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MASTERARBEIT

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„The role of oxidation-specific epitopes on platelet aggregation and function“

verfasst von

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Summary

The role of platelets has been well studied in cardiovascular diseases like atherosclerosis. The aim of this thesis is to investigate the exposure of oxidation-specific epitopes (OSEs) on the surface of platelets upon stimulation with different chemical and physiological agents and their potential role in the context of thrombus formation in atherosclerotic blood vessels. The following experiments show, that the platelet source has an influence on OSE exposure after treatment with ionomycin, as the only valid results could be obtained only with freshly isolated platelets from whole blood, were the activation status is still low and there are no plasma components interfering with OSE detection. Ionomycin is a potent trigger of OSEs on platelets, but not on JURKAT T-cells. Physiological stimuli for platelet activation like ADP, thrombin and arachidonic acid did not trigger a valid exposure of OSEs on the surface of platelets. Additionally to OSE exposure, the treatment of platelets with ionomycin resulted in the loss of OSE exposure on platelets, indicating that they shed parts of their membrane as microparticles. This could be demonstrated by stimulation of platelets with different activation triggers and collection of shed microparticles. The stimulated platelets themselves did only carry OSEs upon ionomycin treatment, but not upon other stimuli, although all platelets were highly activated. Interestingly, all fractions did shed microparticles, which were positive for OSEs with up to 60% of all microparticles carrying OSEs. This finding was not dependent on activation status or used stimulus, as also the unstimulated platelets shed OSE-exposing microparticles. So it was proven that OSE exposure on platelets only occurs through ionomycin treatment, but OSE exposure on microparticles is not dependent on the activation status and a specific stimulus, but can be globally found on microparticles to an extend of around 60% positivity.

Zusammenfassung

Die Rolle von Blutplättchen in kardiovaskulären Krankheiten wie etwa Atherosklerose ist bereits weitgehend erforscht. Das Ziel dieser Masterarbeit ist die Untersuchung von oxidations-spezifischen Epitopen (OSE) auf der Oberfläche von Plättchen nach vorangehender Stimulation mit verschiedenen chemischen und physiologischen Agentien und deren möglicher Rolle im Kontext der Thrombus-Formation in atherosklerotischen Blutgefäßen. Die folgenden Experimente zeigen, dass die Art der Plättchen-Gewinnung einen Einfluss auf die Exposition von OSEs nach einer Ionomycin-Behandlung haben, da nur frisch isolierte Plättchen aus Vollblut gültige Resultate brachte, wobei der Aktivierungs-Status der Plättchen niedrig war und keine zusätzlichen Plasmakomponenten mit der Detektion interferieren konnten. Ionomycin ist ein sehr potenter Auslöser für OSE Exposition in Blutplättchen, jedoch nicht in JURKAT T-Lymphozyten. Physiologische Stimuli wie ADP, Thrombin und Arachidonsäure konnten keine nennenswerte OSE Exposition herbeiführen. Zusätzlich zur OSE Exposition konnte Ionomycin die Plättchen zum Verlust von zuvor fluoreszenz-markierten zellspezifischen Oberflächenmarkern bringen, was vermuten lässt dass Thrombozyten Teile ihrer Membran als Mikropartikel abstoßen. Dies konnte durch eine Stimulation mit verschiedenen Aktivatoren gezeigt werden, wobei die entstehenden Mikropartikel gesammelt wurden. Die stimulierten Plättchen selbst besaßen OSEs nur nach Ionomycin-Behandlung, nicht jedoch nach Behandlung mit den anderen verwendeten Stimuli, obwohl in allen Fällen ein hoher Aktivierungsstatus bestätigt werden konnte. Es konnten aus allen Fraktionen Mikropartikel isoliert werden, die eine Positivität für OSEs von etwa 60% besaßen. Dies konnte ungeachtet des Aktivierungsstatus und des benutzten Stimulus festgestellt werden, da auch unbehandelte Plättchen Mikropartikel produzierten. So konnte in dieser Arbeit festgestellt werden, dass OSEs nur durch Ionomycin-Behandlung auf Plättchen entstehen, jedoch auf allen Mikropartikeln zu etwa 60% zu finden sind, unabhängig von Aktivierungsstatus oder verwendeten Stimulus.

1) Introduction

1.1) Atherosclerosis

Cardiovascular diseases are globally the leading cause of death, according to the WHO, which listed ischemic heart disease and stroke as the top two causes of death within the year 2012.[1]

One of the most common cardiovascular diseases and the most abundant cause for ischemic heart disease, myocardial infarction and stroke is atherosclerosis, a chronic inflammatory disease affecting large and middle-sized arteries. It can develop in arteries throughout the whole body and is related to many diseases that include vessel occlusion – depending on its location within the body - like coronary heart disease, carotid artery disease, peripheral artery disease or chronic kidney disease. In general atherogenesis – the formation of atherosclerosis – is known to be affected by many genetic and environmental risk factors, like hypertension, hypercholesterolemia, diabetes, smoking or too little physical activity. [2]

Within the molecular dynamics of atherogenesis, cholesterol plays an important role. It circulates in the blood, mainly within low-density lipoproteins (LDL), which are phospholipid particles, that contain apolipoprotein B100 (ApoB100), triglycerides and cholesterol. Circulating LDL is able to enter the intima of arterial walls where it gets oxidized by different enzymatic and non-enzymatic processes. Oxidized LDL (oxLDL) is no longer recognized by the original LDL receptor but able to trigger the activation of endothelial cells.[3,4]

At the initial stage, the endothelium gets activated by stimuli including hypertension, hypercholesteremia or pro-inflammatory cytokines, whereupon the endothelial cells start expressing adhesion molecules and white blood cells, most abundantly monocytes, are recruited which enter the intima. Activated endothelium also produces different cytokines and chemokines like macrophage colony-stimulating factor (M-CSF) which triggers the newly-recruited monocytes to differentiate into active macrophages, which then are

able to recognize oxLDL through scavenger receptors like CD36 and other receptors.[5] This recognition of oxLDL leads to its uptake through which the macrophages turn into cholesterol loaded foam cells.[6,7]

The macrophages within the intima further trigger the pro-inflammatory environment through the release of miscellaneous signaling compounds, that attract other cells of the immune system, most prominently T-cells, which themselves become essential compounds of an inflammatory environment.[8] This further causes activation of endothelial cells and the infiltration of smooth muscle cells into the intima. These cells are proliferating and contribute to foam cell formation via uptake of modified lipoproteins. They as well produce extracellular matrix proteins, like fibrin, collagen, proteoglycans and other molecules, that lead to the formation of a fibrous cap. This cap surrounds an atherosclerotic plaque and prevents it from rupturing.[7]

A ruptured plaque would cause the exposure of extracellular components to the blood flow which would trigger the activation of the coagulation cascade as well as adhesion, activation and aggregation of platelets and further the formation of a thrombus that could end in a complete vessel occlusion. While growing, these plaques generate a necrotic core containing dead cells and products of cell-metabolism. The presence of such a necrotic core decreases the stability of the plaque and makes it more prone to rupture.

1.2) Platelets and their role in Atherosclerosis

Platelets are small anucleated cells with an average size from about 2-3µm in diameter. They are part of the myeloid hematopoietic lineage and origin from megakaryocytes situated in the bone marrow, from which they are shed through cytoplasmic fragmentation. Platelets build an essential component of the blood with normal counts ranging from 150 000 – 400 000 cells/µl blood with a life span of about 5-9 days in humans, after which they are cleared from the blood flow by macrophages of the spleen or liver.[9] The main functions of platelets lie within the processes of hemostasis and thrombosis, both related to interactions with vessel walls.

Hemostasis is the fast closure of vascular injury and therefore the physiological arrest of hemorrhage. It is a tightly controlled process involving the vessel wall, platelets and coagulation factors and essential for body maintenance and therefore health.

