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The interaction of procoagulant platelets with neutrophil granulocytes - Potential impact on thromboinflammation

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Abbreviations

Table 1. Abbreviations

ACD-A	Acid citrate dextrose anticoagulant
ADP	Adenosine diphosphate
AF	AlexaFluor
APC (cell)	Antigen-presenting cell
APC (stain)	Allophycocyanin
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAC	COVID-19-associated coagulopathy
CD	Cluster of differentiation
CD11b	Integrin subunit alpha M, part of Mac-1
CD16	F_c receptor ($F_c\gamma$RIII)
CD18	Integrin subunit beta 2, part of Mac-1
CD40	Transmembrane protein, main functions: Costimulation of B cell growth; differentiation and isotype switching
CD40L, sCD40L	CD40 ligand, soluble CD40 ligand
CD41	Integrin α_{IIb}, part of the receptor for fibrinogen and vWF
CD62P	P-selectin
CD63	Lysosomal associated membrane protein 3 (LAMP-3)

citH3	Citrullinated histone 3
COVID-19	Coronavirus disease 2019
DAMP	Damage-associated molecular pattern
DTS	Dense tubular system
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
FC	Flow cytometry
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GP Ibα	Part of the vWF receptor of platelets (GP Ib-IX-V complex)
GP IIb/IIIa	Glycoprotein IIb/IIIa, integrin αIIbβ3 CD41/CD61
GPVI	Glycoprotein IV, collagen receptor
h	Hour(s)
HBSS	Hanks buffered salt solution
HIT	Heparin-induced thrombocytopenia
HMGB1	High mobility group box 1
HOCl	Hypochlorous acid
ICAM-1	Intercellular adhesion molecule 1
IL	Interleukin
ISTH	International Society on Thrombosis and Haemostasis
JAM	Junctional adhesion molecule

LDN	Low-density neutrophils
Ly6g	Lymphocyte antigen 6 family member G, also found on neutrophils
Mac-1	Macrophage-1 antigen complex (CD11b/CD18)
MHC	Major histocompatibility complex
min	Minute(s)
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MPV	Mean platelet volume
MV	Microvesicle
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NOX2	Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2
OCS	Open canalicular system
PAC-1	Platelet-activation complex, active form of glycoprotein IIb/IIIa
PAD4	Peptidyl arginine deiminase 4
PAR	Protease-associated receptor
PBS	Phosphate buffered solution
PE	Phycoerythrin
PF4	Platelet factor 4
PFA	Paraformaldehyde

PLT	Platelet
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear cell
PNA	Platelet-neutrophil aggregate
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1, CD 162
RANTES	Regulated on activation normal T cell expressed and secreted
RBC	Red blood cell
ROS	Reactive oxygen species
RT	Room temperature
S100A8/A9	Calprotectin
SARS-CoV-2	Severe acute respiratory syndrome Coronavirus 2
SEM	Standard error of the mean
SELP	Selectin P, CD62P
SLC44A2	Solute carrier, also known as choline transporter-like protein 2
SSC	Sideward scatter
TF	Tissue factor (III)
T_h	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor

tPA	Tissue plasminogen activator
TRAP-6	Thrombin-receptor activating peptide 6
TxA_2	Thromboxane A_2
VCAM-1	Vascular cell adhesion molecule 1
VTE	Venous thromboembolism
vWF	Von Willebrand factor
WBC	White blood cell
wPLTs	Washed platelets

1 Introduction

1.1 Platelets and Hemostasis

Human platelets are disc-shaped cellular fragments circulating in the bloodstream with a number of 150 - 400x10^3 platelets/mm^3. With a diameter of 2 - 4 µm, platelets are relatively small in comparison to red blood cells (RBCs) and white blood cells (WBCs). Derived from megakaryocytes during the process of thrombopoiesis, platelets lack a nucleus but are equipped with different types of granular contents that may be released upon activation. The mean platelet volume (MPV) of a resting human platelet lies between 7-12 10^{-15} liter. (1,2)

Hemostasis describes a complex process of vast mechanisms aiming to terminate bleeding from a blood vessel. Platelets represent an essential component in hemostatic system. Here, vascular contraction as the first and foremost step aims to limit the extent of the blood loss or at least to reduce the tissue defect in size. Then, secondary hemostasis describes the activation of platelets after their exposure to underlying subendothelial extracellular matrix (ECM). Upon contact with most importantly collagen fibers and von Willebrand factor (vWF), platelets become significantly more adherent to the ECM. Next, they undergo a drastic shape change including the release of their granules and development of pseudopodia, numerous protrusions that increase the surface area and facilitate binding to adjacent platelets. Platelet adhesion and aggregation is mainly mediated by the bridging properties of platelet integrin αIIbβ3 but also other molecules favoring binding such as junctional adhesion molecules (JAMs). (3) Additional platelets are recruited and activated by molecules such as adenosine diphosphate (ADP), arachidonic acid, and thromboxane A_2 (TxA_2) or serotonin. The growing platelet so called white plug may be sufficient to terminate bleeding from small vessels. However, larger vascular defects require the third step of hemostasis to be corrected. Subsequently, a cascade of coagulation factors and enzymes takes place that culminates in the conversion of fibrinogen into fibrin fibers by the catalytic action of thrombin. The initial platelet plug is then stabilized by these fibrin threads and woven with the other cells circulating in the bloodstream.

While this cascade of coagulation is initiated, counterregulatory mechanisms are as well activated to restrict thrombus formation to the site of vessel injury. This tertiary mechanism of hemostasis is called fibrinolysis and relies on formation of the enzyme

plasmin through the action of tissue plasminogen activator (tPA). Later the during tissue repair the clot is resorbed. A schematic representation of secondary hemostasis is provided in **Figure 1**. (4,5)

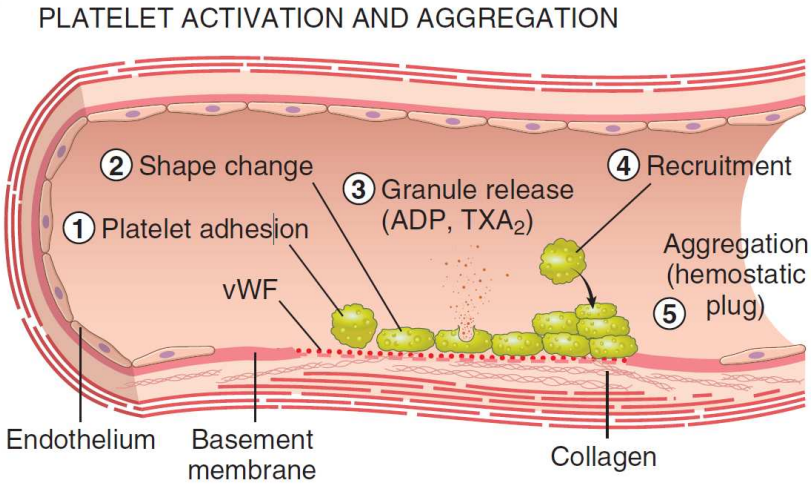

Figure 1. *Secondary hemostasis is represented by activation and aggregation of platelets resulting in a platelet plug. Adapted from: Robbins Basic Pathology, 10th edition, Elsevier 2017. (6)*

As described, adequate platelet count and function are essential for hemostasis. Equipped with a variety of substances, platelets interact with each other and with other cells by secreting or expressing various substances on their surface. Alpha granules take up to 1/10 of the total platelet volume and contain a multitude of proteins including fibrinogen, vWF and platelet P-selectin (CD62P) but also mediators such as platelet factor 4 (PF4) or RANTES (regulated on activation normal T cell expressed and secreted). The latter highlights the importance of platelets for recruiting WBCs and further immune responses. Delta (or dense) granules are a source of phosphonucleotides (ADP or adenosine triphosphate [ATP]) but also incorporate serotonin, calcium and polyphosphates among other substances. (7–9) Degranulation occurs via the ubiquitous open canalicular systems (OCS) providing a pathway towards the exterior. **Figure 2** illustrates the secretive properties of platelets. Additionally, platelets are capable of shedding microvesicles (MVs) which may express and contain certain features of platelets. (10,11)

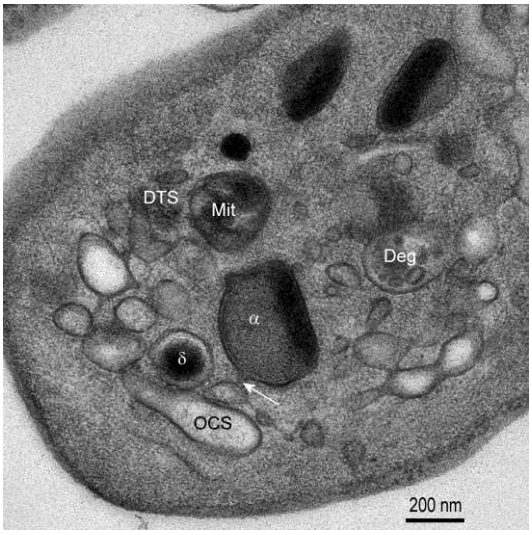

Figure 2. *Main secretory elements of platelets on electron microscopy. Scale bar 200 nm. DTS - dense tubular system, OCS – open canalicular system, α-granules, δ-granules, Mit - mitochondria, Deg - reorganization of the OCS before degranulation. Adapted from: Neumüller et al. Transmission Electron Microscopy of Platelets from Apheresis and Buffy-Coat-Derived Platelet Concentrates. In: The Transmission Electron Microscope - Theory and Applications. InTech 2015. (12)*

To fulfil the phenotypic changes during activation and platelet plug formation the membrane cytoskeleton of platelets is made up of several proteins including spectrin and adducin complexed with filaments of actin lining the intracellular side of the plasmalemma together with microtubules. Contractile motion is mediated by the interaction of phosphorylated non-muscle myosin IIa and actin filaments, but the marginal band constituted by the microtubules also plays a role during the shape changes. (13–15)

In healthy conditions, human platelets are depleted after an average lifespan of 7-11 days human platelets if they have not been consumed for hemostatic purposes before. It is estimated that approximately 10^{11} platelets are turned over each day. Several mechanisms including apoptotic shedding, antibody-mediated elimination and hepatic clearance have been described. (16,17)

Since platelets have plenty of tasks to fulfil in maintaining hemostasis, distinct subpopulations of platelets have been characterized according to their designated function. Typically, flow-cytometric determination of surface molecules expressed on platelets serves as the tool of choice in distinguishing the different subpopulations. Most of the circulating platelets are in a resting state under healthy conditions. Activated platelets, form upon contact with subendothelial matrix or can develop following activation by different mediators released from cells such as leukocytes, endothelial cells but also other already activated platelets.

1.1.1 Resting platelets

The resting state in which the majority of platelets are circulating in the bloodstream is characterized by thin, discoid platelets with a negatively charged outer layer glycocalyx with an asymmetric distribution of membranic lipids including negatively charged phosphatidylserine (PS) residing predominantly on the internal side of the platelet cell membrane. Their cytoskeleton ensures a relatively small and subtle appearance which allows smooth vascular passage. Flow-cytometry of resting platelets reveals a small volume with little forward and side scatter and little to no expression of activation markers such as CD62P or CD63 (LAMP-3). (18,19)

1.1.2 Activated platelets

Exposure of platelets to ECM immediately results in activation of platelets. As this definition is rather broad, several subpopulations of activated platelets have been postulated depicting the different predominating functions and phenotypes of each subclass. (20)

1.1.2.1 Secretory platelets

First, secretory platelets are responsible for the release of signaling molecules into their surroundings. As mentioned above, the contents of alpha and delta granules are rapidly expelled via the secretory system and once released mediate a plethora of cellular responses different purposes. Those include, facilitation of platelet-platelet interactions, stimulation of the plasmatic coagulation cascade and chemoattraction of different immune cells. Flow-cytometric characterization of secretory platelets includes markers for degranulation of α-granules (CD62P) and δ-granules, e.g., lysosomal CD63 (or artificially introduced fluorescence substances such as mepacrine). Platelets not only are involved in regulation of hemostasis but also by their secretive nature appear to play roles in mediating immune responses which yet are not fully understood. (8,21–25)

1.1.2.2 Aggregatory platelets

Platelets with increased binding capacity meaning high expression of active integrin αIIbβ3 are characterized as aggregatory platelets. Integrin αIIbβ3 (CD41/CD61) is the platelets´ fibrinogen receptor and represent its most abundant receptor present with a copy number of up to 80.000/platelet. In its active conformation αIIbβ3 facilitates the binding capacity of platelets to each other but also to fibrin threads. Inhibition of αIIbβ3 is of use during medical interventions such as percutaneous transluminal coronary

angioplasty to prevent the aggregation of platelets. The MPV of aggregatory is described to be within the normal range of a resting platelet. In flow-cytometry they can be detected using procaspase activating compound 1 (PAC-1) antibodies which bind to the active conformation of integrin IIb/IIIa. (25–27)

Following activation, particularly by strong agonists such as collagen, a relevant fraction of platelets undergoes a process called platelet spreading. Platelet adherence to the ECM is followed by structural changes where platelets present several morphological modifications. The initial discoid form converts to a rounder, globular shape with several pseudopods emerging from the platelet center (**Figure 3**, left). These protrusions then elongate further on in the timeline of platelet shape change, leading to the loss of the platelets´ three-dimensional properties as they continuously flatten in the direction of their filopodia (**Figure 3**, center). Consequently, platelets significantly increase their surface area facilitating direct interaction with neighbouring platelets. Finally, platelets reach the fully spread stage where they are said to resemble “fried eggs” (**Figure 3**, right). (19,28)

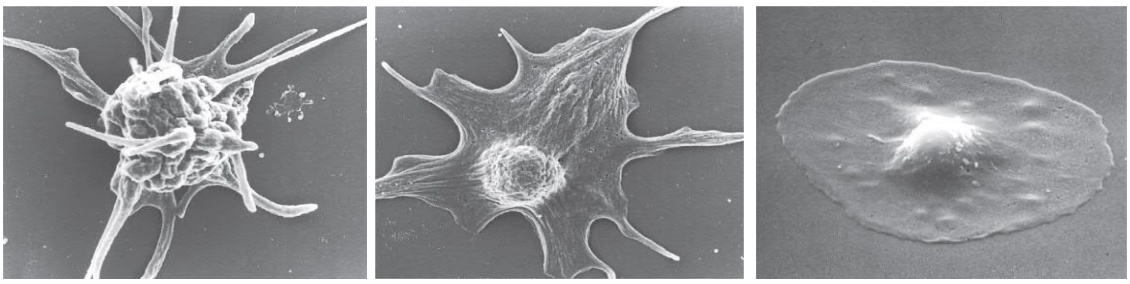

Figure 3. *Platelet spreading after activation on electron microscopy. Varying magnifications. Adapted from Thomas SG. The Structure of Resting and Activated Platelets. (Courtesy of Jim White.) In: Platelets. Academic Press 2019. (18)*

1.1.2.3 Procoagulant platelets

Procoagulant platelets have been described for being a particular subpopulation of activated platelets. The term procoagulant platelets originates from the characteristic membrane properties with particularly PS and other phospholipids externalized on the outer platelet membrane. In non-procoagulant platelets (aggregatory platelets, secretory platelets), PS exposure is usually prevented by the action of flippases which are ATP-dependent enzymes responsible for maintaining membrane integrity with PS concentrated on the cytosolic face. However, during formation of procoagulant platelets externalized PS provides an optimal surface for the coagulation cascade, enabling the assembly of factor IX and VIII tenase complex that has the potential to activate the enzymatic activity of Xa and prothrombin complex culminating in increased thrombin

generation and IIa (thrombin). As the main enzyme of plasmatic coagulation, thrombin then cleaves fibrinogen to fibrin creating a fibrin network. A prolonged state of high intracellular calcium levels recruited from platelets endoplasmatic reticulum and mitochondria paves the way for PS externalization and factor Xa and IIa affinity due to irreversible changes in the platelet membrane, particularly influx of chloride ions and membrane hyperpolarization. As a result, procoagulant platelets undergo ballooning and appear as enlarged, round cells under the microscope. (19,20,29)

Multiple flow-cytometric protocols have been proposed to identify and quantify procoagulant platelets including classical parameters for platelet activation (CD62P), markers from apoptosis protocols targeting PS such as AnnexinV or Lactadherin, the loss of PAC-1 or glycoprotein VI (GPVI shedding), markers of mitochondrial depolarization. (25,29–31) The Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (ISTH) recently recommended the use of a combined approach using CD62P and AnnexinV on a platelet-gated cell population. (32)

Procoagulant platelets have been described to have an essential role in a variety of prothrombotic diseases including COVID-19, heparin-induced thrombocytopenia (HIT), coronary artery disease or cerebral stroke. (30,33–35)

Apart from physiological agonists such as collagen or vWF, platelets can be subjected to several chemical compounds to generate similar intracellular signaling effects after binding to the respective receptor. Thrombin-receptor activating peptide 6 (TRAP-6) is a protease-associated receptor 1 (PAR-1) agonist simulating the effects of thrombin on this specific receptor. Convulxin, a snake venom, is a potent activator of GPVI and therefore has a similar effect as collagen. (25,31,36–38)

1.2 Neutrophil Granulocytes and Innate Immunity

The most abundant cells of the immune system are represented by neutrophil granulocytes. In healthy humans, between 4 - $10x10^3/mm^3$ WBCs constantly circulate in the bloodstream of which 40-70% are neutrophils. As part of the polymorphonuclear cells (PMNs) neutrophils are characterized by their multilobular nuclei and range up to 15 µm in diameter. In general, they are complex cells of the innate immune system with a large defensive repertoire. Namely, neutrophil granulocytes patrol in circulation and are among the first cells that quickly respond to invading pathogens; hence a low neutrophil count

(neutropenia, or less adequately leukopenia) is associated with a high risk of fatal infections.

