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A NON-TRANSCRIPTIONAL ROLE OF NFAT IN REGULATING PLATELETS FUNCTIONS

Valentina Poli

Matr. No. 731629

Tutor: Prof.ssa Francesca Granucci

Co-tutor: Prof. Ivan Zanoni

Coordinator: Prof. Andrea Biondi

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Chapter 1: Introduction

1.1 Platelets: an overview

In 1881, an Italian pathologist, Giulio Bizzozero, described for the first time a novel morphological element in the blood with important roles in hemorrhage and thrombosis. These elements, now known as platelets, were a third morphological element circulating in the blood, different from erythrocytes and leukocytes until then described [1].

Platelets are approximately 2 μm in diameter, circulating, anucleated cells. Their physiological function is to sense any damage at the vessel endothelium and to accumulate at the site of the injury to block the circulatory leak [2], [3]. In the blood of a human adult there are around one trillion platelets circulating, 150-400 × 10^3 platelets/μl of blood, with a lifespan of 8/10 days. In mice, platelet count is higher, 900-1600 × 10^3 platelets/μl of blood, with a lifespan of 4 days [2].

Anucleated cells are found only in mammals. In invertebrates, there is only one type of nucleated cell circulating in the hemolymph, called hemocytes, and this is necessary for all defensive mechanisms, including hemostasis. These cells are similar in structure and function to vertebrate macrophages, they mediate phagocytosis and secrete antimicrobial peptides, but they are also able to aggregate and repair wounds [4].

Nonmammalian vertebrates, such as fish, reptiles and birds, have thrombocytes. They are nucleated cells and are the first cells evolved specialized in hemostasis. In some species, multiple types of thrombocytes have been described characterized by different shapes and sizes [5].

Anucleated platelets are a typical blood element specific of mammalian species, but the evolutionary advantage of this system for the mammalian physiology is still not known.

1.1.1 Megakaryocytes maturation and platelets generation

Platelets are formed and released into the bloodstream by precursor cells called megakaryocytes (MKs), the only polyploid hematopoietic cells, that primarily reside within the bone marrow, but can also be found in the lung and peripheral blood [6].

Pluripotent hematopoietic stem cells (HSCs) can differentiate into a common myeloid progenitor (CMP), that gives rise to megakaryocytes precursors but also generate all the other myeloid cells, such as macrophages, granulocytes, erythrocytes and, in part, dendritic cells. All hematopoietic progenitors express typical surface markers such as CD34 and CD41, but, when cells are committed to the megakaryocytic fate, they can be identified by the expression of CD61 (also known as integrin β3, GPIIIa) and elevated levels of CD41 (also known as integrin αIIb, GPIIb). From the CMP, also referred as colony-forming unit-granulocyte-

erythroid-macrophage-megakaryocytes (CFU-GEMM) arises a bipotential progenitor, called megakaryocyte-erythroid progenitor (MEP), that can generate colonies composed of both megakaryocytic and erythroid cells [7].

MEPs generate diploid precursors that are committed to the megakaryocyte lineage and can be divided into two different colonies based on their capacities: burst-forming unit (BFU) and colony forming unit (CFU) (Fig. 1). The first one has a morphology similar to the small lymphocytes and a high proliferation capacity that can generate 40-500 MKs in a week, whereas the second one is a more mature megakaryocyte progenitor that can generate only 3-50 MKs [7]. From these progenitors the immediate megakaryocyte precursors are generated, they are divided into three different stages of maturation based on their shape, histochemical staining and biochemical markers. The promegakaryoblast is the first recognizable megakaryocyte precursor, that generate the megakaryoblast, a more mature cell with a distinct morphology and two set of chromosomes (4N). The megakaryoblast gives rise to the promegakaryocyte, that terminally differentiate into MKs (Fig. 1) [8].

Thrombopoietin (TPO) and its specific receptor c-Mpl are the major regulators of MKs differentiation and maturation from their precursors. However, also other soluble molecules are involved in these processes, i.e.: granulocyte-macrophage colony-stimulating factor (GM-CSF),

interleukin family proteins (IL-1, IL-3, IL-6 and IL-11), stromal cell-derived factor-1 (SCF-1) and reactive oxygen species (ROS) [9].

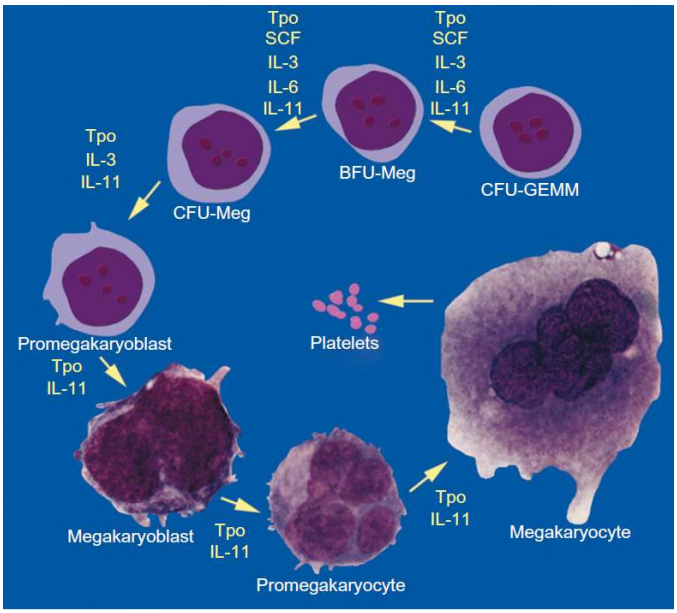

Figure 1| Megakaryocyte development. Schematic representation of different precursor stages of MKs development [8].

MKs are the largest cells in the bone marrow (50-100 µm) and, to generate and release platelets, they become polyploid by endomitosis, a process that consists of repeated cycle of DNA replication without cell division. At this stage, MKs accumulate platelet-specific granules, expand their pool of cytoskeletal proteins and develop a highly invaginated membrane system [10]. Subsequently, MKs undergo a maturation process in which the cytoplasm is packaged into long branching processes called proplatelets. These processes are directly release into the sinusoidal

blood vessels of the bone marrow, where they undergo further fragmentation into individual platelets (Fig. 2) [10].

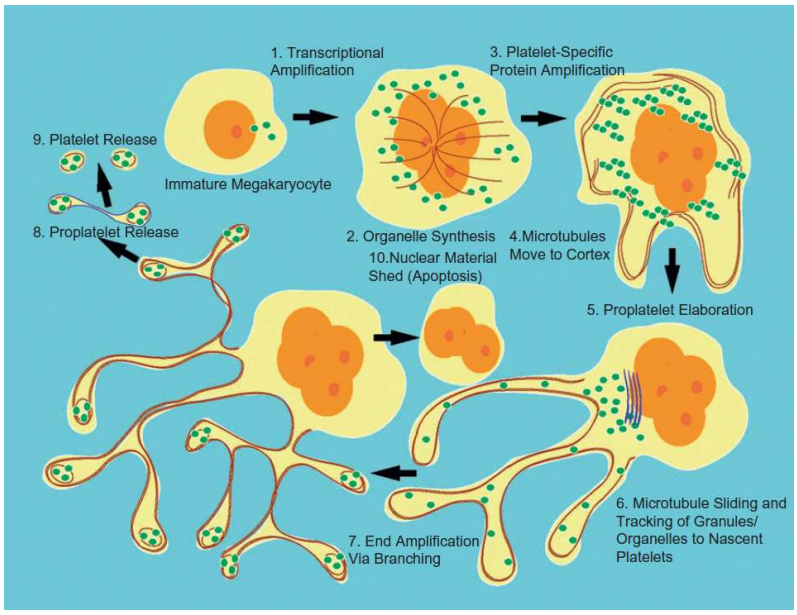

Figure 2| Platelets generation. Schematic representation of the different steps that result in the generation of platelets, from the megakaryocyte to single platelets release [8].

1.1.2 Platelets structure

Platelets are anucleated cells but they contain other organelles and structure found in nucleated cells, such as mitochondria, microtubules, granules, spliceosome and even a functioning mRNA transduction machinery [11].

A platelet at the resting state can be divided into three different zones: peripheral zone, sol-gel zone and organelle zone. The *peripheral zone* includes glycocalyx coat, platelet membrane and submembrane zone and

it contains all the transmembrane receptors necessary for adhesion and aggregation. The submembrane zone is composed of a system of thin filaments resembling actin filaments and is fundamental for shape change and receptors translocation after platelet activation [12].

The *sol-gel zone* contains two different filament systems that are important in shape change, contraction and clot retraction. These two systems are the circumferential coil microtubules, that is a cytoskeletal support system and is involved in maintaining platelet shape, and the actomyosin filament system, that is mainly responsible for shape change and granules shuttling. Moreover, the open canalicular system (OCS) and the dense tubular system (DTS) reside in this zone [13]. The OCS is a network of channels tunneling through the cytoplasm and connected to the surface. It can be considered surface membrane itself because it is the source of extra membrane needed during the spreading process, allowing an increase in platelet surface area up to 420 % of its original size. Moreover, it is the platform through which platelets uptake and transfer products, such as fibrinogen [14]. The DTS is form of residual channels of the endoplasmic reticulum of the originating megakaryocyte and it has a role in modulating platelet activation, for example by serve as calcium storage and hosting prostaglandin and thromboxane synthesis [12].

Finally, the *organelle zone* contains all different types of granules, soluble factors and mitochondria. Platelets contain α-granules, dense granules, lysosomes and peroxisomes. The α-granules are the most numerous,

usually 50 to 80 granules per platelet, ranging in size from 200 to 500 nm, and they account for 10 % of platelet volume. They contain proteins, such as P-selectin, often used as marker of α-granules release, but also hemostatic factors (e.g. von Willebrand Factor and fibrinogen), angiogenic factors (e.g. angiogenin and vascular endothelial growth factor (VEGF)) anti-angiogenic factors (e.g. angiostatin and platelet factor 4 (PF4)) proteases (e.g. MMP2 and MMP9) growth factors (e.g. PDGF, bFGF, SDF1α) and cytokines (e.g. TNFα and TGFβ) (Fig. 3). These proteins can derived from the megakaryocytes or can be internalized by platelets from the circulation [12]. The dense granules are smaller in number and contain small non-protein molecules, such as calcium, adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin and magnesium (Fig. 3). Their secretion can be monitored by measuring ATP release. Platelets contain also lysosomes, that are granules rich in acid hydrolases, necessary to eliminate circulating platelet aggregates [13]. Moreover, it has been shown that platelets contain peroxisomes, but their function in platelet physiology is not well understood yet [15].

	α-Granules	Dense granules
Diameter	200–500 nm	150 nm
Number	50–80 per platelet	3–8 per platelet
Percentage of cell volume	10	~1
Contents	Integral membrane proteins (e.g., P-selectin, αIIbβ3, GPIbα) Coagulants/anticoagulants and fibrinolytic proteins (e.g., factor V, factor IX, plasminogen) Adhesion proteins (e.g., fibrinogen, vWF) Chemokines [e.g., CXCL4 (PF4), CXCL12 (SDF-1α)] Growth factors (e.g., EGF, IGF) Angiogenic factors/inhibitors (e.g., VEGF, PDGF, angiostatins) Immune mediators (e.g., IgG, complement precursors)	Cations (e.g., Ca^{2+}, Mg^{2+}) Polyphosphates Bioactive amines (e.g., serotonin, histamine) Nucleotides (e.g., ADP, ATP)

Figure 3| Platelets granules. The content, frequency and size of α-granules and dense granules found in platelets are summarized in this table. α-granules are more abundant and contain different proteins, while dense granules are loaded with small molecules and are fewer in number (adapted from [16]).

1.2 Platelets activation pathways

Platelets have been discovered for their role in hemostasis more than a century ago. Hemostasis is a complex physiologic process that arrests the bleeding of a damaged vessel and it needs platelets, plasma proteins and endothelial cells to occur. Platelet activation and subsequent plug formation in the hemostatic process can be divided into three different moment: initiation, extension and stabilization. During the initiation phase platelets sense the damage at the endothelium and form a monolayer of activated cells. Once they are activated, they induce the recruitment and activation of other platelets by releasing soluble

mediators; this is called extension phase. At the end of this process, during the stabilization phase, platelets reinforce their interactions in order to prevent rupture and disaggregation of the plug, until the wound is completed healed (Fig. 4).

Initiation phase

Once the endothelium is damaged, von Willebrand Factor (vWF) and collagen are exposed to the blood where they can be bound by circulating platelets. If this interaction remains in place long enough, platelets become activated and form a monolayer that is necessary for further platelets recruitment (Fig. 4) [17]. Platelets bind vWF through a complex composed by glycoprotein (GP) Ib/V/IX, while collagen is the ligand for GPVI mostly but can bind GPIa/IIa too, also known as integrin $\alpha_2\beta_1$. In this initial phase, shear stress and flow rate are important factors that regulate platelet activation. In particular, in static or low flow condition collagen by itself is able to induce platelets activation, while in high flow condition vWF is essential to allow platelets slow down and proper activation [18]. It is not well known how GPIb/V/IX activates a signaling cascade, because it lacks G-protein coupled domains, tyrosine kinase activity or phosphorylable tyrosine residues. Indeed, it has been suggested that GPIb/V/IX can use other adjacent receptors and adaptors [19].

On the other hand, GPVI is constitutively complexed with a FcR_γ chain dimer, that bears an immunoreceptor tyrosine-based activation motif (ITAM) that allows intracellular signaling transduction. Src kinases

activation results in phosphorylation of ITAM tyrosine residues and recruitment and activation of Syk, followed by downstream signaling cascade that involves adaptor proteins like LAT, SLP-76 and Gads, and activates the phospholipase Cϒ2 (PLCϒ2) [17].

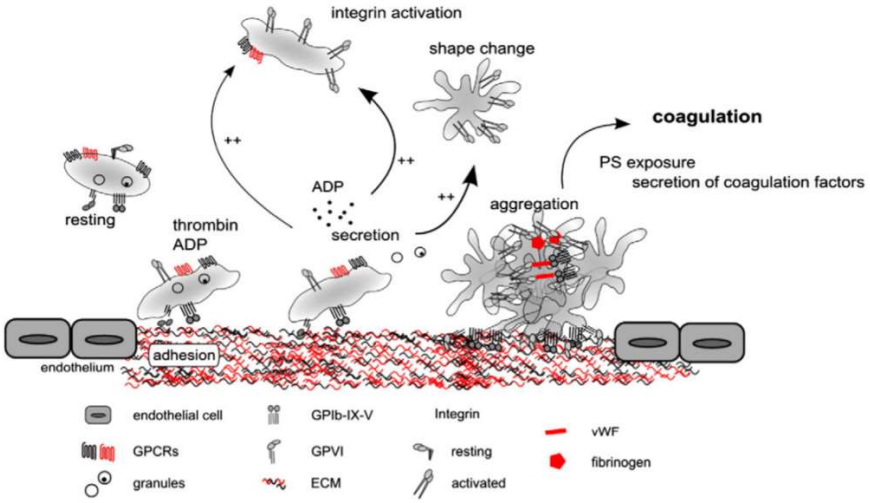

Figure 4| Platelet activation cascade. Schematic representation of the different steps of platelet activation upon vessel injury. The initiation phase is characterized by the adhesion of activated platelets at the damaged endothelium via GPIb/V/IX and GPVI. Subsequently, activated platelets release soluble mediators that mediate further platelet recruitment and activation. The final step is the stabilization of the aggregation with the support of the coagulation cascade [20].

Extension phase

Once platelets have formed the monolayer, they locally release soluble agonists essential for further platelets recruitment and activation. These molecules are mainly thrombin, ADP and thromboxane A_2 (TXA_2) and activate platelets through their respective G-protein coupled receptors (GPCRs) located on the membrane. Upon activation, the intracellular

signaling leads to PLCβ activation, that, in turn, generates second messengers by hydrolyzing phosphatidylinositol-4,5-biphosphate (PIP_2) on the membrane to inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylgylcerol (DAG) (Fig. 5).

IP_3 acts on its receptor on the DTS and leads to calcium release into the cytosol [17].

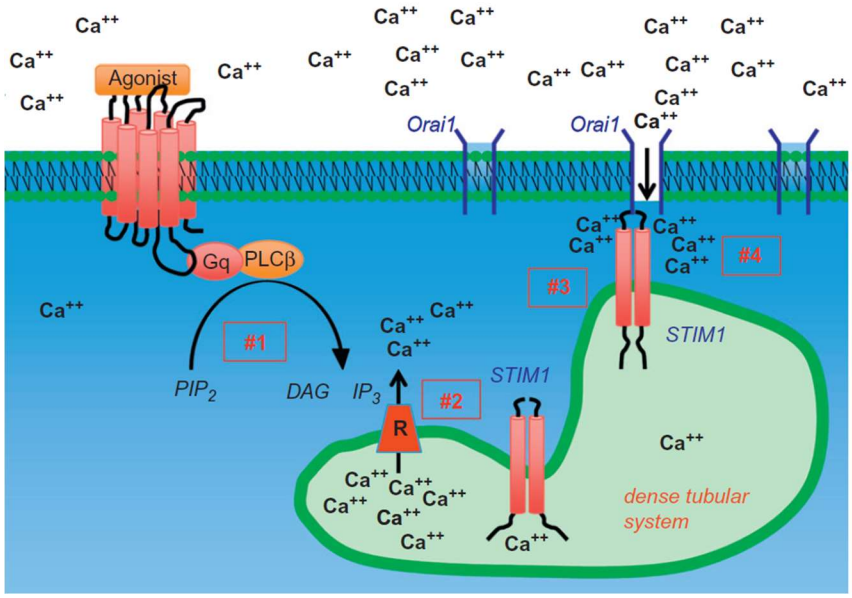

Figure 5| Calcium fluxes in platelets. Upon agonist encounter, GPCRs activate PLCβ that hydrolyzes PIP_2 in IP_3 and DAG (#1). IP_3 binds to its receptor on the dense tubular system and this interaction triggers the release of Ca^{2+}, leading to an increase in the cytosolic Ca^{2+} concentration (#2). Due to the emptying of the dense tubular system, the dense tubular system membrane protein STIM1 changes its conformation and interacts with the calcium channel Orai1 on the plasma membrane (#3), inducing its opening and consequently Ca^{2+} influx (#4) (adapted from [18]).

Changes in cytosolic calcium concentration are critical in platelets activation, in processes like shape change, granule release and aggregation. The first wave of calcium increase is totally dependent on IP_3 signaling and DST emptying, while the second one is mediated by STIM1, a protein in DST that signal calcium depletion, that binds to Orai1 on the membrane. This interaction leads to calcium influx from the extracellular environment (Fig. 5) [21].

On the other hand, DAG is necessary to activate different isoforms of protein kinases C (PKC), that phosphorylate multiple proteins on serine and threonine residues, in order to contribute to stabilize platelets aggregation and activation (Fig. 6) [22].

The increase in cytosolic calcium is also fundamental in integrin $\alpha_{2b}\beta_3$ activation, a necessary step for platelets aggregates formation. Calcium is also needed for granules release that contribute to exposure of several receptors and molecules that enable platelets to reach a fully activated conformation (Fig. 6). For example, P-selectin is relocated on the plasma membrane for interaction with leukocytes and to ensure stability of the aggregates [23]. Moreover, phosphatidyl serine (PS) is flipped from the inner to the outer layer of the membrane to accelerate coagulation with the production of thrombin by serving as a platform for the activity of the prothrombinase complex, composed of coagulation factor Xa and its cofactor factor Va [24].

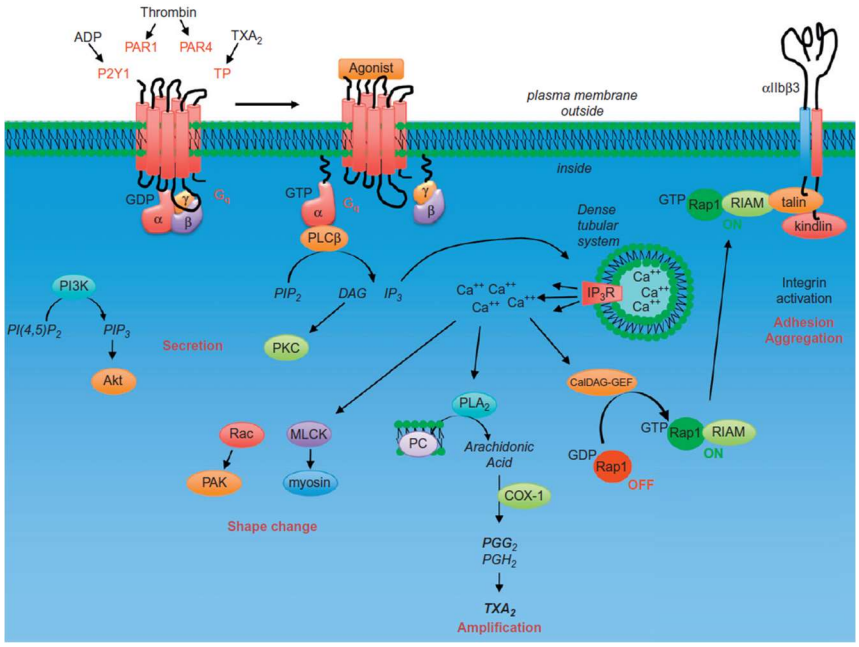

Figure 6| GPCRs signaling in platelet activation. Schematic representation of the activated pathways downstream the GPCRs, necessary to a fully platelet activation. The G protein coupled to the receptor is necessary to activate PLCβ, that generates DAG that in turn stimulate PKC activation. Increase in cytosolic Ca^{2+} concentration leads to activation of different pathways that induce shape change, generation of soluble mediators, such as thromboxane A_2, and integrin $\alpha_{2b}\beta_3$ activation (adapted from [18]).

Stabilization phase

This phase is necessary to strengthen platelets contact and prevent premature disaggregation. This occurs via amplification of intracellular signaling, outside-in signaling downstream integrins and contact-dependent signaling downstream receptors that bind molecules on adjacent platelets surface. In this phase, platelets come in thigh contact sufficiently to allow paracrine action of released molecules and transfer of information, like in neurological and immunological synapses [17].

Once integrin $\alpha_{2b}\beta_3$ binds fibrinogen, it triggers a signaling cascade which leads to cytoskeletal reorganization, stabilization of the aggregates and clot retraction, putting platelets in even closer contact [25].

Other molecules involved in this final stage of platelet activation are junctional adhesion molecules JAM-A and JAM-C, to support cohesive and signaling interactions between platelets [26], CD40 ligand, to interact with other platelets or leukocytes by CD40 [27], Eph receptor for ephrins, to enhance platelet adhesion to fibrinogen [28], and semaphorin 4D and Gas-6, involved in regulation of thrombus formation [29], [30].

Finally, there is the formation of a fibrin network that helps aggregation and thrombus stability. Fibrin is the result of the coagulation cascade that can be activated either by a tissue damage (*intrinsic pathway* of coagulation) or by tissue factor (*extrinsic pathway* of coagulation). Most of the tissue factor circulating in the blood is monocyte-derived and only in small part comes from the vessel walls. The coagulation cascade involves several coagulation factors and cofactors, that in the end activate thrombin enzyme and generate fibrin fibers (Fig. 7). Fibrin is captured within the growing thrombus by integrin $\alpha_{2b}\beta_3$ and P-selectin expressed on activated platelets surface, stabilizing contact-dependent interaction between platelets [17].