Thrombosis on the other side is the pathological formation of blood clots within vessel walls, occurring in certain diseases that all have in common to activate the clot formation non-specifically. This clot can lead to thrombosis and vessel occlusion and further to severe consequences like myocardial infarction, stroke or microvascular thromboembolism, depending on the location of the occlusion and the thrombus.[10]

Platelets possess a unique structure and appearance since they have several special features: They do not possess a nucleus which results in the necessity to already contain all the needed mRNAs and proteins to react to all possible conditions. Since they are small in diameter, their surface area is not very large. Therefore they developed two unique cytoskeletal features: the open canalicular system (OCS) and the dense tubular system (DTS). The OCS is a network of invaginations of the plasma membrane into the interior which builds a great enlargement of the surface area.[11,12] The dense tubular system (DTS) is derived from the smooth endoplasmatic reticulum of the megakaryocytes and serves as storage mainly for intracellular calcium and many enzymes involved

in platelet activation. This channel system is fully internal and not associated with the plasma membrane.[13]

Platelets contain three different types of granules with each type exhibiting different substances and features. These granules are called alpha-granules, dense granules and lysosomes.[14]

Alpha granules are the largest and most prevalent type of granules. They play an important role in hemostasis and thrombosis as they contain many factors involved in adhesion, activation and coagulation, such as P-Selectin, fibronectin, platelet factor 4 or von Willebrand factor. On the granule surface, many glycoproteins are bound that are also expressed on the plasma membrane of platelets.[15]

Dense granules obtained their name because they appear as dense bodies through electron microscopy since they contain high amounts of calcium and phosphate. Additionally they contain among others adenine nucleotides, serotonine and relevant adhesion molecules. They are the smallest granules within platelets and contribute to platelet aggregation and also to local vasoconstriction.[14]

The third type of granules occurring platelets are lysosomes. They are intermediate in size and contain an acidic pH and hydrolytic enzymes. They are released as last of the three types, being a sign for a very strong activation status.[16]

As already mentioned above, platelets have the ability to become activated, which leads to a strong change in platelet shape and function. Activated platelets change their cytoskeleton to form a bigger star-shaped appearance. The surface markers change since granules are released and many adhesion molecules appear on the platelet surface. Signaling molecules, like ADP or arachidonic acid, as well as coagulation factors are released and cause further activation through autocrine loops and binding to endothelium as well as other platelets, resulting in thrombus formation.

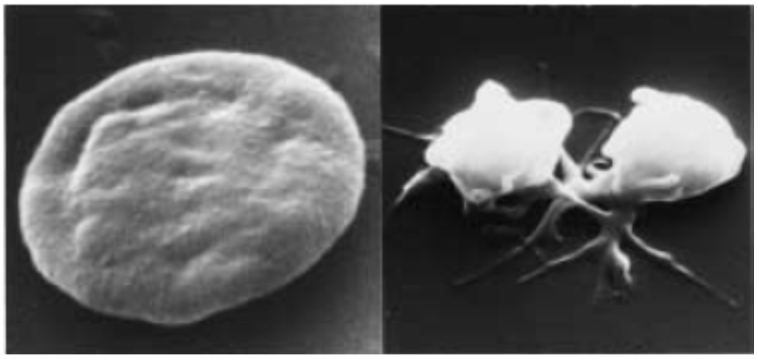

Figure 1: Electron microscopy of resting (left) and activated (right) platelets.[17]

Activation can be triggered through different pathways, like the exposure of extracellular matrix contents like collagen or thrombin to the blood flow or the expression of special adhesion molecules on the surface of endothelial cells that initiate the binding of platelets.

Molecules that trigger activation through binding to receptors on the platelet surface are amongst others ADP, thrombin and arachidonic acid.

ADP binds to the receptors P2Y1 and P2Y12, which are both members of the G-protein coupled seven transmembrane domain receptor family. The response to the P2Y1 receptor results in calcium release from intracellular storage like from the dense tubular system and further in platelet shape change and aggregation, whereas the response to the P2Y12 receptor leads to the inhibition of adenylyl cyclase and is a prominent target for antithrombotic agents, like clopidogrel and ticlopine.[10,18,19]

Platelet response to thrombin is mediated via the protease-activated-receptors (PAR) 1 and 4. The activation of these receptors occurs through proteolytic cleavage which changes the amino termini of the receptors that then build the new ligand.[20] These actions further lead to phospholipase C activation and calcium with the same outcome as described with ADP stimulation.

Arachidonic acid is metabolized through cyclooxygenases (COX1 and COX2) into the cyclic endoperoxide prostaglandin H_2 (PGH_2), which is further processed to thromboxane A_2 (TxA_2) via thromboxane synthase. TxA_2 is excreted and amplifies platelet activation via autocrine and paracrine loops through binding

the thromboxane receptor TP, which is a G-protein coupled receptor as well. It also activates phospholipase C and the further activation mechanisms as already shown for ADP and thrombin.[10]

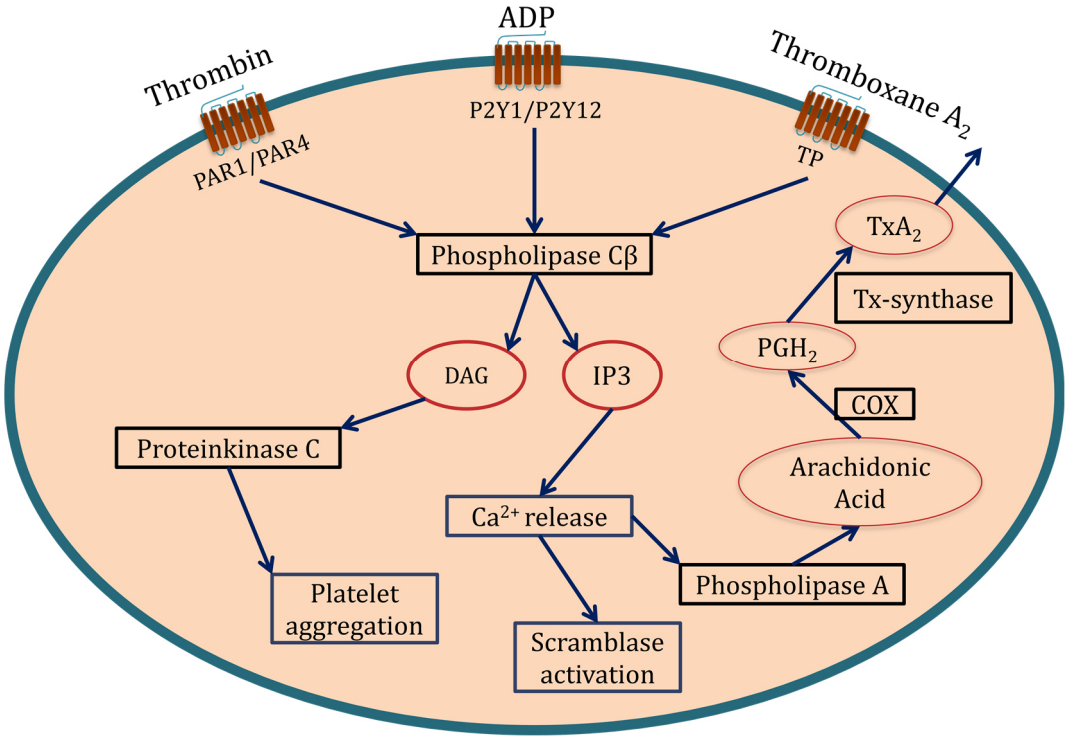

Figure 2: Schematic of the major activation pathways, see text for details

The status of activation can be measured easily through the change of expressed molecules on the platelet surface. Many activation markers and adhesion molecules occur on the surface, mostly through granule release and fusion with the plasma membrane. One of the first proteins occurring on surface of activated platelets is P-selectin.[21]

P-selectin is an adhesion molecule and a member of the selectin family. In platelets it occurs mainly in alpha granules, but it can be also present on activated endothelium and other cells. It is rapidly expressed on the cell surface and the maximum surface expression following stimulation has been reported to occur within 30 seconds to 10 minutes.[22] Therefore P-selectin can be used experimentally as a marker for activation.

Activation of platelets can also be inhibited or postponed for a certain amount of time through different substances. One of the most prominent and heavily used items for this purpose in research is PGI_2, also known as prostacyclin.[23] Prostacyclin is a member of the prostaglandin family, which are all eicosanoids derived from membrane lipids. This molecule has the ability to inhibit platelet activation through the activation of adenylyl cyclase and therefore formation of cAMP.[24] It can also be found within the body as a natural activation inhibitor, which maintains the highly sensible platelet activation status within vessels where the platelets are exposed to turbulences and high shear stress, all possible activation triggers. Prostacyclin loses its effect after some time, as it gets degraded to 6-keto-PGF_1.

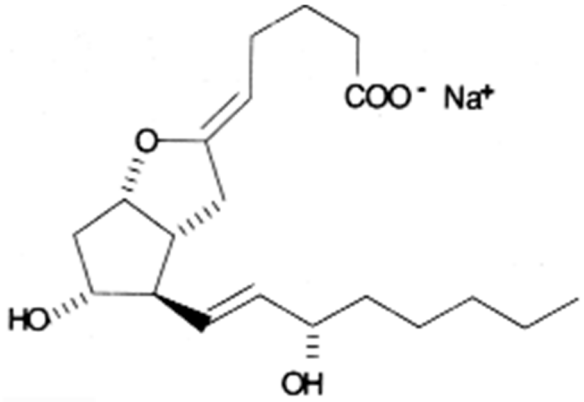

Figure 3: Molecular structure of PGI_2, a platelet activation inhibitor.

The role of platelets in atherosclerosis becomes evident regarding their way of function: Platelets become activated, start to aggregate and form a thrombus through the exposure to extracellular matrix, as it is the case when an atherosclerotic plaques ruptures. The thrombus formed during this process is the direct cause of vessel occlusion and the following actions.

