FVIIa, FXa, acrosin, proteinase 3, but not by thrombin directly. However, thrombin-activated PAR1 can transactivate PAR2. PAR3 gets activated via thrombin, APC, trypsin, and FXa. Lastly, PAR4 is activated via thrombin, trypsin, and cathepsin G, kallikrein-14 (72,74). PAR3 can serve as a cofactor for PAR1 and PAR4.

The main protease, which activates PAR1, PAR3, and PAR4 is the thrombin. While PAR1 can be activated with small concentrations of thrombin, PAR3 and PAR4 can only be activated when thrombin is present at higher concentrations (47). PAR1 and PAR3 have hirudin-like domains that can bind to thrombin exosite I, which enables an efficient activation of these receptors. Despite lacking a hirudin-like sequence, thrombin activates PAR4 and initiates intracellular signaling (74). Thrombin activates the platelets through PAR1 and PAR4 in humans, while mice platelets are activated through PAR3 and PAR4, as PAR1 is not expressed on mice platelets (72). PARs are expressed in many cell types, which are important in coagulation and wound repair.

In addition to that, PARs are also found on $CD4^+$ and $CD8^+$ T-cells, natural killer cells (NK), mast cells, macrophages, astrocytes, neurons, and many cancer cells (76,77). PAR expression can be upregulated during inflammation, injuries, tumor growth, and in many other pathologies (see Table 2) (76).

Pathology	Receptor involved	Cells / tissue affected	Observations
Atherosclerosis	↑ PAR-1	Vascular smooth muscle cells	*In vivo* (Human arteries)
Hypertension (Ang II-induced)	↑ PAR-1	Vascular smooth muscle cells	*In vivo* (Rat aorta)
Aggregating platelets	↑ PAR-1	Vascular smooth muscle cells	*In vitro* (rat and human cells)
Cancer	↑ PAR-1 and –2	Tumour cells	*In vitro* and *in vivo*
CNS inflammation	↑ PAR-1	Neuron and astrocyte	*In vitro*
Neurodegeneration	↑ PAR-1	Neuron and astrocyte	*In vivo* (Wobbler mutant mouse)
Nerve injury	↑ PAR-1, –2, –3, –4	Rat optic nerve	*In vivo*
Arthritis	↑ PAR-2	Murine adjuvant arthritis model	*In vivo*
Hepatitis	↑ PAR-1	Liver mesenchymal cells	*In vivo* (Human)
Ischemia	↑ PAR-1, –2, –3	Rat hippocampus	*In vivo*
Inflammatory myopathy	↑ PAR-1	Human skeletal muscle	*In vitro*
Viral infection	↑ PAR-1 and –2	Mouse airway	*In vivo*
Preeclampsia	↑ PAR-1 and –2	Human umbilical vein endothelial cells	*In vivo* (Human)

Table 2: PAR-mediated pathologies and the expression patterns.

Table modified from Martorell et al. (76). PARs are activated during wound repair, hemostasis, and inflammation. PAR expression is also linked to some pathological conditions as listed above. This suggests the relevance of the thrombin-PAR axis during these conditions (76). CNS= central nervous system; Ang II= Angiotensin II.

1.8 Origin of thrombin

Thrombin plays a role in adaptive and innate immunity, acute and chronic inflammatory diseases, cancer progression and tumor growth, atherosclerosis, angiogenesis, tissue repair, and embryonic development (11,48,78,79). Thrombin fulfills its functions in those mechanisms mainly through PARs (see 1.7). Thrombin precursor prothrombin is known to be mainly secreted from the liver, which produces most of the secreted proteins such as albumin, coagulation factors, phospholipids, angiotensinogen, insulin-like growth factor, and many others (80). Even so, thrombin's actions are not only limited to the vascular compartment; it also mediates non-hemostatic actions on the cells of the extravascular compartment (80). As thrombin is a 72 kDa protein that cannot

simply leave the vascular compartment, the question arises if there are origins of extrahepatic prothrombin expression that account for the well-established action of thrombin on cells in extravascular compartments.

Previous research indicated that thrombin is not only found in the liver and blood but is also in tissues such as the heart, kidney, and lungs (48,81,82). To further investigate the origin of (pro)thrombin expression, Danckwardt laboratory at the university of Mainz generated a new mouse model (called D-Insight) (80). D-Insight transgenic mice allow the monitoring of the non-canonical (extrahepatic) prothrombin expression and secretion dynamics with the help of two luminescence reporters (RLuc and Fluc2CP) and a fluorescence marker (iRFP) in physiological conditions. The specially tailored targeting strategy using “self-cleaving” P2A peptides results in 1:1 co-expression of endogenous RLuc tagged prothrombin and the cell resident markers tagged with iRFP and Fluc (Figure 6) (80,83).

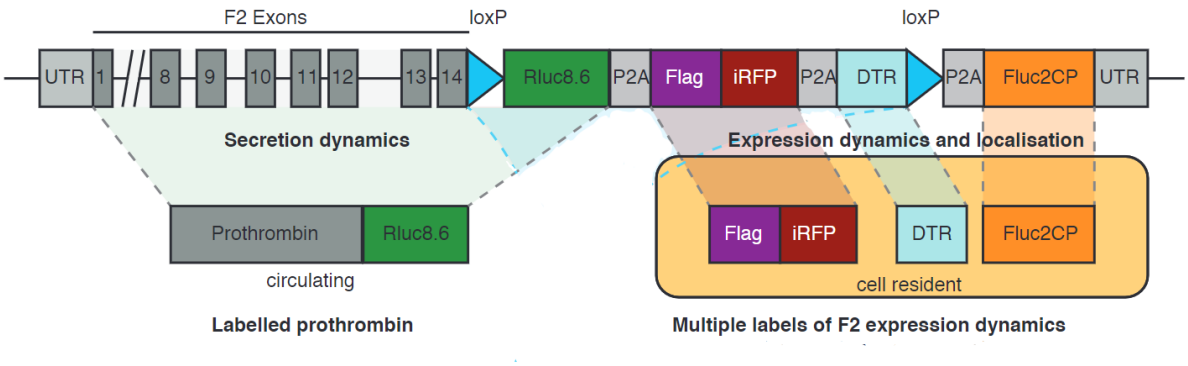

Figure 6: D-Insight imaging for prothrombin expression and secretion monitoring.

Figure modified from Nourse et al. (80). With the help of the D-Insight mice model, our group was able to identify prothrombin expression dynamics. Due to P2A cleavage, the endogenous prothrombin (left side) and the cell resident markers (right side) are expressed in a 1:1 manner, which allowed our group to track the cells which express thrombin.

For the identification of the tissues expressing F2, firefly luciferase (Fluc) activities were measured in B6 mice and D-Insight mice (80). The Fluc activities in the liver, lung, cerebrum, cerebellum, and lymphatic tissues were significantly high. The RNA expressions of F2 also correlated significantly with the Fluc activities in these tissues. The lymphocytes and leukocytes originate from stem cells in the bone marrow and are released into the bloodstream. Finally, lymphoid cells move into the peripheral lymphoid organs (3,84). As lymphatic tissues demonstrated a high Fluc activity, our group created bone marrow chimeric (BMC) D-Insight mice to study whether the cells with high

F2 expression originate from the bone marrow. To that end, irradiated B6 mice were injected with the bone marrow of D-Insight mice, and reconstituted Fluc activities were measured. The liver did not show any reconstitution as the prothrombin in the liver was of hepatic origin, but the lymphatic tissues along with the lung showed an activity reconstitution varying between 80-100% (Figure 7) suggesting that the prothrombin expression in lymphatic tissues originates from the bone marrow-derived cells (83).

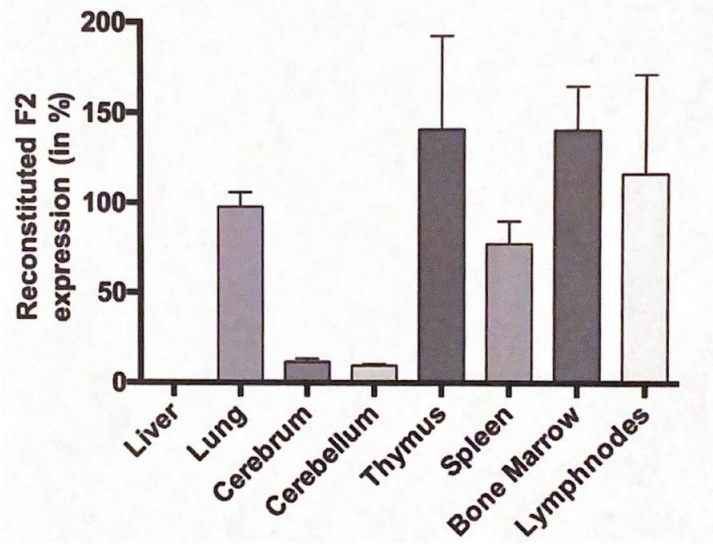

Figure 7: Reconstituted Fluc activity in BMC D-Insight mice.

Figure modified from Khokhar et al. (83). After D-Insight bone marrow transplantation into irradiated B6 mice, the Fluc activities in lymphatic tissues, brain, cerebellum, lung, and liver were measured. All lymphatic tissues (thymus, spleen, bone marrow, and lymph nodes) showed a complete reconstitution of the Fluc activity (compared to the Fluc activity in respective tissues obtained from D-Insight reporter animals).

For further identification of the extrahepatic origin of the F2, bone marrow-derived cells from the spleen were harvested and analyzed using FACS sorting (80,83). The splenocytes from BMC-DI mice were sorted into two groups according to their expression of the major histocompatibility complex-II (MHC-II), which is found on antigen-presenting cells of the immune system, such as B-lymphocytes, macrophages, and dendritic cells (3) and the Fluc activities were measured. The MHC-II negative group showed significantly higher activity than the control group. T-cells are MHC-II negative cells. Thus, splenocytes were sorted for TCRβ chain (T-cell receptor), and the Fluc activities were compared to investigate whether T-cells were responsible for the extrahepatic F2 expression. TCRβ positive cells from BMC-DI mice showed significantly higher activity compared to B6 mice, suggesting that T-cells are expressing extrahepatic F2. The $CD4^+$ and $CD8^+$ T-cell populations both expressed F2 (83). It was also concluded that active ($CD44^+$) T-cells showed a significantly higher F2 expression than not-activated

T-cells ($CD44^-$). These results were validated with in vivo $CD4^+$ and $CD8^+$ T-cell depletion in BMC-DI mice. After the depletion, lymphatic tissues were collected for Fluc activity measurements. Spleen, thymus, and bone marrow showed significantly lower F2 expression after T-cell depletion, confirming the F2 expression in lymphatic organs originates from T-cells (83).

1.9 Aim of the study

Thrombin is a multi-functional serine-protease that plays a crucial role in hemostasis and coagulation. In addition to these functions, it has many non-hemostatic functions, which are mediated mostly by PARs. In recent years, evidence has emerged confirming that thrombin is a key player linking hemostasis to inflammatory events. Depending on its concentration, the responding cell type, and the microenvironment, it can trigger different signaling pathways leading to pro- or anti-inflammatory effects (41,48,58,80,83,85). Thrombin deregulation can lead to hypercoagulation and inflammation in sepsis and hemostatic disorders (48,86). Given that even small alterations in thrombin expression through a prothrombin gene mutation 20210 G>A (1,5-1,7 fold increase) leads to a higher risk of thromboembolic events, fine-tuning of prothrombin expression and maintaining the well-balanced equilibrium is crucial (48,87,88).

The main origin of thrombin is the liver. However, it is also expressed in extrahepatic tissues such as the lung, heart, kidney, brain, and many cancerous tissues (48,81,82,89). Our group's observations confirmed extrahepatic prothrombin expression in the brain, lung, heart, and lymphatic tissues utilizing a novel mouse model (D-Insight, see 1.8) through tracking of the prothrombin expression in vivo (80,83). Lymphatic prothrombin expression could be narrowed down to the activated T-cells (83). In consideration of this, T-cell-derived (pro)thrombin might have many unrecognized roles in physiological and pathological processes in the body.

This study aims to investigate the possible effects of the $CD8^+$ T-cell-derived FII on T-cell expansion and survival. To this end, I made use of genetic and pharmaceutic approaches targeting F2 followed by flow cytometric analysis. As complete F2 knock-out mice die perinatally (65,66), CD4Cre mice (2.5.1) were used, which did not express F2 specifically in the T-cells. I also utilized anticoagulants to inhibit thrombin production in different experimental settings. Finally, I conducted rescue experiments with recombinant prothrombin and thrombin, separately. This study aims to give better insights into the effects of T-cell-derived F2 on T-cells as potential target cells in an autocrine manner and its potential role in physiological and pathological settings.

2. Material and Methods

2.1 Laboratory Equipment and Software

Lab equipment	Manufacturer
Cell-culture incubator	Heracell 150i-, Thermo-Scientific, Waltham, USA
Centrifuge	Heraeus Multifuge 3S-R, Thermo-Scientific, Waltham, USA
Flow cytometers	FACS-Lyric, -LSRII, -Symphony,- Canto, BD-Biosciences,
Light-optical microscope	Carl-Zeiss, Oberkochen, Germany
MACS MultiStand	Miltenyi Biotech, Bergisch Gladbach
Neubauer cell counting chamber	Chamber depth, 0.1mm, Marienfeld, Lauda-Königshofen, Germany
Pipetboy	Integra Biosciences, Zizers, Switzerland
Pipettes	2.5 µL, 10 µL, 20 µL, 100µL, 1000 µL, Eppendorf, Hamburg, Germany
QuadroMACS-Separator	BD-Biosciences, San Diego, USA
Refrigerators, freezers	Liebherr, Thermo-Scientific, Waltham, USA
Sterile bench	Thermo-Scientific, Waltham, USA
Vortex-Genie 2	Sigma-Aldrich, St. Louis, USA

Software	Developer
FlowJo Version 10.5.2	FlowJo, LLC, Ashland, USA
GraphPad Prism Version 8.0.2	Graphpad Software Inc, San Diego, USA

2.2 Consumables

Product	Manufacturer
Cannulas	Sterican Gr.20 ø 0,40 x 20 mm, grey, B.Braun, Melsungen, Germany
Cell culture Multiwell Plates, 96 well	Flat bottom, sterile 96 well, Cellstar Greiner Bio-one, Kremsmünster, Austria

Cell strainer 70µL-Easystrainer	Greiner Bio-one, Kremsmünster, Austria
Falcons and tubes	1.5 mL, 2 mL, 15 mL, 50 mL, Eppendorf, Hamburg, Germany
Filter paper	Thermo-Scientific, Waltham, USA
Flow cytometry tubes	5 mL, Sarstedt, Germany
LS Columns, MACS	Miltenyi Biotech, Bergisch Gladbach, Germany
Pasteur pipettes	150mm, neoLab, Heidelberg, Germany
Pipette tips	0,1-10 µl,100-1000µL sterile, Nerbe Plus, Winsen (Luhe), Germany
Pre-Seperation filters, MACS	70 µm, Miltenyi Biotech, Bergisch Gladbach, Germany
Reagent reservoir	Startub PS, 55 ml, Starlab, Hamburg, Germany
Scalpel	Cutfix, B.Braun, Melsungen, Germany
Serological pipettes	5 ml, 10 ml, 25 ml, Nerbe Plus, Winsen (Luhe), Germany
Skin disinfectant	Desderman, Schülke & Mayr, Norderstedt, Germany
Syringe	InjektF-solo, 1ml, B.Braun, Melsungen, Germany

2.3 Solutions, media, buffers

Product	Manufacturer
7-AAD viability staining solution	2 ml, eBioscience Invitrogen, Thermo-Scientific, Waltham, USA
Annexin V Binding Buffer	10x concentrated, BD-Biosciences, San Diego, USA
Argatroban	50 mg/ml stock, provided by AG Danckwardt
CFSE 5(6) Carboxyfluorescein-diacetat-*N*-succinimidylester	25mg, Sigma-Aldrich, St. Louis, USA
Dimethylsulfoxid (DMSO)	100 ml, Carl-Roth, Karlsruhe, Germany
DNAse I	10mg, Sigma-Aldrich, St. Louis, USA

DPBS 1x, Dubecco's Phosphate Bufffered Saline	500 ml, Gibco, Thermo-Scientific, Waltham, USA
FACS Buffer	5L, all ingredients provided by AG Probst. Composition: 200 ml 0,5 M EDTA; 250 ml BSA (10%); 500 ml 10x concentrated PBS, 4050 ml distilled water
Ketamin / Xylazin	100mg/kg/10mg/kg, Inresa Arzneimittel, Freiburg, Germany
HbSS without calcium, magnesium, and phenol red	500 ml, Gibco, Thermo-Scientific, Waltham, USA
Phenprocoumon	25mg, Sigma-Aldrich, St. Louis, USA
Prothrombin, mouse	100µL, Biomol, Hamburg, Germany
Recombinant mouse IL-2 (carrier-free)	25µg, Biolegend, San Diego, USA
TexMACS cell culture medium	500 ml, Miltenyi Biotech , Bergisch Gladbach, Germany
Thrombin, mouse	50µL, Biomol, Hamburg, Germany
Trypan blue solution	0,4%, Gibco, Thermo-Scientific, Waltham, USA

2.4 Antibodies

Product	**Manufacturer**
Anti-CD28 Monoclonal Antibody	4 mg/ml in PBS, provided by AG Probst
Anti-CD3 Monoclonal Antibody	2 mg/ml in PBS, provided by AG Probst
Anti-Rat and Anti-Hamster Ig κ/ Negative Control Compensation Particles Set	BD-Biosciences, San Diego, USA
APC-Annexin V	25 tests, Biolegend, San Diego, USA
BV510 Rat Anti-mouse I-A/I-E (MHCII) Antibody	50µg, BD-Biosciences, San Diego, USA
CD8a (Ly-2) MicroBeads, mouse	2 ml for total of $2x10^9$ cells, Miltenyi Biotech, Bergisch Gladbach, Germany
CD8a-Monoclonal Antibody (53-6.7), APC	200µg, eBioscience, Thermo-Scientific, Waltham, USA

Fc-Block anti-mouse CD16/CD32	50µL (14,4 mg/ml) Stock, provided by AG Probst, JGU, Mainz
Pacific Blue anti-mouse I-A/I-E (MHCII) Antibody, M5/114.15.2	100 µg, Biolegend, San Diego, USA
Pacific Blue anti-mouse CD8a Antibody (53-6.7)	100 µg, Biolegend, San Diego, USA

2.5 Mouse Lines

Mouse line	**Name**	**Laboratory Animal Company**
C57BL/6JRj	B6-Wildtype	Janvier Labs, Le Genest Saint-Isle, France
CD4Cre x F2tm1c	CD4Cre mice-FII deficiency in CD4+ and CD8+ T-cells	AG Danckwardt/Probst, University Mainz, Germany

2.5.1 CD4Cre x F2tm1c

CD4Cre x F2tm1c mice were generated by AG Danckwardt/AG Probst. This mouse model is a conditional knock-out model, which enables selected genes to be knocked out in specific tissues. Cre/LoxP system (90–94) was used to knock out the F2 gene (prothrombin), specifically in $CD4^+$ (and $CD8^+$) T-cells. T-cells are double-positive ($CD4^+$ and $CD8^+$) before either one of these co-receptors gets downregulated during T-cell maturation. Therefore, the knock-out of the F2 gene by crossing F2 floxed F2tm1c mice with CD4Cre is sufficient to delete the F2 gene in all of the $CD4^+$ and $CD8^+$ T-cells.

Briefly, CD4Cre mice, which had a Cre-recombinase expressed by a promoter that targets the T-cells were bred with floxed *(loxP* flanked) F2 gene-carrying F2tm1c mice. Cre-recombinase recognizes the *loxP* region and cuts it at both ends, thus deleting the F2 gene (Figure 8)(90).

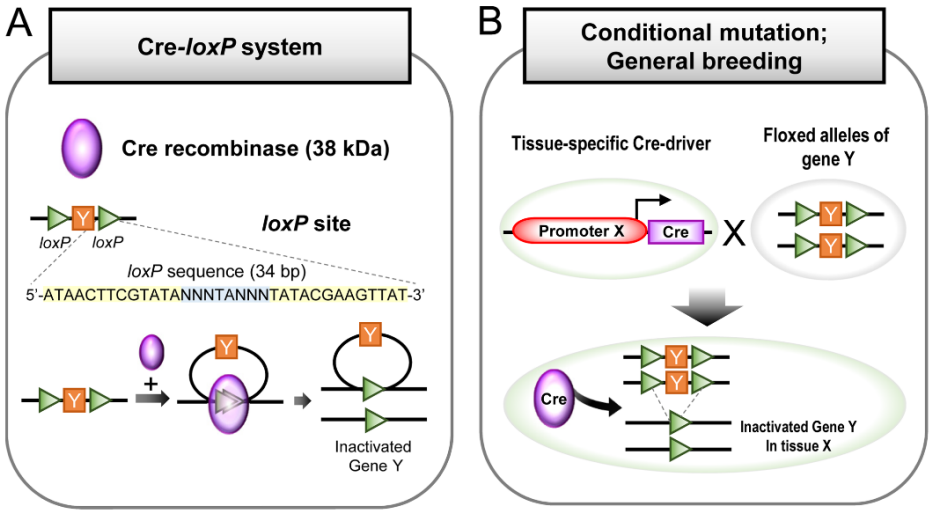

Figure 8. Cre-*loxP* system and gene inactivation.