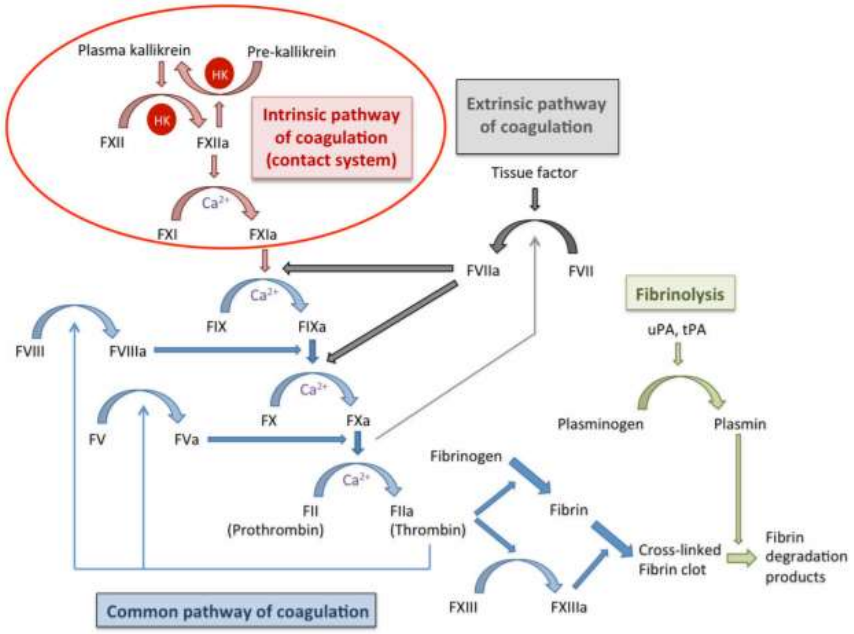

Figure 7| Coagulation cascade. The contact system is the first step of activation of the intrinsic pathway of coagulation and consists of three serine proteinases: coagulation factors XII (FXII) and XI (FXI), and plasma pre-kallikrein, with the nonenzymatic cofactor high molecular weight kininogen (HK). Instead, the extrinsic pathway of coagulation is dependent on tissue factor activation. Subsequent, both the intrinsic and extrinsic pathways activate a cascade of cofactors and clotting factors, that are circulating in the blood as zymogens. The final step of the coagulation cascade is fibrin generation and cross-linking [31].

1.2.1 G-protein coupled receptors: PARs and ADP receptor

Not all the stimuli are equally strong in stimulating platelet response. For example, thrombin induces the largest and fastest increase in cytosolic calcium, on the contrary ADP is weak agonist, it can induce only shape change and reversible aggregation and is dependent on the release of

TXA_2 to obtain a full response. This difference is in part dependent on the guanine nucleotide-binding protein (G protein) coupled to their receptors. Both thrombin and ADP receptors are G-protein coupled receptors, they consist of seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus, that is associated with the G proteins. The G proteins are heterotrimers, composed of α, β and γ subunits. The α subunit is the one that contains the guanine nucleotide binding site, normally occupied by a guanosine 5'-diphosphate (GDP). Once the receptor is activated, GDP is replaced with guanosine 5'-triphosphate (GTP) and causes a conformation change of the α subunit, that is now able to separate itself from βϒ subunits and to interact with different downstream effectors. Hydrolysis of GTP by the α subunit restores the initial conformation [32]. Moreover, the internalization and desensitization of the receptors contribute to modulate the GPCR signaling.

The α subunits can be divided into three different classes Gqα, Giα, G12α and G13α, everyone able to induce a different downstream pathway. Gqα via PLCβ causes increase in cytosolic calcium and subsequent PKC activation. G12α and G13α drive the reorganization of the actin cytoskeleton, inducing filopodia and lamellopodia formation and platelet shape change. Giα suppresses synthesis of the best-known platelet inhibitor cyclic adenosine monophosphate (cAMP) by inhibiting adenylyl cyclase. When cAMP accumulates in presence of nitric oxide (NO) and

prostaglandin I_2 (PGI_2) derived from endothelial cells, Giα inhibits adenylyl cyclase and intracellular concentration of cAMP decreases. Giα, in association with Gβϒ, can also activate other signaling pathways such as phosphoinositide 3-kinase (PI3K)/Akt, Src kinases and Rap1B Ras family, that are important in activation of integrin $\alpha_{2b}\beta_3$ [18].

ADP binds two classes of purinergic GPCR, $P2Y_1$ and $P2Y_{12}$, respectively associated with Gqα, that induce shape change and reversible aggregation, and Giα, and stimulation of both is necessary to induce optimal aggregation response [33]. ADP has an important role in platelets activation because it can be secreted from dense granules to stabilize aggregates and amplify platelets response induced by other agonists [34].

Thrombin is a serine protease, the main effector protease of the coagulation cascade and a strong agonist for platelet response. Thrombin signaling is mediated by a family of protease-activated receptors (PARs), that have the peculiarity to carry their own ligand. The extracellular N-terminus domain (exodomain) is cleaved in a specific site by thrombin, unmasking a new N-terminus domain that serves as a ligand able to bind intramolecularly the body of the receptor and to induce intracellular signaling [35] (Fig. 8).

Four PARs are known in mouse and human. PAR1, 3 and 4 can be activated by thrombin, while PAR2 is activated by trypsin and tryptase or by coagulation factors VIIa and Xa, but not by thrombin [36]. Human platelets express PAR1 and PAR4 and both mediate fully platelet activation. PAR1

can be activated also at low concentration of thrombin, while PAR4 only at high concentration. Moreover, they are activated and turned off with different speed, PAR4 is activated and shut off more slowly than PAR1, but the activity of both receptors is needed to achieve the maximum response in terms of calcium signaling [37].

Murine platelets express PAR3 and PAR4. Like in human platelets, PAR4 is activated at high concentration of thrombin, while PAR3 is necessary for activation at low concentrations of thrombin. Furthermore, PAR3 does not induce by itself a transmembrane signaling, but instead functions as cofactor for cleavage of PAR4 receptor at low concentration of thrombin [35].

PARs are coupled to Gqα, Giα and G12/13α and are able to activate all range of responses, such as shape change, secretion, TXA_2 generation, calcium mobilization and aggregation [17].

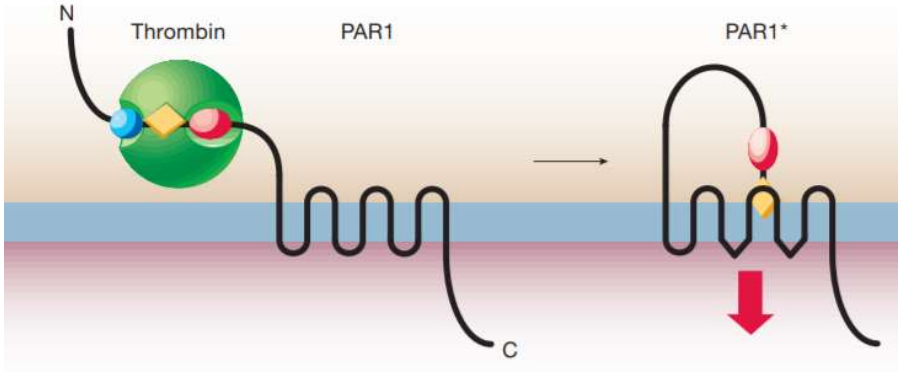

Figure 8| Protease-activated receptor activation. Schematic representation of PAR1 and its mechanism of activation via thrombin cleavage. Thrombin action unmasks a new N-terminus domain that is the ligand of the receptor [35].

1.2.2 Integrin $\alpha_{2b}\beta_3$: inside-out and outside-in signaling

The increase in cytosolic calcium is fundamental in integrin $\alpha_{2b}\beta_3$ activation, a pathway called *inside-out* signaling. Calcium increase activates Ca^{2+}-dependent exchange factor CalDAG-GEF, that, in turn, along with PKC, switches GDP with GTP on the GTP-binding protein Rap1, activating it. Rap1 is then able to bind the adaptor protein RIAM, which interacts with the adaptor proteins bound to the cytoplasmic domain of the integrin α_{2b} and β_3, respectively, talin and kindlin. This signaling cascade causes a conformational change in the integrin extracellular domain from a low-affinity conformation to a high affinity conformation that allows the binding of its ligands [38] (Fig. 9). Activated integrin $\alpha_{2b}\beta_3$ can bind fibrinogen, fibrin and vWF, making tight bridges between platelets within the aggregates.

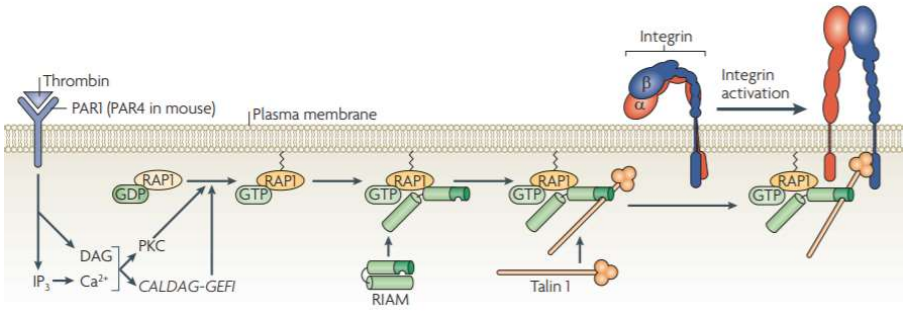

Figure 9| Inside-out signaling. Upon thrombin stimulation, a signaling cascade leads to conformational change of the integrin $\alpha_{2b}\beta_3$ extracellular domain. This cascade is called inside-out signaling and involves exchange factor CalDAG-GEF, small GTPase Rap1 and adaptor proteins RIAM and talin [38].

Once bound to its ligands, integrin $\alpha_{2b}\beta_3$, in turn, induces an intracellular cascade called *outside-in* signaling, that is necessary to sustain platelet activation, full platelet spreading, irreversible aggregation and clot retraction. The first step of the outside-in signaling requires the binding of a Gα13 subunit to the β_3 cytoplasmic tail. This interaction leads to c-Src family kinases and Syk activation, that phosphorylate critical residues on the intracellular tail, necessary to propagate the downstream signaling through the formation of a large protein signaling complex that includes proteins such as focus adhesion kinase (FAK), talin, myosin, and PKCα (Fig. 10).

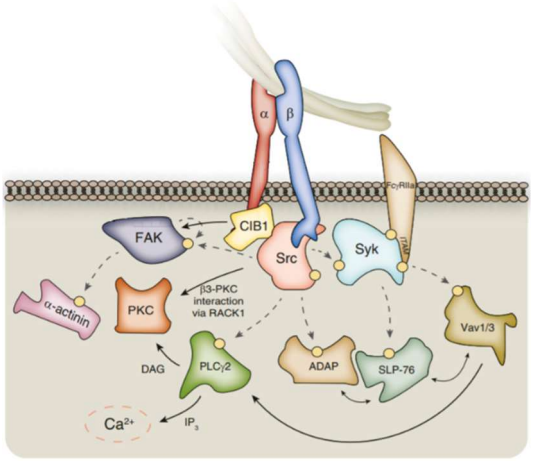

Figure 10| Activation of the outside-in integrin signaling. When integrin $\alpha_{2b}\beta_3$ binds the fibrinogen, the first step of the outside-in signaling cascade is the activation of c-Src family kinases and Syc, that phosphorylate several enzymes and adaptor proteins. This allows the formation of a large protein signaling complex that includes FAK, PLCY2, PKC and ADAP. FAK activation can be supported by CIB-1 bound to the α_{2b} C-terminal tail, and FAK substrates include the actin-binding protein α-actinin. Syk phosphorylates SLP-79 and Vav-family RhoGEFs, which propagate the outside-in signaling. Dashed lines represent phosphorylation events, marked on proteins by yellow circles [39].

Formation of this complex allows activation of the PI3K/Akt pathway, PKC, PLCϒ2, Rac1 and Rap1b, as well as inhibition of RhoA [40]. All these events are important to amplify granule secretion and thrombus growth.

PI3K activates Akt, needed to enhance granule release, maintains high level of PIP_2 and sustains association of myosin heavy chain (MHC) to the cytoskeleton, important for integrin clustering and irreversible aggregation [41]. Rac1 regulates the activation of mitogen-activated protein kinases (MAPK), p38 and ERK, that, in turn, phosphorylate myosin light chain (MLC) to sustain clot retraction [42]. Rap1b activation and, in the late stage, relief of Rho inhibition contribute in clot retraction and full cell spreading [43], [44].

Finally, the phosphorylation of the cytoplasmic tail of integrin leads also to dissociation of talin and subsequent binding of negative regulator proteins not able to signal, turning off the cascade, for example docking protein 1 (DOK1) and integrin cytoplasmic domain-associated protein 1 (ICAP1). Moreover, calcium-activated calpain-1 and -2 proteases negatively regulate integrin signaling via talin and β_3 tail cleavage (Fig. 11) [45].

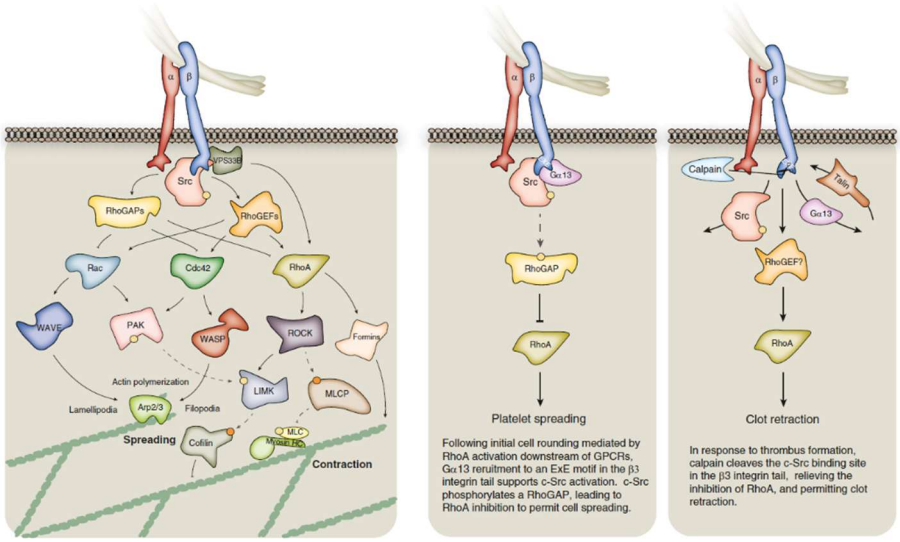

Figure 11| Outside-in integrin signaling. The Rho-family small GTPases Rac, Cdc42 and RhoA are important elements in the outside-in integrin signaling, regulated by GTPase-activating proteins (GAPs) and GTPase exchange factors (GEFs). Cdc42 and Rac can promote Arp2/3-mediated actin polymerization via WAVE and WASP proteins, respectively. RhoA promotes MLC phosphorylation via ROCK-mediated inhibition of MLC phosphatase (MLCP). Actin dynamics is also regulated via other proteins, such as cofilin and formins (left panel). Platelet spreading and clot retraction are tightly regulated by RhoA activity, as shown in central and right panels. Dashed lines represent phosphorylation events, yellow circles activating phosphorylations and orange circles inhibitory phosphorylations [39].

1.3 Platelets and immunity

Since their first discovery, platelets have always been studied for their role in hemostasis and thrombosis, and all the mechanisms related to these functions. However, in the latest decades, emerging evidence suggests that platelets need to be considered also as an integral part of the immune system. Indeed, they can directly recognize pathogens through specific

surface receptors, release antimicrobial peptides and immunomodulatory molecules, and interact with immune cells, in order to modulate the immune response [46]. Due to their unique and diverse capacities, they represent a bridge between thrombotic and inflammatory pathways, revealing new and specific roles for platelets in systemic inflammation, immune process and diseases.

1.3.1 Platelets as immune cells

Due to their physiological function in maintenance of vessel wall integrity, platelets are involved in the earliest step of detection of potential invading pathogens and they have a role in directly and indirectly mediate the immune response.

Platelets express a wide range of receptors on their surface, some of them are related to their role in maintaining the vascular integrity, while others are essential in pathogens recognition. Immune cells are able to recognize and bind Pathogens Associated Molecular Patterns (PAMP) expressed by invading pathogens through specific receptors called Pattern Recognition Receptors (PRR). Some of these receptors are also expressed by platelets, making platelets able to exert an immune surveillance function (Fig. 12) [47]. Among all the PRRs, it has been demonstrated that platelets express, constitutively or inducibly, several functional Toll-like receptors (TLRs). Downstream of these receptors there is the activation of a signaling cascade that includes MYD88 (myeloid differentiation primary response

protein), IRAK (interleukin-1 receptor-associated kinase), TRAF6 (tumor necrosis factor (TNF)-associated factor 6) and AKT signaling, as it occurs in immune cells [48], [49].

Both human and murine platelets express TLR4, through which they can bind lipopolysaccharide (LPS), a component of Gram-negative bacteria's outer membrane. LPS-activated platelets release soluble molecules that enhance platelet aggregation, like thromboxane A2 [49], and that contribute to innate immune cells recruitment and activation, like vWF and PF4 [50]. Platelets also constitutively express TLR2, TLR7 and TLR9, and they can upregulate TLR1 and TLR6 upon stimulation [47], [51].

Moreover, platelets can release molecules with antibacterial properties, platelet microbicidal proteins (PMPs), and cytokines, and are able to modulate the pattern of release based on the type of bacterial ligand encountered (Fig. 12) [52]. Like neutrophils, platelets contain and release granules with different types of microbicidal proteins, including kinocidins (like CXCL4 and CXCL7), defensins, thymosin β4 and fibrinopeptide A and B [53].

In addition, platelets express receptors for effector molecules that are found at the site of invasion, such as immunoglobulins (IgG, IgA and IgE), C-reactive protein and thrombospondin [54]. This aspect confirms their important role at the forefront of the infection and suggests that their immune activity is not a residual capacity from an ancient and different system.

Moreover, they can also generate and release reactive oxygen species (ROS) that play an important role as antimicrobial factors, although the amount is very low compared to neutrophilic ROS production [49].

Platelets contribute to the immune response also by releasing both pro-inflammatory cytokines, such as interleukin-1β (IL1β) and TNFα [55], and anti-inflammatory cytokines, i.e. the transforming growth factor-β (TGF-β) [56].

It has also been proposed that platelets could have a rudimental phagocytic activity against bacteria, but this aspect has not been well explored yet and requires more investigation [57]. It has also been suggested that platelets could exert their immune function by direct coating of pathogens that in turns facilitates phagocytosis by other immune cells, acting like “covercytes” rather than phagocytes [58].

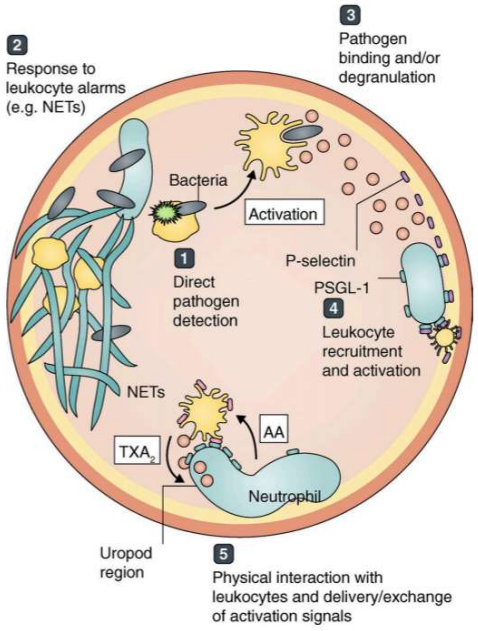

Figure 12| Platelet as immune cells. Platelets exert their immune functions in different ways as shown by this schematic representation (adapted from [59]).

1.3.2 Platelets as immune-modulatory cells

Beyond their capacity to directly fight invading pathogens, platelets have developed an important role in modulating the immune response, by recruiting and promoting both innate and adaptive immune cells activation. Platelets interact with immune cells not only by soluble mediators but also by a direct physical contact. Formation of platelet-leukocyte heteroaggregates has long been known, but their profound biological importance is only recently started to emerge.

P-selectin, a vascular adhesion molecule, also known as CD62P, is one of the major proteins involved in cell-cell interaction, it is moved to the platelet surface upon activation and mediates the binding to P-selectin glycoprotein ligand 1 (PSGL-1 or CD162) on leukocytes (Fig. 13). This interaction provides a first step for adhesion and is important in the context of thrombus formation [60], as well as in directing and facilitating cell-mediate host defenses [57]. For example, it has been demonstrated that P-selectin promotes innate immune response like the release of neutrophil extracellular traps (NETs) by neutrophils [61], but also regulate adaptive immune response like the inhibition of IL-17 production by regulatory T cells [62].

Platelets express and upregulate CD40 and CD40L, through which they bind the cognate receptors expressed by leukocytes and endothelial cells (Fig. 13). This axis has been shown to be important in increasing the cytokine production by monocyte-derived dendritic cells, including IFN_{Υ}, IL-12 and IL-4, and the upregulation of co-stimulatory markers CD80/CD86, resulting in a better induction of T cells response [63]. Moreover, CD40L-expressing platelets interacting with CD40 on endothelial cells results in the upregulation of adhesion molecules like intracellular adhesion molecule 1 (ICAM1) and vascular adhesion molecule 1 (VCAM1) and release of CC-chemokine ligand 2 (CCL2) by endothelial cells, facilitating leukocytes recruitment at the site of inflammation [64].

Other important platelet surface ligands are GPIb, also known as CD42, GPIIb-GPIIIa, also known as integrin $\alpha_{2b}\beta_3$, ICAM2, junctional adhesion molecules (JAM-A, JAM-C) and platelet endothelial cell-adhesion molecule-1 (PECAM-1) through which they establish a contact with immune and endothelial cells [65].

The direct physical interaction between platelets and leukocytes has been shown to be essential also in recruiting and addressing the adaptive immune response in a mouse model of hepatitis B virus infection, where it has been shown that platelets are fundamental in directing CD8 T cytotoxic cells activity against infected hepatic cells [66].

Most of the previous mentioned surface molecules can also be shed and released. This mechanism allows platelets to influence the immune response at a systemic level and not only at the local one. For example, platelets are the principal source of soluble CD40L (sCD40L) in the plasma, and its level can be a marker in different pathological and inflammatory conditions [67], [68].

In addition, in order to prevent pathogens spreading, platelets can modulate the recruitment and trafficking of leukocytes at the site of injury, also by releasing chemokines and microparticle containing attracting molecules. CXCL7 released by platelets is a strong chemoattractant for neutrophils, that bind it via CXCR2, inducing their trans-endothelial migration in the early phase of inflammation [69]. Analogously, CXCL4, also known as platelet factor 4 (PF4), and RANTES

have been shown to be important in leukocytes recruitment in a model of acute lung injury [70]. In addition, PF4 is able to protect monocytes from apoptosis and induce macrophage differentiation [71]. Conversely, the platelet basic protein (PBP) is able to desensitize neutrophils chemotaxis and degranulation, downmodulating neutrophils response [72].

The ability of platelets to modulate the immune response is not unidirectional: leukocyte themselves can influence platelet activation, suggesting a complex relationship between hemostasis and inflammation, rather than an incidental matter. NETs released by neutrophils can induce activation and aggregation of platelets, although it is still debate which element of the NET is responsible for the activation or if the stimulation is due to its web-like structure [73], [74]. Moreover, IL-17A has been shown to promote P-selectin expression on platelets and to enhance ADP-induced aggregation [75].