Neutrophils originate from the process of myelopoiesis, more specifically granulopoiesis that starts with the differentiation of pluripotent hematopoietic stem cells into myeloid precursor cells. Under the influence of growth factors such as granulocyte colony-stimulating factor in the bone marrow, several mitotic and developmental steps are stimulated which result in formation of mature neutrophils. In case of infections or other inflammatory conditions, neutrophil production can be significantly upregulated by cytokines including interleukin-1 (IL-1) leading to the rapid release of neutrophils including immature cells with a non-lobulated, band form nucleus. (39)

As the term neutrophil granulocyte implies, neutrophils are equipped with several different types of granules. Azurophilic or primary granules mainly contain enzymes such as myeloperoxidase (MPO), neutrophil elastase (NE) and cathepsins. Specific or secondary granules on the other hand harbour the antimicrobial protein lactoferrin, several matrix metalloproteinases but also signaling molecules and receptors (e.g., tumor necrosis factor [TNF], fibronectin, or thrombospondin) which can be expressed on the neutrophil membrane upon degranulation. High concentrations of the enzyme gelatinase are found in tertiary (or gelatinase) granules although these granules also comprise lysozyme and the complement receptor Macrophage-1 antigen (Mac-1, CD11b/CD18). Secretory vesicles represent the fourth type of neutrophil granules and serve as a reservoir of membrane-associated proteins, mostly receptors such as Mac-1 or the immunoglobulin gamma F_c region receptor III ($F_c\gamma$RIII, CD16) that binds to the F_c portion of immunoglobulin G. (40,41)

1.2.1 Function and activation of neutrophils

After their release into the bloodstream, neutrophils usually reside only for a few hours in circulation before they extravasate into different tissue compartments. (42) Different chemoattractants (e.g., complement peptides or lipidic leukotrienes) which are released in the vicinity of damaged, infected or otherwise inflamed tissue induce recruitment of additional leukocytes. (43,44) Migration of WBCs from the blood vessels into the surrounding tissues is mediated by the interaction of leukocytes with the blood vessel wall. The traveling velocity of WBCs is reduced as they adhere and slowly “roll” on selectins (e.g., E-selectin, P-selectin) expressed on endothelial cells. Then, leukocytes start to bind to different endothelial glycoproteins such as ICAM-1 (intercellular adhesion molecule 1) or VCAM-1 (vascular cell adhesion molecule 1) using the versatile Mac-1

receptor among others. After firm adhesion, leukocytes traverse the vessel wall in a paracellular manner meaning that they squeeze through the ordinarily tight junctions between two adjacent endothelial cells. This motion, called diapedesis, allows neutrophils to quickly reach and gather at extravascular sites of tissue injury. (45–48)

Being part of the phagocytic system, neutrophil granulocytes can engulf invading pathogens and create so-called phagosomes where intracellular granules and reactive oxygen species (ROS) are attempting to kill the microorganisms during a process known as “respiratory burst”. (49,50)

Next to their ability to engulf invading pathogens, the degranulated antimicrobial enzymes also act in the proximity and facilitate degradation of pathogenic material. As a lysosomal heme-containing enzyme MPO catalyses the production of hypochlorous acid (HOCl). MPO and HOCl have been shown to interact with platelets although they did not appear to be strong platelet activators. (51) However, priming or potentiating effects of MPO on platelets were reported by Kolarova et al. (52) and Kokhan et al. (53). NE on the other hand is a major serine protease and aids in the hydrolysis of several substances including collagen and elastin. (54,55) Similarly to MPO, Chignard et al. reported an enhancing effect of NE on platelet activation that may origin from NE action on integrin αIIbβ3. (56,57) Bykowska et al. had similar findings pointing to a potential role of NE in platelet aggregation. (58) Serine proteases in general have been established to play an important role in hemostasis further emphasizing the ancient bidirectional effects of coagulation and immune responses. (59)

Recognition of mainly bacterial particles occurs via pattern recognition receptors (e.g., toll-like receptor [TLR]) while F_c and complement receptors (e.g., Mac-1) mediate the internalization of already opsonized material. In contrast to the larger monocytes, neutrophils have a limited capacity for phagocytosis and classically do not express MHC II (major histocompatibility complex II), a peptide structure used by antigen-presenting cells (APCs) to present phagocytosed molecules to helper T (T_h, $CD4^+$) lymphocytes. (60–64) However, recent studies have pointed to a function similar to APCs where neutrophils induce pathogen-specific activation of $CD4^+$ T cells and upregulation of CD40. (65,66) Furthermore, secretion of signaling molecules (e.g., TNF) from neutrophil granules primes and recruits additional leukocytes. (67,68)

1.2.2 Formation of neutrophil extracellular traps

As a third property, some neutrophils are known to eject large quantities of neutrophil extracellular traps (NETs) mainly consisting of thin, yet relatively long strands of DNA where antimicrobial enzymes accumulate, and pathogens are trapped. (69)

Although DNA decondensation and expulsion expectedly would necessarily lead to cell death, the Nomenclature Committee on Cell Death (NCCD) emphasized the difference of NET formation with and without cell death in their 2018 consensus. (70) There are hints pointing to a degree of reversibility of NETosis in some certain circumstances. (71) It is still under extensive research what dictates the type of NETosis and subsequently the fate of the neutrophil. The active process of NETosis can be initiated by various biological stimuli such as lipopolysaccharide, immune complexes or as a result of intercellular communication signals (e.g., TLR-mediated) but also artificially with calcium ionophores or phorbol 12-myristate 13-acetate (PMA). (72–74)

In general, NETosis is considered to be a peculiar type of programmed cell death (PCD) different from classical apoptotic or necroptotic pathways. Neutrophils releasing extracellular traps do not express classical apoptotic eat-me molecules on their surface. (75)

In fact, NET formation with cell death or suicidal NETosis is observed upon neutrophil activation by bacteria, viruses, and fungi but also PMA. Suicidal NETosis was shown to be nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2)-dependent with high levels of generated ROS. (76) During the G0 phase in a normal cell, its DNA is densely packed and wrapped around nucleosomes which consist of several proteins including histones. Little is known on the origin of DNA in NETosis and although some DNA may be derived from mitochondria (77), chromatin decondensation is required for nucleic DNA. Here, the enzyme peptidyl arginine deiminase 4 (PAD4) is of importance as it is responsible for catalyzing the conversion of arginine to citrulline. Citrullination of histone proteins reduces their positive charge and therefore loosens the linkage to the negatively charged DNA phosphates. (78–80) As such, citrullinated histones are considered specific for NETs and citrullinated histone 3 (citH3) in particular is a common marker used in commercial ELISA kits or for immunohistochemistry protocols. (81,82) Subsequently, the DNA is thought to exit the cell through large pores that form as result of the slow increase in plasma membrane permeability of neutrophils undergoing programmed cell death with NET formation.

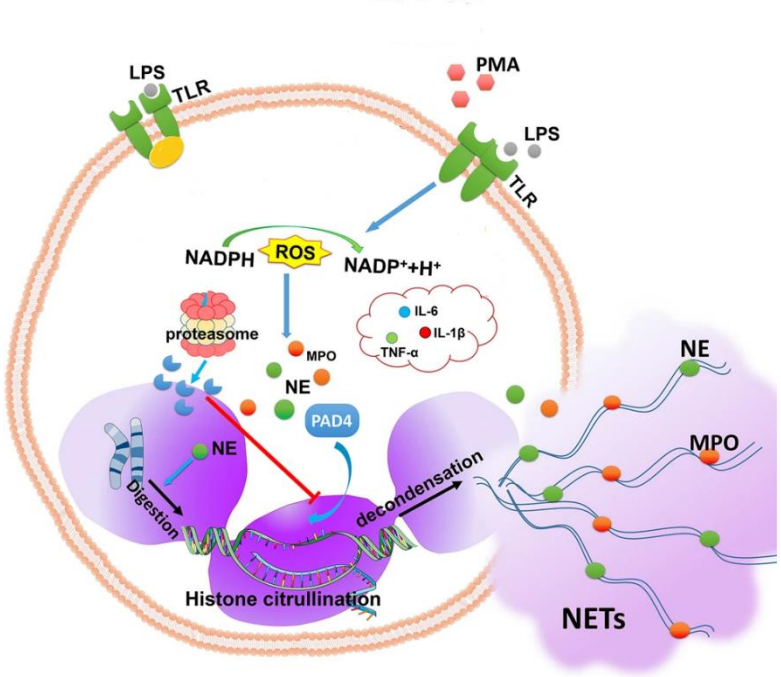

Figure 4. *NOX-2-dependent process of NET formation. NADPH - nicotinamide adenine dinucleotide phosphate, PAD4 - peptidyl arginine deiminase 4, NE – neutrophil elastase, MPO – myeloperoxidase, LPS – lipopolysaccharide, PMA - phorbol myristate acetate, TLR – toll like receptor. Modified from Ou Q et al. Nat Commun 2021. (83)*

NET formation without cell death is known as vital NETosis where neutrophils continue their function after formation of NETs. This process is NOX2-independent (84) and thought to occur after TLR-mediated neutrophil activation. Here, DNA strands leave the neutrophils via exocytosis while the plasmalemma remains intact. (85) Little is known about the relevance of vital NETosis and some evidence also suggests that vital NETosis could be a very rapid response to bacteria which nevertheless ultimately ends with cell death. (86)

1.2.3 NETs in disease

By their nature, NETs play a relevant role in immune defence during severe infections. (87–89) However, their appearance was also observed in non-infectious settings such as rheumatological (77,90,91) or metabolic diseases (92–94). For instance, hyperglycaemia in diabetes type 1 was shown to be linked with the formation of NETs which in turn are associated with the end organ failure in diabetes patients. (95) NETs found in rheumatoid arthritis were shown to negatively affect the inflammatory processes in joints. (96) Elevated levels of NETs have also been described in malignancies where they seem to be interesting targets to limit dissemination and metastatic spread. (97,98) The importance of NETs in vascular pathologies is an already well-established

phenomenon, particularly for cardiovascular diseases. Here, NETs may serve as predictors of mortality after myocardial infarction. (99,100)

Although the detrimental effects of NETs on coagulation have already been described for various prothrombotic conditions before the appearance of SARS-CoV-2, it was the high rate of thrombosis and embolisms observed during the COVID-19 pandemic that directed towards a strong association between hyperinflammation and thrombus formation. In HIT it is already established that formation of life- neutrophil activation and NET release contribute to the onset of thromboembolic events.(81,101)

Various prothrombotic mechanisms have been proposed for NETs. The most obvious property of NETs favoring thrombosis is their large surface providing an ideal assembly side for different prothrombotic coagulation factors. Due to their DNA backbone, negatively charged NETs attract positively charged proteins such as proteinases but also coagulation factors and tissue factor. (102) Additionally, complex interactions between NETs and endothelial cells were described where NETs trigger endothelial injury and chronic inflammation. (103–105) Interestingly, the role of histones in promoting venous thrombosis has been accentuated by some studies (106,107), platelet activation by NETs appears to not necessarily rely on histones. (108)

A distinct subpopulation of neutrophils has been described to have a higher tendency to form NETs (high pro-NETotic potential) than other neutrophil subsets. Those so-called low-density neutrophils (LDNs) were found for having higher prevalence in patients with COVID-19. LDNs which are sometimes also described as CD16 intermediate neutrophils, due to their intermediate surface expression of $F_c\gamma$RIII, were shown to have pro-NETotic gene upregulation and high tendency for spontaneous NET formation in vitro. (109,110)

1.3 Immunothrombosis and Thromboinflammation

Traditional concepts of hemostasis considered activation of platelets and blood coagulation factors as major events that are required for sufficient activation of the coagulation cascade. However, accumulating evidence highlights that several other mechanisms and systems are involved in hemostatic function and dysfunction. This has prompted the development of several concepts including *thromboinflammation* or *immunothrombosis*. Both terms aim to better reflect the complex relationship between the primary hemostatic system and other influencing processes, in particular effects of the immune system.

Tanguay et al. have first conducted experiments to inhibit platelet-leukocyte interaction or in their words reduce “thrombo-inflammatory reactions” in case of restenosis following coronary angioplasty in 2004. (111) Since then, many other links between the hemostatic and immune system have been established. (59,112–114)

Early on in the COVID-19 pandemic, life-threatening thrombotic complications were frequently reported in patients with SARS-CoV-2 infection. (115–118) The overall prevalence of venous thromboembolic embolism (VTE) was estimated to be as high as 20.7% with a doubled risk for COVID-19 cases admitted to the intensive care unit. (119) Subsequently, a significant fraction of these VTE patients complicated with pulmonary embolism further complicating the respiratory insufficiency. For instance, a large retrospective analysis of more than 370,000 COVID-19 patients in England found a 86% risk of such VTE cases to develop pulmonary embolism. (120) This so-called COVID-19-associated coagulopathy (CAC) has so far been explained by hyperactive platelets in combination with prothrombotic effects mediated by immune cells, mainly involving neutrophils and monocytes.

In fact, various direct and indirect factors are of importance in mediating the prothrombotic effects of a dysregulated (hyper-)inflammatory host defence response. Up until now, some established pathways have already been described but there are several hypotheses under research. It is agreed that there are bidirectional effects in action between the hemostatic and immune system resulting in “inflammation-induced coagulation and coagulation-mediated inflammation” (Schrottmaier and Assinger). (121) These connections may stem from more ancient times in phylogeny when organisms were not as sophistically complex as they are now with multiple different cells present in blood. (59,122,123)

1.4 Platelet-neutrophil interaction

1.4.1 Receptor-mediated platelet-neutrophil aggregation

Platelet-neutrophil aggregates (PNAs) represent the first quantifiable endpoint in this interaction between platelets and neutrophils. Elevated levels of PNAs have been observed in infectious diseases such as COVID-19 (124–129) but also non-infectious conditions such as auto-immune or auto-inflammatory diseases (130–132) and chronic cardiovascular affections (133,134). **Figure 5** shows such a PNA in the setting of reperfusion injury following myocardial revascularisation.

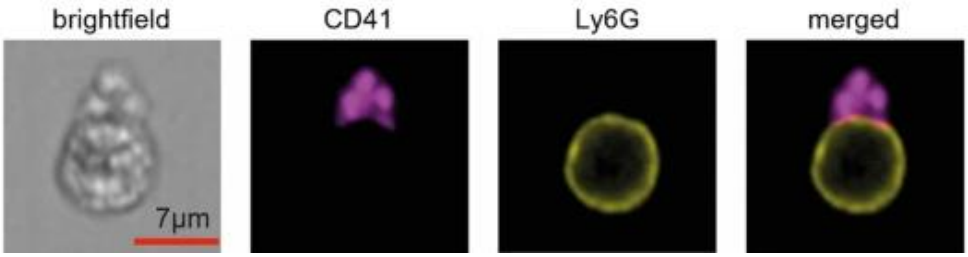

Figure 5. *Platelet-neutrophil aggregate in brightfield and immunofluorescence microscopy. CD41 - Integrin α_{IIb}, Ly6G - lymphocyte antigen 6 family member G. Scale bar 7µm. Adapted from: Starz et al. Basic Res Cardiol 2022 (135)*

Several receptors are known to mediate the binding of platelets and neutrophils. Most importantly, platelet CD62P and neutrophil P-selectin glycoprotein ligand-1 (PSGL-1) serve as the main axis for PNA formation. (136,137) This cellular interaction site is long known and has been extensively studied so that several clinical applications utilize the CD62p-PSGL-1 axis as therapeutic target. In fact several anti-CD62P antibodies have been designed to reduce cell-cell interactions via PSGL-1 and CD62P in sickle cell disease or to inhibit progression of myocardial damage following percutaneous coronary intervention after myocardial infarction. (138,139)

Another relevant docking site for platelets on neutrophils is represented by the macrophage-1-antigen (Mac-1). Despite the name, Mac-1 is also expressed on neutrophils. Made up of the integrins αM (CD11b) and β2(CD18) it serves different purposes during complement binding and leukocyte extravasation. (140) Determination of CD11b may be used as a marker of neutrophil activation besides CD66b. Previously, interaction of Mac-1 with platelet glycoprotein Ibα (vWF receptor) was shown to have a strong influence on thrombus formation in animal models. (141,142) Studies on neutrophils from COVID-19 showed an increased expression of both CD11b and CD18 compared to healthy volunteers. (110,126) Mac-1 also serves as a receptor for different

chemokines excreted from platelets as discussed later. An overview on direct and indirect platelet-neutrophil interaction in the setting of CAC is shown in **Figure 6**.

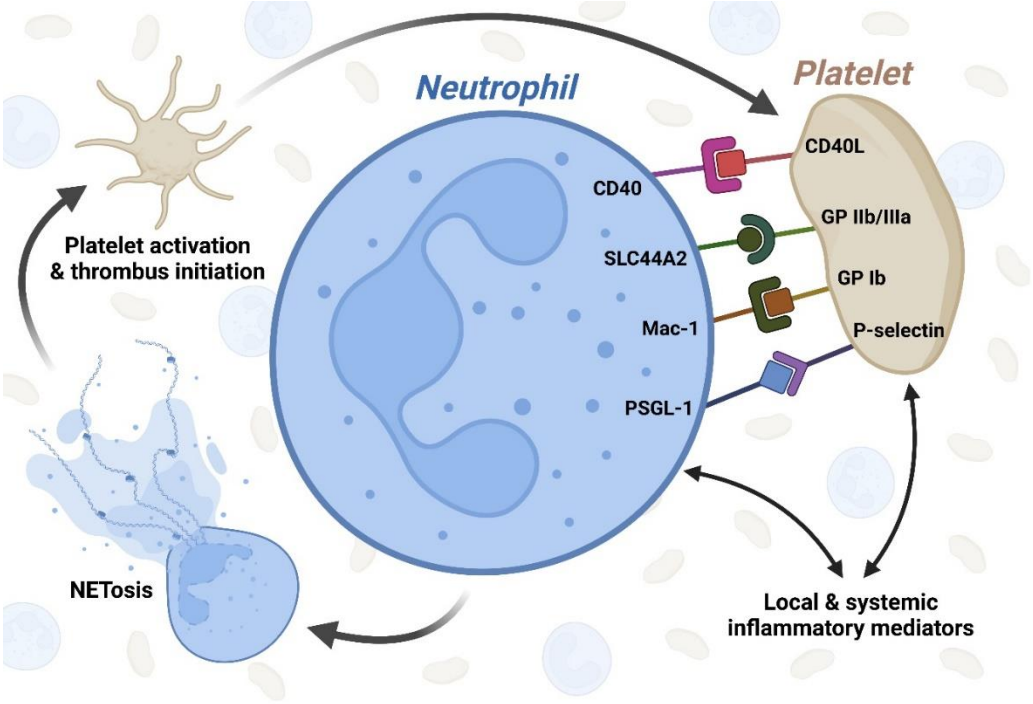

Figure 6. *Direct and indirect pathways of platelet-neutrophil interaction in COVID-19. Adapted from Hirsch et al. Frontiers of Immunology 2023. (143)*

Platelet integrin αIIbβ3 and the widely distributed choline transporter-like protein 2 (SLC44A2) is another relevant axis of platelet-neutrophil interaction. SLC44A2 has already been found to be of importance in hemostasis and particularly VTE. (144,145) Additionally, Constantinescu-Bercu et al. highlighted the neutrophil SLC44A2 – platelet integrin α2bβ3 axis as an important communication channel of NETosis. (146)

As CD40 and its ligand CD40L are involved in the regulation of many immune responses, neutrophils and platelets were also shown to rely on this pathway. (147,148) Animal models found a crucial role of CD40L expressed on platelets and soluble CD40L released from platelets (sCD40L) in activating neutrophils. (149,150) CD40L was established to also bind to Mac-1 with enhancing effects on neutrophil adhesion to platelets. (151,152)

1.4.2 Indirect communication between platelets and neutrophils

Precise analysis of the numerous indirect ways of crosstalk between platelets and neutrophils is challenging. Most importantly, inflammatory mediators excreted from both cell types and MVs are thought to take part in platelet-neutrophil interaction. MVs are

released through membrane budding and usually comprise intracellular contents. Platelets are a well-known source of MVs. Skendros et al. proposed a platelet-neutrophil-tissue factor (TF) axis which could be a critical connector between plasmatic and cellular hemostasis on the one hand and the immune defence system on the other. (153) Both neutrophils and platelets were found to be significant sources of TF-expressing MVs in SARS-CoV-2 infected individuals. (154,155)

A multi-step circuit involving direct interaction via CD62P/PSGL-1 with subsequent platelet-induced arachidonic release from neutrophils is suggested by Rossaint et al. TxA_2 is generated in the platelet after uptake of arachidonic acid. TxA_2 is then released leading to endothelial activation and promotion of leukocyte rolling and diapedesis. (156) Furthermore, NET formation in the pathophysiology of transfusion related acute lung injury (TRALI) has previously been partially attributed to the effects of TxA_2. (157)

The complex cytokine response to SARS-CoV-2 viremia is mediated by multiple cell types including neutrophils and platelets. In severe cases an hyperinflammatory state with massive cytokine release (“cytokine storm”) develops and further aggravates the risks for thrombotic events. As mentioned, next to sCD40L platelets may also secrete other non-cytokine mediators, e.g., the positively charged endogenous chemokine PF4 in COVID-19. (158) PF4 is known to interact with the neutrophil Mac-1 receptor and also directly with NETs due to their anionic nature. The diverse effects of PF4 include chemotaxis, stimulation of NETosis and compaction of NETs. (159–164)

High-mobility group box 1 (HMGB1), a damage-associated molecular pattern (DAMP), also appeared to play a role among the many mediators of platelet-neutrophil signaling. DAMPs including HMGB1 are expectedly potent inducers of NETosis and the importance of HMGB1 in thrombosis has been established in an animal model. (165,166) Findings from Maugeri et al. on patients with acute myocardial infarction indicated that platelet-derived HMGB1 triggers NET release by acting on the RAGE receptor (Receptor for Advanced Glycation End products). (167)

Interleukin 6 (IL-6) has already been identified as a therapeutic target in antagonizing the hyperinflammatory state in severely affected COVID-19 patients. (154,168) Interestingly, *in vitro* blockade of IL-6 with usual concentrations of tocilizumab significantly reduced the number of PNAs and TF^+-platelet MVs. (154)

Furthermore, activated neutrophils may release calprotectin (or S100A8/A9) which is a protein capable of *in vitro* inducing procoagulant platelets via GP Ibα signaling.

S100A8/A9 was shown to be elevated in COVID-19 patients and correlating with disease severity. (169,170)

There is strong evidence for the procoagulant impact of cathepsins and more particularly cathepsin G. Direct effects of this serine protease on platelets is postulated to occur via the thrombin receptors PAR-1 and/or PAR-4. (108,171–173)

1.5 Objectives of this MD thesis

This MD thesis aimed to address the following questions:

- What is the impact of procoagulant platelets on neutrophil granulocytes?
- Are procoagulant platelets different in their influence on neutrophil activation and formation of NETs than activated platelets?
- Is there a difference between platelet-neutrophil aggregates and platelet-induced NET formation?