A: Cre-recombinase is 38 kDa and can recognize loxP sites. Recognized sites are excised, and the floxed gene -F2 (marked as Y) -gets inactivated. **B:** The mice with tissue-specific cre-strain are bred with mice carrying floxed alleles of F2. Cre-recombinase knocks out the floxed F2 gene in the target tissue and inactivates this gene (modified from (90)).

In the CD4Cre x F2tm1c (referred to as CD4Cre) experiments control and CD4Cre mice were gender and age-matched. Mice were 19-23 weeks old, and 25 pairs were used (n=50).

2.5.2 C57BL/6JRj

Wildtype mice were used in argatroban-, vitamin K- and rescue experiments. All of them were female and 19-20 weeks old at the time of sacrifice. We used a total of 125 mice in these experiments.

2.6 FlowJo Analysis

2.6.1 Gating of $CD8^+$ T-lymphocytes

After the acquisition, the samples were analyzed using FlowJo software, as shown in Figure 9.

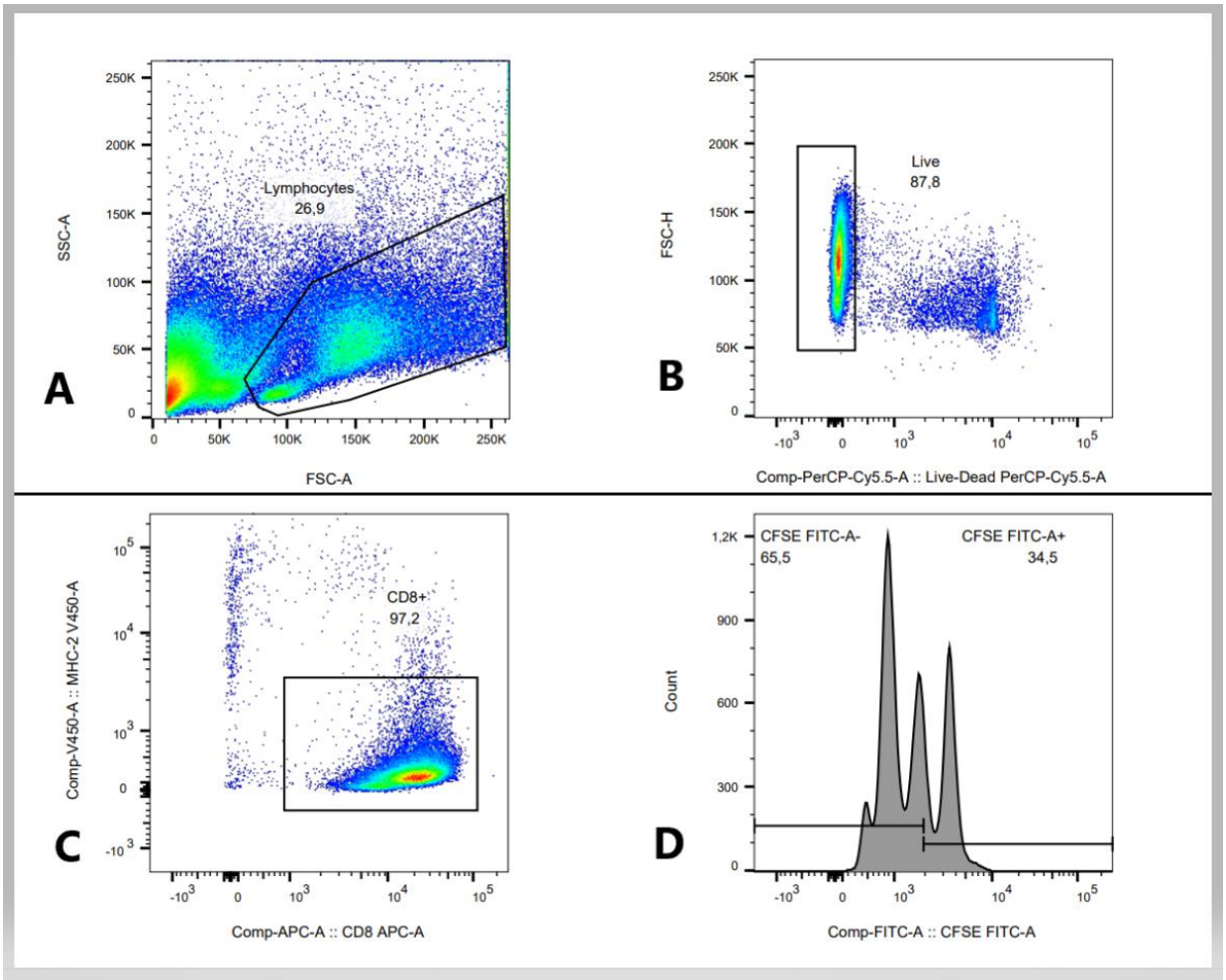

Figure 9. An example of gating for CD8⁺ T-lymphocytes in FlowJo software.

A: Cells were gated by FSC (forward scatter) and SSC (side scatter). FSC intensity is proportional to the cell diameter and SSC shows the granularity of the cell. The population of lymphocytes was selected. **B:** The lymphocyte population selected in A was again gated by 7-AAD, which excites the PerCP-Cy5.5 channel. The cells above 10^3 are assumed to be positive for 7-AAD, which represents the dead cells. For differentiating the living lymphocytes, all cells below 10^3 were selected. **C:** The living lymphocyte population was gated again to select CD8⁺ and MHCII- cells to narrow the population down to CD8⁺ T-lymphocytes. **D:** All living CD8⁺ T-lymphocytes were depicted on a histogram. CFSE-dye excites the FITC channel in BD FACS Lyric. The very first peak on the right side shows the undivided cells. Undivided cells and divided cells were analyzed with FlowJo's proliferation tool (see 2.6.2)) and also gated manually by drawing a line after the first peak. CFSE FITC-A- with 65,5% represents the divided cells among all living CD8⁺ T-lymphocytes. With each division, the CFSE dye in the cells gets halved, and the divisions can be tracked by counting the peaks. In this example, the cells have divided at least three times.

The cells that lie in the gating in Figure 9A are depicted in Figure 9B. Again, the selected cells in Figure 9B were depicted in Figure 9C and so forth. The percentages in figures A, B, C, and D show how many percent of the cells lie within the gate. This is also called "frequent of parent". Additional to these values, the percent of living CD8⁺ T-lymphocytes was calculated by selecting the "frequent of grandparent" for the cells

that lie within the gate in Figure 9C. The value calculated by the software shows the percentage of the cells in the "CD8⁺" gate out of the gate "lymphocytes".

2.6.2 Analysis of proliferation

After gating CD8⁺ T-lymphocytes, FlowJo's proliferation tool was used to get a look into cell biology, as shown in Figure 10.

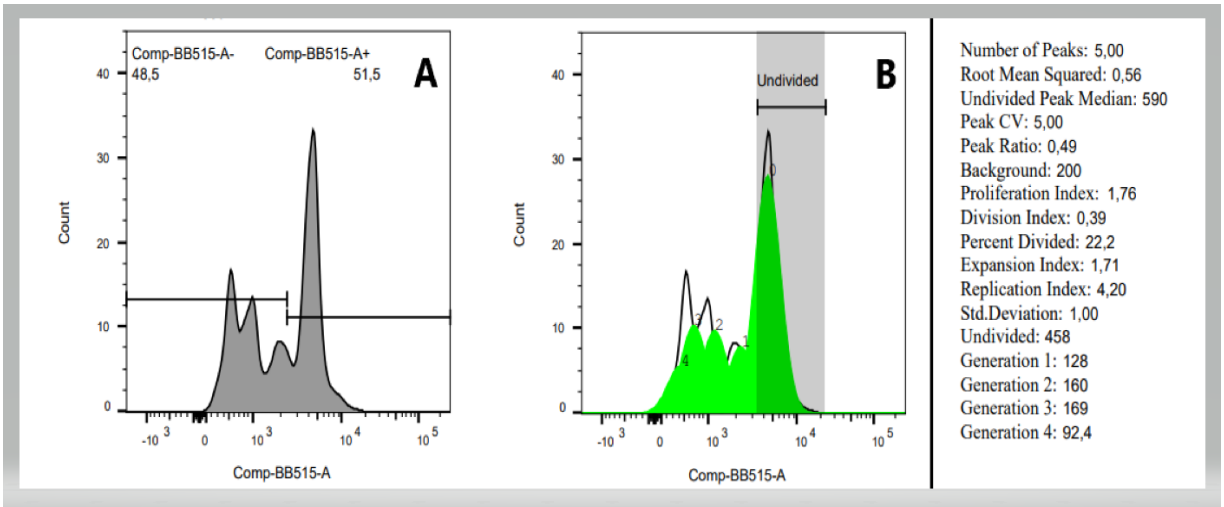

Figure 10. Analysis of proliferation using the proliferation tool in FlowJo software.

An example of a sample analysis from day 3 using FlowJo software. CFSE dye in the CD8⁺ T-lymphocytes excites the BB515 channel in BD FACS Symphony. **A:** The histogram shows 5 peaks, including the first undivided peak. The gate was manually set after the undivided peak. The cells with diluted CFSE are on the left side of the gate, and they make up 48,5% of all acquired cells. **B:** FlowJo´s proliferation tool adjusts a model and calculates several values, including proliferation index, percent of cells that are divided, and the cell count in each generation.

Proliferation data is most commonly reported as the fraction of diluted CFSE (*Dil*) in many papers. *Dil* can be interpreted as the percentage of cells divided in the final cell culture. As in Figure 10A, gating can be manually set after the undivided peak, and the software calculates *Dil.* Other proliferation statistics were calculated using the proliferation tool in FlowJo software. For the proliferation analysis in FlowJo, a model needs to be adjusted (Figure 10B).

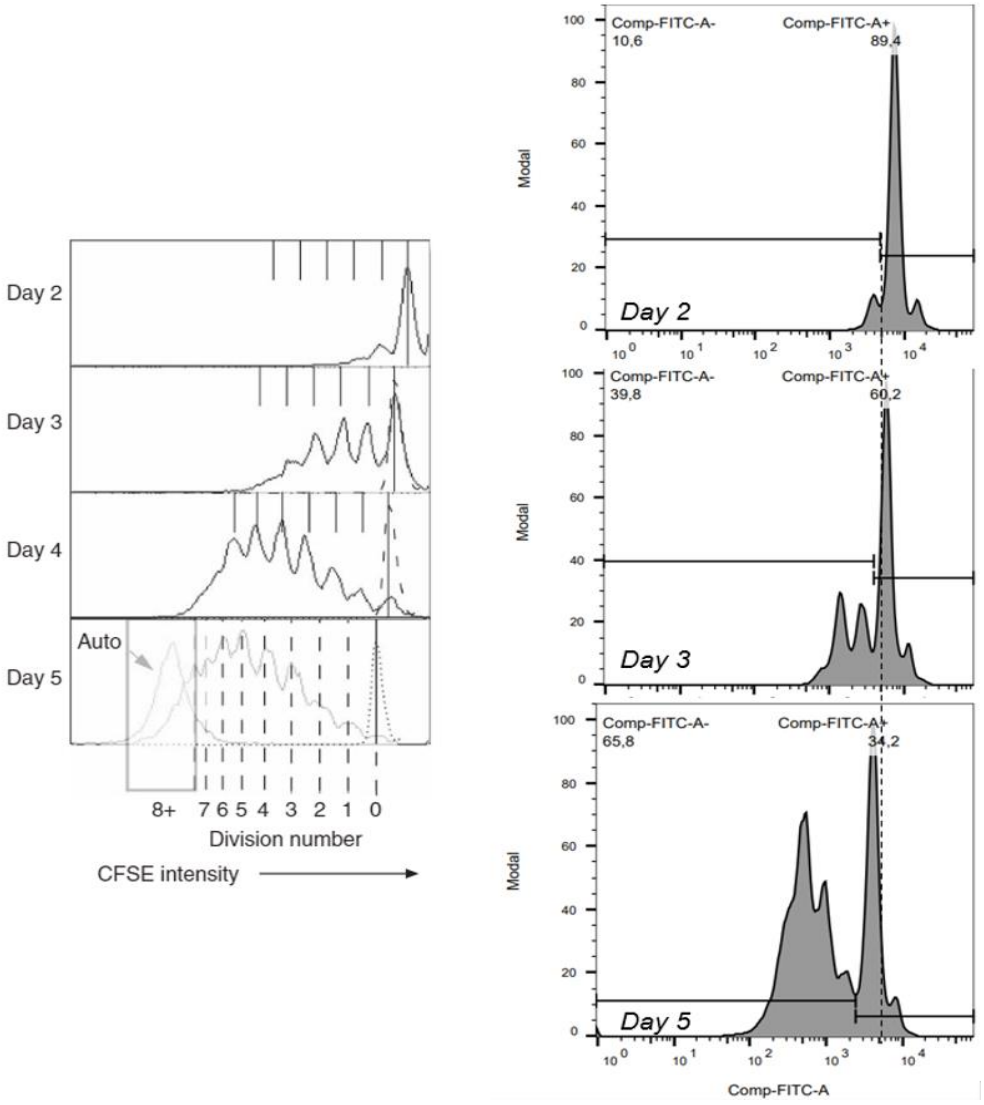

Figure 11. CFSE data in time course.

A: Figure modified from (95). CFSE-labeled and stimulated T-cells were depicted on histograms on subsequent days. The decay of the CFSE dye is depicted with the line cutting through the 0^{th} generation. On day 5 the peaks start to merge with the background so that it gets harder to distinguish peaks and track further proliferation. **B:** An example from a CD4cre-mouse used in the experiments. Histograms from day 2 to 5 show that the undivided peak from day 2 moves towards the left as the proliferation continues. As a result, defining a universal gate for all the samples from different days is not possible while calculating *Dil*. It must be done visually, whereas FlowJo´s proliferation tool recognizes this shift better, and it suffices to define the undivided peak once and apply it to all samples.

First, the undivided peak needs to be manually set at generation 0. The peaks, including the undivided peak, move slowly to the left side as the CFSE dye decays with time, and it becomes difficult to distinguish peaks further than the 7^{th} generation. To that end, the background needs to be calculated using unstained (not CFSE-labeled) cells. The background is the autofluorescence of the cells, and it complicates the visibility of the peaks as they move towards the left during proliferation (95) (Figure 11).

Statistic *All statistics are defined as in FlowJo software.	**Value Range**	**Meaning**
Intrinsic values		
Precursor frequency (PF), %dividing cells	0-1 (0-100%)	Probability of a cell to divide at least once
Proliferation Index (PI)	1-7	The average number of divisions the cells undergo that are responding and have divided at least once, showing the proliferative capacity
Extrinsic values		
Fraction diluted (*Dil),* % of diluted CFSE	0-1 (0-100%)	The fraction/ percent of the cells in the culture that have divided at least once
Expansion Index	1-128	The fold ratio of final cell count to initial cell count
Division Index	0-7	Average division number for all cells in the culture, including non-responding cells
Replication Index	1-128	Fold expansion of the responding cells during cell culture

Table 3. List of proliferation statistics in FlowJo, their characteristics, and value ranges.

Figure modified from (96).

The number of peaks was also set manually by counting the peaks. The peak ratio represents the ratio between the fluorescence of two subsequent peaks (96). Normally the fluorescence of CFSE is expected to get halved in the daughter cells. Therefore it should be 0,5 in a perfect setting. As the divisions in biological systems might not work in such perfection, the peak ratio was adjusted to 0,49. CV is the fluorescent variation within each generation, and it determines the resolution of the peaks. Higher CV values do not allow a good distinction between peaks, and therefore a high CV makes it harder to adjust a proliferation model (96). CV, background, and peak ratios were held con-

stant across all experiments with the same setup. After the model is set, FlowJo computes several values, including precursor frequency (PF), proliferation-, division-, replication-, and expansion index, and how many cells each generation has (Figure 10, Figure 12).

Although it is useful to have many indices and figures, only PI and PF are intrinsic, whereas *Dil,* EI, DI, and RI are extrinsic. Intrinsic values show us pre-programmed biological responses in the cells, while extrinsic values depend on the cultivation time and the initial number of cells in the culture. As *Dil* relies on a visual interpretation of the CFSE histogram, it is also limited. *Dil* can just report how many percent of cells have divided at least once, but it does not give any information concerning how many divisions the responding cells have undergone. This phenomenon can be observed in Figure 12 from Roederer et al. Moreover, EI, DI, and RI are dependent values, meaning that they can be expressed in terms of PF, PI, and *Dil* (96).

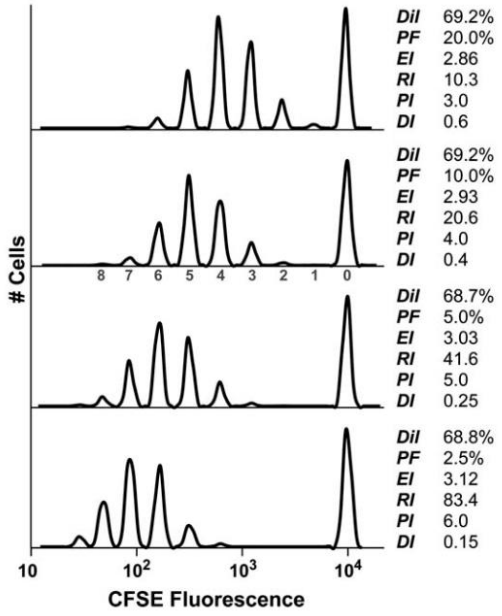

Figure 12. Hypothetical CFSE distributions and proliferation statistics.

Figure from (96). The histograms show different hypothetical CFSE distributions. *Dil* is the same for each histogram, but the precursor frequencies vary from 2,5 to 20%, and the proliferation indices are between 3-6. This figure clearly shows that the visual interpretations can be misleading, taking into account that *Dil* is the same overall, although the biological responses are not the same.

Given the reason that proliferation index and precursor frequency reflect the intrinsic biological processes, do not depend on the factors like initial cell count or the cell culture time, and provide independent information, we chose to introduce only these values along with the percent of living $CD8^+$ T-cells as mentioned above in this work.

2.7 Spleen Extraction

Mice fur was cleaned with 70% ethanol and cut along the left side of the mouse. Afterward, the spleen was extracted and put on a cell strainer. The cell strainer was wetted with TexMacs medium and 1:100 diluted DNAse I (1mg/ml stock) beforehand. The spleen was then mashed with the end of the syringe and washed with the rest of the 5 ml TexMacs (+ DNAse I 1:100) solution into a 50 ml Falcon and centrifuged at 300 g and +4°C. The supernatant was removed, and the cells were resuspended in 12,5 ml of fresh TexMacs.

2.8 CFSE-Labeling

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a novel method, first described by Parish et al. in 1994, which is used for tracking lymphocyte cell proliferation in vivo and in vitro in mice and in vivo in humans. CFSE is initially non-fluorescent and has two acetate groups, which are cleaved as soon as the dye goes into the cell. After this cleavage, CFSE becomes highly fluorescent and cannot leave the cell as the membrane is nearly impermeable against CFSE. During lymphocyte proliferation, the cells divide into two daughter cells, and CFSE dye also gets halved by each division (97).

In our research, to track the proliferation of $CD8^+$ T-cells, we labeled freshly extracted cells from the spleen with 2,5 mM CFSE according to the protocol in the paper by Quah et al. (97). After MACS (see 2.9) and cell culture (see 2.10), we used flow cytometry to track the divisions as shown on Figure 13.

