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The role of platelet-fibrin(ogen) interaction in platelet migration

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Ab	Antibody
ACD	Acid-Citrate-Dextrose
ADP	Adenosindiphosphat
AF488	Alexa Fluor 488
AF594	Alexa Fluor 594
APC	Allophycocyanin
Blb(-)	Blebbistatin, (-)-Enantiomer – active form
Blb(+)	Blebbistatin, (+)-Enantiomer – active form
BSA	Bovines Serumalbumin
CPD	Critical Point Drying
CytD	Cytochalasin D
DIC	Differential Interference Contrast
Fbg	Fibrinogen
FITC	Fluoresceinisothiocyanat
GFP	Green Fluorescent Protein
HMDS	Hexamethyldisalazane
IgG	Immunoglobulin G
ECM	Extracellular Matrix
E. Coli	Escherichia Coli
HEPES	2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethansulfons□aure
HSA	Human Serum Albumin
LPS	Lipopoysaccharide
PBS	Phosphate Buffered Saline
PH	Phase contrast
Plt	Platelets
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PS	Phophatidlyserine
RFP	Red fluorescent protein
rps	Rounds per second
SA	Staphylococcus Aureus
TLR	Toll-like receptor
tmt	Tomato Red
TxA2	Thromboxane A2
vWF	von Willebrand Factor

1 Introduction

In this introduction, I describe the relevance of thrombosis and hemostasis in health and disease, before outlining the two interwoven concepts of the coagulation cascade and the platelet adhesion cascade. Next, I focus on single platelet interaction with the environment, especially mechanosensing, and will briefly elucidate the role of platelets in host defense. After explaining the key elements of cell migration, I will consolidate these aspects to introduce platelet migration as a novel platelet function.

1.1 The (patho-)physiology of thrombosis & hemostasis

Maintaining cellular integrity and homeostasis in a complex structure like the mammalian body is a highly demanding task and required millions of years of evolution [12].

One particularly challenging aspect of homeostasis is to preserve the integrity and function of the circulation, i.e. the blood vessels ranging from large arteries to capillaries. This requires a delicate balance: On the one hand, damaged endothelium and subsequent leakage are a serious threat leading to blood loss and potentially compromising barriers to the exterior environment, creating entry points for pathogens [13]. Therefore, it is of great importance to quickly and efficiently establish a hemostatic plug, highlighted by the dramatic effects of disorders which interfere with hemostasis, ranging from sepsis to coagulation factor deficiencies.

However, hemostasis needs to be spatially and temporally controlled, as the clotting and subsequent occlusion of blood vessels compromises the oxygen supply and therefore integrity of tissue and organs. Indeed, a malfunction of hemostasis leads to critical health issues, as shown by the worldwide impact of thrombotic diseases on health, which are divided in arterial and venous entities.

The single most deadly condition worldwide is atherothrombosis, defined as pathological arterial thrombosis following the rupture of an atherosclerotic plug, leading to either ischemic heart disease or ischemic stroke [14]. In 2012, about 17.5 million individuals died of these two entities, which amounts to 31% of all global deaths [15].

In addition to arterial thrombosis, venous thrombosis is another major health concern associated with pathological thrombosis. Studies report a mean incidence of 0.5 – 1.4 per thousand person years, correlating positively with age. It includes deep vein thrombosis (DVT), as well as pulmonary embolism (PE). PE ensues in one-third of the cases of DVT, with a mortality rate of roughly 10% [16, 17]. As a result, extensive research has been carried out in this field and has led to an increasing understanding of the underlying mechanisms [18-20]. For example, many efforts to selectively target platelet receptors and to inhibit clotting have been made, and have led to successful bench to bedside research [21, 22].

So how is this in most cases flawlessly functioning, highly evolved system organized? In mammals, there are two essential parts involved, (1) the “humoral”, that is protein mediated coagulation cascade and (2) platelets as cellular components. These two parts are intricately linked and work synergistically.

1.1.1 The coagulation cascade

The final and decisive effector of the blood coagulation cascade is thrombin, also named Factor IIa, a serine-protease that is created by proteolytic cleavage targeting the COOH-end of its zymogen, also known as prothrombin or coagulation Factor II [23]. Thrombin executes various biological functions: (1) it strongly activates platelets through protease activated receptors (PARs) [24]. (2) It converts fibrinogen to fibrin and thereby enables the formation of fibrin fibers eventually acting as “glue” sealing off the vascular defect [25]. (3) It maintains a positive feedback loop activating factor V, VIII and IX [26].

Consequently it is essential that thrombin generation is tightly regulated, which is achieved by a complex activation cascade upstream of thrombin. One important checkpoint in thrombin generation is tissue factor. Tissue factor (TF) is a protein present on many cells localized subendothelially and therefore normally not exposed to blood plasma [27]. In arterial hemostasis and thrombosis however, a breach of the endothelium exposes TF to circulating active Factor VIIa, and a complex is formed [28] that can now act on coagulation factors downstream.

In venous thrombosis, there is usually no preceding vessel wall injury. However, monocytes, neutrophils and blood-borne tissue factor carried by microvesicles play a decisive role in initiating intravascular coagulation [18, 19, 29]. Recent findings outline an important role for plasma-derived TF in maintaining the generation of a thrombus extending into the vessel lumen [26, 30].

Tissue factor complex then activates Factors VII, IX and X [31]. Factor IXa then associates with Factor VIII, and this complex in turn also activates factor X [32]. Factor Xa acts

as the final effector of the coagulation cascade by binding Factor V, converting prothrombin to thrombin. Thrombin increases the conversion of factors V and VIII to Va and VIIIa respectively, creating a positive feedback loop and leading to rapid thrombin generation [33, 34].

In parallel, Tissue Factor complex is down-regulated by tissue factor pathway inhibitor, as tenase complex (Factors VIII and IX) and prothrombinase complex (Factors V and X) are becoming sufficient for rapid thrombin conversion [35, 36].

This intricate regulatory mechanism is complemented by the fact that tenase and prothrombinase complexes require negatively charged phospholipid surfaces in order to be activated. These are provided, along with unknown sources, by activated platelets, adding another spatio-temporal checkpoint to coagulation [37].

Once thrombin is formed this protease converts fibrinogen to fibrin which in turn is essential for clot formation and stability [27]. fibrinogen is a large plasma protein (340 kDa) with an average concentration of 2-4 mg per milliliter blood, which makes it one of the most abundant plasma proteins. Its structure is closely connected to its function. fibrinogen is a dimer composed of two identical subunits, which are stabilized by disulfide bonds [38]. Each subunit contains three chains (alpha, beta, and gamma) [39]. First, Thrombin cleaves two fibrinopeptides A from fibrinogen, converting it to fibrin [40] [41]. This leads to the exposure of two new binding sites interacting with two other fibrin monomers, and a protofibril is formed. At the same time, but with a slight delay, fibrinopeptide B is cleaved, creating adaptor sites for the parallel alignment of double-stranded protofibrils [40]. Total internal reflection fluorescence (TIRF) microscopy has demonstrated that the final product, a fibrin fiber, consists of hundreds to thousands parallel protofibrils [42].

Eventually, a fibrin network is formed, which stabilizes the platelet aggregate, seals off a possible vascular defect and stimulates cellular responses like attracting fibroblasts, which is important for processes like reendothelialization and healing [43, 44]. Three-dimensional fibrin networks show extraordinary resistance to mechanic stress [25]. It has been shown that individual fibers withstand an over 5-fold extension without rupturing [45].

However, once more this process has to be tightly controlled, and must be reversible, which is achieved through the Plasmin system. Physiologically, Plasminogen Activators (PAs), namely urokinase type PA (u-PA) and tissue type PA (t-PA) lead to the activation of Plasminogen to Plasmin, which in turn cleaves fibrin at specific sites, dissolving the clot and creating fibrin fragments like D-dimer [46]. D-Dimer is therefore used as a clinically important, yet unspecific marker of DVT [47].

Interestingly, fibrinogen is also considered an acute phase protein, because plasma levels are swiftly upregulated in the context of inflammation [48]. After having been mainly associated with thrombosis and hemostasis in the past, fibrinogen is now seen as a central mediator, effector and signaling molecule in various immunological diseases, ranging from Alzheimer's to bacterial infection [49].

1.1.2 Platelet adhesion cascade

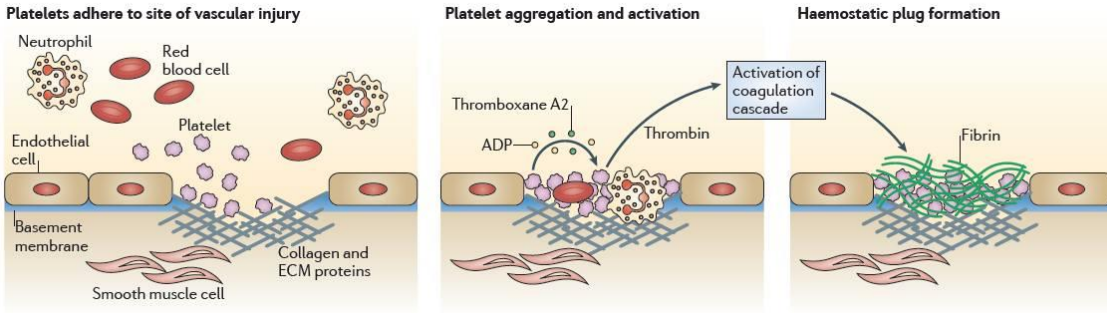
The coagulation cascade closely interacts with its cellular counterpart in the coagulation process, the platelet. Platelets originate in the bone marrow as anucleate cell fragments shed into circulation by megakaryocytes (MKs), large multi-nucleated cells located in the bone marrow [50]. Mature MKs protrude extensions into the vasculature of the bone marrow, and platelets form through shear stress at the very tips of these extensions [51, 52].

They are small, 1-3 µm in diameter in size and circulate for 8-10 days before undergoing pre-programmed apoptosis [52, 53]. In peripheral blood, they are present in high numbers, ranging between 150.000–400.000/µl [54].

Platelets are optimized for their main function, sealing vascular defects, as the following paragraphs will elucidate. However, their distinct arsenal of receptors and effector molecules enables them to participate in many other (patho-) physiological processes, which will be discussed in the following chapter.

As mentioned, Platelets circulate in high numbers in the circulation, permanently scanning the vasculature. When encountering endothelial damage or other vessel abnormalities, e.g. activated endothelium or non-linear flow, they start tethering and become rapidly activated.

Figure 1.1.1 – Platelet adhesion cascade



Adapted from [4]; Illustration of platelet adherence to exposed Collagen/exctracellular matrix (ECM) proteins, followed by aggregation and activation, and the release of ADP, TXA_2 and Thrombin. The coagulation cascade and platelets cooperate to form a hemostatic plug.

vessel wall [55]. Moreover, ligation of GPIb seems to activate platelets via Phospholipase D1 [56, 57].

However, this concept has been broadened by recent findings, which indicate that under very high shear rates (≥20.000 s^{-1}) rheological changes like shear gradients might suffice to induce initial platelet tethering [58]. The prolonged contact of platelets to the endothelium or ECM enables the interaction of the Glycoprotein (GP) VI transmembrane receptor with its substrate, collagen. GP VI cannot provide firm adhesion but rolling, and leads eventually to platelet activation [59].

Next, firm platelet adhesion and stable clot formation requires the activation of platelet integrin receptors. Integrins are heterodimers that are constitutively present on the platelet membrane, but normally adopt an inactive state defined by a "bent" conformation [60]. Binding of talin and/or kindlin to the cytoplasmatic tail of an integrin receptor leads to a change in conformation into the active state [61], necessary for proper integrin-substrate interaction and firm adhesion [62].

The activation of integrins via soluble or bound activators is termed "inside-out" signaling [63]. This limits integrin activation and platelet arrest to areas with high agonist concentrations. Therefore, this elaborate process helps to tightly regulate platelet adhesion and activation to ensure spatiotemporal limitation of clot formation.

Platelets express five integrins, which can be divided in three β1 integrins binding collagen (α2 β1), fibronectin (α5 β1) and laminin (α6 β1), and two β3 integrins, namely αv β3 and αIIbβ3 [64]. αIIbβ3 is the most abundant platelet integrin, with an average expression of 80.000 copies per cell [65]. It´s functional importance is reflected by a severe bleeding disorder which is caused if αIIbβ3 is quantitatively or qualitatively impaired (Glanzmann thrombasthenia) [66].

Mediated by activated integrins, a platelet monolayer is formed [24]. Integrins also transduce signals into the cell after binding substrate, a process that is termed "outside-in" signaling that further amplifies platelet activation.

Next, a robust amplification of activation to ensure full platelet activation is necessary. Activated platelets start to secrete dense and alpha granules containing ADP, thromboxane, serotonin, thrombin, coagulation factors and various other proteins [67]. This causes a positive feedback loop for platelet activation and additionally links it to the coagulation cascade. This link to the coagulation cascade is even further strengthened through the release of fibrinogen and thrombin, exposure of negatively charged phosphatidylserine (PS) and shedding of pro-coagulant membrane particles [37, 62, 68]. In return, the tissue factor dependent coagulation cascade drives thrombin formation (see 1.1.1), which cleaves PAR-1 (PAR3 in mice) and PAR-4

(protease activated receptor) on the platelet membrane. Thrombin is the most potent soluble platelet agonist, acting via G-Protein dependent pathways [69, 70].

In addition to thrombin, second-wave mediators like ADP and TXA_2, act via G-protein coupled receptors (GPCRs), and take effect in an autocrine and paracrine way, ensuring further platelet recruitment, integrin activation and granule release.

ADP activates the platelet receptors $P2Y_1$, which acts through a G_q pathway, and $P2Y_{12}$, acting via G_i. The importance of the second receptor is underlined by the effectiveness of clopidogrel as a platelet aggregation inhibitor although it solely reduces $P2Y_{12}$ signaling by 50-60% [71, 72].

Another positive feedback effector molecule is thromboxane A_2 (TxA_2). TxA_2 is a prostaglandin related lipid, derived from arachidonic acid through cyclooxygenase-1 and thromboxane synthase [24]. Mice deficient in TxA_2-receptor (TP) have prolonged tail bleeding times and form instable thrombi [73].

In addition to secretion and integrin activation, platelet activation leads to shape change within seconds. Platelets rapidly form filopodia and then adopt a spread morphology [74]. By exposing the invaginated canalicular system, a particular form of endoplasmatic reticulum tunneling the plasma membrane, platelets increase their surface area by 2- to 4-fold [75].

The subcellular mechanisms of this dramatic shape change have been elucidated in recent research. They involve the reorganization of the cytoskeleton through the growth of new actin filaments, forming filopodia and lamellipodia [76, 77]. Additionally, the contractile apparatus consisting of myosin IIa is activated, leading to the centralization of granules. It is assumed that these processes increase secretion and adhesion of activated platelets. Subcellularly, involved pathways are comprised of G_i and G_{13} coupled receptors [78], myosin light chain (MLC) phosphorylation via Rho [79, 80] and actin polymerization pathways via Rac, ARP 2/3 and WAVE proteins [81, 82].

Once platelets firmly adhere, are fully activated and have undergone shape change, they can recruit and interact with other platelets, a process that is termed aggregation. Aggregation is defined as the increasing cross-linking of platelets in a growing thrombus through integrin IIb/IIIa [64]. As mentioned before, this integrin needs to be activated, which occurs via G-protein coupled receptors (G_i, G_q and G_{13}) [83] and Rap1/RhoA [84, 85].

1.2 Platelet interaction with the (micro-) environment

The abovementioned steps of platelet activation are well defined and explored in great detail. However, improving *in vitro* and *in vivo* assays and imaging techniques have helped to elucidate another important function of platelets: their reaction to the surrounding environment on the single cell level.

Jackson et al were among the first to define two processes in thrombus formation and platelet aggregation which work synergistically: (1) The well described soluble-agonist mechanism leading to a stable plug and (2) the rheological-biomechanical mechanism forming a loosely packed, dynamically evolving thrombus. The second mechanism seems to be of particular significance in disturbed, non-linear flow conditions. In this case, the thrombus is highly heterogenic and is partially composed of only moderately activated platelets [58].

The latter mechanism also seems to play an important role in disease as dynamic, less stable thrombi are involved in acute coronary syndromes as well as stroke, causing embolization [86-88]. As platelet mechanosensing is necessary for this biomechanical mechanism, it has become increasingly relevant to describe this feature as well as the ensuing response in greater detail [89]. Also, mechanical force exertion on the single cell level is closely associated with this mechanism.

1.2.1 Platelet mechanosensing

Many nucleated cell types integrate mechanical signals from their microenvironment into their functional tasks, ranging from differentiation to proliferation and migration [90, 91].

Platelets do also respond to mechanical signals, for example to shear stress via GPIb-IX-V binding vWF [92]. However, in addition to these hydrodynamic forces, platelets are also subject to the physical properties of the extracellular matrix (ECM).

Only recently, a series of studies have shown that substrate stiffness in general increases adhesion, spreading and activation of platelets. The exact subcellular mechanisms are not well described so far, but integrins, Rac1 and the actin-myosin cytoskeleton seem to be involved [93, 94]. The role of platelet mechanosensing in the various known platelet functions remains to be clarified.

1.2.2 Platelet response

In hemostasis, platelets exert a contractile force on the forming clot, termed clot retraction. Clot retraction ensures a stiff and degradation resistant hemostatic plug, which can be beneficial as it decreases the risk of embolization [95, 96]. Clinically, bulk clot stiffness was found to be significantly higher in platelet rich than platelet poor clots [97, 98]. Due to the very complex properties of a contracting clot, the description of single cell dynamics is necessary for further analysis.

Lam et al. were able to measure single-cell retraction with a modified atomic force microscope setup. They found an average maximum contractile force of 9 nN per cell and adhesion forces of over 70 nN, which is, normalized to cell volume, 10x higher than the force exerted by other non-muscle cell types and is therefore in the range of myoblasts. Interestingly, platelets increased pulling force when exposed to stiffer substrates, hinting at a mechanosensitive relay mechanism to platelet retraction [99, 100]. The authors hypothesize that this behavior is of physiological importance as it facilitates a more uniform clot retraction by leveraging irregular stiffness.

Taken together, this indicates an interesting field of platelet physiology that remains to be elucidated in greater detail.

1.3 Platelets in homeostasis and host defense

Platelets have numerous functions independent of their pivotal role in arterial and venous hemostasis and thrombosis. Through extensive research in this direction, which is currently especially focusing on their role in pathogen defense, light has been shed on these alternative platelet functions. Due to their large numbers and short reaction times platelets are often recruited first in vascular (patho-) physiology (when a disturbance/deviation from normal occurs). They are therefore positioned at the forefront of hemostasis, immune response and tissue homeostasis, possibly helping to guarantee a flawless integration of these three mechanisms [101].

1.3.1 Platelets in homeostasis

Homeostasis is defined as the ability of a system to maintain a certain internal steady state [102]. Platelets contribute to this important concept in a variety of ways. Primarily, platelets contribute to vascular homeostasis through their main function: sealing vascular

defects and preventing blood loss. However, platelet function in homeostasis is not limited to hemostasis.

For example, another aspect of homeostasis is the repair of injury, also termed healing. Tissue repair requires the interaction of extracellular matrix remodeling, cell recruitment, migration, proliferation and differentiation and last but not least angiogenesis [103]. Interestingly, platelets have been shown to contribute to all these processes [104].

In detail, platelets remodel the ECM through the release of metalloproteases [105, 106]. They mediate the recruitment and differentiation of bone marrow derived progenitor cells via SDF1alpha [107, 108] and are important for the initiation of liver regeneration through 5-HT secretion [109]. Platelets release chemokines and growth factors that direct cell migration and differentiation [110, 111], and mediate arterial remodeling through GP1balpha [112, 113].

Additionally, developmental homeostasis is critical for seamless growth and maturation. In 2010, an embryonic role for platelets was found. *Bertozzi et al* report that murine platelets are mandatory for the separation of blood and lymphatic vessels [114]. Platelets also contribute crucially to the occlusion of the ductus arteriosus through remodeling and thrombotic sealing [115].

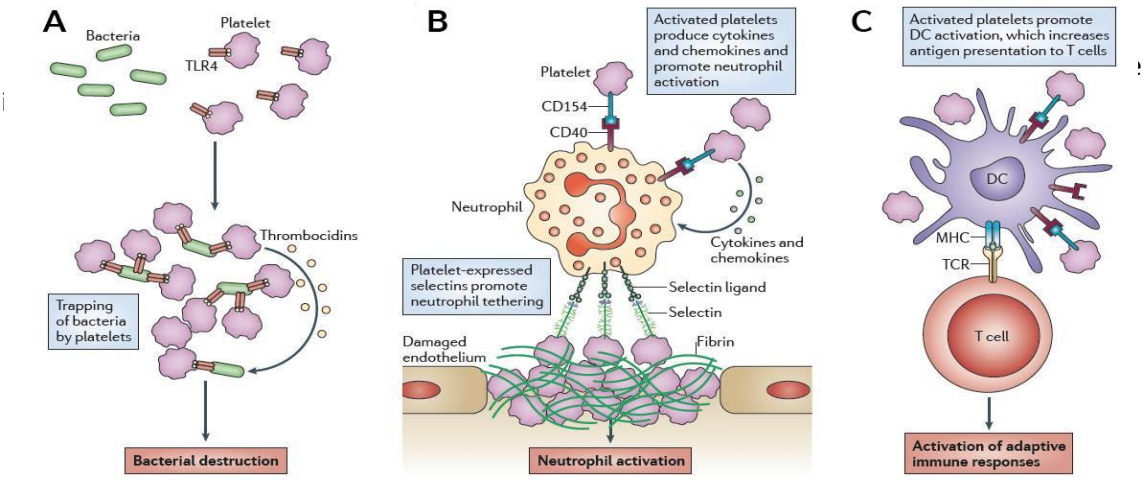
1.3.2 Platelets in host defense

In invertebrates and some early vertebrates, haemocytes are nucleated cells that combine hemostatic and immune functions, whereas in most vertebrates, a further differentiation in “professional” immune cells and hemostatic platelets has taken place [116]. Nevertheless, platelets possess several mechanisms necessary for effective immune function. They contain numerous cytokines and chemokines for immune cell signaling, express receptors that are generally associated with immune cells and even store antimicrobial peptides [117, 118]. Consequently, it does not come as a surprise that platelets contribute to virtually all aspects of host defense: As direct effectors and partners in the innate immune system, as well as contributors to the adaptive immune system.

Important roles for platelets in the innate immune system have been recognized. For example, platelets and pathogens directly interact. They can be activated via toll like receptor (TLR) 2 and 4, responding to lipopolysaccharide (LPS) released from gram-negative bacteria [119, 120]. Platelets bind bacteria directly through their FcrII gamma or GPIba receptor, or indirectly through ECM substrates like fibrin(-ogen) [121, 122]. This has also been shown for parasites and viruses [123, 124]. Plasmodium falciparum, the pathogen causing tropical malaria, has been shown to be directly killed by activated platelets [125].

The interaction of platelets with innate immune cells has recently drawn attention, in particular in hepatic pathogen clearance. Here, platelets guide invading pathogens to resident macrophages called Kupffer cells [126].

Figure 1.3.1 – Platelets in host defense



Adapted from [4]; Three exemplary known mechanisms of platelet contribution to host defense: A) platelets bind bacteria through TLR4, which leads to activation and exocytosis of thrombocidins; B) activated platelets release chemokines and serve as "landing pads" to neutrophils; C) platelets promote APC activation, contributing to the adaptive immune response.

A similar mechanism is observed in the spleen: *Verschoor et al.* could show that platelet-complement trapping of Listeria helps antigen presentation to dendritic cells in the spleen, increasing immunity against this bacterial strain [127, 128]. In systemic inflammation and sepsis, platelets cooperate with neutrophils in pathogen clearance [129, 130].

The second pillar of the immune system is the adaptive immune system. Platelets also support this highly evolved entity. T-cells are central to the adaptive immune system; in this regard, platelets influence CD4 T-cell proliferation and help to arrest patrolling CD8 effector T-cells in the liver [131, 132]. Last but not least, platelets influence B-cell proliferation and phenotype via PECAM-1, as studies with knock-out mice have shown [133].

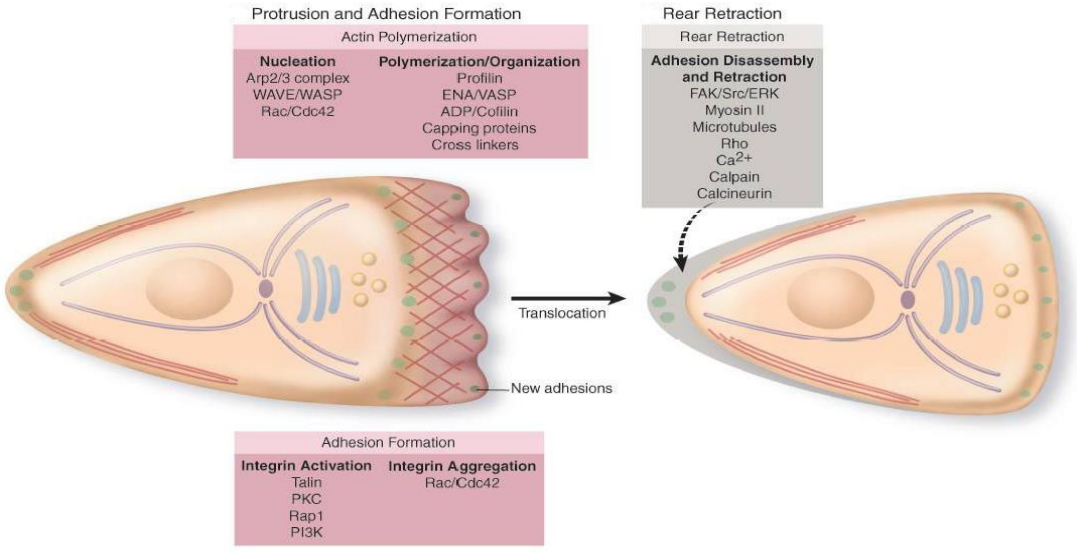
1.4 Cell migration

Cell migration is the process by which living cells move autonomously from one location to another. It is crucial in embryogenesis, tissue renewal and repair, orchestrates the cellular immune response and contributes to metastasis in cancer development [10, 134].

Migration requires polarization, i.e. the formation of a distinct rear and front end. It can be seen as a cyclic process [135].

The first step is adhesion of the cell. The front, also termed leading edge, is normally either composed of filopodia, thin, needle-like protrusions, or lamellipodia, broad sheet-like structures [136]. Filopodia and lamellipodia provide for and are in turn stabilized by integrin-mediated adherence to the ECM [137]. Both are formed by actin assembly, which is also essential for the following protrusion of the cell. F-actin assembly, also called polymerization, exerts pushing forces to protrude the plasma membrane thereby allowing the formation of new integrin binding sites. Naturally, this process has to be tightly controlled, as it determines rate and direction of migration [138].

Figure 1.4.1 – Principles of cell migration



Model of cell migration, illustration slightly adapted from [10]. Protrusion is realized by Actin polymerization, rear retraction is Myosin II dependent; adhesion formation is frequently integrin mediated.

In lamellipodia, actin assembly/branching and therefore protrusion capacity are controlled by ARP 2/3, which in turn is regulated by WAVE/WASP [139]. Upstream of WAVE/WASP, protrusion (in the form of adhesion and actin organization) is centrally regulated by Rho-GTPases, namely Rac and Cdc42 [140]. The latter is a key factor in maintaining cell polarity by limiting protrusion and influencing the position of the microtubule-organizing center (MTOC). MTOC assembly at the front is relevant for chemotaxis and directed cell migration [141, 142].

Therefore, Rac leads to downstream actin polymerization and is a central element of directional protrusion. This process is primarily restricted to the leading edge, as Rac activation is spatially confined [143, 144]. Rac maintains a positive feedback loop through activation by outside-in signaling via integrins, and in turn leads to integrin clustering [145]. Integrin clustering now stabilizes protrusions as it enables the cell to bind extracellular substrate thereby creating the friction required for propulsion, as mentioned earlier.

Retraction of the trailing edge and ensuing release of adhesion is the last step necessary to enable efficient migration. Rear detachment and retraction is realized through myosin IIa contractility acting on actin filaments. Myosin IIa is activated via Rho kinase (ROCK) or the MLC kinase (MLCK) [146]. These regulators are in turn controlled by Rho-GTP and calcium, respectively [147, 148].

1.5 Platelet migration

The first group to report platelet migration was Lowenhaupt et al. in 1973. They showed random platelet migration out of a capillary tube, and described it as directed towards collagen. A second paper by this group showed that the observed re-location of platelets could be inhibited by metabolic inhibitors, which provided further proof that it was an active process requiring cellular integrity and energy [149, 150].

Goetzl et al. developed a trans-well assay to assess random migration. In their setup, adrenergic reagents suppressed platelet migration (through ß-adrenergic receptors), as well as indomethacin (a cyclooxygenase inhibitor) [151].

The first *in vivo* data on platelet migration was provided in 2007. In a mouse asthma model, one group identified platelets in bronchial lavage. They used lung histology and an *in vitro* trans-well assay to conclude that platelets migrate through tissue, involving the Fc Receptor for IgE, and showing chemotaxis towards allergen and anti-IgE [152].

The group around Florian Lang contributed various insights into the mechanisms of platelet migration. They showed chemotaxis of platelets towards Stromal Derived Growth Factor 1 (SDF-1) in a transwell and 2D migration assay. On the cellular level, this seems to be dependent on the receptor CXCR4 and the signal transducer phosphatidylinositol-4,5-bisphosphate 3-kinase, which leads to WASP phosphorylation and ensuing actin polymerization, and is dependent on the serine/threonine-protein kinase Sgk1. Blockade of F-actin polymerization by cytochalasin B abrogates migration. In many migrating cells, migration depends on calcium influx. The calcium channel Orai1 and calcium-dependent SK-4 potassium channel, but not the NPPB-sensitive chloride channel appear to play a decisive role in platelet migration [153-155].

In another in vitro approach, one group found evidence for N-formyl-receptors on platelets, and chemotaxis by platelets towards formyl peptides, which are produced by degradation mainly of bacteria. They concluded that platelet migration plays an important part in host defense [156].