Accordingly, the first objective of this MD thesis was to establish and optimise reliable methods that allowed deeper insights into the interplay of different platelet subpopulations with neutrophil granulocytes. Here, different agonists were used to induce different platelet subpopulations under standardized conditions which enabled systematic investigation of their potential to interact with isolated neutrophil granulocytes.

During the establishment of robust and reproducible protocols, two major readouts in platelet-neutrophil interplay were defined:

First, PNAs resulting from the direct interaction between agonist-stimulated platelets and neutrophils were analysed. Quantification of PNAs was realised using a flow-cytometric (FC) approach and comparisons in PNA formation between differently stimulated platelets were made. This FC model was then used to perform inhibition experiments to further characterize this form of direct platelet-neutrophil interaction.

The potential of platelets to induce formation of NETs represented the second endpoint of interest in this MD project. The objective was to establish microscopic protocols for platelet-neutrophil interaction focusing on visualization and quantification of NETs.

2 Material and Methods

2.1 Materials

Chemicals used for the experiments of this MD thesis are listed in **Table 1**, antibodies and stains are mentioned in **Table 2**. Devices, software, and equipment in the laboratory are comprised in **Table 3** and **Table 4**, respectively.

Table 2. Chemicals

Chemical	Manufacturer
Acid Citrate Dextrose Anticoagulant (ACDA)	TerumoBCT; Lakewood, USA
ACK RBC lysis solution	Life Technologies/Gibco; Carlsbad, USA
Apyrase	Sigma-Aldrich; St. Louis, USA
Bovine Lactadherin	Haematologic TechnologiesProlytix; Essex Junction, USA
Bovine serum albumin (BSA) 20%	Sigma-Aldrich; St. Louis, USA
Calcium chloride ($CaCl_2$)	Sigma-Aldrich; St. Louis, USA
CellFix solution	BD Biosciences; San Jose, USA
Convulxin	Enzo; Farmingdale, USA
Distilled water	Fresenius Kabi; Bad Homburg, GER
Dulbecco´s Phosphate Buffer Solution (PBS)	Life Technologies/Gibco; Carlsbad, USA
EasySep Direct Human Neutrophil Isolation Kit	StemCell Technologies; Vancouver, CA
Fibrinogen	Merck; Darmstadt, USA

Ficoll Paque (density 1.077 g/mL)	GE healthcare; Chicago, USA
Glucose	B. Braun; Melsungen, GER
Hanks buffered salt solution	Carl Roth; Karlsruhe, GER
HEPES	Sigma-Aldrich; St. Louis, USA
Hirudin	Loxo; Dossenheim, GER
Hydrochloric acid (HCl) 1M	VWR Chemicals; Radnor, USA
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich; St. Louis, USA
Paraformaldehyde (PFA)	Morphisto; Offenbach, GER
Penicillin/Streptomycin	Life Technologies/Gibco; Carlsbad, USA
Phorbol 12-myristate 13-acetate (PMA)	Merck; Darmstadt, GER
RPMI 1640 culture medium	Carl Roth; Karlsruhe, GER
Thrombin	Roche; Basel, CH
TRAP-6	HART Biologicals; Hartlepool, UK
Triton X	Sigma-Aldrich; St. Louis, USA

Table 3. Antibodies and stains

Antibody/Stain	Manufacturer
AF488 anti-mouse	Life Technologies/Invitrogen; Carlsbad, USA
AF-647 anti-goat	Life Technologies/Invitrogen; Carlsbad, USA
Anti-AnnexinV FITC	Immunotools; Friesoythe, GER
Anti-CD16 PE	Miltenyi Biotec; Bergisch Gladbach, GER

Anti-CD41 APC	Biolegend; San Diego, USA
Anti-CD62p APC	BD Biosciences; San Jose, USA
Anti-citrullinated histone 3 (anti-citH3), goat	Abcam; Cambridge, UK
Anti-myeloperoxidase (anti-MPO), mouse	Biorad; Hercules, USA
Coralite-555 anti-CD41	Proteintech; Rosemont, USA Biolegend; San Diego, USA
Hoechst 33342 nuclear stain	Life Technologies/Invitrogen; Carlsbad, USA
Inclacumab (anti-SELP)	ProteoGenix; Schiltigheim, FR
Sytox Green Nucleic Acid Stain	Life Technologies/Invitrogen; Carlsbad, USA

Table 4. Devices and software

Device	Company
Axio Observer 7 inverted fluorescence microscope	Carl Zeiss Microscopy; Oberkochen, GER
CELL-DYN Ruby hematological analyzer	Abbott; North Chicago, USA
Flow-cytometer Navios	Beckman Coulter; USA
Freezer (-80°C)	ilshin Europe; Ede, Netherlands
GraphPad Prism	GraphPad Software; Boston, USA
Heraeus 37°C incubator	Thermo Scientific; Waltaham, USA
ImageJ Fiji	

Kaluza	Beckman Coulter; Brea, USA
Microsoft Excel and Word	Microsoft Corporation; Redmond, USA
Refrigerator (4°C)	Siemens; GER
Rotina 46 R Centrifuge	Hettich; Tuttlingen, GER
SevenCompact pH meter S210	Mettler-Toledo; Greifensee, GER
Shaker	Philips; Brussels, Belgium
Vortexer, Reax-Top	Heidolph; Schwabach, GER

Table 5. Laboratory materials

Material	Manufacturer
50 mL Tubes	Greiner bio-one; Frickenhausen, GER
96 well glass bottom plates	Cellvis; Mountain View, CA
9 mL Monovettes	Sarstedt; Nümbrecht, GER
6.5 mL Trisocium Citrate Monovettes	Sarstedt; Nümbrecht, GER
Eppendorf-Cups 1.5; 2 mL	Eppendor; Hamburg, GER
Flow cytometer tubes	Sarstedt; Nümbrecht, GER
Nitril gloves	Paul Hartmann; Heidenheim, GER
Pasteur pipet 5 mL	Carl Roth; Karlsruhe, GER
TipOne XL Graduated Tips 10, 200, 1000 µL	Starlab; Hamburg, GER

2.2 Methods

2.2.1 Isolation of neutrophil granulocytes via density gradient centrifugation

In a first approach, neutrophil granulocytes were isolated using density gradient centrifugation. Here, after written consent was obtained whole blood was withdrawn from healthy blood donors by cubital venipuncture venipuncture (21 gauge) into 6.5 mL citrate monovettes and allowed to rest for 30 minutes (min) at room temperature (RT). Donors were randomly selected at the blood donation center, and no additional data was recorded. The blood was then diluted 1:1 with sterile PBS and carefully layered on Ficoll Paque. After centrifugation (30 min, 400g, RT, no brake) all layers were removed until only PMNs and RBCs remained in the falcon.

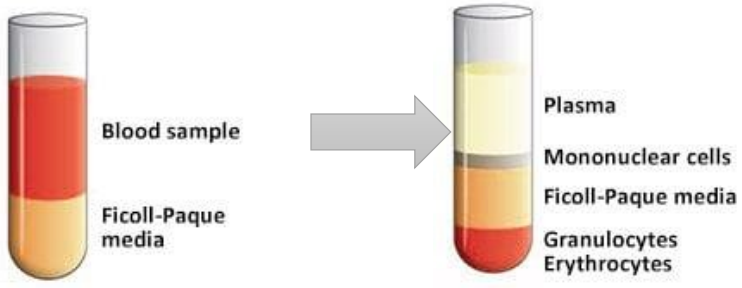

***Figure 7**. Layers before and after centrifugation during density gradient centrifugation. (174)*

Afterwards, RBC lysis was performed twice for 10 min (400g, 10 min, RT, no brake) using ACK RBC lysis solution. Final resuspension of the PMN pellet was performed with 2 mL of RPMI 1640 culture medium which was supplemented with 10 mM HEPES and 100 U/mL Penicillin/Streptomycin. Cell count was adjusted to $5x10^3$ WBCs/μL^3 after automated cell counting at a Cell-Dyn Ruby hematological analyzer. Here, isolation quality in terms of neutrophil purity was also evaluated. Isolated neutrophils were kept at 37°C and used within maximum 1 hour (h).

2.2.2 Neutrophil purification using magnetic beads (negative selection)

Magnetic bead isolation of neutrophil granulocytes was performed with the EasySep Direct Human Neutrophil Isolation Kit according to the manufacturer's instructions (69). Briefly, citrated whole blood was mixed with an antibody cocktail and magnetic beads and then diluted before placing the tube in a magnetic field. The supernatant was

harvested by outpouring the liquid after 5 min within the magnetic field. RBCs and platelets were attracted to the exterior of the tube leaving the PMNs untouched in the center of the mixture.

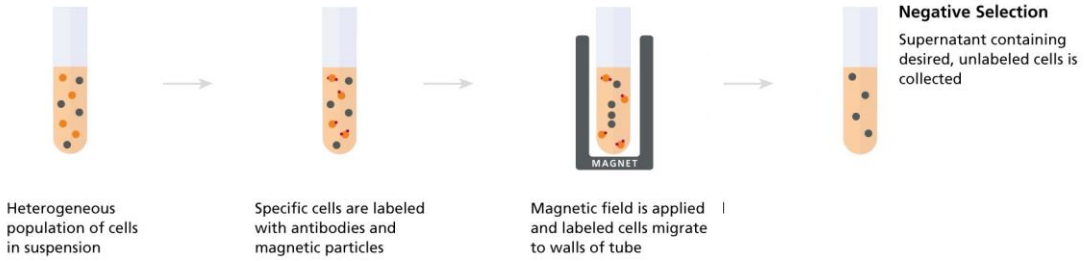

Figure 8. *Schematic representation of negative selection of neutrophils. (175)*

This procedure was repeated once to minimize platelet and RBC contamination and maximize neutrophil purity. After the last step, the purified neutrophil granulocytes were concentrated by centrifuging for 10 min at 400g with no brake. The pellet was then resuspended in RPMI 1640/HEPES/Pen+Strep as described above and adjusted to $5x10^3$ WBCs/μL^3.

2.2.3 Preparation of washed platelets

Washed platelets were prepared as described earlier (17,70). Whole blood was withdrawn by cubital venipuncture into anticoagulant citrate dextrose solution (ACD-A) containing vacutainers and allowed to rest for 30 min at 37°C. The first flow-through was discarded in order to reduce platelet pre-activation via TF and other activating components. Platelet-rich plasma (PRP) was removed carefully with a Pasteur pipette after centrifugation for 20 min at 120g (RT, no brake) and supplemented with 111 µL/mL warmed ACDA and 5 µL/mL warmed apyrase. This was followed by centrifugation at 650g for 7 min (RT, no brake) and resuspension of the platelet pellet in a washing buffer that was prepared from modified Tyrodes buffer (1 mg/mL glucose, 3.5 mg/mL bovine serum albumin, bicarbonate buffer in sterile water enriched with 2 µL/mL apyrase and 2 U/mL of the direct thrombin inhibitor hirudin, pH adjusted to 6.4 with 1 M hydrochloric acid). After 15 min of rest at 37°C a final centrifugation step (650xg, 7 min, RT, no brake) was performed and the obtained platelet pellet was resuspended in a suspension buffer containing the modified Tyrodes buffer with 2 mM $MgCl_2$ and 4 mM $CaCl_2$. After measurement at a Cell-Dyn Ruby hematological analyzer the platelet count was adjusted to $300x10^3$/µL.

2.2.4 Agonist-induced platelet subpopulations

Isolated washed platelets were stimulated with the PAR-1 agonist TRAP-6 (final concentration [c_f]=10 µM, thrombin c_f =1 U/mL), convulxin (c_f =0.1 µg/mL) and in combination (TRAP-6/convulxin, thrombin/convulxin) for 30 min at RT. Ligands were diluted to the c_f in PBS. Afterwards, 10 µL of the platelet suspension were transferred into Hanks buffered salt solution (HBSS) and stained with 1 µL of anti-CD62P-APC and/or anti-AnnexinV-FITC. Flow-cytometric quality control after agonist stimulation was assured via analysis of anti-CD62P-APC /anti-Phosphatidylserine (AnnexinV) signals. Solely activated platelets were considered as $CD62P^+/PS^-$ while procoagulant platelets were defined as double positive ($CD62P^+/PS^+$).

2.2.5 Flow-cytometric assays

2.2.5.1 Quantification of platelet-neutrophil aggregates

Washed platelets (wPLTs) were diluted to 100.000 platelets/µL and incubated with TRAP-6, thrombin, convulxin and combinations of TRAP-6 and thrombin with convulxin for 15 min as described above. Afterwards, $2x10^6$ (20 µL) of each platelet sample was co-incubated with $1x10^5$ (20 µL) of isolated neutrophils for 30 min at 37°C.

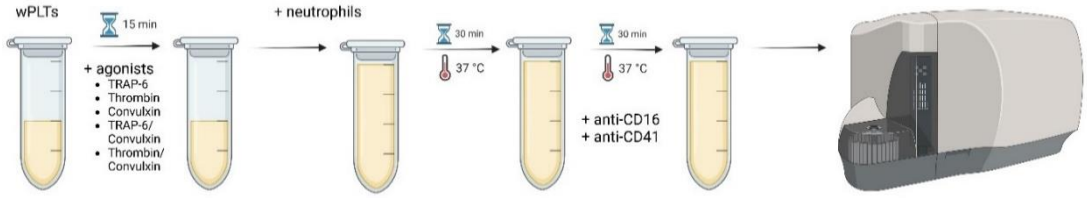

Figure 9. *Workflow for platelet-neutrophil co-incubation. wPLTs - washed platelets. Created with BioRender.com*

Staining with 2 µL anti-CD16 PE and 0.5 µL anti-CD41 APC was performed for additional 30 min in the dark at 37°C. 20 µL of the cell suspension were used and filled up with 480 µL CellFix (1:10 in distilled water) to a final volume of 500 µL for flow-cytometry analysis. A CD16 live gate (2000 events) was used to measure $CD16^+/CD41^+$ events.

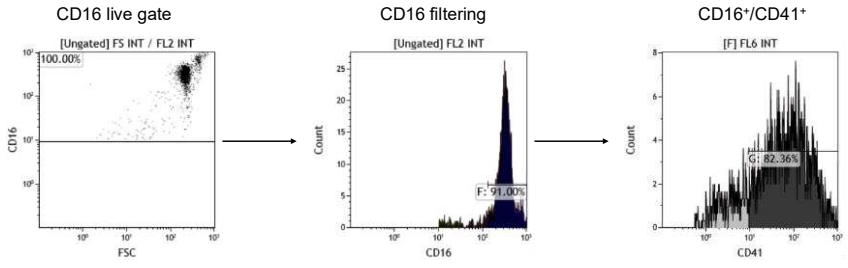

Figure 10. *Gating strategy for quantification of platelet-neutrophil aggregates.*

2.2.5.2 Effect of CD62P inhibition on PNA formation

Platelet CD62P was inhibited with the monoclonal antibody anti-selectin P (anti-SELP) Inclacumab which is currently under evaluation for the treatment of sickle cell anaemia. A serial dilution in PBS was performed to generate a titration curve for the ability of Inclacumab to inhibit CD62P-mediated PNA formation. For this purpose, platelets were stimulated with thrombin to express maximum amounts of expressed CD62P on the platelet surface. Afterwards, cells were incubated with varying concentrations (25 - 0,012 µg/mL) of Inclacumab for 15 min at RT and then incubated with neutrophils as described above. Later, a plateau concentration of 1.5 µg/mL Inclacumab was used to dissect the effect of CD62P blockade on PNA formation for platelets subjected to stimulation with the remaining agonists.

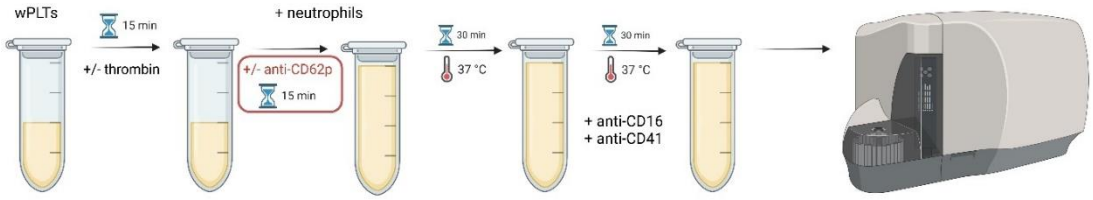

Figure 11. *Schematic representation of CD62P inhibition. Created with BioRender.com*

2.2.5.3 Effects of Phosphatidylserine inhibition on PNA formation

Platelet phosphatidylserine was inhibited with bovine Lactadherin, a phosphatidylserine-binding protein. The serial dilution started with 1000 nM. The experiments were conducted on platelets stimulated with thrombin/convulxin with a maximum induction of procoagulant platelet phenotype as this was where the most residual aggregates were observed (data shown below). After 15 min of lactadherin incubation neutrophils were added as described above.

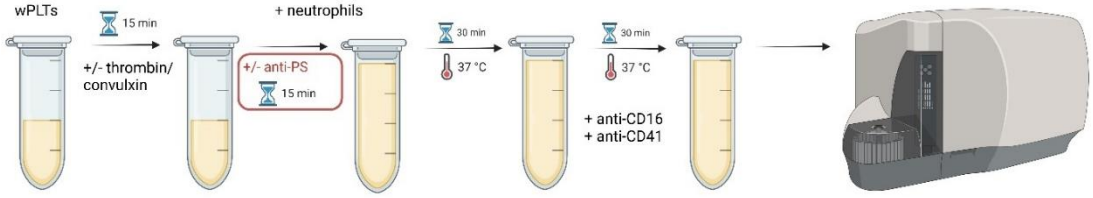

Figure 12. *Schematic representation of PS inhibition. Created with BioRender.com*

2.2.5.4 Combined effects of CD62P and PS inhibition

After dual platelet stimulation with thrombin and convulxin for 15 min, Inclacumab (1.5 µg/mL) was added. To investigate the potential of PS blockade to further decrease PNA

formation beside CD62P/PSGL-1-mediated PNAs the serial dilution of Lactadherin was used. Incubation with Inclacumab and Lactadherin for 15 min was followed by the addition of neutrophils. Staining and FC measurements were performed as described as above.

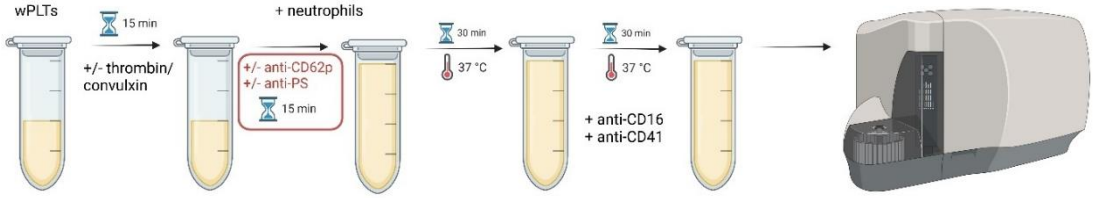

Figure 13. *Schematic representation of CD62P and PS inhibition. Created with BioRender.com*

2.2.6 Microscopic assays for platelet neutrophil interactions

2.2.6.1 Effects of (platelet) agonists on neutrophils

$1x10^5$ isolated neutrophils resuspended in RPMI 1640 medium were incubated on fibrinogen coated wells at 37°C with the following agonists: TRAP-6 (10 µM), high dose thrombin (1 U/mL), low dose or diluted thrombin (0.14 U/mL), convulxin (0.1 µg/mL), TRAP-6/convulxin (10 µM, 0.1 µg/mL), high dose thrombin/convulxin (1 U/mL, 0.1 µg/mL) and low dose or diluted thrombin/convulxin (0.14 U/mL, 0.014 µg/mL). After 2h cells were stained with 167nM Sytox Green Nucleic Acid Stain and 0.1 µg/µL Hoechst 33342 nuclear stain. The number of $Sytox^+$ cells and $Hoechst^+$ cells was quantified manually from 3-5 individual images at 40X magnification and reported as the ratio of $Sytox^+$/$Hoechst^+$ cells.

2.2.6.2 Effect of platelet supernatants on neutrophils

$1x10^7$ washed platelets were subjected to agonist stimulation as described above. After 30 min all samples were centrifuged for 10 min at 1000g with no brake and the supernatant (100 µL) was collected. Afterwards, each well was filled with the supernatant of $1x10^7$ platelets from every condition and incubated for 2.5 h. Staining was achieved with 0.1 µg/µL Hoechst 33342 and 167 nM Sytox Green. Again, the number of $Sytox^+$ cells and $Hoechst^+$ cells was quantified from 4 individual images at 40X magnification and reported as the ratio of $Sytox^+$/$Hoechst^+$ cells.