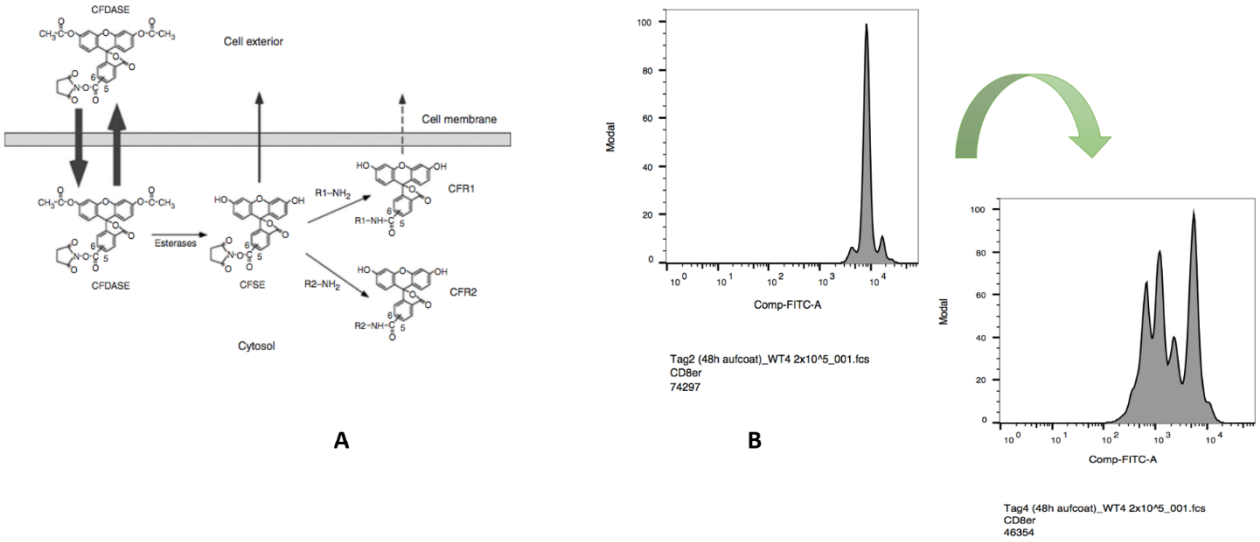

Figure 13. CFSE-dye and tracking proliferation with flow cytometry.

A: CFSE dye goes into the cell, and the esterases in the cytoplasm cleave the acetate groups so that fluorescent CFSE cannot leave the cell (2). **B:** Graphs show the CFSE peaks, analyzed by FlowJo software. The graph on the left side shows the CFSE fluorescent cells on day 2, and the graph on the right side shows the proliferation peaks on day 4. The first peaks represent the cells that are not divided. CFSE dye in the cells gets diluted with every cell division. Every peak represents the cells that went into the next division. In this example, the cells went into 4 to 5 division until day 4.

2.9 MACS

Magnetic-activated cell separation (MACS) is a cell separation technique that was developed by Miltenyi et al. in 1990 (98). MACS utilizes magnetic beads, which are coupled to antibodies, and thereby it can label cells magnetically, which have the corresponding binding sites. When placed into the magnetic field, labeled cells are retained in the MACS column; unlabeled cells flow through. After removing the MACS column from the magnetic field, labeled cells can be collected Figure 14.

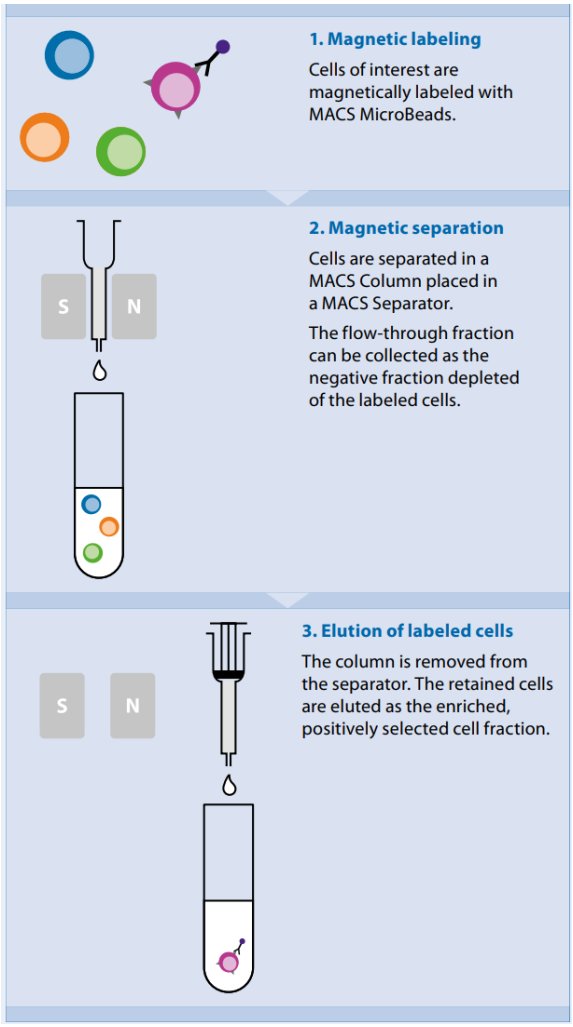

Figure 14. Schematic illustration of magnetic activated cell separation.

Figure modified from Miltenyi Biotech MACS cell separation manual (4). Magnetically labeled cells of interest with corresponding epitopes are retained in the column, which is placed into the magnetic field. When the column is removed from the magnetic field, labeled cells of interest can be collected.

In our experiments, CFSE-labeled cells that are extracted from the spleen were resuspended in 500 µL of HBSS+ 5 mM EDTA solution, 5 µL of Fc-Block was added, and the cell suspension was incubated with 30 µL CD8a (Ly-2) microbeads for 15 minutes in 4°C in the dark.

During incubation, the LS column was placed into the QuadroMACS magnets and then rinsed with cold-degassed HBSS buffer. After the incubation, 1 mL of HBSS + 5 mM EDTA solution was added to the sample. The sample was applied through a 70 µm pre-separation filter onto the LS column. The LS column was then washed twice with degassed HBSS buffer, removed from the magnet, and placed onto the 15 ml Falcon. Fresh 5 ml of HBSS buffer was pipetted onto the column, and the positively selected

$CD8^+$ T-cells were pressed out into the falcon by pushing the plunger firmly. Cell purity after magnetic-activated cell sorting (MACS) was more than 85% for all experiments.

2.10 Serum-free Primary Lymphocyte Cell Culture

Flat-bottomed 96-well cell culture plates were coated with 3 µg/ml anti-CD3 and 10 µg/ml anti-CD28 and incubated for 1 hour at 37°C. After coating, the plate was washed with cold PBS. Freshly sorted $CD8^+$ T-cells were counted using a Neubauer-cell counting chamber and centrifuged at 300 g for 5 minutes. The supernatant was removed, and the cells were resuspended in TexMACS cell culture medium so that there are 10^6 cells per milliliter. 200 µL of the cell suspension was given to each well, resulting in $2x10^5$ cells per well. The cells were transferred onto an uncoated 96-well plate on day 2. Each well was supplemented with 20 ng/ml recombinant carrier-free IL-2 on days 0, 2, and 4 in all experiments. Depending on the experimental setup, the cells were also treated with various substances, as shown in Table 4. $CD8^+$ T-cells were kept until day 5, and cells were analyzed on days 3 and 5 using flow cytometry.

Substance	Experiment	Day given	Concentration
DMSO	• Vitamin-K-Antagonist (VKA)-Experiments (3.1,3.2) • FII/ FIIa Rescue-Experiments (3.2.3)	0, 2, 4	0,05%
Phenprocoumon	VKA-Experiments (3.1, 3.2)	0, 2, 4	1 µg/ml
Argatroban	Direct FII-Inhibitor-Experiment (3.3)	0, 2, 4	25 µg/ml
Thrombin	Rescue-Experiments (3.2.3)	0, 2, 4	11,53 nM
Prothrombin	Rescue-Experiments (3.2.3)	0, 2, 4	11,53 nM

Table 4: Substances given into the cell cultures depending on the experimental setup, their concentrations, and time points of their addition.

2.11 Flow Cytometry

$CD8^+$ T-cells in the culture were analyzed on days 3 and 5. For each day, 2 to 4 replicates of each group were pipetted into a 5 ml FACS tube, and the wells were thoroughly

washed with 200 µL of FACS buffer to get the remaining cells in the well and transferred into the same tube. An additional 2 ml of FACS buffer was given into the same FACS tube, and the cell suspension was centrifuged at 4°C, 300 g for 5 minutes. The FACS staining solution was prepared (see Table 5). The supernatant was removed. The cells were resuspended in 50 µL of FACS staining solution containing antibodies of interest and were then incubated at 4°C for 15 minutes. After the incubation, the cells were washed with 2 ml of FACS buffer and centrifuged at 4°C, 300 g for 5 minutes. The supernatant was removed, and the cell pellet was resuspended in 100 µL of FACS buffer containing 1:100 7-AAD viability staining solution. The samples were acquired for 90 seconds and analyzed in BD FACS Symphony and BD FACS Lyric.

Antibody/Ingredient	**Channel**	**Concentration**	**Amount**
CD8-APC	APC	1:400	5 µL
MHCII-BV421	BV421 / V450	1:400	5 µL
FACS Buffer			Filled up to 2 mL*

Table 5. FACS-staining solution ingredients for 40 samples.

*Each sample was stained using 50 µL of the extracellular staining solution.

2.12 Apoptosis Assay

After harvesting the cells from the cell culture, extracellular staining was performed at 4°C for 15 minutes, as shown in Table 6. After the staining, samples were washed with ice-cold PBS and centrifuged cold in 250 g for 6 minutes. Annexin V binding buffer (10x) was diluted 1:10 with distilled water. The cell pellet was again washed with 500 µL of Annexin binding buffer (1x) and centrifuged again at 250 g for 6 minutes. 40 µL of 1:100 Annexin V-Annexin V binding buffer solution was given to the cell pellet. The cells were incubated for 15 minutes at room temperature in the dark. After the incubation, 2 mL of Annexin binding buffer (1x) was given to the cells, and the cells were centrifuged at 250 g for 6 minutes. The supernatant was discarded, and the cells were resuspended in 100 µL of Annexin binding buffer. 1:100 of 7-AAD dye was added. The samples were acquired by the BD FACS Lyric within 1 hour. Some samples did not receive Annexin V dye, whereas some did not receive the 7-AAD dye as controls (Figure 15A). The gating of the fully stained samples with both dyes is depicted in Figure 15B.

Antibody/Ingredient	Channel (BD FACS Lyric)	Concentra-tion	Amount
CD8-PB	V450	1:400	5 µL
MHCII-BV510	V500	1:400	5 µL
FACS Buffer			Filled up to 2 mL*

Table 6. Staining solution ingredients for apoptosis assay.

*Each sample was stained using 50 µL of the extracellular staining solution. The ingredients on the table are for 40 samples.

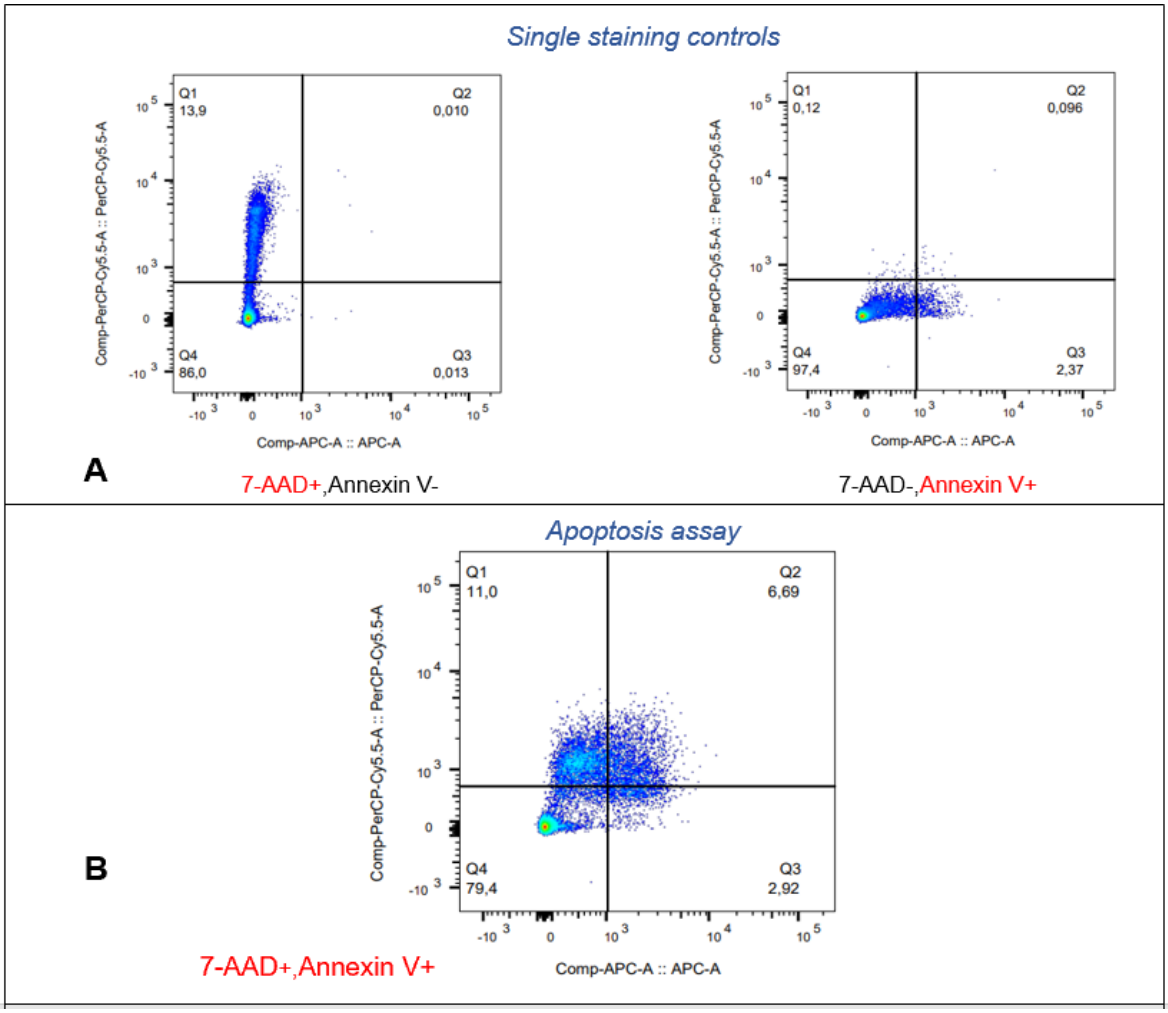

Figure 15. Apoptosis Assay FACS Analysis.

A: Single staining controls were analyzed in BS FACS Lyric. Here, the samples were only stained with 7-AAD on the left side. The graph on the right side shows the samples stained with only Annexin V dye. 7-AAD excites the PerCP-Cy5.5 laser, and Annexin-V excites the APC laser. **B:** An example flow cytometric analysis of a fully stained sample. Q1 represents the cells that are dead, and Q3 shows the cells that are apoptotic but not yet dead. The living cells lie in the Q4 gating. The percentages of each group of cells were depicted on the graph.

2.13 Animal Treatment with Vitamin-K Antagonist

20-week-old female mice were treated with vitamin-K antagonist phenprocoumon to inhibit factor II, VII, IX, and X production. To that end, mice were intraperitoneally injected with 2 mg/kg phenprocoumon for three consecutive days. The injection concentration was determined by treating mice with phenprocoumon varying from 0,2 mg/kg to 2,5 mg/kg. To validate that vitamin-K antagonist treatment worked, we performed tail bleeding assays, and INR was measured.

2.13.1 Measuring INR

Mice were treated with DMSO or various concentrations of phenprocoumon every 24 hours for 3 days. On day 4, 24 hours after the last injection, control and treated mice were anesthetized with 100 mg/kg Ketamine and 10 mg/kg Xylazine. Approximately 500 µL of blood was drawn from the orbital sinus with small capillary tubes. INR values were measured in the central laboratory of the University of Mainz.

2.13.2 Tail bleeding assay

After drawing blood, anesthetized mice were placed on an electrical heating pad in the prone position. The tip of the tail (approximately 1 mm) was clipped with a scalpel and allowed to drop from the pad about 2-3 cm. The stop-clock was started, and the blood drop on the tip of the tail was wiped with the filter paper every 20 seconds to avoid clotting. This was done for a maximum of 30 minutes to avoid death by bleeding. Counting the blood drops on the filter paper allowed us to measure the bleeding time differences between treated and non-treated mice. The mice were then sacrificed with an anesthetic overdose.

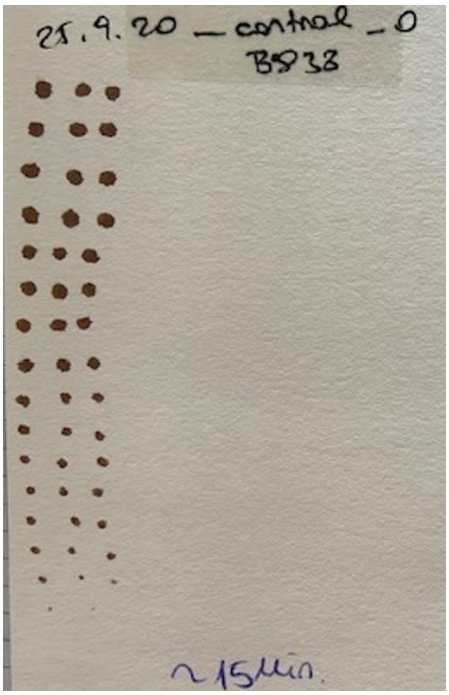

Figure 16. Tail bleeding assay with filter paper.

The blood drop on the tip of the tail was wiped with the filter paper every 20 seconds. As a result, every three blood drops are equal to a minute. Here is an example of a DMSO-treated control mouse for three consecutive days. It takes 10-15 minutes until the blood clots.

2.13.3 Intraperitoneal phenprocoumon treatment

The suitable injection concentration was determined to be 2 mg/kg with the use of INR measurements and tail bleeding assays. Phenprocoumon was available in a 2 mg/ml stock solution solved in DMSO. It was assumed that the mice had 30 grams of body weight. Each mouse received 0,06 mg of phenprocoumon, equaling 30 µL of the stock solution. In order to make the injections easier and tolerable for the mice, the injection solution was filled up to 100 µL with PBS. Control groups also received 100 µL of injection solution containing 30 µL of DMSO solved in PBS. On the fourth day, mice were sacrificed, and their cells were used for the experiments as described above (see 2.6-2.10).

3. Results

Among its numerous functions, thrombin is also considered to be the key regulator linking inflammation and hemostasis (48,58,78,83,85,99,100). Previous findings in our lab using a novel mouse model D-Insight, have shown that thrombin is not only expressed in the liver but also in other tissues, including $CD4^+$ and $CD8^+$ T-cells (83). T-cell activation and expansion in LCMV (lymphocytic choriomeningitis virus) infected mice and LPS-induced sepsis models resulted in the upregulation of prothrombin in lymphatic organs. These findings pose the question of whether extrahepatic prothrombin has a potential role in inflammation and how T-cell-derived prothrombin promotes T-cell proliferation. To this end, we investigated the possible intra-and extracellular effects of the T-cell-derived prothrombin on $CD8^+$ T-cell proliferation and survival. We made use of different approaches in a serum-free environment in this work (Figure 17).

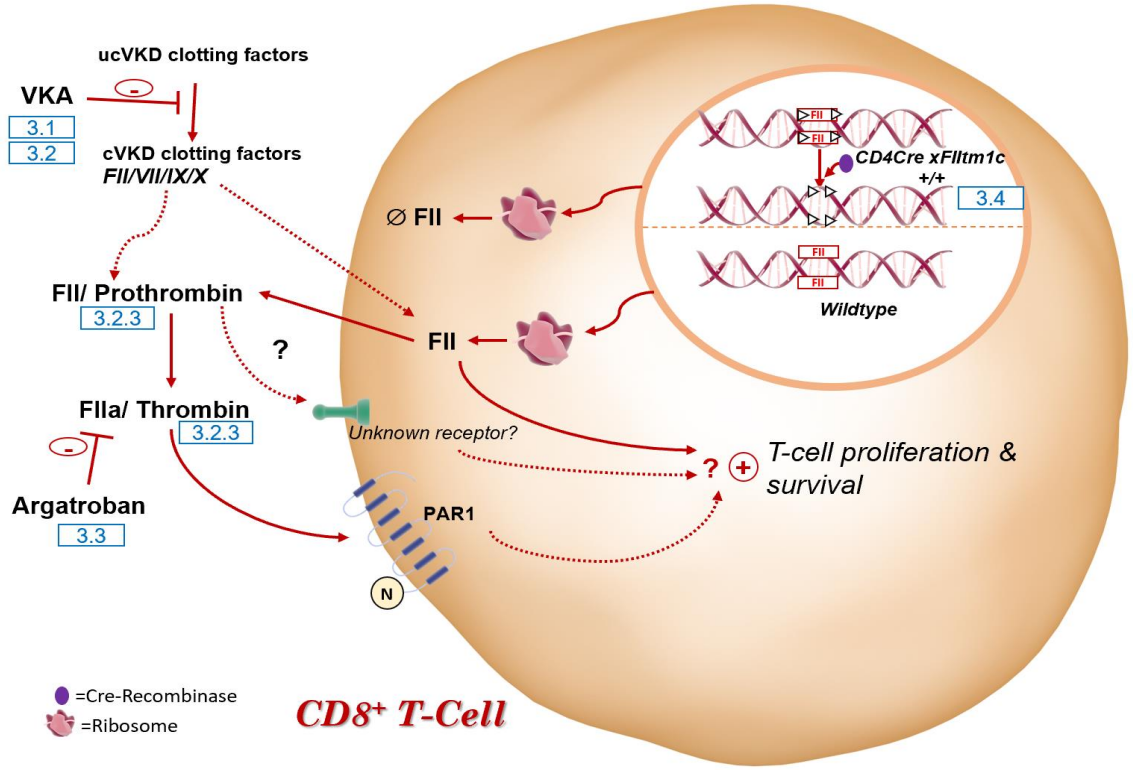

Figure 17: Schematic illustration of our approaches in this work shown on a $CD8^+$ T-cell.