Despite this central role in the course of events of atherosclerosis, the role of platelets regarding the inflammatory microenvironment ion a forming plaque is still a field of deep investigation. It is known that platelets adhering to endothelium release different signaling molecules like cytokines, chemokines

and other proinflammatory molecules, that can recruit and activate proatherogenic monocytes. Platelets can also release growth factors that trigger the proliferation of smooth muscle cells which then can infiltrate the lesions and influence the stability of atherosclerotic plaques. These mechanisms can accelerate the formation of atherosclerotic lesions and are therefore possible targets for medication.[8,25]

1.4) Microparticles

Microparticles are small membrane vesicles of 0,1-1µm diameter and are released from different types of cells under conditions of stress and activation as well as – but not necessarily - upon cell death. They are shed by all kinds of cells and are circulating in the blood of healthy and diseased people. Elevated numbers of circulating microparticles have been found in different states of disease like atherosclerosis, sepsis, diabetes or cancer.[26] The origin of microparticles can be distinguished through the surface markers they are bearing that originate from the cells they are shed from. As they are small membrane particles, they carry the surface markers of their parental cells upon their own surface, as well as other markers like exposed phosphatidylserine.[27] Intact cells possess an asymmetrical lipid bilayer with positively charged aminophospholipids on the inside, like phosphatidylinositol, and neutral phospholipids like phosphatidylcholine and phosphatidylethanolamine on the outside. This distribution is usually provided by a set of enzymes, namely flippase (inward movement of aminophospholipids), floppase (phospholipid-transport from inner to outer leaflet) and scramblase (random movements of phospholipids).[28]

The major part of circulating microparticles in the blood is derived from platelets, which shed small parts of their membrane through activation. During the activation process calcium is released within the platelets from their inner storage compartments (see above). This increase in calcium levels triggers the activation of scramblase, which then starts to scramble the phospholipid bilayer

and phosphatidylserine is exposed on the outside of platelets, which is a key event in platelet activation. These actions also lead to the shedding of microparticles wherever platelets get activated and result in a general high amount of platelet-derived microparticles within the blood.

Microparticles can also derive from several other types of cells like endothelial cells, monocytes, neutrophils or erythrocytes. These types of microparticles are found in blood of healthy individuals at lower levels than platelet-derived microparticles and they usually vary a lot in their composition. Also it is known that in different types of diseases different types of microparticles are elevated in the blood. For example patients with sickle cell disease have elevated levels of monocyte- and endothelial-derived microparticles within their blood flow.[29]

The clearance of microparticles from the circulation takes place in the spleen. This was found when splenectomized wildtype mice had elevated levels of CD42 positive microparticles in their blood compared to control mice.[30] CD42 is the glycoprotein Ib which is a receptor for van Willebrand factor mainly found on platelet surface.

1.3) Oxidation-Specific Epitopes

Biomolecules undergo constant oxidative modifications as part of normal tissue turnover. During certain situations these modifications are even more enhanced, like upon inflammation or increased oxidative stress, and the developing oxidation products start to accumulate. To prevent further damage from these products, nature has evolved several mechanisms for an appropriate response that involves recognition and binding of oxidized epitopes, maintained vastly by the innate immune system.[31]

Oxidatively modified molecules are so-called DAMPs, danger-associated molecular patterns, recognized by various arcs of the innate immune system. DAMPs trigger sterile inflammation, which is an inflammatory state caused by endogenous self-structures that are generated through tissue injury or break down, rather than exogenous structures, which would be represented by PAMPs, pathogen-associated molecular patterns.[32] DAMPs can either be newly generated - like advanced glycation end products (AGEs) - or already present within the inside of cells, where they become DAMPs when exposed outside. These patterns are recognized by the innate immune system via pattern-recognition receptors (PRRs). PRRs are a very heterogeneous group of molecules, named after their evolutionary highly conserved function of binding DAMPs and PAMPs. These molecules can be both, membrane-bound and soluble in plasma.[33]

Oxidation of lipids has been shown to generate specific structures that are recognized by various immune receptors in a hapten-specific manner and are therefore able to be modulated and cleared in a specific response.[34] Lipid peroxidation is prone to affect poly-unsaturated fatty acids (PUFAs), since they contain several double bonds together with highly reactive methylene groups within their structure. PUFAs are highly abundant in phospholipids that build up the cellular membrane. Therefore they are likely to undergo peroxidation upon different biological processes like increased oxidative stress or apoptosis.

Some of these phospholipids prone to oxidation are phosphatidylcholine, phosphatidylserine or cardiolipin.

For example the peroxidation of phosphatidylcholine leads to breakdown products like malondialdehyde (MDA) or 4-hydroxynonenal (4-HNE), structures shown in figure 4. These highly reactive aldehydes can generate covalent adducts with primary amines of proteins and amino groups of lipids in an autologous way, which leads to the formation of so-called oxidation-specific epitopes (OSEs).[33]

These OSEs possess strong pro-inflammatory characteristics and are therefore recognized by several immune receptors like for example complement factor H[35], several scavenger receptors[33], or natural IgM antibodies[36,37], to prevent further damage.

OSEs are formed when cells undergo apoptosis, but could not be found on living cells so far, which gives the immune system the possibility to identify cellular waste and debris and discriminate viable from dying cells.[33]

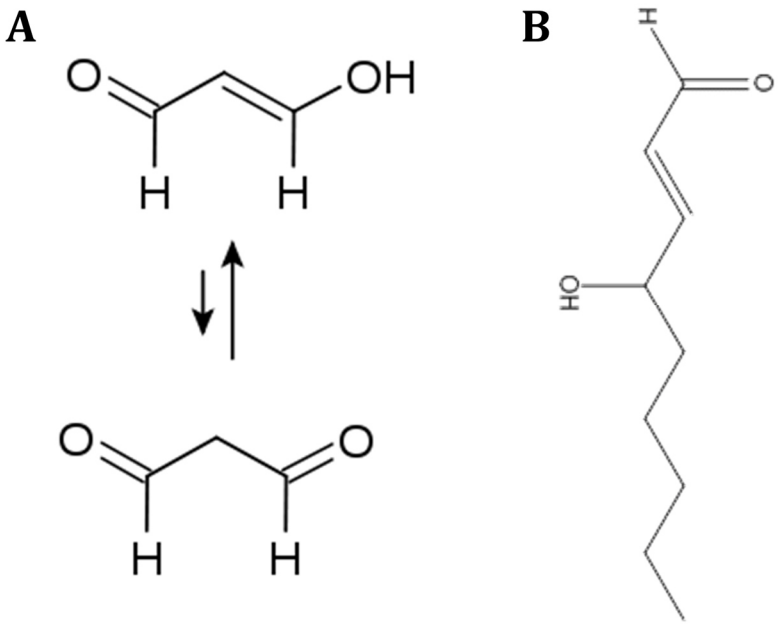

Figure 4: Molecular structures of **A** malondialdehyde (MDA) and **B** 4-hydroxynonenal.

Aim of the thesis

The activation of platelets leads to intense changes in shape and function. The cellular membrane – amongst others – is undergoing heavy turnover as it starts ruffling, the enzyme scramblase gets activated which changes the phospholipid composition and an enormous amount of pro-coagulant molecules starts getting expressed. As already mentioned above, cells undergoing stress expose oxidation-specific epitopes upon their surface, which is a sign of apoptosis. The innate immune system recognized these epitopes as danger signals and as a sign for biological waste that needs to be cleared. [38] The binding of these epitopes is carried out by natural antibodies secreted by the B1 subset of B-lymphocytes. These antibodies belong to the IgM subclass of antibodies.

The starting hypothesis for this project was formed upon the fact that microparticles derived from ionomycin treated platelets exposed OSEs. Thus, a pilot experiment was performed, where platelets treated with ionomycin were found highly activated and positive for OSEs. This exposure of OSEs could be due to activation and therefore may also result from activation with different stimuli. If this hypothesis was found true, this finding could have been a new point of intervention to interfere with platelet aggregation, since the capturing of activated platelets would have been made possible by antibodies binding to these newly exposed epitopes.

Therefore, in this project platelets were treated with different physiological stimuli and exposure of OSEs was evaluated. Additionally the shedding of microparticles was examined and the exposure of OSEs on their surface was studied.

