Platelets shape the immune response of primary human monocytes

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إلى أمي وأبي

dedicated to my mother and father

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List of abbreviations

Adj. p-value	Adjusted p-value
AIM2	Absent in melanoma 2
ALRs	Absent in melanoma-2-like receptors
ASC	Apoptosis-associated speck-like protein containing a CARD
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
CCR2	C-C chemokine receptor type 2
CD	Cluster of differentiation
CDKs	Cyclin-dependent kinases
CLRs	C-type lectin-like receptors
COVID-19	Coronavirus diseases 2019
CSAIDs	Cytokine-suppressive anti-inflammatory drugs
CTB	CellTiter-Blue
CytoD	Cytochalasin D
DAMPs	Danger-associated molecular patterns
DCs	Dendritic cells
DEGs	Differentially expressed genes
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
Ephs	Ephrin receptors
ERK	Extracellular signal-regulated kinases
EVs	Extracellular vesicles
FACS	Fluorescence-activated cell sorting
FC	Fold change
FCS	Fetal calf serum
FS	Full speed
FSC	Forward scatter
GFP	Green fluorescent protein
GO	Gene ontology
GP	Glycoprotein
HET	House of Experimental Therapy
HIV	Human immunodeficiency virus
HTRF	Homogeneous Time Resolved Fluorescence
i.v.	Intravenous
IFN-γ	Interferon gamma
IgG	Immunoglobulin G

IKK	IkappaB kinase
IL	Interleukin
IRFs	Interferon-regulatory factors
ISGs	Interferon-stimulated genes
ITP	Idiopathic thrombocytopenia
JAK	Janus kinase
JNKs	c-Jun N-terminal kinases
KEGG	Kyoto encyclopedia of genes and genomes
Lat-B	Latrunculin B
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MAPKAPKs	Mitogen-activated protein kinase-activated protein kinases
MDSCs	Myeloid-derived suppressor cells
MKKs	MAPK kinases
MKKKs	MAPK kinase kinases
MoPD	Platelet-depleted monocytes
MoStd	Standard monocytes
MPAs	Monocyte-Platelet aggregates
mRNA	Messenger RNA
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor-kappa-B
Nig	Nigericin
NLRs	Nod-like receptors
NLRC4	NLR family CARD domain-containing protein 4
NLRP3	NACHT, LRR and PYD domain-containing protein 3
PA	Protective antigen
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PFA	Paraformaldehyde
PGE1	Prostaglandin E1
PKA	Protein kinase A
PKC	Protein kinase C
Plts	Platelets
PMA	Phorbol-12-myristate-13-acetate
PRP	Platelet-rich plasma
PRRs	Pattern recognition receptors
PS	Phosphatidylserine

PTKs	Protein tyrosine kinases
PVDF	Polyvinylidene fluoride
R848	Resiquimod
RGD	Arginine-Glycine-Aspartate tripeptides
rh	Recombinant human
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
RNA-Seq	RNA-Sequencing
RT	Room temperature
SEAP	Secreted alkaline phosphatase
Siglecs	Sialic acid-binding immunoglobulin-type lectins
SSC	Side scatter
STKs	Serine/Threonine kinases
Sups	Supernatants
TBS	Tris buffered saline
TFs	Transcription factors
TGF-β1	Transforming growth factor β1
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor alpha
UKA	Upstream kinase analysis

1. Introduction

1.1 The immune system

Living organisms are constantly exposed to external noxae. Physical stress, environmental changes, physiological alterations as well as infections and malignancies represent only a few of the challenges that an organism has to combat or adapt to, to ensure survival. One way to do this is through the immune system, acting as a “firewall” controlling the host defense against pathogens and maintaining its integrity. Generally, the immune system can be divided into the innate and the adaptive immune system. Both act in synergistic and complementary ways, as the innate immune system is able to induce the initiation of adaptive immune responses. While the innate immune system recognizes a wide range of pathogen-derived patterns, such as bacterial lipopolysaccharides (LPS) or viral ribonucleic acid (RNA), and rapidly reacts to achieve pathogen clearance, the adaptive immune system is pathogen-specific and characterized by a lag phase until the onset of the cellular and humoral immune responses, followed by the establishment of an immunological memory (Marshall et al., 2018; Nicholson, 2016). Both arms of the immune systems are crucial for host defenses, for which the innate immune system provides the first line of action, supported by the second, more specific adaptive immune responses with a long-term effect (Marshall et al., 2018; Paludan et al., 2021).

1.1.1 Immune responses

A pathogen has to overcome several barriers to invade an organism and disrupts its integrity. Physical and chemical barriers such as the skin, mucosa associated lymphoid tissues, hydrolytic enzymes in the mucus, but also the microbiota populating the skin and the gut provide the initial layers of defense against such invasions. When these barriers fail, the immune system switches to an alert state, and the innate immune cells come into play. The innate immune cells express pattern recognition receptors (PRRs), which sense pathogen-associated molecular patterns (PAMPs), such as LPS, peptides, proteins or nucleic acids derived from the infecting microbes. In response, innate immune cells produce cytokines, such as interleukin (IL) 1β, IL-6 and tumor necrosis factor α (TNFα),

to induce inflammation, and release chemokines to recruit more immune cells to the site of inflammation. Monocytes, macrophages, neutrophils and dendritic cells (DCs) can phagocytose pathogens and lead to their clearance (Marshall et al., 2018). Moreover, neutrophils can induce a defense mechanism, by which they expel extracellular traps (NETs) that can capture and neutralize pathogens (Brinkmann et al., 2004). Macrophages and DCs do not only engulf pathogens, but they also process and present pathogen-derived epitopes to T cells, thus inducing a second wave of the immune response via the adaptive immune system. Natural killer cells also belong to the innate immune system and play an important role in inducing programmed cell death of infected or malignant cells by the secretion of perforins and granzymes, leading to cell death and lysis (Marshall et al., 2018; Nicholson, 2016).

As the innate immune system focuses on achieving a rapid pathogen clearance, it initiates as well the onset of defense mechanisms of the adaptive immune system. The antigen presentation by antigen-presenting cells activates antigen-specific T cells. Cytotoxic T cells are involved in the induction of cell death of infected or malignant cells. Helper T cells provide “help” in activating other immune cell types, among these B cells. In turn, antigen-specific B cells undergo clonal proliferation in the lymph nodes resulting in the production of antigen-specific antibodies, and consequently in the induction of antibody-driven immune responses. After pathogen clearance, the adaptive immune system manifests and maintains an immunological memory, which results in a faster and more efficient immune response to subsequent encounters with the same pathogen (Chaplin, 2010).

1.1.2 Innate immune signaling pathways

For the initiation of an innate immune response, leukocytes first need to detect the invading pathogens, and then transmit a signal to activate downstream effector cascades. PRRs are a group of transmembrane and soluble receptors that can sense a variety of PAMPs, as well as danger-associated molecular patterns (DAMPs). Leukocytes express several PRRs, such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), Absent in melanoma-2-like receptors (ALRs) and C-type lectin-like receptors (CLRs). Each group of PRRs recognizes various PAMPs, resulting in a signal

transduction, for example, via the nuclear factor-κB (NF-κB) or mitogen-activated protein kinases (MAPKs) signaling pathways. As a result, the transcription of pro-inflammatory genes is initiated, leading to the production and release of cytokines and the induction of inflammation (Cui et al., 2014; Li & Wu, 2021).

The diversity of PRRs in their cellular localization and epitope recognition enables the detection of a broad variety of pathogens:

1. **TLRs** are conserved type I transmembrane glycoproteins localized both on the cell surface and in the endosomes. They can recognize a broad spectrum of PAMPs such as LPS, flagellin, lipids, unmethylated CpG deoxyribonucleic acid (DNA), as well as single- or double-stranded RNA. These membrane-associated receptors bind the PAMPs or DAMPs and transmit a signal to activate the NF-κB or interferon-regulatory factors (IRFs) downstream pathways and consequently the transcription of pro-inflammatory genes (Cui et al., 2014; Li & Wu, 2021).

2. **ALRs** and **NLRs** are intracellular receptors and sensors that are able to induce inflammasome-driven immune responses. While ALRs specifically recognize intracellular DNA, resulting in the activation of the absent in melanoma 2 (AIM2) inflammasome, the activators of NLRs include a variety of PAMPs and DAMPs (Cui et al., 2014; Li & Wu, 2021). Activation of both ALRs and NLRs results in the assembly of an inflammasome and consequently IL-1β-/IL-18-driven immune responses. Inflammasomes are large protein complexes consisting of a sensor (an NLR or an ALR), the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and the effector protease (pro-) caspase-1. Some of the inflammasome components are expressed in the inflammasome priming phase upon TLR activation, including NACHT, LRR and PYD domains-containing protein 3 (NLRP3) and pro-IL-1β. The assembly of the macromolecular inflammasome complex is induced in the presence of inflammasome-specific activators such as adenosine triphosphate, crystals or pore-forming toxins for the activation of NLRP3, double-stranded DNA for the activation of AIM2, or flagellin for the activation of NLR family CARD domain-containing protein 4 (NLRC4). The recruited and auto-

activated caspase-1 in the inflammasome complex processes pro-IL-1β or pro-IL-18 into their active forms which are then released through unconventional secretion pathways, initiating an IL-1-driven immune response (Broz & Dixit, 2016; Li & Wu, 2021; Schroder & Tschopp, 2010; Sutterwala et al., 2014).

3. **RLRs** are intracellular receptors involved in the antiviral immune response. They sense viral nucleic acids in the cytoplasm and in turn induce type I interferon signaling (Cui et al., 2014; Li & Wu, 2021).

4. **CLRs** are phagocytic receptors that recognize pathogens' carbohydrates, such as on the surface of fungal cell walls. CLRs thus recognize the pathogen and induce its phagocytosis and elimination (Cui et al., 2014; Li & Wu, 2021).

1.1.2.1 The NF-κB signaling pathway

Inflammation and cytokine-driven immune responses are important host defense mechanisms against infection. The recognition of an invading pathogen via PRRs induces the activation of several downstream signaling pathways, prominently including the NF-κB pathway. The basic NF-κB transcription factor module consists of five subunits (p50, p52, p65, RelB and c-Rel), which are active as dimers and are initially sequestered in the cytoplasm. Upon signal initiation, a cascade of phosphorylation events is induced, resulting in the phosphorylation of IκBα, the inhibitory protein masking the nuclear localization sequence of NF-κB. The phosphorylation of IκBα results in its ubiquitination, dissociation from the NF-κB complex, and subsequent degradation by the ubiquitin-proteasome system. As a consequence, a NF-κB subunit, e.g. p65, can translocate into the nucleus and induce the expression of a range of target genes (Liu et al., 2017; Zinatizadeh et al., 2021).

The NF-κB pathway can follow a canonical or non-canonical activation cascade. The canonical NF-κB activation is triggered by microbes and TLR engagement, or by cytokine receptors such as IL-1R or TNFR and involves the formation of p50/RelA and p50/c-Rel dimers (Karin & Ben-Neriah, 2000; Lawrence, 2009). In contrast, the non-canonical

pathway is induced upon binding of bioactive molecules such as CD40L, lymphotoxin β and RANKL, and is mediated by p100 – the precursor of the p52 NF-κB subunit (Dejardin et al., 2002; Lawrence, 2009; Novack et al., 2003; Senftleben et al., 2001). Generally, the activation of the NF-κB signaling pathway results in the expression of pro-inflammatory cytokines, such as CXCL8, IL-6 and TNFα (Lawrence, 2009; Liu et al., 2017). Moreover, the NF-κB pathway plays a central role in the NLRP3-driven immune response. The NLRP3 inflammasome requires a priming step via TLR- or cytokine receptor-induced NF-κB signaling, which is crucial for the transcriptional induction of the inflammasome sensor NLRP3 and the effector cytokine pro-IL-1β (Broz & Dixit, 2016; Zinatizadeh et al., 2021). Therefore, the NF-κB signaling pathway is one of the most crucial and central signaling pathways for immune responses and host defense.

1.1.2.2 The p38 MAPK signaling pathway

Cells are continuously exposed to external stimuli such as environmental changes, chemical or biological triggers, physical stress and changes in nutrient concentrations. As a consequence, cells respond to stress by the activation of the MAPK pathway, a well-characterized stress-induced pathway, consisting of extracellular signal-regulated kinases 1/2 (ERK1/2), ERK5, c-Jun N-terminal kinases (JNKs), and p38 MAPKs (Cuenda & Rousseau, 2007).

P38 MAPKs are present in the cytoplasm and in the nucleus and are involved in stress-induced cellular and inflammatory responses. There are four different p38 MAPKs in mammals: p38α, p38β, p38γ, and p38δ; out of which p38α is the best characterized. In response to environmental or cellular changes, these proline-directed serine/threonine kinases are activated through a dual phosphorylation by the upstream MAPK kinases (MKKs), which itself was activated by the upstream MAPK kinase kinases (MKKKs). Alteration in nutrient levels, pathogen invasion, tumorigenesis, but also signaling molecules such as the cytokines IL-1β and TNFα can activate the p38 MAPK pathway. As a consequence, p38 MAPKs either remain in the cytoplasm or translocate into the nucleus, where they either directly phosphorylate transcription factors (TFs) or other downstream kinases which in turn phosphorylate various TFs that drive the transcription

of a wide array of gene sets. This divergence of downstream signaling outputs makes the p38 MAPK pathway versatile with pleiotropic outcomes (Canovas & Nebreda, 2021; Cuenda & Rousseau, 2007; Raingeaud et al., 1995).

Several studies focused on p38α. This kinase is involved in DNA repair, cell cycle and survival, as well as in responses to stress by regulating autophagy or cell cycle arrest (Canovas et al., 2018; Lee et al., 2002; W. Li et al., 2017). Furthermore, p38α governs inflammatory responses as it controls the expression of pro-inflammatory genes, such as *COX-2*, *IL-6* and *IL-8 (Olson et al., 2007; Tomida et al., 2015)*, and regulates the transcriptional activity of NF-κB (Olson et al., 2007; Saha et al., 2007). Interestingly, Th1 cells show a defective IL-12-/IL-18-induced interferon-γ (IFN-γ) response in the absence of p38α, implicating its regulatory role in T cell responses (Berenson et al., 2006). Several substrates of p38α are kinases as well, which in turn phosphorylate several downstream targets, resulting in the outbranching versatility of the p38 MAPK pathway. The most important p38α substrates are MAPK-activated protein kinases (MAPKAPKs). For instance, MAPKAPK2 coordinates cellular processes such as cell migration, cell cycle arrest or cell death, and is also involved in LPS-induced secretion of IL-6 and TNFα. This kinase can also interact with adenylate-uridylate-rich element-binding proteins, thus playing a key role in gene expression on the post-transcriptional level by regulating the mRNAs stability, e.g. of cytokine genes (Bollig et al., 2003; Cuenda & Rousseau, 2007).

P38α plays a central role in inflammatory processes, both mediating the downstream signals to cytokine receptor activation and positively modulating the production of pro-inflammatory cytokines. For example, the inhibition of p38 disrupts the secretion of IL-1β and TNFα in stimulated human monocytes (Lee et al., 1994). Moreover, the use of inhibitors of p38 MAPKs such as SB203580 and BIRB0796, and other cytokine-suppressive anti-inflammatory drugs (CSAIDs) paved the way to study the involvement of p38 MAPKs in pathological conditions (Kuma et al., 2005; Lee et al., 1994). The inhibition of p38α in T cells improved their expansion and anti-tumor activity, and enhanced the activity of cytotoxic T cells in colorectal tumors by stabilizing the T cell IFN-α receptor 1; as p38 induces its ubiquitination and subsequent degradation (Bhattacharya et al., 2011; Katlinski et al., 2017). Furthermore, p38α is not only involved in host defense, but also

contributes to the pathological mechanisms of several diseases. Indeed, it was shown that p38 MAPK signaling contributes to the pathogenesis of rheumatoid arthritis, chronic obstructive pulmonary disease, and cardiovascular diseases (Chabaud-Riou & Firestein, 2004; Renda et al., 2008; Reustle & Torzewski, 2018; Schett et al., 2000). Hence, the activities and outcome of p38 are divers depending on the biological context: p38 can be beneficial to host defense, but its activity can also lead to the development of various diseases. Thus, p38 MAPKs in general and p38α in particular play an important role in orchestrating and tuning the type and outcome of immune responses, as they show pivotal roles in cytokine production, responses to cellular stress, as well as in the development of diseases. This pleiotropic nature of p38α makes it an interesting but also challenging target for pharmacological interventions in the clinic.

1.2 Monocytes in health and disease

Human monocytes account for approximately 10% of all leukocytes and are involved in several processes such as tissue repair and homeostasis, inflammatory responses and host defense, but also in pathological mechanisms leading to several diseases (Ginhoux & Jung, 2014; Ziegler-Heitbrock & Hofer, 2013). Monocytes are important blood circulating leukocytes, which rapidly migrate to the site of inflammation and exert pro-inflammatory tasks to enable pathogen clearance and effective host defense (Guilliams et al., 2018). Furthermore, monocytes recruited to the site of inflammation can differentiate towards macrophages and DCs. Indeed, although monocytes were previously considered as precursors of macrophages and DCs, monocytes do not always represent a *bona fide* source of these cell types. Recent evidence has shown that the majority of tissue macrophages descend from an embryonic origin and have the ability to self-renew and replenish the tissue-resident macrophage pool. Classical DCs are derived from a bone marrow precursor (Ginhoux & Guilliams, 2016; Mildner & Jung, 2014). Thus, the traditional view of a monocyte as an immature precursor cell whose major aim is to differentiate towards macrophages or DCs does not represent the whole picture – monocytes are a highly plastic and dynamic effector cell population.

Monocytes can be divided into three subpopulations according to their expression of cluster of differentiation (CD) 14 (TLR4 co-receptor) and CD16 (low affinity immunoglobulin G (IgG) receptor): $CD14^{+}CD16^{-}$, classical monocytes; $CD14^{+}CD16^{+}$, intermediate monocytes; and $CD14^{-}CD16^{+}$, non-classical monocytes. Each of these populations is prone to execute a specific task; however, with high flexibility and plasticity as they are able to take on the functions typical to other subpopulations. Classical monocytes represent 80-90% of monocytes. They are phagocytes with high expression of chemokine receptors and thus have high chemotactic capacity. They also secrete high levels of inflammatory mediators such as IL-1β, IL-6 and TNFα. Consequently, this subpopulation exhibits a strong pro-inflammatory phenotype. Intermediate monocytes account for 2-5% of monocytes. They are able to produce cytokines and are characterized by a uniquely elevated ability to present antigens to T cells. This cell population was shown to be over-represented in several diseases, such as asthma, rheumatoid arthritis and colorectal cancer. Lastly, non-classical monocytes account for 2-10% of the monocyte population and are involved in phagocytosis mediated by the complement system and by Fc gamma receptors, antigen processing, but also in wound healing. Although non-classical monocytes do not secrete cytokine levels as high as classical monocytes, they are associated with overproduction of TNFα, especially in viraemic human immunodeficiency virus (HIV) patients, implicating their significance in viral defense (Kapellos et al., 2019; Thomas et al., 2017; Wong et al., 2011).

Regardless of their phenotype, monocytes are generally rapidly recruited to the site of infection or injury, where they recognize and engulf pathogens, present antigens to T cells, and orchestrate inflammatory responses (Karlmark et al., 2012). However, the activity of monocytes can be a double-edged sword as dysfunctions can lead to an excessive immune response and thus pathological conditions. For example, in liver fibrosis, monocytes were shown to play a dual role. After the exposure of collagen in the liver tissue, monocytes infiltrate the fibrotic tissue in the liver and secrete pro-inflammatory cytokines initiating inflammation. However, infiltrated monocytes differentiate towards macrophages at later points of the disease and secrete matrix metalloproteinases, which degrade collagen-I, thus inducing tissue repair and fibrosis regression (de Souza et al., 2017; Karlmark et al., 2009). In contrast, in the context of atherosclerosis, monocytes play

a central role and rather promote the development of the atherosclerotic plaque. They infiltrate the intima layer of arterial walls, where they store oxidized low-density lipoprotein. Subsequently, they release pro-inflammatory cytokines to recruit more monocytes and other leukocytes, thus driving a chronic baseline inflammatory state. This positive feedback loop of lipid engulfment and enhanced leukocyte recruitment results in a pronounced inflammatory infiltration of the intima and the formation of atherosclerotic plaque. The rupture of the latter results in the clogging of arteries and myocardial infraction and stroke (Bobryshev, 2006).

In other setting, monocytes can also have an immunosuppressive activity. For instance, immunosuppressive monocytes belong to the myeloid-derived suppressor cells (MDSCs). These cells are a heterogenous population predominantly expanded in cancer, where it induces an immunosuppressive state (Gabrilovich & Nagaraj, 2009). Monocyte-derived MDSCs were shown to support the activation of regulatory T cells and a subsequent suppression of anti-tumor T cell activity (Huang et al., 2006). Furthermore, also the activity of monocytes can be impaired by several cues. For example, an immune paralysis can ensue from sepsis or trauma – a condition, by which the immune functions are abolished. Indeed, monocytes can exhibit after trauma an HLA-DR suppression, which is associated with an impaired capacity for antigen-presentation and, consequently, a higher risk of secondary and life-threatening nosocomial infections (Muller Kobold et al., 2000).

1.3 Platelets – beyond hemostasis and coagulation

Platelets are small anucleate cells deriving from mature megakaryocytes in the bone marrow and the lungs (Lefrancais et al., 2017). They are approximately 4 µm in diameter, discus-shaped cells with a lifespan of 5-9 days. Platelets contain three types of granules: α-granules, δ-granules and lysosomal granules; each of these store a different arsenal of proteins and bioactive molecules, which play central roles in platelets' function, hemostasis, immune responses and intercellular communication (Morrell et al., 2014). Platelets are mainly known for their key role in blood coagulation and hemostasis. In the circulation, platelets move in close distance to the vessel wall. Upon external insults such as blood vessel disruption or injury, platelets recognize the exposure of molecules at the

extraluminal side of the blood vessel and rapidly adhere to the site of injury. This is induced after the binding to the von Willebrand factor in the exposed periluminal extracellular matrix via the platelet receptor complex glycoprotein (GP) Ib/V/IX. Moreover, platelet GPVI and αIIβ1 bind to collagen in the exposed periluminal extracellular matrix. As a consequence, the core of the thrombus is formed, which in turn recruits and activates more platelets promoted by platelets' degranulation in the core. These secondarily activated platelets aggregate at the core and form the shell – the outer layer of a thrombus (Ghoshal & Bhattacharyya, 2014; Munnix et al., 2009).