T-cell-derived FII effects on $CD8^+$ T-cells were investigated using several genetic and pharmaceutic approaches. Each experimental setting was numbered according to the sections in this work. For instance, the results of the argatroban experiments can be found in section 3.3. For a better visualization and understanding of the CD4Cre mice and wildtype mice comparison, the nucleus was divided in half. Homozygous CD4CrexF2tm1c mice cannot produce FII because of the genetic deletion in their T-cells.

I performed loss and gain of function experiments. Experiments in which the T-cells were deficient in functional FII (either by genetic deletion or pharmacological inhibition, Table 7, Figure 17) were considered “loss of function” experiments, as we aimed to analyze the changes of T-cell proliferation and survival in the absence of functional FII. We also examined how the addition of recombinant FII/FIIa changes the T-cell behavior, and these experiments are summarized as “gain of function” experiments in Table 7.

“Loss of function”			**“Gain of function”**
VKA *(3.1, 3.2)*	***Argatroban*** *(3.3)*	***CD4Cre x FIItm1c*** *(3.4)*	***Rescue*** *(3.2.3)*
Reduces FII, FVII, FIX, FX	*Antagonizes FIIa*	Genetic deletion of F*II*	*Addition of recombinant FII, FIIa*

Table 7: Overview of experimental settings used in this thesis.

In “loss of function” experiments, FII/FIIa was reduced, inhibited, and deleted by pharmacological and genetic approaches as indicated. In “gain of function experiments,” recombinant FII and FIIa were added to T-cells of anticoagulated, and therefore FII deficient mice to observe if the FII and FIIa addition to T-cells can recover/rescue the initial phenotype.

3.1 In vitro Vitamin-K Antagonist Treatment of CD8$^+$ T-cells

Vitamin-K antagonist phenprocoumon inhibits the production of vitamin-K-dependent coagulation factors X, IX, VII, and II (prothrombin). In order to investigate whether the inhibition of FII generation makes a difference in CD8$^+$ T-cell proliferation patterns and survival, CD8$^+$ T-cells were stimulated with anti-CD3/anti-CD28 for 2 days. 1 µg/ml phenprocoumon was added to the CD8$^+$ T-cell cultures in the treatment group, and as phenprocoumon was solved in DMSO, the control group was treated with DMSO. Both DMSO and phenprocoumon additions were conducted on days 0, 2, and 4 of the cell culture. The cells were acquired on days 3 and 5. Flow cytometry analyzes were done as mentioned above in 2.6 and 2.11.

Phenprocoumon-treated and DMSO-treated (control group) CD8$^+$ T-cells´ proliferation indices were compared after 3 and 5 days of cell culture, as depicted in Figure 18. After 3 days of culture, the control group and the phenprocoumon-treated groups had similar proliferation indices on average (1.08 and 1.07, respectively), and the difference

was not significant (p=0.5). After 5 days of culture, the control group had a significantly higher average proliferation index than the phenprocoumon-treated group (1.21 and 1.16, respectively p=0.01).

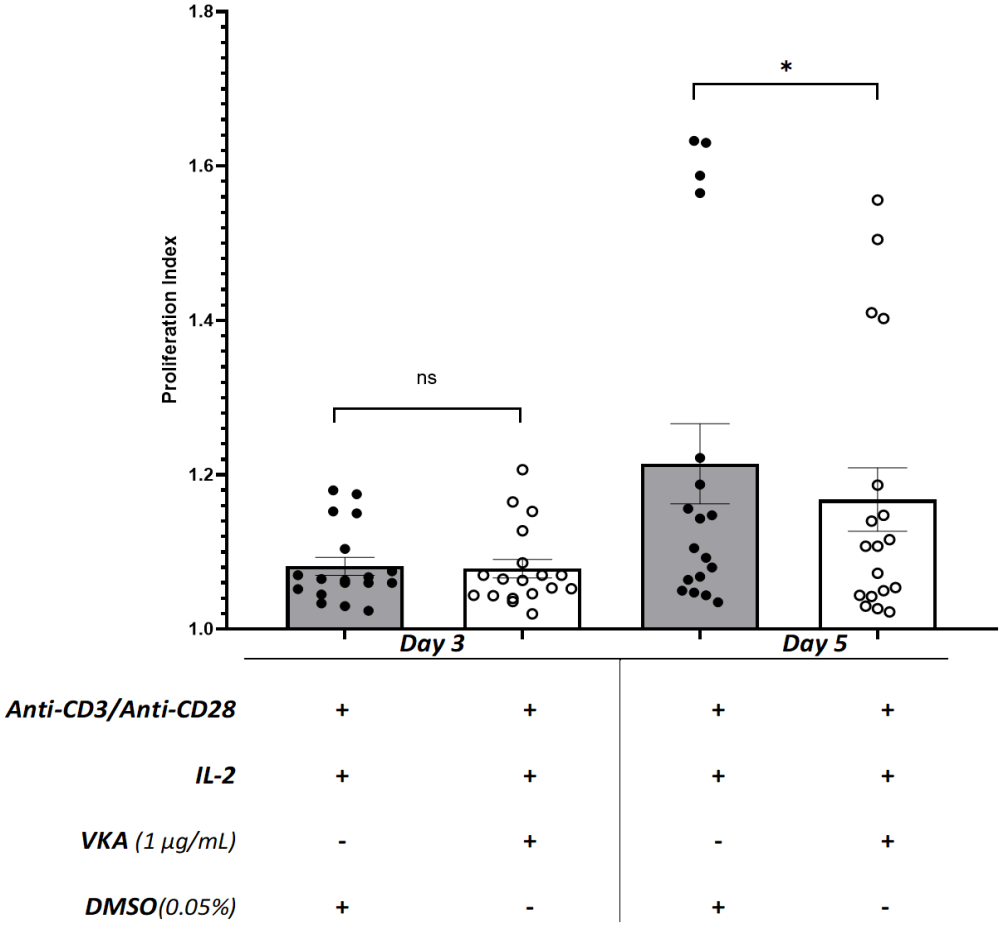

Figure 18: In vitro phenprocoumon treatment leads to a significantly lower proliferation in CD8⁺ T-cells after 5 days.

CD8⁺ T-cells were stimulated with anti-CD3/anti-CD28 and IL-2 and cultivated for 5 days. Each spleen was divided in half. One half was treated with DMSO (control group), and the other half (experiment group) was treated with phenprocoumon in the culture. Each point on the graphs represents the average proliferation index of a mouse. A two-tailed paired t-test was used for the analysis. The bars on the graph represent the standard error of means. N=18. On day 3, the proliferation indices were not significantly different (p=0.552), whereas they were significantly different (p=0.013) on day 5.

Phenprocoumon treatment of the CD8⁺ T-cells did not make a difference in the proliferation index compared to the control group after 3 days of treatment. As mentioned before, the proliferation index shows the average number of divisions of the responding cells which have undergone at least one division. However, after 5 days of Vitamin-K antagonist phenprocoumon treatment, CD8⁺ T-cells in the control group had significantly higher proliferation indices than the treatment group. The seemingly unequivocal

outcome, however, may be attributed to a biological reason that the vitamin-K antagonists usually need up to 5 or more days to show full antithrombotic effects (30). This shows us that the vitamin-K antagonist treatment, which depletes the prothrombin (FII) along with other coagulation factors (factors VII, IX, X), causes a diminution in the average number of divisions of responding $CD8^+$ T-cells and hence, reduces the proliferative capacity.

Figure 19 depicts the survival rates of the same $CD8^+$ T-cells after 3 and 5 days. After 3 days of culture, the control group had significantly higher survival rates of $CD8^+$ T-cells in the culture than the phenprocoumon treated group ($p=0.03$; 31.2% and 28.9%, respectively). After 5 days of culture, the control group had slightly higher survival rates than the phenprocoumon-treated group, but this difference was not significant ($p=0.4$; 14% and 12,7%, respectively). Analyzing the surviving $CD8^+$ T-cell percentages, we concluded that the diminution of the vitamin-K-dependent factors, including prothrombin, causes a significant decrease in survival rates. Even though the differences were not significant after 5 days of culture, this might be the result of the harsh serum-free cell culture conditions, resulting in mass death of T-cells regardless of any treatment.

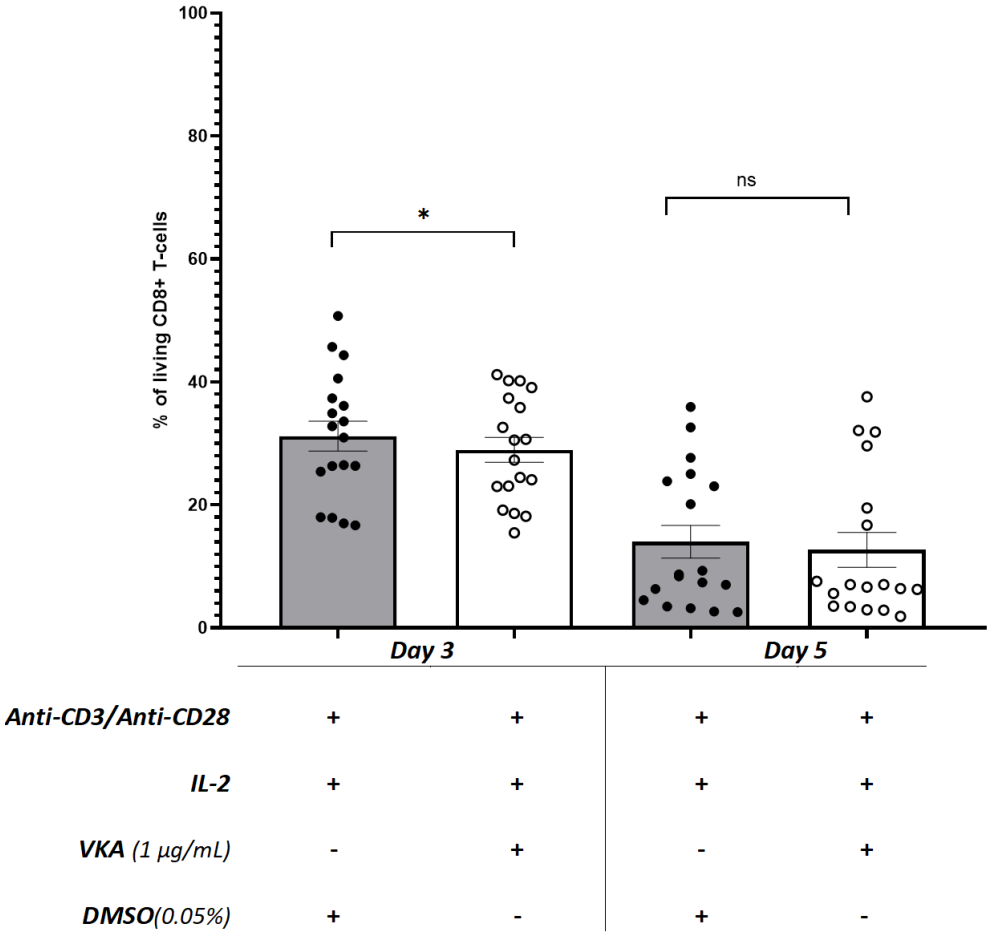

Figure 19: Phenprocoumon treatment of the CD8⁺ T-cells results in lower survival rates.

The same CD8⁺ T-cells as in Figure 18 were analyzed for their survival rates after 3 and 5 days of culture. Each dot represents the average value obtained from a mouse. A two-tailed paired t-test was utilized for the analysis. The standard error of means is shown with error bars. On day 3, the control group had a significantly higher survival rate than the phenprocoumon-treated group. The p-value on day 3 was 0.034 and 0.441 on day 5.

In conclusion, in vitro treatment of the CD8⁺ T-cell cultures with a vitamin-K antagonist for 5 days resulted in a significant decrease in the proliferative cell capacity. The survival rates have also diminished. The effects on the proliferative capacity were only seen after 5 days of treatment (Figure 18), whereas the decrease in the surviving cells was already significant after 3 days of the cell culture (Figure 19).

Even though we observed indications that VKA impairs the proliferation and survival of the CD8⁺ T-cells, retarded functional kinetics of VKA action may account for the somewhat indifferent and seemingly discrepant findings. Therefore, we considered pretreating the animals with VKA before T-cell extraction. This approach may help to generate more robust insights for both proliferation and survival as it allows for minimizing the duration of the T-cell incubation with VKAs in vitro, which appear to be toxic to the cells

in longer incubation setups. Taking this into account, mice were pretreated with phenprocoumon in the next experiments in order to investigate the possible effects of a longer period of anticoagulation on the $CD8^+$ T-cells.

3.2 $CD8^+$ T-cell Proliferation and Survival in Anticoagulated Mice and Rescue Experiments

3.2.1 Anticoagulation of the Mice

In the previous experiments, we showed that in vitro phenprocoumon treatment resulted in a diminution in the proliferation and survival of the $CD8^+$ T-cells. In the next step, we anticoagulated mice systemically by intraperitoneal phenprocoumon injections every 24 hours for a total of 72 hours. We then performed tail bleeding assays (TBA) (2.13.2) and analyzed blood samples for INR values (2.13.1) to validate the effectiveness of the vitamin-K anticoagulation of the mice.

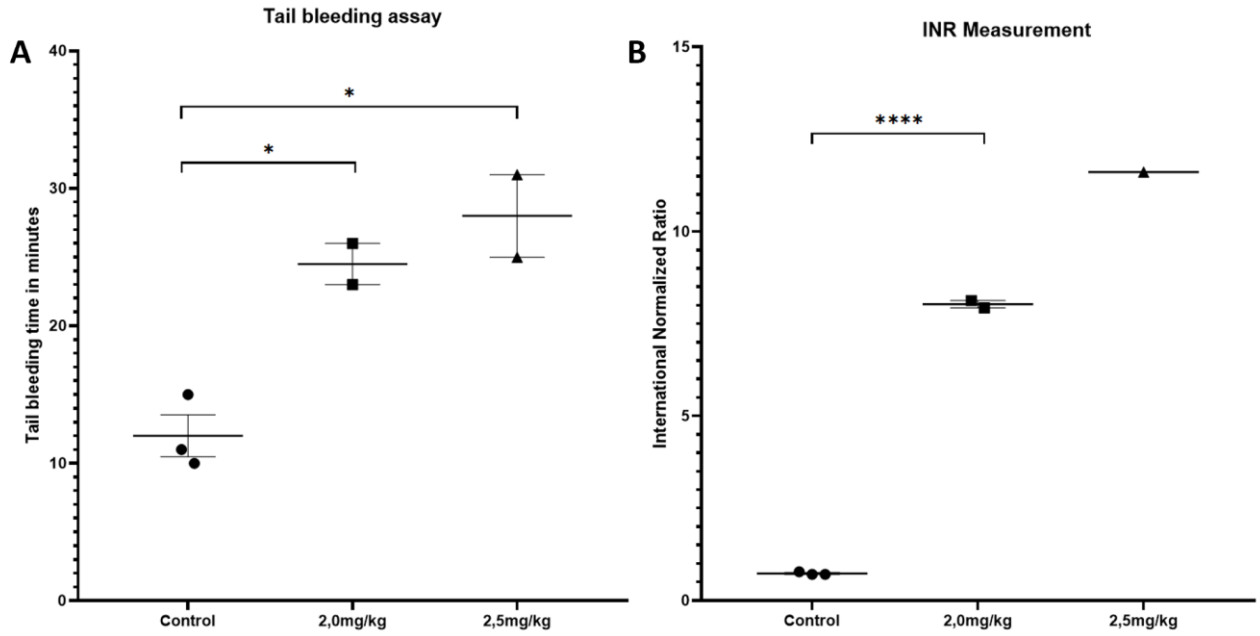

Figure 20: Successful anticoagulation of mice by intraperitoneal phenprocoumon treatment.

Mice were injected i.p. with different concentrations of phenprocoumon for 72 hours. Control mice received only DMSO solved in PBS, whereas treatment groups received two different concentrations of phenprocoumon solved in PBS. n=7. Each point on the graph represents a mouse, and error bars are shown on both graphs. **Figure 20A** shows the results of TBA performed after 72 hours of treatment. Phenprocoumon-treated mice had significantly longer TBT than the control group. A two-tailed unpaired t-test was performed. P-values for the mice who received 2,0 mg/kg and 2,5 mg/kg were respectively 0.011 and 0.012. **Figure 20B** shows the INR-values of the same mice as in Figure 20A. 2 mg/kg phenprocoumon-treated mice had significantly higher INR-values than the control group ($p<0.0001$).

Mice were treated with increasing amounts of vitamin-K antagonist phenprocoumon to test the dose-dependent prolongation of the tail bleeding and the INR (prothrombin time). As depicted in Figure 20, TBT and INR of the control mice were compared to the 2,0 mg/kg and 2,5 mg/kg phenprocoumon-treated mice.

As expected, the pretreatment of mice with increasing amounts of phenprocoumon resulted in a significant dose-dependent prolongation of the bleeding time and the prothrombin time. The average tail bleeding time of the control groups was 12 minutes, whereas the 2 mg/kg and 2,5 mg/kg groups had an average of 24.5 and 28 minutes, respectively ($p=0.01$ for both groups) (Figure 20A). INR is a standardized value for patients using anticoagulants, and it is based on a calculation of the prothrombin time of a standardized thromboplastin reagent. Although it is widely used for human samples, it provided us guidance in comparing the effectiveness of phenprocoumon treatment in mice. The average INR value of the control group was below 1, while 2 mg/kg and 2,5 mg/kg phenprocoumon treated groups had an average of 8 and 11.6, respectively, showing that our phenprocoumon treatment was significantly prolonging the clotting time ($p<0.0001$) (Figure 20B).

Considering the significant changes in INR values and tail bleeding times (TBT) of the phenprocoumon-treated mice, we could confirm that the phenprocoumon treatment succeeded. The mice were sacrificed and autopsied after the final measurements. The mice, who received higher concentrations of phenprocoumon were more prone to bleeding and hemorrhagic complications. Some of them even had minor external injuries, which were bleeding lightly before TBA and INR measurements.

Given that 2 mg/kg phenprocoumon for 3 days was sufficient for the anticoagulation (Figure 20) and the injections in a higher concentration posed a greater risk of internal and external bleeding in the mice, we proceeded with 2 mg/kg intraperitoneal injections in 24-hour intervals in a total of 72 hours for the next experiments.

3.2.2 Proliferation and Survival Patterns in $CD8^+$ T-cells of Anticoagulated Mice in Comparison to the Control Mice

In the previous chapters, we showed that impairing the production of vitamin-K-dependent factors, including prothrombin resulted in a diminution of proliferative capacity and survival. However, the full impact of phenprocoumon can be seen after up to 5 days after the beginning of the treatment, and T-cells cannot endure the cell culture conditions longer than 5 days. Therefore, we used the $CD8^+$ T-cells of priorly anticoagulated mice (see 3.2.1) and DMSO-treated mice as controls, and we continued phenprocoumon (1 µg/ml) and DMSO (0.05%) treatment on the days 0, 2 and 4 in the cultures. The mice were female and age-matched. The cells were acquired on days 3 and 5 of the culture and analyzed with BD FACS Lyric. Flow cytometry analyzes were done as mentioned above in 2.6 and 2.11. The rationale behind this is to start the reduction of vitamin-K-dependent factors before the cell culture time; so that the majority of remaining vitamin-K-dependent factors are used up when the T-cells are taken into the cell culture. This allows us to continue the treatment for a longer time and to observe if this causes further changes in T-cell survival and proliferative behavior.