2) Material and Methods

Material:

Buffers:

- FACS-buffer (for microparticle staining):
  PBS + 0,5% BSA, filtered through 0,1µm filter devices
- Annexin V binding buffer:
  from Annexin V apoptosis detection Kit (Ref 88-8102) , eBioscience Inc., CA, USA; diluted to 1x in sterile ddH2O; Ref 00-0055-56
- Phosphate buffered saline (PBS):
  Obtained from Sigma Aldrich
- Tyrode's buffer:
  134mM NaCl, 12mM NaHCO3, 2,9mM KCl, 0,34mM Na2HPO4, 1mM MgCl2, 10mM Hepes, 5mM glucose, pH 7,4; stored at 4°C

List of used antibodies and staining reagents:

- **Anti-Human/mouse CD62P PE** (eBioscience Inc., CA, USA; Clone Psel.KO2.3, Ref. 12-0626)
- **LEAF™ purified murine IgM Isotype control**, κ chain (Biolegend Inc., CA, USA; Ref. 401604; Clone MM-30)
- **Anti-mouse IgM APC** (Ref. 17-5790, Clone II/41, eBioscience Inc., CA, USA)
- **anti-human CD31 Biotin** (eBioscience Inc., CA, USA; Clone WM59, Ref 13-0319-80, Lot E02494-1631, stock conc. 0.5mg/ml)
- **E06** murine monoclonal antibody (IgM), Specificity: phosphocholine head group of oxidized phospholipids, first published in Horkko et al.[39], (Ref. 330001S, Lot. E06-15, Clone E06, Avanti® Polar Lipids Inc., AL, USA)

- **LR04** murine monoclonal antibody (IgM), Specificity: malondialdehyde, published in Amir et al.[40], kindly provided by Joseph Witztum, Department of Medicine, University of California San Diego
- **NA17** murine monoclonal antibody (IgM), Specificity: malondialdehyde, first published in Chou et al.[31]
- **E014** murine monoclonal antibody (IgM), Specificity: malondialdehyde, first published in Palinski et al.[41]
- **Annexin V PE** (from Annexin V apoptosis detection Kit, eBioscience Inc., CA, USA; Ref 88-8102)
- **7AAD** (from Annexin V apoptosis detection Kit, eBioscience Inc., CA, USA; Ref 88-8102)

List of used machines and software

- Flow cytometer: FACSCalibur™, BD Biosciences, CA, USA
- Analysis software: FlowJo software, Tree Star Inc., OR, USA
- Centrifuge: Allegra® X-I2 Centrifuge, Beckman Coulter, CA, USA
  Microfuge® 18 Centrifuge, Beckman Coulter, CA, USA

Methods

Aphaeresis Platelets

Platelet concentrates isolated by apheresis were obtained from the Department for Blood Group Serology and Transfusion Medicine, Medical University Vienna with the kind help of Dr. Michael B. Fischer.

Platelet-rich Plasma (PrP) from Whole Blood

Venous blood was drawn by a phlebotomist from healthy volunteers into 3,5ml Vacuette® 3,8% sodium citrate tubes. The first tube was discarded. After a short rest of 10 minutes, the tubes were centrifuged for 20 minutes at 120g at room temperature. The upper phase, which is platelet-rich plasma (PrP), was harvested and either immediately used for experiments or processed further.

Isolated Platelets from Whole Blood

PrP was obtained as described above and put to round-bottomed 4ml polystyrene tubes. Prostaglandin I_2 (PGI_2) was added to a final concentration of 1-2µM, depending on the results of a dose finding experiment, performed each time, in which 0,5µM, 1µM, 2µM or 4µM final concentration of PGI2 were added to 100µl PrP in a 1,5ml Eppendorf tube, centrifuged for 60 seconds at 3000g and the pellet resuspended with a P200 pipette. The needed concentration was chosen according to the resolvability of the pellet. The tested amount of PGI_2 was then added to the PrP in the 4ml tube and the tube centrifuged for 10 minutes at 500g with no brakes applied. The supernatant was discarded, the pellet resolved in PBS and the needed amount of isolated platelets added to a V-bottomed 96-well plate. 20-25 minutes after addition of PGI_2 the platelets were ready for further experiments.

Stimulation procedure

Platelets were stimulated with different stimuli in diverse concentrations and time courses. After the stimulation procedure cells were fixed with 1% final concentration of phosphate buffered formalin.

If not indicated otherwise, following reagents and conditions were used:

- **Ionomycin**: incubation in PBS or TBS containing 10mM $CaCl_2$ at room temperature, final concentration of 10µM ionomycin; stimulation stopped with EDTA solution at 5µM final concentration
- **Adenosine-di-phosphate (ADP)**: in PBS at 5µM and 50µM final concentration at room temperature
- **Thrombin**: stock concentration 20 I.U./ml, diluted in PBS to 0,2 I.U./ml or 0,02 I.U./ml
- **Arachidonic Acid**: stock concentration 328mM, diluted in PBS to 100µM or 500µM final concentration

Flow-Cytometry based Analysis of Platelets

Staining was done directly after stimulation and fixation of platelets in a 96-well plate. Antibodies were used at 10µg/ml final concentration in PBS if not indicated otherwise. Cells were incubated with primary antibodies for 15min at room temperature and afterwards directly put to another well containing the secondary antibody (10µg/ml final concentration in PBS, room temperature). After 15 minutes of incubation the whole content of the well was resuspended in 200µl final volume of PBS and transferred to FACS tubes. Analysis was done by flow cytometry and acquired data were analyzed using FlowJo software, where P-selectin positivity was measured as MFI from all cells and OSE positivity was measured as percentage of all events in a blot showing side scatter vs. APC signal. Therefore a gate was drawn within former said blot,

which included all events positive for APC, which was drawn according to the APC-signal in an unstained sample.

Stimulation of JURKAT T-cells

JURKAT cells were cultivated in RPMI + 10% FBS + PenStrep in T75 tissue culture flasks. For stimulation experiments 2 x 105 cells were used per staining condition.

Cells were transferred from the culture flask to a 15ml Falcon tube and centrifuged for 5 minutes at 390g. The pellet was resuspended in 400µl PBS + 2% BSA and the cells put to a V-bottomed 96-well plate. The plate was centrifuged at 390g 6°C for 5 minutes and the cells resuspended in 100µl buffer containing the primary antibodies (10µg/ml; LR04 and Isotype control antibodies used). After 20 minutes incubation at 4°C the cells were washed once with buffer and incubated with the secondary antibody (anti-IgM-APC, 1:800) for 20 minutes at 4°C. After another washing step the cells were stained with Annexin V (5µl per sample 100µl Annexin V binding buffer) and incubated again for 20 minutes at 4°C. Another washing step was performed and the cells resuspended in 250µl buffer. Five µl 7AAD staining solution were added to each sample, incubated for 5 minutes and the samples analyzed directly via flow cytometer and acquired data were analyzed using FlowJo software. OSE-positivity was measured from all cells, within a gate excluding all APC-negative events, that was drawn according to an unstained sample.

Flow-cytometry based aggregation assay

The flow-cytometry based aggregation assay was based on the publication of De Cuyper et al. in Blood in 2013[42].

PrP was collected as described in 2.2.2 but with following changes: Four tubes of venous blood were drawn and after the first centrifugation PrP was collected

(final volume of 6ml) and mixed with 4ml Tyrode's buffer. After pelleting the remaining red and white blood cells by centrifugation (500g 10 minutes at room temperature) the PrP was divided into two parts, one for the stained (6ml) and one for unstained (3ml) conditions. Both tubes were centrifuged at 800g for 20 minutes to pellet the platelets. The pellet for the unstained condition was resuspended in 3ml of Tyrode's buffer with freshly added BSA (3mg/ml). The other pellet was resuspended in 1ml PBS+1% BSA + anti-CD31-biotin antibody (2,5µg/ml final concentration) and incubated for 15 minutes at room temperature. Subsequently the cells were centrifuged at 500g for 10 minutes at room temperature with no brakes applied and taken up in 2ml PBS + 1% BSA. This solution was then divided into two further fractions, 1ml each, and incubated with either avidin-APC or avidin-PE (1:800) for 15 minutes at room temperature in the dark. After another centrifugation step (500g 10 minutes room temperature without brakes) the pellets were taken up in 1ml PBS+1% BSA and pooled to have both fluorescent colours in one tube. For the stimulations 100µl of pooled cell suspension was added per well in a V-bottomed 96-well plate and centrifuged again (same conditions). The cells were resuspended in 100µl PBS+1% BSA per well for PMA stimulation and in PBS+10mM $CaCl_2$ for ionomycin stimulation. The stimulations were done as described in 2.2.4) with different concentrations of PMA (2ng/ml, 20ng/ml, 200ng/ml) and ionomycin (5µM, 0,5µM and 0,05µM) for 20 minutes, either in presence or absence of antibodies against oxidation-specific epitopes or according controls (10µg/ml; 15 minutes incubation at room temperature before stimulation, LR04 and IgM Isotype Control, see 2.1.2. list of used antibodies). When the stimulation was done the plate was centrifuged (same conditions) and the cells resuspended in 250µl PBS+1% BSA. After transferring the suspensions to FACS-tubes the samples were analyzed via flow cytometry and acquired data were analyzed using FlowJo software. Analysis was done within a blot showing PE-signals vs APC-signals. Gates were drawn according to single stained samples, which indicated the borders for positive signals. An unstained sample was used to state the borders for double negative events.