These physical interactions can culminate in a direct exchange of materials between platelets and leukocytes. For example, neutrophils can transfer arachidonic acid to adherent activated platelets by neutrophil-derived extracellular vesicles. Platelets internalize these vesicles via a Mac-1-dependent mechanism, translocate them to compartments enriched in cyclooxygenase enzyme (COX), leading to the production and subsequent release of thromboxane A_2, that, in the end, results in a full neutrophil response [76].

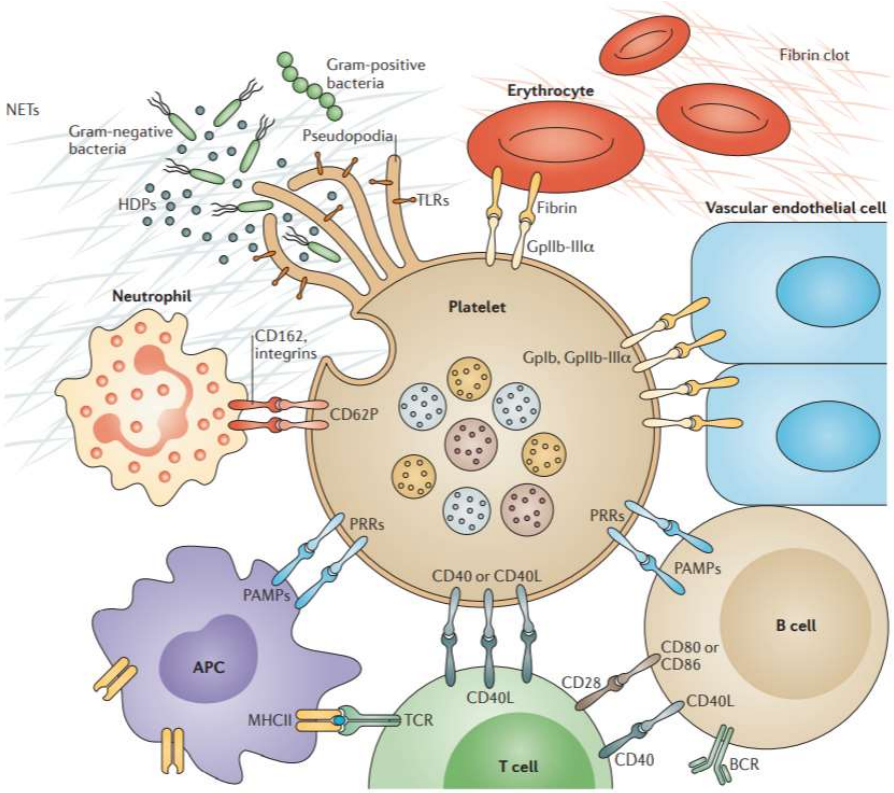

Figure 13| Platelets-leukocytes interaction. Platelets not only can direct recognize invading pathogens, but they can also modulate the immune response by recruiting and interact with immune cells, erythrocytes and endothelial cells. Physical interactions are mainly mediated by CD62P-CD162, CD40-CD40L, GPIb and GPIIb-IIIa [57].

1.4 Platelets-neutrophils interaction

Neutrophils share with platelets their origin within the bone marrow. Neutrophils differentiate from the common myeloid progenitor (CMP), as MKs, and are then release in the bloodstream as mature and fully differentiated cells, characterized by a short lifespan. They are the most

abundant innate immune effector cells and the first immune cell type to be recruited at the site of infection [77].

Platelet-neutrophil interaction is one of the most studied interaction between platelets and leukocytes. Recently it has been shown that platelet-neutrophil complexes (PNCs) can contribute to the etiology of different thrombotic and infectious pathologies [78].

Around 25% of neutrophils circulating in the bloodstream are bound by platelets at the steady state, forming instable complexes that do not compromise the regular blood flowing. Instead, when platelets get activated and these interactions become stable and grow in number, PNCs become a hallmark in a wide range of inflammatory conditions and thrombotic disorders, including sepsis, inflammatory bowel disease and bacterial infection, as well as atherosclerosis and coronary syndromes [78]. The formation of these complexes has been shown to be a reliable marker *in vivo* for platelet activation, even more sensitive than looking directly at P-selectin expression on platelets surface [79].

When damage at the vessel wall occurs, erythrocytes shift their flowing from the central axis of the vessel to the periphery and this causes margination of both platelets and neutrophils facilitating their contact and thus the formation of the PNCs [80]. To form stable complexes, platelets need to be activated, either by soluble factors or directly by activated endothelial cells or injured vessel walls. This activation results in the upregulation of various surface receptors that allow platelets to aggregate

and to deposit on vascular endothelium, with a higher efficiency in condition of high shear rate. This early deposition of platelets then facilitates neutrophils recruitment and adhesion, bridging them with the endothelium [81]. In particular, it has been demonstrated that neutrophils adhere to a specific structure formed by platelets in *in vivo* and *in vitro* condition called flow-induced protrusions (FLIPRs). These are long membrane protrusions that extend from adherent platelets in presence of flow, rich in P-selectin molecules that can be easily bound by PSGL-1 expressed on neutrophils [82].

On the other hand, neutrophils recruited at the injured vessels reorganize their surface receptors in order to rapidly find activated platelets to interact with. Neutrophils extend a domain into the lumen of the vessel where PSGL-1 clusters scan for the presence of activated platelets. Once stable interactions are formed, neutrophils further reorganize the distribution of all the other receptors needed for intravascular migration, NETs generation and inflammation, i.e. Mac-1 and CXCR2 [83].

Platelets interact with neutrophils also by expressing GPIbα, which bind to neutrophil Mac-1, and by release of soluble mediators like CCL5 and PF4. On the other hand, neutrophils release cathepsin G and elastase that further stimulate platelet response by activating PAR receptors on platelets (Fig. 14) [84].

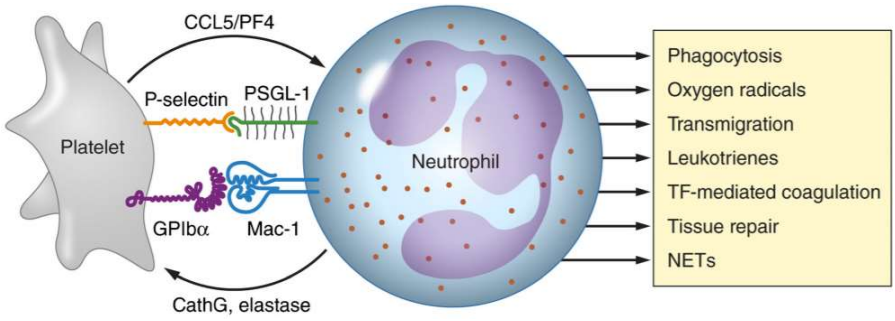

Figure 14| Platelet-neutrophil interaction. Platelets and neutrophils interact by direct binding via surface molecules, such as P-selectin/PSGL-1 and GPIbα/Mac-1, as well as by releasing soluble mediators, like CCL5/PF4 by platelets and cathepsin G (CathG) and elastase by neutrophils. Platelets stimulate neutrophil response, such as phagocytosis, ROS production and NETs release [84].

1.4.1 Platelets influence neutrophils response

Neutrophils are loaded with a wide variety of granules, rich in antimicrobial peptides and proteolytic enzymes, which can mediate killing of invading pathogens. They are able to obstruct microbial invasion also by a combination of phagocytosis, production and release of a large amount of ROS and by the formation of NETs [85]. Platelet-neutrophil interaction results in enhancing all these neutrophilic activities, guaranteeing a full inflammatory response.

Neutrophil interaction with platelets enhances their ability to phagocytose various bacteria *in vitro* [86], while in *in vivo* models of bacterial infection thrombocytopenia results in increase of the bacterial load in the bloodstream [87].

Moreover, platelets can modulate ROS production enhancing the release of ROS or downregulating the oxidative burst depending on the different conditions [84].

1.4.2 Platelet-induced NETosis

NETs are web-like structures composed of chromatin filaments covered with histones, proteases and granular and cytosolic proteins. In particular, they are characterized by the presence of citrullinate histone H3, neutrophil elastase (NE) and myeloperoxidase (MPO) [77]. The majority of DNA in NETs originates from the nucleus, although it has been shown that it can also contain mitochondrial DNA [88].

NETs production and release, also known as NETosis, is a specific feature of neutrophil activation, that enable neutrophils to trap and kill bacteria, preventing their spreading within the host tissues. However, emerging evidences suggest that this mechanism can also occurs in a context of noninfectious sterile inflammation. Depending on the stimulus, NETosis can results in cell death, with a process that takes up to 3-8 hours, but can also occur in non-lytic condition, leading to rapid NETs release within minutes [77].

NADPH-oxidase production of ROS is an early and fundamental step in NET generation. ROS activates protein-arginine deiminase 4 (PAD4), that convert arginine to citrulline on histones, and induces chromatin decondensation in the nucleus. NE and MPO are released from a specific

type of neutrophilic granules, called azurophilic granules, into the cytosol to be translocated into the nucleus, where they promote additional chromatin unfolding. After the nuclear membrane disruption, chromatin is release into the cytosol, where it is decorated with a variety of granular and cytosolic proteins [89]. The exact mechanism of NETs terminal release into the extracellular space still needs to be defined, but the most plausible model is based on the disruption of the plasma membrane [90]. Finally, during infections, NETs last for several days and are thought to be disassembled by the secreted plasma nuclease DNase I [91].

NETosis is a process that needs to be tightly regulated to prevent improper damage and pathological onset. It is thought that the size of the microorganism is one of the several factors that influence NETs release, due to a competition between NETs and phagocytosis in the access to NE. When the pathogen is too large to be phagocytosed, neutrophils preferentially release NET, whereas, when the microorganism is small, it is engulfed into phagosomes, that fuse with azurophilic granules, sequestering NE away from the nucleus, preventing chromatin decondensation [77].

It has been demonstrated that LPS-activated platelets can induce NETs release and this process is beneficial in trapping bacteria in septic blood. Platelet TLR4 appears to be a major regulator involved in platelet-mediated NETosis in a mouse model of endotoxemia [92]. The mechanism underlying this process is not well understood yet; it is not clear if LPS

bound to platelets is also bound by TLR4 on neutrophils, resembling a mechanism of presentation of the stimulus, or if it necessary to trigger platelets response and the release of soluble mediators that, in the end, lead to NET release. A similar mechanism seems to be involved also in a *in vivo* model of LPS-mediated acute lung injury, where platelets are key element in NETs induction and subsequent lung damage [93].

Platelet-induced neutrophils NETs formation requires interaction by P-selectin/PSGL-1 [61] and GPIbα/Mac-1 (also known as CD18) [50], while the contribution of platelet integrin $\alpha_{2b}\beta_3$ seems to be more important in *in vivo* mouse model of lung injury [94] than in the context of human platelets (Fig. 15) [95].

As previously mentioned, also soluble factors are important to integrate the functional interaction between platelets and neutrophils. Chemokines PF4 and CCL5 are necessary not only for neutrophils recruitment but also for inducing NETs release [53]. Moreover, it has been demonstrated that high mobility group box 1 (HMGB1), a damage-associated molecular patter molecule, is a key mediator of NET induction by platelets (Fig. 15). Platelets can release it or present it, inducing autophagy in neutrophils and, in turn, promoting NETosis and inhibiting apoptosis [95]. This mechanism has also been recently observed in systemic sclerosis patients, that show an enrichment in HMGB1-loaded platelets microparticles and NETs in the blood [96].

Furthermore, von Willebrand Factor (vWF) is not only implicated in platelets adhesion to the exposed subendothelium. It has been also observed that vWF is a critical component of NETs, and that citrullinate histone H3 colocalized with this factor [97].

Finally, TXA_2 has been recently included in the list of soluble molecules that contribute to NETs induction. It is a strong platelet activator and it induces strong granules release by platelets that, in the end, results in activation of neutrophils and NETs formation [50] (Fig. 15).

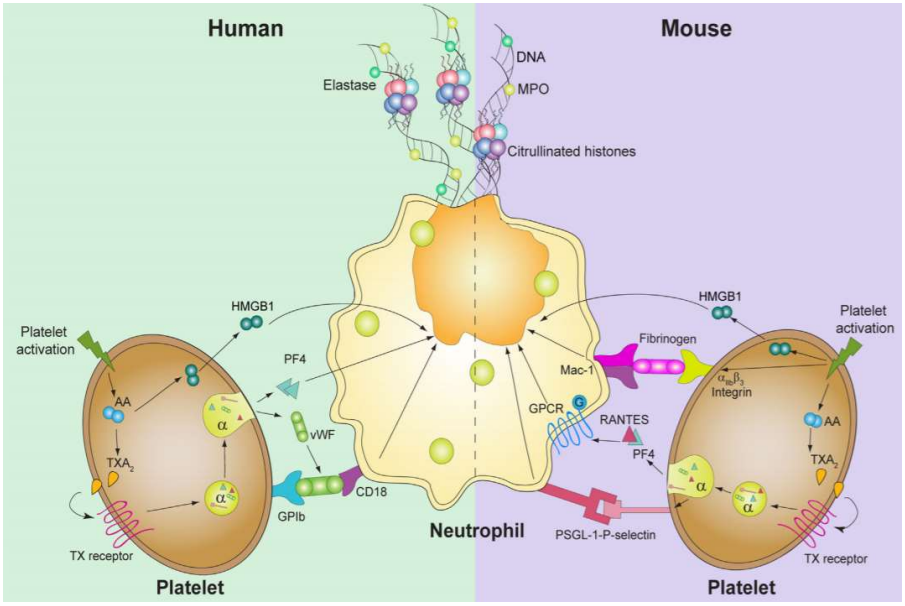

Figure 15| Platelet-induced NETs in human and murine neutrophils. Induction of NETs release by activated platelets is based on slightly different mechanism in human (left) and murine (right) cells. Both direct and indirect interactions are needed to fully induce NETs release by neutrophils [85].

1.4.3 NETs in sterile and infectious inflammations

NETs are critical against fungal and bacterial infections, and it has been shown that they can have also a role in viral response [98]. However, NETs induction can also occur in sterile inflammation condition. Independently of the original cause, when NETs formation is not well controlled, it can become detrimental and affect host survival.

For example, in a mouse model of transfusion-related acute lung injury (TRALI) it has been demonstrated that activated platelets exacerbate NETs release in the lungs, causing endothelial damage. In turn, NETs web structure facilitates platelets accumulation and thrombus formation in the lung microcirculation, increasing lung injury [94].

Another detrimental feature of NETs is occlusion of the vasculature, that promotes deep vein thrombosis (DVT). NETs DNA filamentous structure promotes platelet adhesion, activation and aggregation, enhancing their procoagulant activity, facilitating thrombus formation and growth. Moreover, histones present on NETs are able to induce the release of platelets mediators that, in turn, induce NETosis, creating a positive feedback mechanism [97]. It is thought that NET formation during thrombosis is mainly initiated by hypoxia-induced release of vWF and P-selectin from the endothelium that recruits and activates neutrophils, as well as platelets [99].

Platelets-induced NETs can have a detrimental role also in the context of bacterial sepsis. Sepsis is defined as life-threatening organ dysfunction

caused by a dysregulated host response to infection and characterized by excessive inflammation and immune suppression. The number of cases in the US exceeds 750000 per year and it is estimated that severe sepsis is recorded in 2% of patients admitted to the hospital [100]. Sepsis is a complex syndrome that can be driven by a variety of pathophysiological pathways and so far it has been difficult to develop effective new therapies, leaving mortality still close to 20-30% [101]. Exaggerated platelets activation has been connected to organ injury during sepsis through different mechanisms, including excessive recruitment of leukocytes and inflammation induction, and it has been shown that antiplatelet agents can have a beneficial effect in animal model of sepsis [102]. During endotoxemia, platelets-induced NETs is fundamental in ensnaring bacteria and preventing their spreading into the bloodstream [103], however in a mouse model of bacterial sepsis NETs facilitate platelets aggregation, thrombin activation and fibrin clot formation in the liver vasculature, leading to liver damage and organ failure [104]. Indeed, it is known that severe sepsis is often associated with thrombocytopenia and altered coagulation, caused by both improper activation of the coagulation cascade by tissue factor and impairment of anticoagulant mechanisms, leading to disseminated intravascular coagulation (DIC) [101]. DIC leads to impaired tissue oxygenation and has a major role in causing organs failure, together with hypotension and reduced red-cell deformability, that, in turn, results in patient death. Moreover, thrombus

formation further facilitates NETs release, establishing a detrimental positive feedback. Additionally, it has been observed that plasma from severely septic patients triggers the formation of NETs in the presence of platelets *in vitro* [92].

Another typical feature of sepsis is disruption of the integrity of the endothelial barrier. In response to the infection, both neutrophils and platelets adhere to the endothelium and migrate to the sites of infection, but, in septic condition, exacerbation of this process results in loss of barrier integrity, causing leakage of proteins and plasma in extravascular space, tissue edema and reduced microvascular perfusion [102].

1.5 Nuclear factor of activated T cell: NFAT

As previously described, calcium signaling is fundamental in platelet activation. In a similar manner, in nucleated cells increase in cytosolic calcium drives the activation of the nuclear factor of activated T cells (NFAT).

NFAT is a transcription factor (TF), first discovered in T cells as a DNA-binding factor that binds to the IL-2 (*Il2*) promoter [105]. Initially most research about this TF was conducted in adaptive immune context and it had been shown that NFAT regulates activation and differentiation not only in T cells but also in B cells [106]. Although NFAT is an evolutionarily recent element and, on the contrary, innate immunity is a very ancient form of response to pathogens, later in time it has been demonstrated

that NFAT also contributes to regulate innate immune response in vertebrates [107]. Indeed, it is expressed and functioning in several innate immune cells, such as dendritic cells (DCs), neutrophils, macrophages and mast cells, as well as megakaryocytes [107]. For example, in DCs NFAT is activated downstream of fungal stimuli, via dectin-1 receptor [99], and LPS bacterial ligand, via CD14, through activation of PLCϒ2 and has a role in modulation of cytokine production and apoptosis [109]. Moreover, in megakaryocytes NFAT regulates expression of CD40 ligand [110] and Fas ligand [111].

The NFAT family consists of 5 proteins that are evolutionary related to the REL-NF-κB transcription factor family: NFATc1 (also known as NFAT2 or NFATc), NFATc2 (also known as NFAT1 or NFATp), NFATc3 (also known as NFAT4), NFATc4 (also known as NFAT3 or NFATx) and NFAT5. Every member of the family can have different isoforms derived from different splicing variants [112].

The structure among the various members of the family is conserved: it contains an amino-terminal transactivation domain (TAD), a regulatory domain (also known as the NFAT homology region (NHR)), a highly conserved DNA-binding domain (also called Rel-homology domain (RHD)) and a carboxy-terminal domain (Fig. 16) [107]. The regulatory domain consists of multiple serine-rich regions (SRR1, SP1, SP2, SRR2, SP3 and KTS), that are phosphorylated by NFAT kinases, such as casein kinase 1 (CK1), glycogen synthase kinase 3 (GSK3) and dual-specificity tyrosine-

phosphorylation-regulated kinase (DYRK). Moreover, the regulatory domain contains a docking site for the phosphatase calcineurin (SPRIEIT), a key element in NFAT activation (Fig. 16) [107].

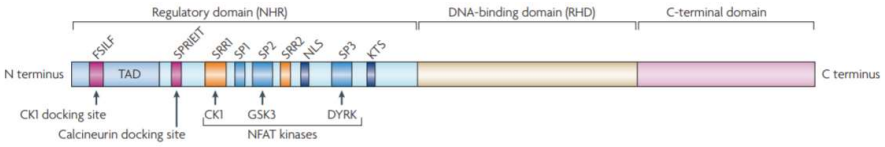

Figure 16| NFAT domains. NFAT is composed of a regulatory domain (NHR), a DNA-binding domain (RHD) and a C-terminal domain. Within the regulatory domain there are several phosphorylation sites that mediate the regulation of NFAT subcellular localization and activity [107].

1.5.1 Calcium-dependent NFAT activation pathway

NFAT5 is the most ancient of the NFAT family, present in both invertebrate and vertebrate, and activated by osmotic stress [113]. All the other members, NFATc1-NFATc4, are present only in vertebrate and are regulated by calcium fluxes, with a signaling that resembles platelet receptor activation pathways. NFATc at a resting state is located in the cytoplasm in a phosphorylated status. When a ligand binds to an immunoreceptor, a receptor tyrosine kinase or a GPCR, there is activation of PLCϒ and subsequent production of IP_3, that induces release of calcium from the endoplasmic reticulum and thereby increasing cytosolic calcium concentration. The calcium sensor protein calmodulin is activated and in turn activates diverse calmodulin-dependent enzymes, including the phosphatase calcineurin (Fig. 17) [106]. Calcineurin binds NFAT at a

specific docking site and dephosphorylates it on multiple phosphoserines in the regulatory domain, leading to the exposure of a nuclear localization signal (NLS) and subsequent NFAT translocation into the nucleus. Once in the nucleus, NFAT, in collaboration with other transcription partners, such as AP1, FOXP2, FOXP3 and GATA, initiates and maintains specific transcriptional programs that are different among different cell types and patterns of stimulation [107].

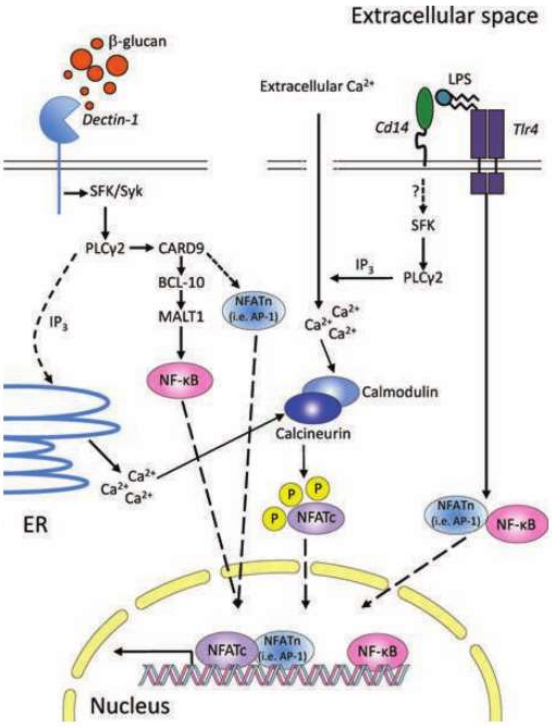

Figure 17| NFAT activation pathway. In DCs, NFAT is activated downstream Dectin-1 or CD14. These pathways lead to a PLCγ2-dependent increase in cytosolic calcium that activates calmodulin, that in turn induces calcineurin-dependent dephosphorylation of NFAT. Dephosphorylated NFAT translocates into the nucleus, where it regulates transcriptional responses with other transcription partners [114].

1.5.2 Regulators of NFAT activity

Phosphorylation status and localization of NFAT are important factors in modulating its transcriptional activity. Indeed, multiple elements are involved in NFAT regulation, not only kinases, but also scaffold proteins and long non-coding RNA.

The phosphorylation status is regulated by multiple kinases, like CK1, GSK3 and DYRK. In particular, these kinases are considered export kinases. They are important when NFAT is in the nucleus and it needs to be rephosphorylated, in order to be relocated into the cytoplasm. Moreover, maintenance kinases act in the cytoplasm to keep NFAT in a fully phosphorylated state and prevent inappropriate translocation [107]. GSK3 phosphorylates NFAT on SP2 and SP3 motifs but it needs a previous phosphorylation by a "priming" kinase that can be PKA or DYRK [115]. CK1 acts both as export kinase and maintenance kinase and phosphorylates the SRR1 motif [116]. Another kinase that negatively regulates NFAT activation, mainly in innate immune cells, is leucine-rich repeat kinase 2 (LRRK2). The existence of a *LRRK2* risk allele associated with Chron's disease which results in slightly less LRRK2 protein and enhanced NFAT activity [114] demonstrates that destabilization of NFAT regulatory circuits can underlie human diseases. Another example is given by *DYRK1A* gene. It resides in the portion of human chromosome 21 that is duplicated in Down's syndrome and this results in reduced NFAT activity and many features of the pathology [114].