A healthy adult produces on average 10^{11} platelets per day, which remain in the circulation for about 5-9 days before clearance (Ghoshal & Bhattacharyya, 2014). Mature platelets are cleared from the blood following several mechanisms. One mechanism involves the desialylation of surface molecules. Indeed, sialic acid residues are determinants for the life-span of platelets (Y. Li et al., 2017; Sorensen et al., 2009). Physiological alterations of sialic acid residues, but also autoantibodies targeting the N-terminal ligand-binding domain of GPIbα, induce the recruitment of neuraminidase-1, which subsequently removes sialic acid residues from the glycoprotein. This leads to an exposure of the underlying carbohydrate moieties and platelet clearance by hepatocytes expressing Ashwell-Morell receptors in the liver (Li et al., 2015).

Platelet clearance can also be initiated by the exposure of "clearing signals", such as phosphatidylserine (PS) at the external leaflet of the plasma membrane. PS^+ platelets and platelet-derived microvesicles are cleared via phagocytosis by macrophages in the liver and spleen (Dasgupta et al., 2009; Ma et al., 2017). Lastly, platelets can undergo apoptosis, similarly to nucleated cells. In this scenario, the balance between the activities of BH3-only initiators of apoptosis, antiapoptotic Bcl-2 family proteins, and pro-apoptotic molecules Bak and Bax plays a central role (Kodama et al., 2011; Zhang et al., 2007). Nevertheless, several studies show that a single depletion or pharmacological inhibition of a single pro-apoptotic factor only has a partial effect of the life-span of platelets, suggesting a more complex network of apoptosis regulation in platelets (Debrincat et al., 2015; Kelly et al., 2010; Kodama et al., 2011; Vandenberg & Cory, 2013).

Over the last decades, the understanding of the role of platelets expanded and their significance in the immune system has been increasingly appreciated. Platelets can detect and interact with pathogens, and they can modulate the immune responses as they also interact with leukocytes, licensing the execution of immune defense mechanisms. Platelets express molecules involved in immune responses, such as TLRs, cytokines, chemokines and co-stimulatory molecules (Ali et al., 2015). These molecules are stored in granules. A high number of proteins are stored in α-granules, which are not only involved in the function regulation of platelets, but also of leukocytes, e.g. CCL5, CXCL12, CD40L, CD62P and platelet factor 4. Platelet δ-granules contain more than 200 types of small signaling mediators such as calcium ions, adenosine diphosphate, adenosine triphosphate, polyphosphates and serotonin, which can influence leukocyte activity. Lastly, lysosomal granules contain glycohydrolases and other degradative enzymes (Ali et al., 2015; Morrell et al., 2014).

Platelets can act as immune effector cells and support the immune system by actively trapping bacteria. They recognize, collect, and bundle bacteria into clusters and consequently restrict their growth and dissemination, in addition to acting as opsonizing cells (Gaertner et al., 2017; White, 2005). Moreover, platelets communicate with leukocytes either via direct physical contact or via bioactive molecules stored in their granules, thus modulating the immune responses of these leukocytes. In fact, platelets guide neutrophils to the site of inflammation and assist their diapedesis as well as NETs release (Stark, 2019; Zuchtriegel et al., 2016). Moreover, platelet-derived soluble factors were shown to enhance the NLRP3-dependent IL-1β response in macrophages (Rolfes et al., 2020). Platelet activity is also linked to the adaptive immune system. Indeed, platelets upregulate CD40L upon activation and enhance antigen presentation by binding CD40 on antigen-presenting cells (Czapiga et al., 2004; Elzey et al., 2003; Han et al., 2020).

In the clinic, platelets serve as a diagnostic biomarker. Platelets were shown to be dysfunctional or altered in numbers or shapes in pathologies such as renal diseases, diabetes, and cardiovascular diseases (Ghoshal & Bhattacharyya, 2014). Platelets from patients with type 1 and 2 diabetes mellitus show hyperaggregation (Adak et al., 2008; Mandal et al., 1993). Furthermore, patients with congestive heart failure have higher

platelet aggregates in the blood (Mehta & Mehta, 1979). Consistent with their diagnostic value, platelets are involved in several diseases with either protective or exacerbating outcomes. For example, circulating monocytes are accompanied by several platelets, together forming monocyte-platelet aggregates (MPAs) and promoting a pro-inflammatory phenotype of circulating monocytes (Passacquale et al., 2011). The numbers of MPAs are increased in cardiovascular diseases and they contribute to their pathogenesis (Allen et al., 2019; Ashman et al., 2003). Similarly, patients with severe coronavirus disease 2019 (COVID-19) have an increased number of circulating MPAs, which is associated with higher mortality (Hottz et al., 2020). Platelet depletion or blocking their interaction with neutrophils using anti-P-selectin antibody ameliorates the outcome of acid-induced acute lung injury (Zarbock et al., 2006). In contrast, the presence of platelets can be protective, as their transfusion improves the liver function in patients with liver cirrhosis (Takahashi et al., 2013). Moreover, platelets show also protective actions against septic shock (Xiang et al., 2013). Thus, platelets are not only an important diagnostic marker to estimate the health of patients, they are also involved in the pathogenesis or remission of several diseases, representing an attractive target for pharmacological and clinical interventions.

1.3.1 Platelet communication with other cell types: soluble mediators, cell-cell contact, extracellular vesicles

Platelets can interact with leukocytes and steer their function via various communication routes, either via soluble factors or direct physical contact. Platelets' granules store a variety of bioactive molecules, which influence the activity of leukocytes. They secrete chemokines, such as CCL5, CXCL4, and CXCL7, which recruit neutrophils and play an important role in neutrophil extravasation in acute lung injury (Brandt et al., 2000; Grommes et al., 2012). Furthermore, platelet-derived releasates were shown to provide additional positive regulatory signals for the NLRP3-inflammasome and enhance the IL-1β-driven immune response in human macrophages (Rolfes et al., 2020).

Platelets can directly interact with leukocytes via cell-cell contact forming platelet-leukocyte aggregates (Li et al., 2000; Susanto & Hickey, 2020). The direct interaction of platelets with monocytes via P-selectin induces the differentiation of the latter towards

dendritic cells with an enhanced ability of cross-presentation and, consequently, more efficient $CD8^+$ T cell activation (Han et al., 2020). Moreover, neutrophils physically "search" for activated platelets using clusters of the P-selectin ligand-1. This interaction results subsequently in signal transduction, initiating neutrophil migration and inducing a neutrophil-driven inflammatory state (Sreeramkumar et al., 2014).

Another layer of intercellular interaction occurs via extracellular vesicles (EVs). Around 80% of circulating EVs in the blood are derived from platelets (Lannan et al., 2015; Ratajczak et al., 2006). These platelet-derived EVs contain bioactive molecules, immunomodulatory proteins and regulatory nucleic acids, which impact the function of the recipient cells (Italiano et al., 2010). Platelet-derived microparticles were shown to have pro-inflammatory effects in rheumatoid arthritis (Boilard et al., 2010). Interestingly, platelet-derived EVs can also carry and transfer transcription factors, thus modulating the transcriptional landscape of the recipient cells (Lannan et al., 2015). Furthermore, platelets do not only release mitochondria to promote inflammation, but they also induce metabolic changes and aid for wound-healing of mesenchymal stem cells by intercellular transfer of platelet-derived mitochondria (Boudreau et al., 2014; Levoux et al., 2021; Marcoux et al., 2017).

Collectively, due to their pleiotropic functions as well as their large excess in the circulation, platelets can be considered as the largest cell population orchestrating hemostasis and immune responses. Better understanding of their functions is therefore of major interest.

1.3.2 Idiopathic thrombocytopenia

Idiopathic thrombocytopenia (ITP) is a heterogenous autoimmune disease characterized by abnormally low platelet counts and a bleeding disorder, leading to complications and potentially to death. ITP is a disease which is diagnosed via exclusion criteria; when platelet counts are lower than 10^5 platelets/µl with an unknown etiology. While primary ITP is described as thrombocytopenia with unknown causes, representing approximately 80% of ITP cases, secondary ITP is a condition where low platelet counts are associated with

infections, e.g. HIV, *Helicobacter pylori* or hepatitis C virus (Samson et al., 2019; Swinkels et al., 2018).

Although the cause of primary ITP remains unknown, dysfunctional platelet production and clearance are the main mechanisms leading to the decreased platelet counts in ITP patients. Dysfunctional megakaryocytes with impaired differentiation, autophagy or thrombopoiesis, or megakaryocyte depletion caused by apoptosis or antibody-mediated destruction are responsible for low counts of platelets (Arnold et al., 2015; Sun & Shan, 2019; Vrbensky et al., 2018). Furthermore, the adaptive immune system plays a central role in the destruction of circulating platelets in the context of ITP. More than 80% of ITP patients have autoantibodies against platelet's glycoproteins, indicating the involvement of autoreactive T and B cells. These autoantibodies opsonize platelets and facilitate their Fc-mediated clearance by spleen macrophages (McFarland, 2002; Norris et al., 2021; Swinkels et al., 2018). Furthermore, antibodies targeting GPIbα induce desialylation of platelet glycoproteins, leading to their clearance in the liver (Li et al., 2015). Nonetheless, some ITP patients do not have antiplatelet antibodies. Instead, these patients show an activity of autoreactive cytotoxic T cells against platelets (Swinkels et al., 2018).

No matter the cause of ITP, pharmacological interventions are required in ITP patients, since only 2% of adults with ITP show a spontaneous remission, whereas 43% of ITP patients develop chronic ITP (McFarland, 2002). The first-line treatments are corticosteroids, usually combined with intravenous immunoglobulin therapy to dampen the autoreactive destruction of platelets. The second-line treatments include rituximab, a monoclonal antibody depleting B cells (Stasi et al., 2001). A further treatment option is splenectomy. Chronic-refractory ITP patients often require a third line of re-treatments with corticosteroids (Samson et al., 2019).

1.4 Research objectives

Platelets are crucial for hemostasis and coagulation. Interestingly, they are increasingly gaining recognition for their thrombo-immunological functions. Platelets modulate the activity of leukocytes and promote the production and release of pro-inflammatory

cytokines (Diacovo et al., 1996; Finsterbusch et al., 2018; Kral et al., 2016; Weyrich et al., 1996). Circulating monocytes are one of the central players in host defense and were shown to form complexes with platelets in both physiological and pathological conditions (Allen et al., 2019; Ashman et al., 2003; Hottz et al., 2020; Thomas et al., 2017). Although increasing evidence shows that platelets can regulate the immune function of monocytes, it was predominantly studied in pathological contexts, and the underlying mechanisms are only partially understood (Passacquale et al., 2011; Rong et al., 2014; Weyrich et al., 1996). Since there is insufficient knowledge of how platelets regulate the immune functions of monocytes, my thesis aimed at filling this gap in knowledge through:

1. establishing an *ex vivo* model of thrombopenic conditions using platelet-depleted primary human monocytes;
2. studying the alterations in cytokine responses of primary human monocytes in the presence or absence of platelets;
3. assessing the platelet-induced changes in the transcriptome, kinase activity profile, and signaling pathways engaged in primary human monocytes;
4. characterizing the molecular mechanisms by which platelets modulate the monocyte-driven immune response;
5. corroborating the *in vitro* findings in blood samples from thrombopenic individuals, such as the patients with idiopathic thrombocytopenia, thus providing a clinical context;
6. studying the impact of *in vivo* platelet depletion on the monocyte transcriptome in the murine system.

The findings in my thesis present platelets as crucial for the pro-inflammatory activity of monocytes. This changes the idea of monocytes being autonomously acting leukocytes and highlight their dependency on platelets for their pro-inflammatory activities. Thus, platelets should be considered as potential pharmacological target to manipulate the outcome of monocyte-driven immune responses.

2. Material and methods

2.1 Material

2.1.1 Consumables

Product	Company
1.5 ml Eppendorf safe-lock tubes	eppendorf
15 ml Tubes	Greiner Bio-One
2 ml Eppendorf safe-lock tubes	eppendorf
384-well microplate, small volume, flat bottom, white	Greiner Bio-One
5 ml polystyrene flow cytometry tubes	Sarstedt
50 ml Tubes	Greiner Bio-One
96 Well Black/Clear Bottom Plate, TC Surface	ThermoFisher
Cell culture flask, surface: Standard, Filter cap	Sarstedt
HTS Transwell 96-well plate, TC treated, 0.4 µm Polycarbonate Membrane	Corning
Immobilon-FL Polyvinylidene fluoride membrane, 0.45 µm	Merck Millipore
NuPAGE Novex 4-12% Bis-Tris Gel	ThermoFisher
Pipette Tips RT LTS 1000µL FL 768A/8	Mettler Toledo
Pipette Tips RT LTS 200µL FL 960A/10	Mettler Toledo
Pipette Tips RT LTS 20µL FL 960A/10	Mettler Toledo
S-Monovette® Citrate 3.2%, (LxØ): 92 x 15 mm	Sarstedt
S-Monovette® K3 EDTA, (LxØ): 92 x 16 mm	Sarstedt
Safety-Multifly® needle, 20G x 3/4", 200 mm	Sarstedt
StableStak™ double-96 rack tips, Sterile unfiltered tips (1 ml, 200 µl, 20 µl)	Mettler Toledo
Tissue culture plates (96-well), sterile, flat-bottom, with lid	Sarstedt
Tissue culture plates (96-well), sterile, round-bottom, with lid	Sarstedt

Whatman® cellulose chromatography paper, 3 mm CHR	Whatman
µ-Slide 8 Well	Ibidi

2.1.2 Chemicals and reagents

Product	**Company**
2-mercaptoethanol	Sigma-Aldrich
Anthrax Protective Antigen (PA), recombinant from Bacillus anthracis	List Biological Laboratories
BAY 11-7082	Sigma-Aldrich
Benzonase® Nuclease	Sigma-Aldrich
Bode Cutasept® F Hautantiseptikum	Hartmann
Buffer RLT	Qiagen
CD14 MicroBeads, human	Miltenyi Biotec
Chloroform	Merck
cOmplete EDTA-free protease inhibitor cocktail tablets	Roche
Cytochalasin D	Sigma-Aldrich
Dimethyl sulfoxide (DMSO), cell culture grade	PAN-Biotech GmbH
eBioscience™ 10X RBC Lysis Buffer (Multi-species)	ThermoFisher
Ethanol absolute pure	AppliChem
FcR Blocking Reagent, human	Miltenyi Biotec
FcR Blocking Reagent, mouse	Miltenyi Biotec
Ficoll® Paque PLUS endotoxin tested	Sigma-Aldrich
Gibco™ RANTES recombinant human protein	ThermoFisher
Glycerol	Merck
GR 144053 trihydrochloride	Tocris
Halt™ Phosphatase Inhibitor (100x)	ThermoFisher
Halt™ Protease Inhibitor Cocktail, EDTA-Free (100x)	ThermoFisher
Heparin-Natrium-25000-ratiopharm®	Ratiopharm
Hoechst 33258	abcam
Hoechst 34580, Invitrogen™	ThermoFisher

LFn-PrgI fusion Protein	Prof. Matthias Geyer and Dr. David Fußhöller (Institute of Structural Biology, University of Bonn, Germany)
LPS-EB Ultrapure from E.coli O111:B4	InvivoGen
M-PER™ Mammalian Protein Extraction Reagent	ThermoFisher Scientific
Methanol	Merck
Neuraminidase from Arthrobacter ureafaciens	Roche
Nigericin, free acid, Invitrogen™	ThermoFisher Scientific
OneComp eBeads™ Compensation Beads, Invitrogen™	ThermoFisher Scientific
PageRuler™ Plus Prestained Protein Ladder	ThermoFisher Scientific
Pam3CysK4	InvivoGen
Paraformaldehyd	Sigma Aldrich
PhosSTOP Easypack Phosphatase Inhibitor Cocktail Tablets	Roche
Prostaglandin E1	Sigma Aldrich)
QIAzol Lysis Reagent	QIAGEN
R848 (Resiquimod)	InvivoGen
Recombinant human CD40L/CD154, soluble	Enzo Life Sciences Inc.
Recombinant Human P-Selectin/CD62P Protein, CF	R&D systems
Recombinant human SDF-alpha (CXCL12)	PeproTech
SB 203580	Tocris
Thrombin from human plasma	Sigma Aldrich
Triton® X-100	Carl Roth
Tween® 20	Sigma Aldrich

2.1.3 Buffers and solutions

Product	**Composition/Company**

Complete RIPA	5 ml RIPA buffer (2x), 3,4 ml H_2O, 400 µl cOmpleteTM EDTA-free protease inhibitor cocktail (50x), 1 ml PhosSTOP™ Easypack phosphatase inhibitor cocktail (10x)
Complete RPMI	10% (v/v) FCS, 1% Penicillin-Streptomycin, 1x (v/v) Glutamax in RPMI
NuPAGE® LDS Sample Buffer (4x)	ThermoFisher
NuPAGE® MES SDS Running Buffer (20x)	ThermoFisher
NuPAGE® Sample Reducing Agent (10x)	ThermoFisher
Permeabilization buffer	2% FCS and 0.2% Triton X-100 in 1x PBS
Pierce® 10X Tris-Glycine buffer	ThermoFisher
Pierce™ 20x TBS Buffer	ThermoFisher
RIPA buffer (2x)	2,4 g Tris base, 8,8 g NaCl, 2 ml EDTA [500 mM], 10 ml Triton X-100, 5 g sodium deoxycholate, 1 g SDS, 100 ml glycerol
TBS-T 0.1%	0.1% Tween® 20 in 1x TBS
Transfer buffer	15% methanol in 1x Tris-Glycin buffer

2.1.4 Cell culture

Product	**Company**
Dulbecco's Phosphate-buffered saline	Gibco™
Fetal calf serum (FCS)	Gibco™

GlutaMax (100x)	Gibco™
HEPES sodium salt	Merck
Penicillin-Streptomycin (10,000 U/ml)	Gibco™
RPMI 1640 Medium	Gibco™
UltraPure™ 0.5 M EDTA, pH 8.0	Invitrogen

2.1.5 Commercial reagent sets (Kits)

Product	**Company**
CellTiter-Blue™ Cell Viability Assay	Promega
HTRF® Human IL-1 beta Kit	Cisbio
HTRF® Human IL6 Kit	Cisbio
HTRF® Human TNF alpha Kit	Cisbio
HTRF® Mouse IL1 beta Kit	Cisbio
HTRF® Mouse IL6 Kit	Cisbio
HTRF® Mouse TNF alpha Kit	Cisbio
HTRF® Phospho-NFkB (Ser536) cellular Kit	Cisbio
Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex™ Panel 1	Invitrogen
nCounter® Human Myeloid Innate Immunity Panel v2	NanoString Technologies
PamChip-4 Protein Tyrosine kinase	PamGene
PamChip-4 Serine-threonine kinase	PamGene
Caspase-Glo® 1 Inflammasome Assay	Promega
EasySep™ Human Monocyte Isolation Kit	STEMCELLTechnologies
Pierce™ LDH cytotoxicity assay Kit	ThermoFisher Scientific

2.1.6 Antibodies

Product	**Company**
Alexa Fluor® 488 anti-human CD41 Antibody (HIP8)	BioLegend
Alexa Fluor® 488 Mouse IgG1, k Isotype Ctrl (FC) Antibody (MOPC-21)	BioLegend
Alexa Fluor® 647 anti-human CD14 Antibody (HCD14)	BioLegend

Alexa Fluor® 647 Mouse IgG1, k Isotype Ctrl (FC) Antibody (MOPC-21)	BioLegend
Anti-mouse CD115 APC (AFS98)	BioLegend
Anti-mouse CD11b PE (M1/70)	eBioscience
Anti-mouse CD45 FITC (30-F11)	BioLegend
Anti-mouse IA/IE BV785 (M5/114.15.2)	BioLegend
Anti-mouse Ly6G BV785 (1A8)	BioLegend
Anti-Puromycin Antibody (12D10)	Merck
Beta-Actin rabbit monoclonal antibody	Li-Cor Biosciences
CD11b Antibody (2LPM19c)	GeneTex
CD14 monoclonal antibody (61D3), APC	eBioscience
CD41a Monoclonal Antibody (HIP8), FITC	eBioscience
CD45 Monoclonal Antibody (2D1), PE	eBioscience
CD62P Monoclonal Antibody (Psel.KO2.3), APC	eBioscience
Human IL-1 beta/IL-1F2 DuoSet ELISA	R&D Systems
Human Siglec-7/CD328 (194212)	R&D Systems
IRDye® 680RD Donkey anti-Mouse IgG Secondary Antibody	Li-Cor Biosciences
IRDye®800CW Donkey anti-Rabbit IgG Secondary Antibody	Li-Cor Biosciences
Mouse IgG2B Isotype Control (20116)	R&D Systems
NF-κB p65 Rabbit mAb (C22B4)	Cell Signaling
Phospho-NF-κB p65 (Ser536) Rabbit mAb (93H1)	Cell Signaling
Polyclonal Rat anti-mouse GPIb for mouse platelet depletion (R300)	emfret Analytics
Polyclonal rat IgG non-immune (C301)	emfret Analytics
Purified Mouse IgG1 k Isotype Control (MOPC-21)	BioLegend

2.1.7 Instruments

Product	**Company**
CASY TTT 60 Cell Analyzer	OLS Omni Life Science

FACS Sorter	FCCF, University hospital Bonn, Germany
MACSQuant® Analyzer 10	Miltenyi Biotech
Odyssey Western-Blot Scanner	Licor
PamStation 12	PamGene
SpectraMax i3, multi-function plate reader	Molecular Devices
TCS SP5 Confocal microscope	Leica
XCell II Blot Module	Novex Life Technologies

2.1.8 Cell lines

Product	**Company**
THP-1 (ATCC TIB-202)	ATCC
THP-1 Dual™ cells NF-kB-SEAP and IRF-Lucia luciferase Reporter (Thpd-nfis)	InvivoGen

2.1.9 Mice

Strain	**Company**
Mus musculus, wild-type (C57BL/6J)	Charles River

2.1.10 Software

Name	**Version**	**Company**
Adobe Illustrator	CS6	Adobe
GraphPad Prism 9	9.3.1	GraphPad Software, LLC
Partek® Genomics Suite®	7.18.0723	Partek®
nSolver™ Analysis Software	4.0.70	NanoString® Technologies Inc.
Fiji (ImageJ)	2.3.0/1.53f	Java

2.2 Methods

2.2.1 Isolation of primary human cells

Human primary cells were isolated from whole blood of healthy volunteers after the signature of an informed consent approved by the Ethics Committee of the University of Bonn (Protocol #282/17) and in accordance with the declaration of Helsinki. Further, in collaboration with Dr. med. Lino L. Teichmann and Dr. med. Lisa Meffert, from the Internal Medicine III – Oncology/Hematology and Rheumatology of the University Hospital of Bonn, we acquired blood from individuals with idiopathic thrombocytopenia provided by Dr. med. Lisa Meffert. Written informed consent was acquired from all subjects according to the Declaration of Helsinki and approval by the Institutional Review Board of the University of Bonn (313/21).