Microparticle generation from platelets

Microparticles were generated from isolated platelets. Platelets were isolated from venous blood collected into Vacuette® tubes containing 3,8% sodium citrate. The blood was centrifuged for 20min at 120g at room temperature. Then the PrP was collected into round-bottomed polystyrene tubes, 1µM final concentration PGI_2 added, and the centrifuged for 10minutes at 500g room temperature without brakes to pellet down the platelets. They were then resuspended in filtered (0,1µm filter) PBS. 10mM final concentration $CaCl_2$ was added to the fraction to be ionomycin-stimulated. Then the platelets were stimulated for 30 minutes with following agents and concentrations:

- ADP: 50µM final concentration
- Thrombin: 0,2 I.U. final concentration
- Arachidonic acid: 500µM final concentration
- Ionomycin: 10µM final concentration

After the stimulation, 200µl of each cell suspension were fixed with 1% phosphate buffered formalin and stained according to the staining procedure described above. The rest was centrifuged for 10 minutes at 500g room temperature to pellet down the remaining platelets and the supernatant containing microparticles was collected into a new tube and stored at -20°C until further usage.

Microparticle staining for flow cytometry

Isolated microparticles obtained through stimulation as described above were stained for flow cytometry analysis to detect OSEs upon their surface.

For the staining, 50µl of microparticle-containing solution was prepared in a 1,5ml Eppendorf tube for each staining condition and centrifuged for 15 minutes at 18000g. The pellet was resuspended in 100µl of primary antibody

solution (40µg/ml antibody final concentration in FACS buffer) and incubated for 20 minutes on ice. Then 80µl of FACS buffer was added to each tube and the samples centrifuged again (same conditions). The pellet is resuspended in 100µl secondary antibody solution (anti-IgM APC used at dilution 1:200) and incubated for 20 minutes on ice. After another addition of 80µl FACS buffer per tube and centrifugation step (same conditions) the microparticles were resuspended in 95µl Annexin V binding buffer (diluted in ddH_2O) and 5µl of Annexin V was added to each tube. The samples were incubated for another 20 minutes on ice, afterwards 80µl FACS buffer were added per sample and centrifuged (same condition). The pellet was taken up in 200µl FACS buffer and analyzed in the flow cytometer and acquired data were analyzed using FlowJo software.

The small size of microparticles makes their detection difficult. To achieve this, a gate was created according to size, which included only events between 1µm and the lower detection limit of the FACSCalibur (0,2µm). Therefore a sample containing only the FACS buffer and another sample containing 1µm beads diluted in FACS buffer were analyzed, to obtain the lower and the upper border of this gate. Additionally, only events that were positive for Annexin V were accepted as true microparticles. Within this gate the positivity for APC was measured, which is the fluorophore coupled to the secondary antibody used to detect OSE-specific antibodies and the according isotype control antibody.

3) Results

Method of platelet isolation

Platelets can be purified from blood in different ways and are used for clinics and research in different preparations. For the following experiments platelets obtained through different methods were measured with respect to OSE exposure and activation status when untreated or treated with ionomycin.

The aim of these experiments was to find the ideal platelet preparation which results in a non-activated negative control and an activated OSE-exposing positive control. For the positive control platelets were treated with 10µM ionomycin as described in "Material and Methods". The status of activation was measured through the expression of P-selectin via an anti-P-selectin antibody conjugated to the fluorophore phycoerythrin (PE). OSE exposure was measured with different antibodies against various OSEs, all of them being generally established and used for detecting OSEs on apoptotic human cells, with the main focus on the antibody LR04 as it gives the highest signal for OSEs on ionomycin treated platelets.

In clinics patient that suffer from thrombocytopenia caused by various diseases and treatments, for example when they undergo chemotherapy, are often given a platelet concentrate which is obtained through apheresis from volunteering donors. This platelet concentrate is treated with different anticoagulants and can be stored for some time until transfusion to the patient.

For the tests discussed here, platelet concentrate obtained through apheresis was kindly provided by Dr. Michael Fischer from the Department of Transfusional Medicine at the Vienna General Hospital. The stimulation and staining procedure is described in "Material and Methods".

The platelets obtained through apheresis showed generally a high activation status already in the untreated samples (data not shown) and gave a slightly

elevated signal for LR04 binding (mean 10,6%). The ionomycin treated samples were positive for LR04 (mean 25,6%) and NA17 (mean 15,6%), as can be seen in Figure 5A.

Compared to this, platelets from Platelet-rich Plasma (PrP) obtained from freshly drawn blood, donated by healthy volunteers, gave different results. The PrP was isolated from venous blood freshly drawn into tubes containing 3,8% sodium citrate as an anticoagulant. These tubes were preferred against tubes that used EDTA as an anticoagulant, since the EDTA has been proven to have a changing effect on platelet shape and function[43].

The isolated PrP was directly used for stimulation and staining as described in "Material and Methods". The activation status of the untreated fraction was elevated with a mean MFI of 69,8 relative units for P-selectin compared to a mean MFI of 216,5 relative units for P-selectin in the ionomycin treated fraction (see Figure 5B). The OSE exposure within these fractions showed a mean percentage of 2,5% for LR04 binding in the untreated platelets, compared to a mean percentage of 8,6% in the ionomycin treated platelets. Additionally, the ionomycin treated fraction was positively stained by NA17 at a percentage of 6% compared to 0% in the unstimulated one (see Figure 5C).

Although these levels of OSE exposure seem already satisfying for proper positive and negative controls, PrP still contains all plasma ingredients like CFH, CRP and other molecules from humoral immune response or all kinds of antibodies that can possibly mask exposed OSEs. Therefore platelets were isolated from PrP and tested as before.

These isolated platelets were kept in PBS and treated with ionomycin as described in "Material and Methods". The treatment with ionomycin led to a high number of LR04-binding platelets (mean 51,9%, see Figure 5D). Although a similarly high percentage (mean 47,5%) of platelets showed apparent positive staining with the isotype control in the ionomycin treated fraction the mean fluorescence intensities were much lower for the isotype control stained fraction compared to the LR04 stained fraction (see Figure 5D & 5E). This

indicates, that there is significantly more binding of LR04 to OSEs than unspecific binding of isotype control antibody.

This strong binding of isotype control antibody can be explained by the activation status of the platelets. The ionomycin treated fraction shows a very high activation, indicated by a very high MFI for P-selectin binding of 1705 relative units compared to 27,8 relative units in the untreated fraction (see Figure 5F).

So this method to obtain isolated platelets was chosen for further experiments, since it gives the least activated untreated control and the highest activated and OSE exposing positive control with a very clean composition as there is no more plasma that could interfere with the treatment and its results.