2 Aim of this study

The reviewed literature on platelet migration has so far failed to convince the scientific audience that platelet migration is (1) an autonomous function of platelets and (2) plays a role *in vivo*. Consequently, most major reviews on platelet function omit platelet migration [13, 62, 89, 116, 118, 157, 158].

There are two major reasons for doubt towards platelet migration. First of all, most of the presented data is restricted to *in vitro* evidence, and the majority of researchers used an indirect measure of transmigration by relying on a Bowden chamber. The pore size was mostly chosen between 3 and 8 µm, which is appropriate for large leukocytes, but allows platelets with a size of 1-2 µm to fall through, and is therefore problematic in proving active migration [151, 152]. Two papers report migration on fibrinogen, but are lacking videos or images to support their findings [149, 153]. Furthermore, an inclusive and thorough synopsis of the data is not provided in these two papers; it is not clear which of the mentioned pathways, receptors and chemotactic agents is most relevant, let alone if researchers looked at them same (patho-)physiological process. Moreover, a detailed explanation of the subcellular mechanisms, like myosin contraction and actin polymerization, is missing.

Another point of general criticism is the lack of substantial proof of *in vivo* relevance. Two papers used histological and one paper *in vivo* fluorescence microscopy to underline their respective *in vitro* findings on platelet migration. From their data, it can be concluded that under certain conditions platelets are found outside of the vasculature. However, direct evidence of migration is not provided. This is particularly problematic in the light of recent insights into leukocyte transmigration, which partially relies on platelet-leukocyte interactions, and is well conceivable to lead to passive "backpacking" of platelets in conjunction with neutrophil and monocyte migration [159, 160].

For the first time, our group around Florian Gärtner was able to establish single cell tracking in multi-color platelet reporter mice in order to visualize that platelets polarize and migrate *in vitro* and *in vivo* [161]. We found that migrating platelets show a characteristic front-rear polarity [162].

Actin-dependent protrusions and adhesions through integrins comprise the leading edge. Movement and polarization seems to be maintained through myosin IIa- based contraction of the trailing edge. We hypothesize that the local microenvironment drives platelet migration and therefore contributes to thrombus reorganization, defining a novel platelet function. However, the exact role of platelet migration in health and disease remains to be explored.

This introduction has delineated (1) the importance of platelets in health and disease, (2) the expanding field of platelet functions in and apart of hemostasis, (3) known aspects of platelet interaction with the microenvironment and in host defense, (4) the concepts of cell migration and (5) our group's previous novel findings concerning platelet migration.

Building on this body of evidence, the study presented here was conceived in order to further integrate known aspects of platelet physiology into the context of platelet migration. In detail, the aim of this study was to:

1. Analyze the interaction of migrating platelets with their substrate
2. Describe the effect of surface characteristics on platelet migration
3. Explore mechanosensing as a crucial component of platelet migration
4. Clarify the subcellular mechanisms involved in migration
5. Evaluate a possible role of platelet migration in host defense

3 Material & Methods

3.1 Material

3.1.1 Biological Compounds

Fibrinogen from human plasma was purchased from Sigma (#F4883). For experiments with fluorescent fibrinogen, either fibrinogen from human plasma, Alexa Fluor 488 or 594 conjugated, from Life Technologies (# F13191) were used. From Sigma Aldrich, we obtained Human Serum Albumin (#A9731), bovine Thrombin (#A456), Plasmin from human plasma (#P1687) and Adenosine 5'-Diposphate (#A2754). Prostaglandin I2 sodium salt was bought from Abcam (#ab120912), Moreover, the thromboxane analogue U46619 was acquired from Enzo (#BML-PG023-0001). Calciumchloride was obtained from Sigma.

3.1.2 Buffers & Solutions

Tyrodes Buffer 10x consisted of 80 g/l NaCl 10.15 g/l NaHCO3 and 1.95 g/l KCl in H_2O. Fresh Tyrodes solution was pipetted by mixing Tyrodes Buffer 10x with Millipore water at a ratio of 1:10, adding 1 g/l Glucose and 1% HEPES Buffer (Sigma) [163]. 0.1 M Potassium Phosphate Buffer consisted of KH_2PO_4 (Sigma) adjusted to a pH of 7.0. ACD-Buffer used in our experiments contained 25 g/l Natrium-Citrate, 13.64 g/l Citric acid monohydrate and 20 g/l Glucose, dissolved in Millipore water.
A solution for 2 in 1 cell Fixation and Permeabilization was prepared by mixing 2% PFA (Thermo Scientific), 0.04% Glutaraldehyde, and 0.05% Non-ident NP-40 in phosphate buffered saline [164].

3.1.3 Antibodies

To detect fibrin formation, the fibrin monoclonal anti-fibrin II chain (#NYBT2G1) antibody was purchased (Accu). To visualize activated GP IIb/IIIa, PAC-1 FITC from

BectonDickinson (#340507) was used. The fluorescent signal was enhanced using Alexa Fluor 488 Versus Fluorescein (FITC), produced by invitrogen. Actin staining was performed with Phalloidin conjugated with AlexaFluor 488 (LifeTechnologies #A12379).

Table 3.1 - Antibodies

Target	Name	ID	Host	Final conc.	Secondary Ab
anti fibrin II chain	Anti-Fibrin II Ab	#NYBT2G1	mouse	1:100	Anti-mouse 594
activated GP IIb/IIIa	Pac-1 FITC	#340507	mouse	1:100	Anti FITC 488
F-actin	Phalloidin AF 488	#A12379	-	1:100	-
β-Tubulin	β-Tubulin Ab	#T8328	mouse	1:200	Anti-Mouse 594
Myosin Phospho-ylated MLC	P-MLC 2	#3674	mouse	1:100	Anti-Mouse 488
GP IIb/IIIa	Hip8 anti GPIIb/IIIa	#ab15021	mouse	1:100	Anti-Mouse 488

Overview over Antibodies used in this study. Secondary Ab was used at a concentration of 1:200.

To show tubulin in fixed platelets, a monoclonal antibody against beta-tubulin was aquired from Sigma-Aldrich (#T8328). Phosphorylated myosin light-chain was detected using a Myosin Phospho-Myosin Light Chain 2 (Thr18/Ser19) Antibody from Cell Signaling (#3674). Rac-1 antibody was bought from Merck Millipore (05-389 Anti-Rac 1 Ab clone 23A8). Anti-human GPIIb antibody, clone Hip8 was purchased from Abcam (#ab15021). Control IgG Mouse was purchased from LifeTechnologies.
Secondary antibodies against the host organism were used to detect primary antibodies, in detail anti-mouse 488, anti-rabbit 594, anti-goat 488 (LifeTechnologies).

3.1.4 Chemicals & Kits

Hexamethyldisilazane was purchased from Sigma (HJO887), HNO_3 20% from Roth (#4337.2). Dimethylsulphoxide solvent was aquired from Sigma-Aldrich (D2438).
Two different Streptavidins were used, namely Streptavidin conjugated with AlexaFluor 488 (S-11223) and a double conjugated Alexa Fluor 488 Streptavidin 5nm colloidal gold conjugate (#A-3230) both produced by Life Technologies. Additionally, we used the pHrodo Red Avidin as a fluorogenic pH sensor (Life Technologies #P35362).

Saponin produced by Sigma (#47063), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) from Thermo Scientific (#20490) and formaldehyde solution 16%, methanol free, also

from Thermo scientific (#28906), were utilized. Also, we purchased ethanol 99.8% from Roth (#9065.1) and glutaraldehyde (#G5882). As a fluorescent mounting medium, we used DAKO S302380-2. For biotinylation assays related to endocytosis experiments, we relied on Thermo Scientific EZ-Link Sulfo NHS-SS Biotinylation Kit (#21945).

3.1.5 Live Bacteria and Bacteria Particles

For pathogen studies, LifeTechnologies AlexaFluor 488 conjugated Staphylococcus Aureus BioParticles were used (#S-23371). These products consist of heat-shocked and formalin-fixed bacteria. E. Coli K12 strain was a kind gift of the Engelmann laboratory (Institut für Klinische Chemie, Ludwig-Maximilians-Universität, Munich, Germany) [165].

3.1.6 Inhibitors

Various inhibitors were used to specify cellular mechanisms involved in platelet behavior in our specific assays. For myosin-blockade, we relied on (-)-Blebbistatin (Calbiochem 203391 CAS856925-71-8) and (+)-Blebbistatin (Calbiochem 203392 CAS1177356-70-5), the positive inactive enantiomer being the control. Upstream and therefore related to myosin function, we blocked Rho-associated protein kinase p160 (ROCK) with Y-27632 (Calbiochem #1254) and the myosin light chain kinase (MLCK) with ML-7 (#4310).

Actin involvement was assessed with Cytochalasin D (PHZ 103 Invitrogen). Integrin GP IIb/IIIa involvement was evaluated with the Fab-fragment Abciximab (Rheopro, Lilly Deuschland GmbH).

Tubulin function was tested with two distinct inhibitors, the stabilizing agent Taxol (Sigma-Aldrich T7191) and polymerization inhibitor Colchicine (Sigma-Aldrich C974).

3.2 Methods

3.2.1 Isolation of human platelets

Due to their small size and aggregate formation, it is technically difficult to image single platelets *in vivo*. Therefore, we analyzed platelet behavior *in vitro*. Consequently, we isolated this cell type from human blood.

Human platelets were isolated as described before [166], with minor variations. Venous blood was drawn from healthy, informed subjects and mixed 7:1 with Acid Citrate Dextrose-Buffer to reduce the pH, prevent clotting and feed the cells, respectively [167]. Next, the ACD containing blood was mixed 1:1 with Tyrodes solution, which was beforehand adjusted to a pH of 6.5. To obtain platelet rich plasma (PRP), the diluted blood was centrifuged at 22 degrees for 35 min at 70 RCF in a centrifuge, without break.

Carefully, platelet rich plasma (PRP) forming a yellow-pale phase on top of the erythrocyte layer was aspirated. PRP was then further diluted by adding Tyrodes solution (1:3) premixed with human serum albumin at a final concentration of 1:1000, and platelet activation was inhibited using 0.1 µg/ml of prostaglandin I2, also known as prostacyclin. After centrifuging this solution at 1200 RCF for 10 minutes, the supernatant was discarded and the formed pellet was re-suspended in Tyrodes solution.

The isolated platelets were used for 6-8 hours, being stored at room temperature. The final concentration was between and 150.000 and 250.000 per µl.
In parallel, PRP was processed by centrifugation (14000 rpm, for 5 min) without any buffer or prostacyclin to obtain platelet poor plasma (PPP).

3.2.2 Slide preparation

Glass slides were obtained from Nexterion (No. 1.5, D263T) and placed in 20% HNO_3 for 60 min on a rocker table, in order to clean the slides. Next, they were washed with autoclaved water and again placed on a rocker table [168].

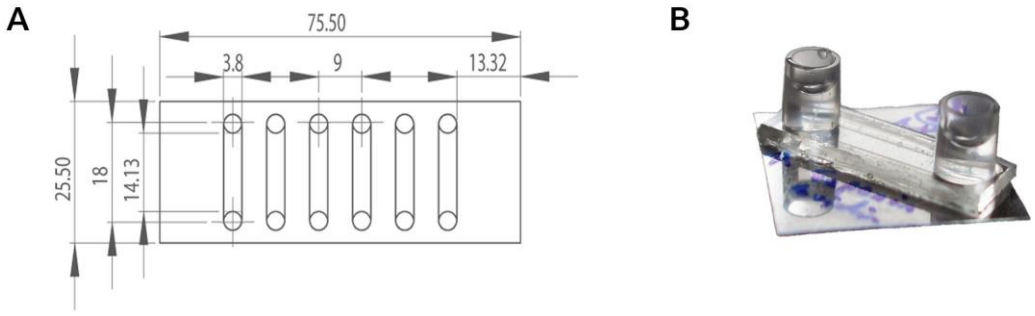
Then, slides were fitted onto a spin coater and remaining water was spun off at 90 rounds per second (rps) for 10 seconds. A small amount of hexamethyldisalazane was superfused onto the glass slides and spin-coated for 30 seconds at 80 rps. This process helped

to optimize the adhesive and anti-adhesive properties of the glass slides, and ensured optimal and equal distribution of protein substrates, coated onto the slides at a later timepoint.

So far, our group had used an open system, gluing the described glass slide to the bottom of a pierced plastic incubation dish, creating a container. This dish could then be filled with up to 200 µl of cell solution, and the open face allowed high-quality image acquisition with a difference-interference-contrast (DIC) objective [169].

However, we became interested in (1) a more differentiated approach, enabling multiple coating and incubation steps and (2) a closed system avoiding surface tension, enabling the use of a high-resolution phase contrast objective.

Figure 3.2.1 – Setup and characteristics of the chamber assay



A) Courtesy of IBIDI, characteristics of Sticky Slides IV$^{0.4}$ [2]. B) Newly established assay using one chamber of the IBIDI Sticky slide IV$^{0.4}$

Therefore we used IBIDI Sticky Slides IV$^{0.4}$, consisting in 6 parallel chambers of 30 µl volume (+ two reservoirs of 60 µl volume) that can be glued on any desired glass slide. We cut these to obtain single chambers, and glued them on our HMDS-treated glass slides. The properties of IBIDI Sticky Slides IV$^{0.4}$ are depicted in Figure 3.2.1.

3.2.3 Coating

3.2.3.1 Fibrinogen/Albumin Coating

Our group around Florian Gärtner previously defined the optimal concentration of HSA and fibrinogen in a coating solution to ensure platelet migration. We adjusted this protocol to use it in our chambers. Concentrations of either compound were optimized to ensure

reproducible maximal migration. Eventually, we used 75 µg/ml fibrinogen and 4 mg/ml of human serum albumin (HSA) in Tyrodes Buffer, and washed this solution with 120 µl Tyrodes solution 5x after 15 minutes incubation at room temperature (RT). Depending on the experiment, we either used unlabeled fibrinogen, biotinylated fibrinogen or fluorescent fibrinogen (AlexaFluor 488/594 conjugated).

3.2.3.2 Cross-Linking

To simulate increasingly cross-linked two-dimensional fibrin networks, we mixed thrombin, calcium, and increasing amounts of platelet poor plasma to gradually change the fibrinogen/thrombin relation. This profoundly influences the fibrin network composition and properties, as described in the literature [25, 170, 171]. To visualize the coating, we added 75 µg/ml fluorescent fibrinogen, AlexaFluor 488 conjugated, and also 4 mg/ml of human serum albumin.

Table 3.2 - Cross-linking assay

Cross-linking:	No	Very low	Low	Intermediate	High
Tyrodes pH 7.2	225.2	220.5	215.5	205.5	185.5
Fbg AF488 1.5 mg/ml	6	6	6	6	6
HSA 8 mg/ml	6	6	6	6	6
Thrombin 100 U/ml	2.5	2.5	2.5	2.5	2.5
PPP	-	5	10	20	40

We determined the addition of 1/6 plasma as sufficient for inducing almost exclusively clot retraction (see 4.2.1.1), and we therefore used this set-up as high-cross linking assay. This then became the retraction reference to migration in various experiments. 1/12 plasma was defined as an intermediate cross-linking assay as the morphology and behavior of platelets consisted of retraction and migration. Coating details are depicted in Table 3.2 - Cross-linking assay

To exclude other factors involved in fibrin network formation and platelet behavior we conducted plasma free experiments by coating the slides with 0.42 mg/ml fibrinogen, 75 µg/ ml fluorescent fibrinogen, AlexaFluor 488 conjugated, and also 4 mg/ml of human serum albumin, Calcium 1 mM and 2 units Thrombin. This assay excluded plasma while maintaining comparable cross-linking (see 4.2.2.2).

3.2.3.3 Covalent Coating

To explore the nature of the fibrinogen-platelet interaction, we covalently coated the substrate fibrinogen onto our glass slides.

To achieve covalent binding of the substrate to the glass surface, we functionalized the glass cover slips. The glass was placed in a PTFE holder and sonicated with ethanol: ultrapure water 1:1 solution. Next, the slide was rinsed and placed in Piranha solution (H2SO4 : H2O2, 1:1) for 30 min at R-T before another washing step. N_2 was used for drying the surface. Now, the aminosilanization process was realized by adding ethanol: ultrapure water : 3-aminopropyl dimethyl ethoxysilane (45:5:1) for 1 hour. After a subsequent washing step with ethanol, the cover slides were baked at 80 °C for 30 minutes. Silanized cover slips retain their ability to interact covalently with protein for up to 6 weeks. Slides were coated overnight with 75 µg/ml fibrinogen and 4 mg/ml of human serum albumin (HSA) in Tyrodes Buffer at a pH 8.5, and washed with 120 µl Tyrodes solution. As a control, the same coating solution was applied to HMDS slides.

3.2.3.4 Mechanosensing Experiments

To explore the possibility of mechanosensing by platelets, we deployed two experimental set-ups. One was a gradual fibrinogen gradient, and the other a sudden drop of the fibrinogen concentration on the slide. We conducted the following experiments with fluorescent fibrinogen, AlexaFluor 488 conjugated, to be able to judge concentration differences and coating quality.

For gradient building, we filled the chamber with Tyrodes Buffer (100 µl), followed by a careful addition of 30 µl highly concentrated coating solution (300 µg/ ml fluorescent Fibrinogen and 26 mg/ml HSA) into one marked reservoir. After 15 min, we removed the coating solution by aspiration and washed the chamber 5x with Tyrodes solution.

To create a steep gradient, from here on coined edge, the procedure that yielded optimal results was the following: A 10 µl drop of Tyrodes buffer containing 75 µg/ ml fluorescent fibrinogen and 4 mg/ml of HSA was pipetted centrally onto the silanized glass slide. Then, the IBIDI Sticky Slide was carefully placed on top, deforming the drop of 20 µl via surface tension into a cuboid water column. After 15 min, the chambers were washed as previously described.

3.2.4 Cell activation and placement

10 µl of washed platelets were, if not indicated differently, suspended in 230 µl Tyrodes Buffer with 200 µM Calcium, 4 µM mM ADP and 2 µM U-46619, directly before the ensuing experiment. Then, 150 µl of this solution was pipetted into the migration chamber.

Calcium seems to be important for migration in platelets. This is not surprising, given the fact that migration in many cell types relies on extracellular calcium. It influences directionality, cytoskeleton reorganization and traction [172]. Especially myosin II activation via MLCK relies on calcium influx [173].

ADP as well as the Thromboxane analog U-46619 were used to activate the platelets. Previous work by our group has shown that a combination of these two agonists leads to stable migration. Interestingly, ADP and thromboxane (TxA_2) are also the two main sources of platelet autocrine signaling and activation and this might explain the fact that despite initial necessity of external stimulation through these agonists, their removal through buffer replacement at a later time point does not affect migration. The notion that these two agonists are central to platelet function is further underlined by the clinical effectiveness of dual antiplatelet therapy with clopidogrel targeting the ADP receptor $P2Y_{12}$ and Aspirin blocking TxA_2 synthesis [174].

3.2.5 Washing procedure

After 15 minutes, the chambers were washed either 2x with 150 µl or by syringe transfusion with warm (pre-heated to 37 degrees) wash solution to avoid further platelet adherence and to replace the chamber fluid with cell free medium for better image quality. The wash solution consisted of 200 µM calcium, 1 mg/ml HSA in Tyrodes buffer. Preliminary experiments showed that this washing procedure did not affect platelet migration negatively (see 3.2.4).

3.2.6 Epifluorescence Microscopy

Fluorescence microscopy is a technical set-up widely used in the life sciences. We relied on an epifluorescence microscope, which in addition to reflection and absorption uses the illumination of probes with light of certain wave lengths (defined by filters) which leads to the excitation of fluorophores present on the sample.

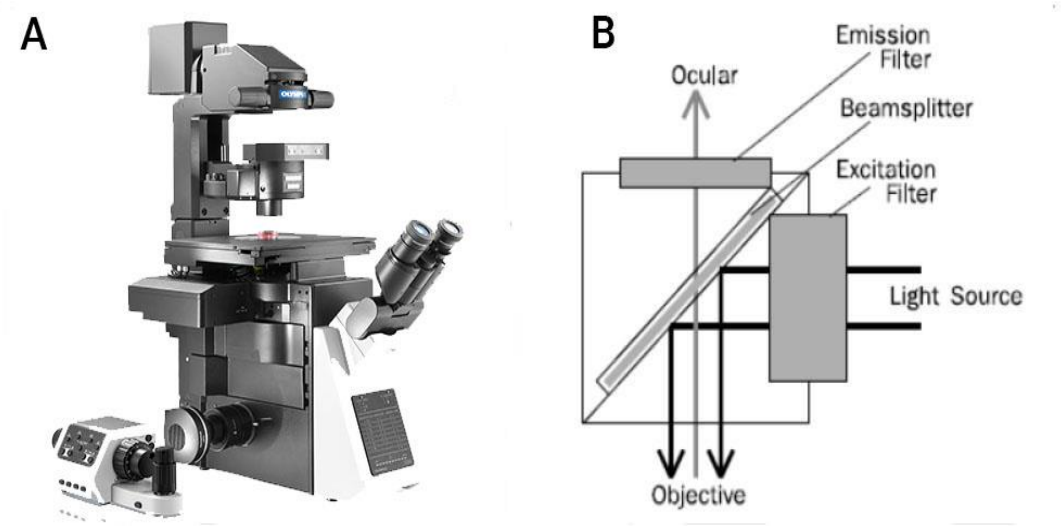
3.2.6.1 Introduction to fluorescence microscopy

For fluorescence microscopy, it is necessary that the imaged specimens are fluorescent. Fluorescence is defined as the emission of light shortly after excitation with a typically shorter wavelength [175]. This shift from excitation to emission wavelength is called Stokes shift and is the reason for the success of this method: the detector can be configured to filter out the

excitation spectrum, and then only detects the emitted signal of interest. The used fluorophores are only excitable at certain wavelengths. Consequently, the two most important properties of any method, sensitivity and specificity (or, in other words, contrast) are very high, allowing for the detection of single fluorophores.

A typical modern epifluorescence microscope (see Figure 3.2.2) uses the simple yet potent mechanism of epi-illumination. In this setup, the objective serves not only its familiar role of magnifying the probe, but also as a condenser for the fluorescence light. Due to the overlap of excitation and emission light path in this setup, the microscope needs a dichroic beam-splitter for separation [176].

Figure 3.2.2 – Epifluorescence microscopy



A) Courtesy of Olympus; a modern Epifluorescence microscope, in this case the Olympus IX 83, also used in this study. B) Adapted from [3], displaying a schema of the filter cube responsible for splitting emission and excitation.

Normally, the beam splitter is composed of a mirror that changes from transmission to reflection between emission and excitation peaks of the used fluorophore, combined with an excitation pre-filter and a barrier filter allowing only the passage of longer wavelengths. This three-component element is often referred to as a filter cube. Logically, each fluorophore with a distinct excitation/emission spectrum requires its own, specific filter set.

3.2.6.2 Epifluorescence setup

To assess platelet behavior in various setups, we relied on fluorescence microscopy combined with difference interference contrast or phase contrast imaging. We used an Olympus IX 83 inverted microscope with a fluorescence lamp and a heated stage. The associated computer provided the necessary control software (cellSens imaging). Two objectives were used: 100 x phase contrast and 40 x difference interference contrast. The microscope was equipped with two fluorescence filter sets, green and red. Standard exposure times were: GFP-Channel =130 ms; RFP-Channel= 300 ms; PH= 118 ms.

3.2.6.3 Time lapse microscopy

For a time-dependent analysis of platelet behavior, we created time-lapse stacks by automatically acquiring images each 12, 20 or 30 seconds, depending on the requirements of the experiment. When using fluorescently labeled compounds, 2- or 3-channel acquisition enabled us to assess the probe in various (frequency or color specific?) channels in parallel.

3.2.6.4 Flow chamber assay

In theory, the IBIDI sticky slides are also functional in a flow chamber assay. However, we had to adapt the set-up greatly to deal with limited space in the heated stage, to avoid loss of focus or instability when live imaging at high resolution, and to avoid air bubbles or other inhomogeneity. We created a 90 degree bent adapter of small size to fit onto the sticky slide reservoir by manipulating a commercially available flow chamber kit, and devised a slide placement avoiding tension and tilt.

A 1 ml syringe prefilled with the appropriate wash solution, pre-warmed in an incubator, was attached to the tube system. Next, the liquid column was advanced to fill the adaptor. A coated IBIDI sticky slide chamber filled with cell suspension was placed in the heated stage. The adaptor was then connected to the reservoir of the IBIDI sticky slide, and a short drainage tube was plugged into the second reservoir. After 15 min incubation, the chamber was flushed using the syringe and time-lapse image acquisition commenced. In some experiments, a second syringe was seamlessly attached and used to add an inhibitor while acquiring images. To assess early steps in platelet migration, in some experiments image acquisition was started right after chamber placement.

3.2.6.5 Still image acquisition

When using fluorescent fibrinogen, conclusions could also be drawn from still images acquired at pre-defined time points. Cell morphology, cleared area and length of cell traces could help to analyze commenced platelet migration retrospectively. Other advantages were the possibility to (1) acquire three images at representative spots per slide, (2) prepare various experiments in parallel and (3) to first fix the cells with paraformaldehyde 4% for later assessment.

3.2.7 Cross-linking & Plasmin Experiments

As described in 3.2.3.2, we performed multiple experiments in which we used varied amounts of plasma with constant levels of thrombin to increasingly cross-link the surface bound fibrin.

3.2.7.1 Cross-linking experiments

Cell suspensions were filled into the coated chambers and incubated for 15 min at 37°C. Then, slides were washed and placed under the microscope. Image acquisition lasted for 40 minutes, recording in the GFP (exp. 130ms) and PH (exp. 118ms) channel, using a 100x Olympus PH objective (30 sec/frame). After 40 minutes, probes were fixed with 1% PFA, incubated for 10 min at 37°C, mounted with DAKO fluorescent mounting medium, and at a later time point three representative overview still images were acquired per slide using the DIC 40x objective.

3.2.7.2 Plasmin reversal

The effect of plasmin on fibrin cross-linking and platelet behavior was assessed by using high cross-linking in two experimental groups in the flow chamber assay (As described in 3.2.6.4). Chambers were incubated for 15 minutes, washed and image acquisition commenced (PH 100x, PH; GFP; 20sec/frame). After 10 minutes, flushing of the chamber with washing solution (Tyrodes, 200 µM Calcium, 1 mg/ml HSA), in one group containing plasmin (mg/ml),

was performed and focus regained/controlled. Time lapse microscopy ended after a total of 150 frames/50 min.

3.2.8 Cell inhibition experiments

We deployed various inhibitors to obtain a more detailed picture of the molecular mechanisms involved in platelet migration. Each inhibitor was tested using two surfaces, one being the standard AlexaFluor488 Fbg-HSA surface, the other being the clot retraction reference coating. The goal of this set-up was to assess the influence of the respective inhibitor on migration and retraction.

The importance of GP IIb/IIIa was assessed by placing the slides with cells into the flow chamber assay as described in 2.1.2. and incubate them for 15 min. Then, the slides were washed as described in 2.4.2. and image acquisition at 10 sec/frame was initiated immediately. After 10 minutes, the slides were again washed with wash solution, containing either C7E3-Fab (10 µg/ml) or Cytochalasin D (2.5 µM) or DMSO as a control. Image acquisition ended after 15 min. This method enabled us to visualize the effect of the inhibitors right after addition, an important tool for inhibitors with rapid and drastic effects.

Myosin activity and upstream regulation as assessed by incubating washed platelets with three different inhibitors 30 min before activation. Blebbistatin (-) is known to entirely and selectively block non-muscle Myosin II activity [177]. Blebbistatin is known to inhibit reversibly and to be phototoxic. For these two reasons we conducted control experiments with the (+)-enantiomer of Blebbistatin that does not show inhibitory properties, and wash-out experiments to compare pharmacodynamics and reversibility in our assay.

In detail, we added washed incubated platelets with 50 µM Blebbistatin (-) or (+) for 30 min to the coated slides which we placed in our flow chamber assay. After 15 min, the chambers were washed with wash solution containing either 50 µM Blebbistatin (-) or (+), or none. Image acquisition lasted for 45 minutes.

Myosin light chain kinase (MLCK) and Rho-associated kinase (ROCK) play an upstream role in platelet myosin signaling. We therefore targeted these kinases with Y-27632 (50 µM) and ML-7 (10 µM), a combination of both and a control containing DMSO and H2O. After incubation, cells were placed in an incubator at 37 °C for 15 minutes, washed with wash

solution containing the inhibitors or DMSO/H2O and then placed under the microscope. Time lapse image acquisition was performed for minutes at a frame rate of 20 seconds.

The role of tubulin was assessed by inhibiting washed platelets with either colchicine (200 µM) or Taxol (10 µM), and EthOh/DMSO as a control. After incubation, cells were placed in an incubator at 37 °C for 15 minutes, washed with wash solution containing the inhibitors or DMSO/H_2O and then placed under the microscope. Time lapse image acquisition was performed for minutes at a frame rate of 30 sec.

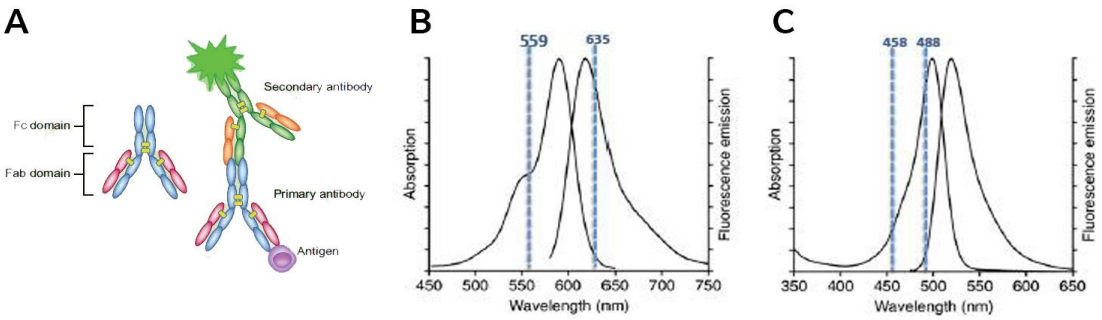
Two to three representative still images of areas not subjected to fluorescence illumination during time lapse image acquisition were taken at a defined time point after image acquisition. The tubulin inhibition experiments were subjected to immunofluorescence (protocol in 3.2.9) and recorded cells were later identified and photographed after tubulin staining.