2.2.1.1 Isolation of peripheral blood mononuclear cells

Human venous blood was collected from healthy volunteers and peripheral blood mononuclear cells (PBMCs) were obtained by density centrifugation: whole blood was diluted 1:3 with phosphate buffered saline (PBS) and layered over Ficoll® Paque PLUS. After a centrifugation at 700 x g for 20 min (acceleration 1, no brake) at room temperature (RT), plasma was discarded and PBMC layer was collected and washed with PBS before the isolation of further leukocyte populations.

2.2.1.2 Isolation of primary human monocytes

Primary human monocytes were obtained from isolated PBMCs via negative selection using the EasySep™ Human Monocyte Isolation Kit (STEMCELL Technologies™) following the manufacturer's protocol. Briefly, blood was collected in S-Monovette® K3EDTA tubes. PBMCs were resuspended in cold PBS containing 2% fetal calf serum (FCS) and 1 mM ethylenediaminetetraacetic acid (EDTA) and incubated with the EasySep™ Monocyte Isolation Cocktail [50 µl/ml] in 5 ml polystyrene tubes. To generate platelet-depleted monocytes, an additional EasySep™ Platelet Removal Cocktail [50

µl/ml] was added. After incubation for 5 min at RT, EasySep™ D Magnetic Particles were added to the cell suspension and incubated for further 5 min at RT. Finally, tubes were placed in an EasySep™ Magnet and cell suspensions containing standard monocytes (MoStd) and platelet-depleted monocytes (MoPD) were collected.

2.2.1.3 Isolation of human platelets

For the isolation of human platelets, venous blood from healthy volunteers was collected in S-Monovette® 9NC tubes. Platelets were isolated as previously described (Alard et al., 2015), however with slight modifications (Rolfes et al., 2020): whole blood was centrifuged at 330 x g for 15 min (acceleration 1, no brake) and platelet-rich plasma (PRP) was collected and transferred into a new 15 ml tube containing 200 nM Prostaglandin E1 (PGE1). PRP was diluted 1:1 with pre-warmed PBS and centrifuged at 240 x g for 10 min to pellet leukocyte contaminants. Platelet suspension was transferred to a new tube containing 200 nM PGE1 and centrifuged at 430 x g for 15 min to pellet platelets. Finally, platelet pellet was washed once with pre-warmed PBS containing 200 nM PGE1 and then resuspended in pre-warmed RPMI.

2.2.2 Generation of platelet-conditioned media

To generate platelet-conditioned media, isolated human platelets ($1x10^8$ cells) were incubated in RPMI either untreated (Plt^{Sups}) or stimulated with 2 ng/ml LPS ($Plts^{LPS\text{-}Sups}$) for 4.5 h at 37 °C and 5% CO_2. Cell suspensions were centrifuged at 3,000 x g for 10 min and supernatants were collected. An additional control LPS-conditioned medium (LPS^{CM}) was generated in the same way, however without platelets.

2.2.3 Mice

C57BL/6J mice (female, 12 weeks) were purchased from Charles River. Animals were housed and experimental procedures took place at the House for Experimental Therapy (HET) of the University Hospital of Bonn. Dr. Lucas Ribeiro (Institute of Innate Immunity, University Hospital Bonn) performed the injections as well as blood drawing from

euthanized mice. Animal handling and experimental procedures used were approved by the regional authority of Rhine-Westphalian state office for the protection of Nature, the Environment and the Consumers (*Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen*, LANUV-NRW, #84-02.04.2016.A487).

2.2.3.1 Sorting of mouse monocytes after platelet depletion *in vivo*

Mice were intravenously (i.v.) injected with 2 mg/kg of polyclonal rat anti-mouse GPIbα for mouse platelet depletion or polyclonal rat IgG none-immune antibody as a control treatment (emfret analytics). After 12 h, mice were challenged i.v. with 2 mg/kg LPS or PBS for 3 h. Blood was collected from euthanized mice and PBMCs were isolated by density gradient centrifugation with Ficoll® Paque PLUS at 700 x g for 30 min at RT. Monocytes were obtained by Fluorescence-activated Cell Sorting at the Flow Cytometry Core Facility of the Medical Faculty at the University of Bonn. Cells were collected in QIAzol lysis reagent and sent for bulk RNA sequencing to GENEWIZ Azenta Life Sciences.

2.2.4 Flow Cytometry

The MACSQuant® Analyzer 10 flow cytometer was utilized to assess the purity of isolated primary human monocytes and platelets, as well as to determine the activation status of isolated human platelets. Cells were incubated with FcR Blocking Reagent for 15 min at 37 °C and 5% CO_2 in a 96-well round-bottom plate. Cells were stained with fluorescently-labelled monoclonal antibodies for 30 min at RT. Finally, cells were washed with PBS and flow cytometry was performed.

2.2.4.1 Purity of isolated primary human monocytes and platelets

To assess the purity of isolated primary human monocytes or platelets, clonal surface markers were used. Cells were stained with anti-CD14 (monocytes), anti-CD45 (leukocytes) and anti-CD41a (platelets).

2.2.4.2 Activation of isolated human platelets

Isolated platelets were stained to assess their activation status and to determine their activation potential. To this end, platelets were stimulated with 1 U/ml Thrombin from human plasma (Sigma-Aldrich) or left unstimulated for 30 min at 37 °C 5% CO_2. Staining with an anti-CD62P antibody was used as marker for platelet activation and anti-CD41a antibody as a clonal platelet marker.

2.2.5 Fluorescence-activated Cell Sorting

Fluorescence-activated cell sorting was used to obtain mouse monocytes. Isolated PBMCs were stained with anti-Ly6G and anti-I-A/I-E antibodies as exclusion markers, and anti-CD45, anti-CD11b, anti-CD115 antibodies as monocyte markers. Additionally, Hoechst 33258 (Abcam) was used as a viability staining. Finally, viable $Ly6G^{-}$I-A/I-E^{-} $CD45^{+}CD11b^{+}CD115^{+}$ monocytes were sorted directly into QIAzol lysis reagent using the BD FACS Aria Fusion (387333827) and BD FACS Aria III (216372545) at the Flow Cytometry Core Facility of the Medical Faculty of the University of Bonn.

2.2.6 Stimulation assays

Monocytes (10^5/well) were seeded in a 96-well flat-bottom. Platelets (10^7/well) were seeded either alone or co-incubated with platelet-depleted monocytes (1:100, Monocyte:Platelets). Cells were stimulated with different TLR agonists: Pam3CysK4 [1 µg/ml] to activate TLR1/2, LPS [2 ng/ml] for TLR4, or R848 [3.5 µg/ml] for TLR7/8 for 4.5 h at 37 °C and 5% CO_2. For inflammasome activation, cells were first primed with a TLR agonist for 3 h followed by an inflammasome activator: for NLRP3, primed cells were treated with Nigericin [10 µM] for 1.5 h, for NLRC4 with PrgI/PA [100 ng/ml / 1 µg/ml] for 1.5 h, or for AIM2 activation with poly(dA:dT) [0.5 µg/ml] and Lipofectamine 2000 for 3 h.

2.2.6.1 Stimulation assay including recombinant human proteins and inhibitors

To explore the interaction between monocytes and platelets, I used either recombinant human proteins to substitute platelets or inhibitors to prevent the interaction between monocytes and platelets. To this end, recombinant proteins substituting the presence of platelets were added to platelet-depleted monocytes prior to priming: rhRANTES [300 or 600 µg/ml], rhCXCL12 [2 ng/ml], rhCD40L [10, 50 or 100 ng/ml], or anti-Siglec 7 [10 µg/ml]. To disrupt the interaction between monocytes and platelets, inhibitors were added independently to either or both cell populations depending on the expression of the targeted ligand/receptor: Arginine-Glycine-Aspartate tripeptides (RGD) [50 µM], anti CD11b antibody [10 µg/ml], GR 144053 trihydrochloride [10 µM], or Neuraminidase *Arthrobacter ureafaciens* (Sialidase) [100 mU/ml] to enzymatically digest sialic acids on platelets.

2.2.7 Cytokine measurement

For measurement of a single cytokine in supernatants, homogeneous time-resolved fluorescence technology (HTRF) was applied using HTRF® Kits (Cisbio) according to the manufacturer's instruction. Cytokine levels were measured with the SpectraMax i3 plate reader (Molecular devices). For a multiplexed measurement of several cytokines, the Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex™ (Invitrogen) was used according to the manufacturer's instructions. Cytokine levels were measured using the MAGPIX® System.

2.2.8 Caspase-Glo® 1 Assay

Caspase-1 activity in supernatants was measured after inflammasome activation assays using Caspase-Glo® 1 Inflammasome Assay (Promega). Briefly, after stimulation, supernatants were collected and incubated with Glo Buffer in a 96-well plate for 30 min at RT. Caspase-1 activity was measured with the SpectraMax i3 plate reader.

2.2.9 HTRF measurement of Phospho-NF-κB

To measure NF-κB activity in primary human monocytes, HTRF® Phospho-NF-κB (Ser536) cellular Kit (Cisbio) was used. Cells were seeded and stimulated with LPS [2 ng/ml] for 2 , 5, 10, 15 or 30 min. Cells were lysed with the supplied lysis buffer and were incubated with phospho-NF-κB detection antibodies under shaking for 30 min at RT. Fluorescence emission of detected phosphorylated NF-κB was measured with the SpectraMax i3 plate reader.

2.2.10 Cell lines

In this thesis, two cell lines were used for experiments: THP-1 cells (ATCC) and THP-1 Dual™ cells (InvivoGen). Cells were cultured at 37 °C and 5% CO_2 in a growth medium of RPMI completed with 10% (v/v) heat-inactivated fetal calf serum, Penicillin [100 U/ml] /Streptomycin [100 µg/ml] and 1x (v/v) GlutaMax. For THP-1 Dual™ cells, the growth medium contained additionally 25 mM HEPES. For expansion, cells were split every second day in cell culture flasks (T-25, T-75 and T-175). For experimental approaches, cells were either left in a monocyte-like phenotype or were differentiated towards macrophage-like THP-1 with 100 nM phorbol 12-myristate 13-acetate (PMA) for 24 h followed by 24 h resting period in complete RPMI without PMA at 37 °C.

2.2.11 Western Blot

Cells were lysed at 4 °C for 30 min with complete RIPA lysis buffer supplemented with Benzonase® Nuclease [25 U/ml] to digest DNA and reduce sample viscosity when whole well lysates were generated. To analyze protein release from stimulated cells, protein precipitation from supernatants was performed using one volume of supernatant homogenized with one volume of methanol and ¼ volume of chloroform. After centrifugation at 13,000 x g for 5 min at RT, upper phase was removed and one volume of methanol was added again and homogenized. Following a final centrifugation at 13,000 for 5 min at RT, precipitated proteins were resuspended in complete RIPA buffer. All

samples were mixed with Laemmli (NuPAGE™ Reducing Agent and NuPAGE™ LDS) and heated at 95 °C for 5 min.

Samples were loaded onto NuPAGE™ Novex 4-12% Bis-Tris gels for 10 min at 80V followed by 1 h and 20 min at 150V in NuPAGE™ MES SDS running buffer. For western blot, semi-wet transfer set (XCell II Blot Module, Novex Life Technologies) was used. Proteins were transferred to an Immobilon-FL Polyvinylidene fluoride (PVDF) membrane in a transfer buffer at 30V for 45 min. Then, membrane was blocked in 3% bovine serum albumin (BSA) [w/v] in Tris buffered saline (TBS) for 2 h at RT. Finally, immunoblotting was performed overnight at 4 °C with primary antibodies diluted in TBS containing 3% BSA [w/v] and 0.1% Tween 20 [v/v] followed by secondary antibodies conjugated to IRDye680 or IRDye800 (1:25000) for 2 h at RT. Membrane was scanned on an Odyssey Imager (Li-Cor Biosciences). Used antibodies: Beta-Actin rabbit Antibody (1:5000), Phospho-NF-kB p65 (Ser536) (93H1) Rabbit mAB #3033 (1:1000) and NF-kB p65 (C22B4) Rabbit mAb #4764 (1:1000).

2.2.12 Confocal laser scanning microscopy

For the visualization of primary human monocytes and platelets, Leica TCS SP5 SMD confocal system (Leica Microsystem, Wetzlar, Germany, project number: 169331223) was used. First, cells were fixed with 4% paraformaldehyde (PFA) for 30 min at 37 °C in µ-slide 8-Well IBIDI chamber or 96 Well Black/Clear bottom plate. Then, cells were incubated in permeabilization/blocking buffer (10% goat serum, 1% FCS and 0.5% Triton X-100 in PBS) supplemented with human FcR Blocking Reagent for 30 min at 37 °C. Finally, cells were stained overnight at 4 °C with Alexa Fluor® 488 anti-human CD41 antibody (Biolegend), Alexa Fluor® 647 anti-human CD14 (Biolegend) and Hoechst 34580 (ThermoFisher).

2.2.13 Cell Viability Assays

To determine cell viability, Lactate Dehydrogenase Assay (LDH) and CellTiter-Blue® cell viability Assay (CTB) were applied. After stimulation, cell supernatants were collected.

While supernatants were used to measure LDH release, cells were used for CTB assay. For LDH assay, collected supernatants were incubated with LDH buffer for 30 min at 37 °C without CO_2. For CTB assay, cells were incubated in the CellTiter-Blue® reagent for 60-120 h at 37 °C with 5% CO_2. In both assays, cells treated with Triton X-100 served as a 100% cell death control. Measurements were performed using the SpectraMax i3 plate reader.

2.2.14 NanoString

To assess gene expression, NanoString® Technologies was applied according to the manufacturer's protocol. After stimulation with LPS or LPS+Nigericin, cells were lysed in RLT Buffer (Qiagen) containing β-Meracptoethanol (10^4 cells/µl). The expression of 770 genes was then determined using the nCounter® Human Myeloid Innate Immunity Panel v2 according to the manufacturer's protocol. In brief, RNA samples were homogenized with CodeSets and left for hybridization overnight. Then, RNA/CodeSet complexes were immobilized on the nCounter cartridge using the nCounter® Prep Station 5s and data collection was performed in the nCounter® Digital Analyzer 5s. The quality of the data was controlled and approved by Dr. rer. nat. James Hembach-Stunden. For data analysis, I performed a normalization of the raw data with nSolverTM analysis software. To this end, genes were normalized to 6 positive controls and 40 housekeeping genes. Data were then visualized using GraphPad Prism 9. Gene Ontology (GO) analysis and visualization were performed using Partek® Genomics Suite®.

2.2.15 PamGene Kinase Activity Assay

Primary human monocytes and platelets from healthy volunteers were seeded in triplicates: MoStd (10^5 monocytes/well), MoPD (0^5 monocytes/well), MoPD (10^5 monocytes/well) reconstituted with Plts (1:100) or Plts alone (10^7 platelets/well). After stimulation with LPS [2 ng/ml] for 15 min at 37 °C and 5% CO_2, cells were consecutively lysed in 60 µl cold M-PER Buffer containing Halt™ Protease Inhibitor (1x) and Halt™ Phosphatase Inhibitor (1x). Cell lysates (5,000 monocytes/µl) were centrifuged at 15,000 x g for 15 min at 4 °C. Supernatants were collected and stored at -80 °C. Kinase activity

assays were performed with Protein Tyrosine Kinase PamChip® (PTKs) and Serine/Threonine Kinase PamChip® (STKs) from PamGene. In pilot pre-experiments (data not shown), I established the required cell number per µl lysate: for PTKs PamChip®, 7.5 µl cell lysate (38,500 cells) were used, for STKs PamChip®, 4.4 µl cell lysate (22,000 cells). PamGene Kinase activity assays were performed according to the manufacturer's instruction using the supplied reagents in the PamGene Assay Kits including washing buffers, BSA, dithiothreitol, PTK-Additive, adenosine triphosphate as well as detection antibodies. Raw data evaluation and data analysis were performed by Dr. Savithri Rangarajan (Field Application Specialist, PamGene) and Dr. Rik de Wijn (Head of Diagnostic Assay Services, PamGene) in 's-Hertogenbosch, The Netherlands, with my scientific input. After peptide quality control and assessment of experimental variation, Dr. Savithri Rangarajan applied a 2-step Combat correction (batch correction algorithm) and performed comparisons depending on the biological question we had. Then, a differential phospho-site statistical analysis with a paired T-test was applied and an Upstream Kinase Analysis (UKA), a PamGene's in-house method, was used to generate a list of corresponding putative kinases. Finally, I visualized the calculated values generated by Dr. Savithri Rangarajan in coral trees using the CORAL online tool (URL: http://phanstiel-lab.med.unc.edu/CORAL/; date accessed: February 2022) developed by the Phanstiel Lab from the University of North Carolina (Manning et al., 2002; Metz et al., 2018).

2.2.16 Bulk RNA sequencing

For bulk RNA sequencing, lysates of mouse monocytes in QIAzol lysis reagent were sent to GENEWIZ (Azenta Life Sciences) and were processed according to the company's pipeline. Briefly, RNA extraction was firstly performed followed by a library preparation. Next, an Ultra-Low Input RNA-Seq was performed using Illumina HiSeq PE 2x150 bp with ~350M reads. Then, sequence quality was evaluated, reads were mapped to the *Mus musculus* GRCm38 reference genome (ENSEMBL) using the STAR aligner v.2.5.2b and unique gene hit counts were generated with the featureCounts from the Subread package v.1.5.2. Finally, standard RNA-Seq analysis was performed, i.e. DEGs identification, significance calculations and Gene Ontology (GO) analysis. Differential gene expression analysis was performed using DESeq2, p-values and log2 fold changes were calculated

by applying the Wald test and genes with adjusted p-value < 0.05 and absolute log2 fold change > 1 (FC > 2) were considered as significantly altered DEGs. Data were visualized using GraphPad Prism 9. GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed and visualized using Partek® Genomics Suite® and the online tool (URL: http://bioinformatics.sdstate.edu/go75/; accessed: February 2022) ShinyGO v0.75 (Ge et al., 2020; Kanehisa et al., 2021; Luo & Brouwer, 2013).

2.2.17 Statistics

Statistical analysis was performed using GraphPad Prism. In experiments containing two variables or more, each with different conditions, two-way ANOVA was applied with Tukey's multiple comparison test with 95% confidence interval. Experiments with one factor with different conditions were statistically analyzed with one-way ANOVA with Tukey's multiple comparison test with 95% confidence interval. P-value < 0.001 (***), 0.01 (**) and 0.05 (*). To test, whether the data are normally distributed, normality and lognormality Tests were used with a GraphPad Prism's default configuration, including Anderson-Darling test, D'Agostino-Pearson test, Shapiro-Wilk normality test and Kolmogorov-Smirnov normality test with Dallal-Wilkinson-Lilliefor P Value. Further, two-tailed T-test was applied either as parametric paired t-Test when data were normally distributed and had two dependent samples or non-parametric unpaired t-Test with Mann-Whitney test when data were not normally distributed and samples were independent.

3. Results

3.1 Platelets govern the pro-inflammatory cytokine secretion in primary human monocytes

3.1.1 Primary human monocytes are surrounded by platelets

Monocytes are circulating leukocytes that can recognize and fight invading pathogens to provide a sterile physiological environment. In the blood, monocytes are constantly surrounded by platelets. To understand the role of platelets (Plts) and their communication with primary human monocytes, I first studied the relationship of platelets and monocytes *ex vivo*. To this end, venous blood was drawn from heathy volunteers and peripheral blood mononuclear cells (PBMCs) were obtained. Monocytes were then further isolated by negative immunomagnetic selection with the EasySep™ Human Monocyte Isolation Kit (STEMCELL Technologies). Thus, I was able to generate two types of monocyte suspensions: i) standard monocytes (MoStd) isolated using the EasySep™ Human Monocyte Isolation Cocktail and ii) platelet-depleted monocytes (MoPD) isolated similarly in the presence of the EasySep™ Human Platelet Removal Cocktail. Finally, flow cytometry (FACS), confocal microscopy as well as the CASY™ cell counter and analyzer were used to characterize monocytes and platelets.

FACS analysis revealed that the cell suspensions of isolated $CD14^+$ monocytes (MoStd and MoPD) were accompanied by a large percentage of $CD41a^+$ platelets (Figure 1). Indeed, I observed three cell populations in each of the suspensions: i) $CD14^+CD41a^-$ Plt-free monocytes, ii) $CD14^-CD41a^+$ free platelets and iii) $CD14^+CD41a^+$ monocyte-platelet aggregates (MPAs). Comparing the frequency of these three populations, I found that the suspension of isolated MoStd consisted of predominantly $CD14^-CD41a^+$ free platelets, $CD14^+CD41a^+$ MPAs and a low percentage of $CD14^+CD41a^-$ monocytes. Interestingly, the suspension of MoPD displayed a shift of MPAs towards the $CD14^+CD41a^-$ gate, indicating an enrichment of the $CD14^+CD41a^-$ Plt-free monocytes. Additionally, the frequency of $CD14^-CD41a^+$ free platelets was decreased (Figure 1A).

Next, I further analyzed the frequencies of these three populations, as detected by FACS, for several isolations, each with blood from a different donor (n=50). I quantified the percentage of Plt-free monocytes, MPAs and free platelets in both MoStd and MoPD suspension. In blood donors, *ex vivo* platelet depletion resulted in the reduction of free platelets from 94.6% to 69.5% and generally in the enrichments of $CD14^+$ monocytes from 5.4% to 30.5%. Importantly, $CD14^+CD41a^-$ platelet-free monocytes were dominating within this enriched $CD14^+$ monocyte population (Figure 1B). Additionally, measurements with the CASY cell counter and analyzer emphasized the decreased counts of platelets upon *ex vivo* platelet depletion, while monocyte counts remained mostly constant (Figure 1C). In agreement with the FACS data, confocal microscopy of MPAs showed that each monocyte was surrounded by several platelets (Figure 1D).