2.2.6.3 Effects of different platelet subpopulations on neutrophils

Direct platelet-neutrophil interaction was assessed on fibrinogen coated wells after 2.5 h of co-incubation. First, $4x10^6$ platelets were subjected to agonist stimulation (buffer,

TRAP-6, thrombin, convulxin, TRAP-6/convulxin and thrombin/convulxin; c_f see above) in Eppendorf tubes each for 15 min. Afterwards $1x10^5$ neutrophil granulocytes were added, and the remaining volume was filled with supplemental RPMI 1640 to reach 100 µL. The samples were introduced in the wells and kept at 37°C for 2.5 h. As a positive control, one sample containing only neutrophils then received 100nM phorbol 12-myristate 13-acetate (PMA) to induce NETosis. Cells and NETs were fixed with 4% paraformaldehyde (PFA) for 15 min, washed and permeabilized with 0.01% Triton X for 5 min. Overnight incubation with primary antibodies (1:500) against MPO (mouse) and citH3 (goat) was followed by addition of the secondary antibodies (1:400) AF488 anti-mouse and AF-647 anti-goat. 0.033 µg/µL Hoechst 33342 nuclear stain and Coralite-555-labeled anti-CD41 were used for tetra-staining purposes after additional washing steps. Double $MPO^+/citH3^+$ structures were counted using a 20X magnification and averaged from 3-5 images from each condition. In total, this experiment was performed 7 times. Representative images of NETs and platelet localization were recorded with the 40X and 100X immersion objective.

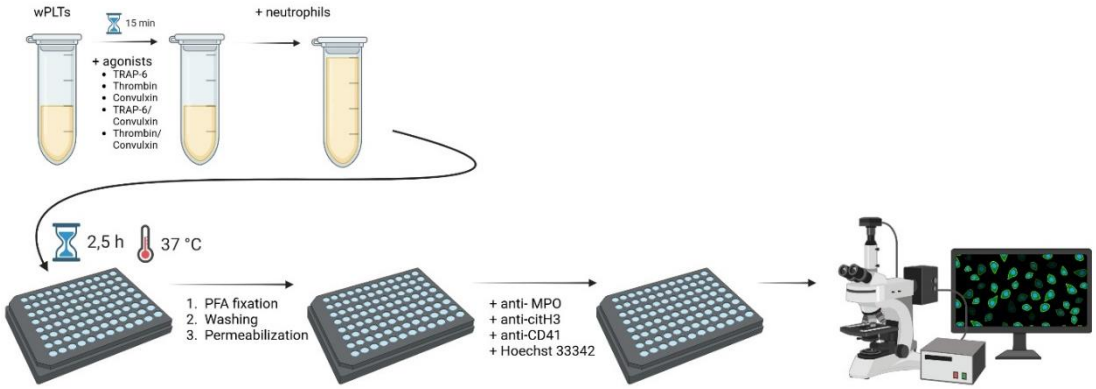

Figure 14. *Workflow for microscopy of platelet-neutrophil interaction. Created with BioRender.com*

2.2.7 Data Analysis and Statistics

For analysis of flow-cytometric data Kaluza software was used. Graphical depiction and statistical analysis were performed with GraphPad Prism 9 while open-source ImageJ Fiji (71) served for image interpretation and scoring of microscopic data. Data storage and transfer was done with MS Excel 360. A p-value <0.05 was assumed to represent statistical significance. Normal distribution was assessed by D'Agostino and Pearson omnibus normality test.

2.2.8 Ethics

All experiments involving human material were approved by the Ethics Committee of the Medical Faculty (140/2022BO2), Eberhard-Karls University, Tübingen, Germany, and were conducted in accordance with the declaration of Helsinki.

3 Results

3.1 Negative selection of neutrophil granulocytes is associated with less particle contamination than density gradient centrifugation

3.1.1 Neutrophil isolation via density gradient centrifugation

To enable the investigation of platelet-leukocyte interaction, platelets and neutrophils were required to be isolated in a highly purified manner to allow the exact dissection of direct and indirect pathways of platelet-neutrophil interaction. First, a conventional density gradient centrifugation protocol was used. Due to massive particle contamination observed in flow-cytometry and microscopy, optimization of the protocol was attempted. With additional centrifugation steps a reduction of particle contamination was possible. **Figure 15** illustrates the degree of particle contamination (including platelets) determined by flow-cytometry for conventional and modified density gradient centrifugation protocols as well as the negative selection method explained next.

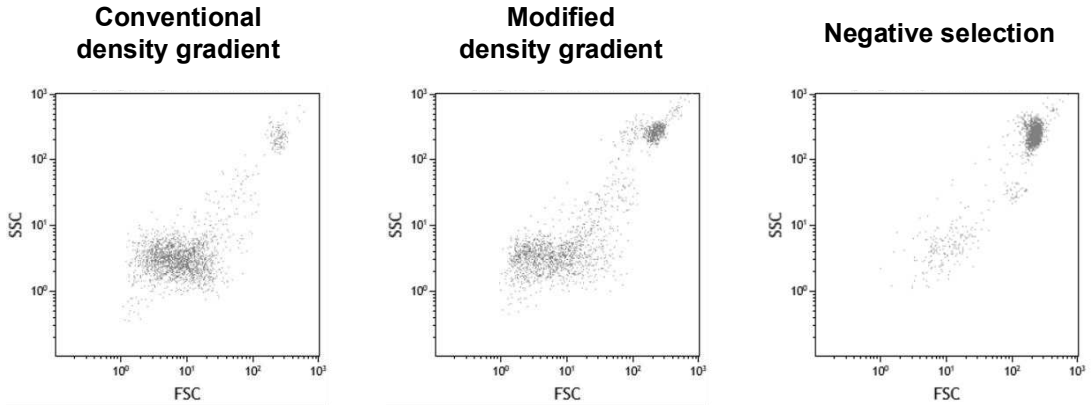

Figure 15. *Exemplary flow-cytometric analysis of leukocyte yield after a single granulocyte isolation with three different protocols. The typical granulocyte window is in the upper right quadrant. SSC – sideward scatter; FSC – forward scatter.*

3.1.2 Neutrophil isolation using negative selection

The second method of neutrophil isolation (EasySep, Stemcell Technologies) used a negative selection antibody mixture and required approximately 50 min in total. In contrast to density gradient centrifugation, only 10 min of centrifugation is required to isolate neutrophils. As shown in **Figure 15**, the yield of neutrophils was largest with this protocol and, most importantly, particle contamination was reduced as compared to

isolation with density gradient centrifugation. Overall, a considerably high mean purity of 94.29% ± 0.33 (standard error of the mean [SEM]) neutrophils (% of WBCs) was achieved with the immunomagnetic selection when comparing the results from automated WBC differential analysis.

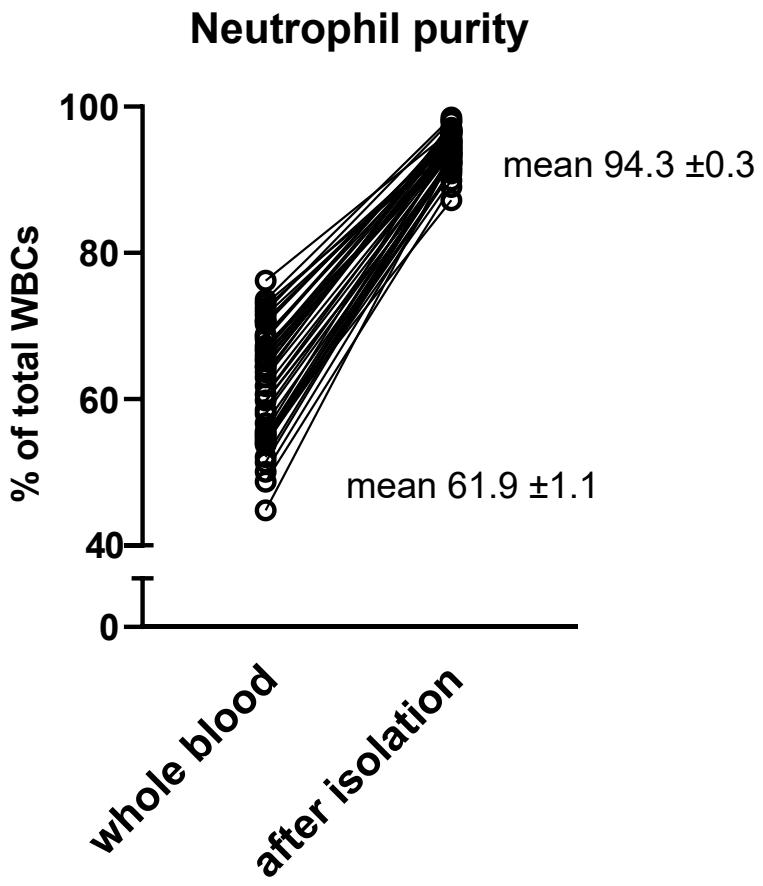

Figure 16. *Percentage of neutrophils before and after negative isolation. Pooled results from automated hematology analyzer CELL-DYN Rub, Abbott. Each data point corresponds to an individual isolation run. N=72.*

For the following experiments neutrophil isolation was performed using the negative selection kit due to the shorter preparation time and subsequently less neutrophil preactivation.

3.2 Induction of activated and procoagulant platelet phenotypes

To induce the formation of different platelet phenotypes, several agonists were used in single and also dual combinations. **Figure 17** demonstrates the flow-cytometric quality control with determination of CD62P and PS (Annexin-V) expression on platelets after

stimulation with of agonists. Unstimulated, isolated platelets were mainly (percentage [%] of $CD62P^-/PS^-$ double negative platelets ±SEM: 82.6% ±1.9. Stimulation of platelets with the PAR-1 agonist TRAP-6 (10 µM) and thrombin (1 U/mL) exclusively caused platelet activation (% of $CD62P^+/PS^-$ platelets: 56.1% ±7.5 and 88.7% ±1.5, respectively. Incubation of platelets with convulxin and dual agonist stimulation with TRAP-6 or thrombin and convulxin resulted in a third platelet phenotype. Besides $CD62P^+/PS^-$ platelets (convulxin: 55.4% ±2.7, TRAP-6/convulxin: 47.8% ±2.8, thrombin/convulxin 42.9% ±4.5), a significant proportion of $CD62P^+/PS^+$ procoagulant platelets formed: 39.2% ±2.8, 45.2% ±2.6, 54.4% ±4.2, respectively.

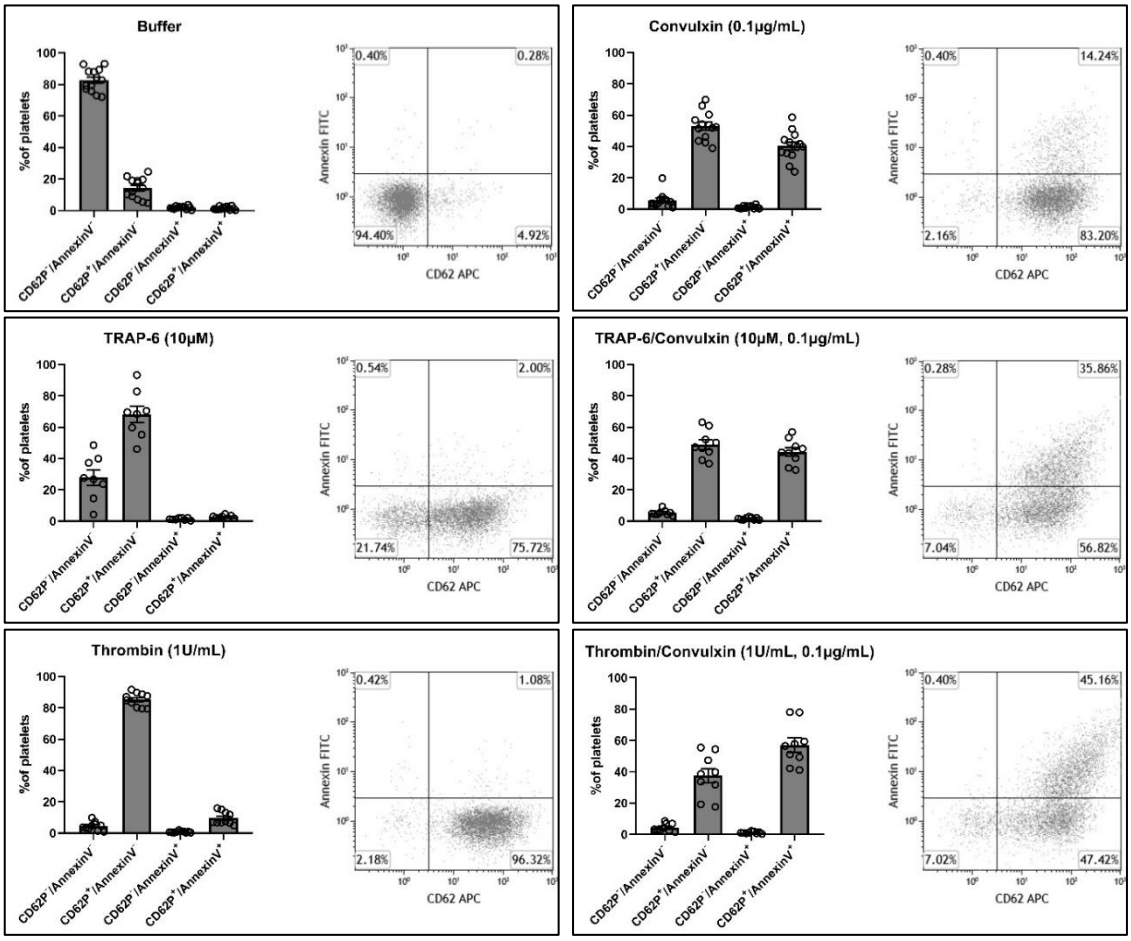

Figure 17. *Platelet phenotypes observed after subjection to different agonists. Expression of CD62P and PS (AnnexinV) were determined using flow-cytometry.*

Based on these data, the three different platelet subpopulations were defined as non-stimulated buffer platelets ($CD62P^-/PS^-$), solely activated platelets ($CD62P^+/PS^-$) and procoagulant ($CD62P^+/PS^+$) platelets. In the following experiments the comparison between single TRAP-6 or thrombin and dual stimulation with convulxin and TRAP-6 or

thrombin was used to investigate the additional effects of procoagulant platelets over activated platelets.

3.3 Both activated and procoagulant platelets have high tendency to mediate increased platelet-neutrophil aggregate formation

3.3.1 Effects of agonist-induced platelet phenotypes on aggregate formation

The impact of different agonist-mediated platelet subpopulations on the interaction with isolated neutrophils was quantified using a flow-cytometric approach. The proportion of $CD16^{+}/CD41^{+}$double positive PNAs was determined to compare the tendency of different agonist-induced platelet subpopulation to form complexes with neutrophils. **Figure 18** shows a microscopic representation of such a PNA resulting from co-incubation of neutrophils with TRAP-6-activated platelets.

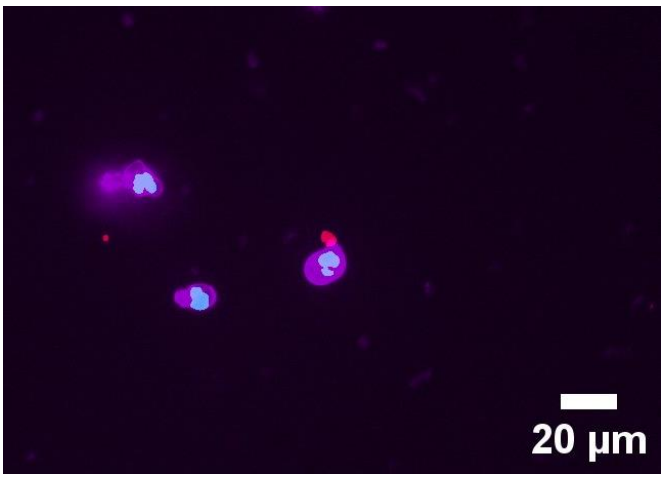

Figure 18. *Microscopic representation of a platelet-neutrophil aggregate. The characteristic multilobulated Hoechst blue nucleus of neutrophil granulocytes (CD16, purple) complexed to a single platelet which appears in red due after staining with anti-CD41-APC. 40X magnification.*

Overall, coincubation of unstimulated neutrophils with agonist-stimulated platelets resulted in a significant increase in PNA formation compared to buffer. An exemplary flow-cytometric plot of CD16 vs. CD41 can be seen in **Figure 19**.

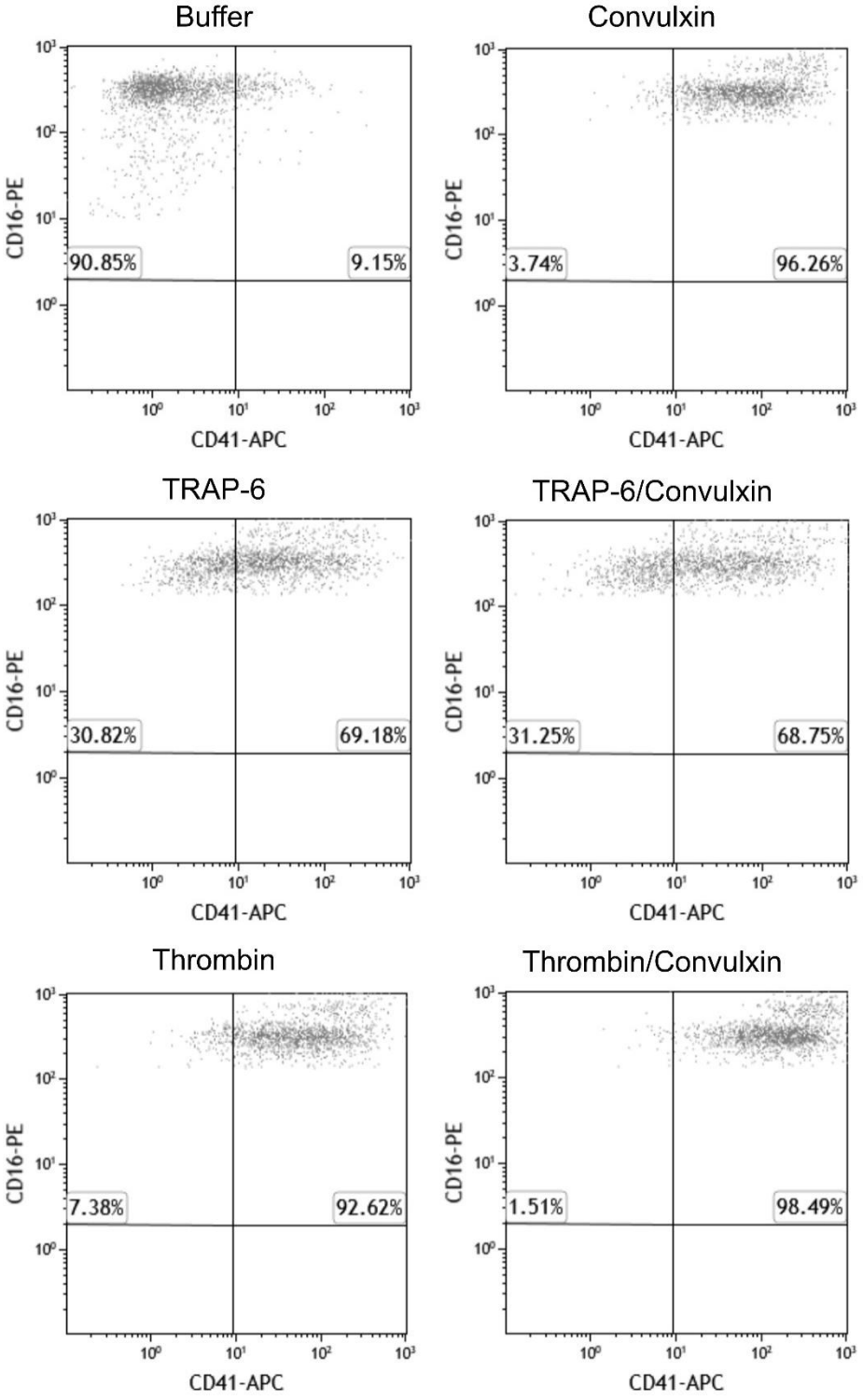

Figure 19. *Flow-cytometric assay for quantification of platelet-neutrophil aggregates. Aggregates were defined as CD16⁺/CD41⁺ double positive events.*

While 21.1% ±1.7 (SEM) $CD16^+$ cells were also $CD41^+$ in unstimulated platelets, TRAP-6 and thrombin pre-treatment of platelets resulted in 49.0% ±5.1 and 85.2% ±3.1 of $CD16^+/CD41^+$ events. Incubation of platelets with convulxin or combinations of convulxin

with TRAP-6 or thrombin showed similar findings (76.5% ±4.8, 73.1% ±4.0 and 79.6% ±3.5, respectively). Here no statistically significant differences were noted between single thrombin and dual thrombin/convulxin stimulation (p=0.129). However, the increase in aggregate formation was more pronounced with TRAP-6/convulxin stimulation than platelet pretreatment with TRAP-6 (p=0.0002). Interestingly some samples appeared to be responsive to TRAP-6 whereas most did not react significantly to TRAP-6 stimulation. As no additional data was collected from the respective donors, no conclusion can be made whether e.g. factors such as age or gender may have an impact on the susceptibility of these platelets.