3.2.9 Indirect Immunofluorescence

Immunofluorescence is one of the most powerful tools in life sciences, and its principles form the basis of many cutting edge technological advances in the field, from intravital multiphoton microscopy to state-of-the-art fluorescence activated cell sorting (FACS).

We used indirect immunofluorescence in our experiments. In brief, specific primary antibodies against epitopes of interest, either on the cell membrane or intracellularly, are added to a probe, after fixating and, for intracellular staining, permeabilizing the cells. Antibodies are protein complexes produced by B-cells that consist of a Fab region which binds the antigen, and an Fc region which allows for interaction with other effectors/components of the immune system.

Figure 3.2.3 – principles of indirect immunofluorescence



A) Adapted from [5], the Fab region of the primary Ab bind the antigen or target; a fluorescent secondary Ab is used to detect the Fc region of the primary Ab. B) and C) are adapted from [9] and display the fluorescent properties of AlexaFluor 594 and 488 respectively. As described in the text, the excitation wavelength is typically lower than the emission wavelength.

For indirect immunofluorescence, the unlabeled primary antibody is detected by a second incubation step with a so called secondary antibody against the constant Fc region of the primary antibody. This secondary antibody is linked to a fluorophore, which allows for example subsequent detection in an epifluorescence microscope [5].

Immunofluorescence was performed by coating the slides either with the standard migration surface (AlexaFluor 488 fibrinogen or unlabeled fibrinogen) or with the reference cross-linking retraction coating (AlexaFluor 488 fibrinogen or unlabeled fibrinogen). Platelets were incubated on the slides for 15 min, washed with wash solution and then fixed after another 15 min with either 2in1 fixation/permeabilization solution (20min) or with 1% PFA in PBS (10min). The second method required subsequent blocking with 2% Glycine (5 min), in some experiments permeabilization with Triton X-100 (0.1%), and blocking with 1% IgG free BSA in PBS.

Next, probes were incubated with the primary antibody (Ab) diluted in PBS containing 1% IgG free BSA. Used Ab concentrations and secondary Ab are indicated in Table 3.1. Slides were washed with DAKO fluorescent mounting medium and fluorescent images were acquired using the IX-83.

3.2.10 Biotinylation assay

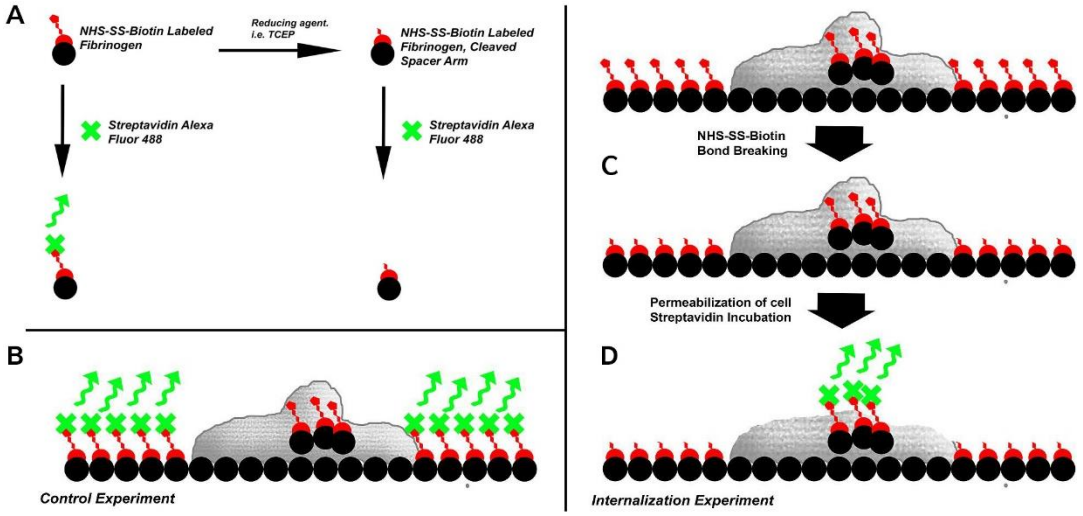
In addition to being an indispensable vitamin in enzyme function, biotin is used in life sciences because of the highly specific and strong binding of (Strept)avidin to biotin. Moreover, biotin is widely available, does hardly interfere with protein function due to its small size, is very stable and there are a wide range of coupling protocols available [178]. Therefore, biotin labeling and subsequent detection with (Strept)avidin proved to be a very potent tool in research for questions ranging from exact protein localization to *in vivo* tracing of cells [179, 180].

The advantage of using NHS-SS-Biotin utilized in our assay is the spacer arm which contains a cleavable disulfide bond [181]. Therefore, labeled proteins can be treated with a reducing agent and are then not able to bind (Strept)avidin compounds anymore. We used a biotinylation assay to assess the fate of fibrinogen being removed from the surface by migrating platelets. The exact experiment set-up and theory is shown in Figure 3.2.4.

Fibrinogen was biotinylated using a kit and following the protocol provided [182]. Briefly, 1 ml of fibrinogen (2 mg/ml) dissolved in PBS was incubated with 180 µl of biotin solution consisting of H_2O and 1.1 mg of NHS-SS-Biotin. After 60 min, desalting columns were used to filter the protein and obtain biotinylated fibrinogen ready to use.

After coating the slides with biotinylated/normal fibrinogen + HSA and placing cells as described, incubation lasted for 10 min at 37°C. Then, probes were fixed with 4% formaldehyde for 10 min at RT and after washing steps, reactivity was quenched using 2% Glycine for 10 min. If applicable, bond breaking ensured by adding Tris(2-carboxyethyl)phosphine (TCEP) 50 mM for 20 minutes to the slides. This mild reducing agent cleaved the disulfide bond of the NHS-SS-Biotin. For labeling, probes were incubated with AlexaFluor 488 conjugated streptavidin (0.02 mg/ml) for 60 min.

Figure 3.2.4 – Biotinylation experiments



The theory of conducted biotinylation experiments. A) shows the basic principle of the cleavable NHS-SS-Biotin, which will not bind fluorescent streptavidin after applying a reducing agent. B) control experiment; if Fbg is internalized no cell based fluorescence C) after cleaving, no fluorescence should be detectable D) in the case of internalization, a subsequent step of cell permeabilization should reveal intracellular, intact NHS-SS-Biotin Fbg and therefore fluoresce.

Depending on the experiment, temporary permeabilization of cell membranes was realized in bond breaking/labeling steps by adding 0.15 % Saponin. Saponin permeabilization ability was tested by assessing internal actin labeling of phalloidin conjugated with AlexaFluor 488 either with or without saponin. All samples were treated with DAKO fluorescent mounting medium and fluorescence signals/distribution were assessed using the IX 83 microscope.

3.2.11 pH-sensing assay

Life Technologies offers pH-Rodo, pH-sensitive dyes that hardly emit any fluorescence at neutral pH and fluoresce vividly in acidic environments. It has been used effectively in the analysis of phagocytosis and endocytosis due to the lower pH in lysosomes/endosomes [183, 184].We used pHrodo Red Avidin, mixed it 1:1 with Streptavidin AlexaFluor488 and added this compound to our slides coated with biotinylated Fbg/HSA at a final concentration of 0.02 mg/ml. We then added platelet suspension and performed time lapse microscopy to obtain images of fibrinogen removal by the cells, recording the green and red fluorescent channel to collect possible fluorescence intensity changes in the RFP-channel (pHrodo) and fluorescence reference in GFP-channel (AlexaFluor488 Streptavidin-Fbg). Additionally, still images of the same areas were acquired before and after adding Tyrodes solution with its pH adjusted to 5.7.

3.2.12 Bacteria platelet interaction assay

We devised a platelet-bacteria assay to study the role of platelet migration in host defense. Due to limited interaction of S.aureus particles and live E. coli bacteria with fibrinogen in preliminary experiments, we used a fibrin coating with virtually no cross-linking (see 3.2.3.2) and then added the S. aureus particles/E. coli bacteria at a concentration of $4x10^8$ per ml to our slide.

Due to the possible interference of E.coli culture medium (LB-Medium, low salt 15.5g/L) with the surface, we centrifuged the bacteria for 10 min at 1.2 G, disposed the supernatant and reconstituted the bacteria in an equal amount of Tyrodes buffer. Additionally, we added 1 mmol of calcium to guarantee physiological calcium levels, before incubating the bacteria/particles for 30 minutes at 37 °C. Afterwards, the slides were washed three times with 150 µl of Tyrodes buffer, and cells were added. After 15 minutes, sufficient time for platelets to spread and start migrating, we washed the slides (see 3.2.5) and incubated them for 45 min at 37 °C.

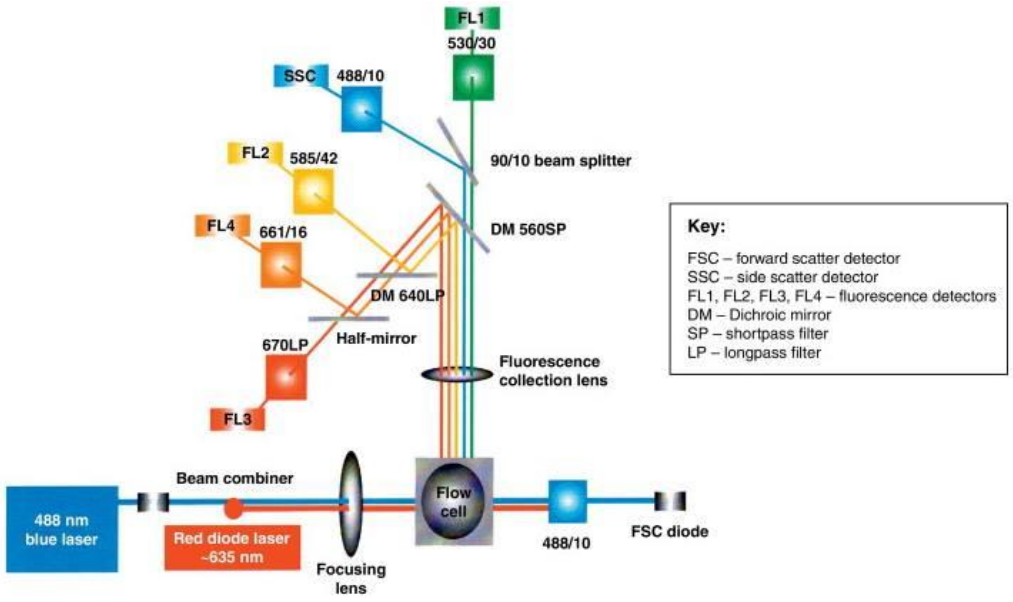
Samples were then rapidly fixated and permeabilized using Fix/Perm solution (see 3.2.9) for 20 min. Then, GP IIb antibody at a 1:100 concentration (20 min) and Anti-Mouse AlexaFluor 488 secondary at 1:200 concentration (20 min) were used for rapid immunostaining of the platelet membranes.

3.2.13 Flow Cytometry

Flow cytometry is widely-used tool for cell counting, characterization and sorting which has revolutionized life sciences as well as hematology and oncology [185, 186]. A flow cytometer consists of a flow cell, which creates a liquid stream of sheath fluid that aligns the probed cells in single file.

In addition to basic properties of the scanned cells, forward scatter (FS) and sideward scatter (SS), which represent size and granularity, respectively, cells can be further differentiated by incubating them with fluorescence marked antibodies before image acquisition. This method allows the identification of populations and characterization of subpopulations combined with quantitative assessment [187].

Figure 3.2.5 – Flow cytometry



Setup of a 4-Laser flow cytometer, adapted from [7]. In a flow cell, single file particles are exposed to the lasers, and forward as well as sideward scatter (FSC and SSC) are recorded, in addition to fluorescent response to the set lasers.

Labelled cells pass the measuring system which consists of a variable number of lasers, and detectors which create digital signals of light scattering as well as fluorescence intensity

signals. These signals are then amplified and visualized through a digital interface. This allows real-time adjustment of parameters like laser voltage and compensation. A modern flow cytometer allows the measurement of thousands of cells/particles per second [188].

3.2.13.1 Sample preparation

To assess binding of E. coli particles to platelets in solution, we designed an assay inspired by similar approaches in the literature [129]. The idea was to use myosin inhibited cells to probe the relevance of myosin for in solution interaction. Because flow cytometry relies on fluorescence signals, and our myosin II inhibitor, Blebbistatin, displays strong auto-fluorescence, we conducted our experiment in three groups: Untreated, Blebbistatin (+) treated and Blebbistatin (-) treated. Blb(+) is the negative, inactive enantiomer with comparable biophysical properties. Optimal concentrations of compounds were found by numerous preliminary experiments.

3.2.13.2 Aquisition

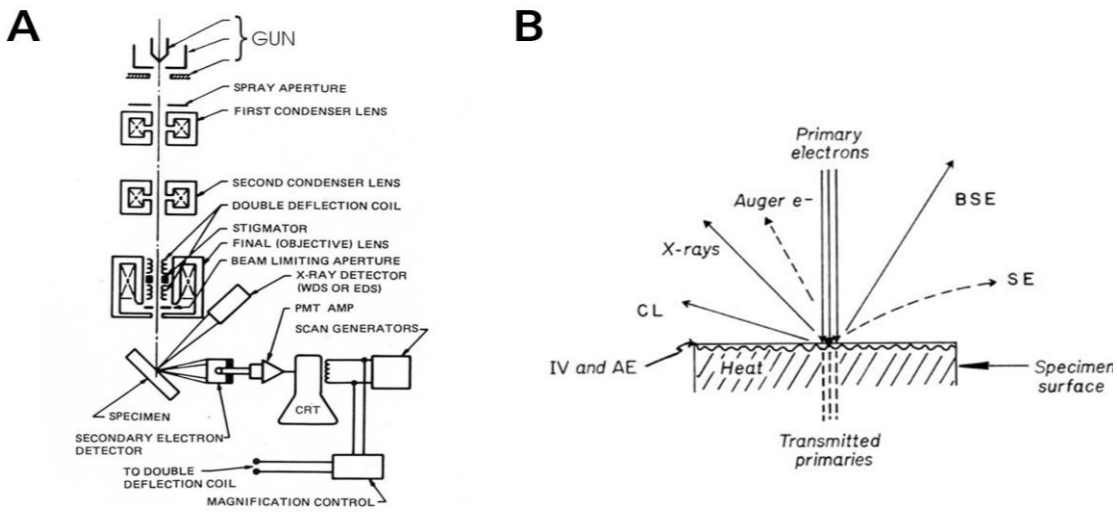
Flow cytometric data was acquired using a Beckman Coulter Gallios 3 Laser/10 colors bench-top flow cytometer. Fluorescence channel 2 (FL2) was used to display live E.coli expressing tmt red. Fluorescence channel 6 (FL6) was used to display platelets incubated with CD42b-APC (near infrared).

3.2.14Scanning Electron Microscopy

The idea behind scanning electron microscopy (SEM) is to use a focused beam of electrons instead of photons for the analysis of a probe, avoiding the diffraction limit of light. This achieves a resolution of higher than one nanometer. Upon interaction with the probe, the electron beam leads to the emission of secondary electrons. These are accelerated and then detected, and through quantitative differences in emission a depth-of-field representation of the scanned probe is created [189, 190].

Due to electrostatic charge of untreated biological specimens interfering with image acquisition, in conventional SEM it is necessary to render the surface of the probe conductive. This is achieved through sputter coating the probe with gold or other conductive metals. Moreover, drying without residues is necessary for optimal image quality [191].

Figure 3.2.6 – Scanning Electron Microscopy



A SEM microscope. A) is adapted from [1]. It shows the pathway of the electron beam and signal detection after interaction with the probe B) adapted from [8] shows the various measurable signals emitted after primary electrons interact with the probe. We relied on the detection of secondary electrons.

2.5 % glutaraldehyde in 0.1 M potassium phosphate buffer. Then, probes were washed three times for 10 minutes with 0.1 M phosphate buffer, and samples were successively dehydrated by 20-40-60-80-99% ethanol. Each step lasted 10 minutes.

For critical point drying, Probes were stored in 99% ethanol overnight. To dry the probes directly before imaging, and ethanol-carbon dioxide exchange, a critical point dryer (CPD; Polaron E3000) was used along with a standard drying protocol [191].

For HMDS drying, probes were treated as described before, with minor adaptations [191]. In detail, samples were incubated for 3 min with 100% HMDS after two 99% Ethanol steps enduring 10 min. Then the supernatant was removed and probes were carefully dried with filter paper. Drying occurred overnight under air flow to avoid moisture.

Immediately before image acquisition, probes were sputter coated with gold (Cressington 108auto) to a total thickness of 6 nm.

3.2.14.2 Image acquisition

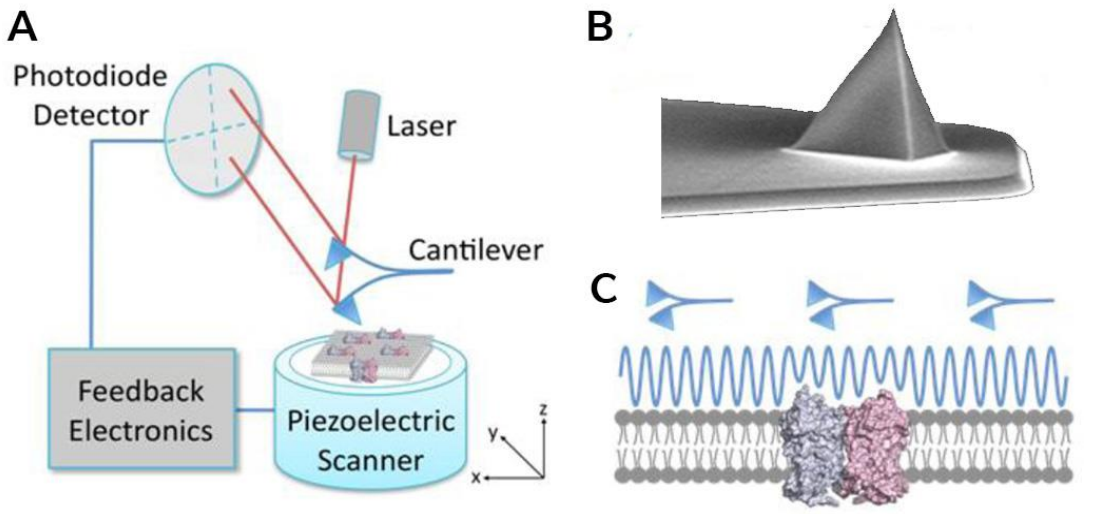
Images were acquired in cooperation with the Nanophysics Group of the Physics Department of LMU München. The used scanning electron microscope was a Zeiss Gemini DSM 982 (LEO, Oberkochen). Electron beam voltage was set at 3 to 8 kV. Maginfication ranged from x1000 for overviews to x92.500 for high-resolution platelet-bacteria interaction representation.

3.2.15 Atomic Force Microscopy

The precursor of the AFM, the scanning tunneling microscope, was developed in 1981, and enabled researchers to image individual atoms on surfaces. This technique is not limited by the diffraction limit imminent to optical solutions, and can be more sensitive than electron microscopy.

By variation of setup, scanned probes no longer needed to be conductive, and the AFM was born [192, 193]. Its strengths regarding biological samples are to operate in fluids as well as air, which avoids the necessity of fixation. Also, modern AFMs can be used in force spectroscopy mode, allowing for manipulation and force measurements [194].

Figure 3.2.7 - Atomic Force Microscopy



Principles of Atomic Force Microscopy, adapted from [6]. A) depicts the components of an AFM. The cantilever scans the surface and gets deflected by its topography. This deflection is detected with a laser and a photodiode detector. B) shows the used Bruker cantilever of the MSNL type, image from [11]. C) displays force tapping mode, the in this case applied technique for surface scanning.

An AFM consists of a cantilever connected to a piezoelectric element, which oscillates the cantilever at its eigen frequency. A defined, very sharp tip of nanometer size is attached to the cantilever and is physically scanning the probe. Properties of the probe are recorded by the displacement of this cantilever, measured by a detector, typically a laser deflecting off the cantilever. More precisely, it records the eigen frequency of the cantilever as a sinusoidal curve defined by frequency, amplitude and phase. Deflections of the cantilever tip change this signal and allow a computational reconstruction of the scanned probe [195].

A computer-controlled stage allows the movement of the probe in x,y,z-directions and therefore an automated surface scan of a given area and size. Integrated software controls acquisition, settings, and visualizes the ongoing measurements. It is therefore ideal to measure surface characteristics like topography and hardness on a nanometer scale [196].

3.2.15.1 Sample preparation

Sample preparation had to be adapted for atomic force microscope (AFM) measurements, because the stage of the used AFM required (1) an open fluid-filled system as the cantilever approaches the probe from the top, and (2) a rather wide opening of at least 2 cm in diameter to fit the AFM device.

Therefore, we adapted our assay accordingly. Spin-coated cover slips were placed into the inverted covers of cell strainers (Biolap, SPL) forming a container and immobilized by paraffin drops in each corner. Next, a IBIDI Culture-Insert with cut-out middle part (now forming one well of 140 µl volume, 8.4 mm x 8.4 mm x 5 mm) was attached centrally and the formed chamber was coated with either standard migration coating, intermediate cross-linking or high cross-linking. After 15 minutes, chambers were washed repeatedly. For surface characterization studies the slides were now washed with PBS. In the case of cell trace analysis, the culture inserts were filled with cell suspension and incubated for 60 minutes. After fixation for 10 min with 1% PFA, they were then washed 5 x with PBS. To finish the preparation in both cases, the culture insert was now removed and the entire container was filled with PBS and sealed for transport.

3.2.15.2 Image acquisition

We retrieved atomic force microscopy images with a MFP-3D AFM (Asylum Research) and cantilevers of the MSNL Type (Type F, triangular, Bruker). The nominal spring constant of

these cantilevers is 0.6 N/m and the resonance (= eigen) frequency in liquid is ~ 30 kHz. Imaging was performed in tapping mode in liquid. Resolution was set at 512 x 512 pixels. The mounted probes were attached to a glass slide fitting into the microscope stage with silica gel. After replacement of sample fluid with equilibrated PBS buffer, probes were placed in the AFM. 10 x 10 µm overview scans were acquired at a line rate of 0.25 Hertz and representative 2 x 2 µm regions were chosen for detailed views (line rate 0.5 Hertz). Images were processed and sections were analyzed with Asylum AFM software and IGOR.

3.2.16 Quantification

Because of the great diversity of the conducted experiments, various methods were used to quantify and describe the acquired data.

3.2.16.1 Single cell tracking & Migration

Platelet migration recorded by time lapse video microscopy was assessed by using FIJI (FIJI Is Just ImageJ). Two manual migration tracking plugins proved to be very useful: Manual Tracking and MTrackJ. (Schindelin et al., 2012, Meijering et al., 2012). Migration was defined by the displacement of a spread platelet of more than one cell diameter. Single, individually migrating platelets were tracked and further analyzed with the Chemotaxis Tool plugin.

Final output parameters consisted of individual cell velocities, cell directionality, distance accumulated and Euclidean distance. The total number of adherent cells was manually counted. The percentage of migrating cells was defined by dividing the number of tracks/total cell number. If fluorescent fibrinogen had been used and tracking was not indicated the GFP and PH channel of the final frame was extracted and analyzed as described in 3.2.16.4. Results were imported into excel and mean values and standard error of the mean were computed.

3.2.16.2 Particle Tracking

Fluorescence labeled fibrinogen could be tracked after being removed from the surface by migrating cells. After tresholding, we manually tracked 5-15 particles moving from the periphery to the center of each cell. Final output parameters consisted of individual cell and particle velocities, cell directionality, distance accumulated and Euclidean distance accumulated. Results were imported into Windows Excel and mean values and standard error

of the mean were computed. Additionally, the mean velocity of the cell for the given recording time was determined. We then identified the 10 fastest, slowest and average cells regarding migration speed and analyzed these groups further considering cell size and particle speed.

3.2.16.3 Form analysis

Form parameters are very important and useful tools to assess *in vitro* cell behavior like spreading and migration [197]. We conducted form analysis by manually defining Regions Of Interest (ROIs) in FIJI, and then using Analyze -> Analyze Particles with “Shape Descriptors” and “Area” checked in Analyze-> Set measurements. This provided us with the output parameters “Area”, “Circularity”, “Aspect Ratio”, “Roundness” and “Solidity”. Further data analysis was performed in Excel.

3.2.16.4 Still Image analysis

Still images were saved in Tagged Image File Format (TIFF) or as Virtual Slide Image (VSI), if containing multiple channels. Images were then imported to FIJI, where various parameters were assessed.

For the cross-linking experiments, we conducted a form analysis (see 3.2.16.3) of the cell form assessed in the DIC channel, as well as a total manual cell count including manual assessment of cell width. In the GFP channel, we performed tresholding (see 3.2.16.6) to measure total cleared area, and also manually marked migration path width and length for migrating cells.

Still images extracted from Time lapse video microscopy could easily be assessed regarding migration by counting the total adherent cell number and counting the number of tracks with more than one cell diameter in length. In biotinylation experiments and fibrin-Ab experiments we manually marked cell and pseudonucleus (PN) outlines as well as an area on each slide that was unaffected by cells, and then performed fluorescence intensity measurements using the Measure dialog. Results were exported to Microsoft Excel.

3.2.16.5 Analysis of flow cytometry data

Analysis of flow cytometric data was performed using Cytobank, an online tool useful for gating, analyzing and generating graphical output of standard lmd files. The negative control

was used to define negative events through setting a dividing gate. Cytobank generated statistics, which were exported to Excel and analyzed further.

3.2.16.6 Thresholding

If necessary, images were tresholded using FIJI. This proved to be very useful whenever overall clearance of fibrinogen was assessed in the GFP channel using fluorescent fibrinogen. First, brightness/contrast were adjusted appropriately, then, the images were converted to 8-bit images. Next, a Gaussian Blur filter (Sigma (Radius = 2.00) was applied, and the plugin "pseudo flat field correction" was used if illumination was irregular. After tresholding, the images were saved and the percentage of the cleared (black) surface to total surface area measured with the "Analyze Particles" dialog. For analysis of platelet-SA interaction, the GFP channel representing SA particles was tresholded and pseudocolored in grey (50% black). Average size was gained through ->Analyze particles. Manually, plt were marked in the corresponding PH channel and pseudocolored in grey (50% black). Image calculator was used for an "addition" operation of the two created images. Resulting black (100%) was tresholded and defined as plt-SA interaction area. This area was measured (->Analyze particles) and related to total SA covered area.

3.2.16.7 Statistics

For statistics, we used IBM SPSS Statistics 22. Data was prepared in Excel and then pasted into the SPSS Data Set panel. After adjusting variable labels, the appropriate statistical tests were performed. For non-parametric independent variables, we used either Kruskal-Wallis Independent Samples test (> two groups), with a Bonferroni post hoc test or Mann-Whitney-U Test (= two groups). For dichotomic independent variables, descriptive statistics with crosstabs, including Pearson's chi-square test, were performed. For non-parametric related variables, we applied Related-samples Wilcoxon Signed Rank Test (= two groups), Related Samples Friedman`s Two-Way Analysis of Variance by ranks (> two groups), or Related-Samples McNemar Test (dichotomic variables). For correlation studies, Spearman's rank correlation coefficient was determined. All indicated ± ranges represent standard error of the mean.

3.2.16.8 Data & Image Processing

Data was visualized with Origin Pro 8 (OriginLab) and Adobe Illustrator 6 (Adobe). The latter was also used for image processing and labeling.

4 Results

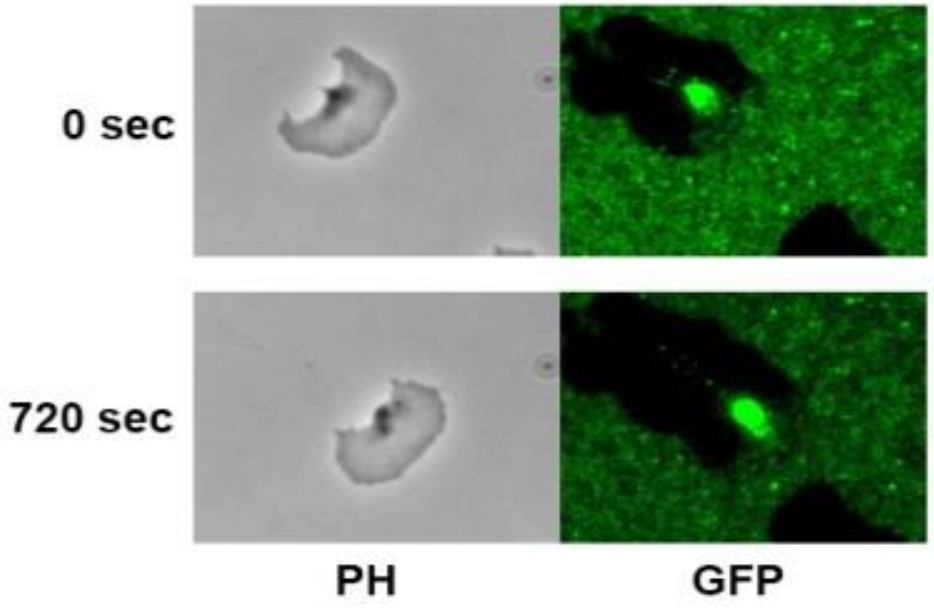
4.1 Platelet migration involves removal of fibrinogen

We started the project with a functioning migration assay and the knowledge that platelets were removing fibrinogen while migrating, leaving fibrinogen-free paths behind [162]. We first set out to analyze this process further.

4.1.1 Characterization of fibrinogen removal

As described in 3.2.3.1, we were able to establish a new migration assay within an IBIDI sticky slide of 0.4mm x 17mm x 3.8mm, which allowed us to better control coating, addition of cells and washing steps. After coating the slides with fluorescent fibrinogen/HSA, we added the cells and identified optimal fluorescence settings to avoid cell damage over the desired time frame and at the same time retain temporal resolution and fluorescence brightness necessary for a closer look at the uptake mechanism (see Figure 4.1.1).