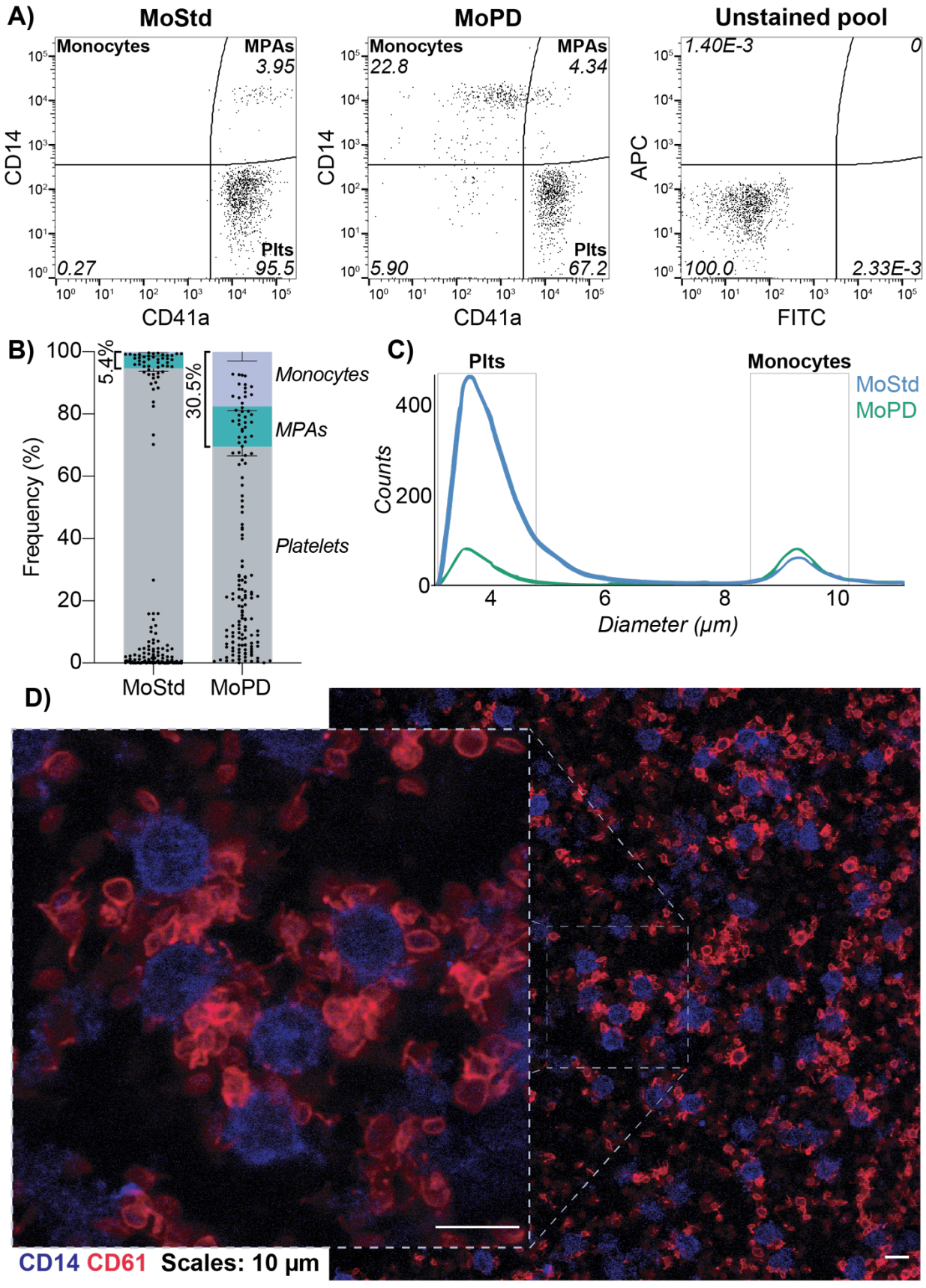
A)
MoStd
MoPD
Unstained pool
Monocytes
MPAs
3.95
Plts
95.5
0.27
22.8
4.34
67.2
5.90
1.40E-3
0
100.0
2.33E-3
CD14
CD41a
APC
FITC
B)
Frequency (%)
5.4%
30.5%
Monocytes
MPAs
Platelets
MoStd
MoPD
C)
Plts
Monocytes
MoStd
MoPD
Counts
Diameter (μm)
D)
CD14 CD61 Scales: 10 μm

Figure 1: Heterotypic interaction between platelets and primary human monocytes. (**A**) Flow cytometry analysis of standard primary human monocytes (MoStd) and platelet-depleted monocytes (MoPD). Cell surface expression of CD14 (monocytes) and CD41a (platelets) was used to assess the frequencies of $CD14^{+}CD41a^{-}$ monocytes, $CD14^{+}CD41a^{+}$ monocyte-platelet aggregates (MPAs) or $CD14^{-}CD41a^{+}$ platelets. (**B**) Quantification of the frequencies of monocytes, MPAs and platelets in MoStd and MoPD in n=50 blood donors. (**C**) Assessment of counts and distribution of monocytes and platelets in MoStd (blue) and MoPD (green) using CASY TTT 60 Cell Analyzer. (**D**) Confocal imaging of MoStd after isolation. CD14 (blue) was used for the identification of monocytes and CD61 (red) of platelets. Scales: 10 µm.

Together, these results show that isolated primary human monocytes are always accompanied by platelets, forming monocyte-platelet aggregates. *Ex vivo* platelet depletion generated "thrombocytopenia-like" conditions with a decreased MPAs frequency and lower abundancy of free platelets.

3.1.2 Platelets are crucial for the production of pro-inflammatory cytokines in primary human monocytes

To understand the impact of platelets on the pro-inflammatory immune response of monocytes, I next investigated the consequences of the platelet depletion on the cytokine responses of primary human monocyte *ex vivo*. In addition to the MoStd and MoPD samples, I established two further experimental groups: i) platelet-depleted monocytes replenished with autologous Plts (MoPD+Plts) as a reconstitution condition and ii) autologous Plts alone to determine the contribution of platelet-derived cytokines.

As monocytes are the major inflammasome-expressing cell population in the blood (Awad et al., 2017), I first measured the release of IL-1β after activation of NLRP3 inflammasome. Further experiments carried out by Agnieszka Demczuk (Master student in our lab, at the Institute of Innate Immunity in Bonn) addressed the effect of platelets on the IL-1β release upon activation of NLRC4 and AIM2 inflammasomes. Cells were primed with LPS [2 ng/ml] for 3 h followed by 1.5 h of nigericin [10 µM] for NLRP3 or PrgI/PA [100 ng/ml / 1 µg/ml] for NLRC4 activation (Figure 2A-B). AIM2 was activated after 3 h of priming with LPS [2 ng/ml] followed by 3 h transfection of poly(dA:dT) [0.5 µg/ml] combined with Lipofectamine 2000 (LF) or LF alone as a control. The priming time of

NLRP3 activation, which was included as a positive control for AIM2 experiment, was adjusted to 4.5 h (Figure 2C). IL-1β levels were measured in cell culture supernatants by homogeneous time resolved fluorescence (HTRF). The results show that MoStd secreted high levels of IL-1β after activation of NLRP3, NLRC4 and AIM2. Surprisingly, platelet-depleted monocytes showed a decreased IL-1β secretion for all tested inflammasomes. Notably, IL-1β secretion was rescued when platelet-depleted monocytes were replenished with autologous platelets (MoPD+Plts). Importantly, platelets alone did not secrete IL-1β, thus excluding an additive effect of platelets (Figure 2A-C). Indeed, the addition of platelets enhanced the secretion of IL-1β from monocytic THP-1 cells and macrophage-like THP-1 cells in a dose-dependent manner (Figure S1). In addition to IL-1β HTRF, I performed measurements of cell viability, LDH release as well as caspase-1 activity. Platelet-depleted monocytes did not only exhibit less cell death after NLRP3 activation, but they also displayed lower caspase-1 activity in their supernatants (Figure S2A-C).

Since these results demonstrate that platelets augment IL-1β secretion triggered by activation of several inflammasomes, I hypothesized that platelets might control inflammasome-independent cytokines as well. To test this, I used the Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex™ Panel 1, a multiplex immunoassay to measure 45 cytokines. Cells were treated, as previously described, with either LPS alone or with LPS followed by stimulation with nigericin (LPS+Nig). Upon stimulation, MoStd secreted high levels of several pro-inflammatory cytokines into their supernatants, whereas MoPD were impaired in their cytokine secretion profile. Interestingly, the reconstitution with platelets restored the capacity of MoPD to produce cytokines. Importantly, platelets alone produced only minor amounts of the measured cytokines (Figure 2D).

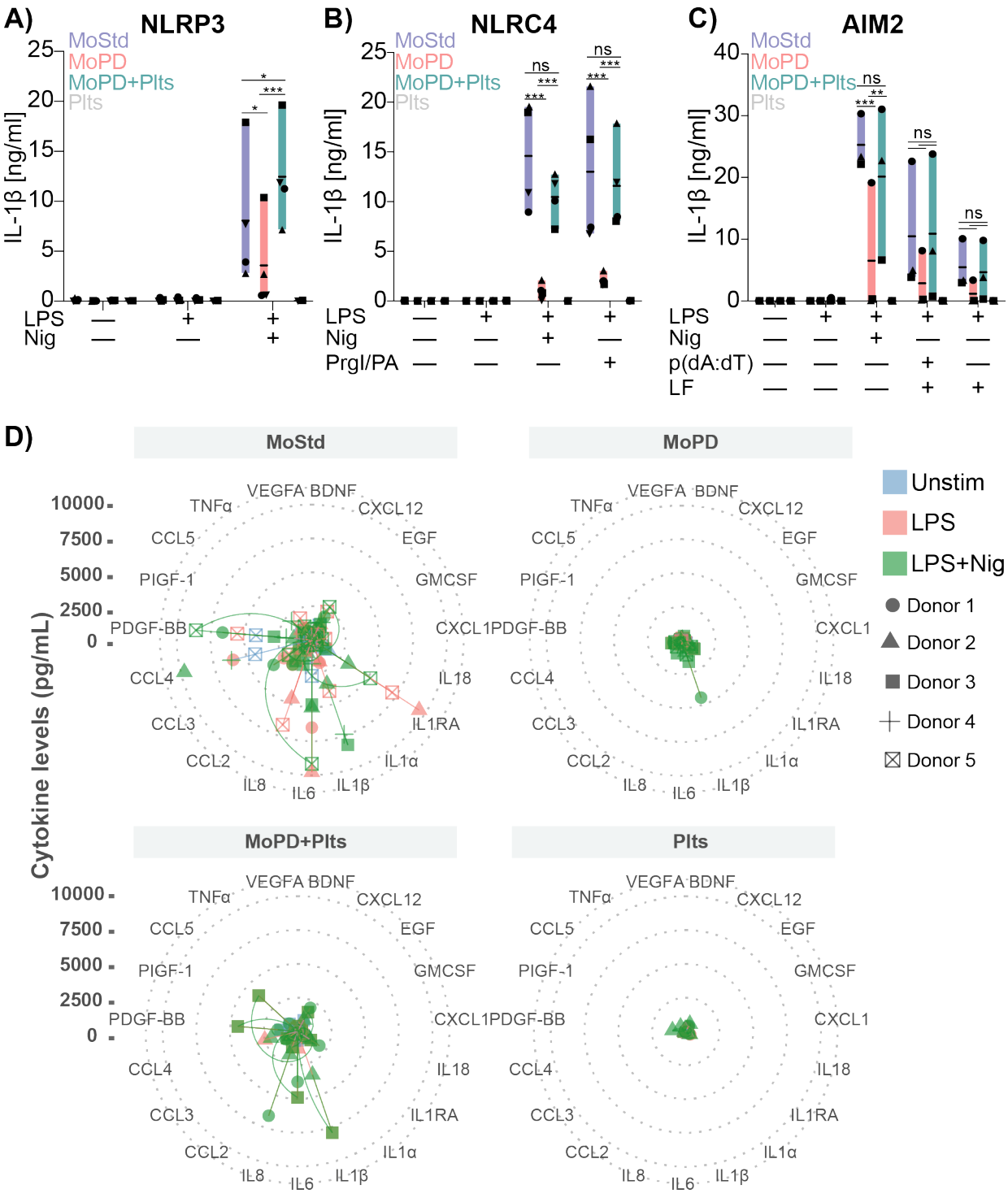

Figure 2: Platelets govern the pro-inflammatory cytokine response. (**A-C**) HTRF measurements of IL-1β secretion by monocytes after LPS [2 ng/ml] priming for 3 h followed by 1.5 h of (**A**) nigericin [10 µM] for NLRP3, (**B**) PrgI/PA [100 ng/ml / 1 µg/ml] for NLRC4, or (**C**) 3 h transfection of 0.5 µg/ml poly(dA:dT) combined to Lipofectamine 2000 (LF) for AIM2 activation or LF as a control. (**B-C**) The experiments of NLRC4 and AIM2 were carried out by Agnieszka Demczuk. (**D**) Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex™ Panel 1 measurement of multiple cytokines. Standard monocytes

(MoStd), platelet-depleted monocytes (MoPD), platelet-depleted monocytes replenished with platelets (MoPD+Plts) and platelets alone (Plts) were stimulated with LPS for 4.5 h (blue) or for 3 h followed by 1.5 h nigericin (red) in n=2-5 donors. Top 19 secreted cytokines are displayed in radar chart generated by Prof. Dr. Bernardo S. Franklin. Floating bars represent max/min values with indication of the mean. Two-way ANOVA with Tukey's multiple comparison test and 95% confidence interval were used. P-value < 0.05 (*). < 0.01 (**) and < 0.001 (***). Each symbol represents one blood donor.

These results highlight the central role of platelets in the pro-inflammatory cytokine response of human monocytes. The presence of platelets did not only amplify the production of IL-1β, but it also was indispensable for the general pro-inflammatory immune response of primary human monocytes.

3.1.3 Platelets affect monocyte cytokine secretion regardless the type of isolation method

The previously described results revealed striking effects on the monocytic function caused by platelet depletion. To exclude the possibility of off-target effects caused by the addition of EasySep™ Human Platelet Removal Cocktail, or due to any other bias related to the use of STEMCELL kit, I decided to isolate monocytes using a second commercially available isolation kit and explore their pro-inflammatory cytokine secretion *ex vivo*. Here, monocytes were isolated side by side with human CD14 MicroBeads (Miltenyi Biotec) via positive selection using magnetic-activated cell sorting (MACS), and the previously described negative selection-based EasySep™ Human Monocyte isolation kit (Figure S3). Monocytes isolated by MACS (MACS-Mo) were compared with MoStd and MoPD obtained with the STEMCELL kit using FACS to characterize MPAs, monocytes and platelets, as well as cytokine measurement to assess the levels of IL-1β, IL-6 and TNFα. Consistent with the results described in section 3.1.1, the FACS data showed that MoStd consisted of a high frequency of MPAs and platelets, whereas these populations were low in MoPD, which had an enriched percentage of platelet-free monocytes. Intriguingly, MACS-Mo obtained with the MACS Miltenyi kit were mainly platelet-free monocytes with strongly decreased platelet counts and few or no MPAs (Figure 3A-B).

Consequently, the MACS monocyte isolation kit (Miltenyi) provided platelet-free monocytes, which were comparable with MoPD population obtained with the STEMCELL kit. To determine whether MACS-Mo behave similarly to MoPD in terms of cytokine secretion, I stimulated cells either with LPS alone [2 ng/ml] for 4.5 h or for 3 h followed by 1.5 h nigericin [10 µM] and collected the supernatants. In agreement with previous findings, MoPD showed a decreased secretion of IL-1β, IL-6 and TNFα. Excitingly, the phenotype of MACS-Mo was more severe than the MoPD, as their cytokine secretion was completely abolished. Consistent with earlier observations, the reconstitution of MoPD or MACS-Mo with autologous platelets rescued cytokine secretion (Figure 3C). Here again, platelets alone did not contribute any cytokines following stimulation.

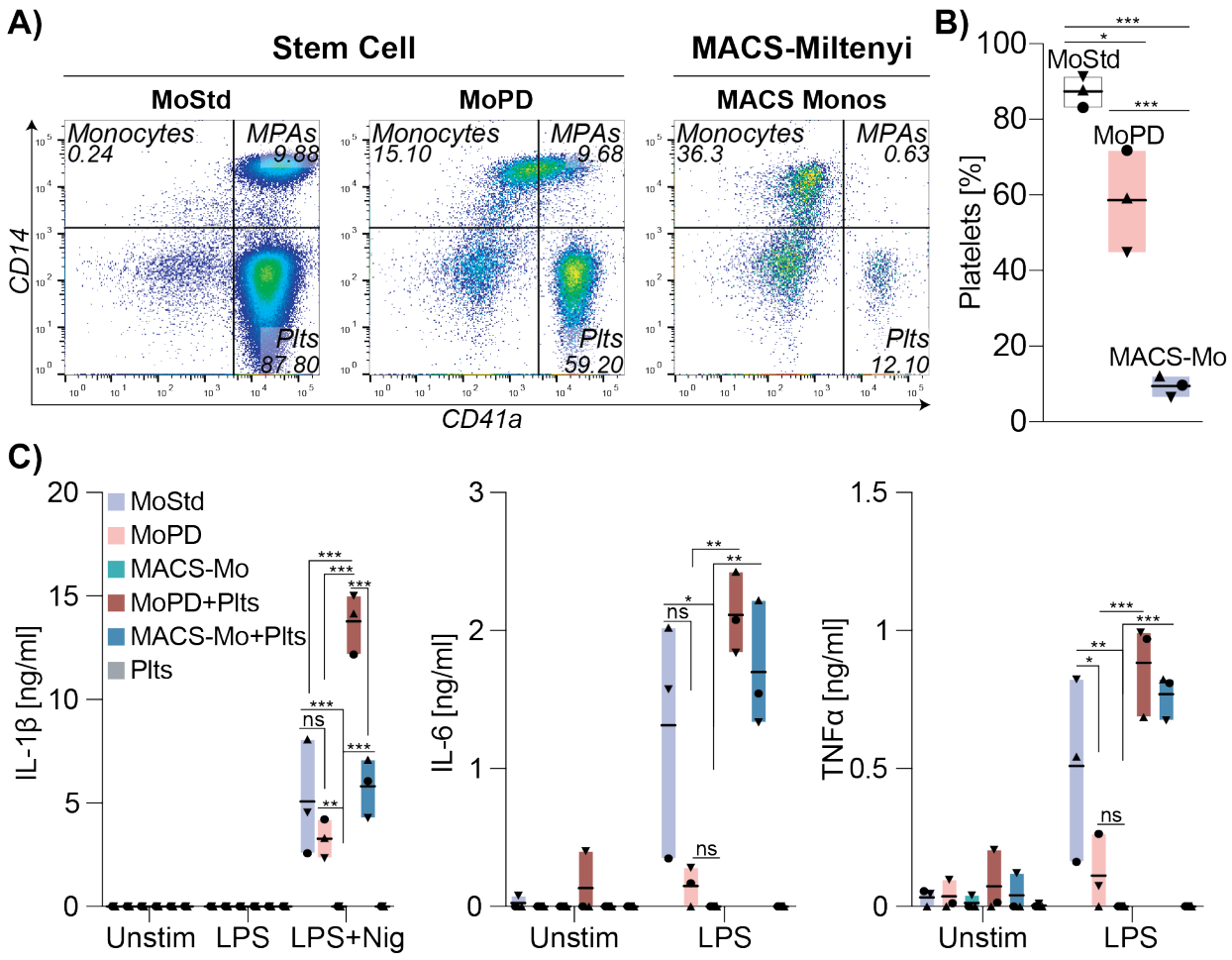

Figure 3: Monocytes isolated with MACS (Miltenyi) are platelet-free and with a disrupted pro-inflammatory cytokine response. (**A**) Representative flow cytometry measurement showing the percentages of monocytes, MPAs and Plts in MoStd, MoPD, and MACS-Mo isolated with MACS (Miltenyi). (**B**) Quantification of platelet frequencies in MoStd and MoPD isolated with the STEMCELL kit, and MACS-Mo isolated with MACS (Miltenyi). (**C**)

Levels of IL-1β, IL-6 and TNFα cytokines measured by HTRF. Cells were either stimulated with LPS [2 ng/ml] for 4.5, or for 3 h followed by 1.5 h of nigericin [10 µM]. All floating bars display the max/min values and indicate the mean. Each symbol represents a different blood donor. For statistics, (**B**) ordinary one-way ANOVA or (**C**) two-way ANOVA were used, each with Tukey's multiple comparison test with 95% confidence interval. P-value < 0.05 (*). < 0.01 (**) and < 0.001 (***).

These results corroborate that platelet depletion disrupts responses to innate immune stimuli and cytokine secretion in primary human monocytes, and excludes the possibility that my observations are the result of an artificial phenomenon caused by the isolation method.

3.1.4 Monocytes from patients with idiopathic thrombocytopenia (ITP) have an impaired capacity to produce cytokines

Ex vivo and *in vitro* experiments have shown that platelet-depleted monocytes have a defective cytokine response. Therefore, I hypothesized that individuals with low platelet counts could exhibit an impaired monocyte-driven immune response. Platelet counts can be affected by several medical disorders. Idiopathic thrombocytopenia (ITP) is a blood disorder characterized by aberrantly low platelet counts (Singh et al., 2021). In collaboration with Dr. med. Lino L. Teichmann and Dr. med. Lisa Meffert from the Internal Medicine III – Oncology/Hematology and Rheumatology Department of the University Hospital of Bonn, I was provided with whole blood from individuals with ITP. To evaluate the monocyte immune responses of these patients, I obtained PBMCs from whole blood of ITP individuals as well as of healthy volunteers, and isolated monocytes using the STEMCELL kit as described before. Importantly, while monocytes from healthy donors were divided into healthy standard monocytes (H-MoStd) and healthy platelet-depleted monocytes (H-MoPD), monocytes from ITP (ITP-MoStd) patients were isolated without any further *ex vivo* platelet depletion.