In Figure 21, phenprocoumon treated and control group $CD8^+$ T-cells´ proliferation indices were compared after 3 and 5 days of cell culture. After 3 days of culture, the phenprocoumon-treated group had a significantly lower proliferation index on average than the control group (respectively 1.13 and 1.18, $p=0.01$). After 5 days of culture, the phenprocoumon-treated group had again a significantly lower average proliferation index than the control group (1.39 and 1.52 respectively, $p=0.03$).

The pretreatment of the mice with phenprocoumon leads to a stronger reduction of vitamin-K-dependent factors and a lower proliferative capacity in $CD8^+$ T-cells. Contrary to the sole in vitro treatment of the cells (see 3.1), proliferation indices of both groups were significantly different in this experimental setting with pre-treated mice as early as on the 3rd day of the cell culture. This shows us that the prior anticoagulation was successful and stronger depletion of the vitamin-K-dependent factors leads to a stronger diminution in the proliferative capacity of the $CD8^+$ T-cells.

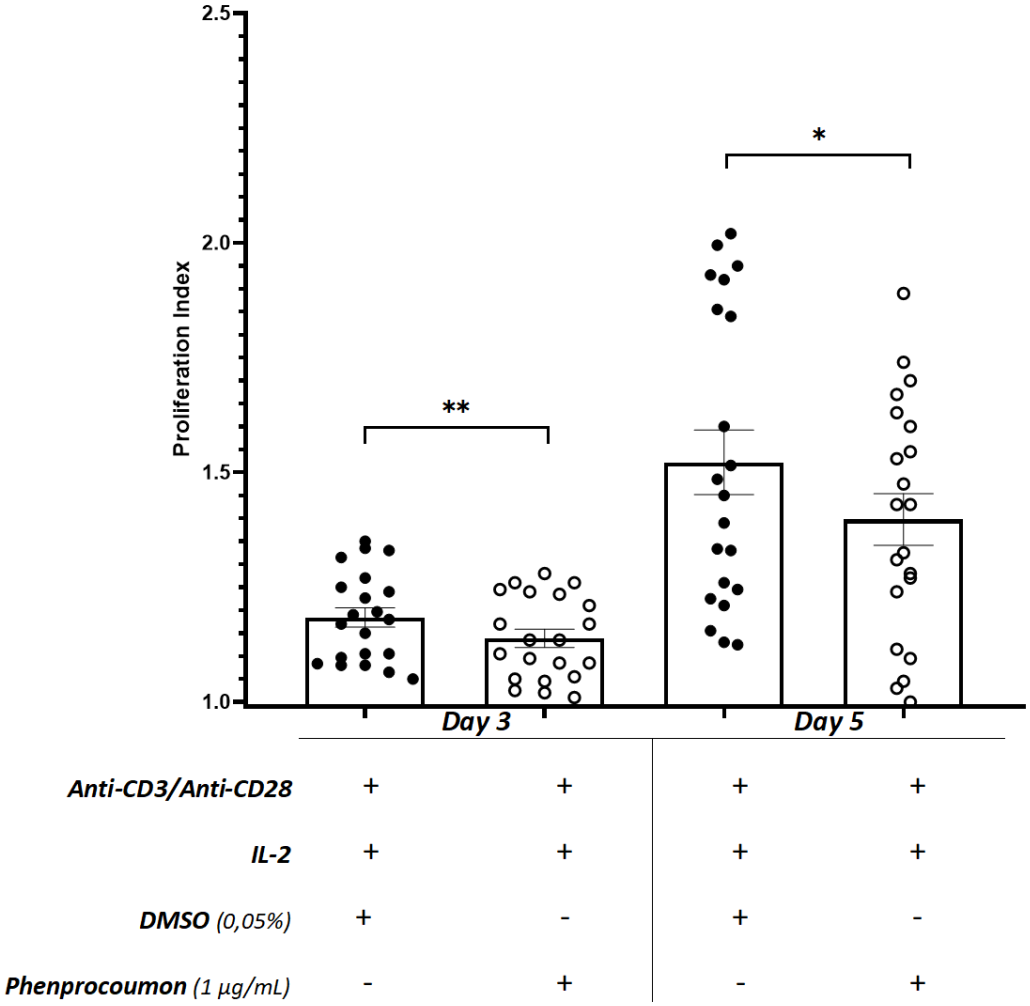

Figure 21: CD8⁺ T-cells of phenprocoumon treated mice show significantly lower proliferation.

Extracted CD8⁺ T-cells of VKA-treated mice and control mice were cultivated for 5 days. On day 3, the control group had significantly higher proliferation indices than the treatment group. N=21. A two-tailed paired t-test was performed, and the p-value=0.011. On day 5, again, the control group had a significantly higher proliferation index than the treatment group. N=21. The P-value of a two-tailed paired t-test was 0.034.

Next, same CD8⁺ T-cells from phenprocoumon and control groups were analyzed for survival rates, as shown in Figure 22. The surviving fraction of the CD8⁺ T-cells were compared after 3 and 5 days of cell culture. After 3 days of culture, the phenprocoumon-treated group had similar survival rates on average as the control group (51.2% and 52% respectively, p>0.05). Again, after 5 days of culture, the phenprocoumon-treated group showed diminished survival rates compared to the control group with a borderline significance (30.3% and 37.7%, respectively p=0.08).

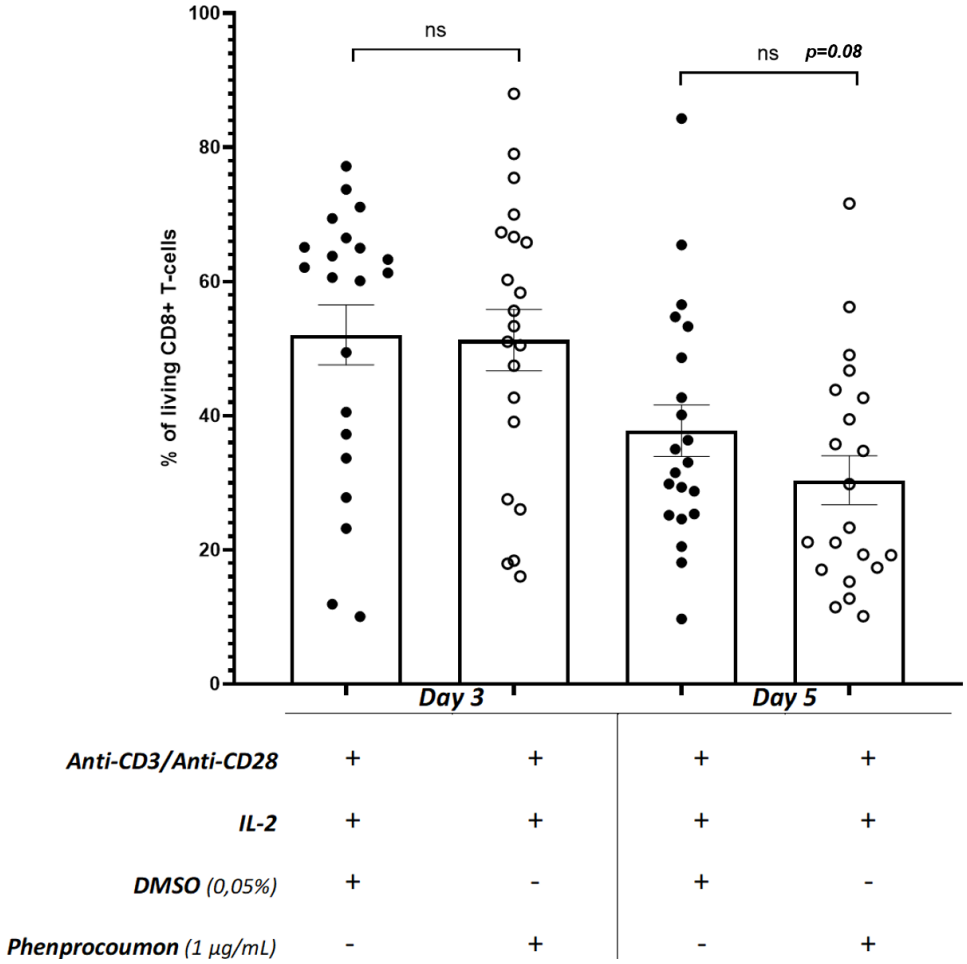

Figure 22: CD8⁺ T-cells of phenprocoumon treated mice show a decreased survival with borderline significance.

Phenprocoumon-treated mice and control mice were compared for survival rates after 3 and 5 days in the cell culture. As expected, survival rates decreased during the cell culture. N=21 and a two-tailed paired t-test were utilized on both days. On day 3, the p-value was 0.8886. On day 5, the average survival rate of the control group was higher than the treated group. However, P-value was borderline significant with 0.0822. The bars on the graphs show the standard error of means.

Altogether, we observed that anticoagulated mice, who had a shortage of vitamin-K-dependent clotting factors, had significantly lower T-cell proliferation indices. Although the survival rates of the non-coagulated mice were higher on average, this difference was borderline significant. These results correlate with the previous experiments in chapter 3.1 and show that inhibiting the vitamin-K-dependent clotting factors results in a significant reduction of proliferative capacity and a trend toward a reduced survival of $CD8^+$ T-cells.

3.2.3 The Effects of Purified Thrombin and Prothrombin on $CD8^+$ T-cell Proliferation by Anticoagulated Mice

After we explored the effects of the vitamin-K-dependent clotting factor depletion (including FII) on the $CD8^+$ T-cell proliferation and survival, we investigated if the addition of prothrombin (FII) and thrombin (FIIa) to the culture of T-cells obtained from anticoagulated mice could reverse those effects. As phenprocoumon inhibits the biogenesis and the secretion of the vitamin-K-dependent factors by the cells, the concentration of the added recombinant thrombin and prothrombin to the cell culture is not affected by the anticoagulation. Provided prothrombin and thrombin have a functional effect on T-cells, this is expected to “rescue” the proliferative capacity and the survival of phenprocoumon-treated $CD8^+$ T-cells.

In the rescue experiments, mice were first anticoagulated with phenprocoumon as described in sections 3.2.1 and 3.2.2, and $CD8^+$ T-cells were extracted from the spleen. $CD8^+$ T-cells obtained from one animal were divided into 3 groups after the extraction. In all groups, the phenprocoumon treatment was given to the extracted cells on day 0 (the day when cells were taken into the culture) and continued on the 2^{nd} and 4^{th} days of the culture. While the first group received phenprocoumon only, the second and third groups were co-treated with recombinant prothrombin and thrombin in addition to phenprocoumon on days 0, 2^{nd}, and 4^{th} day. Flow cytometric analyses were computed on the third and fifth days with BD FACS Lyric as in section 2.11.

The proliferation indices of prothrombin and thrombin-treated groups were compared to the control groups (Figure 23). The control group, which only received anticoagulation, had an average proliferation index of 1.13, which was not significantly different than the average proliferation index of 1.15 after 3 days ($p>0.05$) in the prothrombin group. After 5 days, the averages of the control and prothrombin groups were still not significantly different (1.39 and 1.44, respectively $p>0.05$). In contrast, the addition of the active enzyme thrombin showed a significant difference compared to the control groups on the 3^{rd} and 5^{th} days. On the 3^{rd} day, the control group and thrombin group had average proliferation indices of 1.13 and 1.22, respectively ($p<0.0001$), and on the 5^{th} day, the average proliferation indices were 1.39 and 1.55, respectively ($p<0.0001$).

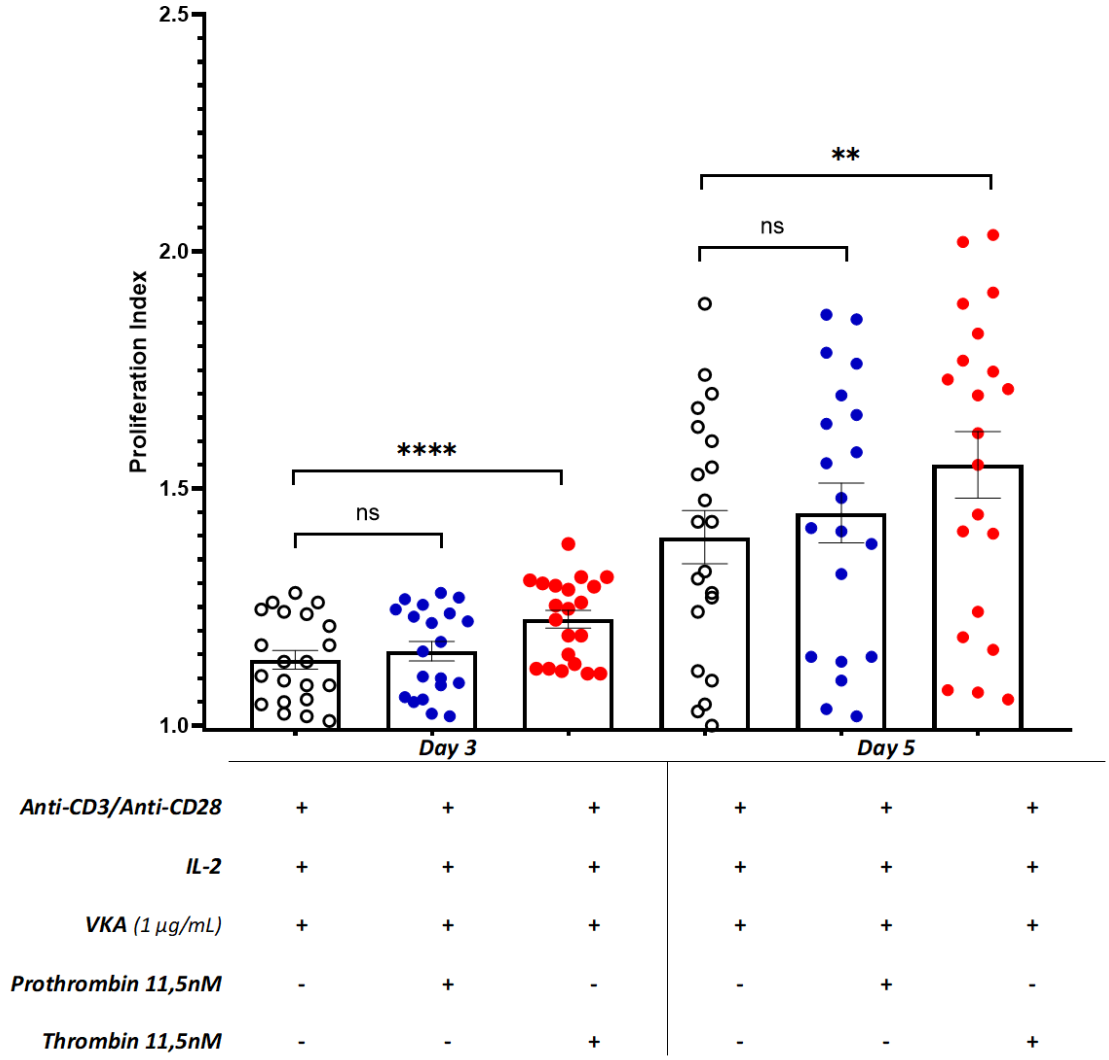

Figure 23: Recombinant thrombin rescues cell proliferation in phenprocoumon treated CD8⁺ T-cells.

Phenprocoumon-treated CD8⁺ T-cells were treated with prothrombin and thrombin and cultured for 5 days. Each dot represents the average value obtained from a mouse. Empty circles show the phenprocoumon-only group, blue dots represent the prothrombin group, and red dots stand for the thrombin group. Two-tailed paired t-tests were performed. The bars on the graphs show the standard error of means. Prothrombin addition did not rescue the CD8⁺ T-cell proliferation; therefore, the proliferation indices were not significantly different than the first group. N=20 and p-values were 0.38 on day 3 and 0.14 on day 5. Thrombin rescued the proliferation; the proliferation indices of the thrombin-treated group were significantly higher than the phenprocoumon-only group on both days. N=21 and p-values were <0.0001 on days 3 and 5.

This implies that the inactive form, prothrombin, is not able to induce CD8⁺ T-cell proliferation. In contrast, the addition of the active enzyme thrombin “rescues” the hypoproliferative phenotype of phenprocoumon-treated CD8⁺ T-cells despite lacking the endogenous vitamin-K dependent coagulation factors, including prothrombin. This suggests that thrombin is an important factor in maintaining and restoring the proliferative capacity of the CD8⁺ T-cells.

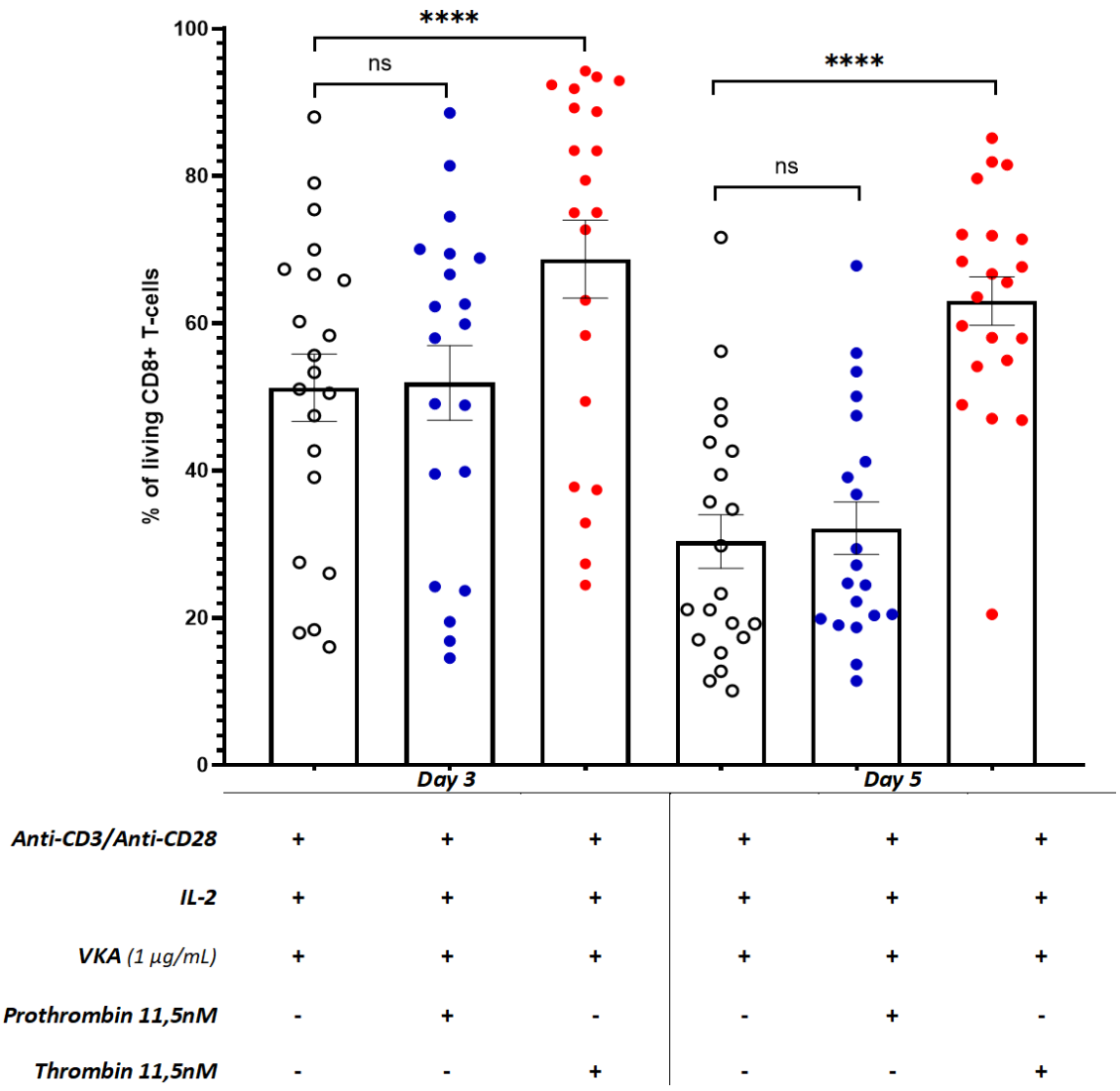

Figure 24: Recombinant thrombin increases the survival of phenprocoumon treated CD8⁺ T-cells significantly.

These data points represent the same mice as in Figure 23. Each dot represents the average value obtained from a mouse. Empty circles show the phenprocoumon-only group, blue represents the prothrombin group, and red stands for the thrombin group. Two-tailed paired t-tests were performed. The bars on the graphs show the standard error of means. Prothrombin did not improve survival rates significantly on both days. P values for days 3 and 5 were respectively 0.8116 and 0.6636. On the contrary, thrombin increased the survival rates of the $CD8^+$ T-cells significantly on both days compared to the phenprocoumon-only group. P values on days 3 and 5 were both <0.0001.