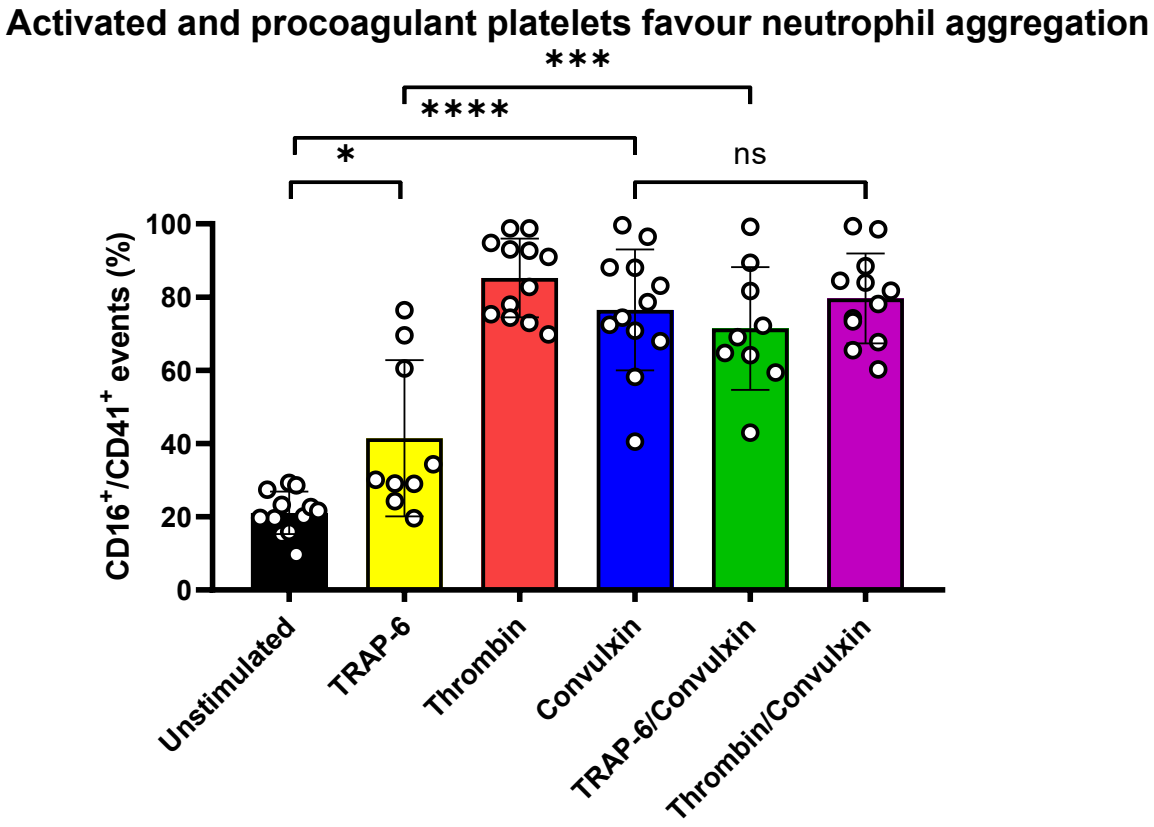

Figure 20. *Tendency of differently stimulated platelets with neutrophils for platelet-neutrophil aggregation as determined by flow-cytometric determination of CD16⁺/CD41⁺ events. Each individual data point corresponds to a separate experiment. Additionally, shown are mean ± SEM. Student's t-test. N=20.*

To investigate potential relationships between CD62P expression and PNA formation, a Pearson correlation analysis was performed between overall CD62P⁺ platelets from the FC quality control and the CD16⁺/CD41⁺ data for all experiments where both data sets were acquired. As expected, there was a strong correlation between CD62P expression

and the proportion of $CD16^+/CD41^+$ (Pearson´s r=0.75, p<0.0001). This emphasizes the importance of the CD62P/PSGL-1 axis in formation of PNAs.

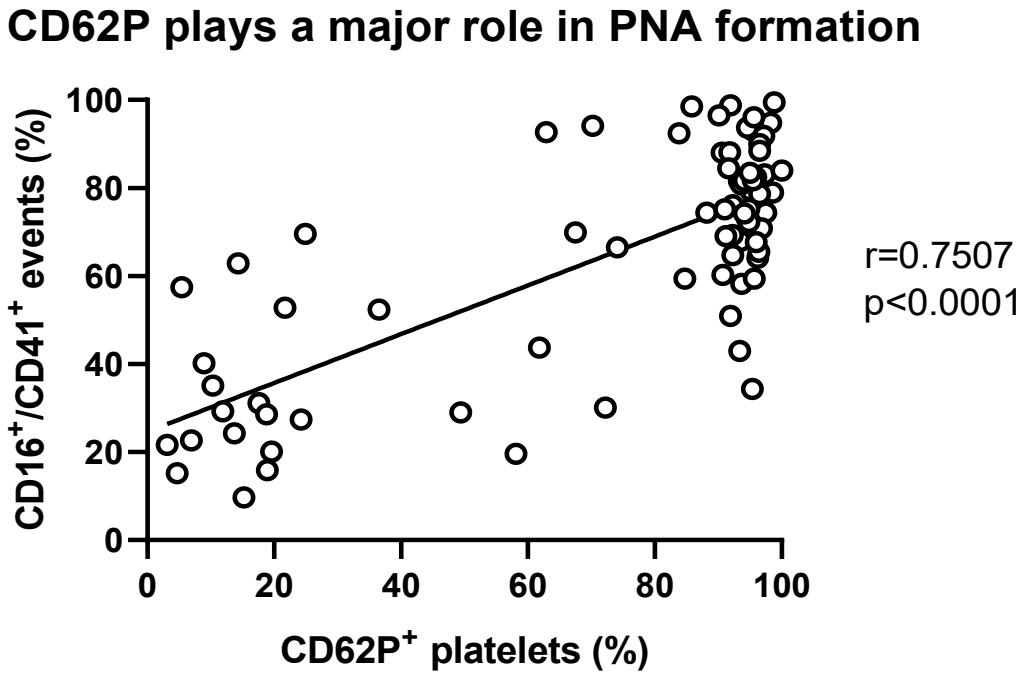

Figure 21. *Pearson correlation between platelet CD62P expression and the tendency to form aggregates with neutrophils. N=80.*

Next, a second analysis was performed to investigate a potential correlation between procoagulant platelets and platelet-leukocyte aggregate formation. Plotting the amount of flow-cytometry-detected $CD62P^+/PS^+$ double positive procoagulant platelets after agonist stimulation versus the proportion of $CD16^+/CD41^+$ events yielded a moderate Pearson´s r of 0.55 (p<0.001). This correlation was weaker than with CD62P only. Of note, the correlation of platelet AnnexinV expression vs. $CD16^+/CD41^+$ events (not shown) did not differ significantly from platelet CD62P+/$AnnexinV^+$ as almost all $AnnexinV^+$ platelets also concomitantly expressed CD62P.

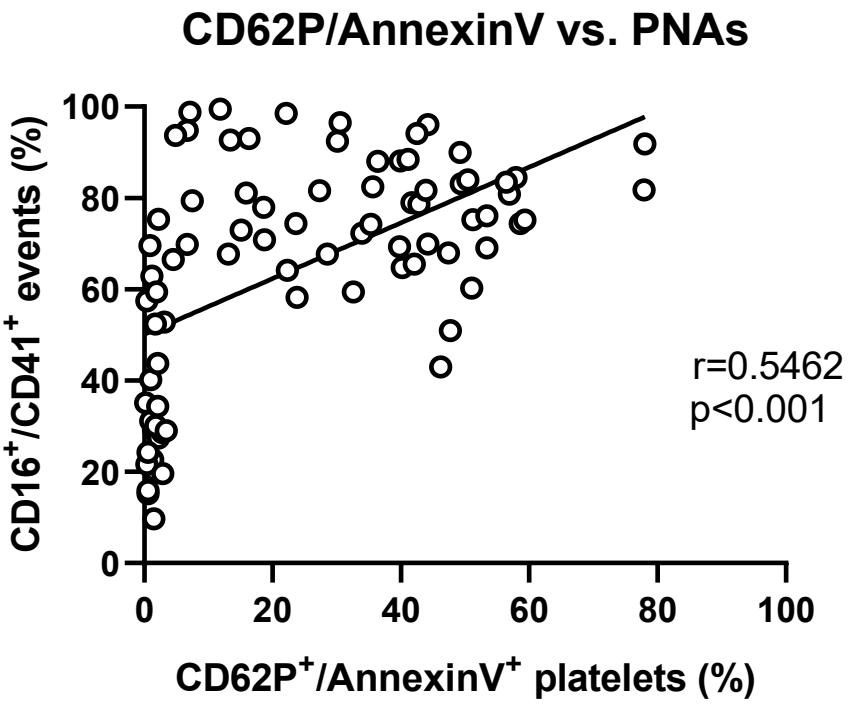

Figure 22. *Pearson correlation between platelet CD62P/PS expression and the tendency to form aggregates with neutrophils. N=80.*

3.3.2 Blockade of aggregate formation with anti-CD62P

To verify whether CD62P inhibition reduced platelet-neutrophil aggregation, the monoclonal anti-CD62P antibody Inclacumab was used. Inclacumab has an FDA approval for sickle cell disease. A serial dilution of Inclacumab was used to systematically analyse its ability to block CD62P/PSGL-1-mediated platelet-neutrophil aggregation. For this purpose, thrombin-stimulated platelets were used as this agonist reliably and exclusively causes a $CD62P^+/PS^-$ phenotype. Activated platelets were incubated with Inclacumab prior to the addition of neutrophils as thrombin reliably caused formation of single $CD62P^+$ platelets. The resulting titration curve is shown in **Figure 23**. The half maximal inhibitory concentration (IC_{50}) was calculated to be 0.15 µg/mL. An inhibition plateau was observed for Inclacumab concentrations starting at approximately 1 µg/mL. Of note, blockade of CD62P did not completely reduce aggregate formation with single thrombin stimulation as the plateau did not reach 100% of inhibition.

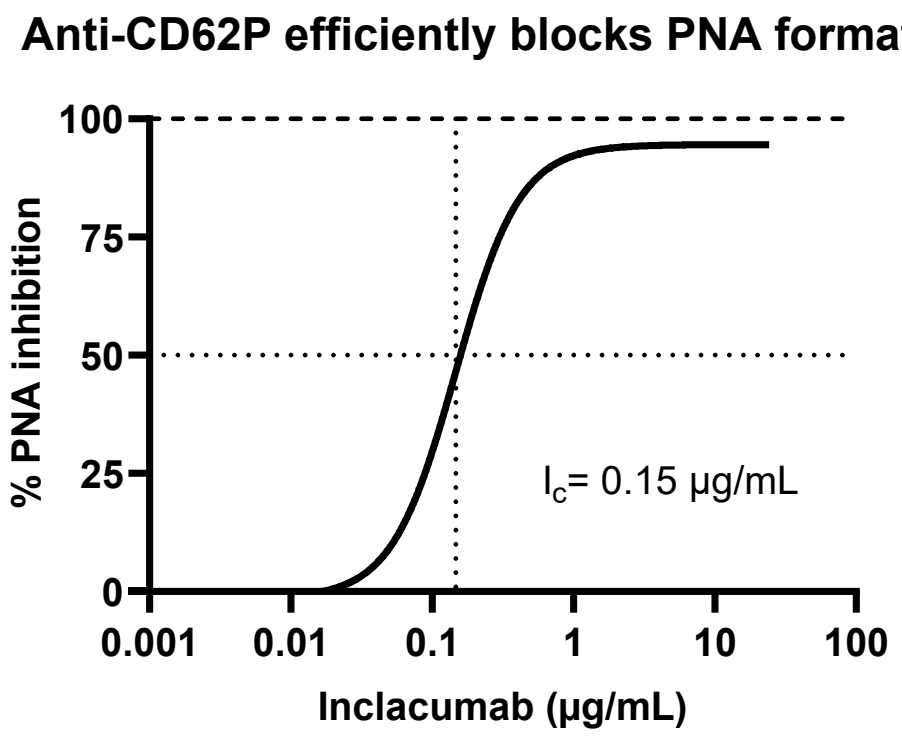

Figure 23. *Titration curve depicting the ability of Inclacumab (anti-CD62P) to block aggregate formation between CD62P$^+$ platelets and isolated neutrophils. Here, 100% of PNA was defined as the proportion of aggregates formed between thrombin-stimulated platelets and neutrophils. IC_{50} - half maximal inhibitory concentration. N=5.*

To confirm the complete blockade of CD62P with Inclacumab, a flow-cytometric quality control was performed that involved incubation of thrombin stimulated platelets with increasing concentrations of Inclacumab before staining and FC analysis of CD62P expression. As visualized in **Figure 24**, previously CD62P positive platelets become CD62P negative after being subjected to Inclacumab concentrations starting with 0.2 µg/mL. This finding indicates that the anti-CD62P antibody Inclacumab has a high avidity to CD62P.

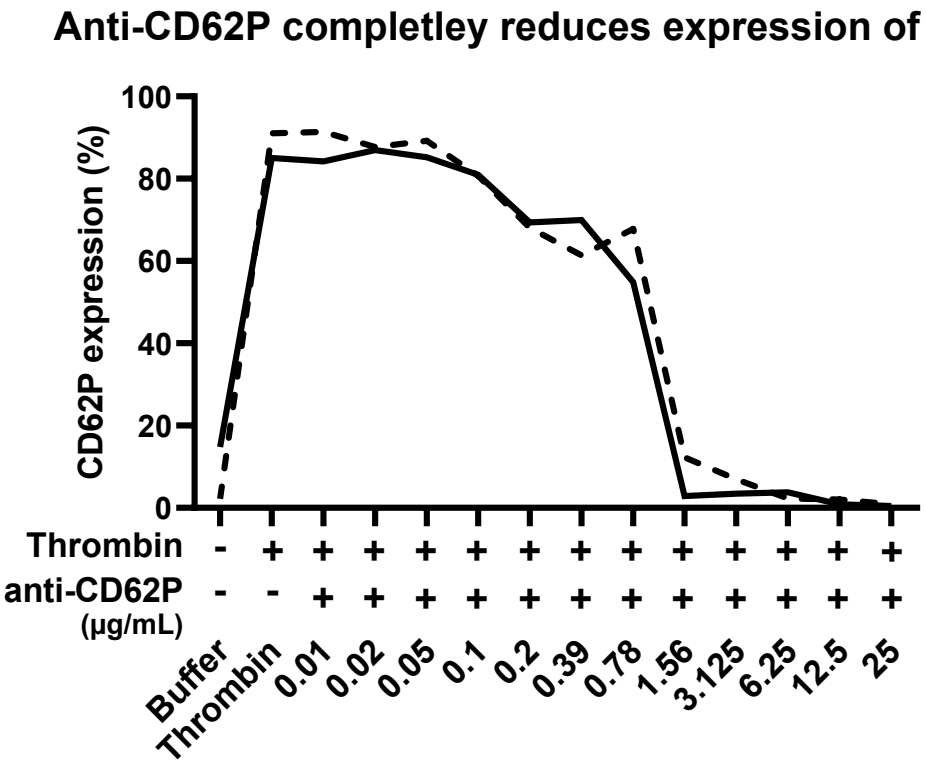

Figure 24. *Flow-cytometric determination of CD62P expression on platelets subjected to stimulation and different concentration of Inclacumab. Both lines correspond to an individual experiment. N=2.*

Next, using the previously determined effective blocking concentration of 1.5 µg/mL Inclacumab was chosen to assess the CD62P/PSGL-1 axis in aggregates formed by convulxin or dual agonist treated platelets. Interestingly, while thrombin or TRAP-6 stimulated platelets were largely prevented from aggregate formation by Inclacumab, aggregates formed by convulxin, and dual agonist treated platelets and neutrophils were less affected by CD62P inhibition as shown in **Figure 25**. The proportion of these residual aggregates was found to be 7.1% ±1.1 (SEM) for TRAP-6 and 17.4% ±1.5 for thrombin while 27.6% ±4.9, 26.8% ±1.4 and 39.2% ±2.5 $CD16^+$ events remained $CD41^+$ after platelet treatment with convulxin, TRAP-6/convulxin and thrombin/convulxin, respectively. The comparison between thrombin and thrombin/convulxin showed a statistical difference between activated and procoagulant platelets (p=0.0121), a finding that was also found to be true for platelet pretreatment with TRAP-6 vs. TRAP-6/convulxin-stimulated platelets (p=0.0058).

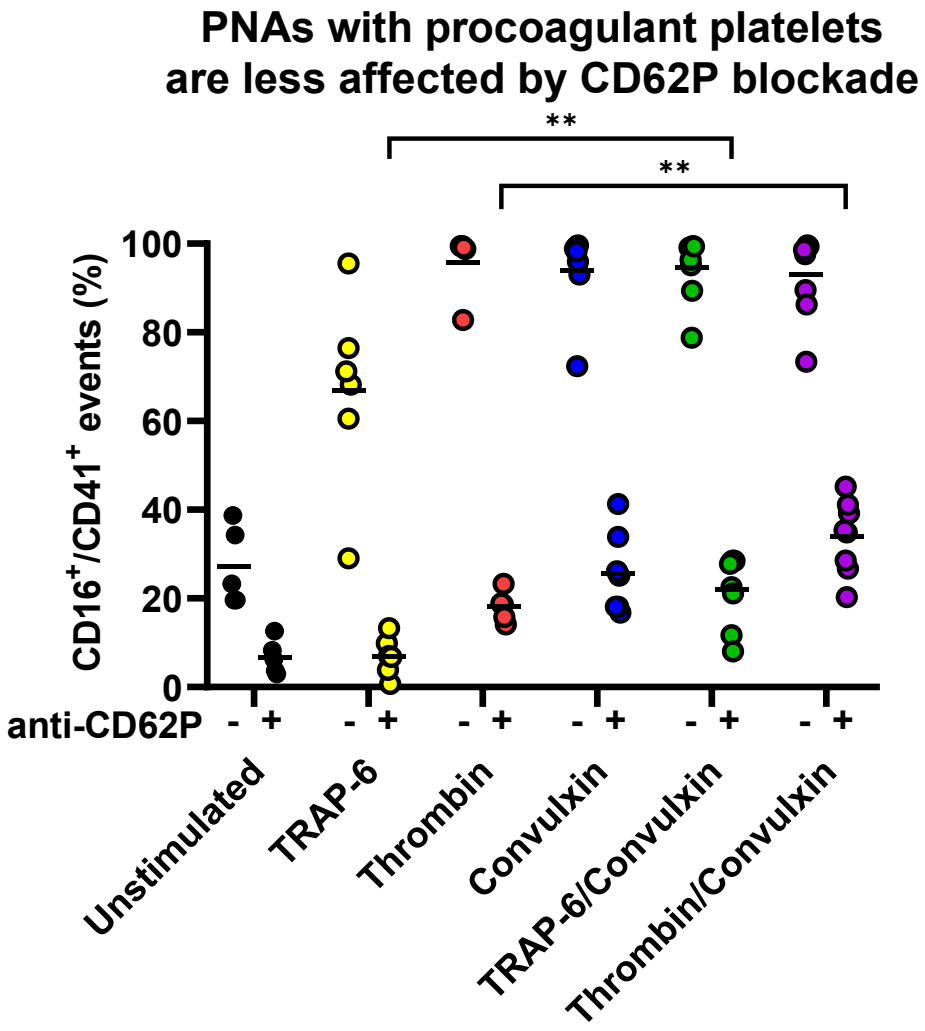

Figure 25. *Inhibition of CD62P with Inclacumab had different effects on PNA formation after agonist stimulation. Each individual two data points correspond to a separate experiment. Additionally, shown are mean ± SEM. Student's t-test. N=7.*

Since a relevant proportion of these convulxin or dual agonist stimulated platelets showed a procoagulant phenotype, these findings direct towards additional (co-) factors mediating platelet leukocyte aggregate formation. This feature is likely to be unique to procoagulant platelets and extends the current consensus on CD62P/PSGL-1 as being the main axis of platelet-leukocyte aggregation. **Figure 26** shows exemplary flow-cytometric representations of the residual PNAs observed after inhibition of CD62P.