In addition to the clinical platelet counts provided by Dr. med. Lisa Meffert (Figure 4A), frequencies and abundancies of platelets and monocytes were measured by FACS and CASY TTT 60 cell counter and analyzer. Comparing the morphological parameters using forward and side scatter (FSC and SSC), I was able to distinguish between PBMCs and

platelets. FACS data show that PBMCs from ITP individuals displayed lower percentage of platelets in comparison to PBMCs obtained from healthy donors (Figure 4C). Furthermore, isolated monocytes from ITP and healthy donors were measured with CASY TTT 60 cell counter and analyzer. As expected, *ex vivo* platelet depletion resulted in reduced platelet counts in H-MoPD compared to H-MoStd. Excitingly, isolated standard monocytes from ITP individuals (ITP-MoStd) showed lower platelet counts in comparison to standard monocytes from healthy donors (H-MoStd). Moreover, ITP-MoStd were comparable with H-MoPD with respect to platelet counts (Figure 4B).

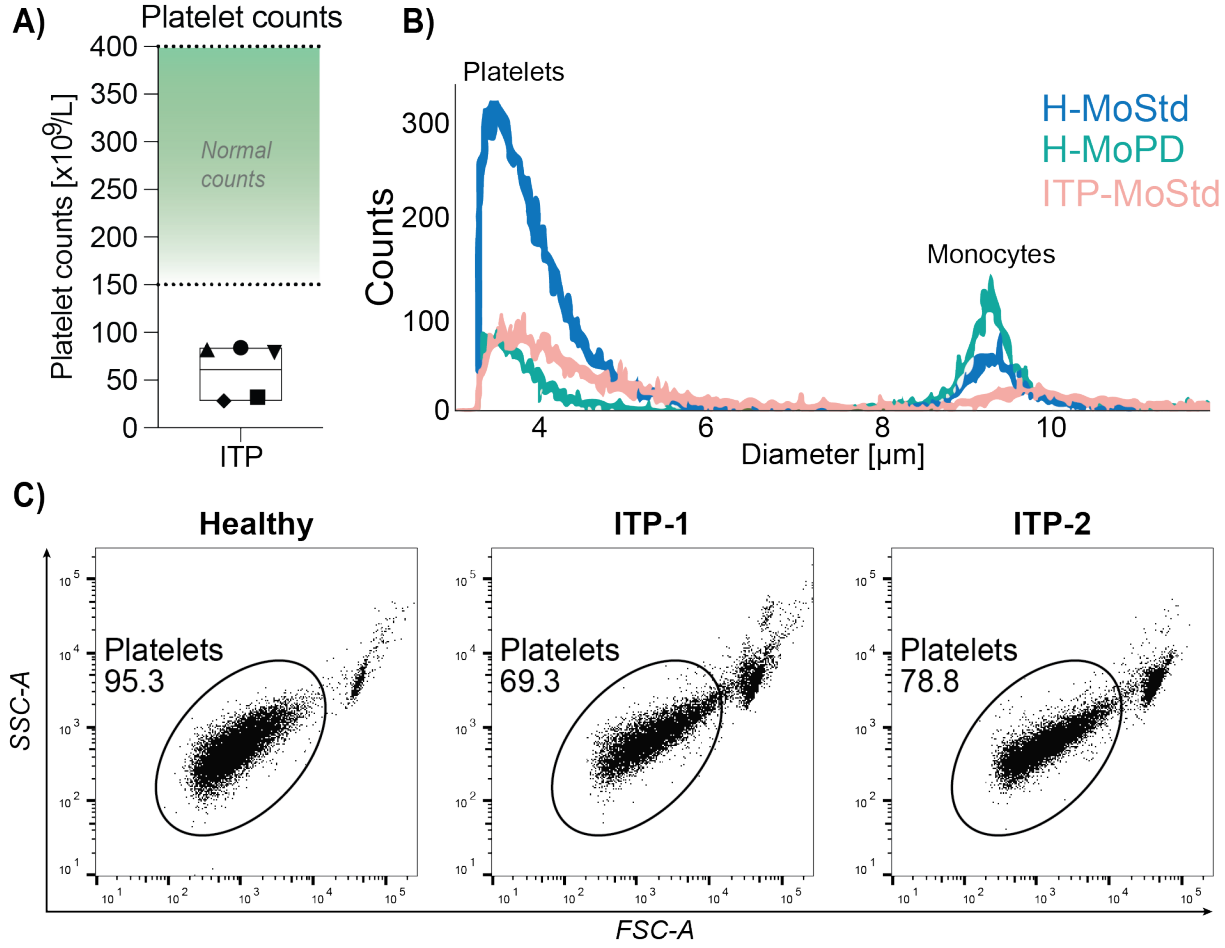

Figure 4: Individuals with idiopathic thrombocytopenia (ITP) display low platelet counts in comparison to healthy donors. (**A**) clinical platelet counts of n=5 ITP patients acquired by Dr. med. Lisa Meffert. Green area in the graph corresponds to the normal platelet counts per liter (Daly, 2011). Each symbol represents one blood donor. (**B**) Data generated with CASY TTT 60 Cell counter and analyzer show cell counts and distribution of monocytes and platelets in cell suspension of healthy standard monocytes (H-MoStd), healthy platelet-depleted monocytes (H-MoPD) and ITP standard monocytes (ITP-MoStd). (**C**)

Representative flow cytometry data display the percentage of platelets (low SSC-A and FSC-A) in PBMCs isolated from healthy and ITP blood.

Since standard monocytes from ITP patients show low proportion of platelets comparable to the *ex vivo* platelet-depleted monocytes from healthy donor, I next measured the concentrations of cytokines secreted from these groups upon stimulation. In addition to the H-MoStd, H-MoPD and ITP-MoStd conditions, platelets from healthy donors (H-Plts) were isolated and served to reconstitute the platelet populations in H-MoPD and ITP-MoStd. Cells were stimulated with LPS [2 ng/ml] alone or LPS followed by nigericin [10 µM] as previously described. In parallel, whole blood from healthy and ITP donors was stimulated with LPS [2 ng/ml] or R848 [10 µM] for 8 h at 37 °C. Concentration of IL-1β, IL-6 and TNFα were quantified in cell-free supernatants using HTRF. Standard monocytes from healthy donors secreted high levels of IL-1β, IL-6 and TNFα, while the levels of these cytokines were decreased in H-MoPD. ITP-MoStd only secreted low levels of IL-1β, IL-6 and TNFα, which were comparable to the cytokine levels of *ex vivo* platelet-depleted monocytes from healthy donors. Importantly, reconstitution of monocytes with H-Plts rescued the cytokine secretion not only in H-MoPD, but also in ITP-MoStd (Figure 5A). Whole blood assays did not show differences in cytokine levels between healthy donors and ITP patients, suggesting a targeted regulatory role of platelets specifically on the immune response of monocytes (Figure 5B).

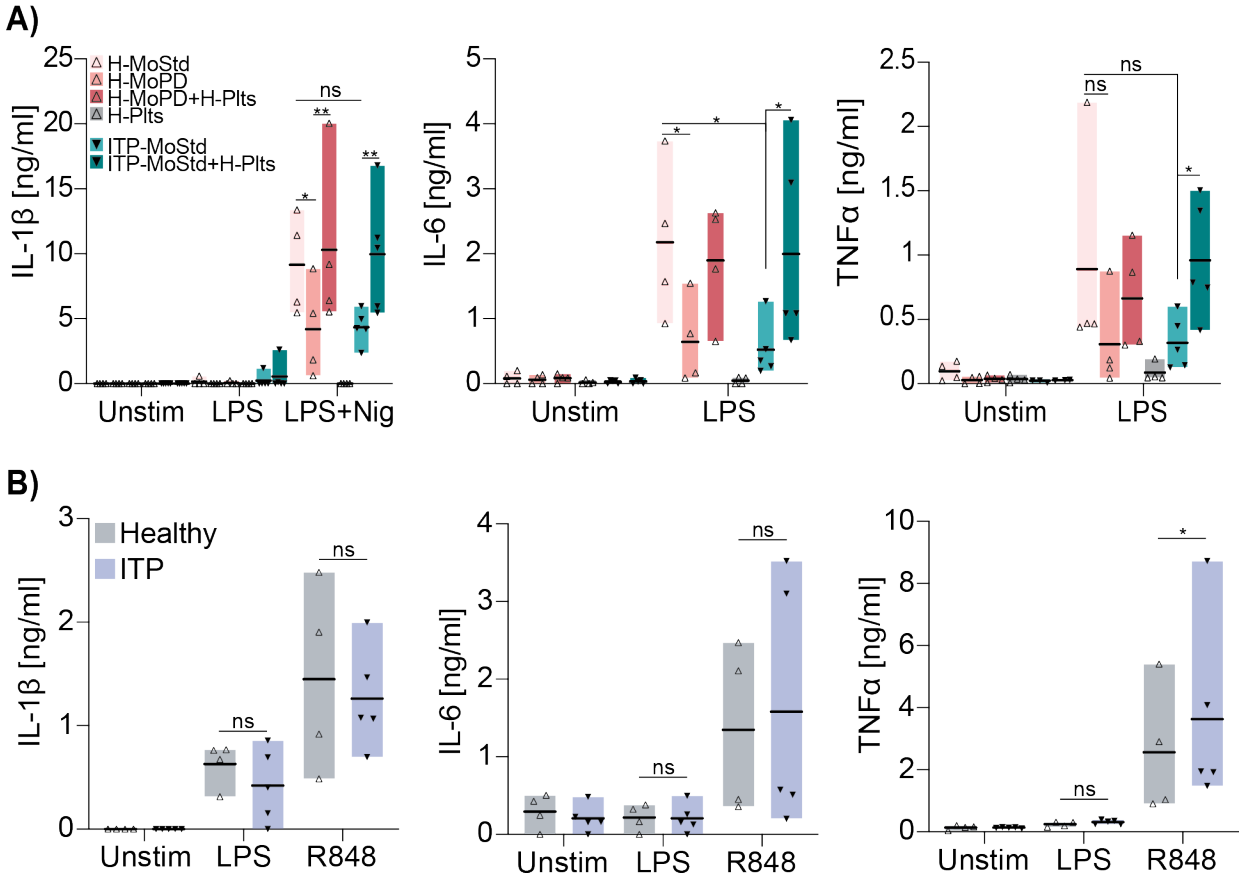

Figure 5: ITP monocytes show an impaired capacity to produce pro-inflammatory cytokines. HTRF measurements display cytokine levels of IL-1β, IL-6 and TNFα in both, (**A**) isolated healthy MoStd, healthy MoPD, healthy MoPD + healthy Plts, ITP-MoStd and ITP-MoStd + healthy Plts, and (**B**) whole blood. Isolated monocytes were stimulated with LPS [2 ng/ml] for 4.5 h or 3 h followed by 1.5 h nigericin [10 µM]. Whole blood from healthy and ITP donors was stimulated with LPS [2 ng/ml] or R848 [10 µM] for 8 h at 37 °C. All floating bars show max/min values with indication to the mean in n=4 healthy and n=5 ITP blood donors. Each symbol represents one blood donor. Two-way ANOVA with Tukey's multiple comparison test and 95% confidence interval were used. P-value < 0.05 (*). < 0.01 (**) and < 0.001 (***).

Together, patients with thrombocytopenia not only suffer from bleeding disorders, but they also show an impaired monocyte-driven immune response. These data not only confirm the fundamental role of platelets in licensing the monocytic immune function, but they furthermore provide a clinical relevance of my *ex vivo* findings.

3.2 TLR signaling and priming event

3.2.1 The platelets' boosting effect is not restricted to a specific TLR

All data so far have shown that platelets induce fundamental changes in the functionality of primary human monocytes, broadly affecting the cytokine response. Hence, I hypothesized that platelets generally affect the TLR signaling network. To test this, I treated MoStd, MoPD, MoPD+Plts and Plts for 4.5 h with Pam3CysK4 [1 µg/ml] to activate TLR1/2, Resiquimod (R848) [10 µM] to activate TLR7/8 or LPS [2 ng/ml] to activate TLR4. To measure IL-1β secretion, cells were primed with the corresponding TLR agonist for 3 h followed by 1.5 of nigericin. Finally, cytokine levels were measured in supernatants using HTRF. Standard monocytes secreted high levels of IL-1β and TNFα after the stimulation or priming with all three TLR agonists. Interestingly, platelet-depleted monocytes showed a decreased cytokine secretion not only after treatment with LPS, but also with Pam3CysK4 and R848. The re-addition of platelets rescued the levels of measured cytokines, regardless of which TLR agonist was used for stimulation (Figure 6). Interestingly, R848 alone induced IL-1β secretion in monocytes with no further requirement of a second signal for inflammasome activation. Also in this setup, monocytes showed an attenuated IL-1β secretion after platelet depletion, which was restored upon platelet replenishment (Figure 6).

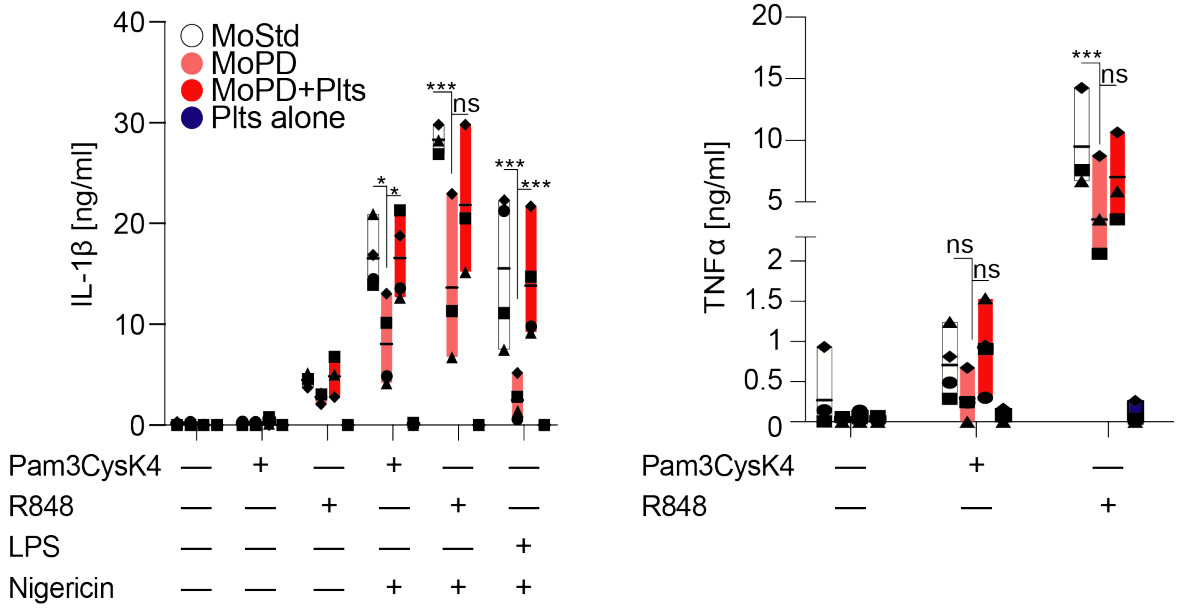

Figure 6: The boosting effect of platelets is not restricted to a specific TLR. HTRF measurement of IL-1β and TNFα secretion. Cells were primed either with Pam3CysK [1 µg/ml], R848 [10 µM] or LPS [2 ng/ml] for 3 h followed by nigericin [10 µM] for further 1.5 h or with TLR agonists alone for 4.5 h. Supernatants were collected and cytokine levels were measured. Floating bars display max/min values with an indication to the mean. Each symbol represents one blood donor. Two-way ANOVA with Tukey's multiple comparison test and 95% confidence interval were used. P-value < 0.05 (*). < 0.01 (**) and < 0.001 (***).

These data indicate that platelets broadly impact the TLR downstream signaling and that this effect is not restricted to a specific TLR.

3.2.2 Platelets act synergistically in the priming of primary human monocytes

Next, I hypothesized that monocytes, in the absence of platelets, produce less cytokines due to an attenuated responsiveness to external stimuli, resulting in low cytokine production and release. To test this, I assessed the cytokine levels not only in the supernatants, but also intracellularly using HTRF. In line with previous data, supernatants from MoStd show high levels of IL-1β and IL-6 secretion, which were abolished after platelet depletion. Intriguingly, the absence of cytokines in the supernatants was connected to the intracellular absence of these cytokines. MoPD showed very low levels of intracellular IL-1β and intracellular IL-6 was completely absent (Figure 7A). Additionally, IL-1β levels were assessed using the WES technology. While monocytes were capable to produce pro-IL-1β (p31) in the presence of platelets (MoStd and MoPD+Plts) during the priming phase and secrete active IL-1β (p17) after inflammasome activation, platelet-depleted monocytes showed an attenuated level of both p31 and p17 (Figure 7B).

Since these results indicate the involvement of platelets in the priming phase, I tested whether platelets only act synergistically with the priming stimulus, or if they could substitute the priming signal for inflammasome activation. To do so, MoStd, MoPD, MoPD+Plts and Plts alone were either left unprimed or were primed with LPS for 3 h. Then, nigericin was added for further 1.5 h for NLRP3 activation, and supernatants were collected for IL-1β measurement with HTRF. In contrast to LPS-primed monocytes, monocytes without the priming signal did not produce high IL-1β levels despite the

presence of platelets. Specifically, MoPD+Plts secreted much more IL-1β when LPS-priming was performed in comparison to MoPD+Plts without prior priming (Figure 7C).

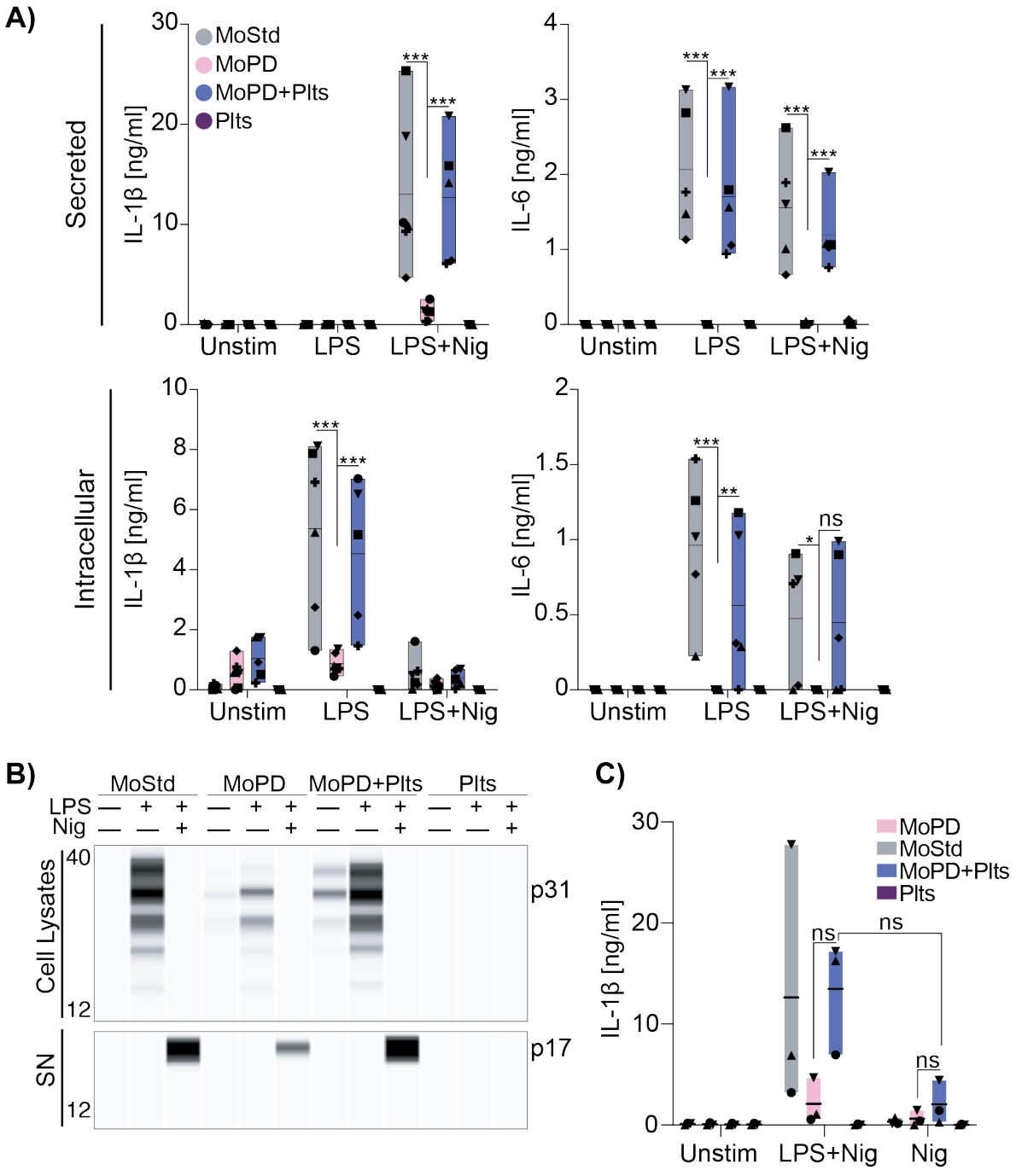

Figure 7: Platelets synergize with LPS priming of primary human monocytes. (**A**) HTRF data showing cytokine levels secreted to the supernatants or produced intracellularly (in cell lysates). Cells were primed with LPS for 3 h followed by nigericin stimulation for 1.5 h, or were stimulated with LPS for 4.5 h. Supernatants were collected and the remaining cells were lysed. Cytokine secretion was assessed in both fractions using HTRF. (**B**) Protein levels of pro-IL-1β (p31) in cell lysates and cleaved IL-1β (p17) in supernatants

(SN) were measured using the WES technology. Cells were stimulated for 4.5 h with LPS or were primed for 3 h with LPS followed by 1.5 h of nigericin treatment. Supernatants were collected for protein precipitation using chloroform and methanol and cell pellet was lysed. MW =molecular weight. (C) HTRF data of IL-1β secretion. Cells were either primed with LPS for 3 h or left unprimed; then treated with nigericin for 1.5 h. Floating bars display max/min values with an indication to the mean. Each symbol represents one blood donor. Two-way ANOVA with Tukey's multiple comparison test and 95% confidence interval were used. P-value < 0.05 (*). < 0.01 (**) and < 0.001 (***).

These data show that monocytes lack intracellular cytokine production in the absence of platelets. Platelets act synergistically to TLR signaling, but do not substitute for an external pro-inflammatory or priming signal.