Figure 4.1.1 - Fbg removal by a migrating platelet

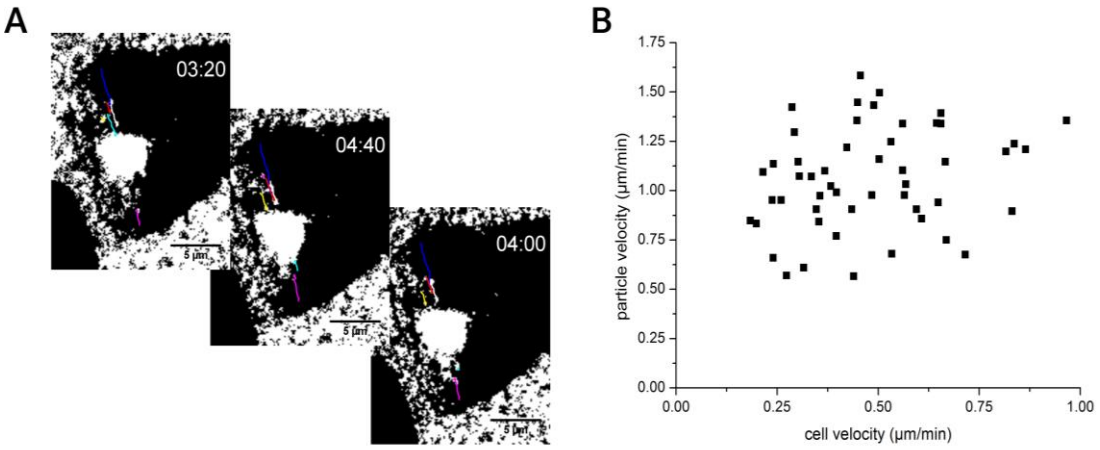


PH and GFP channels represent cell morphology and AlexaFluor488 labeled fluorescent Fbg, respectively. Locomotion and substrate clearance can be visualized by comparing 0 sec to 720 sec of video duration.

4.1.1.1 Dynamics of fibrinogen uptake

Preliminary time lapse video microscopy showed clearly that fluorescent fibrinogen particles removed by migrating platelets could be visualized and tracked over short distances.

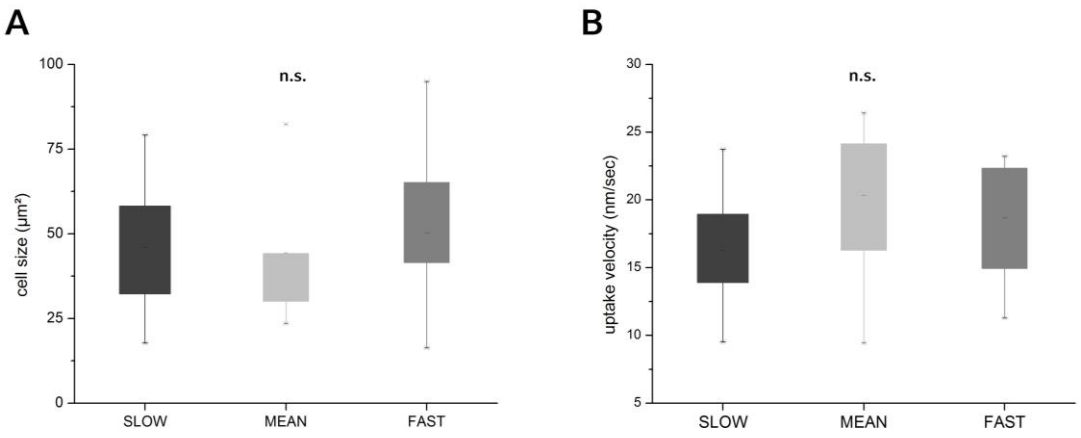
Figure 4.1.2 - Analysis of Fbg removal



A) Tresholded GFP-channel to track individual Fbg particles during uptake; colored lines represent tracked paths. B) plot of cell velocity and average particle velocity per cell, with no apparent correlation. Spearman-coefficient = 0.252

We now started to acquire multiple videos to obtain a sufficiently large sample size for later analysis. Cells included into this analysis migrated more than one cell diameter over the course of image acquisition and remained acceptably in focus. All imaged cells concentrated fibrinogen at their pseudonucleus by radial centralization.

Figure 4.1.3 - Characteristics of Fbg removal



Cell migration velocity distributed into three groups: Slow (0.24 µm/min), mean (0.48 µm/min) and fast (0.78 µm/min). No significant changes regarding cell size (A) and uptake velocity (B) were found in these groups. n=7, Kruskal-Wallis Test, p=0.678 and p=0.111, respectively.

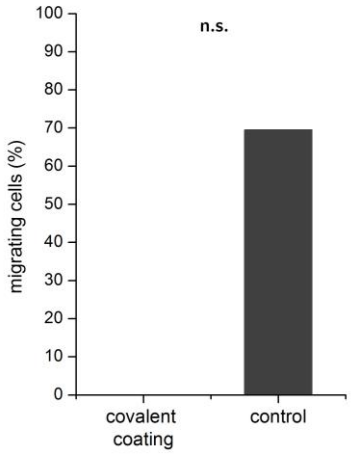
We found average particle centralization speed to be 1.06(±0.36) µm/min. We could not detect a positive or negative correlation between cell size or cell speed and uptake velocity, as depicted in Figure 4.1.2 and Figure 4.1.3.

4.1.1.3 Covalent coating of fibrinogen

Next, we investigated the mechanism of initial fibrinogen removal necessary for the ensuing centralization. We postulated a sheer mechanical force expended by migrating platelets, in contrast to possible enzymatic activity, for example secreted metalloproteases playing a role in interstitial migration of leucocytes [198]. Therefore, we covalently coated fibrinogen to our glass slides and assessed migration. Covalent bonds are intramolecular, strong bonds requiring about 1-2 nano Newton (nN) to be disrupted [199]. Platelets express about 80.000 copies of GPIIb/IIIa, which leads to a necessary force in the range of micro Newtons (µN) to disrupt the bound fibrinogen bonds [65]. In contrast, one platelet can exert on average 29 nN as a whole [100]. We therefore excluded the possibility that platelets could remove the substrate after covalently binding it to the cover slip, if the underlying mechanism is indeed based on mechanical force. We functionalized the cover slip glass with activated carboxyl groups, which covalently bind the Amino-groups of the fibrinogen proteins.

As shown in Figure 4.1.4, migration ceased entirely. We used once again fluorescently labeled fibrinogen to control for reduced or enhanced fibrinogen adsorption influencing our results, but could not detect any evidence for this. We therefore concluded that platelets do not cleave the substrate fibrinogen in our assay but rather remove it entirely from the glass slide.

Figure 4.1.4 - Covalent coating of Fbg

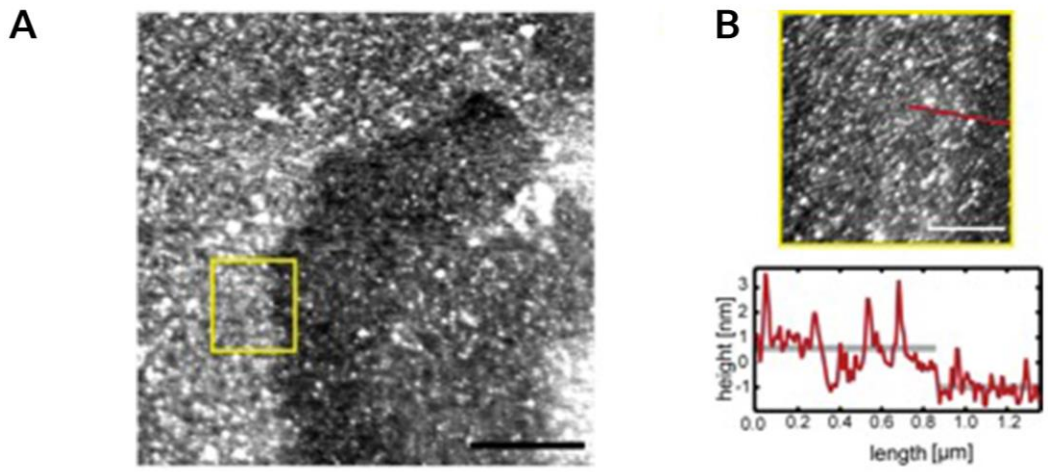


Covalent coating of Fbg to the glass surface to test the hypothesis of a mechanical removal by migrating platelets; indeed, in the control group, an average of 70% of cells migrated, compared to 0% in the covalent coating group. n=2, Mann-Whitney U Test p=0.333

4.1.1.4 AFM representation of migration tracks

We could visualize fibrinogen concentration and loss of fluorescence signals in the paths of migrating platelets. But is fibrinogen really removed by the migrating cells? To answer this specific question, we relied on an atomic force microscope (AFM). An AFM is very well suited for determining surface structure and topography. We used this research technique to determine if cell tracks defined by loss of fluorescence when coating with fluorescent fibrinogen go along with measurable substrate removal by assessing the topography of the fibrinogen coated surface. First, we measured fibrinogen coated slides without the addition of cells, and found the surface to be relatively homogenous within the scanned area of 10x10 µm. A more detailed scan of 2x2 µm in the same area revealed a level surface with alterations around 1 nm. (Figure 4.1.5 - AFM representation of tracks).

Figure 4.1.5 - AFM representation of tracks



A) 10x10 µm AFM representation of tracks in the height image, proving a physical removal of substrate. B) showing 2x2 µm detail of the transition, displaying a visible decline in height in the track.

Next, we looked at a similar coated slide fixated with Formaldehyde after the addition and migration of platelets. Despite of not having a fluorescent epifluorescence objective to

identify areas of interest, we were able to find a relevant spot by rapidly scanning multiple areas of the probe with a high line rate.

We finally imaged an area of 20x20 µm showing two areas of reduced height, compatible with parts of traces left by two migrating platelets (see Figure 4.1.5). We further imaged a high-resolution 2x2 µm height image of the transition zone between the possible trace and unaffected fibrinogen surface, showing an average drop of 1.5 nm in topography. We then identified a large structure in direction of the possible trace, very likely representing the migrated cell, substantiating our assumption. However, due to the limitations of the used AFM, and the large margin between the height-profiles of cell and surface, we could not obtain a high quality topographic image of the cell.

However, due to the very characteristic features and the high congruence between topographic and fluorescent images of comparable slides we concluded that platelet migratory tracks (a) involve the removal of substrate and (b) that this removal is visible topographically.

4.1.2 Internalization or surface transport?

After having confirmed and described the removal and centralization of fibrinogen by platelets, we became interested in one crucial question: After binding fibrinogen, and severing it from the surface, do platelets internalize the substrate, or do they transport it along the surface? In other words, do they use endocytosis to amass fibrinogen intracellularly, or is it surface-transport with a concentration of substrate (and probably receptor) on the cell?

4.1.2.1 Biotinylation assay

One way to address this question is to use a biotinylation assay, frequently used in the field for endocytosis/receptor-recycling research [180, 200]. We hypothesized that internalized NHS-SS-biotin- fibrinogen would not be accessible to a later added fluorescent streptavidin compound, and, at the same time, would be protected from cleavage by reducing agents, which cleave the (strept-)avidin binding site of NHS-SS-Biotin.

If in a next step the cell membrane would be permeabilized, the unaffected internal NHS-SS-biotin- fibrinogen compound should bind the fluorescent Streptavidin (see Figure 3.2.4). We therefore devised a list of experiments and control experiments to test these hypotheses (see Table 4.1 Biotinylation experiments).

First, we assessed if our permeabilization method with the amphipathic glycoside Saponin, which leaves holes in the cell membrane by interacting with cholesterol in the lipid

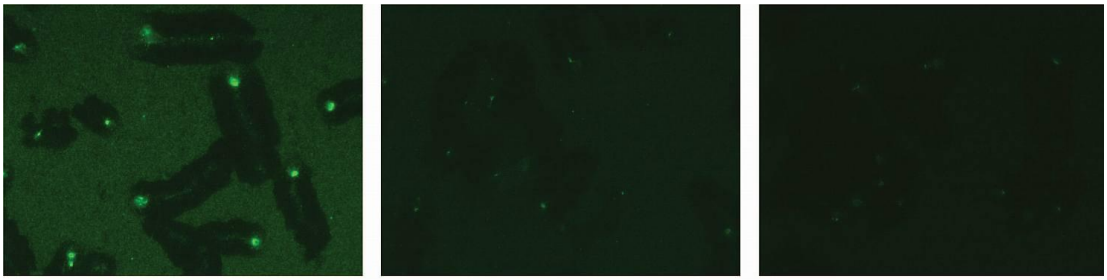
bilayer [201]. We fixated the migrated platelet probes and added a fluorescent Phalloidin-Alexa488 compound, either in the presence or absence of Saponin. Phalloidin is not membrane-permeable and detects specifically actin, an intracellular protein.

Table 4.1 Biotinylation experiments

No	(1) Surface coating	(2) Biotin cleavage (TCEP)	(3) Cell permeabilization (Saponin)	(4) Streptavidin 488
1	Biotinylated Fbg	no	no	yes
2	Biotinylated Fbg	yes	no	yes
3	Biotinylated Fbg	yes	yes	yes
4	Biotinylated Fbg	in combination with permeabilization	no	in combination with permeabilization
5	Non-labeled Fbg	no	no	yes
6	Non-labeled Fbg	yes	no	yes

As expected, fluorescence microscopy revealed only a cell signal above the background signal when incubating the probe with the permeabilizing agent, confirming the potency of Saponin in our setup (phalloidin fluorescence averaged 305.1 MFI in the Saponin treated cells vs. 129.0 MFI in the non-treated cells, n=1, n.s.).

Figure 4.1.6 - Fluorescence in Biotinylation experiments

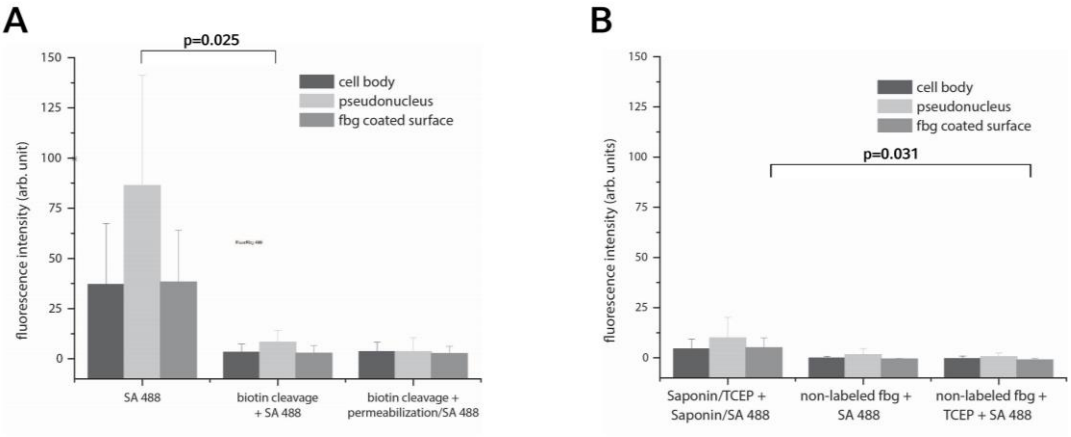


Example of fluorescence signal acquired from the three experimental groups; Fluorescence distribution makes surface transport of substrate likely. Streptavidin is able to bind surface and cell-bound Fbg; after cleavage, there is only globally reduced signal detectable; a subsequent permeabilization does not reveal intracellular biotinylated fibrinogen.

Next we fixated the migrated cells and (1) added fluorescent streptavidin, (2) added a reducing agent and the fluorescent streptavidin and (3) added a reducing agent and the fluorescent streptavidin in combination with the permeabilizing agent Saponin. Fluorescence

microscopy showed increased fluorescence of the Fbg coated surface, pseudonucleus and cell body in (1), with no detectable fluorescence in the other two experiments.

Figure 4.1.7 - Fluorescence distribution in Biotinylation experiments



A) Displays three main experiments also depicted in Figure 4.1.6. There is only a pronounced signal on the cell body, PN and surface in the SA 488 treated group, indicating surface transport of Fbg. n= 4-5, Kruskal-Wallis test: p=0.44(cellbody) p=0.2(PN), p=0.47(surface) significant post hoc test results displayed B) shows three additional control groups, using permeabilization in parallel to cleavage, and using unlabeled substrate to identify unspecific binding. n= 3-4, Kruskal-Wallis test: p=0.44(cellbody) p=0.2(PN), significant post hoc test results displayed.

We therefore concluded that biotinylated fibrinogen is not internalized, as these experiments support this notion. Apparently, added Streptavidin can bind to the centralized biotin-fibrinogen compounds, resulting in a strong fluorescent signal. At the same time, cell bound fibrinogen is also accessible for reducing agents, resulting in no fluorescence of cells (and surface) in experiments (2) and (3).

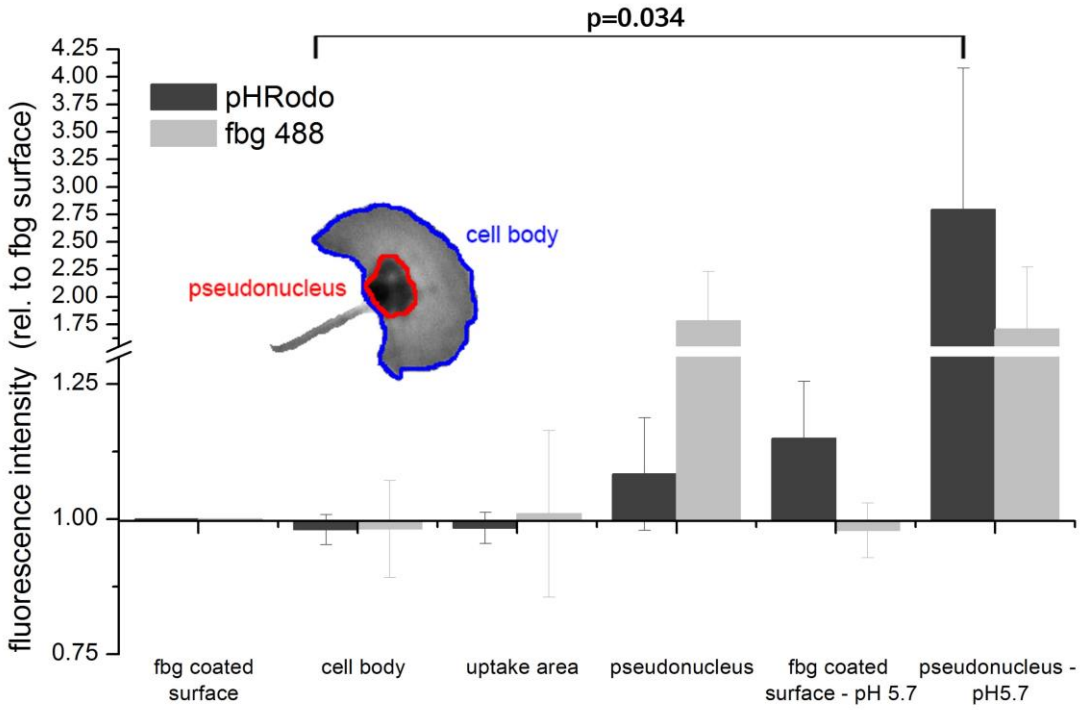
To make sure that streptavidin binds only to biotinylated fibrinogen we also conducted experiments (1) and (2) with unlabeled fibrinogen. As a negative control, we added Saponin to the cleavage and Streptavidin labeling step (see Figure 4.1.7 B). We could not detect any fluorescent signal above the background signal.
We infer that our control experiment setup makes it likely that our assay does allow us to draw conclusions on the internalization of the substrate. It points towards surface transport and concentration of fibrinogen on the surface of migrating platelets.

4.1.2.2 pH-sensing experiments

To further validate our findings, we used a pH sensitive dye, pHRodo Red. This method to assess internalization especially via Lysosomes is based on the lower pH present in Lysosomes (and to a lower degree also Endosomes) [202].

We used it as a compound with avidin, and coupled it to our biotinylated fibrinogen on the cover slip surface. To additionally visualize Fbg in a pH independent manner we in parallel incubated the slides with Streptavidin AlexaFluor488.

Figure 4.1.8 - pH sensing experiments



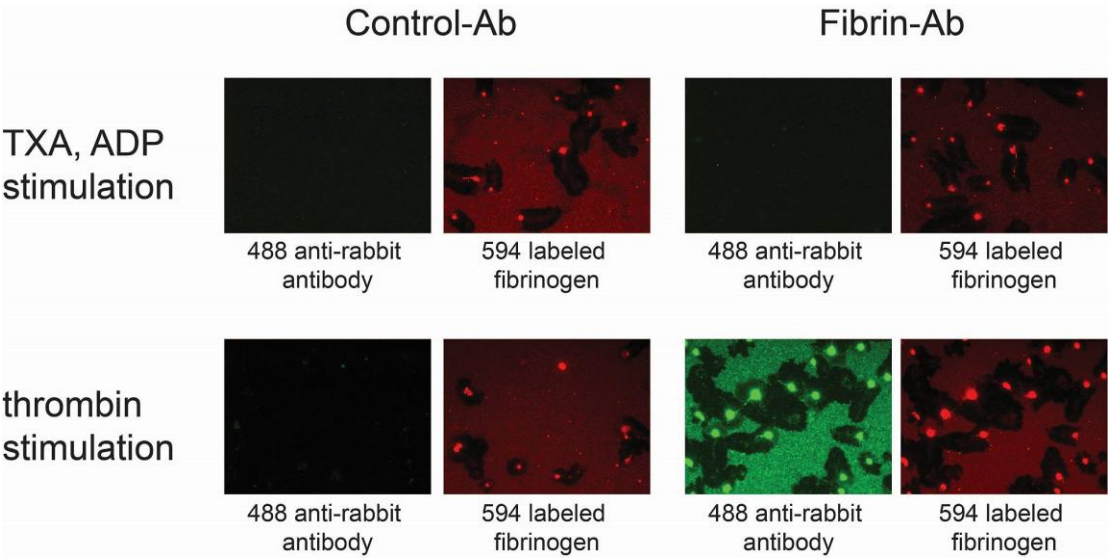
Ensuing time-lapse microscopy revealed unhindered uptake of particles in this setup without a visible change in the RFP channel. As a positive control, we flushed the chambers with buffer of pH 5.7, and compared mean fluorescence of Fbg surface and pseudonucleus. Providing evidence for the principal functionality of the assay, we determined a clear increase of RFP signal by lowering the pH.

We concluded that (1) the change of the signal by the Fbg surface indicates a working pH indicator, (2) Fbg concentrated by the cells remained subject to extracellular pH changes, and (3) Fbg concentrated by the cells remained at a pH above 5.7, about the minimum pH change observed in receptor mediated endocytosis. Consequently, this experiment points at a surface transport and accumulation of fibrinogen.

4.1.2.3 Fibrin visualization

Based on a recent article by *Kasahara et al*, we considered the possibility of fibrin generation on the platelet membrane [203]. It seemed very well possible that migrating platelets could convert fibrinogen and then form a centralized fibrin-clot on their surface, facilitating interaction with other platelets and formation of a thrombus.

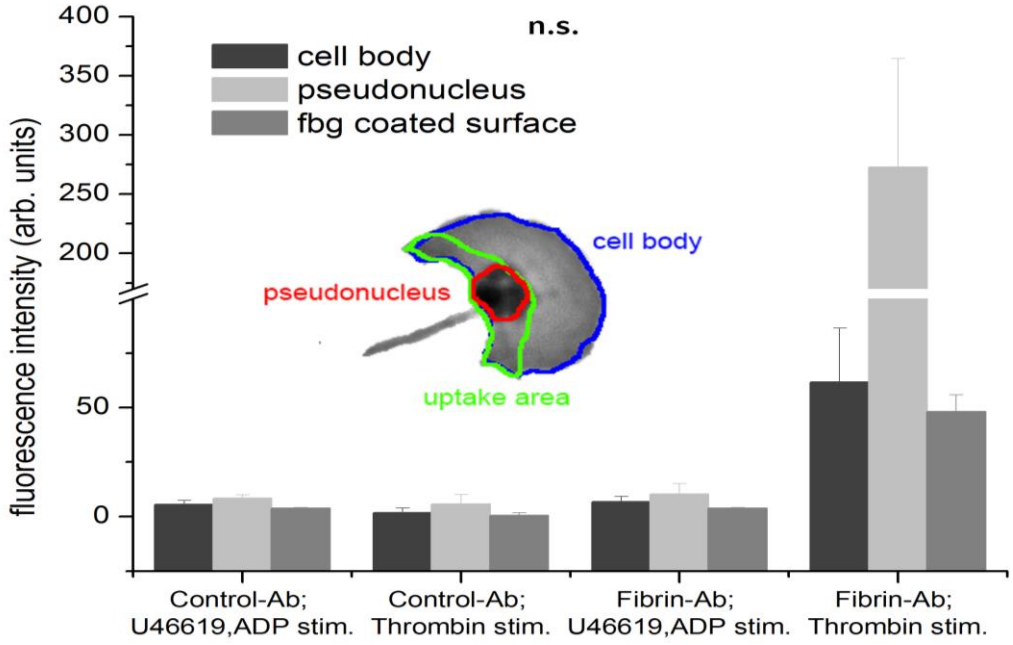
Figure 4.1.9 - Fibrin visualization



We used indirect IF to visualize Fibrin in our assay. In parallel, we used AF 594 Fibrinogen and recorded its distribution in the RFP channel. As expected, the control Ab did not bind to the substrate. The positive control, stimulated with Fibrin generating Thrombin, showed a strong signal. In our TXA,ADP stimulated standard assay we could not detect Thrombin, contradicting the idea that platelets might convert Fbg to Fibrin in our assay. Additionally, the Ab can bind to the concentrated substrate without cell permeabilization, indicating surface localization.

We therefore used the fibrin monoclonal anti-fibrin II chain shown to be specific for fibrin, failing to bind to fibrinogen [204]. To control the specificity in our assay, we devised three control experiments. We stimulated the cells either with U46619, a thromboxane A2 agonist, and ADP, or with thrombin alone. We used thrombin as a positive control being able to convert fibrinogen effectively to fibrin. In both cases, cells were able to migrate as indicated by the tracks of removed fluorescent fibrinogen photographed afterwards. Both groups were then subjected to
immunofluorescence, using the specific fibrin antibody mentioned above and using an anti-IgG control antibody.

Figure 4.1.10 - Fibrin fluorescent signal distribution



Distribution of fibrin signal; there was only pronounced fluorescence detectable in the positive control group, using external thrombin to generate fibrin. See Figure 4.1.9 and text for details. n=2-3, Kruskal-Wallis Test n.s.: p=0.69 (Cell body) p= 0.79 (pseudonucleus) p=0.51 (surface).

Clearly, the only group showing increased fluorescence and therefore specific binding of the fibrin antibody was the positive control stimulated with fibrin. We concluded that (1) our antibody was specific to fibrin in our assay, (2) that platelets in our setup did not convert

surface bound fibrinogen to fibrin, at least not in detectable amounts, (3) and that platelets were able to migrate on, interact with and centralize fibrin as well as fibrinogen.

4.2 The effect of surface characteristics on platelet behavior

The results discussed in 4.1.2.3 were quite surprising to us and offered a new direction in research. We now became increasingly interested how the extracellular microenvironment modulates platelet migration. If platelets were able to migrate on fibrinogen and fibrin monomers, we asked what would happen in the case of fibrin cross-linking. *In vivo*, this cross-linking of Fibrin has vital importance in hemostasis and wound healing.

4.2.1 Fibrin cross-linking

We hypothesized that in our assay, surface characteristics where very important in determining platelet behavior. We therefore devised a set of experiments in which we increasingly cross-linked the fibrin coated to the surface. This would affect binding forces and topography of the surface, and would therefore prove useful in assessing the role of the (micro-) environment on platelet migration.

4.2.1.1 Fibrin cross-linking series

By varying the amount of plasma added to the coating solution (with constant amounts of fluorescent fibrinogen, HSA, calcium and thrombin), we observed different patterns when visualizing the fluorescent fibrinogen (see Figure 4.2.1).

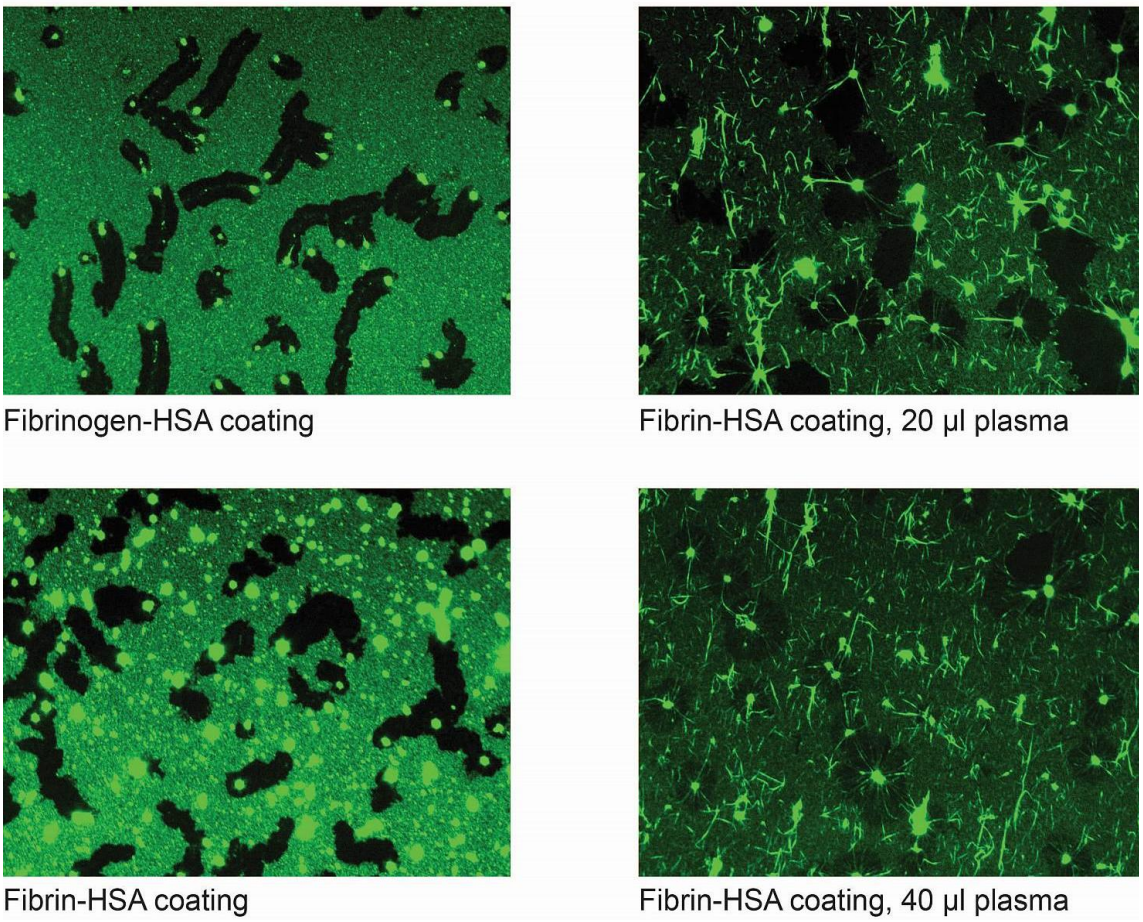
It appeared that increasing amounts of plasma, leading to an increase in fibrinogen (and albumin) concentrations, led to cross-linking visible in the form of fluorescent fibrin strands, as shown in Figure 4.2.1- Fibrin cross-linking surfaces. This is consistent with the notion that the relation of thrombin to fibrinogen is crucial in determining strand formation and thickness. Interestingly, more substrate/less enzyme leads to stronger and thicker strands [205].