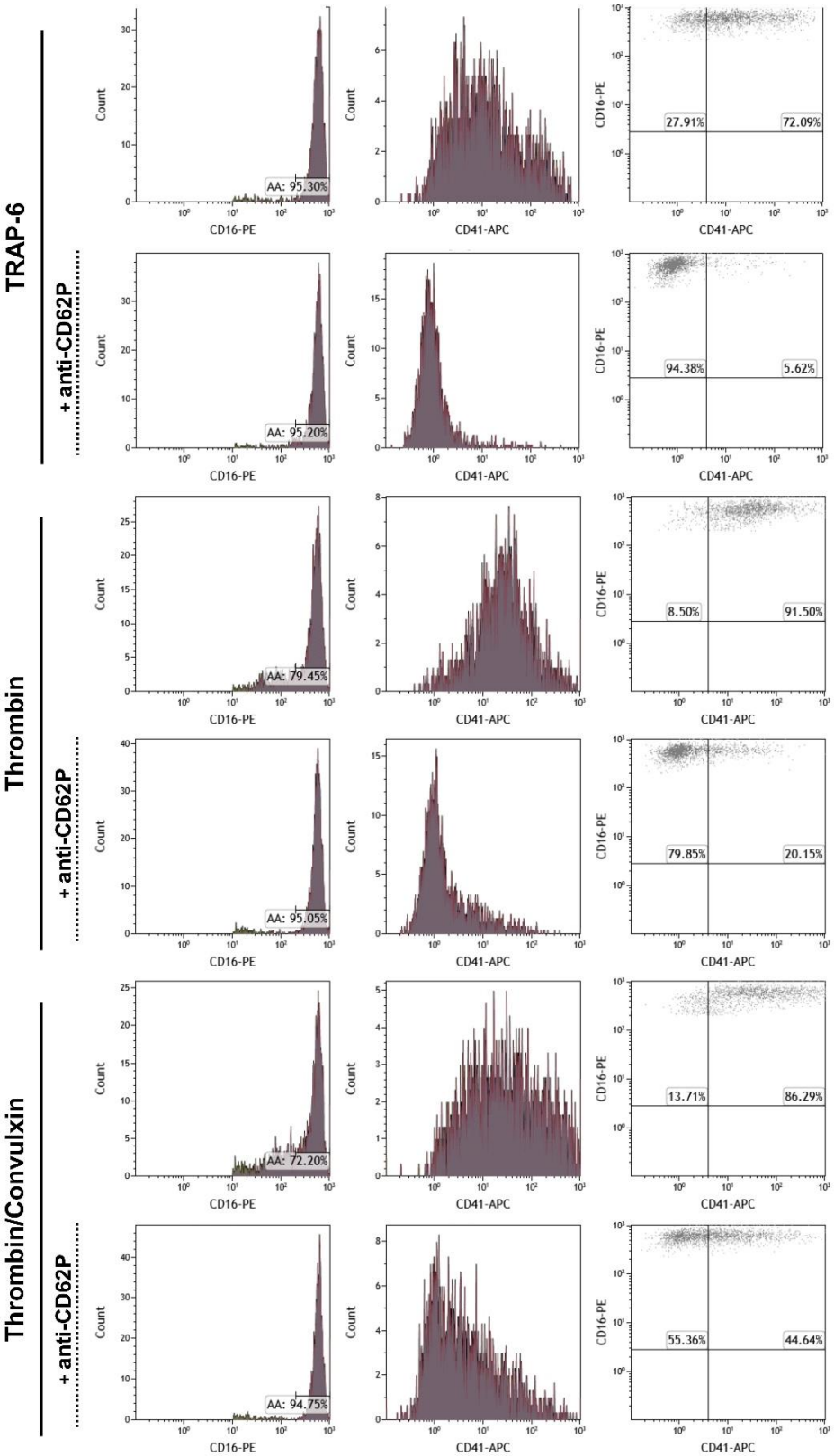

Figure 26. *Exemplary flow-cytometric results after inhibition of CD62P. Only neutrophils (CD16$^+$ events) were recorded and then further analysed in terms of their expression of the platelet marker CD41.*

3.3.3 Effects of phosphatidylserine inhibition

Next, the effects of PS blockade on the surface of human platelets were investigated to further dissect the interplay between procoagulant platelets and neutrophils. In a first step, platelets were stimulated via dual agonist stimulation using 0.1 µg/mL convulxin and 1 U/mL thrombin to induce maximum expression of CD62P and maximum levels of PS externalization on the platelet surface populations. Incubation of treated platelets with decreasing concentrations of the PS-blocking protein Lactadherin was followed by the addition of neutrophils. Afterwards, FC quantification of $CD16^+/CD41^+$ events was performed similarly to the previous experiments.

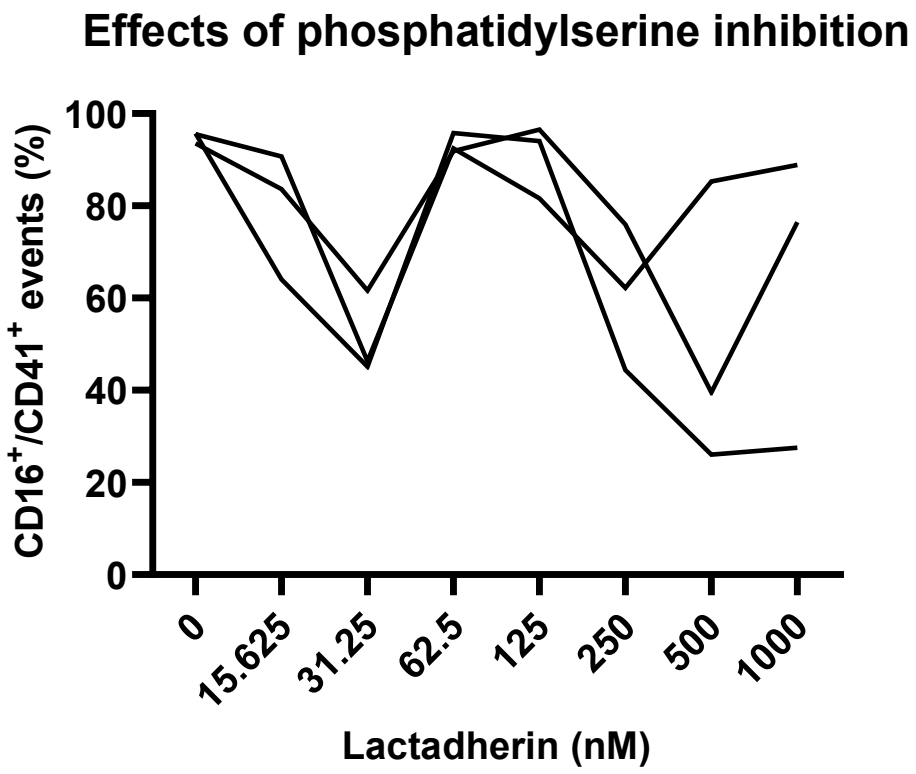

Figure 27. *Selection of 3 individual experiments which were found to show similar characteristics in inhibition of platelet-neutrophil aggregation. N=3.*

Two effective concentrations emerged from this experiment (**Figure 29**). The first concentration of 31.25 nM Lactadherin reduced the proportion of $CD16^+/CD41^+$ events. The mean % of $CD16^+/CD41^+$ events was 51.0% ±5.3. Starting with 250 nM the second decrease in $CD16^+/CD41^+$ events was observed. Here, the reduction appeared to be similarly pronounced with a mean of 60.8% ±9.1 although the SEM was considerably high. At 500 nM Lactadherin two of the selected experiments showed a further reduction

in aggregate formation while in the remaining experiment the proportion of $CD16^+/CD41^+$ events reached baseline levels.

Due to the overall heterogenous picture of inhibition with Lactadherin, no typical titration curve could be derived. The reason for the variation in response to Lactadherin remained unclear.

3.3.4 Impact of simultaneous CD62P and platelet PS blockade on PNA formation from procoagulant platelets

To assess the effects of concomitant inhibition of CD62P and PS expression on the ability of procoagulant platelets to bind to neutrophils, both Inclacumab and Lactadherin were used. While Inclacumab was used at the known plateau concentration of 1.5 µg/mL, a serial dilution was once again used for Lactadherin since no classical titration curve could be generated. Platelets were stimulated with dual thrombin and convulxin stimulation to generate a maximum population of $CD62^+/PS^+$ double positive platelets.

Inclacumab caused a marked decrease in $CD16^+$ cells complexed with platelets as expected. The absolute reduction was even more pronounced than observed in the first experiments with single CD62P inhibition (see **Figure 25**). Here, the percentage of $CD16^+/CD41^+$ events was found to be 30.89% ±1.7 for 1.5 µg/mL Inclacumab.

Interestingly and in contrast to the single inhibition experiments with Lactadherin, the two effective concentrations of 31.25 nM and 250 nM did not appear to have any measurable influence on PNA formation. On the contrary two other concentrations emerged to be capable of further inhibition of aggregate formation. A subtle but statistically relevant decrease in $CD16^+/CD41^+$ events was found for a concentration of 15.625 nM ($p=0.0222$) and 500nM ($p=0.0237$) Lactadherin. 20.09% ±3.7 residual aggregates were observed with 15 nM Lactadherin. At a concentration of 500 nM 24.36% ±2.4 $CD16^+/CD41^+$ events remained.

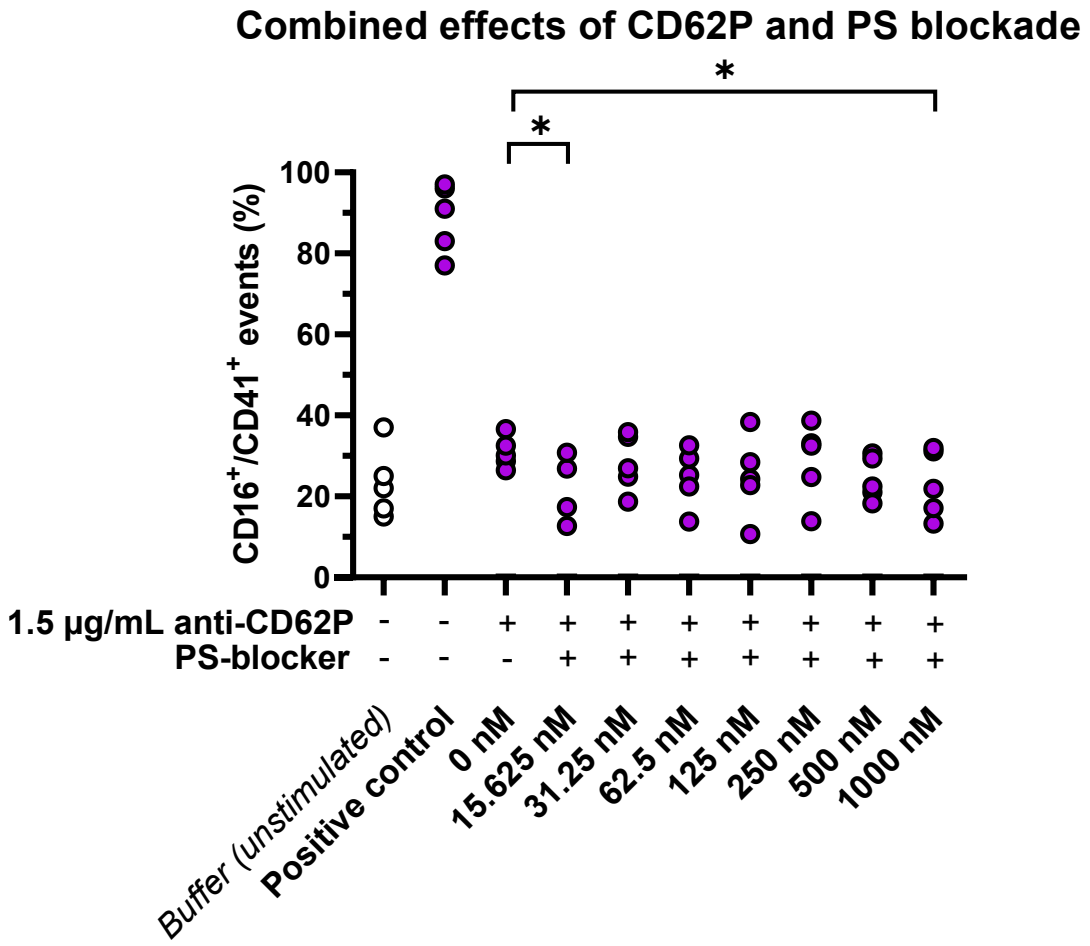

Figure 28. *Determination of CD16⁺/CD41⁺ events after inhibition of CD62P and PS (nanomolar, nM). Buffer (unstimulated) and positive control (stimulated thrombin and convulxin, not inhibited) are presented for reference. Each point corresponds to an individual experiment. Student's t-test. N=5.*

Figure 29 illustrates an example of such a double inhibition experiment. The PNAs formed from mostly procoagulant platelets were reduced after addition of anti-CD62P as described before. Additional anti-PS was able to further reduce the number of PNAs.

These results suggest that next to the strong CD62P/PSGL-1 axis there are additional factors involved which secure the binding between neutrophils and procoagulant platelets. PS appears to be such an influencing factor as PS blockade was able to further destabilize PNA formation.

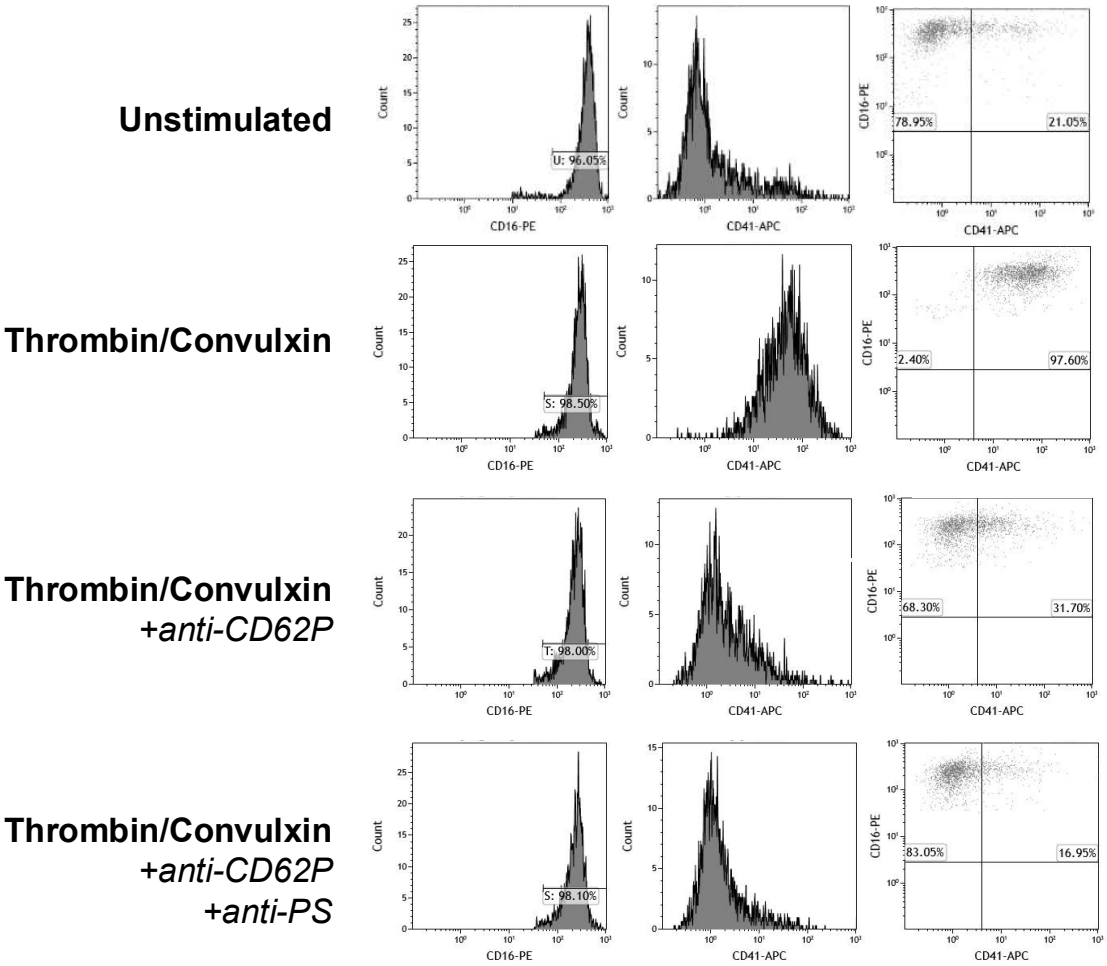

Figure 29. *Consequences of combined blockade of CD62P and PS. Exemplary flow-cytometric plots illustrating the add-on effect of anti-PS for thrombin/convulxin stimulated platelets. CD16-PE – neutrophil marker, CD41-APC – platelet marker.*

3.4 Impact of different platelet subpopulation on neutrophil extracellular trap formation

To investigate the relevance of different platelet subpopulation on NET formation, a microscopy-based model that investigates platelet-neutrophil interaction under static conditions was established to allow quantitative observations on the impact of differently stimulated platelets on neutrophils and more particularly NETs. Several incubation and staining procedures have been evaluated including markers for DNA, degranulation and NETs. **Figure 30** shows an exemplary microscopic image of PMA-stimulated neutrophils releasing NETs and distribution of DNA (Hoechst), MPO and citH3.

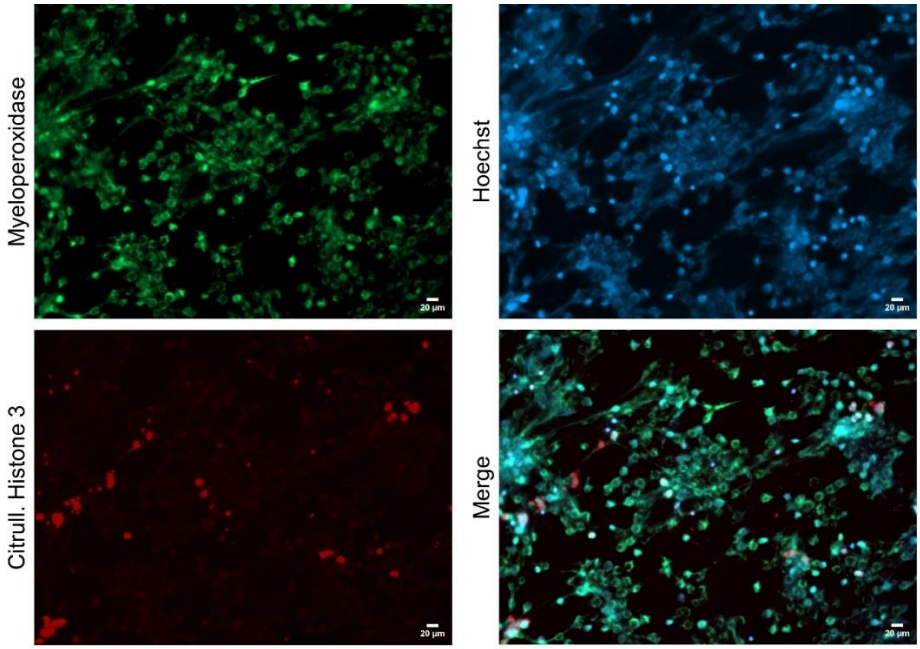

Figure 30. *PMA-induced NET formation. Staining of MPO (green), DNA (Hoechst blue) and citH3 (red) reveals the basic structure of NETs. 20X magnification. Scale bar 20µm.*

3.4.1 Except for thrombin other platelet agonists did not have direct effects on neutrophils

The agonists used to stimulate platelets in the following experiments were TRAP-6, thrombin and convulxin in different combinations. To rule out direct effects of these activating substances on neutrophils a microscopic assay was established. High and low concentrations of the agonists were added to isolated neutrophils with the low concentration corresponding to the final concentration used in the platelet-neutrophil co-incubation experiments later. Due to the protocol of these experiments the agonist-treated platelets were added to the neutrophil suspension undergoing a dilution effect. Staining with the cell death stain Sytox green was performed and compared to the cell permeable Hoechst stain.