3.3 Platelets govern gene expression of primary human monocytes

So far, the presented results emphasize the importance of platelets in the functionality of primary human monocytes. Platelets license monocytes to sense and respond to external stimuli by producing pro-inflammatory cytokines. To gain a more complete picture of this regulation, I focused on the gene transcription to better understand the changes caused by the presence or absence of platelets upstream of cytokine secretion. To address this aim, I used NanoString to determine the impact of platelets on the gene expression in primary human monocytes.

3.3.1 Monocytes show low LPS-induced transcriptional activity after platelet depletion

To understand how platelets lead to the functional changes observed at the level of cytokine secretion, the gene expression profiles of primary human monocytes were investigated. To do so, $1x10^5$ MoStd, MoPD, MoPD+Plts or $1x10^7$ Plts alone were seeded per well, followed by the stimulation with LPS or LPS+Nig as previously described. Then, cells were lysed in RLT buffer containing β-meracaptoethanol. Finally, gene expression was assessed with nCounter® Human Myeloid Innate Immunity Panel v2 (NanoString) according to the manufacturer's protocol. Before proceeding with data analysis, the quality of the acquired data was evaluated by Dr. rer. nat. James Hembach-Stunden. Here,

platelets alone displayed almost no or negligibly low gene expression so no measurement was possible (not shown). Thus, the contribution of platelet-derived mRNA was negligible in the total gene signature of monocyte-platelet heterogenic populations and platelets were only displayed in the principal component analysis (PCA) graph. Next, I performed a normalized and processed the data as described in section 2.2.14.

First, I performed a PCA using Partek® Genomics Suite® to determine the general changes in gene expression according to the clustering pattern of the groups. Platelets formed a distinct cluster on the left side with a low PC #1 value, with a considerable distance to monocytes. In contrast, monocytes clustered on the right side with a high PC #1 value. Generally, unstimulated conditions clustered in the “unstimulated region” with a high PC #2 value, while a stimulation with LPS or LPS+Nig induced a shift to a lowed PC #2 value toward the “stimulated region”. Under unstimulated conditions (green), MoStd (ball) clustered in the “unstimulated region”. Upon stimulation with LPS (orange) or LPS+Nig (red), MoStd displayed a shift toward the “stimulated region” in the PCA. Interestingly, MoPD (pyramid) clustered in the “unstimulated region” and did not display a shift to the “stimulated region” despite the stimulation with LPS or LPS+Nig. Moreover, the re-addition of platelets to MoPD (MoPD+Plts) restored the stimulation-induced cluster shift toward the “stimulated region”. This indicates that after platelet depletion, monocytes do not undergo transcriptional alterations induced by stimulation as detected in standard monocytes (Figure 8).

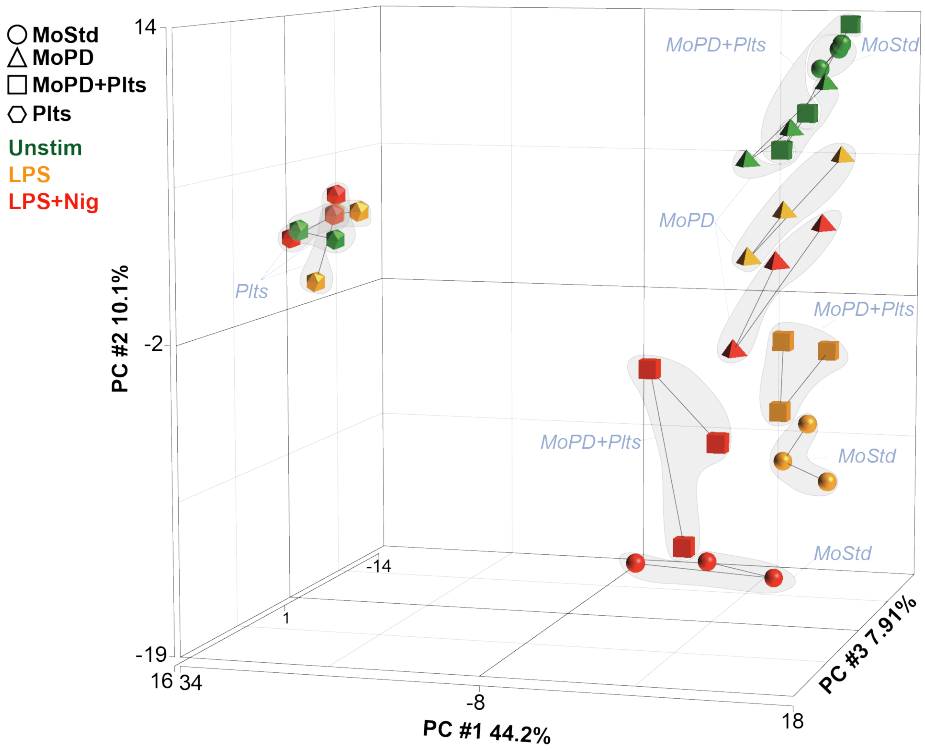

Figure 8: NanoString Principal Component Analysis. PCA displays the distribution of four cell groups according to their gene expression profile. MoStd (ball), MoPD (pyramid), MoPD+Plts (cube) and Plts (hexagonal prism) were left unstimulated (green), treated with LPS for 4.5 h (orange) or with LPS for 3 h followed by 1.5 h of nigericin (red). Each group consists of three symbols, each one representing one of n=3 blood donors.

These data indicate that platelets impact the transcriptional profile of primary human monocytes. Platelet-depleted monocytes seem to be nonresponsive also on transcriptional level.

3.3.2 Platelets facilitate LPS-induced transcriptional response in primary human monocytes

Next, to evaluate the LPS-induced transcriptional alterations between the groups, I analyzed the differentially expressed genes (DEGs) after LPS stimulation within each group of MoStd, MoPD and MoPD+Plts. Then, I quantified the number of unchanged or significantly altered genes with fold change ≥ 2 and p-value < 0.05. Pie charts show that LPS stimulation induced 154 DEGs in MoStd, while in MoPD only 4 genes were

significantly changed. Interestingly, the re-addition of Plts to MoPD (MoPD+Plts) increased the number of DEGs to 86 (Figure 9).

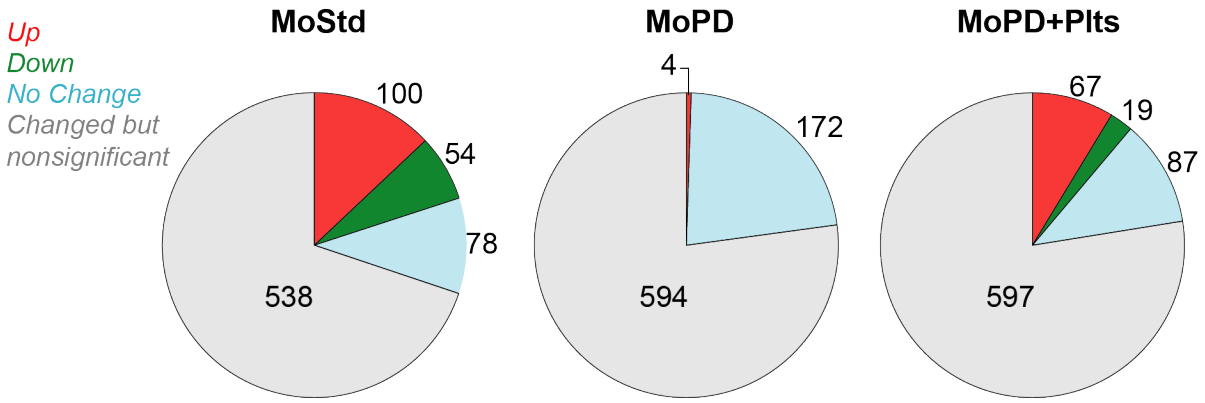

Figure 9: Quantification of LPS-induced differentially expressed genes (DEGs). Pie charts display the number of DEGs after LPS treatment of MoStd, MoPD and MoPD+Plts. Each pie chart displays the number of gene sets that were significantly upregulated (red), significantly downregulated (green), nonsignificantly changed (gray) or remained unchanged. Significantly changed genes were altered genes with fold change ≥ 2 and p-value < 0.05.

These data indicate that platelets significantly alter the transcriptional profile of primary human monocytes. To further elucidate the role of platelets in the regulation of transcription in primary human monocytes, I explored the DEGs (fold change ≥ 2 and p-value < 0.05) I previously identified in Partek® Genomics Suite® by generating volcano plots. Therefore, I log-transformed the values of the fold change (Log2) and p-values (-Log10) and displayed these values in a volcano plot in GraphPad Prism.

The NanoString data show that LPS induced a specific gene expression profile in MoStd. Pro-inflammatory genes were upregulated, such as interleukins (e.g. *IL6*, *IL1A*, *IL1B*), NF-κB-related genes (*NFKB1*, *NFKBIA*), co-stimulatory molecules (e.g. *CD80*) and chemokines (e.g. *CCL2*). In contrast, the expression of other mRNAs was downregulated, such as chemokine receptors (e.g. *CCR2*), the anti-inflammatory *MERTK*, or *FCGR3A* which is involved in Fc-dependent cellular cytotoxicity (Mellor et al., 2013) (Figure 10). As previously shown in the pie charts (Figure 9), LPS significantly induced the expression of only 4 genes in MoPD: *TNFAIP8*, *RIPK2*, *TRAF1*, and *BTG2*. Interestingly, when platelets

were added to MoPD, the impaired transcriptional capacity was reversed and a set of LPS-induced genes was partially restored (Figure 10).

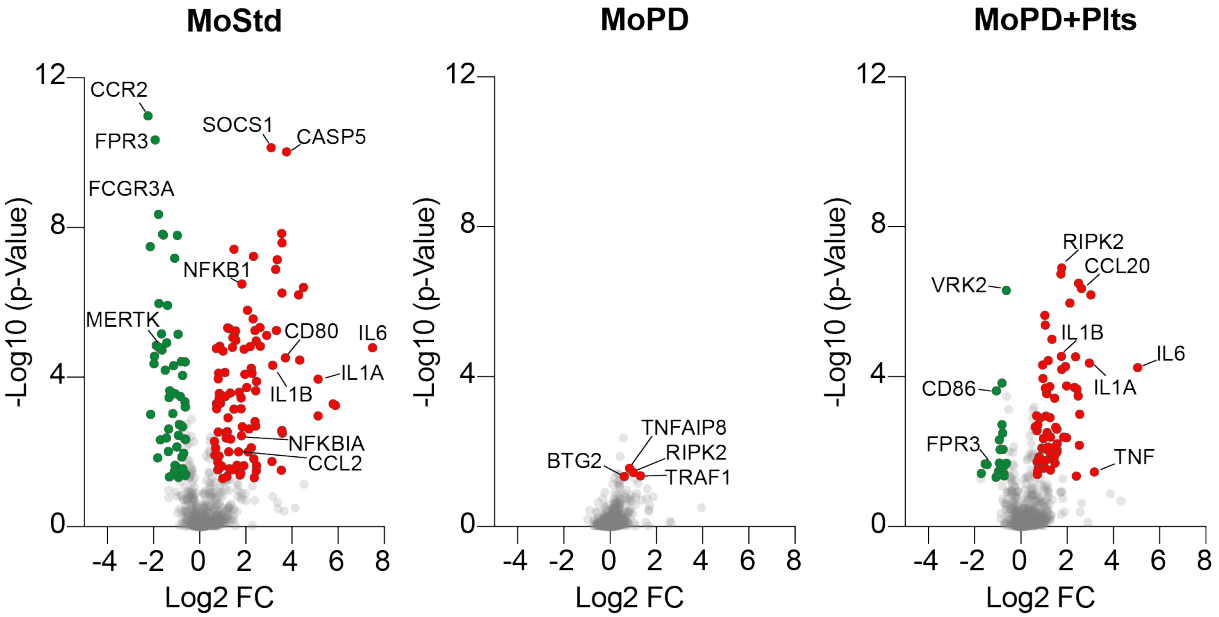

Figure 10: LPS-induced differentially expressed genes (DEGs). Volcano plots display the significantly upregulated (red) and downregulated (green) genes in MoStd, MoPD and MoPD+Plts upon stimulation with LPS [2 ng/ml] for 4.5 h (n=3 blood donors). Significantly changed genes fulfilled the criteria of fold change ≥ 2 and p-value < 0.05.

Together, these findings suggest that the magnitude of transcriptional responsiveness of monocytes stimulated with LPS is positively controlled by platelets. The abolished LPS-induced transcriptional response can be restored upon platelet replenishment, implying that the impact of platelet depletion on the transcriptional response of monocytes is reversible.

3.3.3 Platelet depletion induces transcriptional reprogramming in primary human monocytes in steady state

To investigate if platelet depletion induces transcriptional changes in primary human monocytes in steady state, MoStd and MoPD were compared under unstimulated conditions. Here, I generated a list of DEGs (fold change ≥ 2 and p-value < 0.05) using Partek® Genomics Suite®. Data were then log-transformed as described previously and

plotted in GraphPad Prism. Interestingly, NanoString data show that the absence of platelets per se induces changes on the transcription level of several genes. There were 43 and 44 significantly up- and downregulated genes, respectively (Figure 11A). I plotted all genes in a volcano plot and highlighted the genes significantly up-regulated (red) and down-regulated (green) upon platelet depletion (Figure 11B). Interestingly, several hits of the significantly altered genes play immune regulatory roles in monocytes: downregulated genes included genes required for sensing of chemokines or external stimuli such as *CCR2*, *FCGR3A* and *CD14*. Similarly downregulated were genes encoding kinases involved in pro-inflammatory signaling such as *BTK* and *MAPK14* (p38-α) (Herlaar & Brown, 1999; Weber et al., 2017), or *RAB3D*, a GTPase involved in vesicular trafficking pathways (Pavlos et al., 2005). In contrast, some other genes were upregulated, among them transcription factors e.g. *EGR2*, *PPARG*, as well as NF-κB repressor genes such as *ATF3* and inhibitors of MAPK signaling pathway such as *SPRY2* (De Nardo et al., 2014; Tsavachidou et al., 2004) (Figure 11B).

Finally, to obtain a biological interpretation, I performed gene set analysis (GO enrichment) in Partek® Genomics Suite®. Significantly changed genes were analyzed using Fisher's Exact test for groups consisting of more than 2 genes. Enrichment scores were displayed as a bar chart and a forest plot (Figure 11C-D). GO enrichment shows that platelet depletion caused an enrichment of gene sets involved in i) response to stimuli, such as detection and response to environmental changes and external stimuli, ii) immune system processes, such as activation of immune response or tolerance induction, iii) locomotion and taxis, and iv) general biological processes, e.g. interaction and biological regulation (Figure 11C). Forest plot deconvoluted the number of up- and down-regulated genes in each of the classified GO terms sorted by the number of sufficiently downregulated genes. The "biological regulation" group contained the highest number of downregulated genes (Figure 11D).

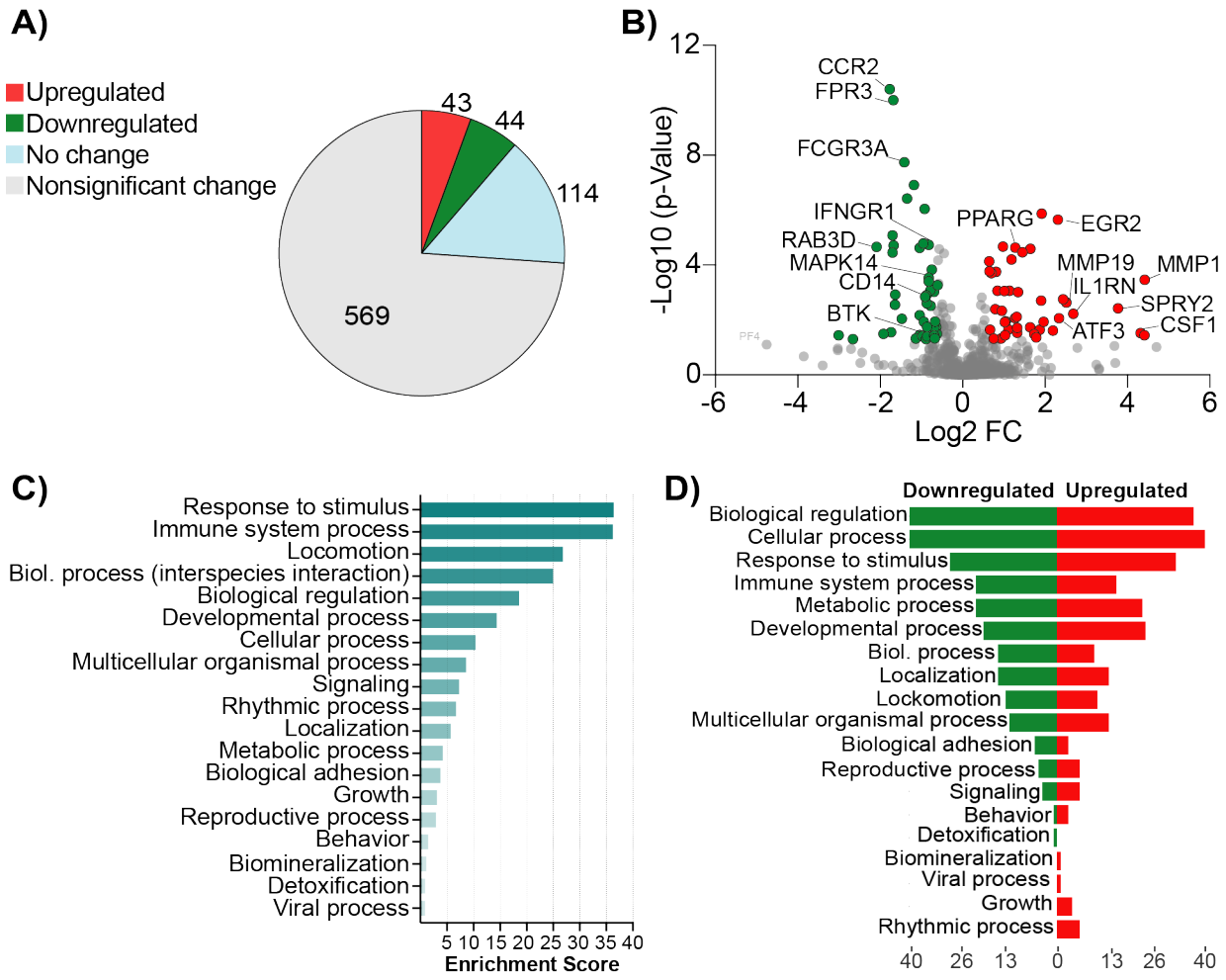

Figure 11: Platelet depletion induces transcriptional reprogramming in primary human monocytes. (**A**) Pie chart representing the number of significantly upregulated (red), downregulated (green), non-significantly changed (gray) and unchanged genes (blue) upon platelet depletion. (**B**) Volcano plot displays differentially expressed genes induced by platelet depletion alone comparing unstimulated MoPD and MoStd. Volcano plot shows significantly upregulated (red) and downregulated (green) genes. (**C**) Gene ontology (GO) analysis displays enriched gene sets categorized in biological processes that were changed upon platelet depletion. (**D**) Forest plot presents GO analysis showing the proportions of down- (green) and upregulated (red) enriched gene sets in each GO category. Alterations with fold change ≥ 2 and p-value < 0.05 are considered as significant.

These data emphasize that the absence of platelets alone is sufficient to change the transcriptional fingerprint of primary human monocytes. In the absence of platelets, genes involved in pro-inflammatory signaling such as MAPK14 (p38-α) and BTK are downregulated, while other mRNAs encoding regulatory proteins involved in monocyte-differentiation processes, DNA methylation, or repressors of NF-κB are induced. Thus, platelets shape the gene expression profile of primary human monocytes.

3.4 Bulk RNA sequencing of murine monocytes after platelet depletion *in vivo*

So far, the experimental outcomes of platelet depletion *ex vivo* have emphasized the key role of platelets in governing the gene expression of primary human monocytes. To determine how these effects translate into a more physiological environment, I performed *in vivo* mouse experiments with the help of Dr. Lucas Ribeiro (Institute of Innate Immunity, University Hospital of Bonn), followed by bulk RNA sequencing (RNA-Seq) performed by GENEWIZ (Azenta Life Sciences).

3.4.1 Platelet depletion *in vivo* elevates LPS-induced cytokine levels in the blood

To understand how platelets modulate the immune response of monocytes *in vivo*, mouse experiments were performed. Here, Dr. Lucas Ribeiro (Institute of Innate Immunity, University Hospital of Bonn) performed the injections of mice and subsequent blood collection. Twelve-week old female C57BL/6J mice were intravenously (i.v.) injected with 2 mg/kg polyclonal IgG fraction from non-immunized rats as a mock group or 2 mg/kg polyclonal rat anti-mouse GPIbα for mouse platelet depletion. After 12 hours, mice were challenged i.v. with LPS [2 mg/kg] or PBS for 3 h. Finally, blood was collected in tubes containing heparin [5,000 U/ml]. Using density gradient centrifugation, I obtained PBMCs and collected plasma for cytokine measurements. PBMCs were stained with markers for a monocyte-specific expression profile of surface molecules ($Ly6G^{-}IA/IE^{-}CD45^{+}CD11b^{+}CD115^{+}$). Monocytes were sorted at the Flow Cytometry Core Facility (FCCF) of the University Hospital of Bonn directly into QIAzol lysis reagent for further processing and bulk RNA-Seq at GENEWIZ (Figure 12A).

To confirm the efficiency of the *in vivo* platelet depletion, I performed FACS measurements and quantified the frequencies of platelets in whole blood. Representative FACS plots confirm platelet depletion after the application of the anti-mouse GPIbα antibody (Figure 12B). Furthermore, the analysis of data from n=3 experiments shows that the frequency of platelets decreased by 91.7% upon platelet depletion *in vivo* (Figure 12C). Finally, the levels of IL-1β, IL-6 and TNFα in collected plasma were quantified by HTRF. LPS treatment induced the secretion of IL-1β, IL-6 and TNFα in mock-treated and platelet-

depleted mice. Interestingly, the platelet-depleted group had a stronger LPS-induced cytokine response compared to mock-treated mice (Figure 12D).