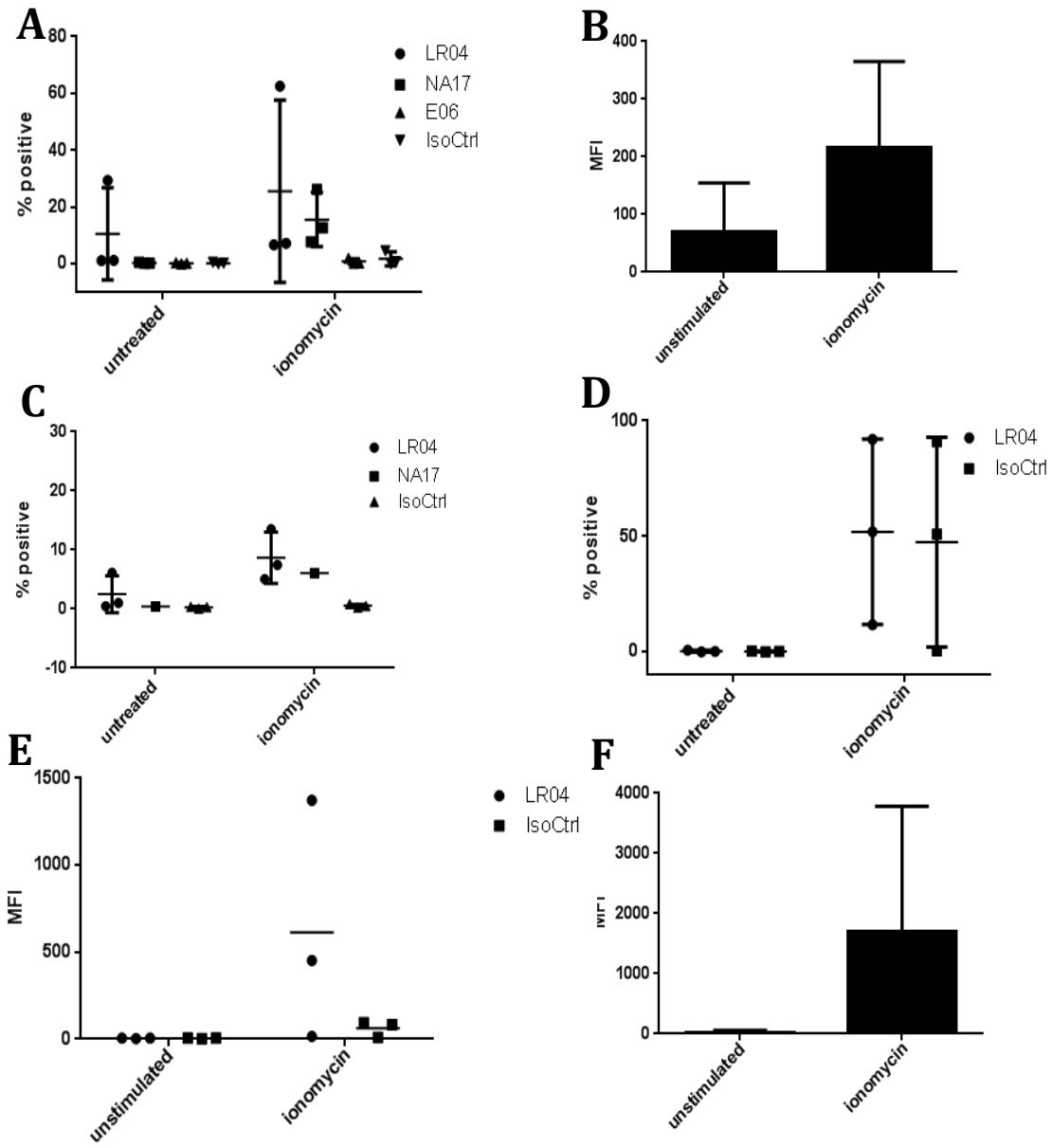

Figure 5: **Analysis of platelets obtained through different methods**. **A** OSE exposure on platelets obtained through apheresis. (n=3) **B** P-selectin expression and **C** OSE exposure on platelets obtained as platelet-rich plasma (PrP; n=3). **D** OSE exposure, **E** MFI of OSE exposure and **F** P-Selectin expression on isolated platelets (n=3), all stimulated with 10µM ionomycin for 30 minutes.

Effect of ionomycin on different cells

As a next step, the treatment with ionomycin was further established. Isolated platelets were stimulated with 10µM final concentration of ionomycin for a time course of 5 – 30 minutes. As ionomycin is a calcium-ionophore calcium has to be present during the stimulation in order to render ionomycin active. So 10mM final concentration of $CaCl_2$ was added to the platelet fraction that was to be stimulated.

After the stimulation the platelets were directly fixed and stained as described in "Material and Methods". All ionomycin treated fractions showed a clear positivity for OSE exposure as they were positively stained for LR04 as well as E014 (see Figure 6A). On average the highest OSE expression could be seen after 10 minutes with LR04-binding around 37% of all platelets and a P-selectin expression with a MFI of around 1300 relative units, as depicted in Figure 6B. The following time points of 20 minutes and 30 minutes did not show such a high positivity of P-selectin with MFIs of around 1000 relative units for both time points, respectively. Although the cells definitely were activated, it is not very likely that platelets stimulated for 10 minutes with ionomycin were more activated than platelets stimulated for 20 minutes. The decreasing signal for antibody binding could be due to platelets already shedding parts of their membrane and therefore losing the P-selectin molecules as well as possibly occurring OSEs.

To investigate the effect of ionomycin on other cells present in the blood flow, T-cells from the cell line JURKAT were stimulated with ionomycin at similar conditions as performed with platelets. It is already known that lymphocytes get activated when treated with ionomycin.[44,45] Therefore, JURKAT T-cells were incubated with different concentrations (1µM, 5µM or 10µM) and for different incubation times (10 minutes, 30 minutes or 4 hours) with ionomycin and then stained for OSEs. Additionally to this staining, a viability test using Annexin V and 7AAD was performed that showed that there were no differences regarding viability between untreated and treated cells (data not shown). The staining did not show any positivity for OSEs on Ionomycin activated JURKAT T-cells (see

Figure 6C), giving only a positive signal for unspecific binding by the isotype control antibody.

These results suggest, that only platelets and not other cells relevant for the immune system expose OSEs on their surface when treated with ionomycin.

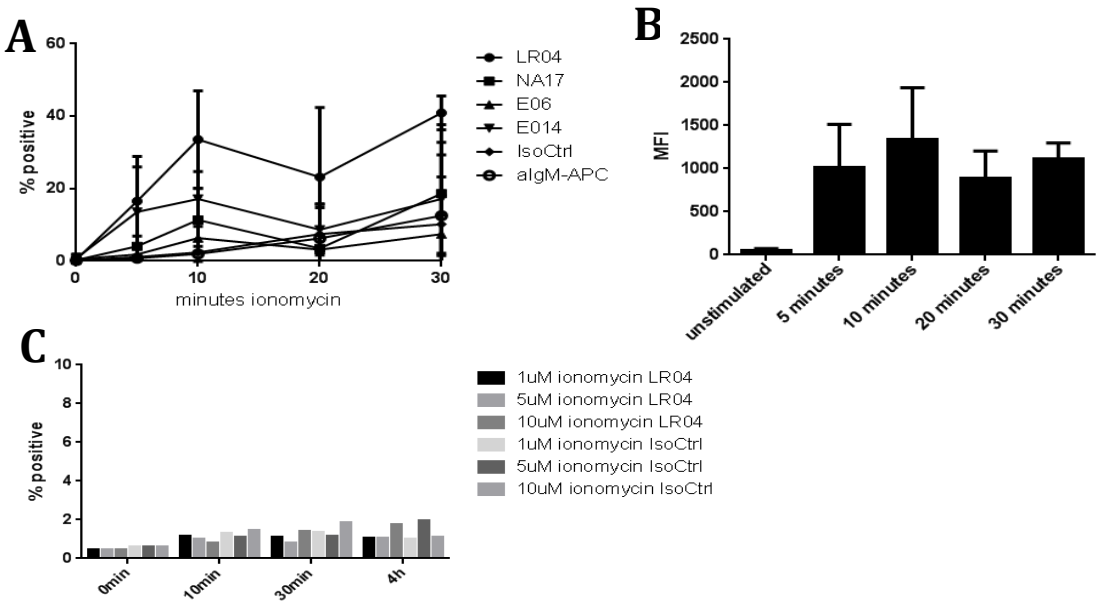

Figure 6: **Ionomycin time course analysis on platelets and JURKAT T-cells**. **A** OSE exposure and **B** P-selectin expression on platelets after ionomycin (10µM) treatment. (n=3) **C** OSE exposure on JURKAT T-cells after ionomycin treatment.

OSEs on differently stimulated platelets

To further test the occurrence of OSEs on activated platelets, stimulation assays were performed with different physiological stimuli and the platelets were tested for activation status as well as staining with different OSE-specific antibodies.

The first stimulation assay was performed with two different concentrations of ADP, 5µM and 50µM final concentration, at a time course of 5 – 30 minutes incubation. Both concentrations were potent activators as they both induced a robust P-selectin expression on platelets (see Figure 7A). It is clearly visible that even with a ten times higher amount of ADP there was a similar outcome in P-selectin expression than with the lower concentration, indicating that the maximum of activation, that is possible with this stimulus, was achieved.

Although the platelets were activated, no OSEs could be identified on the surface. Only the staining with the E06 antibody, which recognizes oxidized phosphatidylcholine, seems to give minimal positivity of 2-4%, but these elevated mean values are derived from a single outlier (see figure 7B).

Similar results were obtained when platelets were stimulated with different concentrations of thrombin, using 0,2 I.U. or 0,02 I.U. . The stimulation resulted in very high P-selectin expression, reaching almost a MFI of 1500 with the higher thrombin concentration (see Figure 7C). Still, the exposure of OSE was almost negative, reaching maximum around 1% for LR04 in one condition, which also has a very high error indicating this is not a representative result (see Figure 7D).

Similar results could be observed after stimulation with arachidonic acid (see Figures 7E and 7F). A minimal positivity for E06 staining of around 1,5 - 2% occurred on stimulated but also on unstimulated platelets.