Competition between NFAT and cytoplasmic scaffolding proteins for the access to calcineurin is another mechanism to control NFAT activity. It has been demonstrated that cytoplasmic scaffold proteins HOMER2 and HOMER3 can bind calcineurin at the same binding site of NFAT, thus prevent NFAT dephosphorylation [118]. Moreover, NFAT activity is modulated by a long non-coding RNA, a non-coding repressor of NFAT, called NRON, that was identified as a specific inhibitor of NFAT nuclear trafficking using a library of short hairpin RNAs (shRNAs) [119]. Additionally, phosphorylated NFAT interacts in the cytoplasm with a scaffold protein called IQGAP1, which serves as platform for a bigger complex. In fact, NFAT is not freely scattered in the cytoplasm, but is associated in an RNA-protein complex that includes all the above-mentioned regulatory elements, calmodulin, calcineurin, CK1, GSK3, DYRK, LRRK2, NRON and IQGAP, which promotes efficient localization of NFAT to its regulators in the cytoplasm and better controls NFAT activation (Fig. 18) [120].

Interestingly, some of the previously mentioned regulatory elements of NFAT are also present in platelets where they have an active role in regulating platelet activation. For example, GSK3β is a negative regulator of platelet function and thrombosis downstream GPCRs [118] and it acts with CK1 in the canonical Wnt-β-catenin pathway [122]. Moreover, IQGAP1 negatively regulates platelet procoagulant activity under conditions of mechanical shear stress [123].

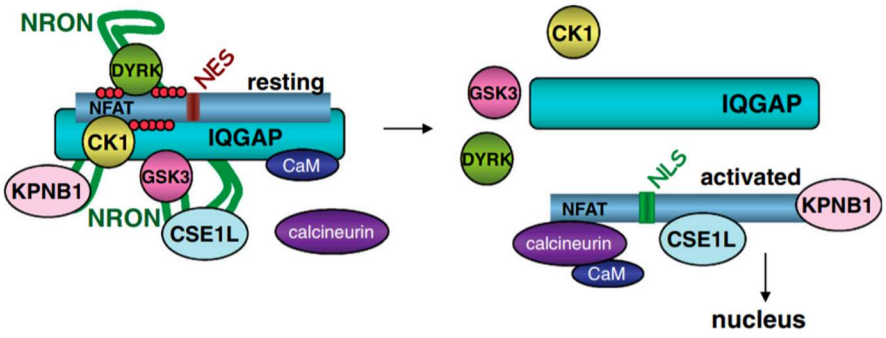

Figure 18| NFAT complex. In the cytoplasm phosphorylated inactive form of NFAT, characterized by the exposure of the nuclear export signal (NES), is associated in an RNA-protein complex that includes several of NFAT regulatory elements. This complex consists of calmodulin, the scaffold protein IQGAP, the long non-coding RNA NRON, kinases such as CK1, GSK3 and DYRK, and proteins involved in nuclear transport KPNB1 and CSE1L. Upon calcineurin activation, dephosphorylation of NFAT occurs, leading to the exposure of a nuclear localization signal (NLS) and NFAT translocation to the nucleus [120].

1.6 Transcription factors in platelets

Although platelets do not have a genomic DNA, they contain TFs. Until recently, these elements were thought to be vestigial remnants from megakaryocytes, but we now know that they have important, if only partially understood, functions.

The idea that platelets can contain TFs is a relatively new concept that has led to the discovery of a large number of TFs in platelets, including NF-κB, PPARY, STAT3 and others (Fig. 19) [124]. Numerous TFs have a non-genomic role, both in platelets as well as in nucleated cells. Moreover,

platelets can transfer TFs to vascular and immune cells through platelet microparticles, modulating the activity of cells nearby [125].

Transcription factor	Activation of transcription factor	Agonist-induced activation
p65	Phosphorylation	↑ Aggregation, spreading, clot retraction, GPIBα shedding
PPARγ	Ligand binding (15d-PGJ_2, TZDs)	↓ Aggregation, CD40L, TXB_2, P-selectin
	Phosphorylation	↑ Collagen-induced activation and granule secretion
PPARβ/δ	Ligand binding (GW501516, PGI_2)	↓ Aggregation
PPARα	Ligand binding (fenofibrate)	↓ Aggregation
	Phosphorylation	↑ Activation
LXRβ	Ligand binding	↓ Collagen-induced aggregation
RXRα	Ligand binding (9cRA)	↓ Activation
GR	Ligand binding (prednisolone)	↓ Activation
	Ligand binding (dexamethasone)	Unknown
AHR	Ligand binding	↑ Activation
STAT3	Phosphorylation	↑ Aggregation, P-selectin, thrombosis

Figure 19| Transcription factors found in platelets. List of all TFs discovered to be present in platelets. For each TF is listed type of activation and function in platelet activation upon agonist stimulation [124].

1.6.1 NF-κB signaling in platelets

NF-κB (nuclear factor kappa β) is a master TF in immunity and has an important role in regulating inflammation and immune response. It is a heterodimer composed of p50 and p65 subunits, tightly bound to inhibitory proteins of the IκB family. However, not many years ago, its presence and function in platelets have been described [126]. Indeed, NF-

κB is present in platelets and its inhibitors impair platelet activation, aggregation and spreading on fibrinogen. However, the precise mechanism of action is still unclear. Few years later, it was demonstrated that the majority of NF-κB family members are present in platelets, such as p50/p65 subunits, RelB and c-Rel, IκB proteins and IKK members, which regulate NF-κB activation [127].

In platelets NF-κB is activated downstream of the thrombin receptor PAR and it acts as a positive mediator of platelet response. Nevertheless, the complete signaling pathway that leads to NF-κB activation and its specific mechanism of action are still not known. In the latest years, several papers described this signaling pathway, using NF-κB inhibitors and knock-out mice [126]–[129].

It has been suggested that p38 signaling is upstream NF-κB, while ERK pathway is downstream. ERK is able to activate phospholipase A_2 (PLA_2), that in turn induce TXA2 synthesis to sustain platelet aggregation [128]. Additionally, human PAR4 stimulation leads to ceramide production by sphingomyelin phosphodiesterase (Smase), which in turn activated p38 and NF-κB, while PAR1 signaling is ceramide-independent (Fig. 20) [129].

A model has been suggested where, through PAR stimulation, the NF-κB regulators IKKβ is phosphorylated and activated. This in turns results in the phosphorylation and degradation of the NF-κB inhibitor IκBα. Degradation of IκBα leads to p65 release from the inhibitory complex, with induction of platelet aggregation and granule release (Fig. 20) [126].

GPIb shedding also seems to occur downstream of PAR stimulation with a mechanism dependent on the NF-κB pathway and on the sheddase ADAM17. ADAM17 is located on the cell surface where, following activation by proteolytic cleavage to its mature and active form, it cleaves various substrates, such as GPIb. IKKβ deficiency inhibits ADAM17 maturation, resulting in delayed shedding of platelet GPIb [130].

However, it has been also described a mechanism by which NF-κB appears to fine-tune platelet response by an inhibitory feedback signaling. After p65 dissociation, from the same complex PKA is released and it induces vasodilator-stimulated phosphoprotein (VASP) phosphorylation, which mediates platelet inhibitory signaling [131].

Moreover, it has been demonstrated that, during the second wave of platelet activation, IKKβ is important in driving synaptosomal-associated protein-23 (SNAP23) activation to modulate granule release. SNAP-23 is a regulator of granule secretion and member of the target soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (*t*-SNARE) complex. Activation by phosphorylation allows it to interact with vesicle-associated membrane protein (VAMP or v-SNARE) to facilitate granule release [132].

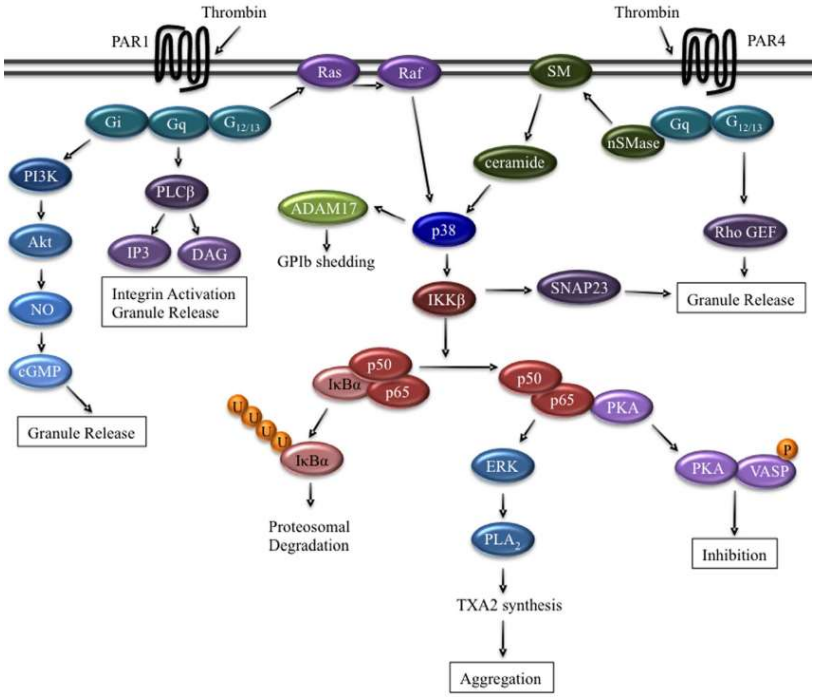

Figure 20| Activation pathway of NFκB. Downstream human and murine PAR stimulation, activation of NF-κB occurs through p38, that in turn activates IKKβ. IKKβ phosphorylates and induces degradation of IκBα, that releases NF-κB leading to platelet aggregation and modulation of platelet response via PKA [124].

NF-κB plays an important role in regulating platelet function and it has been shown that NF-κB can be phosphorylated in platelets as it occurs in nucleated cells. However, the effect of NF-κB direct inhibition or deletion has not been characterized yet, revealing a lack of knowledge in what its actual non-transcriptional role is.

A very interesting aspect of a non-nuclear role of p65 and IκBα is that they are found in the mitochondria of nucleated cells [133]. It has been

demonstrated that their regulation in this location is completely distinct from the one that occurs into the cytoplasm and they respond to stimuli different from the canonical NF-κB pathway [134]. In a similar way, another example of non-transcriptional role of a TF is given by signal transducer and activator of transcription 3 (STAT3), member of STAT protein family. STAT3 activity is stimulated in response to cytokines and growth factors and plays a key role in several cellular processes, such as proliferation, apoptosis and differentiation, as well as modulation of immune responses [127]. As shown for p65 and IκBα, a small portion of STAT3 pool is found in mitochondria. In this location, it has a function in modulating cellular respiration. In particular, STAT3 is a regulator of the complex I and II of the electron transport chain (ETC) and it influences ATP and ROS mitochondrial production [136].

1.7 Platelet dysfunction in human pathology

Platelet activity is essential to maintain vascular integrity and it needs to be tightly regulated to avoid improper bleeding and thrombus formation. Platelet dysfunction can be associated to alteration of their number or impairment in different aspects of their activity, such as defects in adhesion, activation, secretion or aggregation. Generally, patients with congenital and acquired abnormalities of platelet number or function have mucocutaneous bleeding of variable severity and excessive hemorrhage after surgery or trauma. Platelet disorders can be classified

based on platelet the component that is affected, for example defect in platelet receptor for adhesive molecules or soluble agonist, defect in platelet granules, defect in signal transduction pathway or defect in procoagulant phospholipids [137].

1.7.1 Glanzmann Thrombasthenia

Glanzmann Thrombasthenia (GT) is a rare (1:1,000,000 people) autosomal recessive disease, caused by defective or absence of one of the two components of the integrin $\alpha_{2b}\beta_3$ complex. Characteristic of the disease is lack of platelet aggregation in response to any agonist, mucocutaneus bleeding, such as epistaxis, gingival bleeding, menorrhagia and gastrointestinal bleeding, and bleeding after trauma or surgery. GT platelets can normally bind the subendothelium but they fail to form stable aggregates, spread and build up thrombus. On the contrary, the heterozygotes do not show impairment in platelet response [138]. In severe form of GT platelets lack of fibrinogen in their α-granules, due to their inability to uptake it from the circulation through an $\alpha_{2b}\beta_3$-dependent mechanism [139].

The genes encoding for the two glycoproteins are *ITAG2B* and *ITGB3.* The expression of *ITAG2B* is restricted to megakaryocytes lineage, while *ITGB3* is also expressed in other cell types as a component of the vitronectin receptor (αVβ3). Mutations can affect synthesis of the glycoproteins or their trafficking to the cell surface. Usually, mutations, such as non-sense

mutations, that result in the generation of truncated proteins, are associated with more severe forms of GT [137].

There are a few possible treatments for GT. These include: fibrinolytic inhibitors, to delay cleavage of the fibrin fibers, recombinant coagulation factor VIIa, to promote coagulation, and platelet transfusion. Platelet transfusion is reserved for patients with severe bleeding who are unresponsive to other therapies, because the high risk of alloimmunization that can limit subsequent responses to platelet transfusions [140]. Moreover, there are a few reports of correction of GT with allogeneic bone marrow transplant [141]–[143].

In general, therapeutic options for GT patients are few and not curative, especially when alloimmunization occurs. Although, gene therapy could be a future useful approach for these patients [144], better understanding of platelet response and mechanism of activation can be beneficial in the design new types of drugs that can enhance platelet responsiveness.

1.7.2 Hermansky-Pudlak syndrome

Hermansky-Pudlak syndrome (HPS) belongs to a heterogenous group of platelet disorders with defects in granule secretion. HPS is a rare autosomal recessive disease caused by mutation in *HPS* genes involved in production and control of subcellular lysosome-related organelles, such as melanosomes, dense granules and lysosomes. HPS patients also have impairments in specialized secretory cells such as melanocytes, platelets,

lung alveolar type II epithelial cells and cytotoxic T cells. These defects result in phenotypic features of HPS patients, including albinism, cellular storage disorders, like pulmonary fibrosis and granulomatous colitis, and prolonged bleeding time. Depending on the mutation that affect the patient, the severity of platelet abnormalities is variable [145].

HPS is considered a rare pathology in the general population but occurs with relatively high frequency in certain isolated groups, such as in the northwestern region of Puerto Rico and in an isolated village in the Swiss Alps [137].

There are nine HPS subtypes, based on the gene that is mutated: HPS-1 (caused by mutation in *HPS-1* gene), HPS-2 (mutation in *AP3B1*), HPS-3 (mutation in *HPS-3*), HPS-4 (mutation in *HPS-4*), HPS-5 (mutation in *HPS-5*), HPS-6 (mutation in *HPS-6*), HPS-7 (mutation in *HPS-7*), HPS-8 (mutation in *BLOC1S3*) and HPS-9 (mutation in *pallidin*). HPS-1 seems to be the most severe form of HPS. All *HPS* genes encode for proteins that are components of one of four protein complexes involved in generation, maturation and trafficking of the lysosome-related organelles: biogenesis of lysosome-related organelles complex (BLOC)-1, BLOC-2, BLOC-3 and adaptor protein complex-3 (AP-3) [145]. For example, BLOC-3 is composed of HPS1 and HPS4, and pallidin is a component of BLOC-1 complex with BLOC subunit 3 (BLOS3). Moreover, HPS-7 results from the mutation in the dysbindin protein, a member of BLOC-1 complex (Fig. 21) [137].

As GT, therapeutic approaches for HPS patients are limited and they are mainly based on transfusion with normal platelets, anti-fibrinolytic agent and recombinant factor VIIa or desmopressin, a synthetic analogue of the antidiuretic hormone vasopressin, that increases the plasma levels of factor VIII and vWF [137].

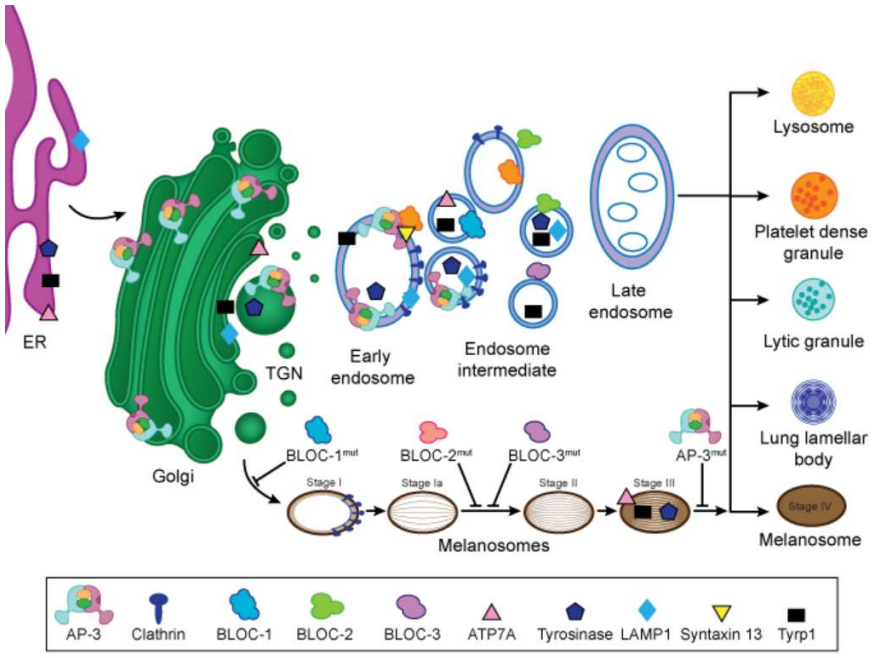

Figure 21| Lysosome-related organelles maturation. The HPS genes encode for proteins involved in formation of complexes BLOC-1, BLOC-2, BLOC-3 and AP-3, that are necessary for generation and maturation of lysosome-related organelles, such as lysosomes, dense granules, lytic granules, lung lamellar bodies and melanosomes. BLOC1 interacts with AP-3 and they are involved in the early steps of vescicle trafficking from the early endosome, while BLOC-2 interacts with BLOC-3 and they are involved in later stages [147].

1.8 Scope of the thesis

Although platelets are anucleated cells, it is known that they contain TFs. It has been clearly demonstrated that these TFs are not vestigial remnants from megakaryocytic precursors, but they have a non-transcriptional role in regulating platelet response. Furthermore, platelets are now considered an integral part of the immune system for their role in participating and modulating immune response and, as other immune cells, it has been demonstrated that NF-κB activation controls specific functions in platelets.

The scope of this thesis is to assess the functions in platelets of other immune TFs different from NF-κB, and in particular we focused our attention on NFATc. Indeed, few studies in the past reported that calcineurin inhibitors used in immunosuppressive treatment of transplanted patient affect platelet activity [148]–[151]. Considering that NFATc activation and platelet signaling pathways share calcium dependency, we hypothesized that NFATc could regulate platelet responses.

The elucidation of novel mechanisms by which TFs can regulate the activation of platelets could highlight the complexity of these anucleated cells and offer new possible ways of intervention against pathologies associated with platelet functions and dysfunctions.

1.9 References

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Chapter 2: A non-transcriptional role of NFAT in regulating platelets functions

Although platelets are anucleated cells, in the latest decade several papers demonstrated that they contain transcription factors (TFs), that are not vestigial remnants from megakaryocytes, but have a non-genomic role in regulating platelet functions. Among all the TFs expressed in platelets, it has been shown that NF-κB is present and regulates several aspects of platelet activation [1]. In immune cells NF-κB is a key transcription regulator: it is involved in modulating survival, proliferation and inflammation but it can also have non-genomic role in mitochondria [2].

Another important transcription factor in immune cells is nuclear factor of activated T cells (NFAT), initially discovered in T cells, but now known to be also expressed in innate immune cells, such as dendritic cells and macrophages. There are 5 different NFAT proteins: NFATc1, NFATc2, NFATc3 and NFATc4 regulated by calcium fluxes, and NFAT5 regulated by osmotic stress. Inactive NFATc resides into the cytoplasm in a phosphorylated status, bound to a bigger complex that include some of its regulatory kinases, such as GSK3, CK1 and DYRK [3]. In response to cell stimuli that increase cytosolic calcium, the calcium sensor protein calmodulin activates the phosphatase calcineurin that, in turn,

dephosphorylates NFATc. Dephosphorylation of NFATc results in exposure of a nuclear localization signal (NLS) that allows NFATc to translocate into the nucleus and to modulate gene transcription [4].

Calcium fluxes are a key element also in mediating platelet activation. A similar calcium-dependent signaling pathway is activated downstream of transmembrane platelet receptors of all canonical stimuli, including thrombin, ADP and collagen. When thrombin activates its protease-activated receptor (PAR) on platelet surface, activation of PLCβ occurs and generates second messenger IP_3 that, in turn, releases calcium from the dense tubular system. This increase in cytosolic calcium induces several calcium-dependent mechanisms, such as integrin $\alpha_{2b}\beta_3$ activation, granule release, actin remodeling and spreading [5].

Because platelets play a critical role not only in hemostasis and thrombosis, but also in early steps of immune response against invading pathogens, we decided to unveil whether NFATc is also expressed in platelets and could play a role in regulating platelet physiology. A possible role for NFATc in regulating platelet physiology is suggested by 1) similarities in signaling pathways that lead to activation of NFATc and activation of platelets and 2) the observation that calcineurin inhibitors, such as cyclosporin A (CsA) and tacrolimus (which are used as immunosuppressive drugs in transplanted patients), alter platelet functionality [6], [7].

2.1 Results

2.1.1 NFATc2 is expressed in platelets and it is activated upon PAR4 stimulation

First, we wanted to assess the presence of NFATc in platelets. We tested the expression of NFATc1, c2, c3 and c4 in murine platelets, compared to T cells, bone marrow-derived dendritic cells (BMDC) and macrophages (BMDM) as control. Moreover, in order to exclude that the signal in platelets was due to contaminant white blood cells (WBC), we purified protein from a number of WBC identical to the number of contaminant cells (< 2%) found in our platelet preparation. As shown in figure 1A, platelets do express NFATc1 and NFATc2, but not NFATc3 or NFATc4. We were not able to detect any band in the WBC lysate, confirming that NFATc1 and NFATc2 bands observed in platelets preparation is indeed due to the expression of these NFATs by platelets and it didn't result from any other contaminant leukocytes. As a control, we also confirmed the presence of NF-κB subunit p65 as previously described [1].

To better appreciate the different pattern of NFATc1 and NFATc2 expression in different cell types, we loaded differential amount of proteins from different cell types. We observed that platelets express a single isoform of NFATc1 and NFATc2, while T cells, BMDC and BMDM show two or three different isoforms of these proteins (Fig. 1B).