Next, we analyzed the cell survival of the $CD8^+$ T-cells in the identical experimental setup (Figure 24). Here, we observed that the prothrombin does not rescue cell survival of phenprocoumon-treated $CD8^+$ T-cells. The control group, which received phenprocoumon only had an average survival rate of 51.2%, which was not significantly different compared to the average survival rate of 51.9% after 3 days ($p>0.05$) in the prothrombin-treated group. Similar results were obtained for the experimental course over 5 days (30.3% and 32.1% respectively, $p>0.05$).

In contrast, thrombin addition led to a significant improvement in the cell survival of T-cells during 5 days of the cell culture and rescued the cells successfully. This change was significant as early as the 3rd day of the cell culture. After 3 days, the thrombin group had 68.7% living $CD8^+$ T-cells on average, whereas the control group had only 51.2% of the cells alive ($p<0.0001$). On the 5th day, the control group had an average of 30.3%, and the thrombin group still had an average of 63% living $CD8^+$ T-cells ($p<0.0001$). Apart from the significant difference between the thrombin group when compared the control group, it is also remarkable that the control and the prothrombin groups had an approximate cell loss of 20%, whereas the thrombin-treated group had only a 5% loss between the 3rd and the 5th day of the cell culture. This suggests an important role for thrombin in the survival and maintenance of $CD8^+$ T-cells.

In this section, we showed that the $CD8^+$ T-cells from the mice who received vitamin-K antagonists went through fewer divisions and had fewer surviving cells during 5 days of cell culture. We also showed that this effect could be reversed when $CD8^+$ T-cells were treated with active thrombin, while the cells that received enzymatically inactive precursor prothrombin did not show any differences in proliferation and survival after the treatment. This, together with the effect seen after treatment of the T-cells with phenprocoumon, suggests that T-cell intrinsic thrombin expression may stimulate the proliferation and survival of T-cells in an autocrine manner. Next, we aimed to corroborate this further by inhibiting thrombin activity with a specific direct thrombin inhibitor.

3.3 Effects of Direct FII Inhibitor Argatroban on $CD8^+$ T-Cell Proliferation and Survival

In the next experiments, we investigated if “loss of thrombin function” would make a difference in the proliferation and survival patterns of the $CD8^+$ T-cells. As the treatment with vitamin-K antagonists inhibits the production of all vitamin-K-dependent coagulation factors (factor II, VII, IX, X) by the cell, it is not possible to identify which factor is responsible for the diminution of proliferative capacity and survival of the cells after treatment with phenprocoumon (see 3.1, 3.2.2). Considering thrombin’s ability to rescue this effect in anticoagulated $CD8^+$ T-cells (see above), we used a direct thrombin inhibitor, argatroban to investigate the effects of thrombin inhibition on T-cell proliferation and survival in further detail.

CD8⁺ T-cells from wild-type mice were divided into 2 groups. Both groups were stimulated with anti-CD3/anti-CD28 for 2 days. Argatroban (25 µg/ml) was added to the CD8⁺ T-cell cultures in the treatment group. As argatroban was solved in DMSO, the control group was treated with DMSO. DMSO and argatroban additions were conducted on days 0, 2, and 4 of the cell culture. The cells were acquired on days 3 and 5 of the culture and analyzed with BD FACS Lyric. Flow cytometry analyses were done as mentioned above in 2.6-2.11.

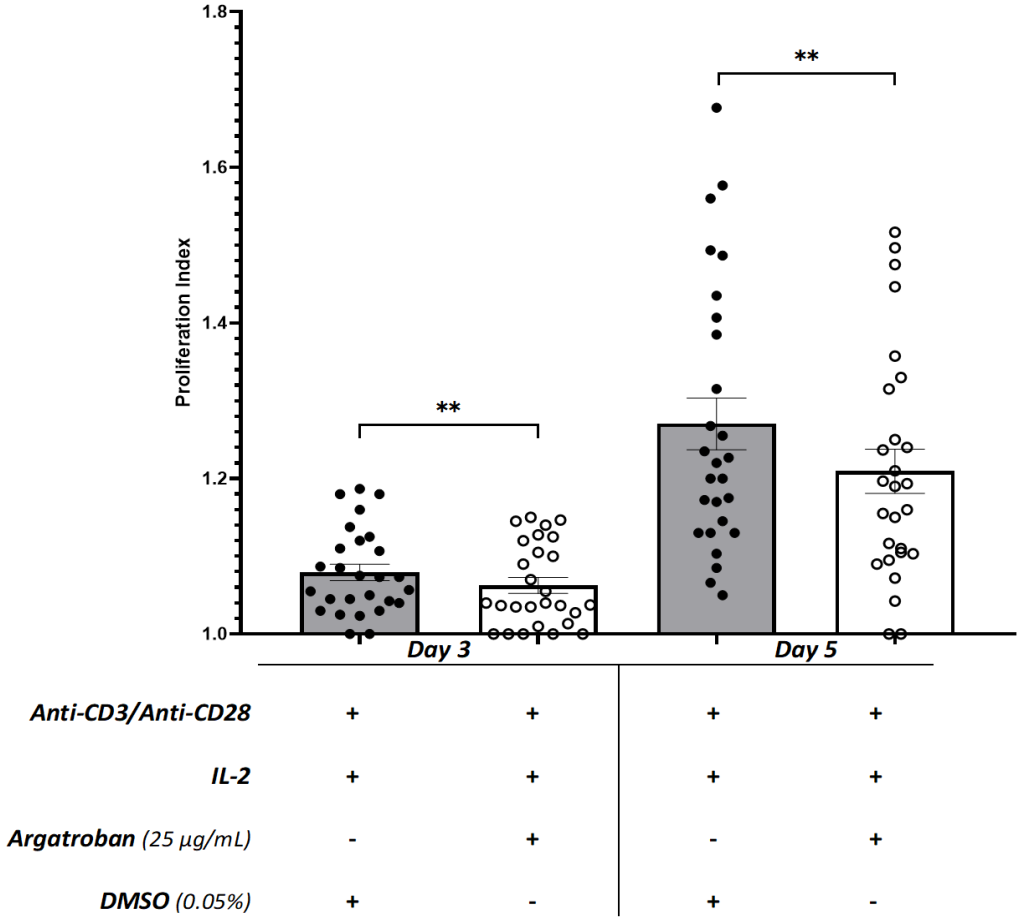

Figure 25: Argatroban treatment results in significantly lower proliferation in CD8⁺ T-cells.

Argatroban-treated CD8⁺ T-cells were compared with DMSO-treated cells of the control group. Both populations were cultured for 5 days. Each dot represents the average value obtained from a mouse. The empty circle shows the Argatroban-treated group, and black represents the control group. Two-tailed paired t-tests were performed. The bars on the graphs show the standard error of means. N=27. On both days the control group had significantly higher proliferation indices than the Argatroban-treated group. On days 3 and 5, p values were respectively 0.0039 and 0.0035.

In Figure 25, the proliferation indices of the argatroban and control groups are depicted. After 3 days of culture, the control group and argatroban-treated groups had slightly, albeit significantly (p=0.003) reduced proliferation indices (1.07 and 1.06, respectively). After 5 days of culture, the control group had a significantly higher average proliferation

index than the argatroban-treated group (1.27 and 1.20 respectively, p=0.003). These results suggest that thrombin inhibition reduces $CD8^+$ T-cell proliferation. We next analyzed the cell survival of $CD8^+$ T-cells after 3 and 5 days (Figure 26). After 3 days of culture, the control group had slightly higher survival rates of $CD8^+$ T-cells compared to the argatroban-treated group, and this was significant (21.7% and 20% respectively; p=0.04). After 5 days of culture, the control group had higher survival rates than the argatroban-treated group (14% and 12.7% respectively, p=0.001).

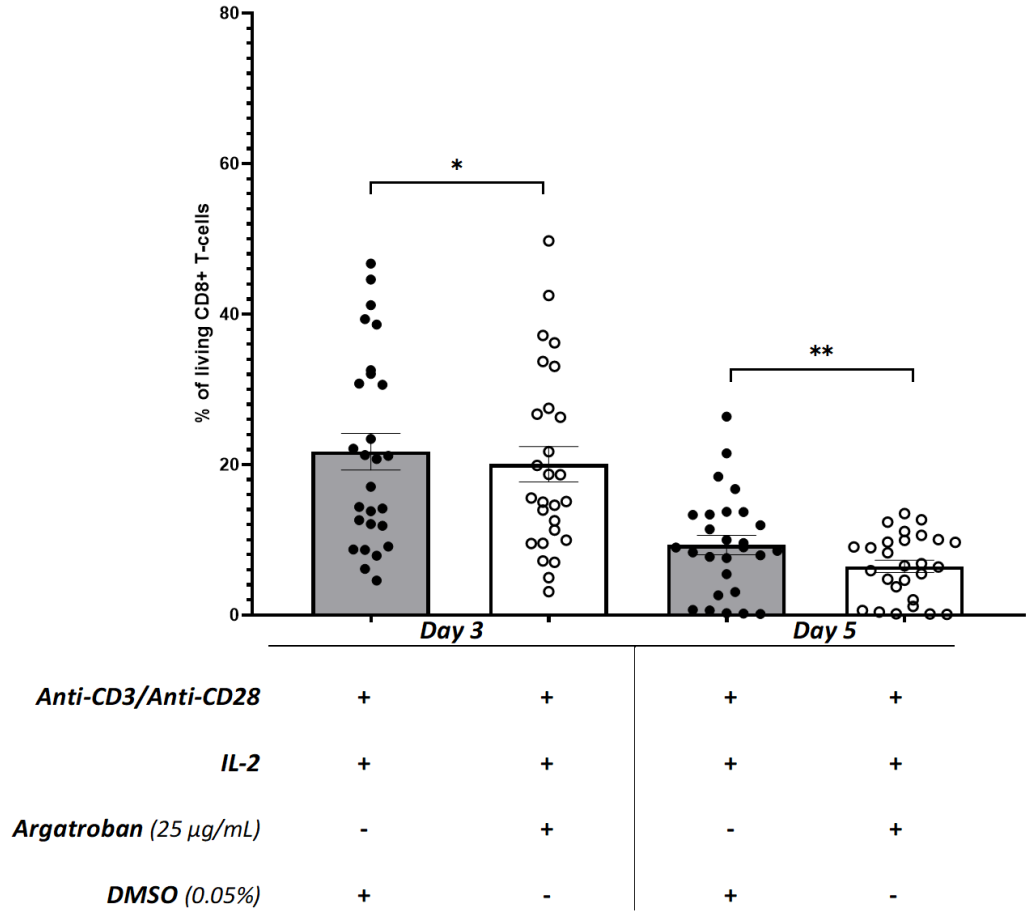

Figure 26: Argatroban-treated $CD8^+$ T-cells show lower survival rates.

Argatroban-treated $CD8^+$ T-cells were compared with DMSO-treated cells of the control group. Both populations were cultured for 5 days. This data represents the same mice as in Figure 25. Each dot represents the average value obtained from a mouse. Empty circles show the argatroban-treated group, and black dots represent the control group. Two-tailed paired t-tests were performed. The bars on the graphs show the standard error of means. N=27. On both days the mice in the control group had significantly higher survival rates than the Argatroban-treated group. On days 3 and 5, p values were 0.0483 and 0.0015, respectively.

Altogether, in line with the preceding observations, the direct thrombin inhibitor argatroban reduced the proliferation and survival of $CD8^+$ T-cells in the cell culture. Next, we aimed to investigate the outcomes after the genetic deletion of thrombin (see 3.4).

3.4 A Novel FII Knockout Mice Model and the Effects of T-cell Derived FII Deficiency

3.4.1 $CD8^+$ T-Cell Proliferation and Survival Patterns in T-Cell Derived FII Knockout Mice

The above-described functional data based on pharmaceutical compounds and in re-complementation assays with recombinant (pro)thrombin suggest FII as an important actor in $CD8^+$ T-cell survival and proliferation. To further corroborate the effect of FII on T-cell function, a new mouse model lacking FII in T-cells was created. For this purpose, a T-cell specific “cre” expressing mouse line (CD4Cre) was crossed with a line that contains a floxed F2 allele (F2tm1c), and the effects of the lack of $CD8^+$ T-cell derived FII on proliferation and survival were observed in vitro (details in 2.5.1). $CD8^+$ T-cells from age-matched sibling mice that did not express the cre-recombinase gene served as controls. The cells were acquired on days 3 and 5 of the culture and analyzed with BD FACS Lyric. Flow cytometry analyzes were done as mentioned above in 2.6-2.11.

The proliferation indices of the prothrombin lacking $CD8^+$ T-cells compared to the wild-type mice are depicted in Figure 27. After three days of the cell culture, the $CD8^+$ T-cells from the wildtype mice had significantly higher proliferation indices compared to prothrombin lacking $CD8^+$ T-cells from CD4Cre mice (1.18 and 1.10, $p=0.007$). This difference remained significant also after day 5, where $CD8^+$ T-cells from wildtype mice showed significantly higher proliferation rates than those obtained from CD4Cre mice (1.61 and 1.41 on average respectively, $p=0.02$). This suggests that genetic thrombin ablation caused a diminution of the proliferative capacity comparable to drug-induced thrombin depletion or its functional inhibition. Since the $CD8^+$ T-cells cannot produce their own (pro)thrombin, they were not able to divide as much as the wildtype $CD8^+$ T-cells. This finding suggests that these cells require thrombin to efficiently produce new daughter cells in vitro. The same cells were also analyzed for survival rates in vitro to address the question of how genetic ablation of (pro)thrombin affects the viability of the $CD8^+$ T-cells in the culture. In accordance with the preceding findings, $CD8^+$ T-cell

survival from wildtype mice was higher compared to CD4Cre mice. However, this difference had a borderline significance (47.8% and 40.7% respectively, p=0.06, not shown here).

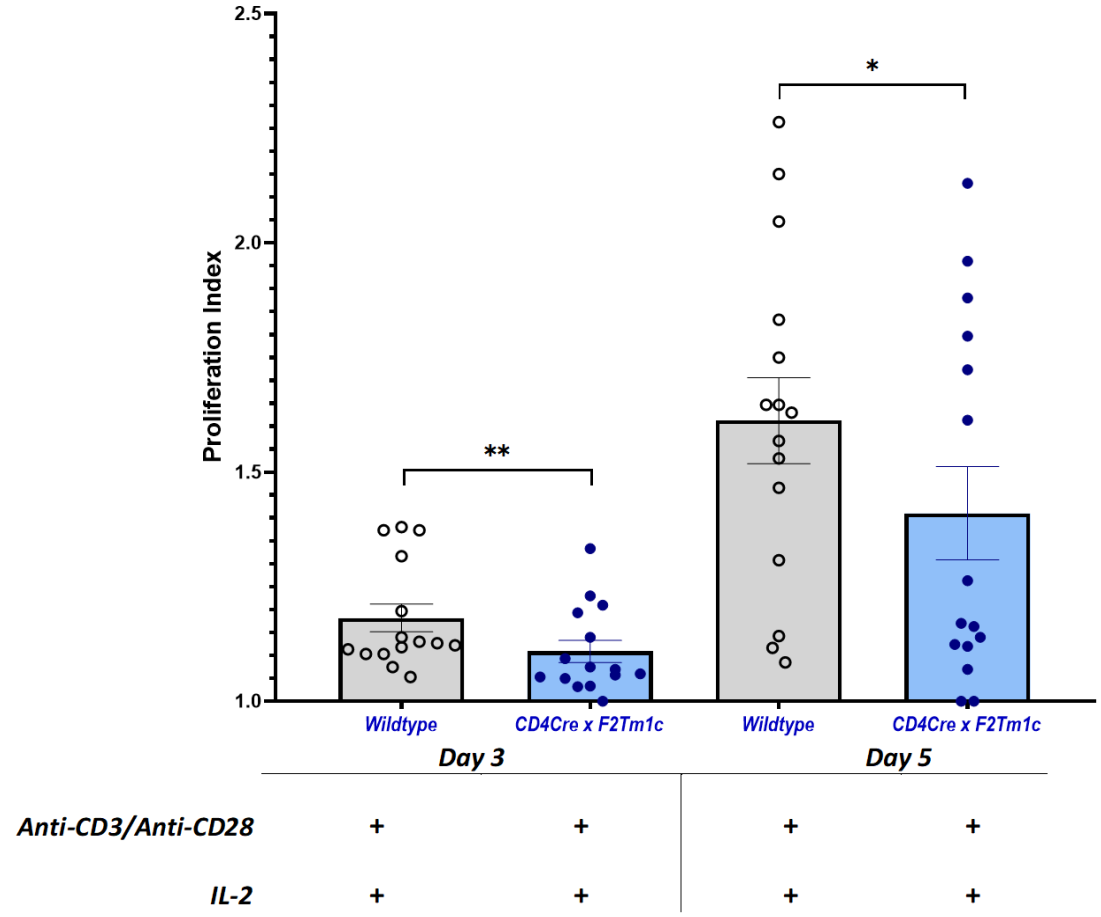

Figure 27: The lack of prothrombin gene in mice leads to a significant reduction of CD8⁺ T-cell proliferation.

Wildtype mice and CD4Cre mice, who lack FII in the CD8⁺ T-cells were analyzed for the proliferation index. Every dot represents the average proliferation index of a mouse. The empty circles represent the data points of the wild-type mice, while the blue dots represent the data from the CD4Cre mice. Two-tailed paired t-tests were performed. The bars on the graphs show the standard error of means. N=15. Wildtype mice had significantly higher proliferation indices on the 3rd and 5th days. The p-values on days 3 and 5 were respectively 0.0077 and 0.0224.

CD8⁺ T-cells obtained from wild-type mice had a slightly higher surviving fraction of cells than those from the prothrombin knock-out mice (35.1% and 28.3%) on day 5 (not shown here). In complementation to these analyses, we finally analyzed the apoptosis rates of CD8⁺ T-cells with and without genetic depletion of prothrombin in these cells.

3.4.2 Apoptosis Assays in CD4Cre Mice

In the previous experiments, we showed that $CD8^+$ T-cells that do not express prothrombin show a lower proliferative capacity as compared to T-cells obtained from wildtype mice and tend to survive for a shorter time in the cell culture conditions. We decided to analyze the apoptosis rates to understand the underlying reasons for the decreased survival rates in mice lacking the prothrombin gene in the T-cells. T-cells from CD4Cre mice, who lack prothrombin in their T-cells were cultured and harvested on the 3rd, 4th, and 5th days. After special staining for apoptosis markers (details in 2.12), $CD8^+$ T-cells were analyzed in BD FACS Lyric (see 2.6-2.11).

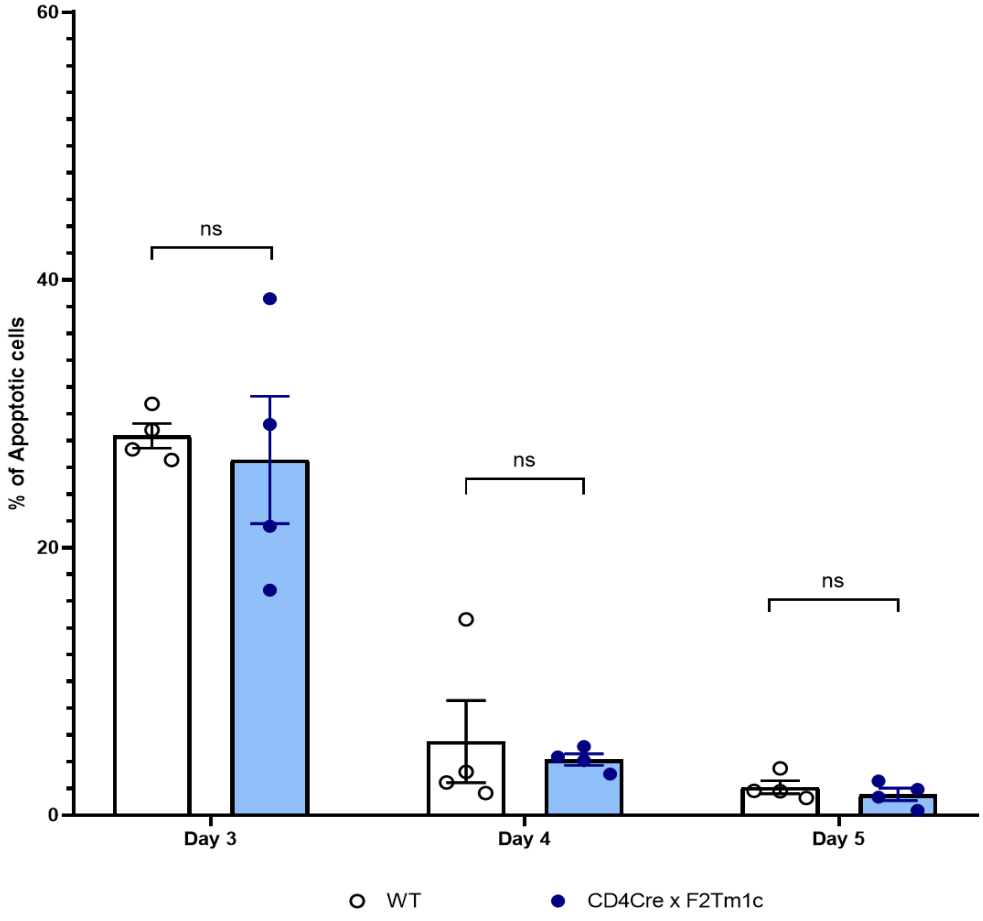

Figure 28: The lack of prothrombin gene in mice does not have a significant effect on apoptosis rates.