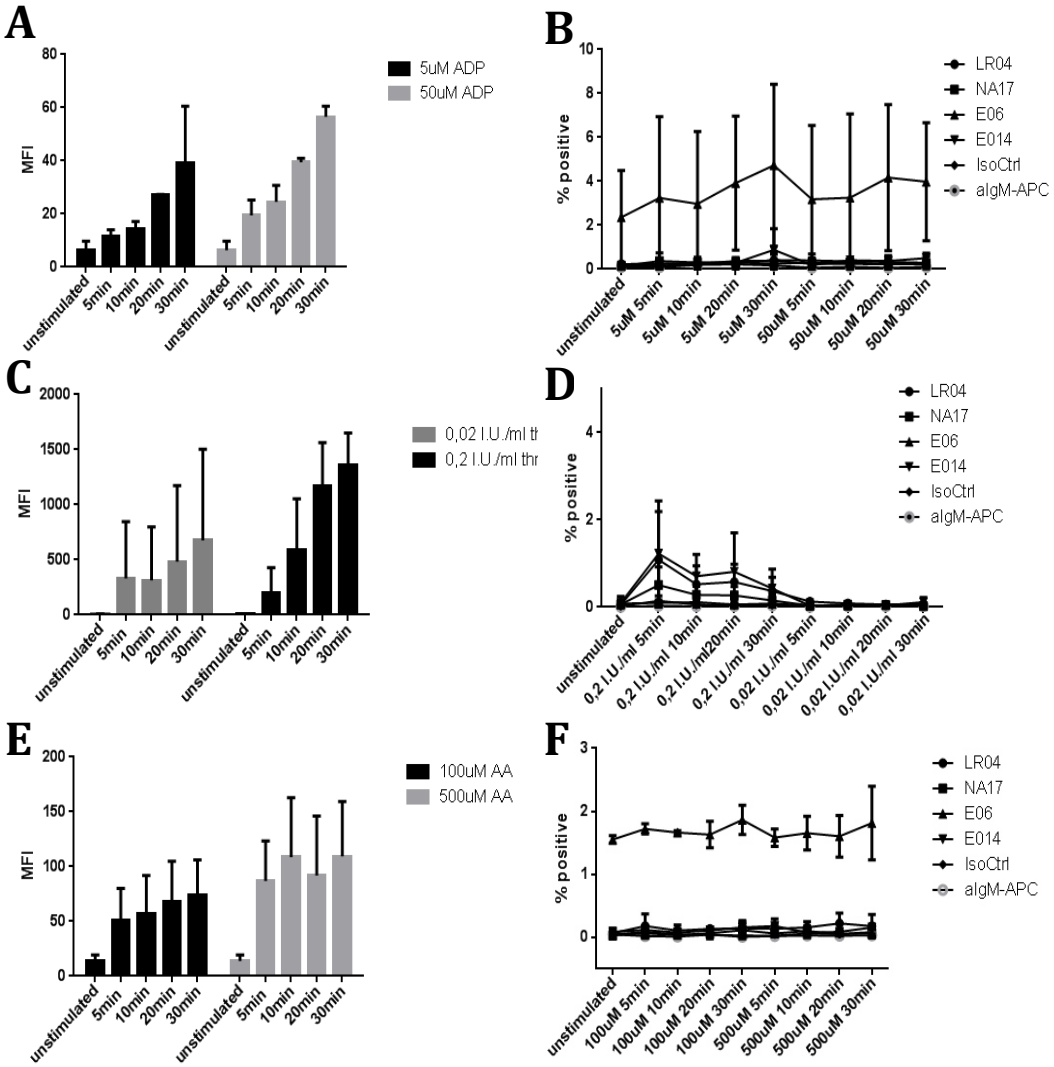

Figure 7: **Analysis of OSE exposure on differently stimulated platelets.**
A P-Selectin expression and **B** OSE exposure on ADP stimulated platelets. (n=3)
C P-Selectin expression and **D** OSE exposure on thrombin stimulated platelets. (n=3) **E** P-Selectin expression and **F** OSE exposure on platelets stimulated with arachidonic acid. (n=3)

Ionomycin treated platelets loose antibody labeling

Additionally to the stimulation assays, a novel FACS-based aggregation assay was performed, first described by de Cuyper et al. in 2013[42]. The exact method is described in "Material and Methods".

For this assay, isolated platelets were stained in two different fractions with the same antibody, but conjugated to a different fluorophore. Then the fractions were either left unstimulated or stimulated with different concentrations (5µM, 0,5µM or 0,05µM) of ionomycin. After stimulation, the differently stained fractions were mixed and immediately analyzed by flow cytometry. Aggregates formed by platelets from different fractions were visible as double positive events for both fluorophores. Aggregates formed between platelets stained with the same fluorophore were visible as single positive events in the FACS blot.

Figure 8A shows the FACS blot of untreated platelets that were stained in two fractions and mixed without stimulation. Almost all platelets were either single or double positive for fluorophores, leaving only 2,9% of all events double negative. This blot showed the validity of the method, as all platelets were stained and aggregates were formed already in the untreated platelets as they became activated through the procedure of antibody staining. In figure 8B FACS blots of platelets treated the same way as in figure 8A are shown, with the difference that ionomycin was added at different concentration after the staining and mixing of fractions. These blots show, that the majority of cells treated with ionomycin move to the double negative panel, giving a value of over 50% within this section. As figure 8A proves, all platelets and formed aggregates carried at least one fluorophore, leading to the conclusion that upon ionomycin treatment platelets are able to lose this labelling.

As ionomycin is commonly used to generate microparticles the hypothesis was formed that this loss of labelling occurred due to microparticle formation that included the labelling. This way the platelets would have been able to lose the staining and become double negative within a FACS analysis.

This shedding of microparticles was further thought to be a direct effect of platelet activation, as ionomycin has been proven in this work to trigger a highly activated status, whereas unstimulated platelets stay at a very low or not activated status. Activation of platelets triggers vast changes in shape and membrane composition, making it very likely that microparticles are shed during this process. This was tested through stimulating platelets with different compounds and collecting the supernatant in which microparticles are found. Then the platelets themselves as well as the derived microparticles were tested for exposure of OSEs.

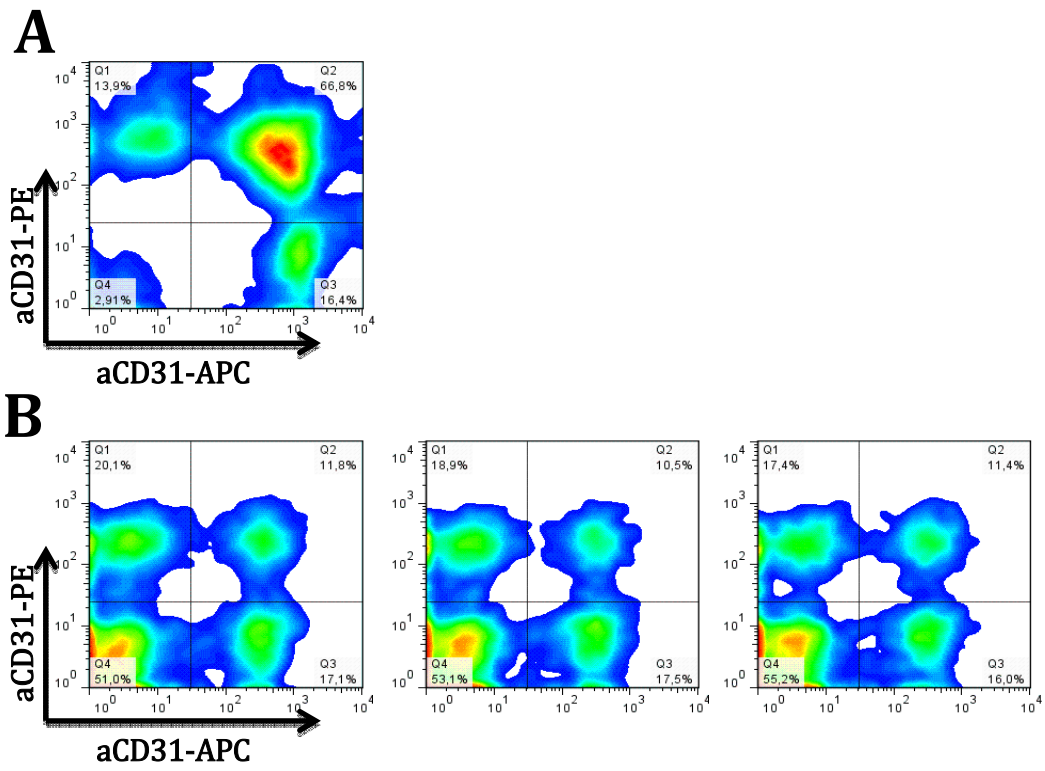

Figure 8: **FACS based aggregation assay**. **A** untreated platelets, one fraction stained with aCD31-FITC and another fraction stained with aCD31-PE , afterwards mixed and analysed **B** stained fractions mixed and treated with 5µM (left panel), 0,5µM (middle panel) or 0,05µM (right panel) ionomycin for 20 minutes.

Platelets shed microparticles upon stimulation

Microparticles were generated from isolated platelets using the highest used concentrations of every stimulus, which was 50µM ADP, 0,2 I.U. thrombin, 500µM arachidonic acid and 10µM ionomycin. Each stimulus was incubated for 30 minutes. Directly after stimulation, some platelets were fixed and stained for P-selectin and OSE exposure according to the standard protocol described in "Material and Methods". The results from this staining are shown in panels A and B of Figure 9.

Figure 9A shows that only platelets treated with ionomycin give a positive signal for LR04 binding and therefore OSE exposure, as already seen before in Figures 6 and 7. The stimulation in general was successful as the high expression of P-Selectin on each stimulated fraction shows (Figure 9B). The highest stimulation could be achieved through ionomycin treatment, giving a mean MFI of 5400 compared to a mean MFI of 33 in the unstimulated fraction. All other stimuli had minimum a five times higher expression of P-Selectin than the unstimulated, indicating successful platelet activation.

The microparticles were isolated from the supernatant and stored at -20°C. Since there were only small numbers of microparticles within some samples, a valid quantification of these could not be done. Microparticles were directly stained for OSEs. This staining showed that although there were vast differences in activation status and OSE exposure on the platelets themselves, the OSE exposure on the derived microparticles was very similar within each condition (see Figure 9C). Around 60% of all microparticles had OSEs upon their surface, regardless of activation status and stimulation procedure.