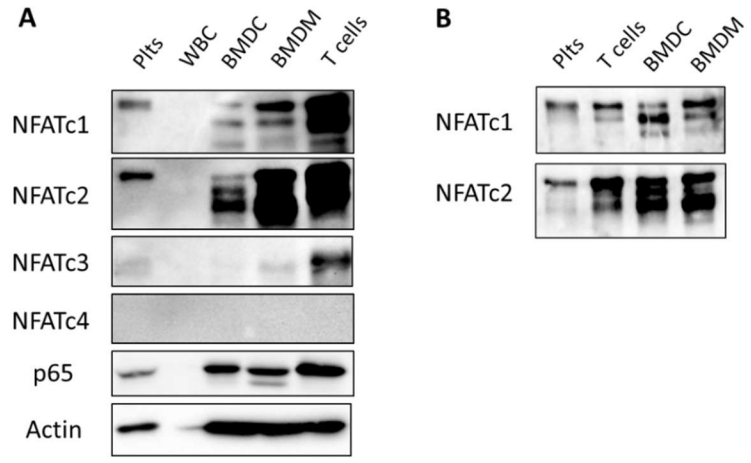

Figure 1| NFATc1 and NFATc2 are expressed in platelets. A) Western blot analysis of NFATc1, NFATc2, NFATc3, NFATc4 and p65 of 20 µg of lysate from murine platelets (Plts), WBC, BMDC, BMDM and T cells. NFATc1 and NFATc2 proteins have been developed at the same time. B) Western blot analysis of NFATc1 and NFATc2 of different amount of protein in the indicated cell types, specifically 20 µg of platelet lysate, 2 µg of T cells lysate, 10 µg of BMDC and BMDM lysates.

Our western blot analysis revealed that in platelets NFATc2 is more abundant than NFATc1 (Fig. 1A). Moreover, NFATc1KO mice have an embryonal lethal phenotype due to failure in developing normal cardiac valves and cannot be maintained in homozygosity [8]. Because of these reasons, in this work we focused our attention on NFATc2, although we cannot exclude the contribution of NFATc1 to some of the processes we describe.

Because NFATc2 activation in nucleated cells is dependent on its dephosphorylation, we tested if also in platelets NFATc2 becomes

dephosphorylated upon stimulation with PAR4-AP, a synthetic peptide that, as the thrombin enzyme, induces activation of PAR4 signaling. To address this question we used a Phos-Tag western blot technology, that allows to separate protein not only based on their molecular weight but also on their phosphorylation status. Activation of platelets with PAR4-AP resulted in a time-dependent and calcineurin-dependent dephosphorylation of NFATc2, while pretreatment of platelets with the calcineurin inhibitor FK506 completely abolished this phenotype with the accumulation of hyper-phosphorylated NFATc2 (Fig 2A).

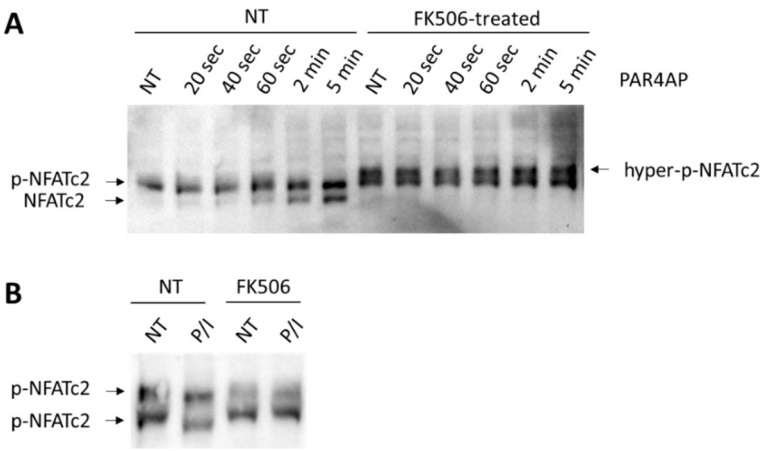

Figure 2| NFATc2 is dephosphorylated upon PAR4 stimulation in platelets. A) Phos-tag western blot analysis of platelets pretreated or not with FK506 stimulated with PAR4-AP 150 µM for the indicated time points. Arrow indicates different phosphorylated status of NFATc2. B) Phos-tag western blot analysis of T cells pretreated or not with 1 µM FK506 (90 min) stimulated with PMA/ionomycin (P/I) for 30 minutes.

As control, stimulation of T cells with PMA/ionomycin induced a shift in both NFATc2 bands observed, suggesting the presence of two isoforms that are dephosphorylated upon T cells activation, while FK506 pretreatment totally inhibits this change (Fig 2B).

2.1.2 Calcineurin inhibition or genetic absence of NFATc2 enhances platelet aggregation upon stimulation

A small number of reports suggested that calcineurin inhibition, used as immunosuppressive treatment for transplanted patients, affects platelet functionality [6], [7], [10]. To investigate this possibility, we treated mice *in vivo* with FK506 for 14 days and tested platelet aggregation in whole blood. As shown in fig. 3A, FK506 treatment enhances platelet aggregation compared to non-treated platelets upon stimulation with PAR4-AP, confirming a role of calcineurin in the signaling cascade elicited by PAR4 stimulation in platelets. In order to directly correlate the observed effect to platelet physiology, we treated the mice *in vivo* and then performed an aggregation test using platelet rich plasma (PRP), in absence of any other cellular component present in the blood. FK506 treatment enhanced platelets aggregation upon PAR4 stimulation as observed in whole blood (Fig. 3B). Moreover, to exclude the influence of any other soluble plasma component, we repeated the same experiment using washed platelets (WPs). WPs from FK506-treated mice stimulated with PAR4AP showed an hyperaggregated phenotype, even stronger than

the one observed in the context of whole blood or PRP (Fig. 3C). This data demonstrates that calcineurin inhibition with FK506 *in vivo* directly affects platelet activation itself, rather than modulating it through other cellular or soluble components in the blood.

Additionally, we observed that the hyper-aggregated phenotype was restricted to PAR4 stimulation and did not occur upon ADP receptor stimulation (Fig. 3D), suggesting a specific role of calcineurin downstream the PAR4 receptor.

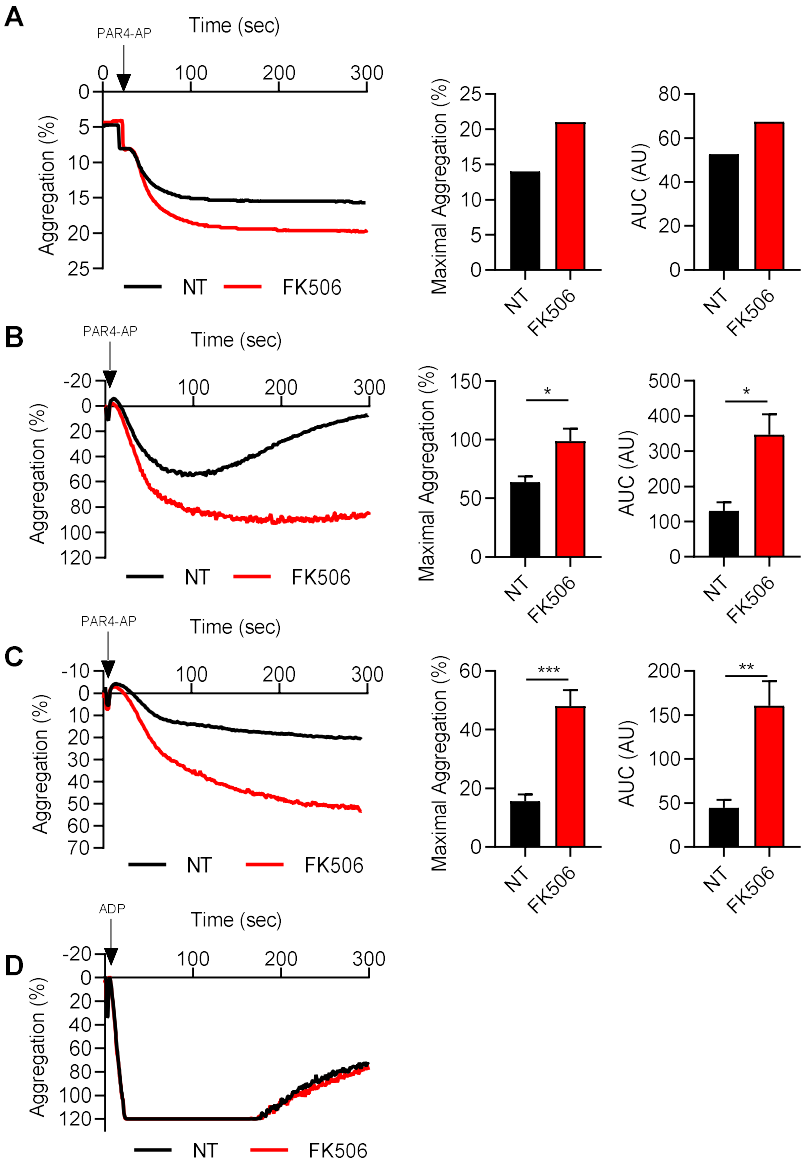

Figure 3| Calcineurin inhibition *in vivo* enhances platelets aggregation upon PAR4 stimulation. A) Whole blood from non-treated or FK506-treated mice was stimulated with 200 µM PAR4-AP. Aggregation curves and histograms of maximal aggregation

reached and area under curve (AUC) are shown. B) PRP and C) WPs from non-treated or FK506-treated mice were stimulated with 100 µM PAR4-AP. D) PRP from non-treated or FK506-treated mice was stimulated with 10 µM ADP. Data are represented as mean ± SEM (n = 5). Unpaired two tailed *t*-test was used for statistics. *$P < 0.05$ and ***$P < 0.001$.

To exclude that the phenotype observed was due to a bystander effect of the drugs on other cellular blood components or on megakaryocytes, instead of platelets themselves, we treated PRP and WPs *in vitro* with FK506. In both PRP and WPs, FK506 treatment induced platelets to aggregate more compared to the non-treated counterparts (Fig. 4A and 4B), confirming that calcineurin inhibition directly alters platelet response.

As previously observed, stimulation with different doses of ADP did not result in different profiles of aggregation between FK506-treated and non-treated platelets (Fig. 4C and 4D), further proving that calcineurin modifies the signaling cascade activated in response to PAR4 activation.

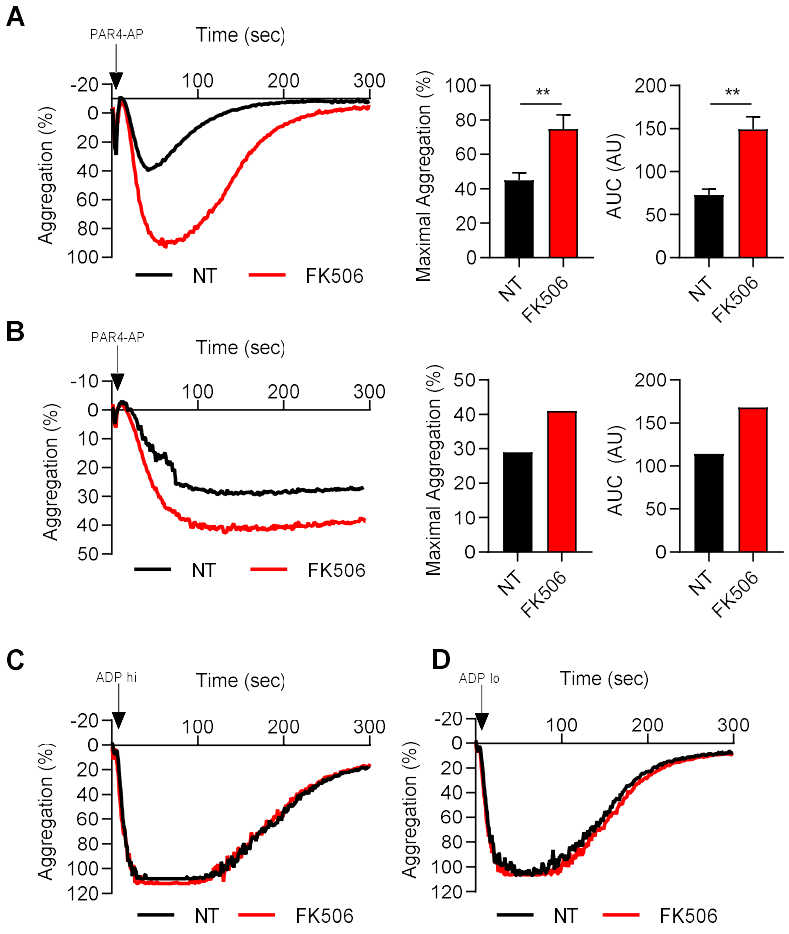

Figure 4| Calcineurin inhibition *in vitro* with FK506 enhances platelets aggregation upon PAR4 stimulation. A) PRP and B) WPs were incubated with 1 µM FK506 or vehicle (NT) for 1 h and then stimulated with 150 µM PAR4-AP. D) PRP pretreated or not with FK506 was stimulated with 20 µM ADP (ADP hi) or 10 µM ADP (ADP lo). Aggregation curves are shown. Data are represented as mean ± SEM (n= 3). Unpaired two tailed *t*-test was used for statistics. ***P* < 0.01.

To verify that the observed phenotype was due to calcineurin inhibition and not to an off-target effect of FK506, we treated PRP and WPs *in vitro* with another calcineurin inhibitor, cyclosporin A (CsA). When PRP or WPs were used, inhibition of calcineurin with CsA enhanced platelets aggregation upon PAR4 stimulation (Fig. 5A and 5B), suggesting that the hyperactivated phenotype observed upon PAR4 stimulation is directly due to calcineurin inhibition and it is not due to an off target of FK506.

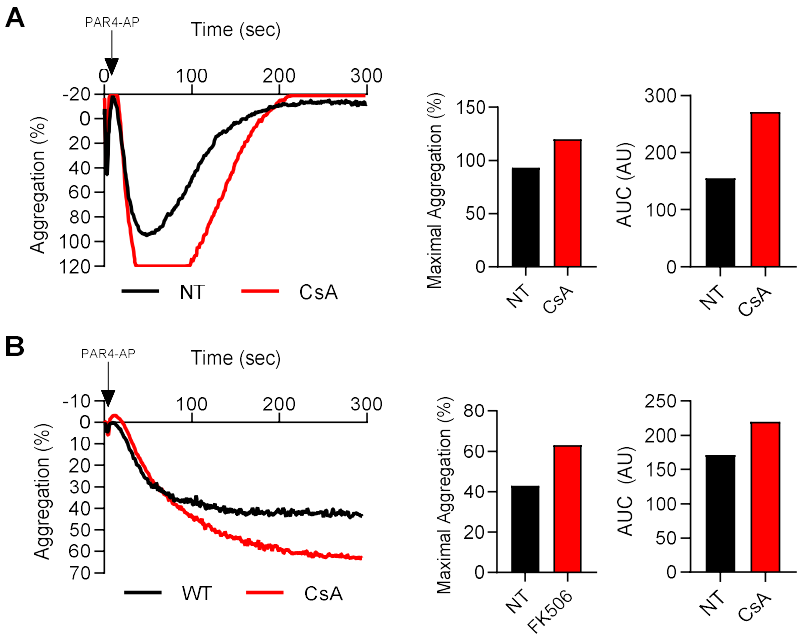

Figure 5| Calcineurin inhibition *in vitro* with CsA enhances platelets aggregation upon PAR4 stimulation. A) PRP and B) WPs were incubated with 250 ng/ml cyclosporin A (CsA) or vehicle (NT) for 1 h and then stimulated with 100 µM PAR4-AP. Aggregation curves and histograms of maximal aggregation reached and area under curve (AUC) are shown.

In figure 3 we demonstrated that calcineurin inhibition enhances platelet response and affects NFATc2 dephosphorylation (Fig. 2), thus we tested whether genetic absence of NFATc2 could affect platelet activation upon PAR4 stimulation. PRP and WPs from NFATc2KO mice stimulated with PAR4-AP showed an hyperaggregated phenotype compared to wild-type platelets (WT) (Fig. 6A and 6B), and similar to what observed in FK506- or CsA-treated platelets. All these data support the conclusion that in platelets a calcineurin/NFATc2-dependent inhibitory pathway is activated downstream PAR4 to regulate platelets aggregation.

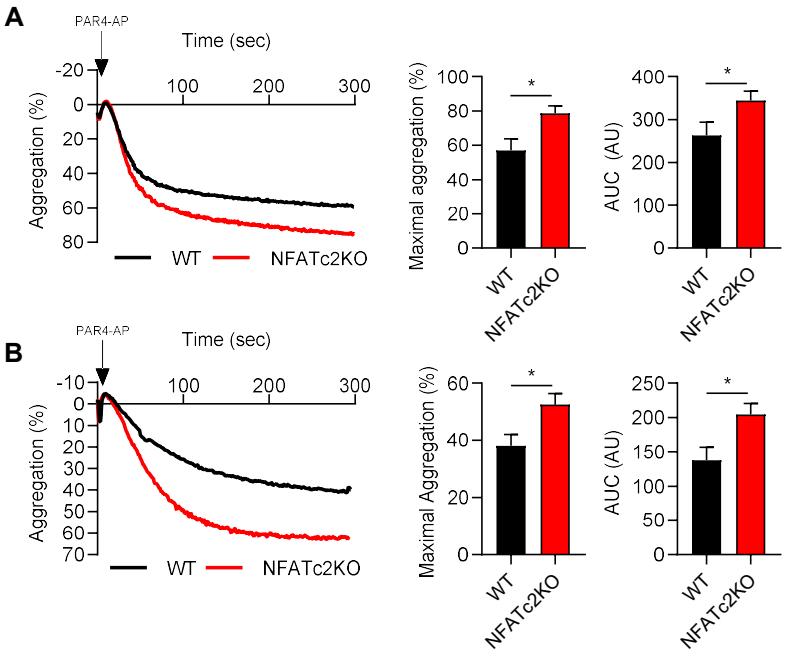

Figure 6| Genetic absence of NFATc2 enhances platelets aggregation upon PAR4 stimulation. A) PRP and B) WPs from WT or NFATc2KO mice were stimulated with 150

μM PAR4-AP. Aggregation curves and histograms of maximal aggregation reached and area under curve (AUC) are shown. Data are represented as mean ± SEM (n= 6). Unpaired two tailed *t*-test was used for statistics. *$P < 0.05$.

Next, we tested the activity of this pathway in human platelets. We pretreated human PRP *in vitro* with FK506 or CsA at different concentrations and then we treated it with TRAP, the peptide that stimulate human PARs. We found that also human platelets are sensitive to calcineurin inhibition and drug treatment resulted in an hyperaggregated phenotype upon PAR stimulation at different concentrations of agonist (Fig. 7A, 7B and 7C). In particular, we obtained a more consistent effect when PRP was pretreated with a high dose of CsA (1000 ng/ml) and it was stimulated with a low concentration of TRAP (3 μM) (Fig. 7D). These data confirm the presence of a calcineurin/NFATc2 regulatory pathway also in human platelets, as we have previously shown for murine platelets.

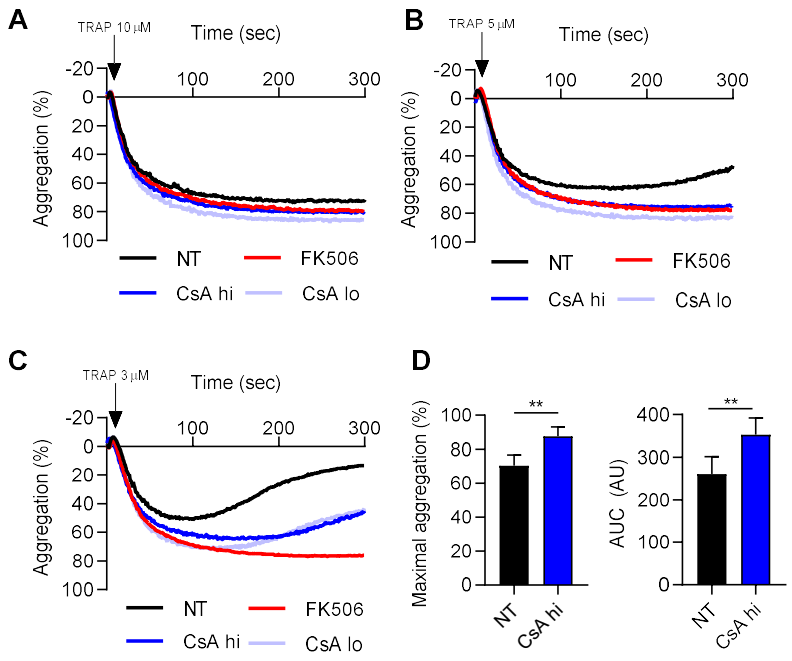

Figure 7| Calcineurin inhibition enhances human platelets aggregation upon PAR stimulation. A-C) Representative aggregation curve of PRP from healthy donor pretreated for 2 h with FK506 10 µM, CsA 1000 ng/ml (CsA hi) or 500 ng/ml (CsA lo) or vehicle (NT) and then stimulated with 10 µM (A) 5 µM (B) and 3 µM (C) of TRAP. D) Histograms representing maximal aggregation reached and area under curves (AUC) of human PRP pretreated or not with CsA hi and stimulated with 3 µM TRAP. Data are represented as mean ± SEM (n= 16). Paired two tailed *t*-test was used for statistics. ***P* < 0.01.

2.1.3 Calcineurin inhibition or genetic absence of NFATc2 enhances upregulation of platelet activation markers

To evaluate whether calcineurin inhibition affects also other features of platelets activation, such as inside-out integrin $\alpha_{2b}\beta_3$ signaling and α-granules release, we analyzed respectively integrin $\alpha_{2b}\beta_3$ activation and P-

selectin exposure in murine platelets by FACS analysis. FK506 and CsA treatment of murine platelets *in vitro* increased both integrin $\alpha_{2b}\beta_3$ activation (detected with the activation-dependent monoclonal antibody JON/A) and P-selectin exposure upon PAR4 stimulation but did not affect response to ADP (Fig. 8A and 8B), consistent with what we observed with the aggregation test.

Analogously, we analyzed these parameters in NFATc2KO platelets and we observed a similar phenotype. NFATc2KO platelets showed higher level of integrin $\alpha_{2b}\beta_3$ activation and P-selectin exposure compared to WT platelets when stimulated with PAR4-AP but not with ADP (Fig. 8C).

To test that the phenotype observed with the inhibition of calcineurin was due to the inhibition of NFATc2 activation, and not of other NFATs or off targets of the drug, we treated NFATc2KO platelets *in vitro* with FK506. We found that FK506 treatment had not an additive effect on NFATc2KO platelets (Fig. 8D), suggesting that calcineurin treatment mimic the deficiency in NFATc2.

These data demonstrate that in platelets, upon stimulation of PAR4, activation of a calcineurin/NFATc2 pathway occurs and it negatively regulates both inside-out integrin $\alpha_{2b}\beta_3$ signaling and α-granule release.