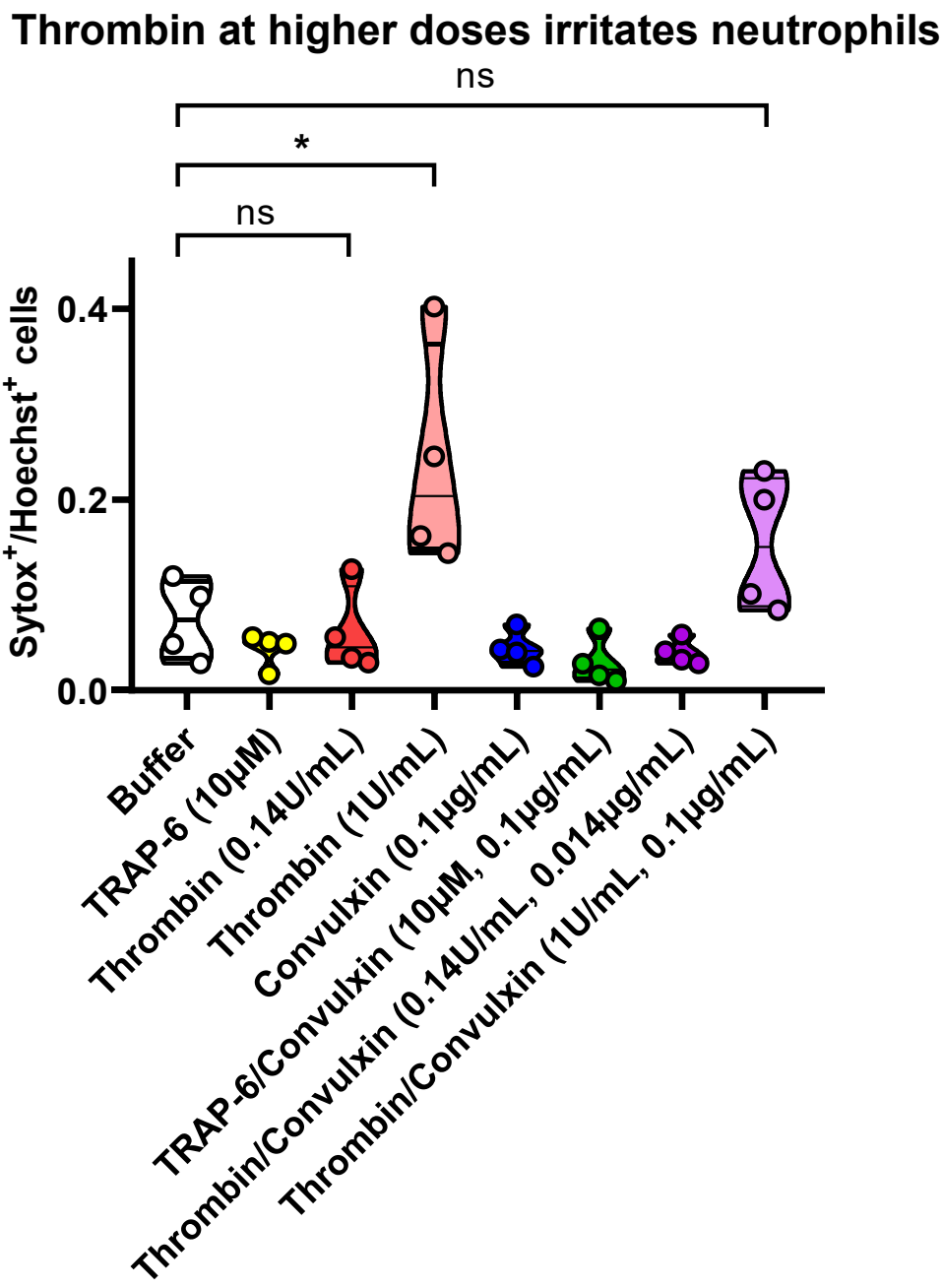

Figure 31. *The isolated agonist effects on neutrophils highlight the potential of thrombin to activate neutrophils at high concentrations. These effects were deemed negligible for the following experiments with lower concentrations due to dilution. Data shown as individual points and truncated violin plots. Student's t-test. N=4.*

As expected, TRAP-6 and convulxin did not cause any significant increase in membrane permeabilization (proportion of Sytox green$^+$ cells) and may be considered platelet-specific even at the high (undiluted) concentrations. Thrombin on the other hand, was found to irritate neutrophils and resulted in Sytox influx when used in high doses of 1 U/mL. Compared to buffer, this difference was statistically significant (p=0.0385).

However, as in the following experiments the platelets were always pre-treated with agonists before co-incubation with neutrophils, the final concentration of each respective agonist was lower. At this lower concentration of 0.14U/mL the direct effects of thrombin on neutrophils were not observed. **Figure 32** includes exemplary microscopic images of the differences in Sytox^{+} neutrophils after incubation with the agonists.

As an interesting minor finding, high dose thrombin combined with high dose convulxin also resulted in an increase of neutrophil membrane permeability even though this effect was lower than with thrombin alone and statistically insignificant.

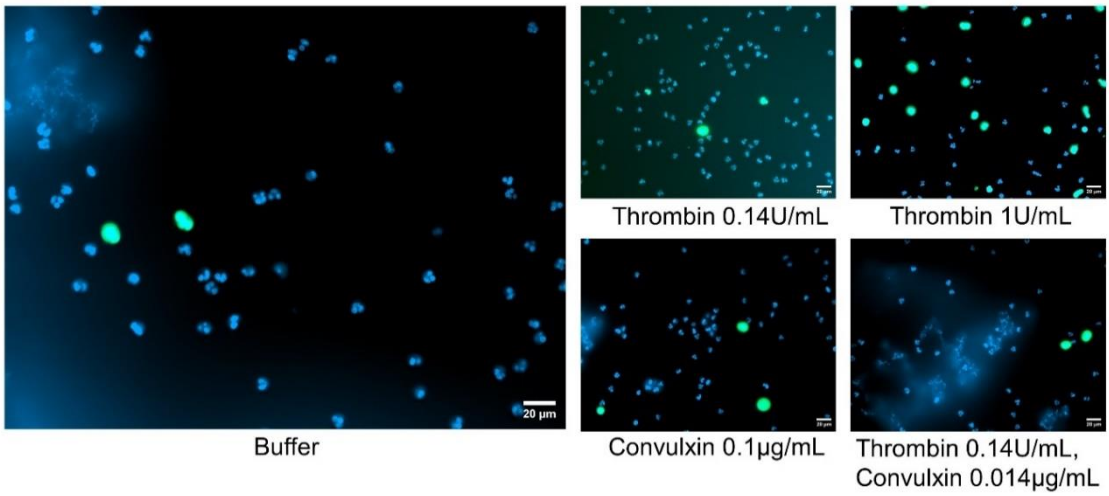

Figure 32. *Exemplary microscopy of neutrophils treated with platelet agonists. Double staining with the nuclear dyes Hoechst (blue) and Sytox (green) was used to assess the consequences on neutrophils. 40X magnification, 20 µm scale bar.*

3.5 Effects of platelet releasate on neutrophils

To further investigate and visualize other aspects in platelet-neutrophil interplay, a microscopic assay was developed. First, the impact of platelet supernatants on neutrophils was assessed. The releasate of $1x10^7$ platelets was harvested after stimulation with different agonists and added to $5x10^4$ neutrophils. This ratio of 1:200 was chosen to first appreciate the potential of substances secreted from platelets undergoing different phenotypic changes. In contrast to the physiologic ratio of approximately 1:20-1:40 in the bloodstream, different ratios could be found at sites of vessel injury where the rapidly forming plug forms rapidly from an innumerable number of platelets. **Figure 34** shows the effects of platelet releasates from differently stimulated platelet samples. Exemplary microscopic images of the effects of platelet releasates on neutrophils can be seen in **Figure 33**.

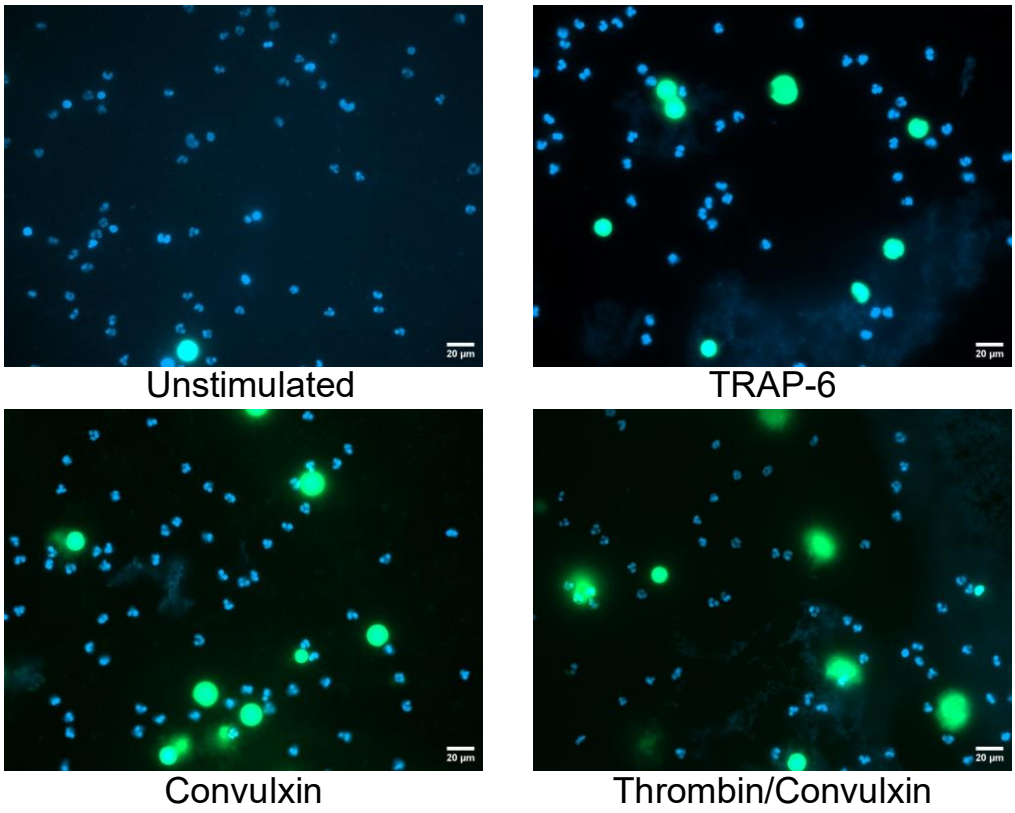

Figure 33. *Exemplary microscopic images of neutrophils subjected to different platelet supernatants. Staining of nucleic acids with Hoechst (blue) and Sytox (green) of which Sytox at 167nM enters only cells with membrane dysfunction. Images at 40X magnification.*

A significant difference was observed between unstimulated platelets and platelets subjected to agonist treatment (e.g., unstimulated vs. TRAP-6 p=0.0068). However, this assay failed to show any differences among the particular agonists. Thus, no clear deduction can be made regard the impact of supernatant from activated versus procoagulant platelets.

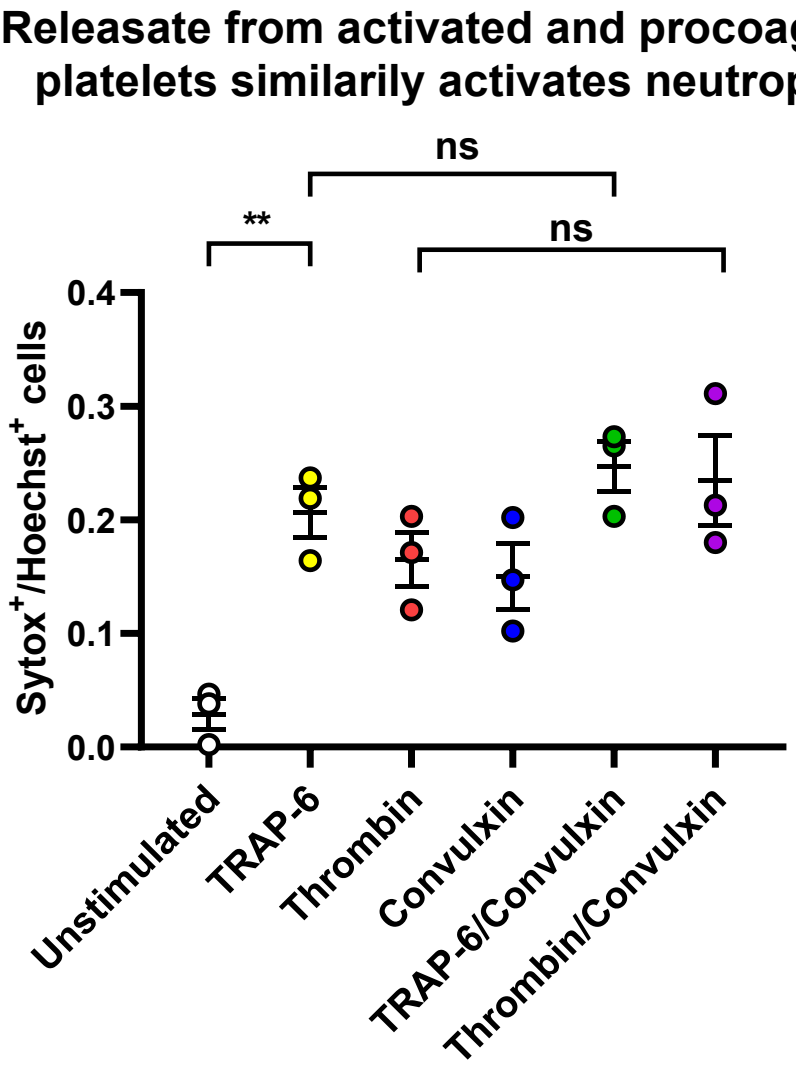

Figure 34. *Impact of platelet supernatants on isolated neutrophils. The ratio between Sytox⁺ and Hoechst⁺ cells was calculated for 4 individual images per condition and experiment and then averaged to allow comparison. Data shown as individual experiments (points) and mean + SEM. Student's t-test. N=3.*

These findings highlight the significant impact of non-cellular components such as singular substances but also MVs in the interplay between neutrophils and platelets.

3.6 Platelet-induced formation of NETs

Ultimately, the potential of different platelet subpopulations to stimulate the release of NETs from isolated neutrophils was investigated using a microscopic approach. First, platelets were subjected to agonist stimulation as described before. Afterwards, platelets and neutrophils were co-incubated for 2.5h, fixed and stained for the presence of NETs.

All stimulated conditions appeared to have more NETs than unstimulated platelets. The number of NETs increased from baseline (1.7 ±0.2) to 4.3 ±0.9 with TRAP-6, 5.6 ±0.7 with thrombin, 8.3 ±0.8 with convulxin and 7.1 ±0.9 with dual thrombin/convulxin stimulation. The maximum of 9 ±1 NETs was observed with platelets pretreated with TRAP-6/convulxin.

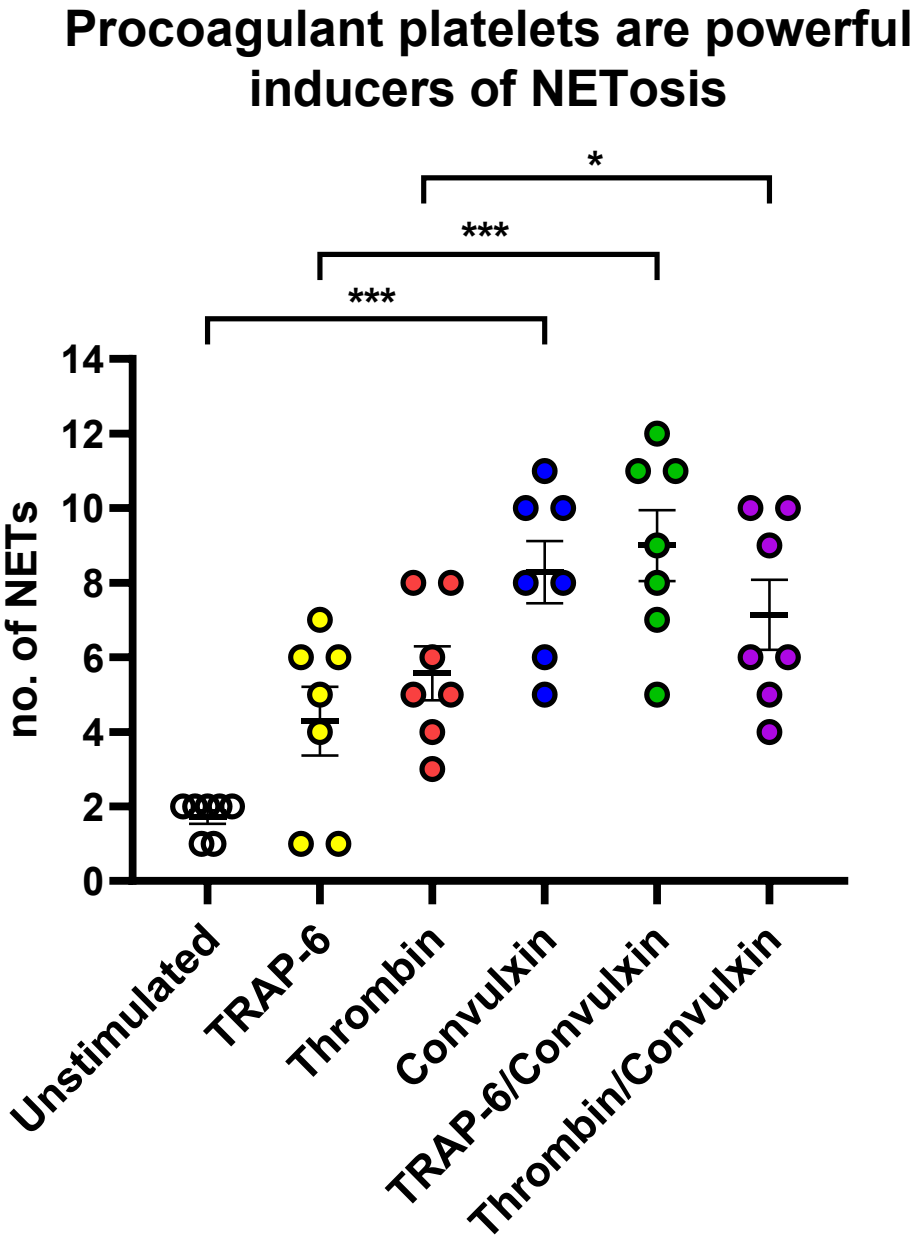

Figure 35. *Ability of agonist-treated platelets to induce release of NETs. NETs were defined as $MPO^{+}/citH3^{+}$ double positive structures identified and manually counted on microscopy. Of note, the co-incubation was performed on fibrinogen-coated wells and therefore samples with thrombin stimulation expectedly manifested with fibrin strands. Data shown as individual points and mean + SEM. Student's t-test. N=7.*

A statistically significant difference was found between TRAP-6 activated platelets and the TRAP-6/convulxin dual stimulated platelets ($p<0.001$). Differences between thrombin and thrombin/convulxin were expected to be at least similarly high but since the co-incubation of platelets and neutrophils was performed on a fibrinogen coating, fibrin strands formed and presumably overlaid some NETs resulting in an underreporting in both conditions. Still, a statistically significant difference was observed between thrombin single and dual thrombin/convulxin stimulation ($p=0.0248$).

Overall, these findings point to an increased formation of NETs with procoagulant platelets when compared to NET formation initiated by exclusively $CD62P^{+}/PS^{-}$ activated platelets. **Figure 36** shows a microscopic image of platelets residing within a NET after platelet stimulation with convulxin.