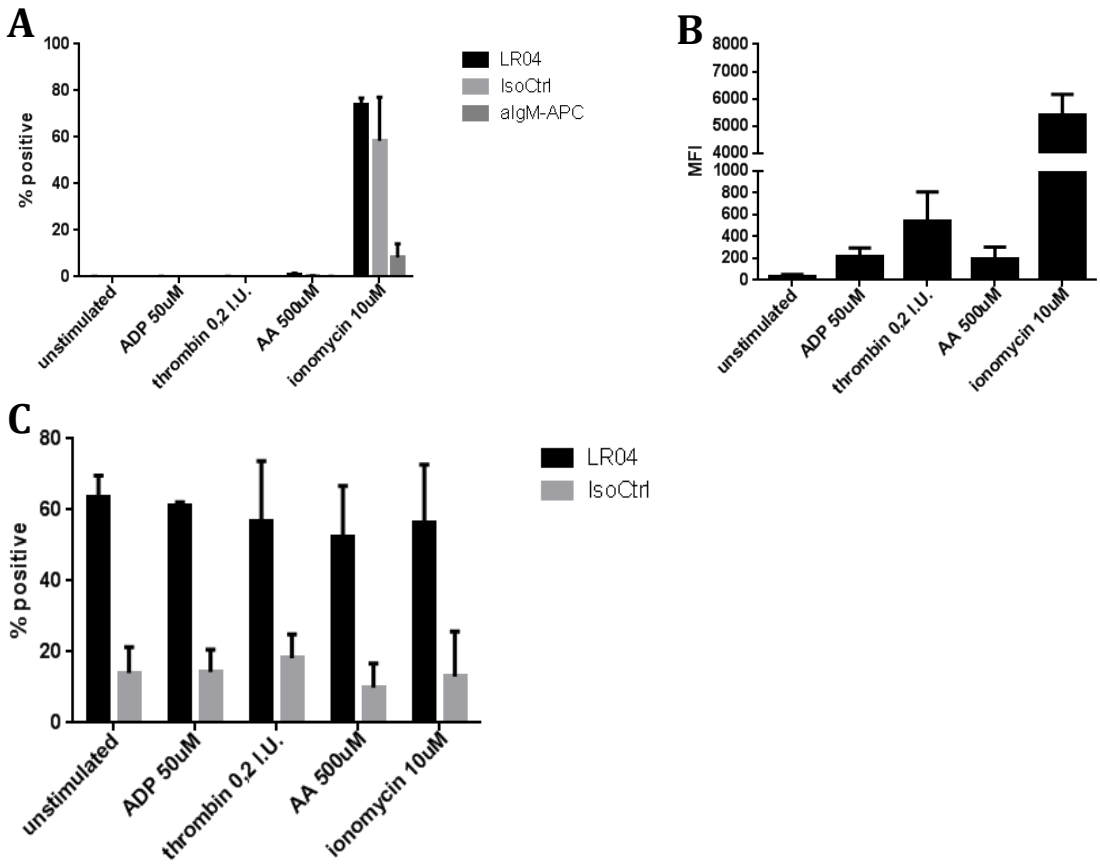

Figure 9: **Analysis of platelets after microparticle generation**. **A** OSE exposure and **B** P-Selectin expression on platelets after microparticle generation. **C** staining for OSEs on isolated microparticles from each stimulation condition. (n=3)

4) Discussion

Platelets are known to play a very important role in many diseases, especially related to thrombus formation and subsequent clinical events, like ischemic heart diseases and stroke. The changes undergoing within platelets upon activation have been already well characterized. This thesis provides further characterization of membrane composition upon activation in relation to oxidation specific epitopes. These epitopes have been found important in several malignancies and inflammatory settings and are proven to be recognized and bound by the immune system as danger-associated molecular patterns.

Within this work, it could be shown that treatment of ionomycin on platelets triggers not only activation but also the exposition of oxidation-specific epitopes on the membrane surface, in contrast to unstimulated platelets where these epitopes could not be found. Therefore ionomycin treatment served as a positive control for further investigation of OSE occurrence on differently treated platelets. It could be shown, that the isolation method from which platelets are obtained has an impact not only on OSE expression but also on activation status and detection mechanisms. Platelets obtained through apheresis showed an activated state already without any treatment, whereas platelets in platelet-rich plasma showed a very low activation status. Due to the additional ingredients within plasma, that can possibly mark the epitopes under investigation, platelets were isolated and kept only in PBS for further experiments. This method showed the most efficient OSE detection on ionomycin treated cells, whereas untreated cells still kept a non- or very low activated status, thereby serving as a valid negative control.

OSEs could be detected on all ionomycin treated platelets, but not on activated JURKAT T-cells serving as example for other immune cells within the blood stream. Therefore this exposure is unique to platelets, although ionomycin is known to activate a variety of cells.

Platelets stimulated with different physiological stimuli did not trigger OSE exposure, although they get activated through the treatment to different extents. Only minor binding of E06 could be detected in some cases, which was not concluded to be representative due to high error or not depending on activation status, as it is the case when stimulated with arachidonic acid. These experiments showed, that OSE exposure is not due to activation and could not be detected on all activated platelets. Therefore the original hypothesis of this thesis was denied and binding of OSEs by natural IgM antibodies cannot be used to prevent aggregation of activated platelets.

Anyhow, platelets treated with ionomycin were shown to lose a before present antibody staining, indicating that they shed parts of their membrane during this treatment. This shedding was proven to occur as microparticle formation.

Microparticles were shed upon all used treatments, including ionomycin, ADP, thrombin and arachidonic acid, as well as on untreated platelets. A quantitative relation could not be proven due to difficulties in microparticle isolation and quantification methods caused by the small amounts of microparticles shed in some conditions. Anyway, a subjective evaluation of the researcher performing the experiments, estimated that the highest amount of microparticles could be obtained from the ionomycin treated samples, followed by treatment with arachidonic acid and thrombin, whereas untreated platelets only gave a very low number of microparticles. Albeit this subjective assumption, a staining of all microparticles for OSEs revealed, that although the platelet themselves did only carry them upon stimulation with ionomycin and not the other treatments, all microparticles showed a positivity for OSEs of around 60%, independent of what triggered platelet activation.

These findings indicate, that although OSEs cannot be used as a marker to identify activated platelets, the microparticles derived from activated platelets display a possible target of antibodies and agents directed against OSEs. This binding of microparticles through OSE specific agents opens many possibilities to interfere in pathologies and diseases that are triggered or worsened by the presence or uptake of circulating microparticles. As microparticles have already

been shown to be enriched in several diseases, in inflammatory environment and in acute and chronic violations of tissue and are thought to have a pro-inflammatory and worsening effect, the possibility to bind and therefore neutralize them by binding to OSEs present on their surface, gives possibilities for treatment of these diseases and pathologies.

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6) Appendix

Table of Figures

Curriculum Vitae

Karin Harold, BSc

Date of Birth: January 21st, 1991

Place of Birth: Horn, Austria

EDUCATION:

2012-2014:	Masters course "Immune biology and Microbiology", University of Vienna
2009-2012:	Bachelors course "Molecular Biotechnology", University of Applied Sciences FH Campus Wien, Vienna
2001-2009:	Bundesgymnasium Rechte Kremszeile, Krems an der Donau

PROFESSIONAL EXPERIENCE:

March 2013 – October 2014: diploma student and research assistant at
CeMM Research Center for Molecular Medicine
of the Austrian Academy of Sciences,
Supervisor: Univ.-Prof. DDr. Christoph J. Binder

January - May 2012: laboratory training at
Wenner Gren Institute for Cell Biology,
University of Stockholm, Sweden
Supervisor: Prof. Roger Karlsson

Different trainings during my education at the university in the fields of Biochemistry, analytical chemistry, cell biology, microbiology and immunology as well as in science related Austrian and European legislation and project management.

LINGUISTIC COMPETENCES:

German:	Fluently (mother tongue)
English:	fluently (C1/C2)
French:	basic knowledge (A2)

PROFESSIONAL COMPETENCES

Experience in different methods of cell and molecular biology, especially:

FACS, cellular assays, cell culture, molecular assays like ELISA, western blot, transfection, light and fluorescence microscopy, intracellular and extracellular staining, histological sample preparation and staining, gel electrophoresis, PCR, etc.

SOCIAL AND ORGANIZATIONAL COMPETENCES:

Competences in teambuilding, conflict management, stress and time management, communication and motivation management, presentation skills and project management obtained through interactive courses at University and leadership of an official youth organization.

ADDITIONAL COMPETENCES:

Basic skills in Austrian law, specifically public law, pharmaceutical law and science specific law. Knowledge in quality management, GMP/GLP/GSP obtained through education at university.

Basic knowledge in business management, especially organizational and human resources, innovation and technology management and marketing through additional courses at the Department of Business Administration at the Vienna University.

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