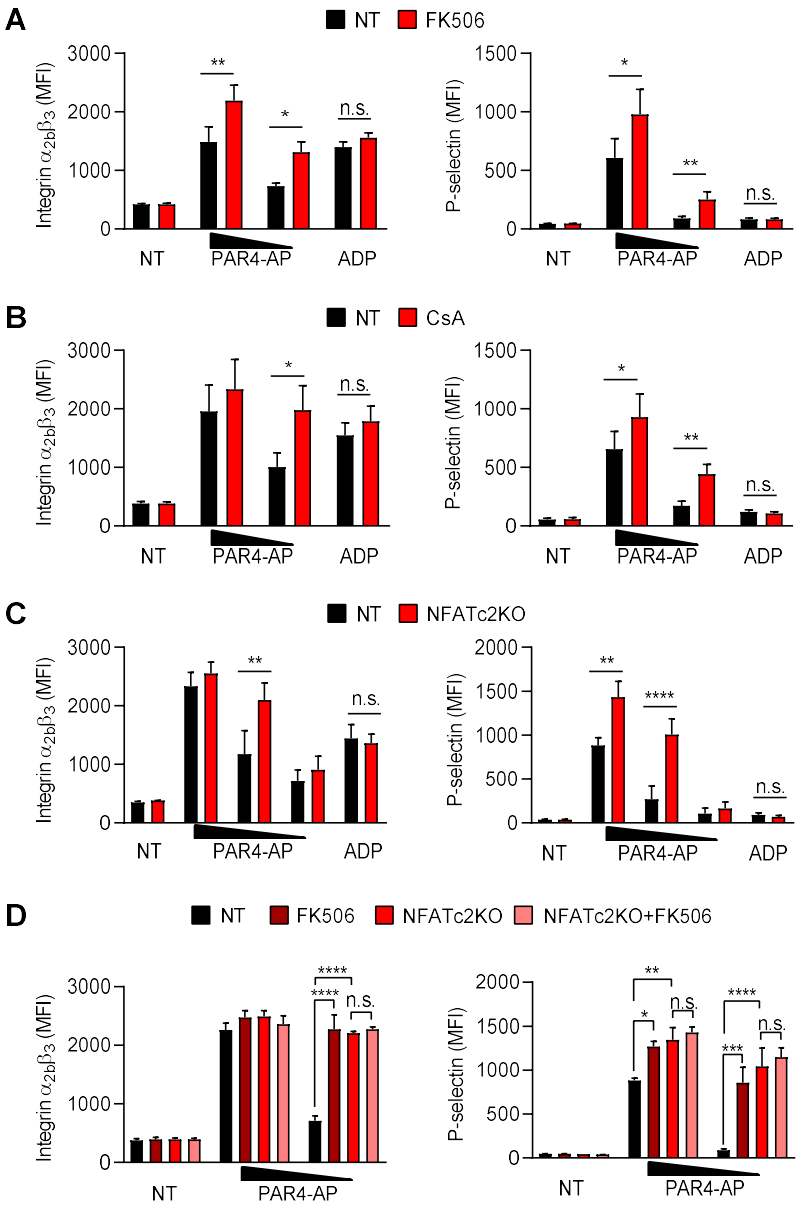

Figure 8| Calcineurin inhibition or genetic absence of NFATc2 enhances integrin $\alpha_{2b}\beta_3$ activation and P-selectin upregulation upon PAR4 stimulation. PRP was incubated with

1 µM FK506 (A) or 250 ng/ml CsA (B) for 1 h and then stimulated with PAR4-AP at different concentrations (150 µM and 100 µM) or ADP 20 µM. Integrin activation $\alpha_{2b}\beta_3$ and P-selectin exposure were analyzed by FACS. Data are represented as mean ± SEM (n=10). C) PRP from WT or NFATc2KO mice was stimulated with PAR4-AP at different concentrations (200 µM, 150 µM and 100 µM) or ADP 20 µM. Data are represented as mean ± SEM (n=5). D) PRP from WT or NFATc2KO mice, pretreated or not for 1 h with 1 µM FK506, was stimulated with PAR4-AP at different concentrations (200 µM and 150 µM). Data are represented as mean ± SEM (n=3). A two-way ANOVA test was used for statistics. N.s., not significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

Similar experiments were also performed in human platelets. We pretreated human PRP *in vitro* with different concentrations of FK506 or CsA and tested for integrin $\alpha_{2b}\beta_3$ and P-selectin upregulation upon TRAP stimulation. As for murine platelets, calcineurin inhibition enhanced integrin $\alpha_{2b}\beta_3$ activation and P-selectin upregulation upon stimulation with non-saturated concentration of TRAP (1.5 µM) but had no effect at high concentration of TRAP (10 µM) (Fig. 9). In particular, a high dose of CsA is more effective in inducing the hyperactivated phenotype, as observed for aggregations (Fig. 7D).

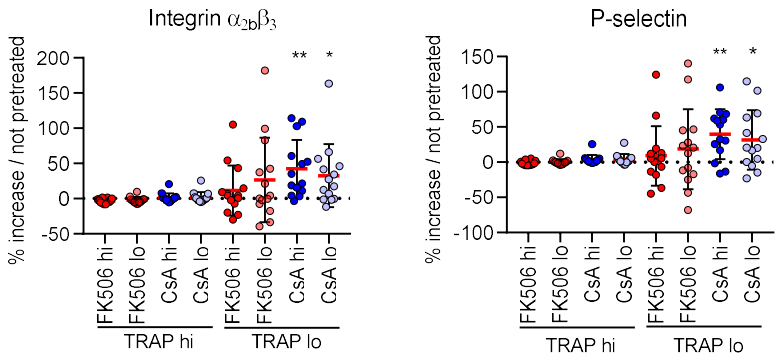

Figure 9| Calcineurin inhibition enhances integrin $\alpha_{2b}\beta_3$ activation and P-selectin upregulation upon TRAP stimulation. Human PRP was pretreated for 2 h with 20 µM FK506 (FK506 hi), 10 µM FK506 (FK506 lo), 1000 ng/ml CsA (CsA hi) or 500 ng/ml CsA (CsA lo) and then stimulated with 10 µM TRAP (TRAP hi) or 1.5 µM TRAP (TRAP lo). Integrin $\alpha_{2b}\beta_3$ activation (left) and P-selectin exposure (right) were analyzed by FACS. Data are represented as percentage of increase compared to the response of platelets not pretreated with any drugs for every single donor. Mean is depicted as red bar ± SD (n=14). One-sample t test was used for statistics. N.s., not significant ($P > 0.05$), $*P < 0.05$ and $**P < 0.01$.

Finally, we tested if dense granule release was also affected by calcineurin inhibition or NFATc2 deficiency. To test dense granule release we measured ATP released (by luminescence) over time during activation upon stimulation with the PAR agonist. We found that ATP release was enhanced upon stimulation in FK506-treated platelets (Fig. 10A), NFATc2KO platelets (Fig. 10B) and human platelets pretreated with CsA (Fig. 10C). Overall, these data demonstrate that the calcineurin/NFATc2 pathway negatively regulates not only α-granule release but also dense granules release in both murine and human platelets.

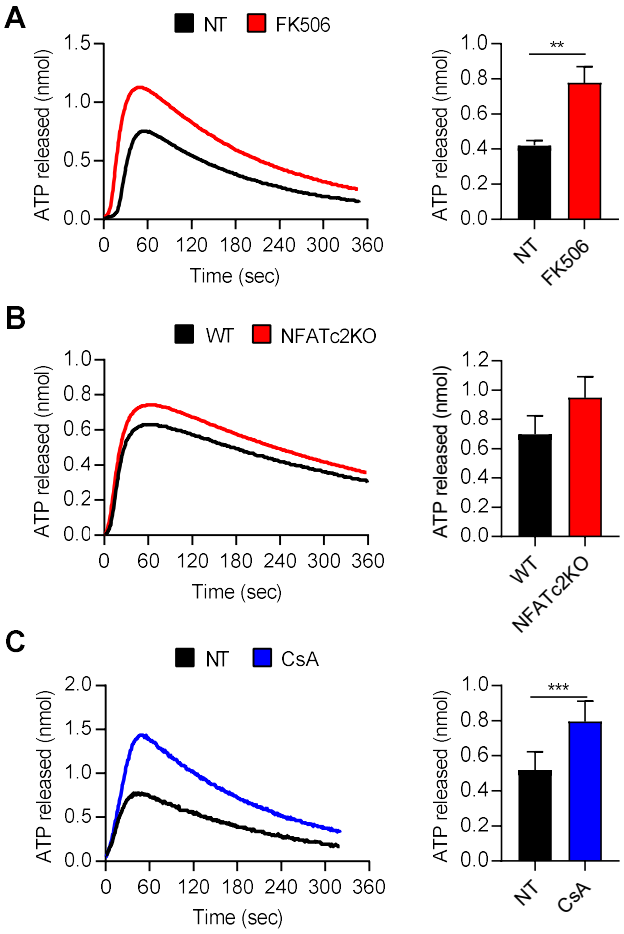

Figure 10| Calcineurin/NFAT pathway regulates ATP release in platelets. ATP release was measured upon stimulation of FK506-treated PRP (A), NFATc2KO PRP (B) or CsA-treated human PRP (C). Curves represent ATP release overtime. Histograms represent mean ± SEM (n=5 for murine experiments, n=10 fur human experiments). Unpaired two tailed *t*-test was used for statistics. ***P* < 0.01 and ****P* < 0.001.

2.1.4 The hyperactivated phenotype of platelets results in more efficient spreading on fibrinogen

Integrin $\alpha_{2b}\beta_3$ activation is necessary for fibrinogen binding and platelet spreading [11]. To assess whether enhancement in integrin $\alpha_{2b}\beta_3$ activation correlates with higher ability to bind to and spread on fibrinogen, we tested platelets spreading on fibrinogen coated coverslips. WPs were plated on fibrinogen coated coverslip and stimulated for 45 minutes with different PAR agonist or ADP. The platelet area (before and after stimulation) was quantified by fluorescence microscopy by staining for actin. The percentage of area increase after stimulation was calculated.

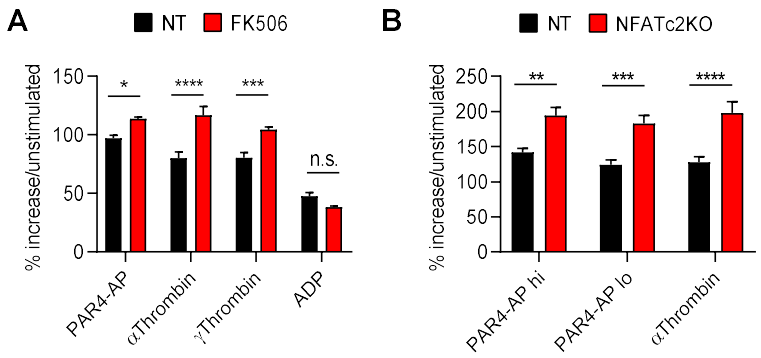

Figure 11| Calcineurin inhibition or genetic absence of NFATc2 leads to more efficient spreading on fibrinogen. A) WPs from FK506-treated mice were placed on fibrinogen coated coverslips and stimulated for 45 minutes with PAR4-AP 100 µM, α-thrombin 0.1 U/ml, γ-thrombin 240 nM, or ADP 20 µM. B) WPs from NFATc2KO mice were placed on fibrinogen coated coverslips and stimulated for 45 minutes with PAR4-AP 200 (PAR4-AP hi), PAR4-AP 100 µM (PAR4-AP lo) or α-thrombin 0.1 U/ml. Results are shown as percentage of increase of the covered area compared to area of non-stimulated platelets. Data are represented as mean ± SEM (n=5). A two-way ANOVA test was used

for statistics. N.s., not significant (P > 0.05), *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

Both WPs from FK506-treated mice and from NFATc2KO mice showed more efficient spreading compared to their control groups (Fig. 11A and 11B), indicating that calcineurin inhibition or genetic absence of NFATc2 induces upregulation of integrin activation that directly results in a higher ability to bind fibrinogen and in increased platelets spreading.

2.1.5 Calcineurin/NFATc2 pathway regulates hemostasis and thrombosis

To evaluate whether the hyperactivated phenotype due to calcineurin inhibition or NFATc2 deficiency affected also platelet functionality *in vivo*, we used two different *in vivo* models to test platelet functionality in hemostasis or in thrombosis.

Using a tail bleeding model, we found that FK506-treated mice and NFATc2KO mice had shorter bleeding time compared to their control groups (Fig. 12A and 12B), suggesting that the hyperactivated phenotype observed *in vitro* occurs also *in vivo* and affects platelets functions during hemostasis.

Analogously, FK506-treated mice and NFATc2KO mice showed higher pathology score in a thromboembolism model when a non-lethal dose of platelet agonists were injected (Fig. 12C and 12D), indicating an hyperactivated status of platelets in these backgrounds.

Taken together these results support the hypothesis that the calcineurin/NFATc2 pathway is an inhibitory pathway that modulates platelets activation both *in vitro* and *in vivo*.

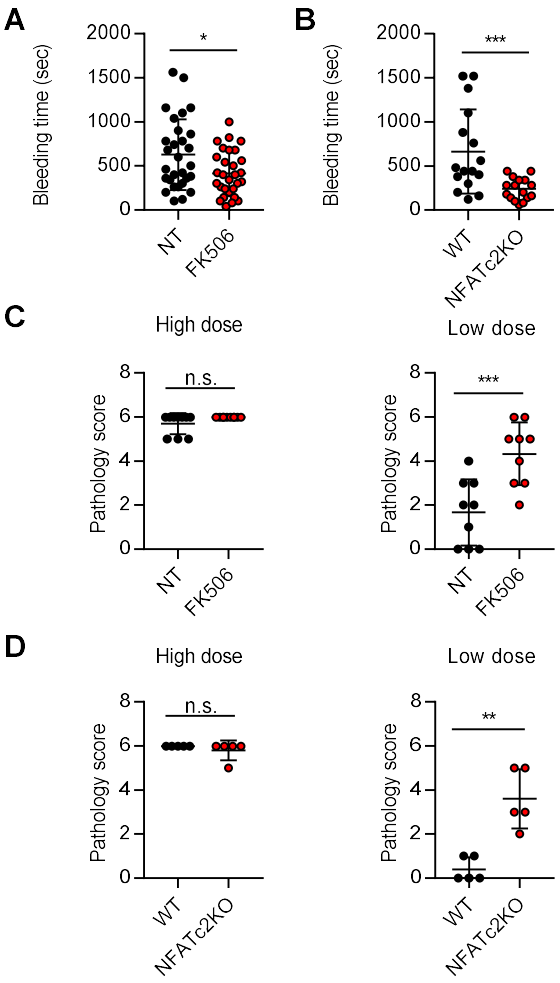

Figure 12| Calcineurin inhibition and NFATc2 absence affect platelet functionality in *in vivo* physiology. Tail bleeding test was performed on mice treated or not with FK506 (A) or on WT and NFATc2KO mice (B). Bars represented mean ± SD (n=29 for FK506-treated

mice, n=16 for NFATc2KO mice). C) Mice treated or not with FK506 were injected with high (80 µl) or low (30 µl) amount of a mixture of collagen and epinephrine and were scored for a pathology index. Bar represented mean ± SD (n=10). D) WT or NFATc2KO mice were injected with high (80 µl) or low (50 µl) amount of a mixture of collagen and epinephrine and were scored for a pathology index. Bar represented mean ± SD (n=5). Each dot represents a mouse. Unpaired two tailed *t*-test was used for statistics. N.s., not significant ($P > 0.05$), $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

2.1.6 The calcineurin/NFATc2 pathway is partially dependent on ADP receptor stimulation but not on thromboxane production

Platelet activation is a two-step process. The first wave of activation is directly dependent on the exogenous stimulus, while the second one is activated by autocrine stimuli released by platelets themselves [5]. We next investigated whether the calcineurin/NFATc2 pathway modulates platelet activation during the first wave of activation (directly downstream the PAR4 receptor) rather than during the second wave of activation (downstream of other receptors that amplify platelet response, such as thromboxane (TXA_2) receptor or ADP receptor).

To evaluate the role of TXA_2 receptor, we inhibited TXA_2 production with the COX inhibitor indomethacin. We found that integrin $\alpha_{2b}\beta_3$ activation and P-selectin exposure in FK506-treated platelets were not affected by indomethacin pretreatment, while in non-pretreated platelets indomethacin reduced upregulation of both markers (Fig. 13A). Similar results were obtained in human platelets: indomethacin pretreatment had no effect on unstimulated platelets (data not shown) and, importantly, it did not rescue the effect of CsA treatment on integrin $\alpha_{2b}\beta_3$

activation (Fig. 13B left) and had only a marginal effect on α-granules release compared to the control (Fig. 13B right). These data demonstrate that in both murine and human platelets the phenotype driven by calcineurin inhibition does not depend on thromboxane production.

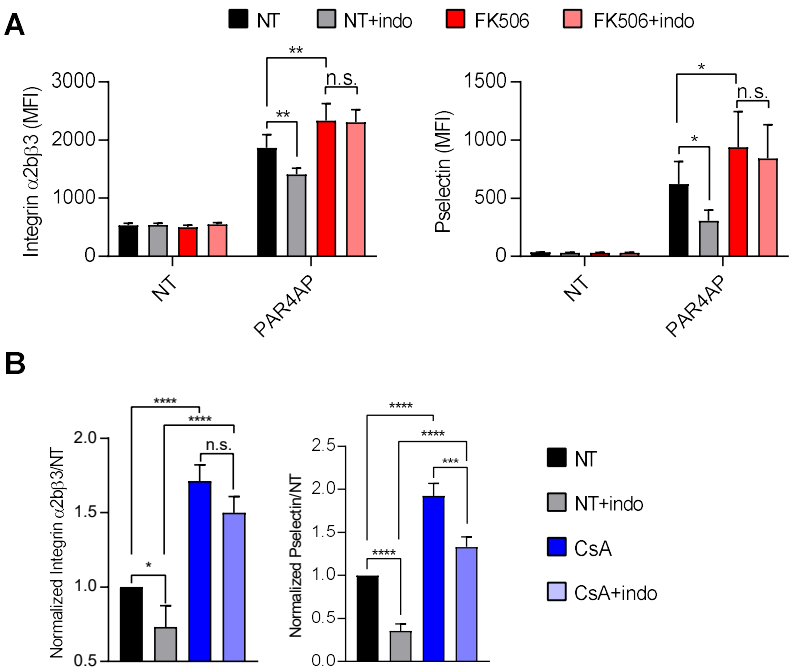

Figure 13| Calcineurin inhibition phenotype is not dependent on thromboxane production. A) Murine PRP was incubated or not for 1 h with 1 µM FK506 and pretreated or not for 10 min with indomethacin 100 µM (indo), then stimulated with 100 µM PAR4-AP. Integrin activation $\alpha_{2b}\beta_3$ and P-selectin exposure were analyzed by FACS. Data are represented as mean ± SEM (n=5). A two-way ANOVA test was used for statistics. B) PRP from healthy donors was incubated or not 2 h with 1000 ng/ml CsA and pretreated or not 10 min with indomethacin 100 µM (indo), then stimulated with 1.5 µM TRAP. Data are normalized over NT. Data are represented as mean ± SEM (n=4 different donors). Two-way ANOVA was used for statistic taking in account non-stimulated samples (not shown). N.s., not significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

ADP receptor activation is a key step in the second wave of activation, and it is necessary to amplify and stabilize platelet response upon PAR stimulation [12]. To determine the role of ADP receptor in calcineurin/NFATc2 pathway, we inhibited ADP receptor activation with a specific inhibitor of the ADP receptor, ARC-66096, and tested the upregulation of activation markers upon stimulation. We observed that the FK506-induced hyperactivated phenotype was partially abolished when platelets were also pretreated with ARC-66096. We found that integrin $\alpha_{2b}\beta_3$ activation and P-selectin exposure levels of FK506- and non-treated platelets were comparable when pretreated with ARC-66096 and stimulated with low concentration of agonist (Fig. 14A). Similarly, we found that the hyperactivated phenotype of NFATc2KO platelets was significantly decreased when platelets were pretreated with the ADP receptor inhibitor (Fig. 14B), suggesting that calcineurin/NFATc2 pathway is partially dependent on ADP receptor stimulation to enhance integrin $\alpha_{2b}\beta_3$ activation and α-granule release.

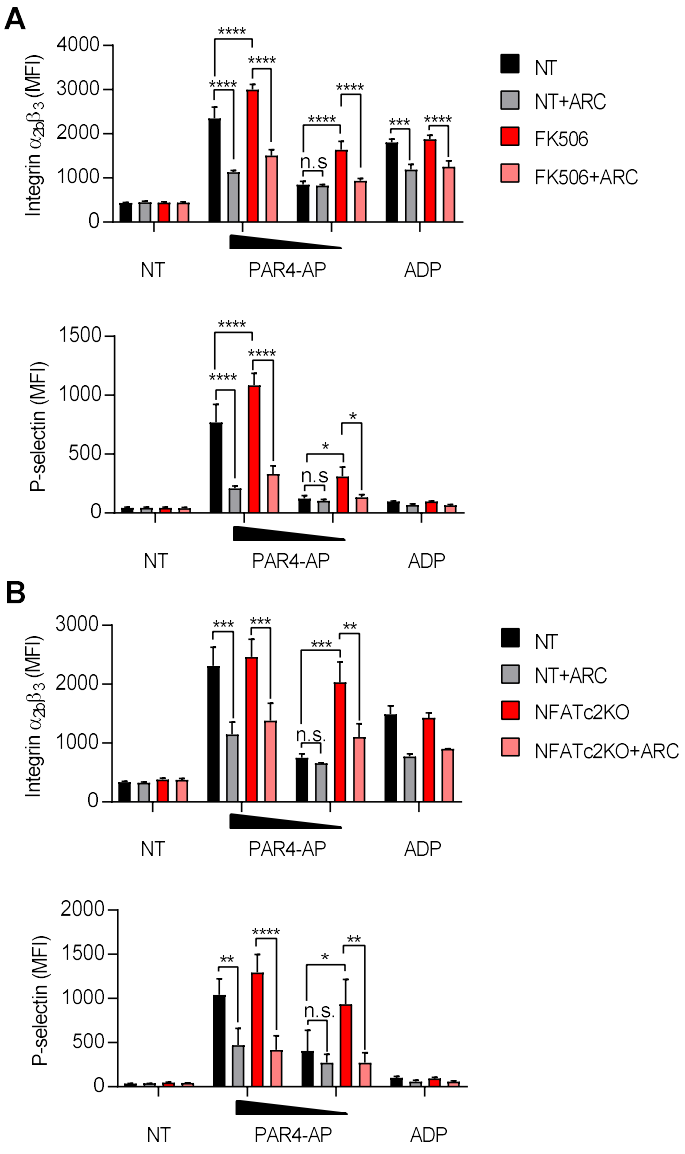

Figure 14| ADP receptor inhibition abolishes the hyperactivated phenotype induced by calcineurin inhibition and genetic absence of NFATc2. A) PRP was incubated or not for 1 h with 1 µM FK506 and pretreated or not for 10 min with 3 µM ARC-66096 (ARC), then stimulated with 150 or 100 µM PAR4-AP. Data are represented as mean ± SEM (n=6). B)

PRP from WT or NFATc2KO mice was pretreated or not 10 min with 3 µM ARC-66096 (ARC), then stimulated with 150 or 100 µM PAR4-AP. Data are represented as mean ± SEM (n=5). A two-way ANOVA test was used for statistics. N.s., not significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

To further investigate the involvement of ADP receptor in calcineurin/NFATc2 pathway, we tested the effect of ARC-66096 on platelet aggregation. We found that ARC-66096 treatment affected FK506-treated platelets aggregation during the second phase of the aggregation curve, but the use of the inhibitor did not restore the level of aggregation of non-treated platelets (Fig. 15A). The maximal aggregation reached in FK506-treated platelets was not altered by ARC-66096 treatment (Fig. 15B), suggesting that calcineurin inhibition potentiates the first phase of platelet aggregation in an ADP independent manner. This is in agreement with our previous funding that the maximal aggregation of ADP-treated platelets is not altered by calcineurin inhibition (Fig. 4C and 4D). Intriguingly, although ADP receptor inhibition resulted in significant reduction of final aggregation level of FK506-treated platelets (Fig. 15C), the percentage of inhibition of platelets treated with ARC-66096 alone is significantly higher compared to platelets treated with FK506 and ARC-66096. These data suggest that the capacity of FK506 to increase the maximal aggregation is not dependent on the ADP receptor activation. The reduction observed during the second wave of platelet response in FK506-treated platelets could be either related to a partial dependency

on ADP receptor stimulation of the calcineurin/NFATc2 pathway or to the physiological dependency of this second phase on the ADP signaling.