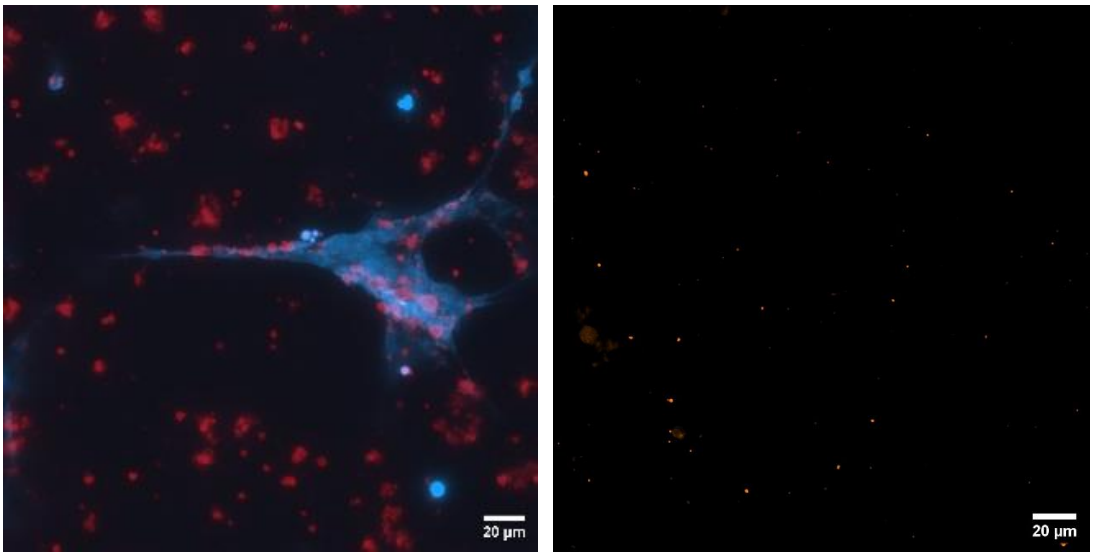

Figure 36. *Left: co-localization of NETs (Hoechst blue) and platelets (CD41-APC, red) in a NET induced by convulxin-treated PLTs. Right: no NETs are induced by platelets pre-incubated with TRAP-6 (negative control). Scale bar 20 µm.*

4 Discussion

Thromboinflammation is a relatively new concept aiming to further describe the close interaction between the primary hemostatic and the immune system. Little is known about the exact mechanisms, but current research is accumulating. As hyperinflammatory conditions with thrombotic complications are not uncommon and usually are associated with life-threatening situations, more insights would significantly improve patient outcome.

The first objective of this MD thesis was to establish a solid protocol to investigate platelet-neutrophil interaction. Depending on the isolation method used, phenotype and functionality in terms of responsiveness of the neutrophil population may differ significantly. (176) For our purpose, we found that negative selection of neutrophil granulocytes was associated with less particle contamination than conventional density gradient centrifugation (**Figure 15**). In the latter, optimal segregation of the WBCs into PMNs and PBMCs requires a total centrifugation time of 30-40 min. Additional centrifugation is required during the RBC lysis steps. The cumulative centrifugation time of >30 min may leave the isolated cells more agitated and activated. (177) Considering the relatively short lifetime of neutrophils, the optimal period for experiments including neutrophils is restricted to a few hours. Efforts to minimize platelet contamination during density gradient centrifugation by adding additional centrifugation steps further complicated the fine line between neutrophil purity and baseline activity. Negative selection on the other hand is quicker, but is known to have higher rates of contamination, most notably by platelets. (178), and is limited in the total amount of cells to be isolated. The difference in yield after isolation in this MD thesis point towards a superiority of the negative selection kit used (EasySep Direct Human Neutrophil Isolation Kit from StemCell Technologies). This is supported by the widespread use of this system by several working groups. (81,129,179,180) Particle contamination is a well-known phenomenon complicating negative selection procedures (178) and was also encountered during the experiments of this doctoral thesis. Another advantage when compared to positive selection cell sorting protocols is that by negative selection the desired cells do not need to bind to specific antibodies, minimizing the risk of cellular activation or receptor blockade. Neutrophil purity reached high, acceptable levels although published data suggests a potential for even higher purities with and without protocol modifications. (181,182)

Secondly, in line with the literature we induced platelet activation with different agonists. Platelet exposure to TRAP-6 or thrombin alone resulted in a mainly $CD62P^+$ platelet population, while stimulation via GPVI by convulxin with and without concomitant PAR activation leads to formation of a significant amount of $CD62P^+/PS^+$ platelets. (36,183,184)

To rule out confounding direct effects of the chosen platelet agonists on neutrophils, a set of side experiments was conducted. Here, a high final concentration of 1 U/mL thrombin was found to statistically significant increase the proportion of $Sytox^+$ cells while the other agonists appeared to have no measurable impact. Only few studies which have addressed the potential of thrombin to influence neutrophils pointing to chemoattractant, pro-adherent and activating properties of elevated concentrations of thrombin. For instance, Zimmermann et al. showed an increase in leukocyte adhesion at 2 U/mL thrombin while Baranes et al. observed dose-dependent degranulation with thrombin concentrations ≥1 U/mL. (185–187)

For TRAP and convulxin little to no literature was available for comparison. (36,37,188) It remained unclear whether the stimulating effects of thrombin were reduced when combined with convulxin. The design of the experiments in this thesis allowed platelet stimulation prior to incubation with neutrophils with a subsequent dilution effect of the agonists when platelet and neutrophil samples were joined.

As a quality control of the experiments of this MD thesis, platelet phenotype after agonist treatment was analysed using flow-cytometry in parallel to the main assays on interaction between platelets and neutrophils. The generation of different platelet phenotypes according to standardized protocols is well established in our laboratory. Other groups also rely on agonist stimulation in order to trigger procoagulant platelet formation. The flow-cytometric findings of these quality control experiments were consistent with the literature on resting, activated and procoagulant platelet. (189–192)

A multitude of research has already been conducted on the interaction sites implied in platelet-leukocyte interplay. The importance of the axis consisting of CD62P and neutrophil PSGL-1 has been highlighted extensively. (136,137) In line with this, the proportion of $CD16^+$ neutrophils complexed to platelets ($CD16^+/CD41^+$) increased in the experiments of this MD thesis when platelets were stimulated beforehand.

Blockade of platelet CD62P with a monoclonal antibody significantly reduced the number of PNAs formed as expected based on the RCT by Schmitt et al. (94) However, procoagulant platelets appeared to be less influenced by CD62P blockade than solely

activated platelets (see **Figure 25**). This points to additional factors and/or axes in the direct interaction between neutrophils and procoagulant platelets. As a next step, the relevance of platelet PS was investigated as presence of PS on the platelet surface is one hallmark of procoagulant platelets. A heterogenous picture was encountered (**Figures 27, 28**). One potential reason could be the complex interaction between PS and the blocking substance lactadherin. Lactadherin not only inhibits the procoagulant role of PS on the platelet surface but also may facilitate linkage to phagocytic cells. (29) Here, further experiments with lactadherin and other PS-blocking chemicals could be helpful.

Concomitant blockade of CD62P and PS was performed to investigate whether the remaining $CD16^{+}/CD41^{+}$ events after CD62P blockade were sensitive to PS inhibition in thrombin/convulxin-treated platelets. A subtle but statistically significant effect of dual inhibition was observed which supports a certain relevance of PS in the strong formation of PNAs. With the definition of procoagulant platelets evolving steadily, other surface markers could be assessed, too.

The indirect effects of platelets on neutrophils observed in the supernatant experiments emphasize the powerful secretive properties of platelets (**Figures 33, 34**). Although not further investigated, MVs also may play a crucial role in mediating the effects of activated platelets on neutrophil granulocytes. (10,11)

One of the main findings of this MD thesis was the massive potential of procoagulant platelets to induce NETosis observed upon microscopy of platelet-neutrophil interaction (**Figure 35**). A significant increase in NET formation compared to unstimulated conditions was observed when platelets were activated with TRAP-6 or thrombin. Increased induction of NETs from TRAP-6-activated platelets has already been described. (157,193,194) Interestingly, dual stimulation of platelets with TRAP-6 and convulxin or single convulxin treatment resulted in an even higher number of NETs. This clearly demonstrates the great potential of procoagulant platelets to induce NET release.

Certainly, the effects of effects of procoagulant platelets on neutrophils are partly mediated by CD62P (81,195), but other factors are very likely to be involved. Particular additional mediators are needed to explain the further increase in NETosis from procoagulant platelets when compared to activated platelets. Whether procoagulant platelets exert these unique properties in a direct neutrophil-binding manner or by indirectly secreted substances remains to be investigated. Based on the results from the

supernatant experiments the latter is likely to play a relevant role further highlighting the massive secretive properties of platelets.

Future investigations should focus on other axes of communication in the bi-directional relationship of platelets and neutrophils. While static models are easier to establish, efforts are needed to design dynamic experiments to account for the numerous processes taking place inside and outside of the cells. In a clinical setting, samples of patients with pathologies known for their increased prothrombotic capacity such as HIT or disseminated intravascular coagulation (DIC) e.g. in septic shock could be further analysed in this regard.

Translation of laboratory data into real life care would bring major benefits to patients suffering from thromboinflammation. Especially antibody-mediated thrombosis may be targeted by newly designed pharmaceuticals. (196–198) Deeper understanding of immune thrombosis as a relevant factor of mortality would clearly lead to better treatment of critically ill patients.

Limitations of this study include the natural inter-individual variability of the cellular samples. Blood specimen stemmed from varying donators and therefore comparability of the neutrophils and/or platelets was compromised. Study protocols also focusing on recording general data from the donors could help to identify the influence of gender, age, stress level or other factors on the pre-activation state and response to stimuli of both isolated platelets and neutrophils. In the beginning platelets and neutrophils were isolated from the same donor but due to the significantly longer preparation time for platelet washing the experiments often had to be aborted because of already NETotic neutrophils. Blood collection from the same person at two times could be a solution to overcome this obstacle. Little is known about states of pre-activation of neutrophils or platelets in healthy individuals and how this could affect interaction of the two cell types. Moreover, a relatively static protocol was used in this approach to dissect the interaction between platelets and neutrophil granulocytes. Future research should account for the dynamic properties of both platelets and neutrophils in mediating their primary functions as well as their direct and indirect ways of communication.

This experimental MD thesis embarked with an unidirectional approach i.e. focusing on the effects of different subpopulations of platelets on neutrophil granulocytes. As the relationship between these cell types very likely is more complex and certainly bidirectional, next experiments should be designed to elucidate the pathways originating from the neutrophil point of view.

Besides the interplay between platelets and neutrophils, the broad idea of immunothrombosis includes other cell types and mediators to be involved. Of particular interest for further research could be endothelial cells and other inflammatory cells such as monocytes. Other APCs (e.g., dendritic cells) may also play an important role in eliciting the hyperinflammatory prothrombotic state. As mentioned, other PS blocking agents should be developed and tested to investigate the docking sites of procoagulant platelet PS on neutrophil granulocytes. Additional mediators between platelets and neutrophil granulocytes to be assessed in future experiments could be the already established IL-6, PF-4, HMGB1, calprotectin and for instance cathepsin G. The large entity of MVs surely represents another promising target.

5 Summary

The aim of this MD thesis was to investigate the interaction of procoagulant platelets with neutrophil granulocytes. This interaction was recently proposed to play an important role in the setting of immunothrombosis, where (hyper-)inflammatory diseases trigger a pro-thrombotic setting with increased risk of thrombosis and embolism. For the purpose of this MD project, laboratory models were developed that allowed further quantification of platelet-neutrophil interplay. Washed platelets were subjected to established agonists in order to induce different platelet subpopulations. The resulting distinguishable platelet subpopulations included resting, activated and procoagulant platelets. The procoagulant effects of the latter have at least partially been attributed to the presence of PS on the platelet surface. Basically, two endpoints in platelet-neutrophil interaction were defined. First, platelet-neutrophil aggregate (PNA) formation was assessed in a flow-cytometric approach. A microscopic assay was then established to visualize and quantify the effects on release of NETs. Activated and procoagulant platelets appeared to have a similar potential in stimulating PNA formation. CD62-P plays a key role in mediating PNA formation although interestingly, subsequent inhibition experiments with anti-CD62P and anti-PS point towards at least one additional factor involved in aggregation. Namely, procoagulant platelets demonstrated to be less affected by CD62P blockade and to a certain degree showed sensitivity to PS inhibition. Ultimately, release of neutrophil extracellular traps (NETs) from neutrophils was observed to be higher when co-incubated with procoagulant platelets as compared to NET release by stimulation from solely activated platelets. The obtained data in this MD thesis support the hypothesis that procoagulant platelets are equipped with additional unique pro-NETotic properties which may favour pathological thrombus formation.

Summarized overview of this MD thesis

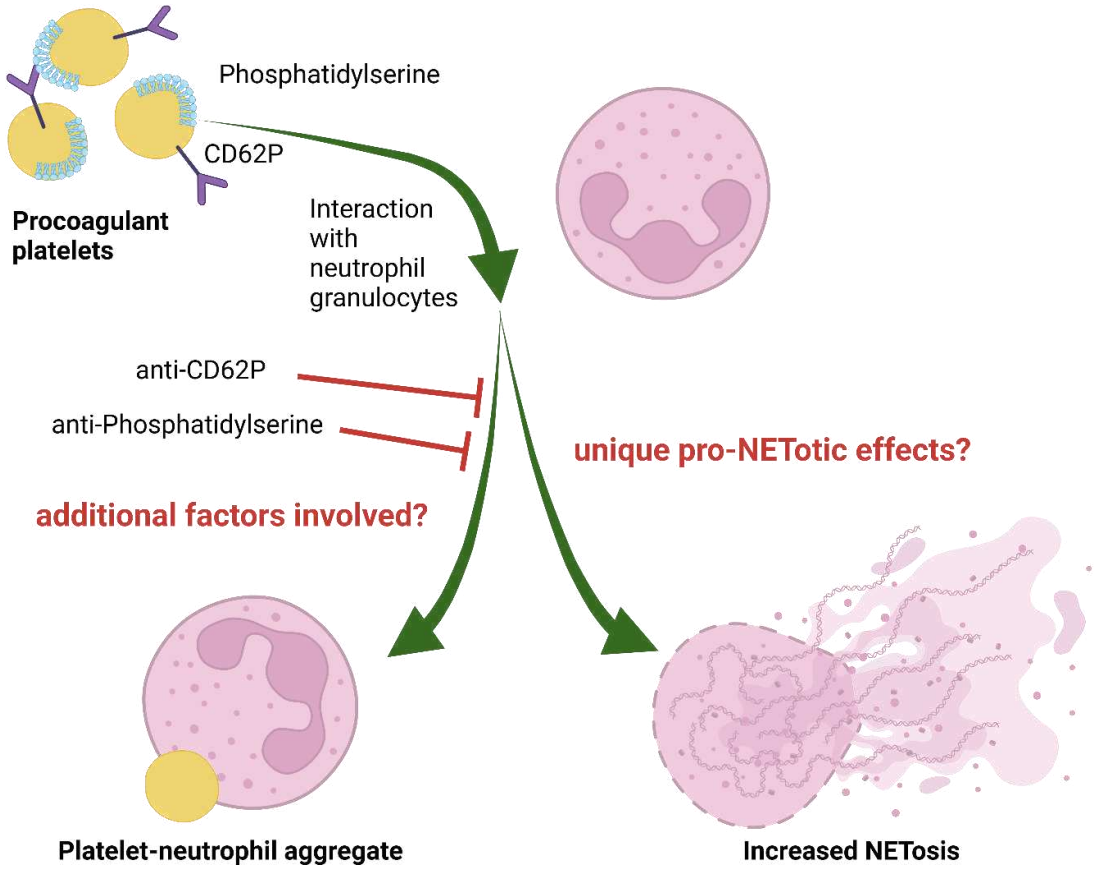

Figure 37. *Summarized overview of this MD thesis. CD62P – Platelet P-Selectin, NET – neutrophil extracellular trap. Created with Biorender.com*

Zusammenfassung

Das Ziel dieser Dissertation war die weiterführende Untersuchung der Interaktion zwischen prokoagulanten Blutplättchen und neutrophilen Granulozyten. Dieser Interaktion wurde in letzter Zeit eine wichtige Rolle in der Entstehung von Thrombosen und Embolien im Rahmen (hyper-)entzündlichen wie z.B. COVID-19 zugeschrieben. Zu diesem Zweck wurden Labormodelle entwickelt, die die Quantifizierung der Interaktion zwischen Blutplättchen und Neutrophilen erlaubten. Gewaschene Blutplättchen wurden einer Stimulation mit bekannten Agonisten unterzogen, um verschiedene Phänotypen von Blutplättchen zu erzeugen. Hier wurde zwischen ruhenden, aktivierten und prokoagulanten Blutplättchen unterschieden. Die prothrombotische Wirkung der letzteren Blutplättchen-Subpopulation wird vor allem wegen dem im Rahmen der Entstehung von prokoagulanten Blutplättchen externalisierten Phopshatidyslerin (PS) zugeschrieben. Grundlegend wurden zwei Endpunkte der Interaktion zwischen Blutplättchen und Neutrophilen unterschieden. Zuerst wurde die Aggregatbildung von Blutplättchen und Neutrophilen in einem Durchflusszytometrie-Modell untersucht. Ein mikroskopischer Ansatz diente dann der Visualisierung und Quantifizierung der Auswirkungen der unterschiedlichen Phänotypen von Blutplättchen auf die Sekretion von sog. neutrophilen extrazellulären Fallen (neutrophil extracellular traps, NETs). Aktivierte und prokoagulante Blutplättchen zeigten ein ähnlich hohes Potenzial, die Aggregatbildung aus Blutplättchen und Neutrophilen zu stimulieren. Das Oberflächenmolekül CD62-P spielt hier eine entscheidende Rolle in der Vermittlung der Aggregatbildung, auch wenn Hemmexperimente mit anti-CD62P und anti-Phosphatidylserine interessanterweise mindestens einen zusätzlichen anderen Faktor nahelegen. Prokoagulante Plättchen zeigten sich durch die CD62P-Blockade weniger stark gehemmt, was die Aggregatbildung betraf, jedoch bestand eine gewisse Sensitivität für PS-Hemmung. Die Freisetzung von NETs von Neutrophilen war in den Experimenten mit prokoagulanten Blutplättchen höher als in den Vergleichsexperiment mit aktivierten Blutplättchen. Die gesammelten Daten dieser Dissertation unterstützen die Hypothese, dass prokoagulante Blutplättchen mit zusätzlichen besonderen pro-NETotischen Merkmalen ausgestattet sind, die wiederum die Entstehung krankhafter Thrombosen begünstigen.

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7 Erklärungen zum Eigenanteil

Die vorliegende wissenschaftliche Arbeit wurde am Institut für Klinische und Experimentelle Transfusionsmedizin durch Univ. Prof. Dr. Tamam Bakchoul betreut.

Die Konzeption der Studie und Versuche erfolgte unter Anleitung von Herrn Univ. Prof. Dr. Tamam Bakchoul und Herrn Dr. Jan Zlamal. Teile der Daten aus der Studie sind publiziert auf einem Poster, das in der Poster Session auf *der 56. Jahrestagung der Deutschen Gesellschaft für Transfusionsmedizin und Immunhämatologie (DGTI)* im Jahr 2023 präsentiert wurde. Ebenfalls ist im Rahmen der Literaturrecherche ein Review entstanden, welches in *Frontiers in Immunology* erschienen ist.

Die Versuche wurden nach Einarbeitung durch Herrn Dr. Jan Zlamal von mir eigenständig durchgeführt.

Die statistische Auswertung erfolgte nach initialer Einweisung von Herrn Dr. Jan Zlamal eigenständig durch mich.

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den 12.12.2025

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Johannes Hirsch

8 Publications and Presentations

Paper: Hirsch J, Uzun G, Zlamal J, Singh A, Bakchoul T. Platelet-neutrophil interaction in COVID-19 and vaccine-induced thrombotic thrombocytopenia. Front Immunol. 2023;141186000. doi:10.3389/fimmu.2023.1186000 PMID 37275917. (143)

Poster: Hirsch J, Zlamal J, Bakchoul T. The impact of different platelet subpopulations on platelet-neutrophil interaction. PS-2-29. Poster Session auf der 56. Jahrestagung der Deutschen Gesellschaft für Transfusionsmedizin und Immunhämatologie (DGTI) 2023 (199)