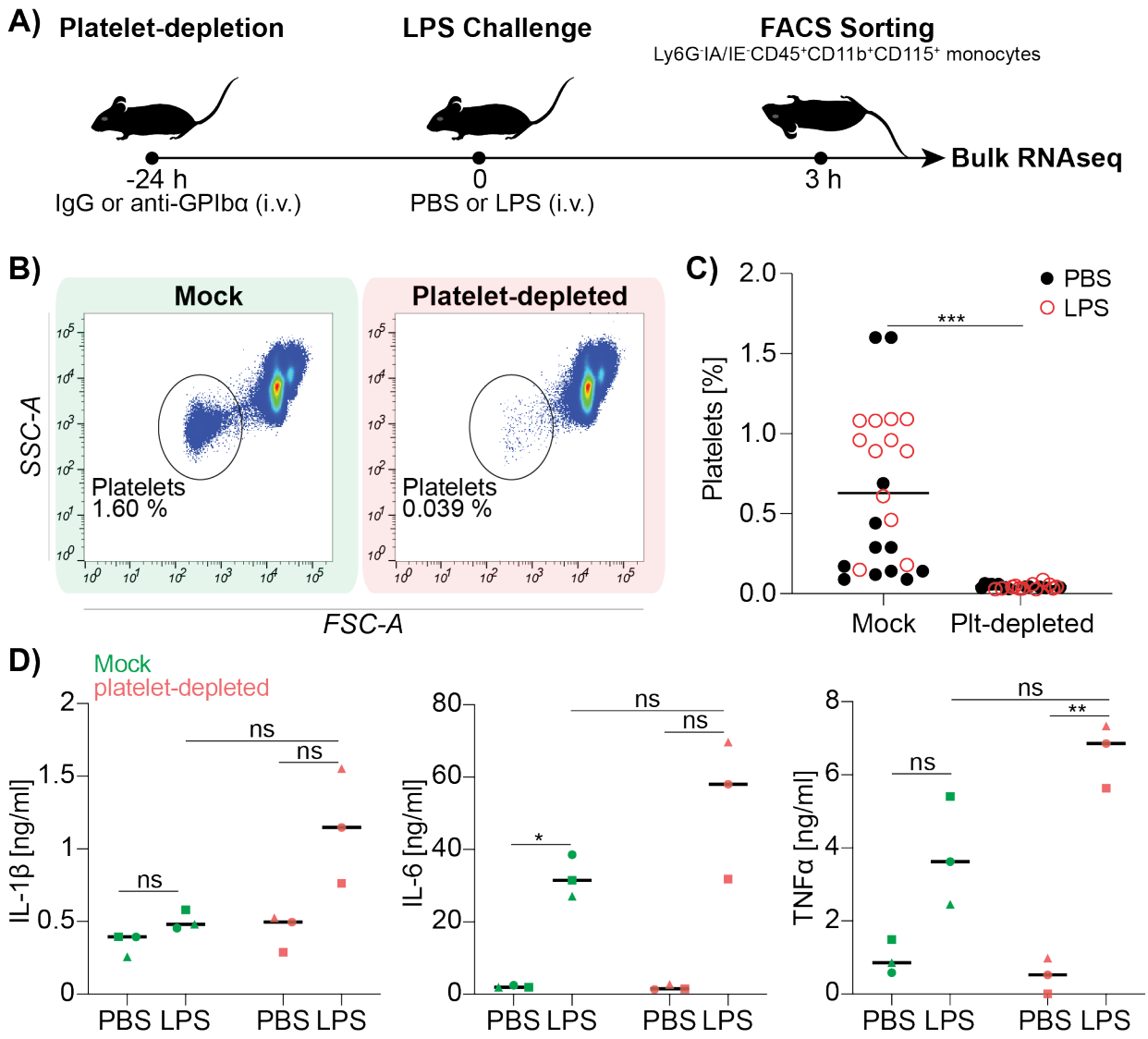

Figure 12: Platelet depletion *in vivo*. (**A**) Schematic illustration of experimental settings. (**B**) Representative FACS plot displaying the percentage of platelets in whole blood of mock-treated or platelet-depleted mice. (**C**) Quantification of FACS data displaying the percentage of platelets in whole blood of mock-treated mice vs. platelet-depleted mice (n=24 per condition). (**D**) HTRF data show the concentrations of TNFα, IL-1β and IL-6 in collected plasma from mock-treated and platelet-depleted mice. Each symbol represents pooled blood from four mice in one of n=3 independent experiments. For statistical analysis (**C**) nonparametric two-tailed unpaired t Test with Mann-Whitney Test or (**D**) two-way ANOVA with the Geisser-Greenhouse correction and Tukey's multiple comparisons were applied.

These data demonstrate that platelet depletion using anti-mouse GPIbα induced thrombocytopenia in mice. In comparison to mock-treated mice, platelet-depleted mice showed higher plasma levels of IL-1β, IL-6 and TNFα after LPS challenge, suggesting a protective role of platelets during host response to LPS in the mouse system. However, statistical analysis did not reach significance.

3.4.2 Platelet-depleted mouse monocytes show an enhanced LPS-induced transcriptional response in comparison to monocytes from mock-treated mice

To determine the role of platelets in modulating the function of monocytes, bulk RNA sequencing was performed. The yield of sorted Ly6G$^-$IA/IE$^-$CD45$^+$CD11b$^+$CD115$^+$ monocytes was in the range of 500 – 10,000 cells in total per run. Interestingly, fewer monocytes could be isolated from mice treated with LPS (Figure 13A). For RNA-Seq, samples were sent to GENEWIZ (Azenta Life Sciences) and were processed according to the company's workflow.

Initially, I was interested in the differences between monocytes from platelet-depleted mice and mock-treated mice after LPS challenge. Therefore, I compared the number of significantly changed LPS-induced genes in both groups and displayed these in Venn diagram (Figure 13B). These data show that LPS induced transcriptional changes of 3.2 times as many genes in monocytes from platelet-depleted mice when compared to monocytes from mock-treated mice. Furthermore, ~84% of significant DEGs in mock-treated mice were common with the significant DEGs in platelet-depleted mice. Monocytes from platelet-depleted mice uniquely expressed 3008 DEGs, whereas monocytes from mock-treated mice expressed 202 unique DEGs (Figure 13B).

Next, I plotted the DEGs in a volcano plot, highlighting the significant ones (as calculated by GENEWIZ): values with adjusted p-value (adj. p-value) of less than 0.05 and the log2 fold change greater than 1 were upregulated DEGs (red), whereas values with adj. p-value of less than 0.05 and log2 fold change of less -1 were downregulated DEGs (green) (Figure 13C-D). The challenge with LPS induced an upregulation of pro-inflammatory

gene sets (such as *Cxcl10*, *Ccl12*, *Il1a*, *Ly6c*, interferon-stimulated genes (ISGs) and *Il12a*) as well as the downregulation of several genes (such as *Cx3cr1*, *Ccl6* and *Card10*) in both groups (Figure 13C-D). Surprisingly, the magnitude of the fold change induction of the 1080 commonly expressed genes was similar in both groups (Figure S5).

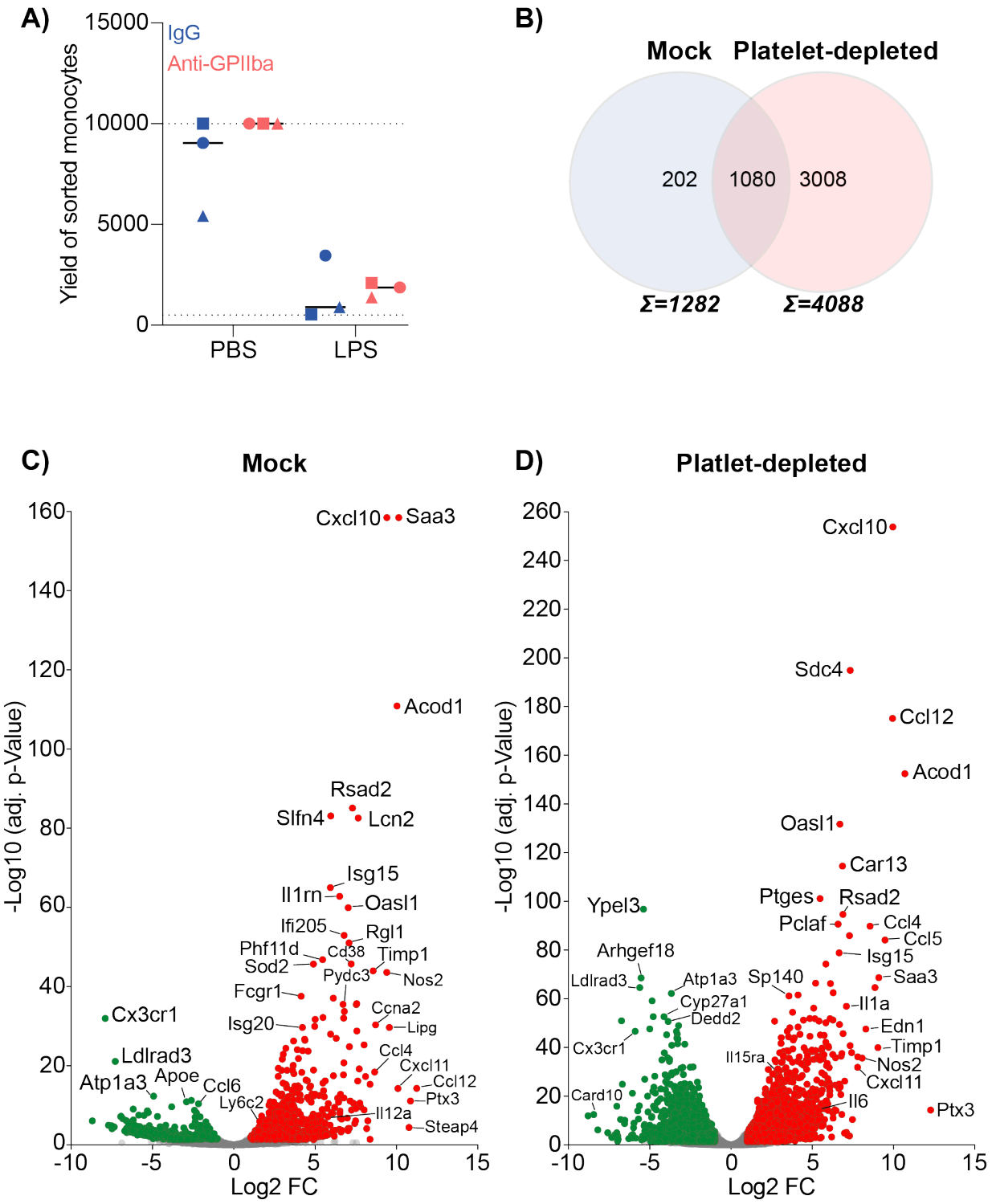

Figure 13: LPS-induced transcriptional response in mouse monocytes of mock-treated and platelet-depleted mice. (**A**) Presentation of monocyte yield after sorting in n=3 independent experiments. (**B**) Venn diagram displays the number of differentially expressed genes after LPS challenge in mock-treated or platelet-depleted mice. The number presented in the intersection represents the number of DEGs that were commonly significantly expressed in both mouse groups. Non-intersecting parts display the number of uniquely expressed DEGs in either mock-treated or platelet-depleted mice. (**C-D**) Volcano plots display significant differentially expressed genes in (**C**) mock-treated and (**D**) platelet-depleted mice with adjusted p-value < 0.05 and log2 fold change > 1.

These data show that monocytes from mock-treated and platelet-depleted mice express different transcription profiles upon challenge with LPS. Although LPS-induced pro-inflammatory genes (e.g. cytokines) were commonly expressed to a similar extent, monocytes from platelet-depleted mice expressed ~15 times more unique genes. This suggests a generally broader transcriptional response, which corresponds to the classes of altered uniquiely expressed genes rather than their expression magnitude.

3.4.3 Platelet depletion *in vivo* polarizes mouse monocytes towards a "cell maintenance" state

The previous RNA-Seq data indicate that platelet depletion in mice does not alter the magnitude of monocytes' response to LPS, but it rather induces an enrichment of distinct gene classes. For a biological interpretation of this phenomenon, gene ontology analysis of the significant DEGs was applied by GENEWIZ (Azenta Life Sciences) using the Fisher exact test, and GO terms were assigned using the software GeneSCF v.1-p2 software.

First, I quantified the number of GO terms identified in monocytes from mock-treated and from platelet-depleted mice upon LPS challenge and determined the common GO terms (Figure 14A). Interestingly, the majority of GO terms identified in monocytes from mock-treated mice represented immune, inflammatory and defense functions. In monocytes from platelet-depleted mice, these were rather processes associated with cell cycle, cell division and apoptosis (Figure 14B-C). Similarly, when comparing the common GO terms shared by the two mouse groups, monocytes from platelet-depleted mice displayed an enrichment in cellular processes such as cell cycle, chromosomal segregation, cell

division and mitotic cytokinesis, and less enrichment in immune system processes, viral defense and the response to lipopolysaccharide (Figure 14D).

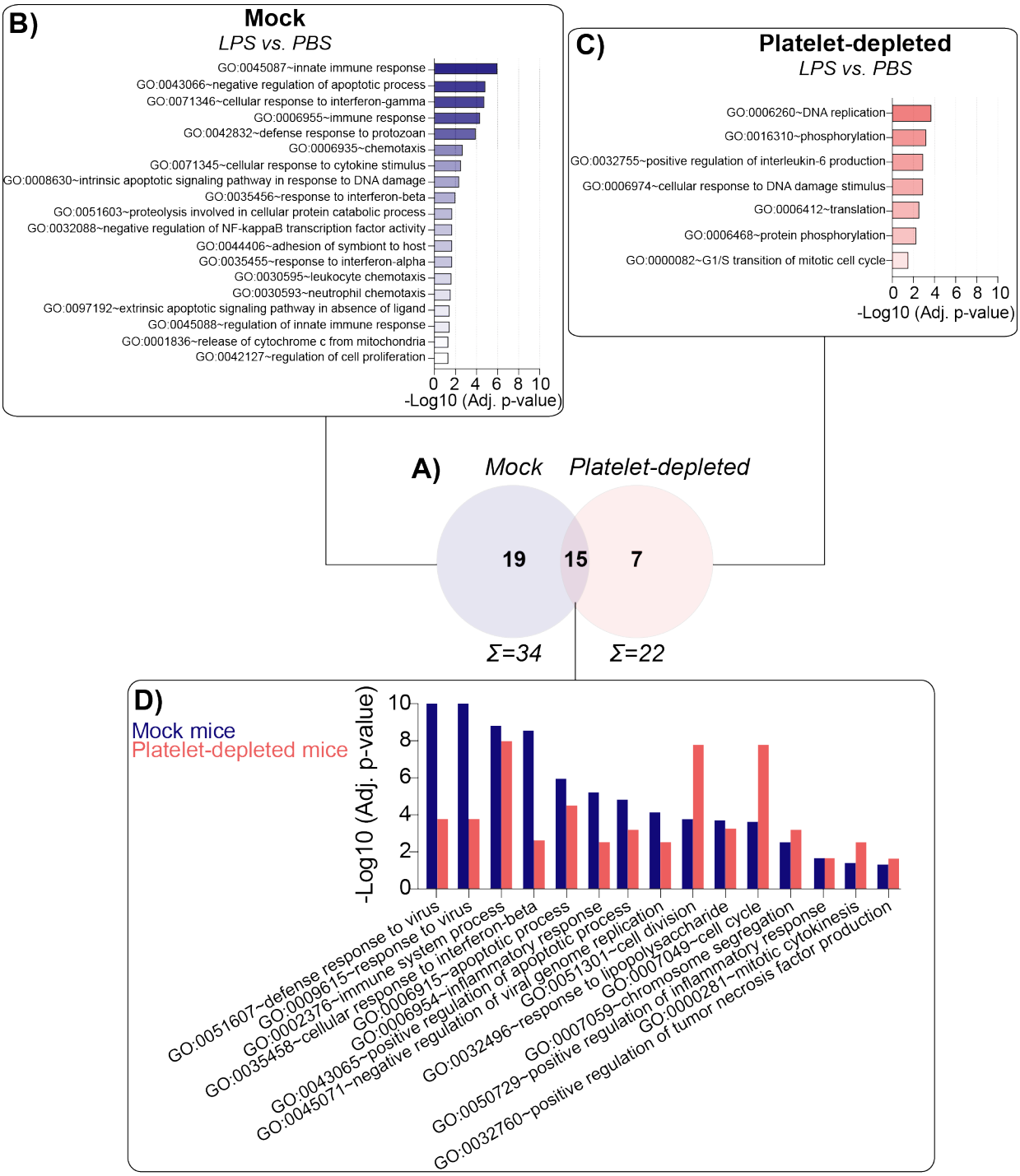

Figure 14: Gene ontology analysis of LPS-induced significant DEGs in monocytes from mock-treated and platelet-depleted mice. (**A**) Venn diagram displays the number of GO terms that are common (intersection) or unique for either mock-treated or platelet-depleted mice (non-intersecting). (**B-D**) Bar plots display GO terms enriched either uniquely in

monocytes from (**B**) mock-treated or (**C**) platelet-depleted mice, or (**D**) are common between both groups.

These data suggest that although monocytes in platelet-depleted mice maintain their pro-inflammatory capacity, platelet depletion induces transcriptional reprogramming leading to their polarization towards “cell maintenance” state.

3.4.4 Platelet depletion is sufficient to induce transcriptional changes of genes involved in signal transduction and immune response

To understand how platelets govern the transcriptional gene signature of monocytes at steady state, I evaluated the significant DEGs (provided by GENEWIZ) induced by platelet depletion alone. Interestingly, monocytes from thrombopenic mice showed predominantly a downregulation of platelet-related genes (such as *Pf4*, *Tubb1*, *Myl9*, *Gp9*, *Ppbp*, *Treml1* and *Clu*), confirming the depletion of platelets; whereas genes encoding the components of the complement system (such as *C1qa*, *C1qb* and *C1qc*) and other genes involved in cell activation processes (e.g., *Htra3* and *Mertk*) were upregulated (Figure 15A).

Next, I analyzed gene ontology enrichment of significantly altered DEGs using GO Process of the online tool (URL: http://bioinformatics.sdstate.edu/go75/; accessed: February 2022) ShinyGO v0.75 (Ge et al., 2020; Kanehisa et al., 2021; Luo & Brouwer, 2013). The top 20 enriched GO terms mainly showed changes in blood coagulation, platelet and cell activation, complement activation as well as humoral immune response and immune system processes (Figure 15B). To strengthen the analysis, I additionally used the database of Partek® Genomics Suite®. Here, I applied GO enrichment analysis to the significant DEGs using Fisher’s Exact Test. Platelet depletion *in vivo* induced changes in gene sets involved generally in biological regulation, cellular processes, locomotion, developmental processes and immune system processes. When GO terms were considered in detail, GO term hits such as cell activation, signal transduction, transmembrane transport, immune response, cellular response to stimulus and leukocyte activation were among the mostly drastically changed between the two groups (Figure 15C).

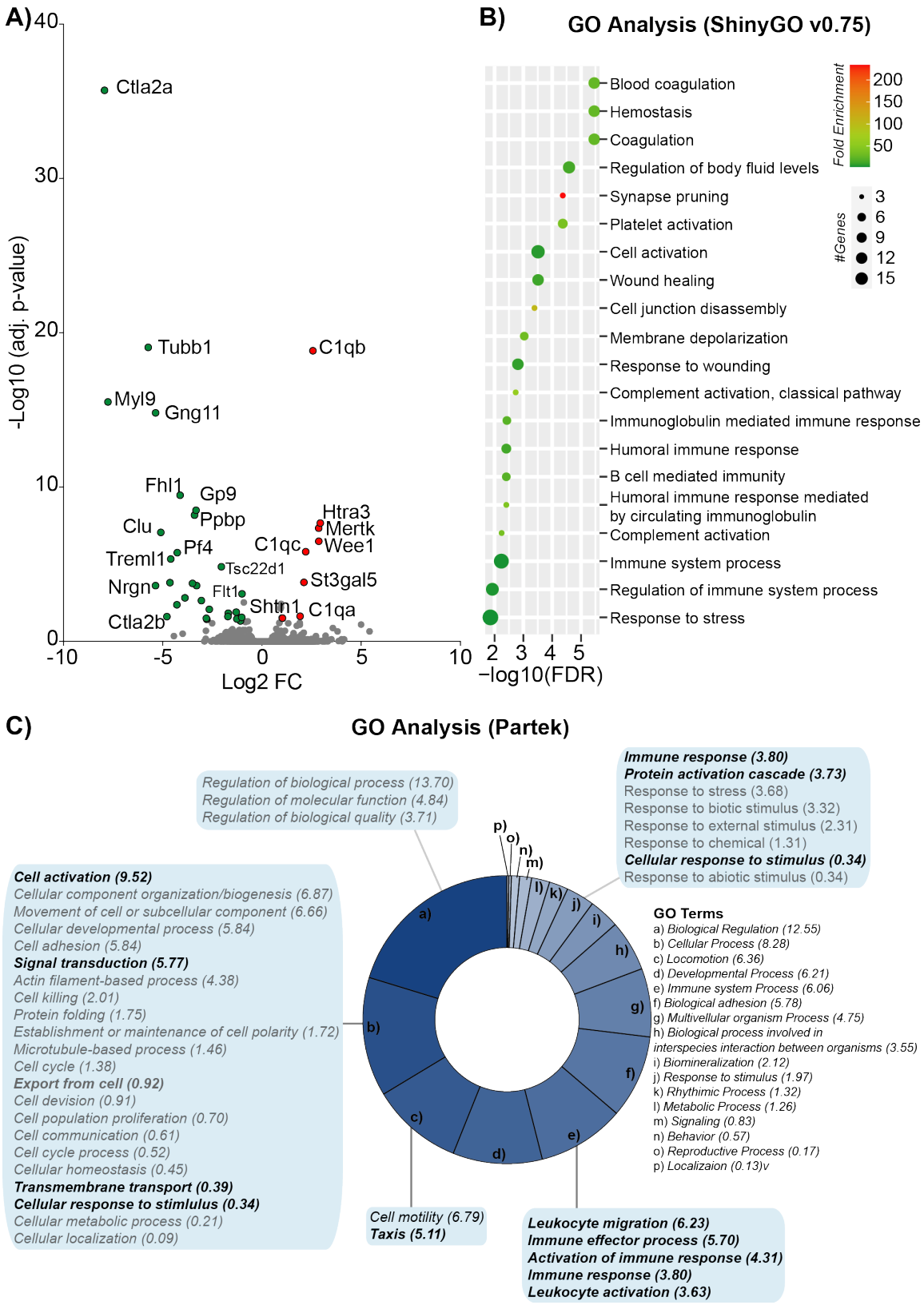

Figure 15: Platelet depletion-induced transcriptional alterations. (**A**) Volcano plot displays the significantly upregulated (red) and downregulated (green) genes upon platelet depletion without further stimulation. (**B**) GO Analysis performed with the online tool (URL:

http://bioinformatics.sdstate.edu/go75/; accessed: February 2022) ShinyGO v0.75 displays the top 20 enriched gene ontology terms (Ge et al., 2020; Kanehisa et al., 2021; Luo & Brouwer, 2013). (**C**) Detailed GO analysis generated in Partek displaying the gene sets enriched upon platelet depletion. Highlighted are biological processes involved in immune responses, cell motility and transport machinery.