Considering all the results obtained on platelet activation markers and on aggregation profiles, we hypothesize that the effects observed with the inhibition of the calcineurin/NFATc2 pathway are only partially dependent on ADP receptor stimulation. We, instead, posit that the calcineurin/NFATc2 pathway inhibition results in higher amount of dense granule release, that, in turns, enhances ADP receptor stimulation, that subsequently amplifies and stabilizes platelets response.

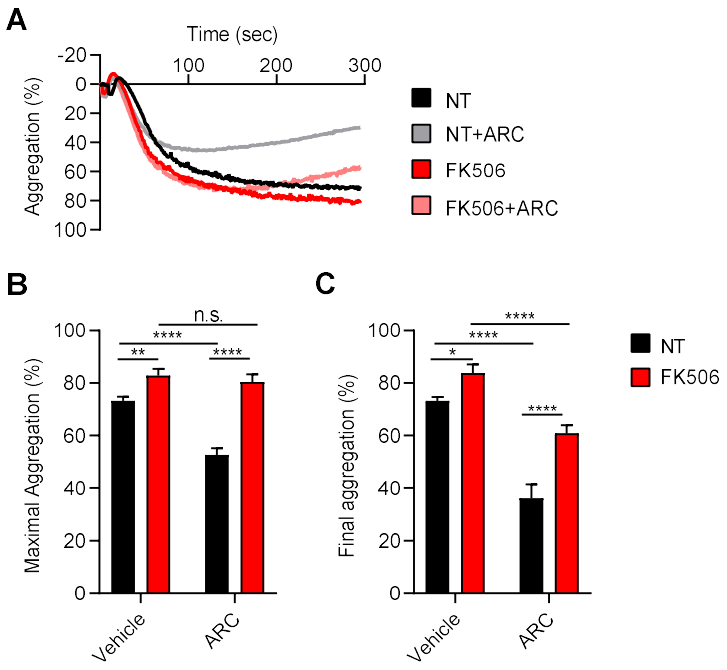

Figure 15| Calcineurin inhibition-induced hyperaggregation is only partially dependent on ADP receptor activation. PRP was incubated for 1 h with 1 µM FK506 or vehicle (NT) and pretreated or not for 10 min with 3 µM ARC-66096 (ARC), then stimulated with 150 PAR4-AP. (A) Aggregation curves, (B) histograms of maximal aggregation reached and (C) area under curve (AUC) are shown. Data are represented as mean ± SEM (n= 6). A two-way ANOVA test was used for statistics. N.s., not significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

Next, we tested the relationship between ADP signaling and the calcineurin/NFATc2 pathway in human platelets. Inhibition of ADP receptor had no effect on unstimulated platelets (data not shown) but, similar to what observed in murine platelets, in human platelets the effect of CsA treatment on integrin $\alpha_{2b}\beta_3$ activation and P-selectin exposure

upon stimulation with PAR agonist was dependent on ADP receptor activation (Fig. 16A). Compared to murine platelets, the aggregation curves of untreated platelets with or without treatment with ARC-66096 suggested a more central role for the ADP receptor during the physiological aggregation of human platelets, especially during the second wave of the activation (Fig. 16B). Despite this strong dependency on the ADP, we observed that ARC-66096 only partially reduced the maximal aggregation reached by CsA-treated platelets. Calcineurin- and ADP receptor-inhibited platelets (CsA+ARC) maintained a significant difference in their aggregation compared to the response of platelets treated with ARC alone (NT+ARC), confirming that part of the process regulated by the calcineurin/NFATc2 pathway is not dependent on the stimulation of the ADP receptor (Fig. 16C). Notably, the second phase of the aggregation curve in human platelets was totally dependent on the ADP receptor stimulation in both groups (Fig. 16D), confirming a central role for the ADP signaling in this phase, and, consequently, on the effects driven by the inhibition of the calcineurin/NFATc2 pathway.

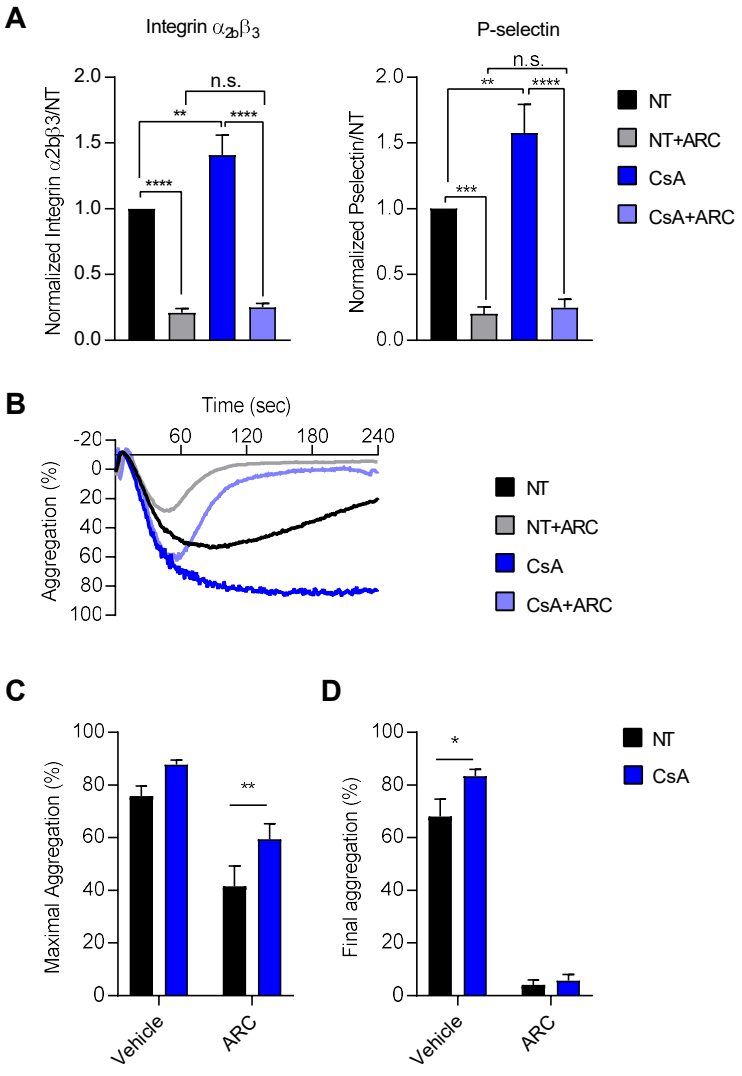

Figure 16| In human platelets calcineurin inhibition phenotype is dependent on ADP receptor stimulation A) PRP from healthy donors was incubated or not for 2 h with 1000 ng/ml CsA and pretreated or not 10 min with 3 µM ARC-66096 (ARC), then stimulated with 1.5 µM TRAP. Data are normalized over NT. Data are represented as mean ± SEM (n=8). One-way ANOVA was used for statistics. B) Aggregation curves and histograms of (C) maximal aggregation reached and (D) final aggregation level of platelets stimulated

with 3 µM TRAP are shown. Data are represented as mean ± SEM (n= 6). A two-way ANOVA test was used for statistics. N.s., not significant (P > 0.05), **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001.

2.1.7 The inhibition of the calcineurin/NFATc2 pathway is a new therapeutic approach for the Hermansky-Pudlak syndrome

Although in transplanted patients calcineurin inhibition has many side effects, the capacity of this drug to enhance platelet response could be harnessed to design new therapeutic ways of intervention in pediatric patients with life-treating defects in platelet activation. The Hermansky-Pudlak syndromes (HPS) are a group of genetic diseases, characterized by defects on dense granules maturation and release due to mutations in different genes involved in this process. We wanted to test whether the inhibition of the calcineurin/NFATc2 pathway could be beneficial in inducing a better response of platelets in HPS patients. We analyzed PRP from a patient with HPS type 2 and found that CsA treatment enhanced aggregation upon stimulation with different concentration of TRAP (Fig. 17A). Upon stimulation with a non-saturating concentration of TRAP the maximal aggregation reached was significantly enhanced by CsA-pretreatment (Fig. 17B). Additionally, integrin $\alpha_{2b}\beta_3$ activation and P-selectin exposure were also significantly increased upon CsA-treatment (Fig. 17C). Moreover, PRP from two twin patients with HPS type 1 showed a significant increase in maximal aggregation reached upon TRAP

stimulation with CsA-pretreatment, although without consistently altering the second phase of aggregation (Fig. 17D and 17E).

The absence of dense granules in platelets from HPS patients confirms that the inhibition of calcineurin/NFATc2 pathway enhances the aggregation and the upregulation of platelet activation markers with a mechanism that is only partially dependent on ADP signaling, as observed for murine platelets. Taken together all these data suggest that also in human platelets calcineurin/NFATc2 pathway acts directly downstream the thrombin receptors, where its inhibition results in boosting platelet aggregation, integrin activation and α- and dense granules release in a partially ADP-dependent manner.

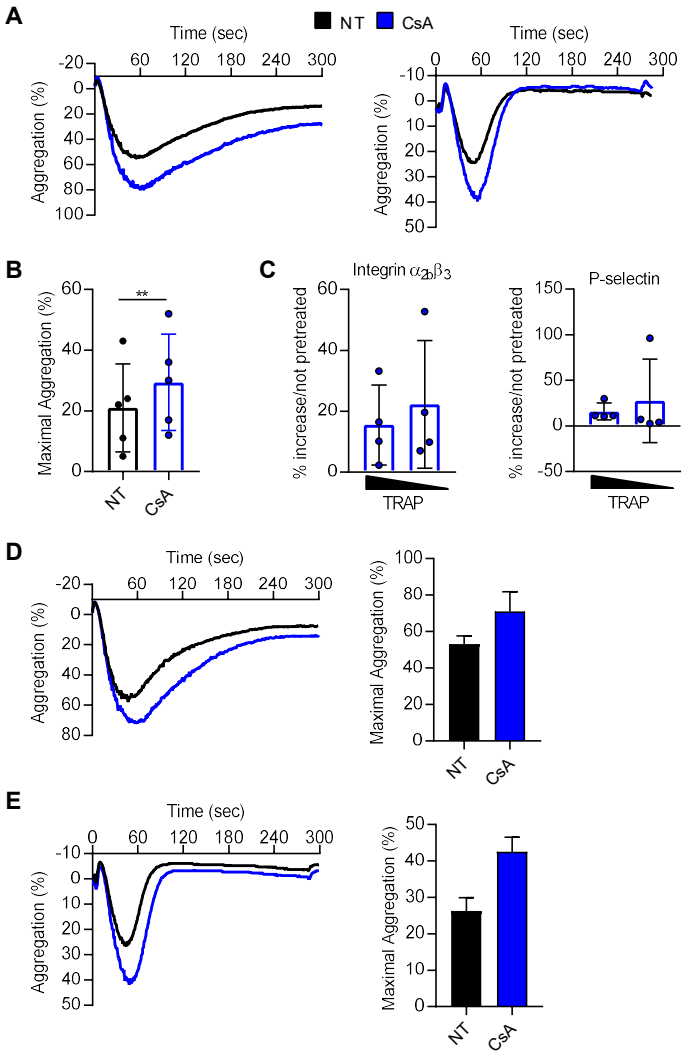

Figure 17| Calcineurin inhibition enhances HPS patients' PRP activation upon TRAP stimulation. A) Aggregation curves of PRP from HPS type 2 patient pretreated for 2 h with 1000 ng/ml CsA (CsA) and stimulated with 5 µM (left) or 3 µM (right) TRAP. B) PRP from HPS type 2 patient was pretreated for 2 h with 1000 ng/ml CsA (CsA) and stimulated with 3 µM TRAP. Data are represented as mean ± SD (n=5 independent blood drawing from the same donor). Paired two tailed *t*-test was used for statistics. ***P* < 0.01. C) PRP from HPS type 2 patient was pretreated for 2 h with 1000 ng/ml CsA (CsA) and stimulated

with 3 or 1.5 μM TRAP. Integrin activation (left) and P-selectin exposure (right) are represented as percentage of increase compared to the response of platelets not pretreated with CsA. Each dot represents an independent blood drawing from the same donor. One-sample t test was used for statistics. *$P < 0.05$. D) Aggregation curves of PRP from HPS type 1 patient #1 and (E) patient #2 pretreated for 2 h with 1000 ng/ml CsA (CsA) and stimulated with 3 μM TRAP. Histogram of maximal aggregation is represented as mean ± SD (n=4 technical replicates).

2.1.8 The calcineurin/NFATc2 pathway is partially dependent on the outside-in integrin $\alpha_{2b}\beta_3$ signaling

During the first wave of platelet activation, the inside-out integrin signaling pathway leads to a conformational change of integrin $\alpha_{2b}\beta_3$ to a high affinity fibrinogen binding conformation. Once integrin is activated, its binding to the fibrinogen activates the outside-in integrin $\alpha_{2b}\beta_3$ signaling [11]. This pathway is fundamental in stabilizing platelet aggregation, activation and thrombus formation. We analyzed if the outside-in signaling pathway was involved in the regulation of platelet functions downstream of the calcineurin/NFATc2 pathway. We found that inhibition of outside-in signaling, with the integrin $\alpha_{2b}\beta_3$ inhibitor eptifibatide, partially rescued the hyperactivated phenotype of FK506-treated murine platelets, as measured by P-selectin exposure (Fig. 18). This hints that the calcineurin/NFATc2 pathway modulates α-granules release with a mechanism partially dependent on the outside-in integrin signaling pathway.

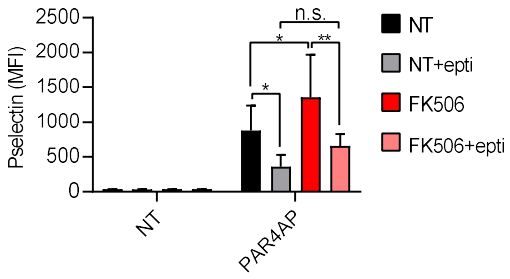

Figure 18| Calcineurin inhibition effect on α-granules release is dependent on the outside-in integrin signaling. Murine PRP was incubated or not for 1 h with 1 µM FK506 and pretreated or not for 10 min with 3 µg/ml eptifibatide, then stimulated with 150 µM PAR4-AP. Data are represented as mean ± SEM (n=3). A two-way ANOVA test was used for statistics. *$P < 0.05$ and **$P < 0.01$.

We further investigated the role of the outside-in signaling in the calcineurin/NFATc2 pathway in human platelet using blood from Glanzmann thrombasthenia (GT) patient, that lack the expression of integrins $\alpha_{2b}\beta_3$. We found that calcineurin inhibition is still able to slightly enhance aggregation upon stimulation with different concentrations of TRAP (Fig. 19A and 19B). These results suggest that the calcineurin/NFATc2 pathway modulates aggregation in human platelets with a mechanism independent on the outside-in integrin $\alpha_{2b}\beta_3$ signaling, diversely to what observed for α-granules release regulation in murine platelets.

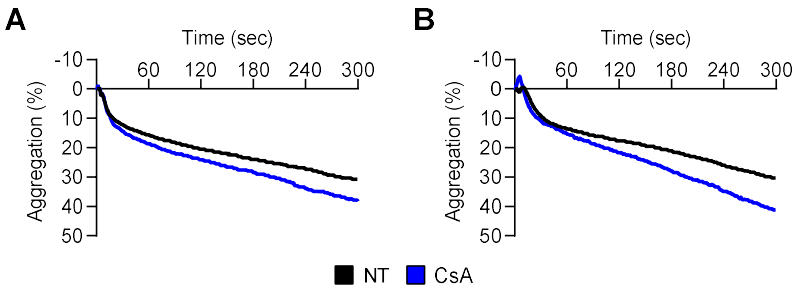

Figure 19| Calcineurin inhibition enhances platelet aggregation in Glanzmann thrombasthenia patient's PRP. Aggregation curves of PRP from GT patient pretreated for 2 h with 500 ng/ml CsA (CsA) and stimulated with 20 µM (A) or 10 µM (B) of TRAP.

2.1.9 The calcineurin/NFATc2 pathway modulates platelet-induced NET formation *in vitro*

It is well known that platelets interact with immune cells and they are also able to modulate the immune response. In particular, platelet-neutrophil complex (PNC) is one of the most characterized heterotypic interaction and it plays a very important role during inflammation or in thrombotic disorders, such as sepsis and bacterial infection, as well as atherosclerosis [13]. P-selectin on platelet surface has a key role in mediating the physical interaction between neutrophils and platelets and the formation of these heteroaggregates [14]. In the complexes composed by platelets and neutrophils, the two cell types modulate each other reciprocally: platelets can stimulate neutrophilic functions, including ROS production and NETs formation, and, in turns, neutrophils can further enhance platelets responses [15].

Considering the close relationship between these two cell types, we assessed whether the calcineurin/NFATc2 pathway plays a role in modulating the ability of platelets to interact with neutrophils. To test the effect of calcineurin inhibition on platelet binding to neutrophils, we analyzed the formation of PNC *ex vivo* upon PAR4 stimulation. Whole blood from mice treated or not with FK506 was stimulated with PAR4-AP and analyzed for PNC formation by FACS analysis. We found that, upon stimulation, the percentage of neutrophils positive for the platelet marker CD41 is only slightly higher in the FK506-treated group compared with the non-treated one (Fig. 20A). Notably, we found that the MFI of CD41 on the cell surface of neutrophils is significantly higher in the FK506-treated group (Fig. 20B), suggesting that the number of neutrophils bound by at least one platelet in the two groups is similar but that, upon the inhibition of calcineurin, there are more platelets bound per single neutrophil.

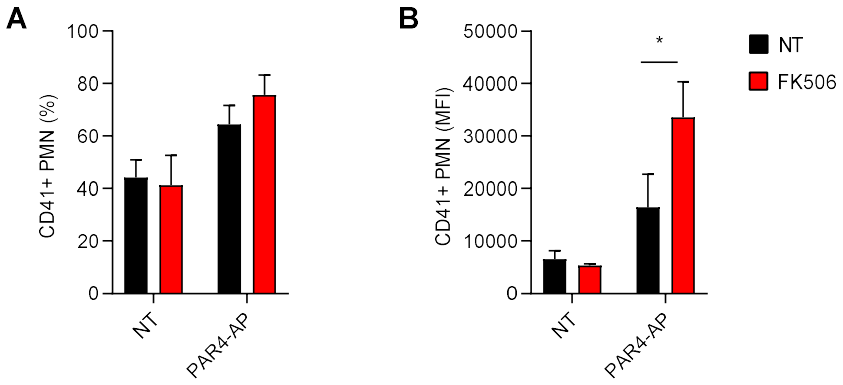

Figure 20| Calcineurin/NFATc2 pathway modulates platelets-neutrophils physical interaction. Whole blood from mice treated or not with FK506 for 14 days was

stimulated with 100 µM PAR4-AP. PNC formation was analyzed by FACS. Results are represented (A) as percentage of neutrophils (Ly6G+CD11b+) CD41+ and (B) as MFI for CD41 in Ly6G+CD11b+ gate. Data are represented as mean ± SEM (n=4). A two-way ANOVA test was used for statistics. *$P < 0.05$.

These data indicate that the enhancement observed in P-selectin exposure upon calcineurin inhibition correlates with a higher ability of platelets to interact with neutrophils in our *ex vivo* experimental setting.

In different experimental settings it has been demonstrated that platelets activated with their canonical stimuli, like thrombin, or bacterial ligand, like LPS, can induce release of NETs by neutrophils [16]–[18], we next tested if the increased interaction observed between platelets and neutrophils results in a better induction of NETs release. We tested platelet-induced NETs release by neutrophils *in vitro* using an immunofluorescence assay. After stimulation of neutrophils with activated platelets, we fixed the cells and stained nuclei with DAPI (blue) and NETs with an anti-citrullinate histone H3 antibody (red) (Fig. 21A). We found that FK506-treated platelets stimulated with PAR4-AP, PAR4-AP plus LPS, or LPS alone were able to better induce NETs release compare to non-treated platelets (Fig. 21B). These data demonstrate that the hyperactivated phenotype of platelets due to calcineurin inhibition well correlates not only with a better ability to interact with neutrophils but also with the capacity of platelets to stimulate the release of NETs.

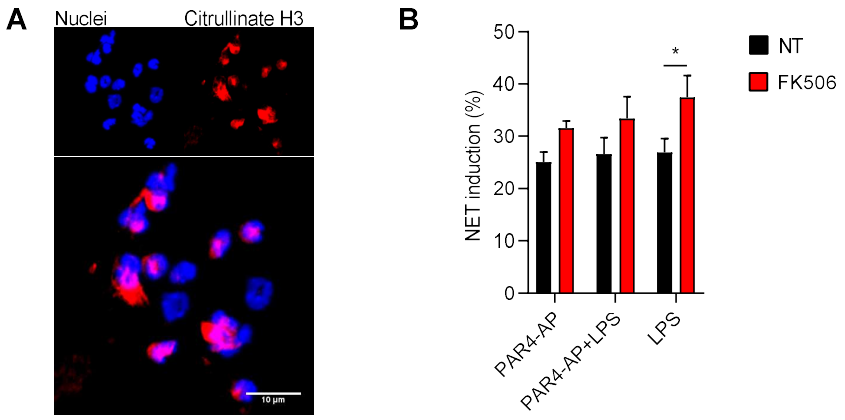

Figure 21| Calcineurin/NFATc2 pathway modulates platelet-induced NETs release by neutrophils. A) NETs formation was analyzed by immunofluorescence. Nuclei were stained with DAPI (blue), NETs were stained with anti-citrullinate histone H3 antibody (red). B) Quantification of NETs as percentage of neutrophils positive for citrullinate histone H3. Data are represented as mean ± SEM (n=4). A two-way ANOVA test was used for statistics. *$P < 0.05$.

2.1.10 The calcineurin/NFATc2 pathway modulates platelet-neutrophil interaction in pathological conditions

Exaggerated platelet-induced NETosis has been shown to be detrimental in the context of sepsis, causing intravascular coagulation and organ failure [16]. We wanted to further analyze the effect of the inhibition of the calcineurin/NFATc2 pathway in platelets on platelet-neutrophil interaction in two *in vivo* models of sepsis. We used a platelet transplant model to have mice in which only platelets were treated with FK506. We found that mice transplanted with FK506-treated platelets were more susceptible to LPS-induced sepsis, showing a more accentuated body

temperature drop (Fig. 22A) and higher mortality compared to the control group (Fig. 22B). Moreover, the levels of the DNA in the plasma, often used as an indirect marker on NET release in the bloodstream, was significantly higher in mice reconstituted with FK506-treated platelets compare to mice reconstituted with non-treated platelets (Fig. 22C).

To visualize NET release by neutrophils we used an *E. Coli*-induced sepsis model in mice reconstituted with platelets treated or not with FK506. We used an imaging-based protocol that allowed us to visualize *ex vivo* NETs formed *in vivo*. We found that FK506-treated platelets were able to better induce NET formation as observed both into the peritoneal lavage (Fig. 22D and quantify in Fig. 22F) or on the peritoneal wall (Fig. 22E and 22G). Moreover, this enhancement in NET release correlated with a higher amount of citrullinate histone H3 in liver of mice reconstituted with FK506-treated platelets (Fig. 22H). Taken together these data demonstrate that the inhibition of the calcineurin/NFATc2 pathway results in an hyperactivated state of stimulated platelets that are more efficient not only in physically interacting with neutrophils but also in inducing NETosis.