Wildtype mice and CD4Cre mice (lacking the prothrombin gene) were cultured and analyzed for apoptosis rates on days 3, 4, and 5. Every dot represents the average $CD8^+$ T-cell apoptosis rates of a mouse. The empty circles represent the data points of the wild-type mice, while the blue dots represent the data from the CD4Cre mice. Two-tailed paired t-tests were performed. The bars on the graphs show the standard error of means. N=4. The differences between wildtype and CD4Cre mice were not significant throughout the cell culture. Apoptosis rates decreased from day 3 to 5. The p-values on the 3rd, 4th, and 5th days were respectively 0.6698, 0.6959, and 0.4517.

The apoptosis rates of the $CD8^+$ T-cells harvested from the wildtype mice and the CD4Cre mice were compared in Figure 28. T-cells from the wildtype mice showed a trend towards higher apoptosis rates than those from the CD4Cre mice on all days. However, these differences were not significant ($p>0.05$).

Together, the comparison between the $CD8^+$ T-cells obtained from wildtype mice and CD4Cre mice showed that prothrombin deficiency leads to a reduction of the proliferative capacity of $CD8^+$ T-cells. The prothrombin-producing $CD8^+$ T-cells also show a tendency to survive longer in the cell culture. These findings together with the preceding observations utilizing genetic and pharmaceutical approaches (as shown in chapters 3.1, 3.2, and 3.3) show that the T-cell-derived prothrombin is an important factor for $CD8^+$ T-cell proliferation and survival.

4. Discussion

In addition to its hemostatic functions in coagulation and injury, thrombin has a host of direct actions on different cell types (47). Thrombin is generated at vessel injury sites and leads to the activation of various cells. Along with the formation of a stable clot, it also contributes to wound healing. Many cells including immune cells have protease-activated receptors, which are activated upon cleavage of thrombin (47,57). Studies in recent years made clear that thrombin plays role in inflammation by activating platelets, inducing the proinflammatory cytokine secretion by endothelial cells, and the recruitment of inflammatory cells through PARs (101). The link between inflammation and coagulation has primarily been attributed to thrombin's effects on the innate immune system (48,50,53,79,99). However, thrombin's possible effects on the cells of adaptive immunity, especially on T-cells remained largely unknown.

Among its roles in innate and adaptive immunity, thrombin is also central in acute and chronic inflammatory diseases due to its impact on the effectors of the immune system. Thrombin can be considered a physiologic mediator of inflammatory events. Inhibition of thrombin via hirudin enhances the pathology and reduces the leukocyte infiltration in a model of glomerulonephritis and improves the condition of mice with arthritis (85). Thrombin also plays a role in CNS inflammation and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis. Thrombin disrupts the BBB (blood-brain barrier) and increases the permeability leading to brain inflammation (102–104). It can activate microglia and contribute to scar formation (see 4.2.2). In Alzheimer's disease, direct thrombin inhibitor dabigatran was shown to have beneficial effects, reducing inflammation, oxidative stress, and tau pathologies in vivo (81). In Parkinson's disease, thrombin injection into the substantia nigra resulted in a loss of dopaminergic neurons and elevated neuroinflammation (81,105). Thrombin inhibition was shown to be beneficial in *S. aureus* skin infection and endocarditis (106,107) and during systemic hyperinflammation and sepsis (108,109). There is also evidence that thrombin can also improve host defense and survival by limiting bacterial outgrowth and enhancing platelet-neutrophil interactions (110).

Thrombin plays a role in tumor initiation, progression, and metastasis through its interactions with innate and adaptive immune functions (48,78,79). The association be-

tween cancer and pathologies of the hemostatic system (thrombosis, Trousseau's syndrome, DIC) are known for more than 150 years (111,112). Recent studies demonstrate that elevated hemostatic factors and procoagulants expressed by tumor cells correlate with poor prognosis in lung, breast, colon, and pancreatic cancer patients. TF expression from tumor cells and the following thrombin generation support the malignancy (113). Impaired blood coagulation and especially prothrombin diminution were shown to reduce the metastatic potential in mice and diminish the invasive growth of the tumor cells (48,113,114). Thrombin can contribute to tumor dormancy, supporting tumor growth after thrombin has reached the critical concentration and it is also involved in neoangiogenesis during tumor development (111).

4.1 Thrombin in adaptive immunity

Thrombin is known to be mitogenic for various types of cells such as fibroblasts, endothelial cells, and mesenchymal cells (47). Joyce et. al suggested that thrombin may modulate T-cell activation at hemostatic stress sites and showed that the thrombin modulation mobilized Ca^{+2} in and activated PKC-signaling in T-cells (115). T-cell activation starts an intracellular signaling pathway that brings about T-cell proliferation, effector function, and death depending on the signals (116). Our group has previously shown that thrombin is also expressed in extrahepatic tissues and the lymphatic origin of thrombin is T-cells. T-cell-derived thrombin showed upregulation during sepsis, suggesting a role in T-cell activation (80,83).

In this work, we show that depletion of T-cell-derived thrombin (by genetic and pharmaceutic means) leads to a reduction of proliferation in purified $CD8^+$ T-cells in serum-free culture (see 3.2.2, 3.3, 3.4). We also show that this effect could be reversed by the addition of thrombin (3.2.3). Vitamin K antagonist-treated $CD8^+$ T-cells showed a reduction of proliferation, but this was observed after 5 days of cell culture (see 3.1, Figure 18). To investigate, if the phenprocoumon treatment for a longer period would further diminish the proliferation, we injected wild-type mice with phenprocoumon for 3 days and treated the cells for 5 further days in the cell culture. The results showed a significant reduction of $CD8^+$ T-cell proliferation as early as the third day in the culture, suggesting that longer phenprocoumon treatment diminished the proliferation even more (see 3.2.2, Figure 21). This was not unexpected, as phenprocoumon is a vitamin-K antagonist, which inhibits the gamma-carboxylation of the coagulation factors like

thrombin (1.4.1) and it takes approximately 5 days until active coagulation factors are exhausted and antithrombotic effects set in (30).

A direct thrombin inhibitor argatroban was utilized to reduce thrombin activity in the cell culture, which resulted in a diminution in the proliferation of $CD8^+$ T-cells (Figure 25). CD4Cre mice that do not have prothrombin gene expression in their T-cells also showed a reduction in proliferation compared to the wildtype pairs (Figure 27). These results were in line with our findings from previous experiments with phenprocoumon (3.1, 3.2.2) and also with previous results from our group (83). Our findings indicate that thrombin inhibition or depletion leads to the reduction of $CD8^+$ T-cell proliferation. These results are in line with previous studies by Gorski et. al concluding that thrombin inhibitor heparin suppresses T-lymphoproliferative responses. Blood sample analysis of patients with low-dose heparin therapy showed lower expansion rates of $CD4^+$ and $CD8^+$ cells after being treated with PHA, a mitogen for T-cells (117). A recent study by Wang et. al. utilized the same method in this work, a $CD4^+$ T-cell assay with CFSE dye-dilution method. This study showed that T-cell-derived FV (118,119) suppressed lymphocyte proliferation, whereas thrombin addition prevented the suppression of the proliferation and direct thrombin inhibitor hirudin also enhanced the $CD4^+$ T-cell proliferation. The researchers concluded that heparin may aggravate the suppression of the adaptive response. Though it is unclear if T-cell-derived thrombin production and its concentration were taken into account during the study, this work underlines the thrombin's proliferative effects on T-cells (120).

Considering that loss of thrombin function resulted in a reduction of proliferation, we co-incubated T-cells obtained from anticoagulated mice with recombinant prothrombin or thrombin. Through the treatment with prothrombin, we tested if prothrombin could activate $CD8^+$ T-cells via another unknown receptor or a non-canonical pathway. Prothrombin-treated cells did not show a significant difference, whereas thrombin co-incubation led to a highly significant increase in proliferation (see 3.2.3, Figure 23). Prothrombin is the inactive version of thrombin, and it needs to be activated to function as a serine-protease (see 1.3.2). $CD8^+$ T-cells express PAR1 (57,101) and thrombin was shown to induce a PAR1 internalization (49), confirming that thrombin activates PAR1. Studies with PAR1 knockout mice models have a delayed inflammatory response in sepsis, arthritis, Th2-mediated colitis, and lung injury (121). Thus, $CD8^+$ T-cell prolifer-

ation following canonical PAR1 activation through thrombin addition might have protective properties during inflammatory diseases. Previous work was also in line with our results. Naldini et. al showed an IL-2-dependent $CD4^+$ and $CD8^+$ T-cell proliferation upon thrombin treatment in anti-CD3 stimulated cell cultures (122) and previous work from our lab also gave consistent results (83).

Thrombin can have protective and disruptive effects depending on its concentration. While low-thrombin concentrations protect the endothelial barrier and induce anti-inflammatory responses, higher concentrations of thrombin disrupt the endothelial barrier and induce pro-inflammatory events (59,123). Thrombin also functions as a pro- or antiapoptotic agent depending on its concentration. Low-thrombin concentration can initiate heterodimerization of the PAR1 and PAR3 receptors, enhancing cytoprotection. Low-concentrated thrombin can couple to thrombomodulin, to activate EPCR on the EC membrane. EPCR turns PC to APC, which in turn activates PAR1 and shifts signaling towards cytoprotective and regenerative effects (59,71). On the other hand, high thrombin concentration induces PAR1-PAR4 heterodimerization, which causes inflammation, diabetic vasculopathy, and cancer (59). Preliminary work has shown that the proliferation enhancement of T-cells via thrombin is also concentration-dependent (122).

Our results have shown that the control group had comparable results to the anticoagulated mice, who received thrombin rescue (see 3.2.2, 3.2.3). The average proliferation index of the thrombin-rescued group was slightly higher than the control group on both days, and this difference was significant on day 3 ($p=0.02$) (not shown here). FII produced by $CD8^+$ T-cells might be in lower concentrations than the purified thrombin concentration, which was given to the culture media. This might result in a higher proliferation in thrombin-rescued cells on day 3. The $CD8^+$ T-cells in the control group might catch up to the rescue group, resulting in a similar proliferation index on day 5, due to a positive feedback mechanism, where the further proliferation leads to a consequent increase in FII-secreting cells. To address the validity of this hypothesis, T-cell-derived prothrombin and thrombin concentrations can be measured in serum-free cultures in future studies.

Thrombin is capable of modulating various T-cell responses. Preliminary work showed that thrombin downregulated IL-12 production and upregulated IL-6, IL-8, and IL-10 production when it activates T-cells via PAR1. IL-12 is an important proinflammatory

mediator for Th1 response and IFN-γ production (122,124). Upregulation of IL-6 and IL-10 stimulates Th2 responses and the Th1 response gets suppressed. Thus, thrombin is a regulator of Th1/Th2 responses and can contribute to avoiding prolonged inflammation (124). Dendritic cells are antigen-presenting cells and link innate immunity to adaptive immunity. They have PAR1 on their surfaces and thrombin can stimulate blood DCs. Thrombin-stimulated DCs enhance $CD4^+$ T-cell proliferation and IFN-γ production. This shows that thrombin can indirectly induce T-cell proliferation and is a crucial factor in T-cell development (125).

Thrombin-induced PAR-signaling upregulates cytokine expression in $CD8^+$ T-cells, especially of IFN-γ (101,126). IFN-γ can induce anti-tumor and anti-viral effects of $CD8^+$ T-cells, induce cell motility and enhance cytotoxic cell-mediated apoptosis of MHCI expressing cells during inflammation (127). Another study also confirmed that the presence of thrombin or PAR1 agonist TFLLR induces $CD8^+$ T-cell function and proliferation of IFN-γ secreting $CD8^+$ T-cells. Surprisingly, in the presence of platelets, $CD8^+$ T-cell proliferation and IFN-γ production were inhibited despite thrombin treatment. Platelets also express PAR1 and thrombin activation of platelets induces TGF-β secretion, which negatively regulates T-cell inflammation. This mechanism inhibits T-cell activation in platelet-present environments like blood vessels (126). We speculate that, as soon as $CD8^+$ T-cells migrate in peripheral tissues, T-cell-derived thrombin might jump in to activate the proliferation and enhance their effector functions for an adequate T-cell-mediated immune response. The function impairment in $CD8^+$ T-cells via platelet-derived TGF-β was shown to reduce the tumor-infiltrating lymphocyte numbers and function (TILs) in ovarian cancer. TIL presence was associated with higher survival rates and the experimental groups with higher platelet extravasation developed bigger tumors (128).

Especially $CD8^+$ TILs are crucial for tumor clearance with their cytotoxicity and aggressive tumor microenvironment along with other inhibitory factors like platelet presence decrease their numbers and T-cell functionality. In our study, we investigated the possible effects of T-cell-derived thrombin on $CD8^+$ T-cell survival. Inhibition of thrombin production with phenprocoumon resulted in a slight decrease in survival rates of $CD8^+$ T-cells. This difference was more prominent on day 5, though it was not significant ($p=0.08$) (see 3.2.2, Figure 22). $CD8^+$ T-cells isolated from the CD4cre mice line also

showed diminished survival rates compared to wildtype mice, though it was not significant. To investigate this even further, we measured apoptosis rates in wildtype and CD4Cre mice. Surprisingly, although there was no significant difference in the apoptosis rates, CD4Cre mice tended to have lower apoptosis rates (see 3.4.1, Figure 28). The direct thrombin inhibitor-treated $CD8^+$ T-cells showed a significant decrease in T-cell survival on days 3 and 5 ($p=0.04$ and $p=0.0001$ respectively) (see 3.3, Figure 26). The fact that argatroban-treated $CD8^+$ T-cells showed a significant decrease but the CD4Cre mice did not, can be due to incomplete depletion of (pro)thrombin or unknown factors, which contribute to survival and/or apoptosis of these cells. There might be other genes, which compensate for the thrombin function in survival. Another possibility would be the existence of thrombin-like proteases, which might be inhibited by argatroban-treated mice, but not in the other experimental settings. $CD8^+$ T-cells of anticoagulated mice had higher survival rates upon thrombin addition, while prothrombin addition did not make a significant increase (see 3.2.3, Figure 24). These results are in accordance with the study of Cantrell et. al, who showed that thrombin stimulation of $CD8^+$ T-cells leads to an increase in survival and cytokine production in a PAR1-independent manner (129). Thrombin was previously shown to activate MCP-1 production via PAR1 in T-cells (130). MCP-1 is crucial for effector $CD8^+$ T-cell survival, proliferation, and T-cell differentiation. MCP-1 blockade was shown to reduce the survival in T-effector memory cells and the proliferation and migration of T central memory cells (131). The reduction of the survival rate upon inhibition of T-cell-derived thrombin and the increase in surviving T-cells indicate undiscovered roles of thrombin in T-cell survival and apoptosis. Our study did not address whether this effect is PAR1-dependent or involves another mechanism. Therefore, further studies are needed to unveil underlying mechanisms.

T-cells are major drivers in the establishment of inflammatory responses and the generation of memory. Peripheral T-cells consist of naïve T-cells, memory T-cells, and regulatory T-cells. After antigen encounters in peripheral lymphoid organs, they differentiate into effector cells and migrate to diverse tissues to clear the pathogens (Figure 29) (132). Effector cells are short-lived and only 5-6% of them persist after the infection, turning into memory cells (133). The total T-cell complement of the human body is estimated to be $26x10^{10}$ and only 2-2,5% of these cells are found in circulating blood (134). T-cells populate every tissue in the body including primary and secondary lym-

phoid tissues, mucosal sites, barriers, exocrine organs, the brain, CNS, and many others (132). T-cells have distinct roles in every anatomic compartment and memory T-cell subsets are defined by their migration sites and tissue residence.

Tissue-resident memory T-cells (T_{RM}) are retained in tissues and mediate rapid protection against diverse infections. The peripheral T-cell count and composition are controlled tightly through hemostatic mechanisms. T-cell hemostasis is balanced via proliferation and apoptosis rate changes and dysregulation could lead to inflammatory disorders. In the periphery, the T-cells must maintain reactivity and survive (135). Our findings suggest that T-cell-derived thrombin is a factor, which regulates the proliferation and survival of $CD8^+$ T-cells. Previous studies have shown that thrombin inhibitor heparin disturbs lymphocyte circulation by impeding the entry of lymphoid cells into lymph nodes and Peyer's patches (136). Heparin was also shown to derange lymphocyte trafficking by causing an inability to mediate to the reaction site (137). Thus, T-cell-derived thrombin could be meaningful in maintaining T-cell hemostasis in peripheral tissues and may be involved in T-cell-mediated immune responses.

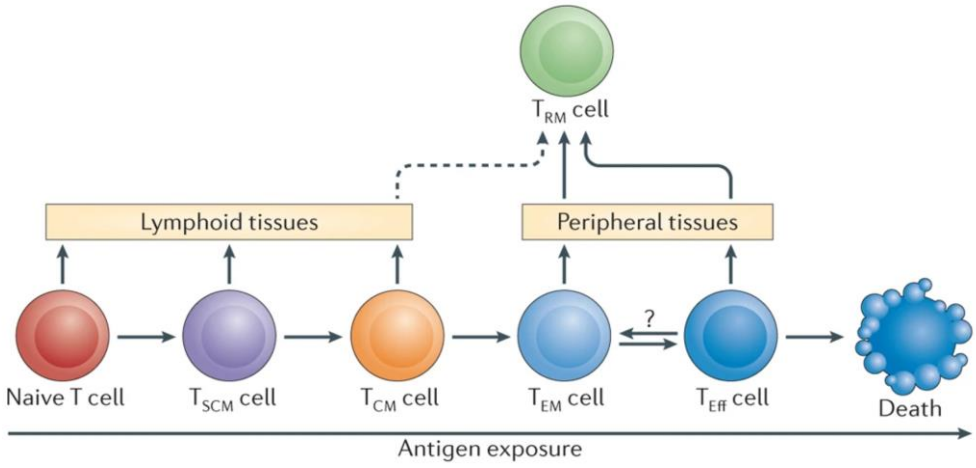

Figure 29: Memory T-cell differentiation.

The figure from Farber et. al (134) shows a model for memory T-cell differentiation. T_{SCM}=stem cell memory, T_{CM}=central memory, T_{EM}=effector memory cells, T_{Eff}=effector cells. T_{EM} and T_{Eff} can migrate to peripheral tissues. T_{RM} cells can originate from T_{EM} and T_{Eff} migration to these sites. T_{CM} cells are found in circulation and lymphoid sites, while T_{EM} cells can migrate to peripheral tissues. After subsiding infection, most of the T_{Eff} cells die, T_{RM} cells remain in the tissues and they may adopt a dual role of protection and regulation through secretion of different chemokines, but also by triggering anti-inflammatory responses to prevent overactivation (132,134).

The expression of PAR1 was found to be associated with T-cell differentiation and $CD8^+$ T-cells were found to be expressing more PAR1 in comparison to $CD4^+$ T-cells. Among T-cell subsets, the highest expression was found on antigen-experienced

memory effector T-cells, which are long-lived and able to migrate to peripheral tissues searching for potential antigens. Thrombin is not a potent chemoattractant, but it was shown to induce PAR-1-dependent chemokinesis and cytoskeleton reorganization in $CD8^+$ T-cells. It also promotes a transformation in the $CD8^+$ T-cell shape, transitioning these into a “flexible” form. This allows a fast direction change of T-cells and the patrolling at the vascular injury sites and neighboring sites (101). Thrombin stimulates $CD8^+$ T-cell degranulation and T-cell mediated cytotoxicity, which can be inhibited by PAR1 antagonist, suggesting that thrombin-mediated PAR1 signaling is involved in this mechanism (138). Thrombin was also shown to promote T-lymphocyte recruitment in an NF-KB dependent way (139). High F-actin levels and following cap formation are suggested to induce lymphocyte extravasation in lupus erythematosus patients. Thus, thrombin-producing T-cells might induce several mechanisms in peripheral tissues as described in previous studies, where thrombin functions on the same $CD8^+$ T-cell and surrounding T-cells in an auto- or paracrine manner, leading to lymphocyte recruitment, extravasation, and enhancing immune responses at inflammation sites in various ways. The migrating and thrombin-producing T-cells could also serve as a potential thrombin “carrier” to the extravascular compartment of peripheral tissues, which were identified to have thrombin expression in previous studies (80,83).