Next, we placed activated platelets on our engineered surfaces and visualized migration and fibrinogen interaction in parallel. We discovered that increased cross-linking reduced migration in a gradual manner, leading to total abolishment of migration at a certain level of cross linking.

To gain more insight into cell behavior on representative surfaces, we devised experiments consisting of increased cross-linking, with fibrinogen as a control and maximum

cross-linking defined as a plasma-thrombin ratio completely abolishing migration. The results are visualized in Figure 4.2.2. We analyzed the percentage of migrating cells, the area cleared per cell, the track width per cell, the reach per cell, and two cell form parameters, the aspect ratio and cell size.

Figure 4.2.1- Fibrin cross-linking surfaces



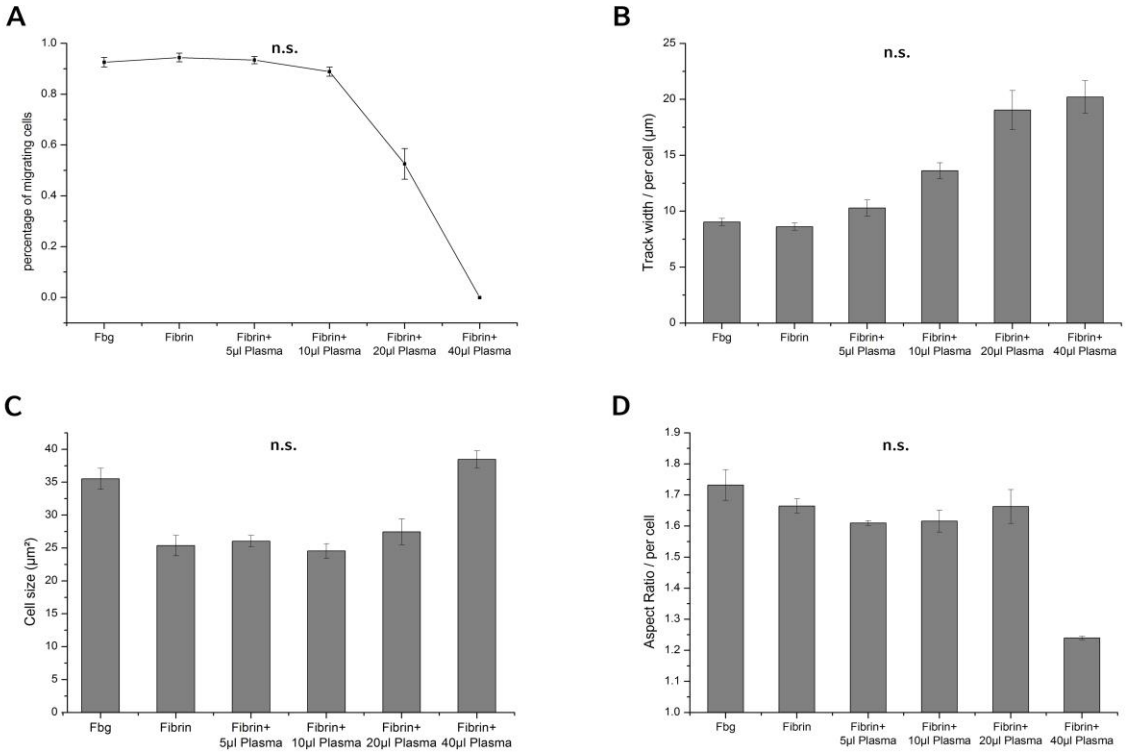
Fibrin cross-linking series. Normal migration on the fibrinogen -HSA as well as the fibrin-HSA coated surfaces, as indicated by cell tracks. Intermediate cross-linking (20 µl of plasma) shows some cells migrating and others retracting. High cross-linking (40 µl plasma) leads to retraction of cells.

The percentage of migrating cells remained consistently over 90(±2)% of spread platelets, decreasing sharply to 53(±6)% and then to 0(±0)% in the two highest cross-linking

experiments. Cross-linking seems to seriously affect migration at some level, disabling this platelet feature. We conclude that mechanical properties altered through fibrin strand formation changes platelet behavior and can limit migration.

Another interesting parameter of platelet activity measured was the area cleared per cell. Slight cross-linking (5-10 µl Plasma) seems to slightly increase the area cleared. Strikingly, even substrate composition diminishing or completely abolishing migration is cleared, albeit to a smaller extent. Under these conditions, cells seem to retract the substrate in a circular manner.

Figure 4.2.2 - Cell response to cross-linking



Cell behavior dependent on increasing cross-linking. A) percentage of migrating cells drops sharply at intermediate cross-linking to 0% with on a highly cross-linked surface. B) The track with of cells increases. C) cell size shows a sharp increase at high cross-linking. D) Aspect ratio shows a sharp decrease on surface leading to retraction. See text for details. n=3, Kruskal-Wallis test, n.s.

We defined the reach per cell as the track width per cell divided by its greatest diameter. This parameter proved useful to assess platelet-substrate interaction under increasingly cross-linked conditions. We observed that cross-linking enabled platelets to interact with substrate not directly in contact with their receptors by acting not on single fibrin(ogen) molecules but on increasingly larger mechanically connected substrate sections. This offered a plausible explanation of the increased area cleared under slight cross-linking conditions. The reach per platelet seemed to grow with increasing cross-linking, reaching a maximum at intermediate cross-linking (20 µl Plasma) and decreasing slightly at higher cross-linking. This might be due to changes in the two factors influencing the substrate in this regard: The increasing area of interconnected substrate and the increasing resistance of the substrate.

We also assessed two cell form parameters. The cell size was relatively constant across different experiments, being elevated on fibrinogen and high cross linking. The results for the aspect ratio (AR) were more varied. AR is a parameter for the relation between width and length of a cell. The aspect ratio decreased sharply in the group of non-migrating platelets retracting the substrate, compared to the other experiments. In our assay, the AR seems to be a morphological correlate to changed cell behavior, switching from migration to retraction. This observation fits well into the conception of cell polarization, leading to an increased AR, as a prerequisite for migration.

We conclude that we can observe two distinct patterns of platelet behavior, migration and retraction, by altering the surface properties. This change in behavior seems to a degree gradual, although the sudden change in AR shows a clear phenotype associated with migration and pure retraction.

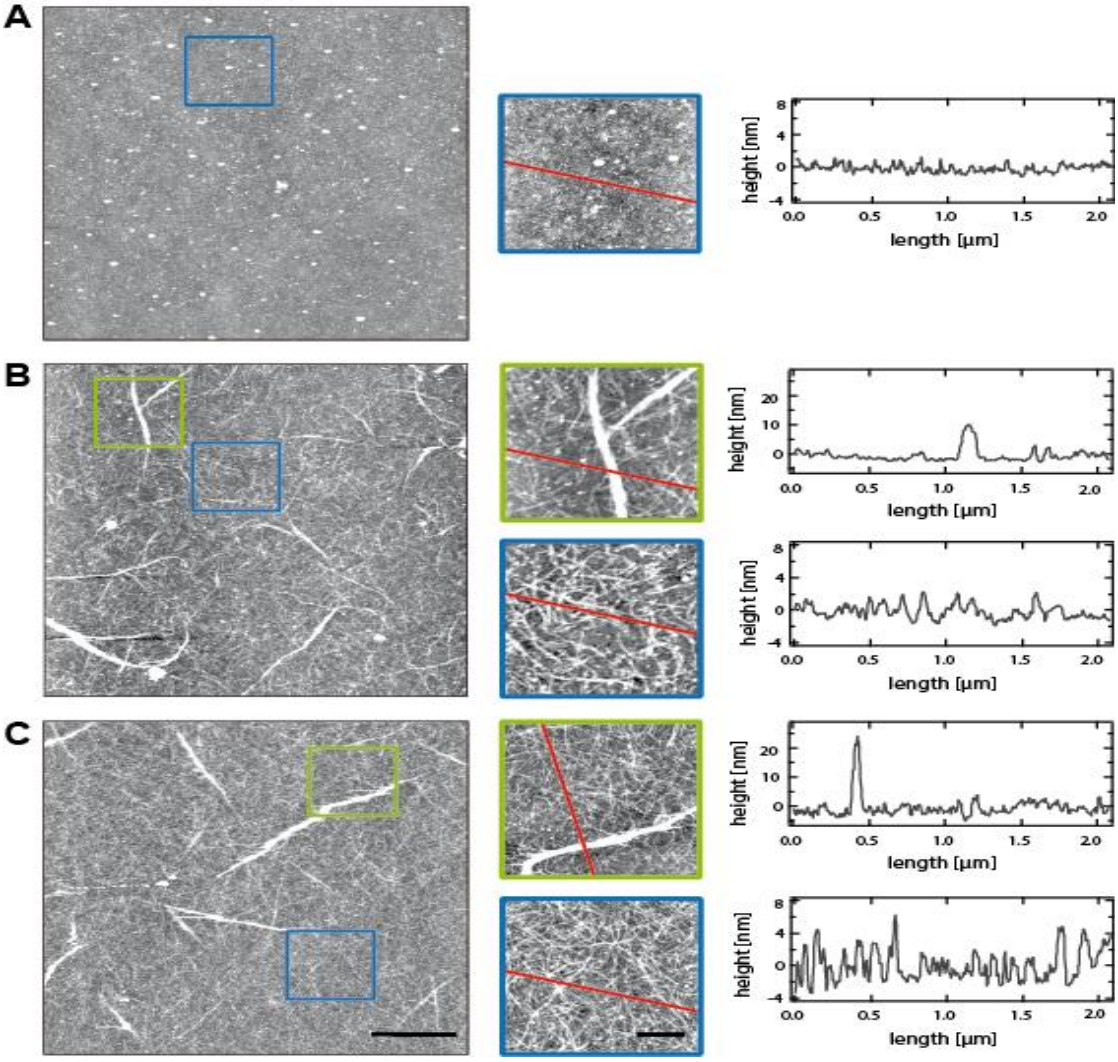
4.2.1.2 Characterization of the surface with AFM

We now suspected the surface properties to be highly influential in determining platelet behavior. Therefore we wanted to further characterize our engineered fibrin(ogen) surfaces with increasing cross-linking. Of particular interest to us was fibrinogen (93 % migrating cells), intermediate cross-linking (20 µl Plasma, 53% migrating cells) and high cross-linking (40 µl Plasma, 0% migrating cells), representing all the observed platelet phenotypes, including the transition from migration to retraction. We chose to use an atomic force microscope, as this device can topographically scan surfaces and produce a height-image.

The results fitted perfectly to our epifluorescence images, regarding surface structure and fibrin strands. The fibrinogen surface appeared homogenous, with single aggregates showing increased height. An analyzed section revealed about +/- 1 nanometer alterations in the height profile.

In contrast, the intermediate cross-linked probe showed fibers of very heterogeneous size. A detailed acquisition of a large fiber revealed a height of about 10 nm. Interestingly, an area without large fibers still revealed a cross-linked ultrastructure, with fibers of about +/- 2 nanometers.

Figure 4.2.3 - AFM characterization of cross-linking



AFM surface scans of A) Fbg-HSA, B) intermediate cross-linking and C) high cross-linking surfaces. Representative 10x10 µm images were acquired and two 2x2 µm details were imaged to assess fiber height (green) and ultrastructure (blue). Prominent differences were visible. Of particular relevance for cell response seems to be the ultrastructure, as all cells on a given slide display comparable behavior independent if in contact with bigger fibers or not.

The high cross-linking probe revealed even larger fibers of over 20 nm in height, in combination with a highly cross-linked ultrastructure with fibers of +/- 4 nanometers. Especially the ultrastructure was of great interest to us, as the resolution of our regular epifluorescence microscope could not reveal these details.

4.2.2 Plasmin reversal of fibrin cross-linking

In vivo, fibrin containing clots in hemostasis and thrombosis are constantly formed and dissolved [89]. Consequently, activated platelets can encounter changing and reversible substrate properties. We have discovered that platelet behavior is dependent on the substrate – absence of/minimal cross-linking triggering migration, high cross-linking leading to circular retraction of substrate. As a logical conclusion, we asked if platelet behavior would be reversible with changing substrate properties.

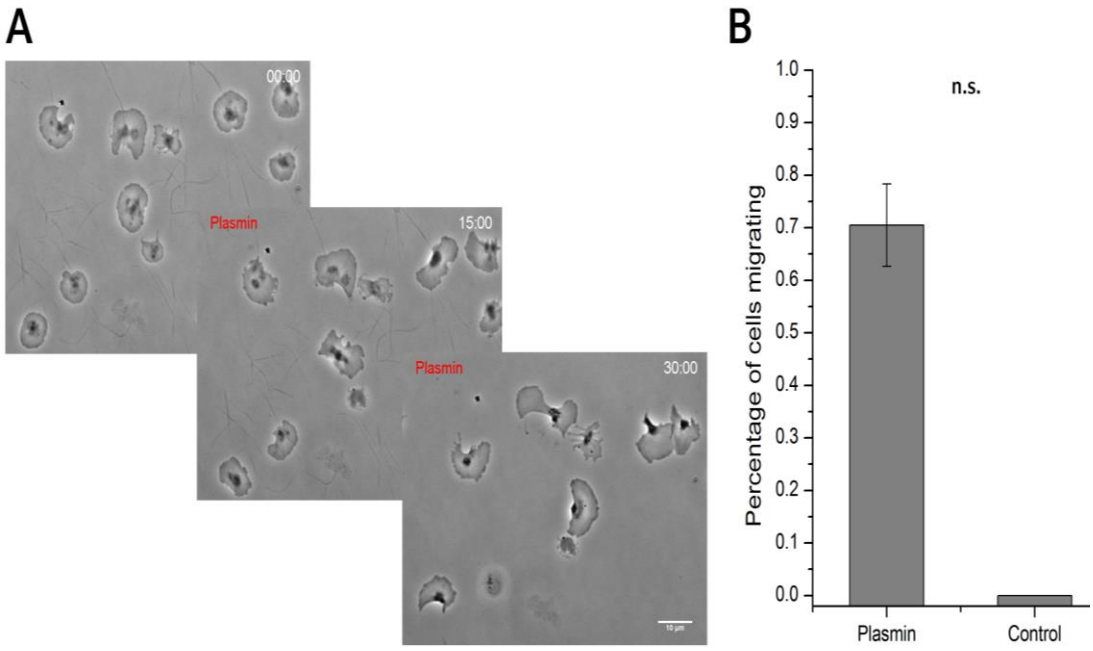
4.2.2.1 Reversal of high cross-linking

We tried to answer this question by devising a strongly cross-linked Fibrin substrate with no migration visible in previous experiments. In one group, we added Plasmin, which cleaves Fibrin fibers (see 1.1.1), in the other only Tyrodes solution as a control. Preliminary experiments helped us to establish a working concentration of Plasmin with gradual cleavage of Fibrin in 10-15 minutes, assessed through the waning of the GFP signal (We used Fbg AF 488 for coating) over time.

In the Plasmin treated group, an average of 70(±7.9)% of visualized platelets fulfilled the criteria of migration after being exposed 35 min to plasmin, versus 0(±0)% in the control group. But not only migration was significantly enhanced by Plasmin treatment; aspect ratio increased, indicating polarization of the cell, similar to the results described in 4.2.1.1.

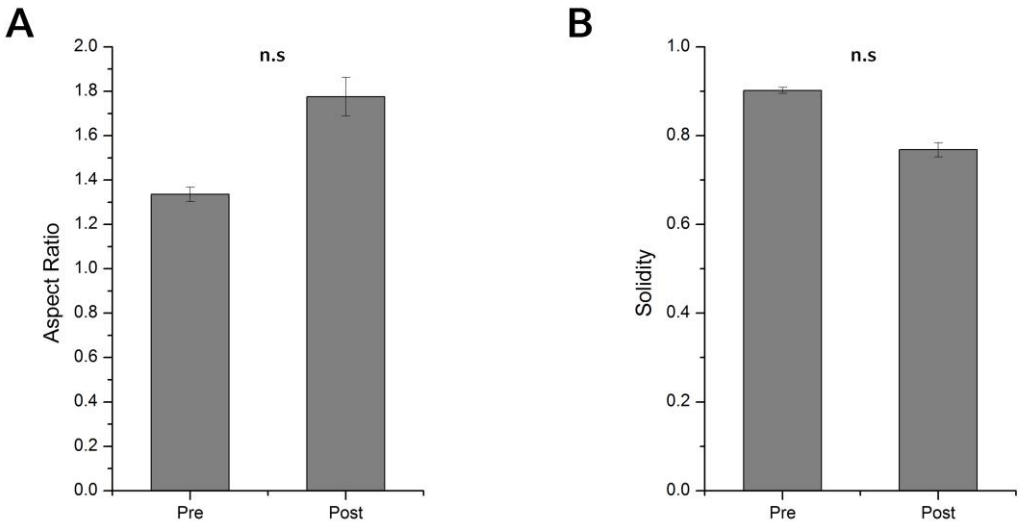
Solidity is defined as the area of the actual cell divided by the area of a polygon connecting all membrane points. It is an indicator of the compactness of a cell. Solidity decreased significantly with reversal of cross linking by addition of plasmin. The decrease can be explained by the more homogenous surface after plasmin treatment. We conclude that platelet behavior in our assay is (1) indeed predominantly dependent on surface characteristics and (2) can be adapted rapidly to changes in the local microenvironment.

Figure 4.2.4 - Plasmin reversal



Reversibility of platelet behavior through Plasmin. A) cells retract on highly cross-linked substrate. Still images after Plasmin addition at 15 and 30 mins show morphological change and migration. B) An average of 70% of platelets migrate after Plasmin treatment, compared to 0% in the mock treated control group. n=3 Mann-Whitney U Test, p=0.1 n.s.

Figure 4.2.5 - Effect of Plasmin reversal on cell morphology



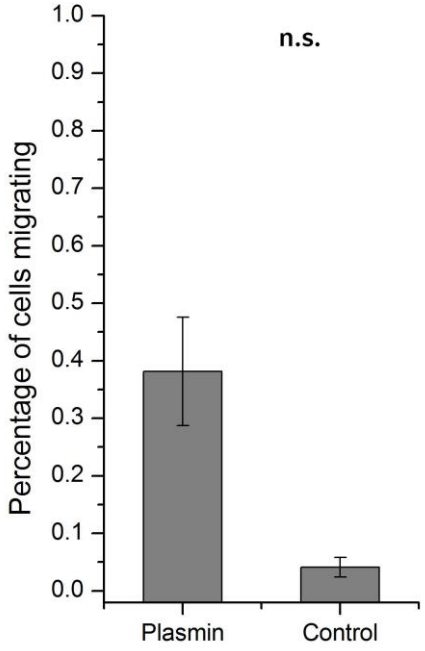
Aspect ratio (A) and solidity (B) assessed just before (pre) and after (post) plasmin treatment. There is a trend to an increase in AR and a decrease in solidity. n=3, Wilcoxon Signed Rank Test, n.s.

4.2.2.2 Reversal of Fibrin cross-linking without plasma

However, one weakness of our cross-linking assay is the use of plasma for fibrin fiber generation. Plasma not only contains coagulation factors but also platelet activators, enzymatic regulators of hemostasis and adhesive proteins other than fibrinogen (e.g. fibronectin, vitronectin); it therefore multiplies the possible confounders in our experiment. As the experiment described in 4.2.2.1is both crucial and central to our hypothesis of specific microenvironment-induced and reversible platelet behavior, we conducted the same experiments in an altered setup avoiding plasma.

As *Gabriel et al.* have pointed out, the decisive factor in fibrin fiber thickness and structure is the thrombin/fibrinogen ratio [170]. We concluded that the main effect of plasma addition lay in the additional Fbg (and maybe albumin) added to the coating solution. We computed the average amount of Fbg, Albumin and Calcium present in plasma volume to be replaced, and used these substances instead.

Figure 4.2.6 - Plasmin reversal of Plasma free cross-linking assay



Experiment analog to Figure 4.2.4, but without the use of Plasma to exclude plasma specific effects. An average of 38.2% of cells, compared to 4.1% in the control group, migrate. n=3, Mann Whitney U Test, p=0.1

Analysis of the experiments conducted exactly as the described plasmin reversal experiments with the slight alteration of plasma-free coating, yielded similar results overall. It seems that plasma factors apart from Fbg, Albumin and Calcium do not have a significant influence on our assay. However, the migration ratio of the plasmin treated group declined to 38.2(±9.4)%, with 4.1(±1.7)% of control platelets migrating as well. We attribute the less pronounced differences between Plasmin and Control groups to the lack of optimization of the coating, described for the Plasma cross-linking in 4.2.1.1.

4.3 Mechanosensing of migrating platelets

We had now gained insight into the strong influence of surface characteristics on platelet behavior, and on migration in particular. It was clear that these environmental clues must somehow be transferred to the internal of the cell, where they would influence the signaling loops controlling spreading, migration, and retraction. As the AFM and reversibility studies hinted at a solely “mechanical” difference of cross-linking causing profound differences in platelet response, we attributed a central role to mechanosensing. Mechanosensing is an important feature of various cell types, and of particular importance in migration [206]. If mechanotaxis in our case relies on changes of elasticity/rigidity (durotaxis), adhesiveness (haptotaxis), or a combination of the two, remains to be seen.

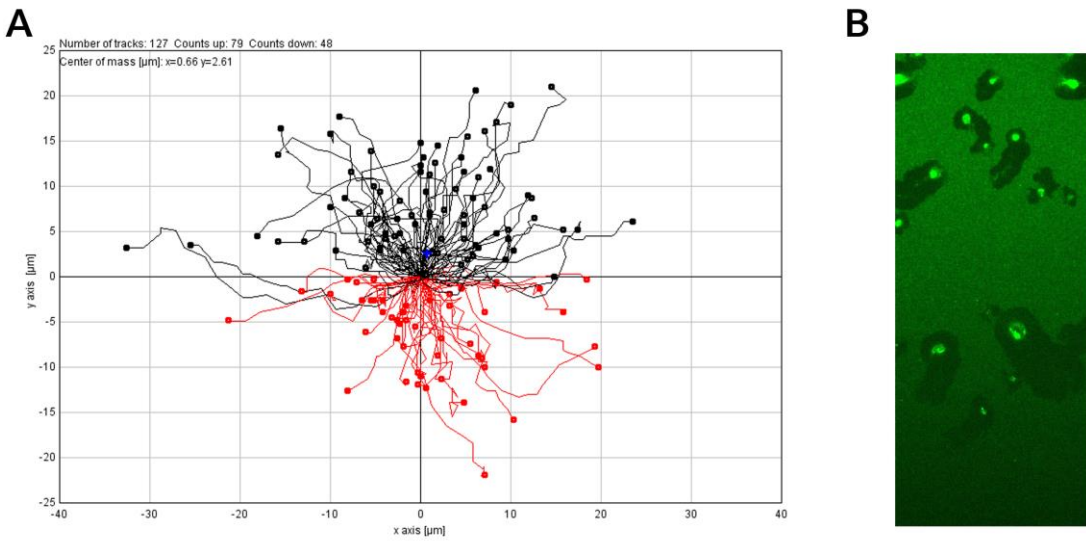
In the context of migration, mechanosensing is generally not used to explain overall cell behavior, but considered a mechanism leading to directionality. We became interested in the effect of matrix inhomogeneity on platelet migration, and assessed its effect with two experimental setups – a fibrinogen gradient and a steep change in coating density (edge).

4.3.1 Platelet response to fibrinogen gradient coating

There are countless protocols and commercial solutions to creating gradients *in vitro*. However, due to our two component coating - Fbg and Albumin – and the HMDS treatment of our slides, most common methods were not applicable. Fluorescence controls and cell migration indices showed the most efficient coating method to be the addition of highly concentrated coating solution to one reservoir. The slow diffusion of Fbg/Alb created a visible decline of fluorescence intensity in the chamber proportional to the distance to the reservoir. In a next step, we added activated platelets and incubated them for a total of 60 min at 37°C, as described before (see 3.2.4 and 3.2.5).

We then acquired still images of areas that showed linear decline of fluorescence. To avoid differences in coating density, pictures were taken on a line perpendicular to the diffusion gradient. Migration tracks were digitalized and analyzed in a 2D matrix. We found that of a total of 127 analyzed cells, 79 (62.2%) migrated toward higher fibrinogen densities, and only 48 (37.8%) towards lower densities. We reasoned that obviously platelets are mechanosensitive towards changes in substrate density, and that they preferably migrate towards higher densities.

Figure 4.3.1 - Effect of a Fbg gradient on cell directionality



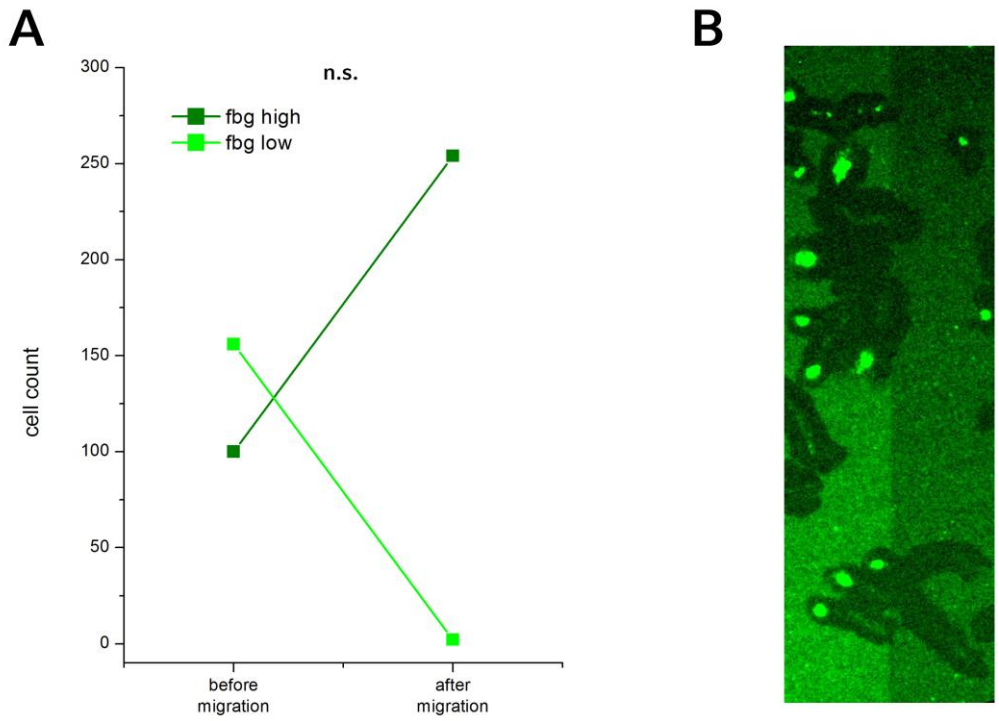
Assessment of the effect of a fibrinogen gradient on cell directionality. A) depicts cell tracks of 127 cells over 60 mins in a 2D space. 79 migrate in the direction of the gradient, 48 against. B) shows the fluorescent control for visualizing the gradient through Fbg AF 488 signal.

However, our data does not provide a black and white picture as there is still a large population of platelets not reacting to the change in density in a comparable way. The reason for this might be that other signals not measured play a similarly important role, or that our coating method and its assessment through fluorescence intensity is too crude a tool for the detection of subtle effects/coating irregularities. Moreover, it is possible that the gradient involved is not steep enough to create a stereotypical response.

4.3.2 Platelet response to fibrinogen edge coating

This possibility led us to the idea of creating an extremely steep gradient of under one micrometer, more or less a “step” or “edge” in fluorescence intensity. To achieve this, we tried various coating procedures, for example to pipet a drop of 2x concentrated coating solution onto the slide before attaching the IBIDI chamber. This approach created a visible edge of fluorescence intensity. We acquired fluorescence still images showing the tracks of migrated platelets as well as the intensity edge. We limited our analysis to cells that during their course of migration had been in contact with the edge.

Figure 4.3.2 - Effect of a Fbg edge on cell directionality



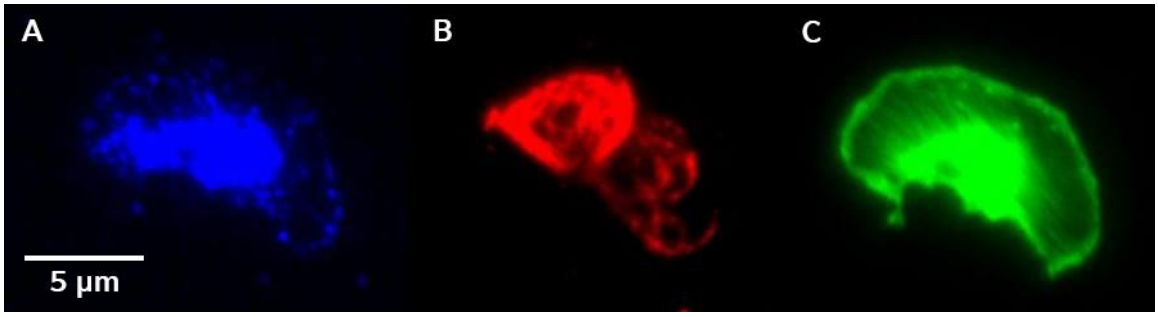
A) Cell positions were analyzed regarding fbg high and low coated areas before and after cell migration of platelets that came into contact with the edge. n=4, Related Samples Wilcoxon Signed Rank Test, p=0.68 B) Fluorescent representation of the steep drop in fluorescence intensity attributed to a drop in fbg density.