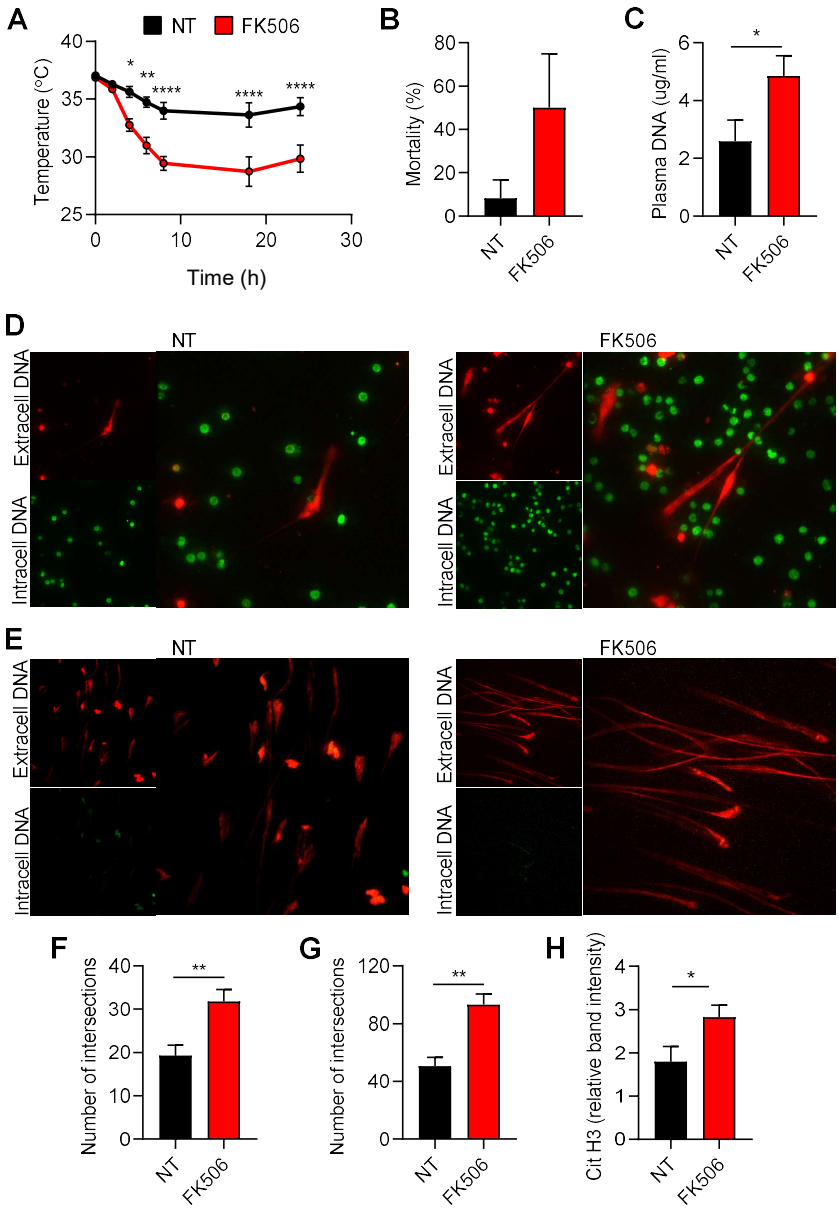

Figure 22| Calcineurin/NFATc2 pathway inhibition exacerbates outcome of two murine models of sepsis. Platelet-depleted mice transplanted with platelets treated or not *in*

vitro for 1h with 1µM FK506 were injected with 10 mg/kg LPS. (A) Body temperatures, (B) mortality rate and (C) plasma DNA content are shown. Data are represented as mean ± SEM (n=12, three independent experiment). A two-way ANOVA test and unpaired two tailed *t*-test were used for statistics. Platelet-depleted mice transplanted with platelets treated or not *in vitro* for 1h with 1µM FK506 were injected with 1×10^8 *E. Coli*. Representative images of (D) peritoneal lavage or (E) peritoneal tissue are shown. Extracellular DNA were stained with Sytox orange (red) and intracellular DNA with Syto green (green). Quantification of extracellular DNA in (F) peritoneal lavage or (G) peritoneal tissue from different images. Data are represented as mean ± SEM (n=7 and n=4 respectively). H) Quantification of relative band intensity in western blots for citrullinate histone H3 from liver extracts (n=8, two independent experiments). Unpaired two tailed *t*-test were used for statistics. $*P < 0.05$, $**P < 0.01$ and $****P < 0.0001$.

2.2 Materials and methods

Antibodies and chemicals. Antibodies for immunoblot (NFATc1, NFATc2, NFATc3, NFATc4 and p65) were purchased from Cell Signaling. Actin antibody for immunoblot and all chemicals were purchased from Sigma-Aldrich unless otherwise specified. Cyclosporin A was purchased from Enzo life sciences, α and ϒ thrombin were from Heamatologic Technologies Inc. ARC-66096 was purchased from Tocris. All antibodies for flow cytometry (PE/Cy7 anti-mouse CD41, APC anti-mouse CD62P, PE anti-human CD62P and APC anti-human CD42b) were purchased from BioLegend. Antibody for the active form of the integrin $\alpha_{2b}\beta_3$ was purchased from Emfret Analytics for murine isoform (PE-conjugated, clone JON/A) and from BD Bioscience for human isoform (FITC-conjugated, clone PAC1).

Murine platelets isolation. Murine blood was obtained from retro orbital plexus of wild-type (WT) or NFATc2KO mice using sodium citrate as anticoagulant. For *in vivo* treatment with FK506 mice were injected daily with 4 mg/kg of FK506 for 14 days. From whole blood, platelet-rich plasma (PRP) was obtained by centrifugation at 100 × *g* for 10 minutes without brake at room temperature. Washed platelets (WPs) were obtained by washing PRP with an equal volume of Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 2.8 mM D-glucose monohydrate, 0.4 mM NaH2PO4, 5 mM HEPES, pH 7.4) with 0.02 U/ml apyrase and 140 nM PGI_2 at 2000 × *g* for

10 minutes and by resuspending the pellet in Tyrode's buffer with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Where indicated PRP was pretreated *in vitro* for 1 h with 1 µM FK506 or 250 ng/ml cyclosporine A (CsA). Platelet purity and count were assessed by flow cytometry. The platelet concentration was calculated using CountBright Absolute Cell Counting Beads (Thermo Scientific) according to the manufacturer's instructions.

Human platelet isolation. Human blood was obtained from healthy adult volunteers, who have not taken medication within a week before donation, or patients with Hermansky-Pudlak syndrome or Glanzmann thrombasthenia thanks to the collaboration with the Center for Platelet Research Studies (Boston). PRP was obtained by centrifugation at 110 × *g* for 12 minutes without brake at room temperature. Where indicated PRP was pretreated in vitro 2 h with different doses of FK506 or CsA. Platelet purity and count were assessed by flow cytometry.

Immunoblotting. 20×10^6 WPs were stimulated for the indicated time points at room temperature in low-binding Eppendorf tube and then were lysed using 5X Laemmli buffer. Samples were boiled for 5 minutes at 95°C and resolved on 10 % SDS-PAGE or precast 7.5 % SuperSep Phos-Tag gels (Wako Pure Chemical Corporation) where indicated. Primary antibodies were used at a 1:1000 dilution in TRIS buffer saline (TBS)-0.1% tween 5 %

BSA. Secondary antibodies were diluted 1:5000 in TBS-tween 5 % non-fat dry milk.

Platelet aggregation and secretion. 240 µl of PRP adjusted with platelet-poor plasma at a concentration of 1.5-2 × 10^5 plts/µl was stimulated in a Model 700 Aggregometer (Chrono-Log) with 10 µl of the indicated stimuli. Prior stimulation, 10 µl of luciferin-luciferase Chrono-Lume were added to measure secretion of dense granules content. Aggregation curves and luminescence were recorded for 5 minutes. At the end of the test, an ATP standard was added to quantify the amount of ATP released.

Platelet activation markers assay by flow cytometry. 10 µl of PRP adjusted with Tyrode's buffer at a concentration of 1-1.5 × 10^5 plts/µl were stimulated with 5 µl of the indicated stimuli. Simultaneously, samples were stained with 10 µl of antibody mix for CD41, CD62P and active form of integrin $\alpha_{2b}\beta_3$. After 15 minutes, samples were fixed with 400 µl of 1 % formaldehyde in Tyrode's buffer. Data were acquired using a BD FACS Canto II and analyzed with FlowJo software.

Platelet spreading assay. Glass coverslips were coated with fibrinogen (150 µg/ml) overnight at 4°C in a 24 multiwells. 2 × 10^6 WPs in 400 µl of Tyrode's buffer were plated on coverslip, stimulated or not with the indicated stimuli and let adhere for 45 minutes at room temperature.

Samples were fixed with 4 % paraformaldehyde and permeabilized 10 minutes with 0.2 % BSA 0.5 % Triton X-100 in PBS. Then, samples were kept in blocking solution (2 % BSA in PBS) for 30 minutes and actin was stained with phalloidin AlexaFluo488 (LifeTechnologies) in blocking solution for 20 minutes. Samples were mounted and were imaged using a fluorescence wide-field microscope. Images were analyzed with ImageJ software (NIH) and area of single platelets was measured in 5 different field of views per sample.

Tail bleeding time. Mice were sedated with 100 µl of a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml) and the tail was cut 4 mm from the distal tip. Blood drops from the tail were collected on blotting paper every 20 seconds until the complete stop of the bleeding. The bleeding time was defined as the time point at which there was no visible sign of blood on the blotting paper.

Thromboembolism model. Mice sedated with a mixture of ketamine and xylazine were injected with the indicated volume of a combination of collagen (250 µg/ml) and epinephrine (1.5 µg/ml). Mice were observed for 60 minutes and scored for a pathology index as following described: 6 = dead, 5 = asleep without paw reflex, 4 = asleep with paw reflex, 3 = awake without paw reflex, 2 = awake with paw reflex, 1 = moving without paw reflex, 0 = moving with paw reflex.

Investigation of platelet-neutrophil complexes. Whole blood from WT or FK506-treated mice was diluted 1:2 in Tyrode's buffer, then stimulated with PAR4-AP for 30 minutes at room temperature and, at the same time, stained with antibody anti-CD41, anti-Ly6G and anti-CD11b. Samples were fixed with FACS lysing solution (BD Bioscience). Data were acquired using a BD FACS Canto II and analyzed with FlowJo software.

***In vitro* platelets-induced NET formation.** Naïve neutrophils from the bone marrow of WT mice were purified over a 62.5% Percoll gradient (GE Healthcare) in Ca^{2+}- and Mg^{2+}-free HBSS as previously described [19]. Neutrophils were plated on fibrinogen-coated coverslip and stimulated 1 h at 37°C with an equal volume of PRP previously stimulated or not with PAR agonist and/or LPS for 5 minutes. Stimulation of neutrophils with platelet-poor plasma (PPP) and phorbol myristate acetate (PMA) were used, as negative and positive controls respectively. Samples were fixed adding an equal volume of 4 % paraformaldehyde for 30 minutes at 37°C. Then, cells were blocked with 2 % BSA in PBS for 45 minutes at room temperature and stained for NETs with anti-citrullinate histone H3 overnight at 4°C (Abcam ab5103), followed by anti-rabbit Alexa 568 secondary antibody. Nuclei were stained with DAPI. Samples were mounted and were imaged using a fluorescence wide-field microscope. Images were analyzed with ImageJ software (NIH) and neutrophils were

considered positive for NETs formation when DAPI colocalized with citrullinate histone H3 signal.

Platelet transplant model. Platelet depletion was induced in 10-12 weeks old WT mice by retro-orbital injection of anti-CD41 antibody (clone MWReg30, Biolegend), 5 μg/mouse 2 days before platelet reconstitution and 2 μg/mouse 1 day before platelet reconstitution. Peripheral platelet counts were determined by flow cytometry 12 hours after platelet depletion. For reconstitution, washed platelets from several donor mice were pooled and 10^9 platelets, treated or not with FK506, were transferred i.v. retro-orbitally into platelet-depleted WT mice. Peripheral platelet counts were determined by flow cytometry 30 minutes after platelet transfer and mice were subsequently subjected to sepsis induction.

LPS-induced sepsis model. Mice were injected intra peritoneally with 10 mg/kg of lipopolysaccharide (LPS O55:B5 serotype, Sigma). Body temperature was measured every two hours. Survival was assessed 18 hours later. and DNA plasma content was quantified by Quant-iT™ PicoGreen™ dsDNA Reagent (Invitrogen).

***E. Coli*-induced sepsis model.** Mice were i.p. injected with 1×10^8 CFU/mouse of *E. Coli*. NETs formation was assessed 8 hours later in the

peritoneal lavage and on the serosal surface of the peritoneal tissue, as previously described [20]. Briefly, mice were sacrificed in a CO_2 chamber and the peritoneal fluid and membrane samples harvested. The peritoneal cavity was rinsed with sterile PBS solution (200 µl per side) and analyzed for *in vivo* NET formation. Peritoneal fluids and membranes were placed on poly-L-lysine coated coverslip and stained for intracellular and extracellular DNA, with Syto Green and Sitox Orange (Invitrogen) respectively. NETs were qualitatively and quantitatively analyzed using confocal microscopy, followed by standardized grid analysis of 5 randomly selected fields of views per sample (ImageJ, NIH). From the same mice, presence of citrullinate histone H3 in liver extracts was assessed by western blots. Relative quantification is expressed as relative band intensity of citrullinate histone H3.

Statistical analysis. GraphPad Prism statistical software (version 7) was used to analyzed results. All data are presented as the mean ± SEM. For parametric data, statistical significance was analyzed using Student's *t*-test or ANOVA, following determination of normal distribution and equal variances. Sample sizes for each experimental condition are provided in the figures and the respective legends. Asterisks were used as follows (also indicated in figure legends): *P < 0.05, **P < 0.01, ***P < 0,001 and ****P < 0,0001.

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Chapter 3: Final considerations

3.1 Summary

Platelets have been known as key players in the hemostatic process but, more recently, it was demonstrated that they have an important role also in modulating the inflammatory response by establishing direct and indirect interactions with immune cells [1].

Interestingly, although platelets are anucleated cells, they contain functional transcription factors (TFs) that modulate their activation via non-transcriptional functions. In particular, it has been shown that platelets contain NF-κB, a TF that controls important functions of immune cells during the inflammatory process, and that NF-κB regulates platelet activation [2].

In this work, we investigated the possibility that other TFs different from NF-κB might play regulatory roles during platelet activation. We focus our attention on a TF typically expressed by immune cells: nuclear factor of activated T cells (NFAT). NFAT family contain 5 different proteins: NFATc1, NFATc2, NFATc3 and NFATc4 regulated by calcium fluxes and NFAT5 regulated by osmotic stress [3]. Inhibition of the NFAT pathway, especially in T cells, using calcineurin inhibitors, is crucial in the immunosuppressive treatment in transplanted patients to avoid organ rejection. However, it has been reported that calcineurin inhibition with cyclosporin A and tacrolimus (FK506) affects platelet functionality in transplanted patients

[4], [5]. Moreover, calcium fluxes, that mediate NFATc activation in immune cells, are also an essential element in platelet activation processes [6].

We found that, upon PAR4 stimulation, the calcineurin/NFATc2 pathway is activated and regulates platelet functions. This pathway is activated both in murine and human platelets, and its inhibition results in enhancement of platelet aggregation, integrin activation, granules release and spreading on fibrinogen. By using murine *in vivo* models, we demonstrated that NFATc2 activation in platelets is involved in modulating hemostasis and thrombosis. Moreover, platelet NFATc2 activation regulates the interaction between platelets and neutrophils and impacts the severity of sepsis development. Our *in vitro* experiments using different chemicals inhibitors suggest a model in which NFATc2 acts downstream of PAR4 via a mechanism that involves both the ADP receptor and the outside-in integrin $\alpha_{2b}\beta_3$ pathway.

Finally, we found that inhibition of the calcineurin/NFATc2 pathway partially rescues platelet activation defects in two rare human platelet pathologies, the Hermansky-Pudlak syndrome and the Glanzmann thrombasthenia.

3.2 Conclusions and future perspectives

The discovery that a transcription factor family, NFATc, regulates the activation of platelets reveals the complexity of these anucleated cells and confirms that platelets are not simply megakaryocytic fragments. As previously mentioned, since their first discovery as “the third morphological element of the blood” [7], platelets have been mostly studied for their function in maintaining the integrity of the vessels walls. However, in the latest decades, it has been revealed that platelets are also an integral part of the immune system and that they constitute a bridge between hemostasis and inflammation. Platelets are the first element present at the site of vessel injury, they can promptly bind possible invading pathogens and release antimicrobial peptides. Moreover, they can recruit and interact directly or indirectly with immune cells and modulate the inflammatory response [1].

The ability of platelets to exert all these different functions seems to be in contrast with the lack of a nucleus, an element that is considered the final container of all the information and programs of a cell. Nevertheless, it has been shown that platelets contain mRNA, ribosomes, and all the components necessary for protein translation and that they can modulate protein production based on their needs [8]. Moreover, platelets do not have a nucleus, but they contain TFs that are not vestigial remnants inherited from megakaryocytes. These TFs appear to have a role, only partially understood, in regulating platelet functions [9]. Among other TFs

previously investigated to control platelet functions, we described for the first time that platelets do contain NFATc2 and that this TF has a role in regulating platelet activation upon PAR stimulation. We showed that inhibition of the calcineurin/NFATc2 pathway, both in murine and human platelets, results in a hyperactivated phenotype, by measuring different aspects of platelet activation, such as aggregation, α- and dense granules release, integrin $\alpha_{2b}\beta_3$ activation and spreading on fibrinogen. This unveils that the calcineurin/NFATc2 pathway negatively regulates platelet response upon PAR stimulation and that NFATc2 is necessary to turn off the signaling to avoid excessive platelet activation. This pathway not only is involved in modulating platelet responses in *in vitro* experimental settings, but we found that it is also required to regulate platelet functionality in hemostasis and thrombosis *in vivo*, in a tail bleeding model and in a thromboembolism model respectively. Our data suggest that the calcineurin/NFATc2 pathway is actively involved in modulating all the aspects of platelet activation and interactions with other platelets and immune cells present in the blood.

In particular, we focused our attention on platelet-neutrophil interaction. It is well known that platelets and neutrophils interact with and modulate each other; activated platelets can stimulate neutrophils response, such as NET release, and, on the other hand, neutrophils can further stimulate platelet activation, for example through NET components or by releasing soluble mediators [10], [11]. Uncontrolled formation of platelet-

neutrophil complexes contributes to the etiology of different thrombotic and infectious diseases [12]. In the context of sepsis, improper platelet activation results in disseminated intravascular coagulation (DIC) that enhances inflammation and neutrophils activation. Subsequently, NETs released by neutrophils further stimulate platelet activation and thrombi formation, contributing to poor tissue oxygenation and organ failure [13]. We found that the hyperactivated phenotype resulting from the calcineurin/NFATc2 inhibition correlates with a higher ability of these platelets to interact with and stimulate neutrophils response, both in *in vitro* experimental set ups and *in vivo* bacterial sepsis models. In these models, we found that the hyperactivation of platelets exacerbates the pathology, inducing more consistent NETs release and leading to a poor outcome. These results corroborate the concept of a close relationship between platelets and neutrophils and the importance of the regulation of this interaction in pathophysiological conditions.

From a molecular perspective, we found that the calcineurin/NFATc2 pathway acts via the ADP-receptor and the outside-in integrin $\alpha_{2b}\beta_3$ pathway. However, further studies are needed to understand the exact molecular mechanism through which NFATc2 exerts its non-transcriptional role. In nucleated cell, cytosolic NFATc2 is in a big complex that includes all its regulatory elements, such as calcineurin, calmodulin, kinases that are involved in NFATc2 phosphorylation and dephosphorylation, such as GSK3, CK1, LRRK2, DYRK, the long non-coding

RNA NRON and the scaffold protein IQGAP [14]. Some of these elements are also present in platelets, where they have specific roles in modulating platelet activation. For example, IQGAP1 is involved in modulation of calcium intracellular level and phosphatidylserine (PS) exposure, and it is considered a negative regulator of platelets procoagulant activity [15]. GSK3 is another negative regulator of platelet function [16], its inhibition in both murine and human platelets leads to enhanced aggregation and ATP release upon PAR stimulation [17]. Moreover, CK1 is present in platelets, it is involved in the canonical Wnt-β-catenin pathway [18] and it can also be released to phosphorylate plasma proteins [19]. Future aim of this projects is to evaluate wheatear this NFATc2 complex is also present in platelets and, if so, which proteins are involved in the regulation of platelet functions. Therefore, we suggest that the non-transcriptional role of NFATc2 could be related to its ability to interact or not with other elements, present in the complex or involved in this regulatory process, and that in this way NFATc2 modulates the activity of platelets in a specific spatiotemporal window upon PAR stimulation. Our experiments with NFATc2KO platelets and with FK506 suggest, respectively, that it is important not only the presence of NFATc2 but also its ability to be activated by the calcineurin. In this new scenario, we found that, as in nucleated cells, NFATc2 is dephosphorylated upon PAR stimulation. It is still to be determined whether in platelets this dephosphorylation reflects a required step to activate NFATc2, or it is only a concomitant and

dispensable event not related to its non-transcriptional function. On the other hand, recent studies demonstrated that TFs can have a non-transcriptional role in nucleated cells too, as exemplified by STAT3 in the mitochondria [20]. Analogously, in the future it could be very interesting to dissect the presence of the non-transcriptional function of NFATc2 also in nucleated cells.

Moreover, to understand NFATc2 functionality it could be useful to address its specific subcellular localization prior and after stimulation. Indeed, it has been shown that in platelets different subcellular localization of different elements can be used to modulate their interaction and functionality, for example, mRNAs and eukaryotic initiation factor eIF4E reside in different compartments of platelets until activation mediate their relocalization to the cytoskeleton that leads to their interaction and subsequent mRNA translation [21]. It could be possible that NFATc2 occupies different subcellular localization before and after the stimulation, and this relocalization regulates its interaction with other proteins in its pathway in order to modulate and turn off the PAR signaling pathway.

A more profound understanding of the activation pathways in platelets can be incredibly useful for the development of new therapeutic ways of intervention. Nowadays human pathologies that affect platelet functionality have few therapeutic possibilities and none of them is curative [22]. Thanks to the collaboration with the Center for Platelet

Research Studies (Boston, USA) we had access to blood samples from patients with vary rare platelet disorders. We found that inhibition of the calcineurin/NFATc2 pathway improves platelet response in blood samples from patients with the Hermansky-Pudlak syndrome and the Glanzmann thrombasthenia. Our data strongly suggest that the calcineurin/NFATc2 pathway can be targeted to develop innovative therapeutic approaches to treat platelet dysfunctions with poor prognosis. In this context, the drugs currently available to block NFATc2 activation act on the phosphatase calcineurin with consequent off targets effects. Identification of the exact non-transcriptional function of NFATc2 could help in the design of new molecules to specifically inhibit this pathway in platelets and reduce side effects.

In conclusion, our work reveals for the first time that the activation of the calcineurin/NFATc2 pathway in platelets has a non-transcriptional role and it is a key negative regulator of platelet responses. Further studies are needed to characterize the mechanism of action through which NFATc2 exerts its non-transcriptional function in modulating platelet activation and to understand if NFATc2 could have a non-transcriptional role also in nucleated cells.

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