4.2 Thrombin in extrahepatic tissues

Thrombin is a 72 kDa molecule, which has comparable dimensions to the albumin in the blood vessels. Albumin weighs 69 kDa and the permeation of molecules through the endothelial barrier is known to have an inverse relation to the molecular weight (140). Smaller proteins can permeate through the endothelial barrier easier compared to bigger proteins like albumin. Albumin permeability through the vessels is 0.001 of the water permeability (141). Capillary endothelium may have different properties in different tissues. For instance, it is fenestrated in some organs like intestines, kidneys, and endocrine organs or even discontinuous in the bone marrow and the liver, allowing a greater permeability for macromolecules. It is moderately dense in skeletal muscles, skin, and lungs; and very dense in the testis and brain, where it forms the blood-testis barrier and blood-brain barrier respectively (3). Although the majority of the thrombin is produced by the liver and secreted into the blood circulation, there is evidence of thrombin in extrahepatic tissues like the brain, testis, and lymphatic tissues (47,48,80,83). There is also evidence of thrombin in the interstitium (80,142). Thus,

our findings could suggest a potential role for T-cells in the (pro)thrombin production in the extrahepatic tissues.

4.2.1 Thrombin in interstitium

Interstitial space consists of the spaces between the cells and makes up to one-sixth of the total body volume (141). It has the largest volume, excluding the intracellular fluid and it has the components that are needed for the activation of prothrombin to the active thrombin (143). De Ridder and colleagues suggest that there is no evidence of thrombin passing the endothelial barrier into the extravascular space (142). Prothrombin may thus be expressed locally in extrahepatic tissues for a functional role on cells residing in extravascular compartments such as the interstitial space and other organs. T-cells can exit the vascular compartment and migrate into interstitial spaces of non-lymphoid tissues (144). There are many possible ways for the thrombin generation in the interstitium such as the presence and activation of the TF from neutrophil traps, macrophages, and microparticles (MP), the activation of the intrinsic coagulation pathway through contact with lymphatic endothelium, MASP-1/-2, and the histone release from necrotic cells (142). Another fibrinogen-like protein Fgl-2 is secreted by $CD4^+$ and $CD8^+$ T-cells and regulatory T-cells (T_{reg}) and it is capable of activating prothrombin into thrombin in the interstitium (142,145). T-memory cells that are defined by their migration capacity and tissue residence express more Fgl-2 compared to naïve T-lymphocytes (146). Prothrombin activity and presence were also measured along with the other hemostatic factors in lymphatic fluid in previous studies (147,148), where the immune cells including $CD8^+$ T-cells are present. These pieces of evidence hint that the activated T-cells might have largely undiscovered roles in thrombin generation in the interstitium. T-cell-derived thrombin could participate in various physiological and pathological processes, which need to be examined further.

4.2.2 Thrombin in brain

The blood-brain barrier (BBB) consists of endothelial cells, astrocyte end-feet, and pericytes. The endothelial cells have tight junctions between them, which form a diffusion barrier preventing the entry of substances from the blood. It allows the entry of the O_2, CO_2, nutrients, and some large molecules like insulin, leptin, and transferrin (149). In physiological conditions, thrombin is localized in neurons and glial cells in CNS. Prothrombin expression was found in the cerebral cortex, hippocampus, and cerebellum (80,81,83). Thrombin receptor PAR is expressed in oligodendrocytes, microglia, and

astrocytes, suggesting possible thrombin functions in these cells. Shavit et. al showed that LPS treatment resulted in inflammation and the activation of coagulation in the brain. The LPS treatment induces an upregulation of the thrombin, PAR1, EPCR, and PC expression in microglia and the hippocampus region (41). Higher concentrations of thrombin cause brain damage, whereas low-thrombin conditioning of the cells has a neuroprotective influence on CNS (81). Thrombin was demonstrated to protect rat primary astrocytes and neurons from cell death in various stress conditions in a PAR-dependent manner, while thrombin treatment in high concentrations resulted in death (150). PAR1 activation is responsible for thrombin's actions in CNS and thrombin was shown to be highly regulated during physiological and pathological processes. Thrombin's actions on CNS are the greatest when the blood-brain barrier (BBB) is disrupted.

Thrombin increases BBB permeability by activating endothelial cells, allowing inflammatory cells and inflammatory mediators to enter the brain tissue (102). During infections, many immune cells migrate to and proliferate in the inflamed tissues. LCMV-induced acute meningitis models showed that specific $CD8^+$ T-cells produce proinflammatory cytokines that can affect blood-brain barrier integrity. Chemokine upregulation was shown to be linked with IFN-γ upregulation and this mechanism was suggested to be an important step in the amplification of chemokine expression and T-cell recruitment to the brain. In previous work, Catalfamo et al. demonstrated that thrombin activation of $CD8^+$ T-cells results in IFN-γ production (151). According to our group's results, $CD8^+$ T-cells produce thrombin, which enhances their survival and proliferation. These findings might suggest an important role for thrombin-activated IFN-γ producing T-cells in inflammatory incidents in the brain. They might contribute to the T-cell recruitment and their own activation in a positive feedback manner. Migrating $CD8^+$ T-cells could also act as a thrombin carrier through the blood-brain barrier.

Thrombin in the brain is implicated in neuronal development, altering cell morphology and cell migration patterns (81). It plays a role in synaptic plasticity and contributes to repair processes following cerebrovascular injury (150). A recent study showed that $CD8^+$ T-cells infiltrate the brain in aging people and Alzheimer's disease. Alzheimer's disease model mice that lacked $CD8^+$ T-cells demonstrated altered neuronal and synapse-related gene expression patterns in the hippocampus. Ablation of the $CD8^+$ T-cells in the blood, spleen, and brain led to altered gene expressions concerning synaptic plasticity (152). Given that thrombin also plays a role in synaptic plasticity, T-cell-

derived thrombin might be the key to this process, however further studies are needed. T-cell presence increases during neurodegenerative diseases such as Parkinson's disease (153), ALS (154), stroke (155), multiple sclerosis (156), and autoimmune encephalitis (157). Thus, understanding the role of T-cell-derived thrombin and elucidating the mechanism of how a thrombin-related increase in survival and proliferation of the T-cells in brain tissue influences these pathological disorders could be essential for understanding the underlying pathophysiology.

4.2.3 Thrombin in testis

The testis is the male organ, where spermatogenesis takes place. Spermatogenesis is regulated through many hormones such as FSH, LH, testosterone, and estrogen and can take up to 65 days until mature germ cells are produced (3). During the development of mature spermatids, sperms pass through tightly coiled seminiferous tubules in which the epithelium contains different stages of germ cells (Figure 30). The testis has one of the tightest barriers in the mammalian body: the blood-testis barrier, which is formed by Sertoli cells and divides the seminiferous tubules into basal and adluminal compartments. Spermatogonia and primary spermatocytes are found in the basal compartment, while other stages of developing germ cells such as secondary spermatocytes and spermatids reside in the adluminal compartment, which provides a unique milieu for germ cell development. The blood-testis barrier consists of tight junctions, ectoplasmic specializations, desmosomes, and gap junctions. Tight junctions function as a barrier preventing the passage of water, solutes, and large molecules between the paracellular space and they limit the protein and lipid movement, whereas gap junctions allow the diffusion of molecules smaller than 1 kDa (158).

The blood-testis barrier separates the germ cells in the adluminal compartment from lymphatic and circulatory systems and it creates an immunologically privileged environment for meiosis, as sperm membranes can contain antigenic proteins (158,159). There are also other mechanisms like systemic immune tolerance and local immunosuppression, which harmonically work with BTB to maintain the immunoprivilege (160).

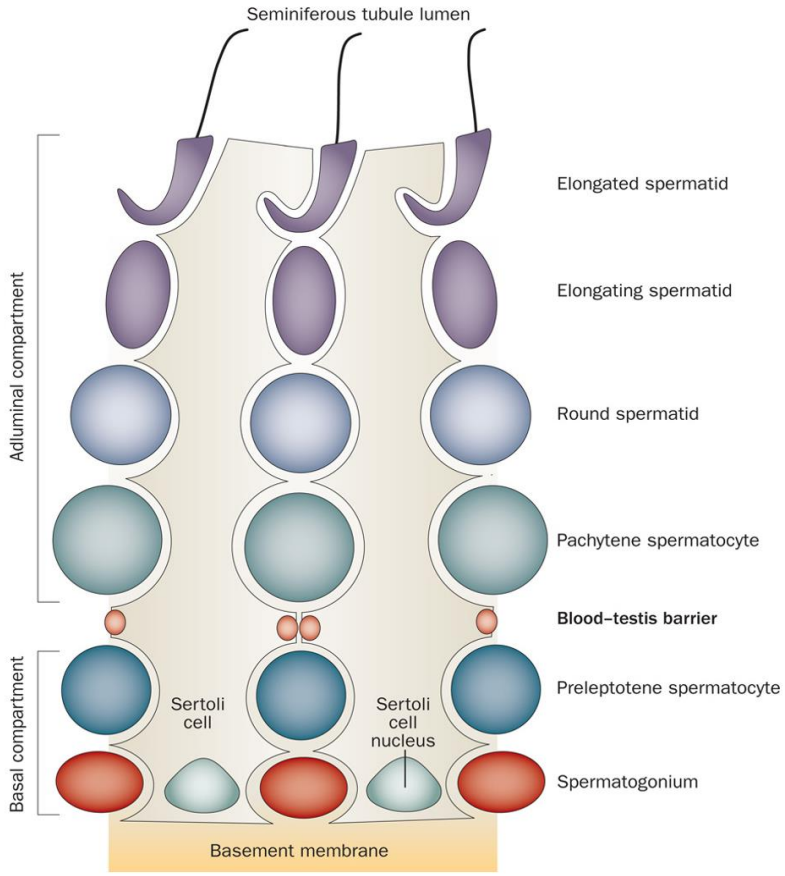

Figure 30: Development stages of germ cells in mammalian testis.

Figure modified from Cheng et al (161). Seminiferous tubules contain different stages of developing germ cells. The blood-testis barrier (BTB) separates seminiferous epithelium in basal and adluminal compartments. Preleptotene spermatocytes turn into leptotene spermatocytes, and these are followed by zygotene spermatocytes. Zygotene spermatocytes differentiate into the pachytene spermatocytes that have traversed the BTB (162).

Although the mammalian testis is an immune-privileged site, it has many afferent lymphatic vessels and it harbors many immune cells such as macrophages, DCs, mast cells, and lymphocytes (160). Approximately 15% of the adult immune cells in the rat testis were shown to be $CD8^+$ T-cells (163). T-cells are crucial for the maintenance of immune homeostasis and pathological diseases of the testis (159).

The blood-testis barrier (BTB) is known to be one of the tightest barriers and its permeability was put to test in previous studies. Haverfield et al. investigated the differential BTB permeability using different tracer dyes with 0.6 kDa, 70 kDa, and 150 kDa weight in rat testis. 150 kDa dye permeated up to preleptotene spermatocytes and could not reach and pass BTB. The dyes weighing 0.6 kDa and 70 kDa were able to diffuse up to leptotene spermatocytes, but they also could not reach and traverse the BTB (164). Given that prothrombin weighs 72 kDa, this study shows that prothrombin

should be unlikely to permeate through the blood-testis barrier. However, the existence of prothrombin in mice testis was shown by our group (80). We can conclude in light of these studies that the prothrombin in the testis must be expressed locally and/or transported through an unknown mechanism.

The function of prothrombin in the testis and how it is produced or potentially transported into the testis remains unknown and requires further investigation. A possible function of thrombin could be the enhancement of immunological properties through its effects on $CD8^+$ T-cell survival, maintenance, and proliferation. As the prothrombin is not likely to permeate the BTB by simple diffusion, prothrombin production by the resident $CD8^+$ T-cells in the testis could be a possible way for thrombin to pass the BTB. However, these theories need further investigation. The permeation studies mentioned here were conducted with rats, and not with mice. The behavior of the mice testis can be investigated under the same conditions and in the existence of the prothrombin to understand the mechanisms of the prothrombin transport in the testis. In future studies, prothrombin expression in basal and adluminal testis compartments can be quantified separately to understand the exact prothrombin localization in the testis. Resident T-cells in the testis can be depleted and prothrombin expression can be measured after the depletion to unveil their possible role in the prothrombin production or/and transport within the testis. For a better understanding of the function of T-cell-derived prothrombin, further studies can be conducted with T-cells that are cultivated with different stages of germ cells and these can be treated with different anticoagulants and (pro)thrombin.

4.3 Clinical implications

Newly discovered evidence from the latest studies points out that prothrombin is produced extrahepatically in T-cells (80,83). The whole scale of T-cell-derived thrombin's functions is not yet completely understood. Thrombin ties coagulation to inflammation and it is an important factor in the pathology of many diseases. Hence understanding the effects of T-cell-derived thrombin could help enlighten the underlying mechanisms in diseases and pathologies involving T-cells and prothrombin concentration shifts.

HIV (Human immunodeficiency virus) is a virus, which attacks the immune system, most specifically the T-helper cells. HIV infection is associated with a $CD8^+$ T-cell expansion and inverted $CD4^+/CD8^+$ ratios. The expansion of the $CD8^+$ T-cell pool occurs

in the early infection phase and numbers increase despite the antiretroviral therapy (ART) throughout the years. Even ART-receiving patients with suppressed HIV levels in the blood, especially patients with suboptimal $CD4^+/CD8^+$ T-cell ratios show elevated levels of inflammation and coagulation (126). Therefore, elucidating possible functions of T-cell-derived thrombin is important for understanding underlying mechanisms of higher inflammation (165) and thrombotic events such as venous thromboembolism (VTE) (166) in HIV-infected individuals.

PAR-1 expression on the surface of $CD8^+$ T-cells increases during the HIV infection, which leads to an increase in the effector function of $CD8^+$ T-cells. $CD8^+$ T-cell population co-expressing $PAR1^+$ and $CX3CR1^+$, which is localized in vascular endothelium and can be activated by thrombin, expands in HIV patients regardless of ART. CXCR1 is found in atherosclerotic plaques and is likely to be involved in atherogenesis (167). Mudd et al. have concluded that the persistence and expansion of the $CX3CR1^+$ $CD8^+$ T-cell population could be predisposing HIV patients to cardiovascular events (126). T-cell-derived, thrombin-mediated enhancement of $CD8^+$ T-cell survival and proliferation as shown by our study could be involved in $CD8^+$ T-cell lymphocytosis in HIV patients. However, further in vivo and in vitro investigations must be done to verify this and shed light on detailed mechanisms of T-cell expansion and HIV-related diseases.

Thrombosis is defined as the formation of a blood clot in a vessel, decreasing or blocking the blood flow in the vessel. It is a common disease, which is affecting at least 1 of 1000 individuals in the general population (168). Thrombosis occurs when the tightly controlled balance between coagulation and anticoagulation shifts towards the procoagulant side due to genetic and environmental factors. Familiar clustering and higher susceptibility of certain individuals led the researchers to look for common genetic polymorphisms related to thrombosis. The two most common causes of thrombophilia are the factor V Leiden mutation and the G20210A mutation in the prothrombin gene (169). Activated factor V (FVa) acts as a cofactor of factor X, which converts inactive prothrombin to thrombin and helps clot formation (see Figure 1). During fibrinolysis, activated protein C (APC) cleaves the FVa and downregulates thrombin generation. This limits the clotting and initiates the breakdown of the formed clot. However, in factor V Leiden mutation, APC is unable to cleave and inactivate FVa due to a single amino acid replacement in the protein structure. As a result of this change, APC loses the

anticoagulant effect on FV, thrombin generation continues, and blood clot keeps growing pathologically (170). The second most common cause of thrombophilia occurs through a G to A substitution at nucleotide position 20210 in the 3´untranslated region of the prothrombin gene. This single nucleotide change leads to an enhanced 3´ end processing and therefore to an upregulation of prothrombin synthesis by 1.5 to 1.7-fold and an increase in plasma prothrombin levels (48,171,172). G20210A mutation is a major risk factor for complications in pregnancy, and thromboembolic events (3-fold increased risk), showing that even the slightest changes in the prothrombin expression cause a clinically significant tendency to thrombophilia (172–175).

Evidently, mutations causing increased prothrombin/thrombin expression and clotting might result in serious clinical disorders such as deep vein thrombosis and deadly thromboembolic events. However, little attention has been paid to the possible outcomes of thrombophilic mutations in inflammatory settings such as sepsis, infection, and chronic inflammatory diseases (169,176). Researchers hypothesized that factor V-Leiden mutation might have been positively selected and preserved in populations due to its protective effects during inflammation. Kerlin et al. demonstrated that the heterozygous FVL mutation carrier mice were shown to produce more thrombin and had a survival advantage compared to wild-type mice in LPS-induced sepsis models (177). Therapeutic heparin was shown to abolish the survival advantage of heterozygous FVL mice during sepsis. The survival advantage of heterozygous FVL mutation was also confirmed in a large clinical trial with sepsis trial (PROWESS)(178) and another small study with patients in acute respiratory distress syndrome (179), which strongly indicated a possible beneficial role of moderately elevated thrombin levels in severe inflammation. Although some of the other studies showed contradicting results (180–183), the evidence of the survival advantage through elevated levels of thrombin in two different species remains important. The study groups could not provide evidence for cytokine differences or altered coagulation as an explanation for better survival rates in FVL patients and mice. Earlier findings conclude that LPS might induce T-cell activation (184,185) and our group’s recent findings indicate that LPS-stimulated T-cells secrete thrombin and the lack of thrombin ceases the T-cell activation in sepsis (83). In light of these studies, patients with thrombophilic mutations might profit from increased T-cell-derived thrombin secretion during inflammatory conditions through better T-cell response, activation, and proliferation. However, this hypothesis remains to be proved by further large-scale studies in different populations and patient groups.

5. Summary

Thrombin (FIIa) is a multifunctional serine-protease, which is formed by the activation of prothrombin. Thrombin plays a central role in primary and secondary hemostasis by activation of platelets, leading to the formation of the platelet plug and it converts fibrinogen to fibrin monomers. Other than its broad functions in hemostasis and clot formation, thrombin has pleiotropic effects such as angiogenesis, tissue repair, embryonic development, and cancer progression, which are mediated through PARs. In recent years, increasing evidence from studies identified thrombin as a key regulator between inflammation and hemostasis.

Previous findings in our lab with a novel mouse model have shown that thrombin is not only expressed in the liver but also originates from extrahepatic sources, one of those identified as T-cells. The upregulation of T-cell-derived thrombin during acute inflammation hints at its many unrecognized roles in physiological and pathological processes in the body. In this study, we investigated the possible effects of the $CD8^+$ T-cell-derived FII on T-cell proliferation and survival using serum-free cell cultures from primary $CD8^+$ T-cells extracted from mice spleen. CFSE-labeled $CD8^+$ T-cells were observed over five days and their proliferation and survival rates were analyzed using flow cytometry. We targeted FII with genetic and pharmaceutic approaches. The systematic and in vitro phenprocoumon treatment of $CD8^+$ T-cells from wild-type mice showed a significant reduction in survival and proliferation. The addition of thrombin after phenprocoumon treatment rescued the T-cells and restored their ability to survive longer and proliferate more, whereas prothrombin did not. The treatment with the direct thrombin inhibitor argatroban decreased survival and proliferation in $CD8^+$ T-cells. Genetic ablation of FII in T-cells resulted in a significantly lower $CD8^+$ T-cell proliferation in transgenic CD4Cre mice but it did not affect survival rates. These results suggest that T-cell-derived thrombin is a regulator of T-cell proliferation and survival.

T-cells can act as potential thrombin carriers, and FII originating from T-cells may regulate immune responses through the proliferation and trafficking of T-cells in certain tissues, where liver-produced thrombin is normally excluded. Discovering the effects of T-cell-derived FII in T-cell proliferation and survival is important for uncovering its previously unknown role in extrahepatic compartments during acute and chronic inflammatory diseases.

6. Acknowledgment

7. References

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Curriculum vitae