Of a total of 256 analyzed migrating platelets, 39.1% spread in the high fibrinogen coated, 60.9% in the low fibrinogen coated areas. After 60 minutes of migration, 99.2% of these cells were localized in the highly coated areas. This clearly shows stereotypical response of platelets to steep gradients in surface substrate, with a pronounced directionality towards higher substrate densities. Especially pronounced inhomogeneity on a µm scale, which can be sensed by a single adherent platelet at a given time point seems to provoke a strong mechanosensitive response.

4.4 Cellular mechanisms of migration and retraction

Migration and retraction rely on very specific intracellular signaling pathways and effector molecules. For other cell types, this has been described in great detail, but migration of platelets is a novel, not well described function [10]. Consequently, we tried to evaluate the role of major cytoskeletal components as well as the prominent platelet integrin GPIIb/IIIa in platelet migration.

Figure 4.4.1 - Cytoskeletal components in migrating platelets



IF of migrating platelets, pseudocolored; A) displays active, phosphorylated Myosin II, B) shows β-tubulin, C) F-Actin bundles

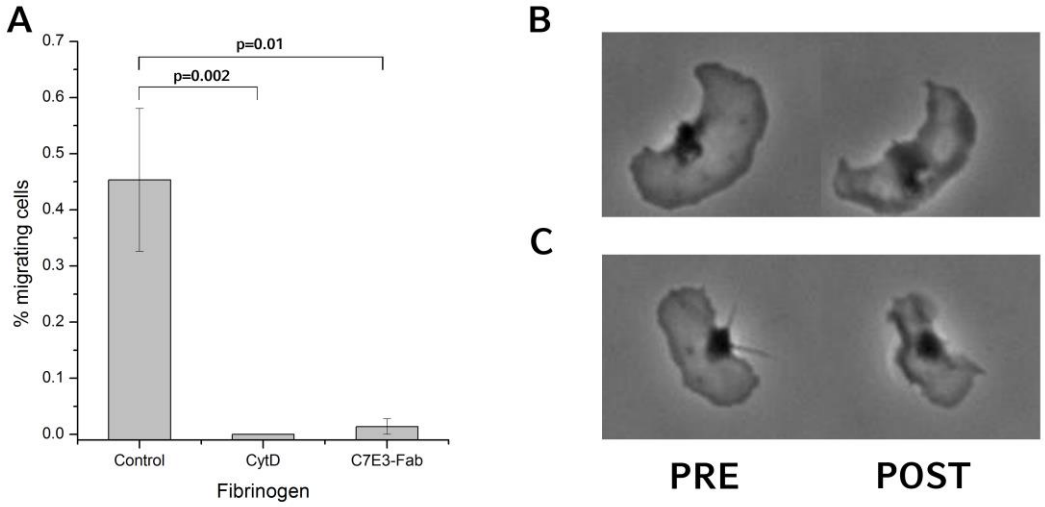
4.4.1 The role of actin and integrins

Actin is the most central component of the cytoskeleton and is involved in almost all aspects of platelet function, from aggregation and retraction to adhesion and shape change. Its role is so central that for example thrombelastometric measurements that exclude platelet function use the potent actin polymerization inhibitor Cytochalasin D to virtually block every aspect of platelet function [207, 208].

The most abundant integrin that is also central to platelet function is CD41, also called GPIIb/IIIa. This integrin mediates platelet adherence to fibrinogen and fibrin, and was therefore a central candidate for the mediation of adhesion in our assay [209]. Indeed, preliminary experiments had shown the necessity of functional GPIIb/IIIa in our setup, however, a precise comparison of its function in retraction and migration had not been performed. Luckily, there is an effective and well-described inhibitor available: A monoclonal antibody fragment, C7E3-Fab, also known as Abciximab. Abciximab is actually used to treat a subset of patients undergoing acute percutaneous coronary intervention due to its strong effect on platelet function, underlining its specificity [210].

We tested the effect of Cytochalasin D and C7E3-Fab on migrating and retracting platelets by adding the inhibitors after initiation of migration/retraction. This was crucial as we did not want to analyze the already known effect of actin/integrin inhibition on platelet spreading, which precedes migration. Results are shown in Figure 4.4.2 and Figure 4.4.3.

Figure 4.4.2 - Effect of Actin and GPIIb/IIIa inhibition on migrating platelets



A) % of migrating cells in Control, CytD (Actin inhibition) and C7E3-Fab (GPIIb/IIIa inhibition) treated groups. n=5, Kruskal-Wallis test p= 0.001, post hoc test results displayed. B) effect of CytD on the morphology of migrating platelets: lamellipodium and PN change reflection C) effect of C7E3-Fab: Flapping back of lamellipodium indicates inability to form new/loss of adhesions.

4.4.1.1 The role of actin

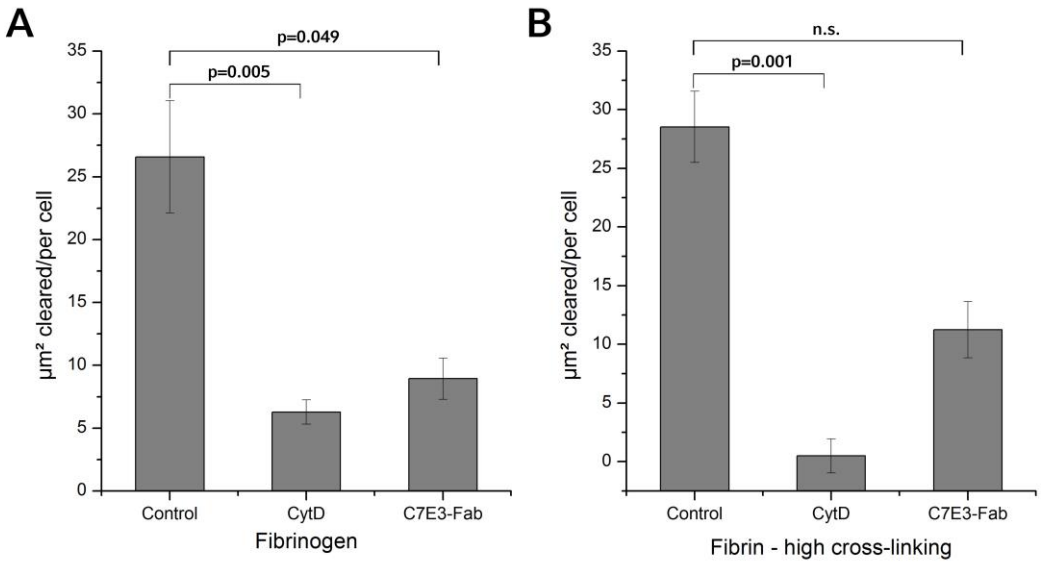
As expected, platelets treated with Cytochalasin D (CytD) quickly stopped forward motion. Additionally, the constant ruffling of the lamellipodium stopped abruptly, indicating that this is dependent on actin polymerization. This was the case with all coatings. Actin polymerization seems to be central to migration as 0(±0)% of platelets on fbg were able to migrate compared to 45.3(±12.7)% in the control. Moreover, substrate clearance per cell stalled in all three groups. The low percentages of migrating cells in the control group can be explained by the short imaging period of only 12 minutes in total.

We conclude that actin is central to migration and retraction of individual platelets.

4.4.1.2 The role of integrin IIb/IIIa

Integrin IIb/IIIa inhibition also virtually abolished migration (1.4(±1.4)% migrating cells/spreaded cells) but the effect on the area cleared per cell seemed less pronounced than in the Cyt D groups, especially on high cross-linked fibrinogen. We observed that fibrin(ogen) already bound to active GPIIb/IIIa would be centralized even after C7E3-Fab treatment, and , because of the increased reach of platelets placed on cross-linked fibrin, remaining activity could explain a comparatively higher clearance per cell in these groups.

Figure 4.4.3 - Effect of Actin and GPIIb/IIIa inhibition on substrate clearance



Substrate removal in Control, CytD (Actin inhibition) and C7E3-Fab (GPIIb/IIIa inhibition) treated groups, displaying a visible effect of CytD and C7E3-Fab in both groups. A) on fibrinogen surface (migration) n=5, Kruskal-Wallis test p= 0.005, post hoc test results displayed. B) on fibrin high cross-linking (retraction) n=5, Kruskal-Wallis test p= 0.001, post hoc test results displayed.

Morphologically, the effect of C3E7-Fab is quite distinct from the CytD effect. Platelets exhibit an increased oscillation of the lamellipodium, probably due to the inability to form new adhesions with the substrate. Traction force generation is key to migration. In most cases, it is dependent on integrin function linking ECM to cytoskeleton [10]. We report that this seems also true for migrating platelets, and in the case of Fibrin(ogen) as substrate, specifically GP IIb/IIIa is necessary for retraction and migration.

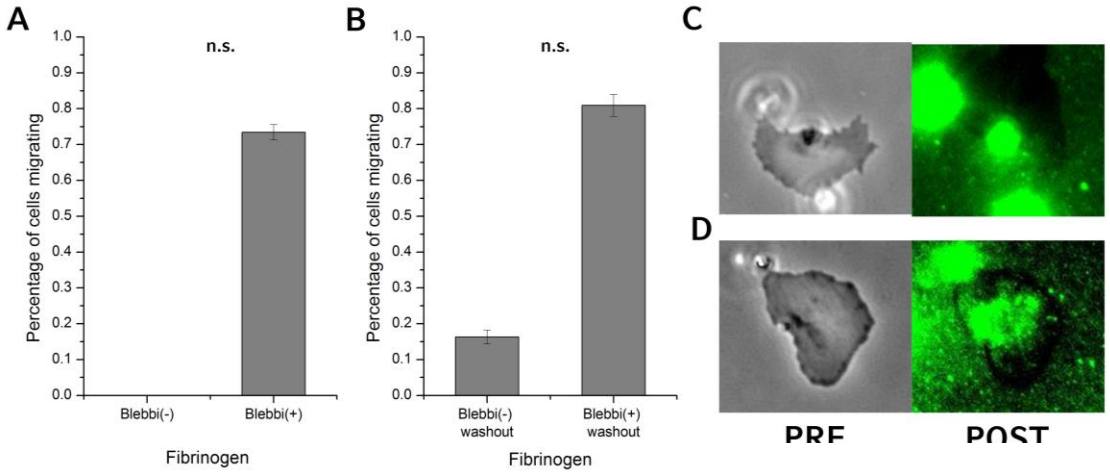
4.4.2 The role of myosin

In migrating cells, retraction of the trailing edge necessary for detachment and propulsion is often dependent on myosin activity [211]. Platelets contain functional Myosin IIa which has a central role in shape change, spreading and also hemostatic function, but does not influence aggregation [212]. It also mediates retraction in platelets [173]. Myosin IIa function is regulated through two pathways: The myosin light chain Kinase (MLCK) and the Rho associated Kinase (ROCK) pathway. Taken together, we became interested in the role of myosin IIa and its activators in our migration and retraction assay.

4.4.2.1 Complete non muscle Myosin II inhibition

Unfortunately, we did not have blood from a May-Hegglin anomaly, Fechtner or Sebastian syndrome at our disposal. These three syndromes are all based on a genetic MHY9 mutation. MHY9 is the gene coding for Myosin IIa in platelets, monocytes and granulocytes [213].

Figure 4.4.4 - Effect of Myosin inhibition on migrating platelets



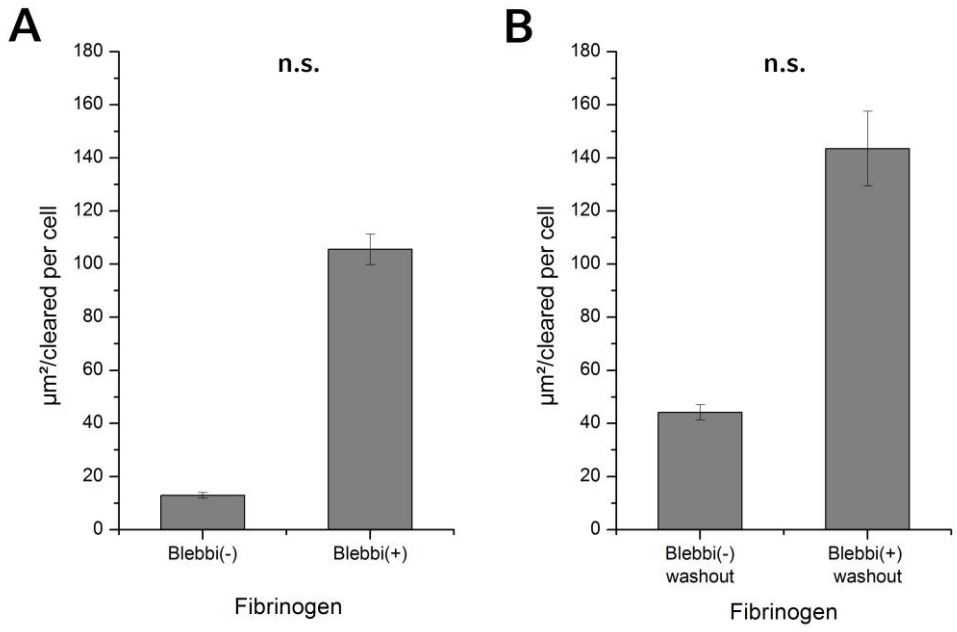
A) Exposure of cells to the active Blb(-) and the inactive Blb(+) conformation of the myosin inhibitor, displaying the percentage of migrating cells. n=3; Mann Whitney U Test; p=0.1 B) Wash out of the inhibitor. The effect of myosin inhibition is partly reversible. n=3; Mann Whitney U Test; p=0.1 C) shows the phenotype and Fbg removal of a control cell, D) of a Blb(-) inhibited cell. Note the strong additional fluorescence of the inhibitor and its control.

We therefore relied on the potent small molecule myosin II inhibitor (-)-Blebbistatin (Blb(-)) [177]. This compound exhibits a strong auto-fluorescence and might therefore have unwanted side effects in an epifluorescence microscopy assay. We therefore used the inactive (+)-Enantiomer of Blebbistatin (Blb(+)) as a control.

Blebbistatin completely inhibited migration on fibrinogen, whereas the percentage of migrating cells in the Blb(+) group was 73.4(±2.1)%. Moreover, it limited the cleared area to 12.9(±1.07) µm²/ per cell compared to 105.6(±5.73) µm²/ per cell in the Blb(+) group. Morphologically, platelets appeared spread, but could not remove the substrate they came into contact with, showing virtually no trace or mark in the GFP channel (which represents AlexaFluor 488 labeled fbg). This profound effect makes it likely that Myosin II is crucial for force generation and therefore centralization of substrate, in this case Fbg.

The effect was also pronounced in the retraction assay (Fibrin high cross-linking). Platelets cleared on average only 1.1(±0.5) µm²/ per cell versus 53.53(±5.4) µm²/ per cell in the Blb(+) control group. Once again, cells appeared spread and seemed unable to generate force.

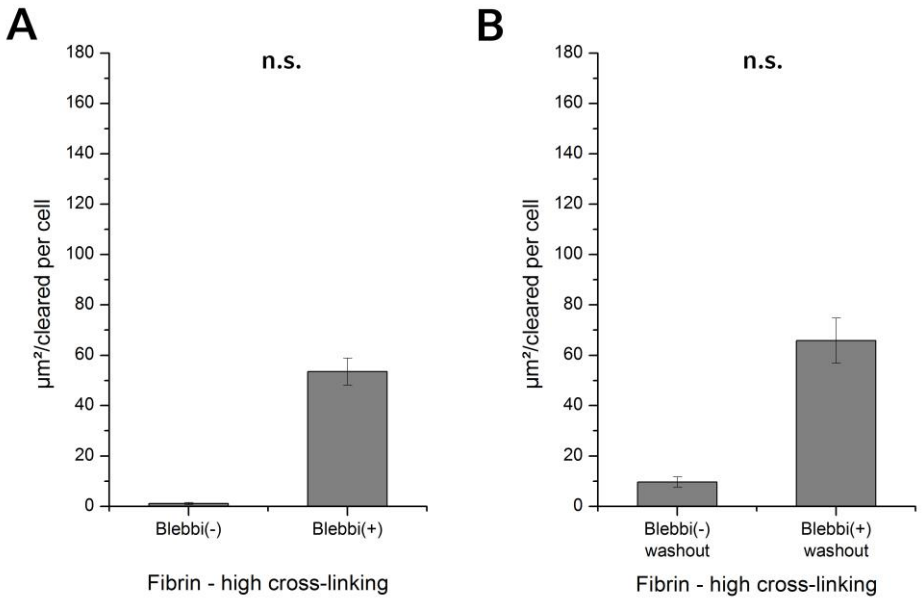
Figure 4.4.5 - Effect of myosin inhibition on substrate removal by migrating platelets



Analysis of substrate removal of migrating cells in Myosin inhibited (Blebbi(-)) and Control (Blebbi(+)) groups. A) displays constant exposure. n=3; Mann Whitney U Test; p=0.1. B) shows the effect of washing the inhibitor out.. n=3; Mann Whitney U Test; p=0.1.

Additionally, we investigated the reversibility of Myosin inhibition. We incubated the cells for a total of 45 minutes with the inhibitor/control, including 15 min activated and placed on the slides. Then, we replaced the supernatant with inhibitor free Tyrodes containing albumin and calcium. Interestingly, we observed a partial reversibility of the inhibitor's effect. 16.3(±1.9)% of the Blb(-) treated cells showed migration compared to the earlier mentioned 0(±0)% under continuous exposure. The same trend was visible for substrate clearance in Fibrinogen and Fibrin high cross-linking assays. Blb(+) treated cells subjected to wash-out also increased performance in all measured variables, indicating a unspecific effect of the compound.

Figure 4.4.6 - Effect of Myosin inhibition on retracting platelets



Analysis of substrate removal of retracting cells in Myosin inhibited (Blebbi(-)) and Control (Blebbi(+)) groups. A) displays constant exposure. n=3; Mann Whitney U Test; p=0.1. B) shows the effect of washing the inhibitor out.. n=3; Mann Whitney U Test; p=0.1.

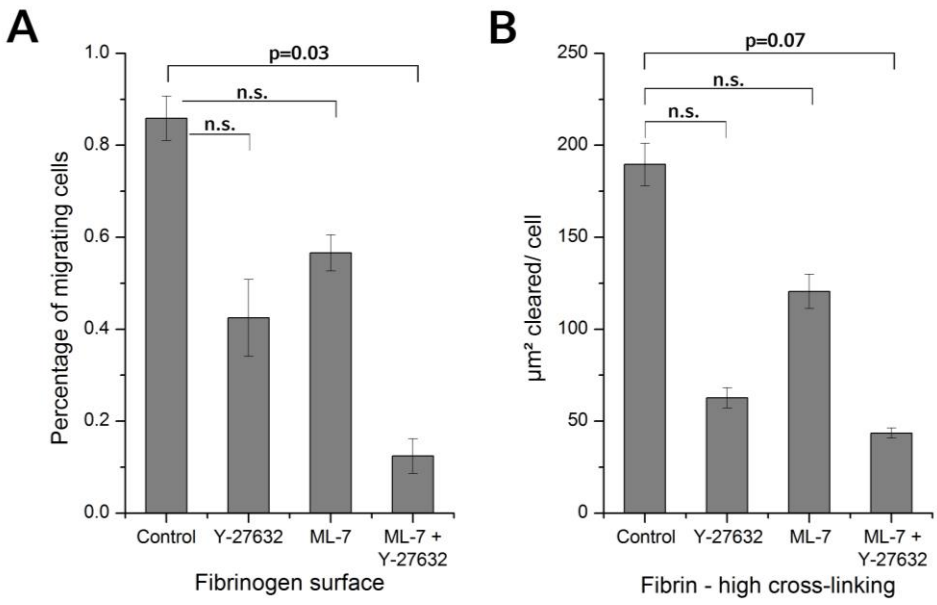
We reasoned that in both retraction and migration, Myosin II functions seems to be central to force generation. Because of the relative selectivity of Myosin inhibition (no effect on aggregation), Myosin II might be an interesting target for *in vivo* experiments exploring the role of platelet migration. However, a detailed review of the literature reveals numerous "side

effects" now attributed to defects in Myosin IIa function in megakaryocytes and platelets [212-214]. Moreover, Myosin inhibition inhibits retraction as well as migration of individual platelets, two mechanisms that we would like to dissect further.

4.4.2.2 Upstream manipulation of myosin

As mentioned, there are two major activating pathways upstream of Myosin IIa function, ROCK and MLCK. Luckily, small molecule inhibitors for both of these pathways are available, ML-7 blocking MLCK and Y-27632 inhibiting ROCK [215, 216]. This helped us to distinguish the influence of these two pathways on retraction and migration. We devised 4 groups: Control, Y-27632, ML-7 and a combination of both inhibitors to test the hypothesis that these two pathways stand alone in synergistic Myosin II activation.

Figure 4.4.7 - Effect of upstream myosin inhibition



Effect of ROCK-Inhibitor Y-27632 and MLCK-Inhibitor ML-7 on migration and retraction. A combination of both inhibitors shows a signifcant decrease in migration/retraction. A) Percentage of migrating cells. n=4, Kruskal Wallis test p= 0.001, post hoc test results displayed. B) area cleared per retracting cell, n=4, Kruskal Wallis test p= 0.001, post hoc test results displayed.

Migration percentages for the 4 mentioned groups were 85.9(±4.8)%, 42.5(±8.4)%, 56.6(±3.9)% and 12.4(±3.8)% respectively. This clearly shows that both pathways play a role in migration, and, moreover, a combined inhibition seems to have an additive effect. The difference between Blb(-) and Y-27632+ML-7 inhibited cells regarding migration shows that there is possibly at least a limited activation of Myosin II apart from MLCK/ROCK. However, we consider this data to favor the notion that (1) these two pathways regulate Myosin II and (2) are both almost equally involved in migration.

Cells placed on Fibrin of high cross-linking, i.e. our retraction assay, exhibited a pronounced effect of both inhibitors on retraction, with a combination of both inhibitors increasing the effect (see Figure 4.4.7 B). However, in this setup, MLCK seems to play a less relevant role than ROCK, as inhibition of the second pathway reduced migration by almost double the amount compared to MLCK inhibition.

This unbalanced effect on retraction not visible in migration experiments might be based on the fact that ROCK is also involved in other pathways via Rho signaling, which may play a more significant role in this setup [147]. Generally, however, this finding makes differential pathway activation in platelet response, dividing migration and retraction also on a subcellular level, more likely.

4.4.3 The role of tubulin

Tubulin is another key element of the cytoskeleton. β1-tubulin, the dominant form of tubulin in platelets, is not only of vital importance for platelet production, but also plays a role in platelet morphology [217, 218]. Moreover, it is an important player in cell migration, where it is for example involved in directional migration [10]. To shed light on the role of tubulin in migrating and retracting platelets, we conducted experiments with microtubule inhibitors.

4.4.3.1 Effect of tubulin inhibition on migration and retraction

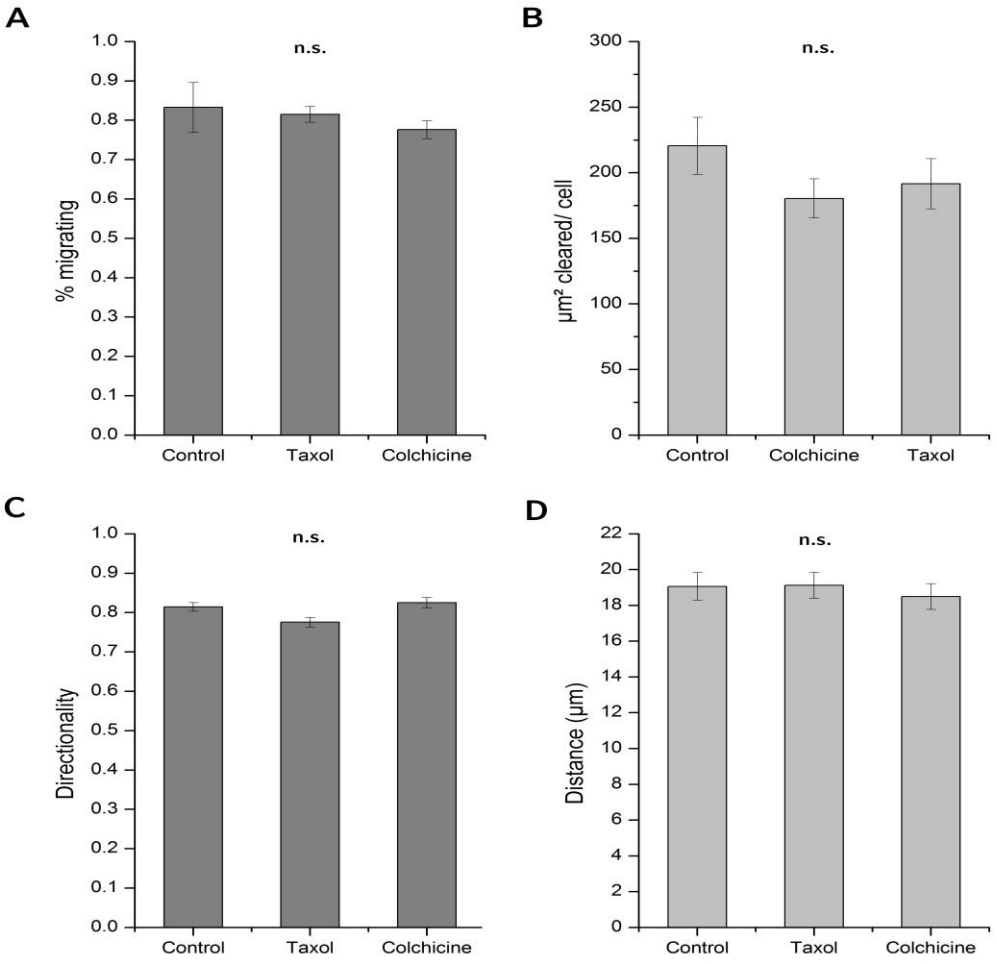
We used two distinct inhibitors to test our hypothesis that tubulin plays a role in platelet migration and retraction. We used Taxol, a microtubule stabilizing agent, and Colchicine, an inhibitor of microtubule polymerization, that both have found their way to clinical use [219].

Interestingly, we could see no significant effect of either inhibitor on the percentage of migrating cells (migration) or the area cleared per cell (retraction). Because of the above mentioned role of tubulin in migration, we performed an additional quantitative analysis of

migration specific aspects. However, we could not determine a significant difference in velocity, distance covered per cell, and directionality.

These results were a surprise for us, as it is intuitive that the circular microtubule coil of non-activated, spherical platelets would be a major factor effecting shape change, spreading, and ultimately polarization, preceding migration.

Figure 4.4.8 - Effects of tubulin inhibition on platelet migration and retraction



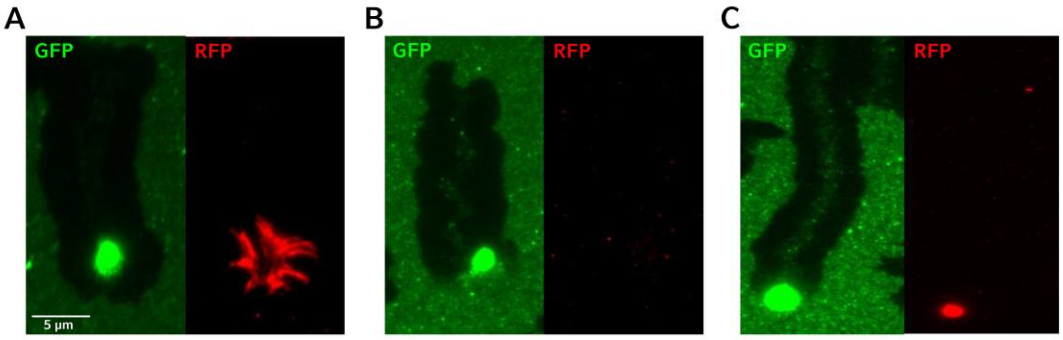
Results obtained from experiments with the microtubule stabilizing agent Taxol and the polymerization inhibitor colchicine, compared to controls. n=3 A) percentage of migrating cells on fibrinogen. Kruskal Wallis test p=0.67 B) effect on area cleared per cell in retraction (high-cross linking) Kruskal Wallis test p=0.56 C) directionality of migrating cells Kruskal Wallis test p=0.25 D) distance accumulated by migrating cells. Kruskal Wallis test p=0.73

4.4.3.2 Effects of tubulin inhibition on tubulin distribution

Because of these results, that in some ways also contradict some papers in the literature [217], we decided to use indirect immunofluorescence of beta-tubulin to visualize the expected effect of the inhibitors.

However we had difficulties in visualizing the tubulin network in control experiments; after trying another monoclonal tubulin antibody clone, which did not change image quality, we concluded that the standard staining protocol established in our lab (1% Glutaraldehyde for fixation, 0.1% Triton for permeabilization) somehow degraded the delicate tubulin structures of spread platelets. We then exhaustively scanned the literature for alternative staining methods. After trying different approaches, we established a slightly modified protocol adapted from the literature [164]. It relied on a fixation combination of glutaraldehyde and formaldehyde, combined with NP-40 for permeabilization, all in one single step, which additionally saved time.

Figure 4.4.9 - Effect of inhibitors on tubulin distribution



Fluorescent images displaying AF488 labeled Fbg in the GFP channel, and anti-β-tubulin Ab in the RFP channel. A) control group, showing extensive polymerization of tubulin in the migrating platelet B) absence of tubulin signal in the colchicine group C) Taxol fixated tubulin, and therefore prevented changes in tubulin structure in spreading and following migration.

We devised this IF as an optical control of inhibitor function. Directly after conducting live microscopy videography, we fixated and permeabilized the probes as described in the previous paragraph. The following visualization of tubulin content revealed a profound effect of both inhibitors: Whereas the control platelets expressed a complex, previously reported

microtubule structure [220], the stabilizing effect of taxol led to a point like, central signal, whereas the polymerization inhibitor colchicine led to no fluorescence signal at all, indicating a complete de-polymerization of tubulin. We conclude that our assay indeed induced reproducible inhibition of tubulin.

Italiano et al. were able to show that platelets of beta1-tubulin (-/-) mice were still able to perform their hemostatic tasks despite their spherical form. The authors concluded that the prominent marginal band of platelets might be a “side effect” from pro-platelet generation, where tubulin plays a central role [221]. The lacking effect of tubulin inhibition on migration is surprising; however, we observe random migration in our assay, and a possible effect on directed migration (unfortunately we have not developed a functional assay for this aspect yet) requires further investigation.

4.5 Platelet-bacteria interaction in migration

As discussed in 1.3.2, there is growing evidence for the role of platelets as immune cells in host defense, and improving imaging techniques have helped to show *in vivo* interaction of platelets with bacteria [13, 126]. Migration is a key feature of many immune cells as it allows an efficient translocation to inflamed areas, and helps immune surveillance as well as pathogen clearance [160, 169]. We therefore formed the hypothesis that migration as a newly discovered function of platelets might contribute to their role in immunity.

We decided to focus on the well-known phenomenon of platelet-bacteria interaction. It has been shown that platelets bind various pathogens in the circulation, but we were interested on migration-dependent effects [127]. This requires the binding of bacteria to a substrate that allows platelet migration. A relevant comparable *in vivo* situation is easily conceivable: In infective endocarditis, platelets, bacteria (mostly staphylococcus aureus) and fibrin(ogen) form tightly packed aggregates [222, 223]. We resorted to inactivated commercially available S. Aureus Bioparticles (SA) and non-pathogenic live E. coli.

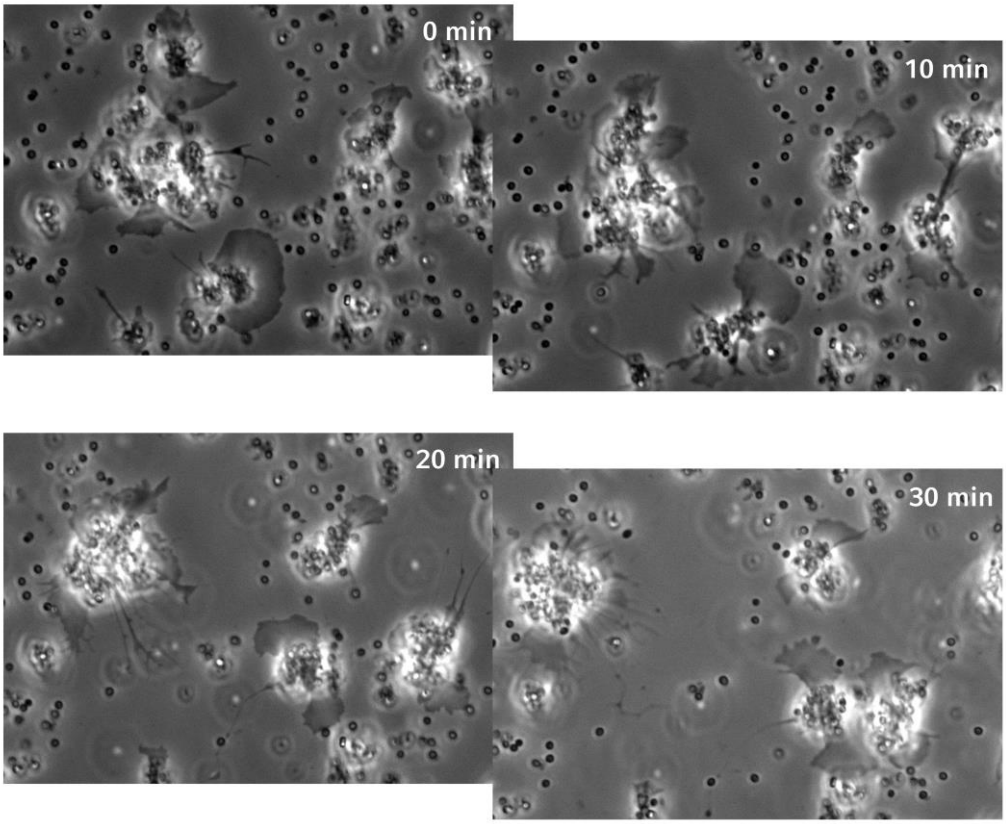
4.5.1 Interaction S. Aureus Bioparticles with migrating platelets

By acquiring AlexaFluor 594 (RFP channel) fluorescent SA bioparticles, we could combine their use with AlexaFluor 488 labeled fibrinogen. We could therefore display fibrin clearance and SA localization in parallel. We decided to first coat the surface as described, and then add SA bioparticles. Then, adhesion and distribution was assessed using epifluorescence microscopy. Preliminary experiments showed an increase of SA binding when using non cross-linked fibrin. Ideal SA incubation time was found to be 30 min at 37 °C. After a subsequent washing step, slides were incubated with activated platelets.

4.5.1.1 Imaging of interaction

To visualize live interaction of platelets and bacteria, we acquired time lapse videos. Platelets migrated as previously described, and effectively took up SA particles bound to surface fibrinogen. This process appeared to be highly effective. Fibrinogen-devoid migration paths were also cleared of SA fluorescence signal, hinting at a simultaneous take-up.

Figure 4.5.1 - Clearance of S. aureus particles by migrating platelets



Uptake of S. Aureus bioparticles by clearly distinguishable migrating platelets over time.

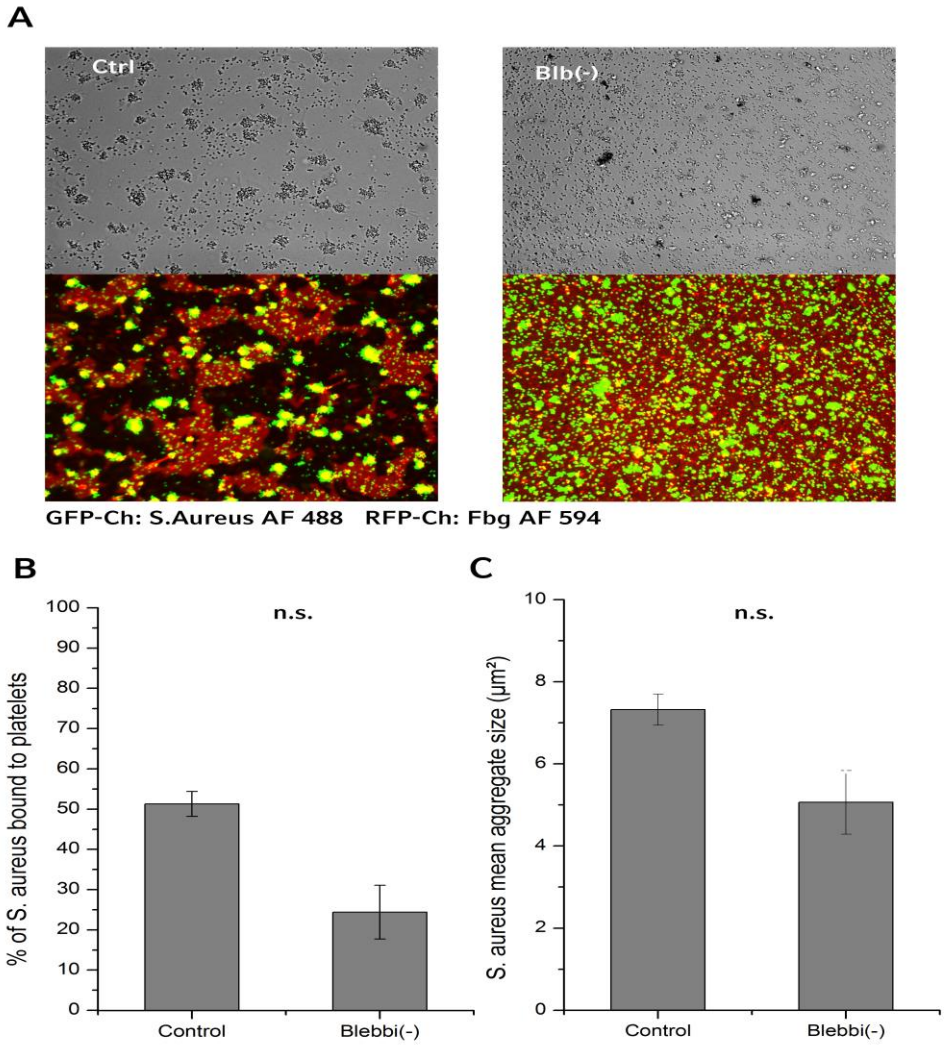
Due to the similar kinetics and distribution of fibrin and SA particle take up, and because of evidence in the literature [224], we hypothesize that in our assay, the interaction of platelets with bacteria particles is mediated through fibrinogen and therefore GP IIb/IIIa. More insight could be gained by additional studies, for example looking at the effect of integrin inhibition on the binding strength of already centralized bacteria.

4.5.1.2 Distribution patterns

Similar to fibrinogen, platelets centralized the bacteria, leading to the redistribution of pathogens. Fluorescent images before and after addition of activated platelets clearly showed

an overall effect on pathogen distribution. We then inhibited migration with Blb(-), a myosin II inhibitor, to distinguish migration/retraction effects from an effect mediated by mere presence of platelets.

Figure 4.5.2 - Effect of platelet migration on S.Aureus binding and aggregate size



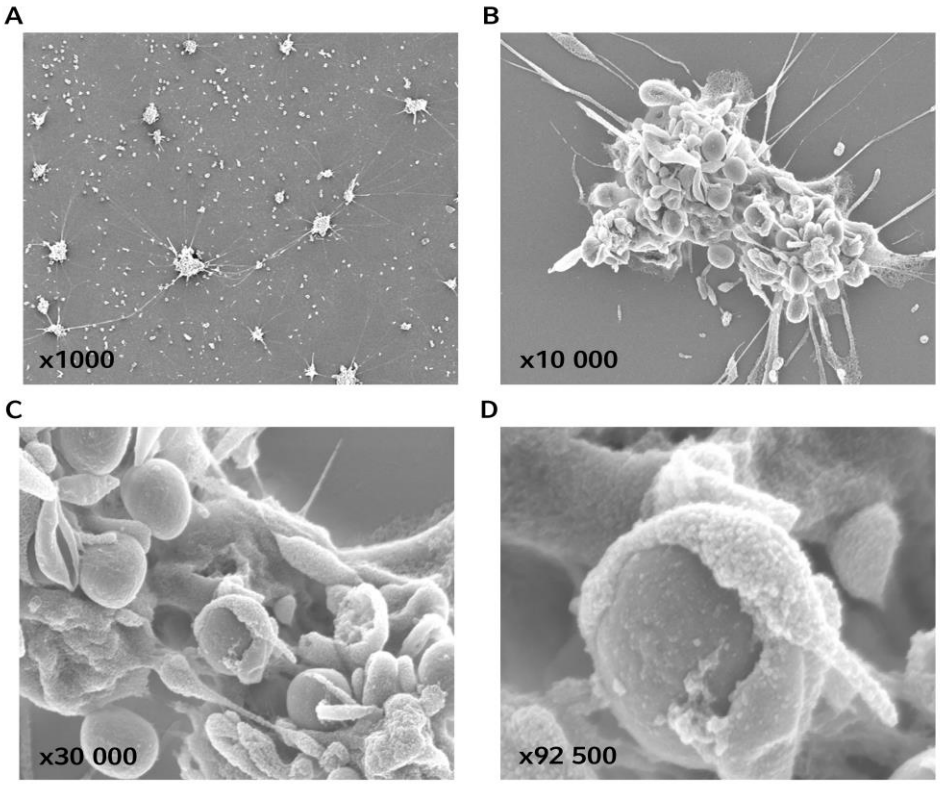
Inactivated S. aureus labeled with AF488 was incubated on fibrin AF 594, before addition of control or myosin inhibited (Blebbi(-)) platelets. n=3 A) PH and GFP/RFP channels displayed; visible aggregate formation in the migration group. B) Effect of migration on the percentage of S. Aureus bound to platelets. Mann-Whitney U test, p=0.1 C) Effect of migration on mean S. Aureus aggregate size. Mann-Whitney U test, p=0.4

Myosin inhibition abolished (1) as expected migration and (2) also attenuated pathogen redistribution. In the control group, a mean of 51.3(±3.1)% of bacteria were bound to platelets, compared to only 24.4(±0.7)% in the myosin inhibited group. In parallel, average S. Aureus aggregate size was 7.32 (±0.38) versus 5.07 (±0.77) μm^2. We conclude that in our assay, platelet migration plays a vital role in platelet-bacteria interaction and seems to contribute to the effective formation of platelet-bacteria aggregates. Nevertheless, it remains unclear if this helps for example pathogen clearance by neutrophils or hinders bacteria proliferation. Once again, further experiments are required.

4.5.1.3 Scanning Electron Microscopy

To gain insight into the nature of platelet pathogen interaction, we conducted scanning electron microscopy allowing nanometer scale resolution. Platelets could be visualized interacting with numerous bacteria, at a platelet – bound bacteria ratio of probably 1:100. High resolution detail revealed that SA particles are not internalized but rather bound to the surface. However, bound bacteria seem to be tightly attached, as some were partly encompassed by platelet membrane.

Figure 4.5.3 - REM visualization of platelet-S.Aureus aggregates



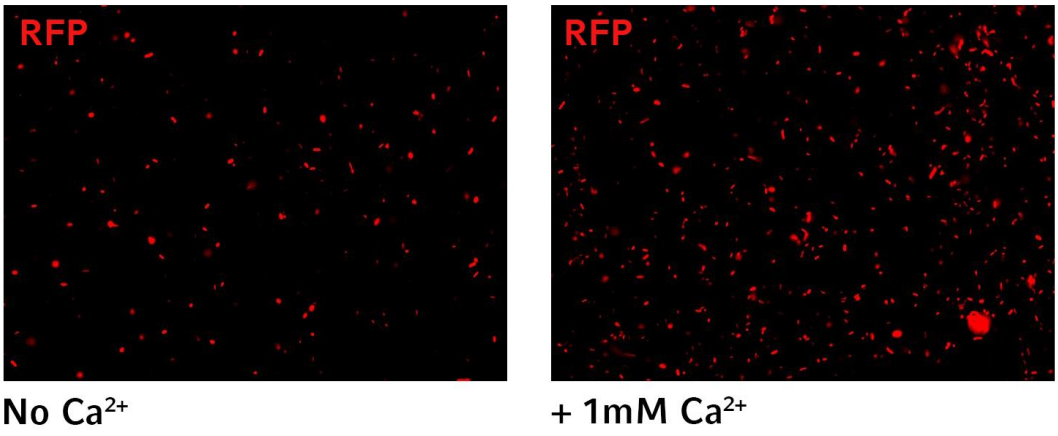
Representative REM images of the assay described in Figure 4.5.2. A) Overview showing platelets and S.Aureus distribution B) Single platelet concentrating more than a dozen bacteria C) detailed view of bound bacteria D) ultra- structure of S.A. particle and engulfing platelet membrane

4.5.2 Interaction of platelets with live Escherichia coli

We could show that platelets interact with S. Aureus bioparticles in an *in vitro* migration assay. However, despite preserving surface structure of the pathogen, these bioparticles are not comparable to an active pathogen in infection, which is proliferating, releasing enzymes and toxins, and actively interacts with its microenvironment.

As a consequence, we next used live Escherichia coli expressing the fluorophore tomato red (tmt), in our assay. Preliminary experiments showed that calcium seems to play a role in E. coli – fibrin interaction, as the addition of calcium led to a denser RFP signal on the fibrin surface, indicating increased binding of bacteria (see Figure 4.5.4).

Figure 4.5.4 – Effect of Ca^{2+} on E. Coli fibrin binding

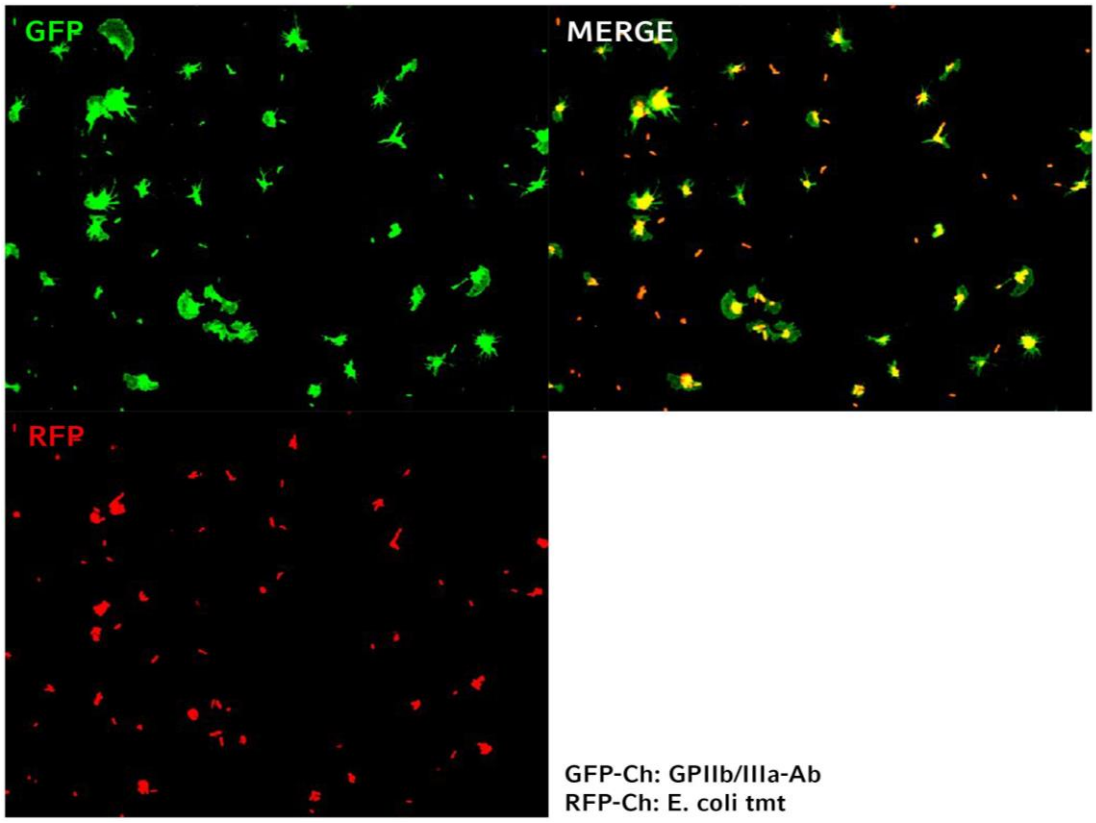


Representative fluorescence images of live E. Coli expressing tomato red (tmt), coated on fibrin with and without 1mM Calcium.

We then decided to use non-fluorescent fibrin as substrate, because this allowed us to later use indirect immunofluorescence staining of GPIIb/IIIa in the GFP channel (AlexaFluor488 secondary). As the prominent integrin of platelets, GPIIb/IIIa staining effectively marks the

entire platelet membrane. This would help us in quickly and accurately identifying platelet-bacteria conjugates.

Figure 4.5.5 - Platelet interaction with live E.Coli in migration



Representative fluorescence image of platelets identified with GPIIb/IIIa (GFP-Channel) and tomato red (tmt) expressing live E.Coli (RFP-Channel). MERGE indicates overlay of these two channels. Formation of platelet-bacteria aggregates are visible.

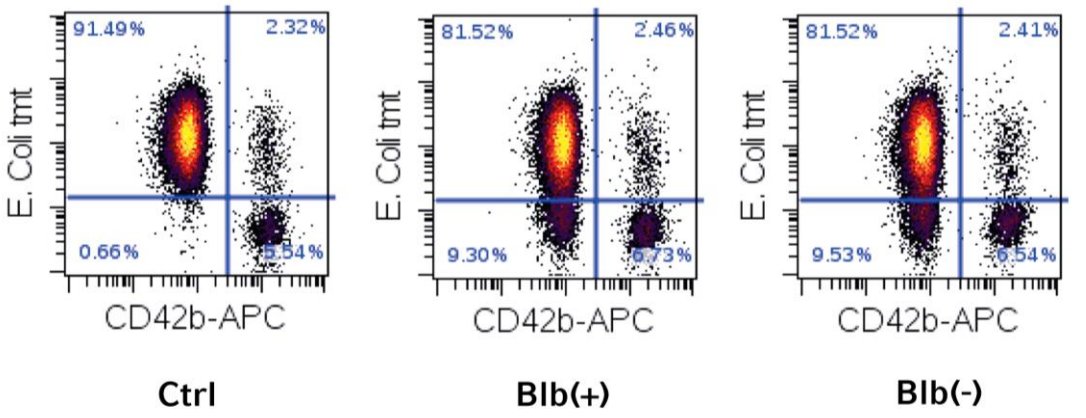
The conducted experiments yielded comparable results to the ones involving SA particles. Once again, effective platelet-bacteria aggregate formation occurred through the mechanism of platelet migration. We reason that this effect is also relevant in the interaction with live bacteria. We propose a possible role of this mechanism in infective endocarditis, sepsis and catheter biofilms, which all involve fibrin(ogen) disposition and platelet involvement.

Additionally, similar experiments with pathogenic bacteria of clinical relevance are indicated, like S. aureus or Streptococci.

4.5.3 Bacteria – platelet binding in solution

One might ask what the effect of myosin inhibition might be on platelet-bacteria aggregate formation in solution, a common phenomenon taking place in circulation. We hypothesized that Myosin inhibition distinguishes platelet migration and retraction from platelet functions in the blood stream, independent of surface contact. Therefore, we devised a flow cytometric assay to assess aggregate formation in solution, with or without myosin inhibition. After incubation of washed platelets with E. Coli tmt, in the presence of calcium and fibrinogen, we used a CD42b (GPIb)-APC antibody to label platelets. We used this antibody because (1) GPIb is platelet specific and (2) seems not to be relevant in our assay.

Figure 4.5.6 - Analysis of platelet-bacteria aggregate formation in solution



Flow cytometric experiment displaying platelets (CD42b-APC + E.Coli tmt -), E. coli expressing tmt red (CD42b-APC - E.Coli tmt +) and plt-bacteria aggregates (CD42b-APC + E.Coli tmt +). Aggregates can be visualized in all groups with comparable incidence, which provides support for the thesis that platelet myosin II function is negligible for in solution binding of pathogens

We distinguished CD42b + tmt + platelet-bacteria aggregates by plotting FL3/FL6 channels. Clearly, the control group revealed a double positive population, as well as two populations single positive of CD42b and tmt, representing platelets and bacteria not

interacting. To support our hypothesis, Blb(-) inhibition did not diminish the double positive population. This indicates that platelet myosin does not significantly affect bacteria interaction in solution, in contrast to its pivotal effect on platelet clearance of surface bound bacteria. This opens the door for *in vivo* experiments with MYH9 (-/-) platelets, looking for a specific effect of platelet migration/retraction in immune response.

Nevertheless, our flow cytometric assay need to be refined, with the goal to (1) increase platelet bacteria interaction, (2) define receptors and platelet cytoskeletal components relevant for bacteria binding.

5 Discussion

Aim of the study. This study was designed to clarify aspects of platelet migration, a feature that has so far not been recognized as a platelet function. Our goal was to analyze cell-fibrinogen interaction, describe the role of extracellular microenvironment on platelet migration, explore mechanosensing as a possible aspect of directional guidance and evaluate a possible physiological role of migration in immunology. In the light of very limited publications on the topic, it is relevant to gain insight into this novel platelet function. So far, there are only eight publications covering platelet migration, with the earliest being published 1973, the latest in 2010 [149-156]. For example, *Pitchford et al* claim that allergens induce migration of platelets to the lung, using histopathology and in vitro transwell assays. Limitations of their study include the *in vitro* use of a large 3 µm pores that can be easily passed by 3 times smaller sized platelets without active cytoskeletal rearrangements, and the lack of alternative considerations for platelet extravasation in inflammation like increased endothelial permeability, or even fixation artefacts [152]. *Lowenhaupt et al* used a 2D migration assay similar to our approach over 40 years ago. However, microscope technology then did not allow single cell tracking due to limited resolution [149]. Moreover, none of the mentioned studies assessed if 2D migration criteria from a cellular biology standpoint were fulfilled, as described in 1.4 - Cell migration.

In summary, none of these papers could establish a reproducible and validated *in vitro* assay of platelet migration, or show credible platelet migration *in vivo*. The missing impact on the field of the novel and exciting data presented in these papers indicates doubt of the scientific community. As a consequence, platelet migration has never found its way into high impact reviews, reflecting the controversial role it occupies with experts of the field [225, 226].

This work is based on our group's development of a novel *in vitro* assay for the study of platelet migration. We think that we established for the first time a credible and reproducible method to study platelet migration *in vitro*. In combination with technological advances, we were able to dissect observed migration in great detail. However, limited preceding knowledge of platelet migration warrants a general description of the phenomenon first, limiting the depth and thoroughness of our experiments in regard of detailed activation and signaling pathways. As in many other pioneering studies, it will be necessary to close these gaps in follow up studies and refine some key experiments, and it will be interesting to see other groups' contributions to the field.

Used methods. We mainly relied on the described *in vitro* migration assay (see Methods). In general, a functional *in vitro* approach offers many advantages. It is as close as life sciences get to a perfect experimental setup with one independent and one dependent variable, minimizing confounders and other unknown or uncontrollable variables. This makes *in vitro* findings reproducible, exact and internally valid. Moreover, such assays can often give first hints at otherwise indiscernible *in vivo* mechanisms and functions. Given the small size, large number and rapid aggregate formation of platelets *in vivo*, the use of *in vitro* approaches, especially regarding a newly discovered function, seem intuitive.

At the same time, *in vitro* approaches come with some serious caveats and limitations. Due to the artificial setup, a 1:1 transfer of *in vitro* findings to the *in vivo* situation is possible in only a very limited number of cases. Each approach should be used to its strengths, for example *in vitro* experiments for the discovery of (subcellular) mechanisms and *in vivo* approaches for showing resulting function and relevance. We tried to adhere to this concept, and virtually all findings presented here need to be confirmed *in vivo* using appropriate mouse models.

We mainly used isolated human platelets in our experiments. The advantages were a reduction of mice sacrificed for our research, and the easy, inexpensive and quick availability of human blood material. Moreover, mouse and man do vary substantially, particularly in immunological function. For example, mouse platelets do not express the immune Fc gamma RII receptor [123]. As it is the long term goal of medical research to show relevance in humans, it makes sense to include experiments based on human probes. However, the above mentioned necessity of corresponding *in vivo* experiments makes an initial *in vitro* analogy with isolated murine platelets necessary.

We established an adapted migration assay. We devised a chamber based technique, improving the original, open system [162]. We could now perform multiple coating and fluid exchange steps, in comparison to the before practiced addition of substrate, cells and activators in one step. This increased reproducibility and control dramatically. Another significant change was the use of fluorescent fibrinogen, introduced after the discovery of substrate removal by migrating platelets. This led to three major advantages. First, we were now able to trace migrated platelets and determine certain migration indices even after fixation. Second, optical control of fibrinogen gradients and fbg density steps were possible, allowing for mechanosensing experiments. Last but not least, we could now image the interaction of non-migrating, retracting platelets with fibrin. Importantly, we could compare migration and retraction with the newly defined index area cleared/per cell.

It needs to be mentioned that we did not explore 3D migration of platelets. Interaction of cells with three-dimensional ECM is very relevant and frequent in the extravascular space *in vivo*, and it has been shown that many migrating cells exhibit a profoundly changed morphology and behavior in 3D matrixes [227]. However, possible intravascular platelet

migration is likely to be two-dimensional, as is leukocyte intravascular crawling. Additionally, our assay did not cover an important aspect of cell migration: directionality towards chemical signals, also called chemotaxis. Also considering the four publications focusing on platelet chemosensing and chemotaxis, it is only logical to explore the possibility of a similar function in our assay, avoiding the artefact prone transwell assay used in the literature. On the other hand, not chemotaxis but durotaxis, the migration along rigidity gradients, could be predominant in platelet migration, as discussed in conjunction with the results of the fibrinogen gradient experiments.

Platelets act their part in the circulation, exposed to very high to low shear rates. Notably, rheology and shear stress are of vital importance in platelet function [89, 228]. We conducted our experiments in stable conditions without continuous flow. The reason for this was that we preferred to first describe important aspects of platelet migration in a stable, uncomplicated environment before switching to a flow chamber approach. In addition, preliminary experiments in our group showed surprising resistance and unchanged behavior of migrating platelets against flow [162].

We studied subcellular components and pathways involved in migration by using mainly small molecule inhibitors. Small molecule inhibitors are useful thanks to their quick diffusion to their targets, the high specificity of their action and the low concentrations necessary for effective inhibition [229]. We complemented all inhibition experiments with corresponding controls, and in the case of Blebbistatin, we even used the inactive enantiomer of the same compound as a control. This helped to monitor any adverse effect of Blb related to its other chemical properties like auto-fluorescence. Nevertheless, the gold standard to study subcellular processes is the use of genetically modified mice, for example with a conditional knock down, knock out or overexpression of target molecules. Despite disadvantages (for example off target effects of genetic manipulation or long term compensation through alternative pathways), it is indicated to repeat central inhibition experiments with murine platelets from transgenic mice.

One of our first research questions was the nature of fibrin(ogen) removal of migrating platelets. Did we witness internalization or surface transport? Relevant literature was compatible with both possibilities [203, 230, 231]. We used up-to-date *in vitro* methods to clarify the mechanism: A pH sensitive dye coupled to fibrinogen, a sophisticated biotinylation assay and indirect immunofluorescence (with and without cell permeabilization). Despite the unambiguous results won by these three distinct methodical approaches, we have to take into account the possible insensitivity towards for example a minor percentage of substrate internalized. It would therefore be useful to use transmission electron microscopy to resolve any uncertainty.

We explored a possible role of migration in immunology, specifically host defense, by adding live bacteria (E. Coli) and inactivated bacteria particles (S. Aureus) to our assay. This approach allowed us to image single cell-pathogen interactions, and we were even able to acquire Scanning Electron Microscopy images showing the exact localization of bacteria on platelets. We conclude that despite the mentioned limitations of *in vitro* assays regarding *in vivo* function, it would be hard to impossible to accomplish comparable resolution in a living organism. That said there is still room for improvement of the used assay, for example the variation of surface, or additional experiments in a three dimensional fibrin network.

In the field of host pathogen interactions, very defined and sophisticated in *vitro* methods are used. Especially activation markers and surface receptor expression, as well as chemokine release and cell or respectively pathogen integrity are important outcome parameters. Similar methods have been put into practice with platelets [119, 125]. It will be important to use a subset of these approaches to clarify the role of platelet migration in host defense.

Obtained results.

We first analyzed the relation of radial fibrinogen removal and platelet migration characteristics. Fbg removal and radial centralization showed kinetics independent of cell size and migration speed, making an independent, stereotypical subcellular mechanism likely. There are various papers showing actin flow and myosin contraction to be relevant in cell migration and particularly trailing edge contraction [232-234]; we therefore hypothesize a comparable mechanism in migrating platelets. We could show that myosin dependent substrate removal is required for migration in our assay. In the future, it would be useful to visualize life actin flow, for example using a LifeAct mouse that expresses an actin binding GFP-peptide [234].

We examined fibrinogen removal by using epifluorescence microscopy combined with AlexaFluor 488 Fbg as substrate. We excluded the possibility of an isolated cleavage of the fluorophore or only partial substrate removal by immunofluorescence showing no signal in migration paths and by the visualization of topographic tracks using AFM. However, we could not answer the question of absolute quantitative take-up of substrate, and, related to this, satiability of migrating platelets. In the literature, various helpful techniques can be found that could be implemented, for example ultracentrifugation to identify possibly endocytosed receptors or substrate. This method would allow for tracking of intracellular fibrinogen by fractionation of intracellular departments [235].

Three of the used assays (pH sensing, indirect IF and biotinylation) pointed out that fibrin(ogen) is not internalized as expected but rather transported on the surface of migrating platelets, to be amassed close to the pseudonucleus. There are various reports showing

fibrinogen internalization of platelets, claiming that a major percentage of platelets' fibrinogen content is indeed acquired through internalization in the circulation [230, 231, 236]. This seemed to play no role in our setup; rather we propose a remotely analog mechanism to the one observed by *Kasahara et al*. They postulate an axis of actomyosin, lipid rafts containing GPIIb/IIIa and surface bound Fibrin(ogen) to serve as the main linkage from ECM to cytoskeleton in clot retraction [203]. It is intuitive to conceive the concentrated Fibrin(ogen), as a landing pad for additional platelets or other circulating cells, or it might play a so far unrecognized role in signaling.

We then focused on the role of the extracellular microenvironment and its effect on platelet migration. By increasingly cross-linking fibrin we could show that platelet behavior gradually switches from migration to retraction. These findings were particularly relevant for the project as we were now able to relate heavily doubted migration behavior of platelets to well-known individual retraction of platelets, collectively called clot retraction [100]. Platelets spread and retracted on highly cross-linked fibrin but were unable to migrate due to their inability to remove the substrate. This highlights the importance of the mechanical properties of the substrate for the initiation of platelet migration. Indeed, platelets bound to highly crosslinked fibrin started to migrate after fragmentation of the fibrin-network by plasmin-digestion. We could also show that platelets migrating or retracting show significant differences in morphology as well. In summary, platelet migration and retraction both rely on myosin II function, but seem to be differentially regulated separate functions of adherent platelets. Interestingly, we could identify surface characteristics, i.e. degree of cross-linking, to be the only discernible factor deciding if platelets retracted or migrated.

It will now be a research priority to provide a relevant explanation and possible *in vivo* relevance for this novel function. To approach this question it might be useful to further refine the used migration assay by moving it closer to physiological conditions. For example, experiments with whole blood or cultured endothelial cells have proved to be very useful in determining platelet related mechanisms [58, 237].

A logical conclusion of behavioral change by platelets induced through a mere cross-linking event was that these cells sense physical properties of their microenvironment in our assay. There are only few publications on the subject of platelet mechanosensing, but two recent papers showed the importance of this mechanism for platelet activation, spreading and adhesion on different substrates [93, 94]. We manufactured an Fbg gradient as well as a substrate edge (steep gradient between low and high fibrinogen concentration) to show that migrating platelets indeed sense their environment and migrate towards higher densities of substrate. We reason that mechanosensing might also play a vital role in *in vivo* migration, for example allowing platelets to follow fibrin(ogen) gradients towards the epicenter of a vascular breach. It remains to be elucidated if elasticity/rigidity (durotaxis), adhesiveness (haptotaxis), or a combination of both defines directionality. In the field of cell migration, highly

sophisticated assays have been devised to study related questions, and should in the future be applied to gain further insight [238]. Moreover, subcellular regulatory mechanisms already demonstrated in other migratory cells need to be shown for migratory platelets [239, 240].

We found actin and GP IIb/IIIa to be indispensable for platelet migration. The role of actin is not surprising as this component of the cytoskeleton is central to platelet function in general. It has been shown in platelets that actin polymerization is necessary for shape change, spreading, aggregation and force generation [76, 207, 208]. As we used fibrin(ogen) as substrate, it is just as conceivable that GP IIb/IIIa as on the one hand the most abundant integrin and on the other hand the one responsible for fibrin(ogen) binding, does affect migration. It would be interesting to establish migration assays on other substrates, that would possibly show other surface receptors on platelets to be central to force transmission to the ECM; for example GP VI on collagen or GPIb-V-IX on vWF coated surfaces [55, 166].

Even more interesting was the dependence of migration on myosin IIa; myosin IIa has been shown to be important for integrin outside-in signaling and cell contraction, but does not affect other functions like aggregation and secretion [212]. Searching for a migration specific inhibitor, myosin knock out platelets offer a first opportunity for preliminary assessment of *in vivo* function of migration. Nonetheless, we could confirm the reported effect of myosin inhibition on platelet contractility. Therefore, MYH9 deficient platelets are not able to migrate nor able to retract. This makes the crucial distinction between migration and individual platelet retraction via myosin inhibition/MYH9 deficient mice impossible [173, 241].

We also looked into tubulin function in migrating platelets. By using IF as optical control and two tubulin inhibitors based on entirely different molecular mechanisms, we could show a negligible role of tubulin in our migration assay. Reviewing the literature, this is not particularly surprising as *Italiano et al* were able to show that despite morphological changes, other platelet functions remained virtually unaffected by β-tubulin knockdown [221].

Last but not least, in the search for a role of platelet migration *in vivo*, we discovered pronounced interaction of live E. coli and inactivated S. aureus particles with platelets. This interaction was facilitated and optimized through platelets' ability to migrate, as subsequent myosin inhibition demonstrated. Preliminary flow cytometric data revealed that myosin inhibition had no effect on platelet-bacteria interaction in solution, an important finding that could allow to differentiate soluble and surface-dependent interaction via Myosin inhibition/knockdown. This is particularly relevant because of recent evidence showing the importance of platelet-bacteria aggregate formation in the circulation for immune function [127].

In our assay, we quantified bacteria distribution and platelet-bacteria aggregate formation. However, outcome parameters need to be refined to clarify the effect of platelet-

fibrin(ogen)-bacteria aggregate formation and therefore the role of migration in host defense. Reports in the literature point towards opposite directions: Some bacteria seem to exploit platelets in infection, whereas in other contexts platelets contribute to pathogen clearance [126, 242]. For example, the addition of other immune cells like neutrophils would allow the measurement of pathogen clearance/neutralization. Moreover, it would be interesting to test the viability, for example via the ability to reproduce, of bacteria bound to platelets. Furthermore, as fibrin(ogen) plays a central role in our assay, it would be of great use to focus on live bacteria strains that are known to avidly bind fibrin(ogen), or even use the coagulation cascade for sustaining infection like staphylococcus aureus [224, 243]. This bacterium contains proteases for initiating and maintaining blood coagulation, therefore protecting itself from an effective immune response [243]. To sum it up, a more physiological approach could clear the way to finding an *in vivo* role of platelet-bacteria interaction.

This work is based on our group's development of a novel *in vitro* assay for the study of platelet migration. We think that we established for the first time a credible and reproducible method to study platelet migration *in vitro*. In combination with technological advances, we were able to dissect observed migration in great detail. However, limited preceding knowledge of platelet migration required a general description of the phenomenon as first step, limiting the depth and thoroughness of our experiments in regard of detailed activation and signaling pathways. As in many other pioneering studies, it will be necessary to close these gaps in follow up studies and refine some key experiments, and it will be interesting to see other groups' contributions to the field.

6 Summary/Zusammenfassung

Background. Platelets are not only critical players in hemostasis but also play an increasingly recognized role in host defense. Despite a well described model of the platelet adhesion and activation cascade *in vivo*, platelet function on the single cell level is not fully understood. This study is conducted in the light of a novel *in vitro* platelet migration assay developed by our group, which allows for the first time to gain insight in the unrecognized and doubted migratory ability of platelets.

Results. We could show that migrating platelets remove the substrate by radial centralization, creating substrate-free migratory tracks. The removal of fibrin(ogen) is accomplished through surface transport and not internalization. Platelets migrated on fibrinogen as well as on fibrin. However, a gradual increase in cross-linking of the substrate, monitored by fluorescence microscopy, could gradually abolish migration, leading instead to substrate retraction of individual platelets. AFM confirmed qualitatively altered surface characteristics in the high cross-linked assay that triggered retraction. We therefore concluded that surface characteristics suffice to determine platelet behavior ranging from migration to retraction. We provided further proof of this principle by application of plasmin to retracting platelets, which led to the reversal of cross-linking as well as the reestablishment of migratory behavior (migration index of 70(±7.9)% in the plasmin treated vs 0(±0)% in the control group) and morphology (significant increase in aspect ratio and decrease in solidity). Platelets seem to sense their microenvironment and respond accordingly. To validate this finding and furthermore determine platelet response to mechanical cues in migration, we established a fibrinogen gradient as well as an edge - a steep transition of low to high fibrinogen coating density. Both assays showed the active translocation of platelets to higher fibrinogen densities, with a more dramatic effect seen in cells that interacted with the edge, prompting the localization of 99.2% of monitored cell into the high density area. Inhibition of cytoskeletal components and pathways established the dependence of migration on actin and integrin GPIIb/IIIa, and, more interestingly, on myosin IIa, whereas tubulin did not play a discernible role. Searching for a role of platelet migration *in vivo*, we discovered pronounced interactions of live Escherichia Coli and inactivated Staphylococcus Aureus particles with platelets. This interaction was facilitated and optimized through platelets' ability to migrate, as subsequent myosin inhibition resulted in the absence of platelet-bacteria-aggregates and diminished pathogen clearance.

Conclusion. This study clarified the interaction of migrating platelets with fibrin(ogen) and identified surface characteristics as a crucial regulatory mechanism of platelet migration. Related to this we discovered directional migration of mechanosensing platelets steered by the

adhesiveness of the substrate. Our results underline myosin II as a central component of migration as well as a possible target to specifically block migration in vivo. Ultimately, we demonstrated for the first time a possible role of platelet migration in host defense against bacteria.

Hintergrund. Thrombozyten spielen nicht nur eine zentrale Rolle in der Hämostase, sondern scheinen auch zusätzlich immunlogische Funktionen zu übernehmen. Obwohl ein gut beschriebenes Modell für die Thrombozytenadhesion und –aktivierung *in vivo* existiert, sind einige Aspekte des Thrombozytenverhaltens auf Einzelzell-Ebene nicht restlos geklärt. Diese Studie steht im Zusammenhang mit der Entwicklung eines neuen *in vitro* Versuchsverfahrens durch unsere Gruppe, der es ermöglicht, das erste Mal die bezweifelte und weitestgehend unbekannte migratorische Fähigkeit von Blutplättchen näher zu beschreiben.

Ergebnisse. Wir konnten zeigen, dass migrierende Thrombozyten das Substrat durch radiale Zentralisierung entfernen und dadurch substratfreie Spuren hinterlassen. Die Entfernung von in unserem Fall Fibrin(ogen) wird durch Oberflächentransport und nicht Internalisierung realisiert. Thrombozyten migrieren sowohl auf Fibrinogen als auch auf Fibrin; allerdings führt eine schrittweise Zunahme an Vernetzung, die wir mit Fluoreszenzmikroskopie kontrollierten, zu einer graduellen Abnahme der Migration, und löst stattdessen eine Substrat-Retraktion von einzelnen Blutplättchen aus. AFM konnte die qualitativ veränderten Eigenschaften der progredient vernetzten Oberflächen bestätigen, welche zur Retraktion führten; wir schlussfolgerten daher, dass veränderte Oberflächeneigenschaften ausreichen, um das Verhalten von Retraktion bis Migration festzulegen. Wir konnten einen weiteren Beleg für diesen Grundsatz liefern, indem wir retrahierende Thrombozyten mit Plasmin behandelten. Dies führte zur Auflösung der Oberflächenvernetzung und gleichzeitig zum Wiederaufteten des Migrationsverhaltens (70(±7.9)% migrierende Zellen in der Plasmin-Gruppe im Gegensatz zu 0(±0)% in der Kontrollgruppe) und -morphologie (signifikante Zunahme der AR und Abnahme der Solidität) der Plättchen. Blutplättchen scheinen ihre unmittelbare Umgebung wahrzunehmen und darauf spezifisch zu antworten. Um dies zu validieren und außerdem die Reaktion von migrierenden Blutplättchen auf mechanische Eigenschaften ihrer Umgebung zu bestimmen, etablierten wir einen Fibrinogen-Gradient sowie eine Substratkante –einen scharfen Übergang von höherer zu niedriger Substratkonzentration. Beide Experimente zeigten eine aktive Translokation von Thrombozyten zu Gebieten mit einer höheren Dichte an Fibrinogen, mit einem stärker ausgeprägten Effekt bei denjenigen Zellen, welche in Kontakt mit der Substratkante kamen. Eine Interaktion führte bei diesen Versuchen zu einer Lokalisation von 99.2% der beobachteten Zellen in den Bereich höherer Substratdichte. Die Hemmung von Bestandteilen des Zytoskeletts und zellulären Signalwegen zeigte eine Abhängigkeit der Migration von Aktin und dem Integrin GP IIb/IIIa, und, noch interessanter, von Myosin IIa, während dagegen Tubulin keine erkennbare Rolle spielte. Auf der Suche nach der Rolle von

Plättchenmigration *in vivo* endeckten wir eine Interaktion von lebenden Escherichia Coli und inaktivierten *Staphylococcus aureus* Partikeln mit Plättchen. Diese Interaktion wurde durch die Fähigkeit von Thrombozyten, zu migrieren, gefördert und optimiert. Hemmung von Myosin IIa führte zum Fehlen von Bakterien-Thrombozyten-Aggregaten und senkte die Rate der Erreger-Klärung.

Schlussfolgerung. Diese Studie beleuchtete die Interaktion von migrierenden Thrombozyten mit Fibrin(ogen) im Detail und identifizierte Oberflächeneigenschaften als entscheidenden Regulationsmechanismus der Thrombozytenmigration. In engem Zusammenhang damit konnten wir durch „Mechanosensing“ vermittelte direktionale Migration von Blutplättchen beschreiben. Unsere Ergebnisse unterstreichen Myosin II als zentrale Komponente der Thrombozytenmigration und zusätzlich als mögliche Zielstruktur in spezifischen Knock-Out Experimenten. Außerdem konnten wir das erste Mal eine mögliche Rolle der Thrombozytenmigration in der Abwehr von Bakterien aufzeigen.

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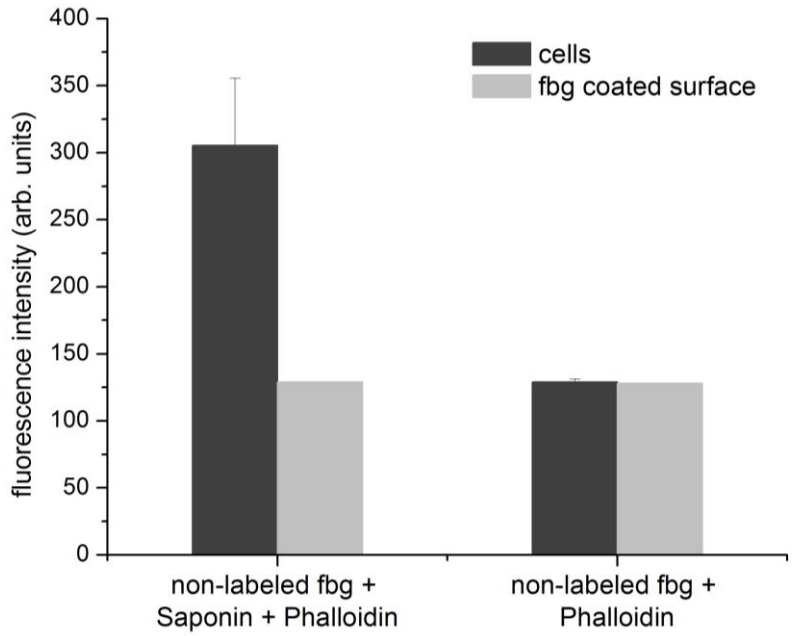
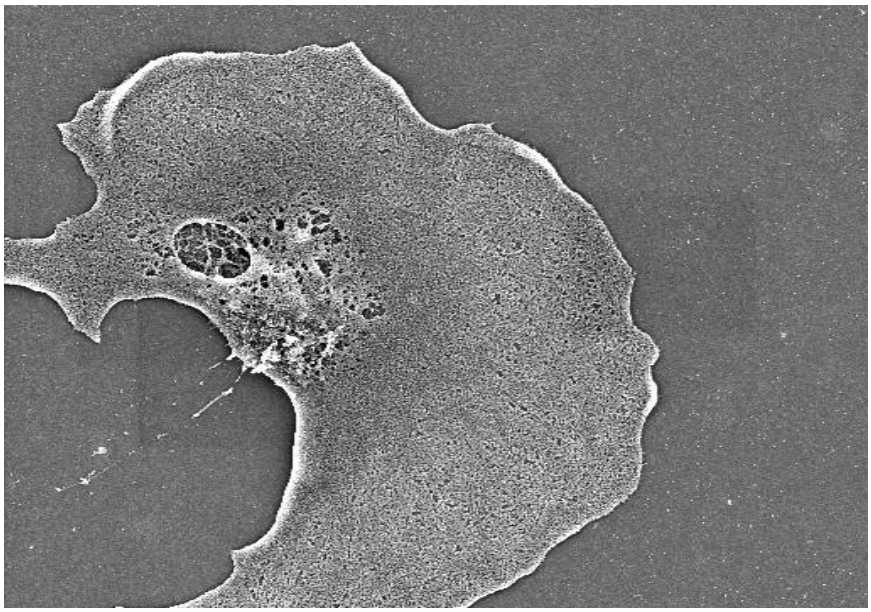
8 Appendix

Saponin permeabilization control experiment

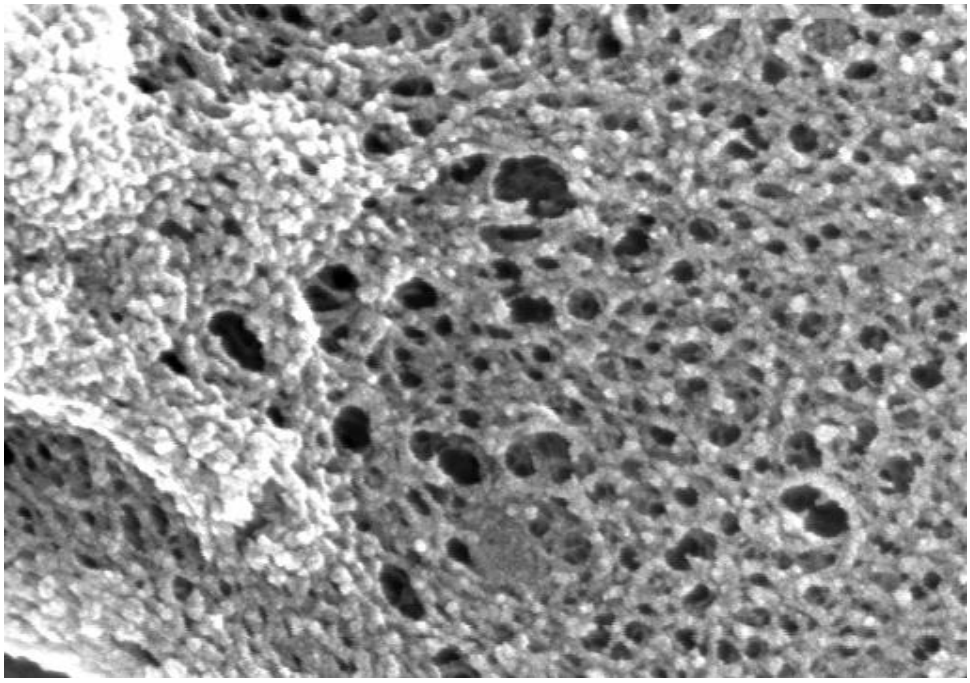


There is an increase of fluorescence of cells after permeabilization, when actin is visualized with Phalloidin AF 488, indicating a functional permeabilization. n=2, Mann Whitney U test, n.s

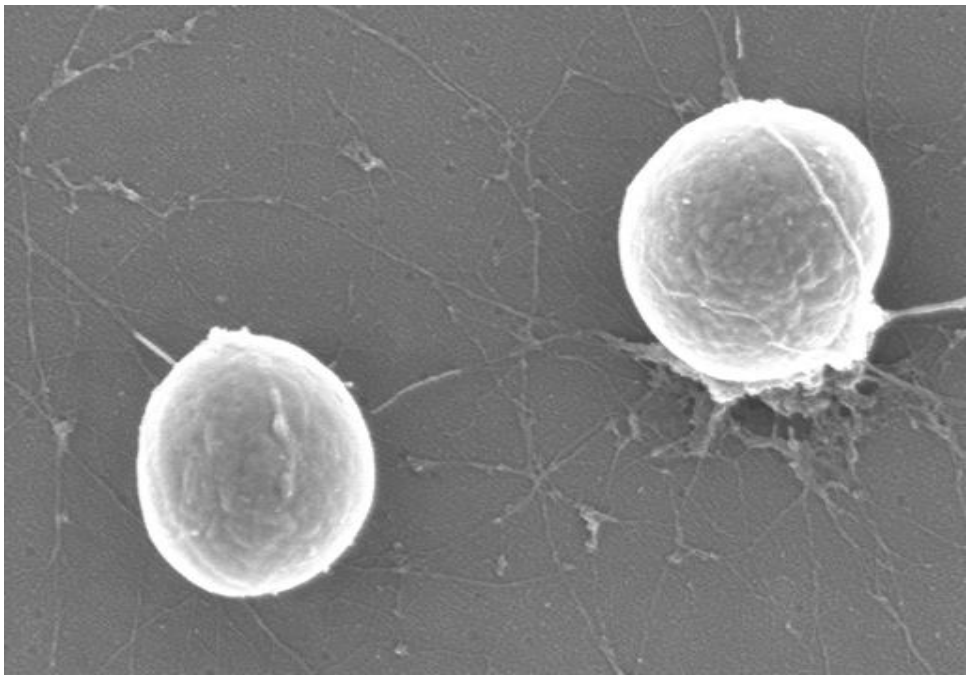
Migrating platelet, SEM, x10 000

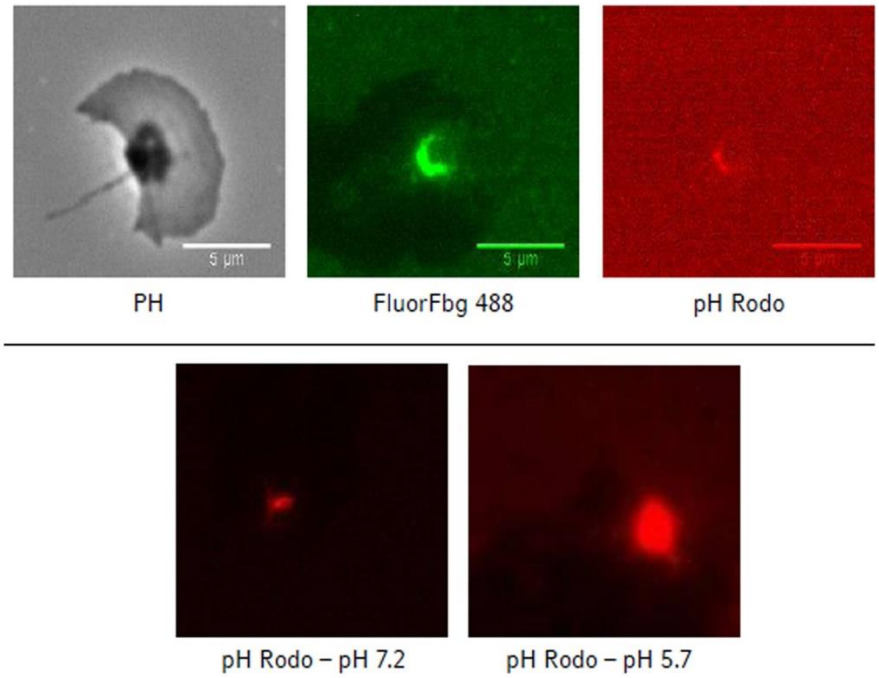


Platelet ultrastructure, SEM, x100 000

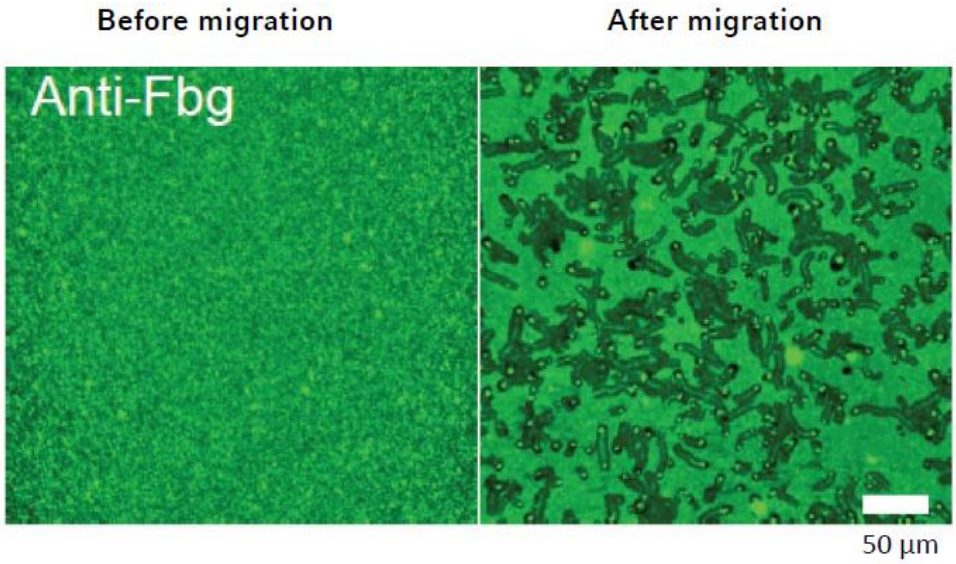


SA particles bound to fibrin, SEM, x50.000

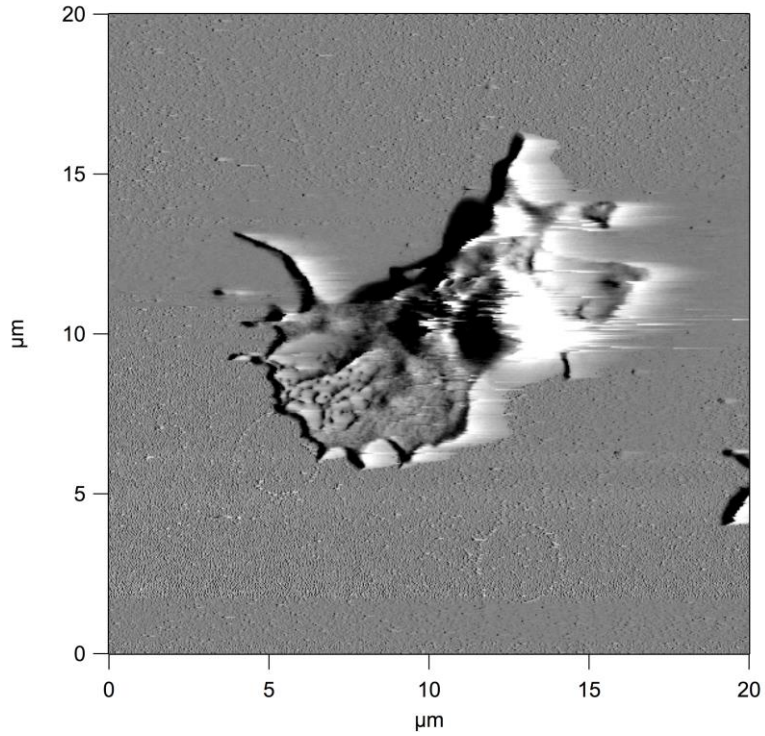




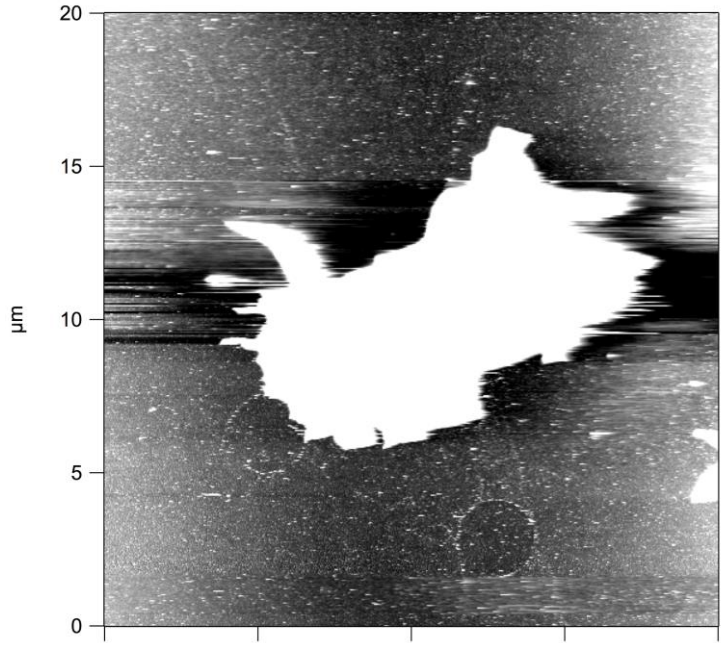
Fibrinogen-Ab visualizing cell tracks



Platelet retrace image, AFM, 20x20 µm



Platelet height image, AFM, 20x20 µm



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10 Eidesstattliche Versicherung

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Ich erkläre hiermit an Eides statt,

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München, 18.12.2017

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