Finally, I performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using the online tool (URL: http://bioinformatics.sdstate.edu/go75/; accessed: February 2022) ShinyGO v0.75 (Ge et al., 2020; Kanehisa et al., 2021; Luo & Brouwer, 2013) and Partek® Genomics Suite®. Pathway analysis showed high enrichment in complement and coagulation cascades, disease-associated pathways, chemokine signaling pathway, Rap1 signaling pathway and PI3K-Akt signaling pathway induced by platelet depletion *in vivo* (Figure 16A-B).

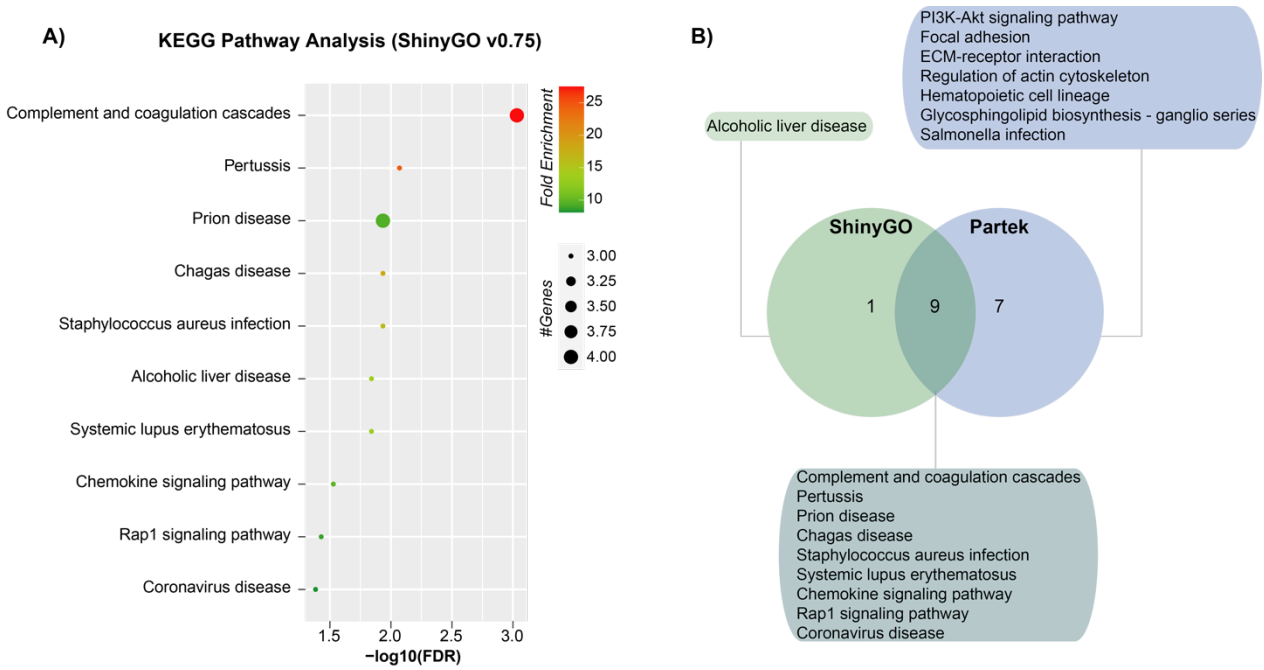

Figure 16: KEGG Pathway analysis of DEGs in mouse monocytes upon platelet depletion. (**A-B**) KEGG Pathway analysis performed on significant DEGs upon platelet depletion. (**A**) Dot-plot diagram generated in ShinyGO v0.75 displays top 10 enriched pathways. (**B**) Venn diagram displays KEGG Pathways identified by ShinyGO v0.75 and in Partek. Data analyzed with ShinyGo v0.75 were generated online (URL: http://bioinformatics.sdstate.edu/go75/; accessed: February 2022) (Ge et al., 2020; Kanehisa et al., 2021; Luo & Brouwer, 2013).

Together, these data show that platelet depletion alone is sufficient to induce transcriptional changes in mouse monocytes. As a consequence, monocytes show alterations in gene sets involved in coagulation and platelet activation, the complement system, immune responses, leukocyte activation and signal transduction such as PI3K-Akt pathway, Rap1 pathway or chemokine signaling pathway.

3.5 Primary human monocytes show an altered kinase activity profile in the absence of platelets

Until now, all presented data indicated that monocytes undergo functional re-programming in the absence of platelets, affecting not only the immune response and cytokine secretion, but also the transcription of genes involved in other cellular pathways. To determine how platelets globally affect the activation of signal transduction cascades in monocytes, I performed a kinase activity assay using the PamGene technology. Monocytes (MoStd, MoPD, MoPD+Plts) and platelets (Plts) were isolated from healthy donors and treated with LPS for 15 min. Cell lysates were then generated and applied in kinase activity assays Protein Tyrosine Kinases (PTKs) PamChip® and Serine/Threonine Kinases (STKs) PamChip® and measured at the PamStation12. Finally, peptide quality control and bioinformatic data analysis was performed by Dr. Savithri Rangarajan (PamGene) and Dr. Rik de Wijn (PamGene, ‘s-Hertogenbosch, The Netherlands).

First, when looking at the raw data, platelets alone displayed very high intrinsic kinase activity. This high platelet-derived “background” masked signals derived from monocytes. As a consequence, platelet-depleted monocytes displayed a clearly lower signature of kinase activity signals, while all other groups containing platelets (MoStd, MoPD+Plts and Plts) were dominated by the platelet-derived signal. To be able to analyze the kinase activity in monocytes, we decided to get the help of Dr. Rik de Wijn to subtract the masking signal coming from the platelets from all datasets. Dr. Savithri Rangarajan thus further processed the data using an algorithm to subtract the platelets’ signature. These processed datasets were used for further analysis and biological interpretations.

I first was interested whether the presence or absence of platelets influence the kinase activity of monocytes after LPS treatment. Here, the LPS-induced changes in MoStd, MoPD, MoPD+Plts and Plts were assessed by comparing the LPS condition to the corresponding unstimulated condition within each group. I visualized the calculated values generated by Dr. Savithri Rangarajan in coral trees using the CORAL online tool (URL: http://phanstiel-lab.med.unc.edu/CORAL/; date accessed: February 2022) developed in the Phanstiel Lab from the University of North Carolina (Manning et al., 2002; Metz et al., 2018). Platelets alone displayed a distinct pattern of kinase activities following the stimulation with LPS (Figure 17). Standard monocytes showed a decreased activity for the majority of tested kinases after LPS treatment. In contrast, platelet-depleted monocytes noticeably increased their kinase activity after LPS treatment. Interestingly, the re-addition of platelets to monocytes induced an intermediate kinase activity signature, in which mainly STKs (lower branches) showed a decreased kinase activity while PTKs mainly remained active (Figure 17).

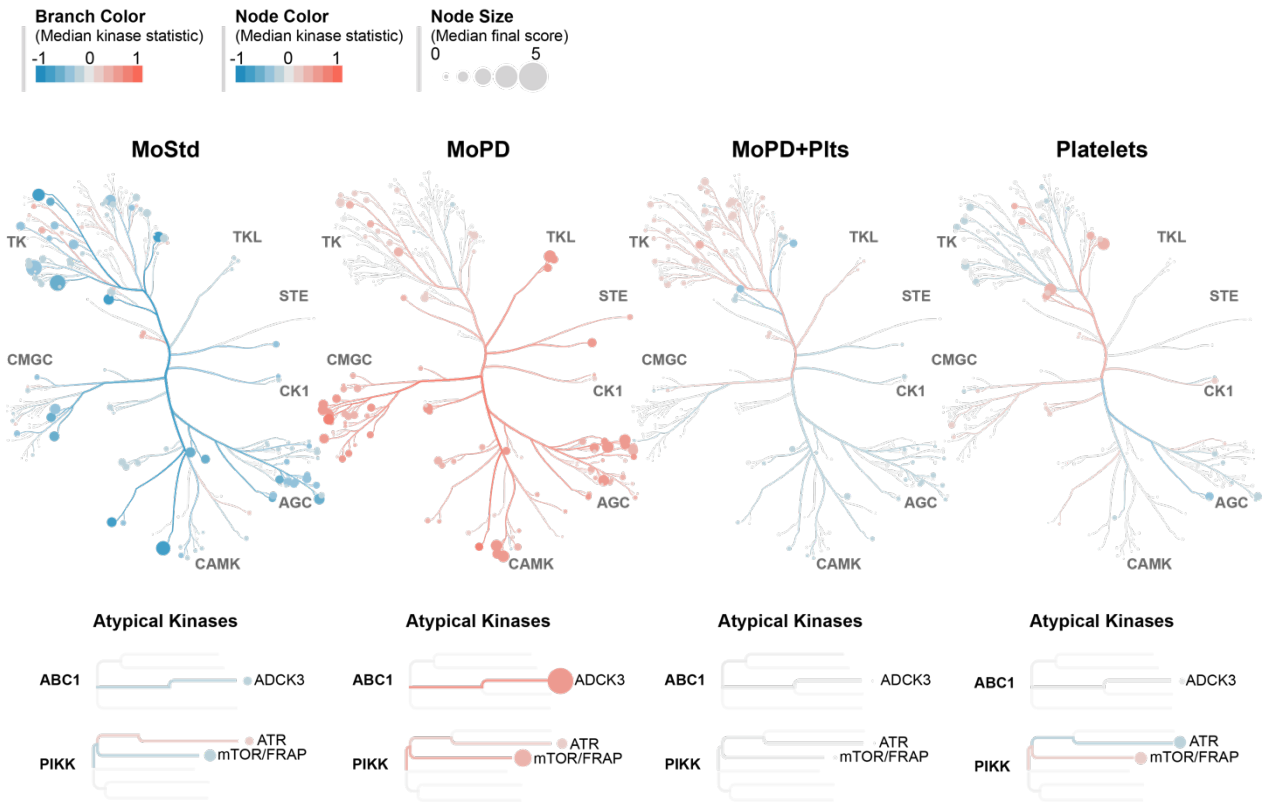

Figure 17: LPS-induced kinase activity. Coral trees display the activity of protein tyrosine kinases and serine/threonine kinases in MoStd, MoPD, MoPD+Plts and platelets upon LPS stimulation for 15 min (n=5), measured at the PamStation12. Kinase families with

higher activity are displayed in red, while a decreased kinase activity is shown in blue. Node size displays the ranking factor (median final score) calculated from the sensitivity (difference between LPS and unstimulated) and the specificity score (assignment of kinases from PamGene databases to a set of peptides). TK: tyrosine kinase group, TKL: tyrosine kinase-like group, CMGC: CMGC group, STE: STE group, CK1: cell kinase 1 group, AGC: protein kinase A, G and C families, CAMK: calcium and calmodulin-regulated kinases. Coral trees were generated using the CORAL online tool (URL: http://phanstiel-lab.med.unc.edu/CORAL/; accessed: February 2022) developed in the Phanstiel Lab from the University of North Carolina (Manning et al., 2002; Metz et al., 2018).

These data suggest that platelet-depleted monocytes have a generally altered signature of LPS-induced kinase activity in comparison to standard monocytes, indicating changes in signaling.

Previous transcriptomic data have shown that the platelet depletion alone was sufficient to induce transcriptional reprogramming in monocytes. To determine whether this is also true for the kinase activity of primary human monocytes, I compared the kinase activity of platelet-depleted monocytes and standard monocytes from five different donors under unstimulated condition. Interestingly, platelet depletion was sufficient to induce changes in the activity of kinases in the monocytes. Platelet depletion induced a decreased activity for the majority of tested kinases such as several erythropoietin-producing human hepatocellular receptors (Ephs), Bruton's tyrosine kinase (BTK), p38 mitogen-activated protein kinases (MAPKs), cyclin-depended kinases (CDKs), mitogen-activated protein or kinase-activated protein kinases (MAPKAPKs). Only few kinases increased their activity after platelet depletion, among these, IkappaB kinases (IKK) and Janus kinases (JAK) (Figure 18A).

To generate a more biological interpretation of the collected results, Dr. Savithri Rangarajan applied a pathway and network analysis. This pathway analysis revealed that platelet depletion induced changes in kinase sets involved in signal transduction associated with NF-κB activation, immune responses via CD40 signaling as well as other immune signaling pathways (Figure 18B). Furthermore, network analysis showed enrichments in cellular processes such as hemopoiesis, cell cycle and regulation of translation initiation. Platelet depletion also induced changes in networks involved in immunological processes such as signal transduction, cell adhesion, anti-apoptotic

signals mediated via MAPK and JAK/STAT signaling and other immune response cascades (Figure 18C).

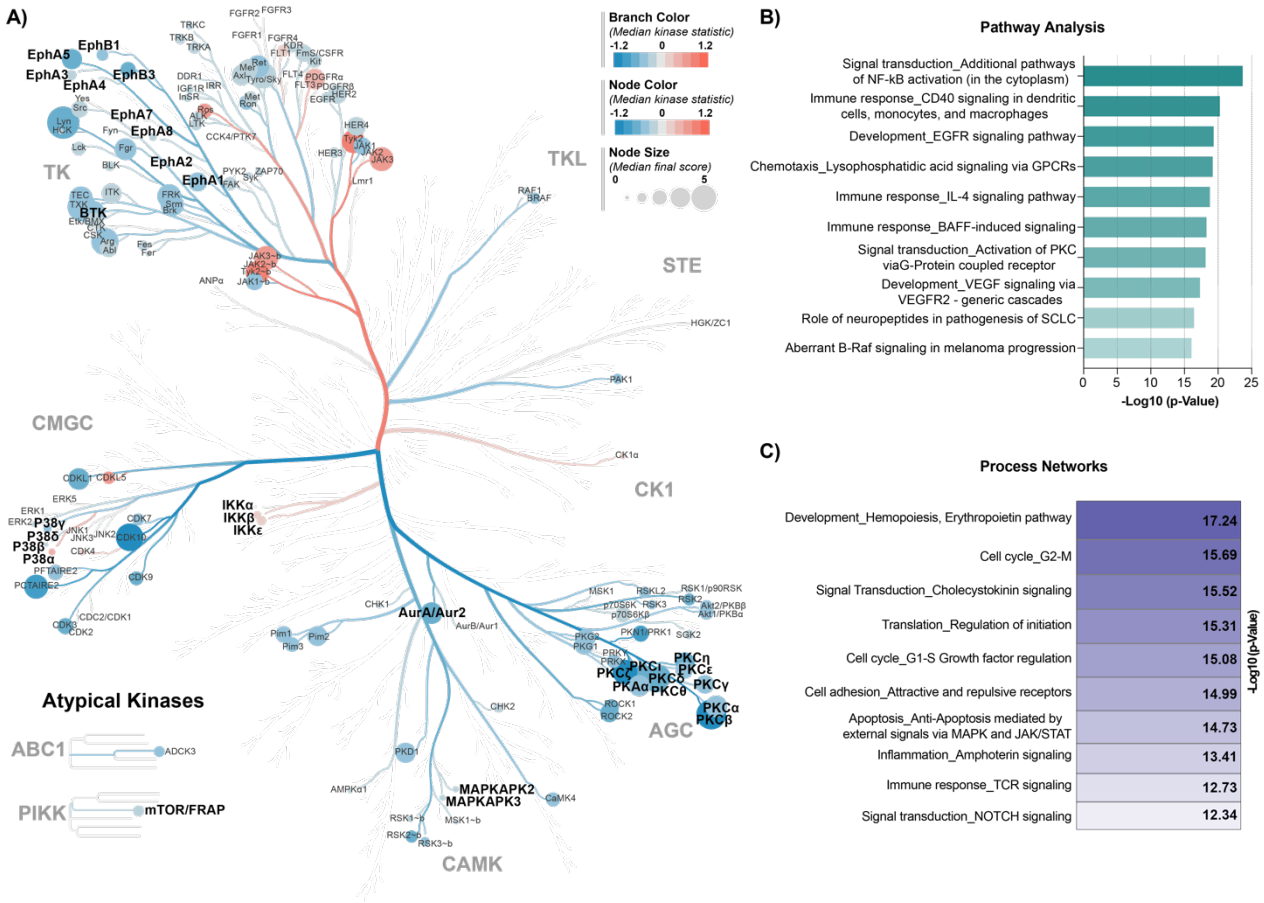

Figure 18: Platelet depletion-induced alterations in kinase activity. (**A**) Coral tree displays changes in kinase activity in primary human monocytes (n=5) induced by platelet depletion. Platelet depletion alone resulted in enhanced (red) or dampened (blue) kinase activity. (**B**) Pathway analysis and (**C**) process network analysis were performed with by Dr. Savithri Rangarajan from PamGene displaying top 10 enriched pathways and networks. Coral trees were generated using the online CORAL tool (URL: http://phanstiel-lab.med.unc.edu/CORAL/; accessed: February 2022) developed in the Phanstiel Lab from the University of North Carolina (Manning et al., 2002; Metz et al., 2018).

Together, these data emphasize the fundamental role of platelets in shaping the monocytic function on the level of signal transduction. These findings suggest that platelets may act as a “switch” for signal transduction of several pathways, among them p38-MAPK, in monocytes.

3.6 Direct cellular access is required for the communication between platelets and monocytes

3.6.1 Platelets actively license cytokine secretion in primary human monocytes

To better understand how platelets govern the cytokine secretion of primary human monocytes, I asked whether platelets act as passive carrier of co-stimulatory molecules or whether they actively induce the rescue of cytokine secretion in monocytes. To test this, cell fixation was performed to conserve and cross-link protein structures in platelets. I fixed freshly isolated platelets ($Plts^{Fixed}$) with 2% PFA for 30 min at 37 °C and gently washed the PFA with PBS. Following fixation, platelets no longer exposed CD62P to their surface following stimulation with thrombin, confirming the successful fixation (Figure S4). Next, $Plts^{Fixed}$ or untreated Plts were co-incubated with MoPD and cells were stimulated as previously described. In line with previous observations, HTRF data show a decreased secretion of IL-1β, IL-6 and TNFα upon platelet depletion. As expected, reconstitution with untreated platelets rescued the cytokine secretion in MoPD. In contrast, $Plts^{Fixed}$ were not able to replenish the capacity of MoPD to secrete cytokines (Figure 19).

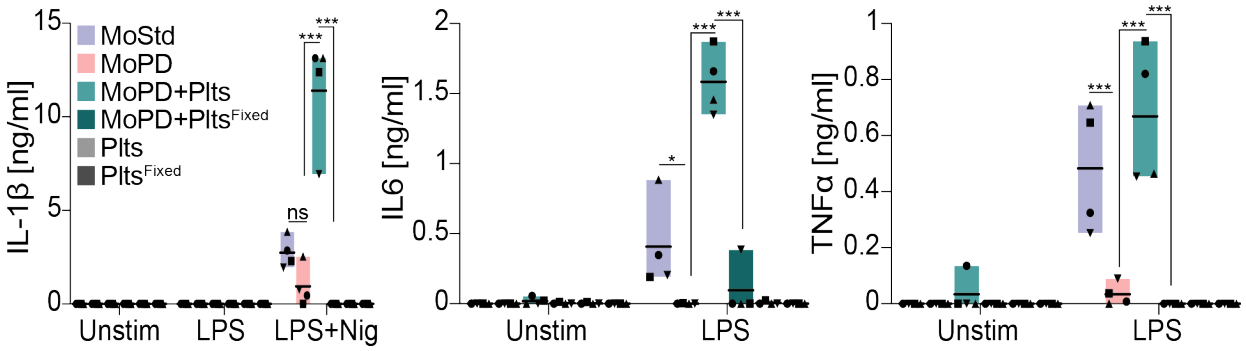

Figure 19: Platelets need to be viable to rescue the cytokine secretion in primary human monocytes. HTRF data display secretion levels of IL-1β, IL-6 and TNFα. Cells were stimulated with 4.5 h of LPS [2 ng/ml] or 3 h with LPS followed by 1.5 h nigericin [10 µM]. Floating bars display the max/min values and mean. Each symbol represents one of n=4 blood donor. Two-way ANOVA with Tukey's multiple comparison test and 95% confidence interval were used. P-value < 0.05 (*). < 0.01 (**) and < 0.001 (***).

These data suggest that platelets need to be viable to fulfil their role in the rescue of cytokine secretion in primary human monocytes and do not act as "inert vehicles" that

passively carry stimulatory molecules. Thus, platelets actively boost cytokine secretion in primary human monocytes.

3.6.2 Platelets boost cytokine secretion in monocytes independent of CD62P, CD40L, CCL5 or CXCL12

To investigate, how platelets rescue the cytokine secretion in primary human monocytes, I focused on possible immune modulatory factors expressed by platelets and tested their involvement. It has been reported that platelets can govern leukocyte function via CXCL12 and recruit and activate leukocytes, e.g., neutrophils, by the secretion of CCL5, one of the abundantly secreted platelet chemokines (Chatterjee et al., 2015; Hwaiz et al., 2015). Furthermore, platelets express CD40L and CD62P, which are involved in the interaction and activation of leukocytes (Kral et al., 2016). To test the involvement of these stimulatory factors in the platelet-induced boosting of monocyte cytokine secretion, MoPD were treated with rhCD62P, rhCD40L, rhCCL5 or rhCXCL12 to substitute for platelets. After stimulation with LPS or LPS+Nig, supernatants were collected and cytokine secretion was measured. MoStd secreted high levels of IL-1β. As shown previously, platelet depletion abolished the production of these cytokines in MoPD. None of the administered candidate proteins was able to replenish the cytokine secretion in MoPD, indicating that these molecules are not involved in the platelet-induced rescue of cytokine secretion in monocytes (Figure 20A-C).

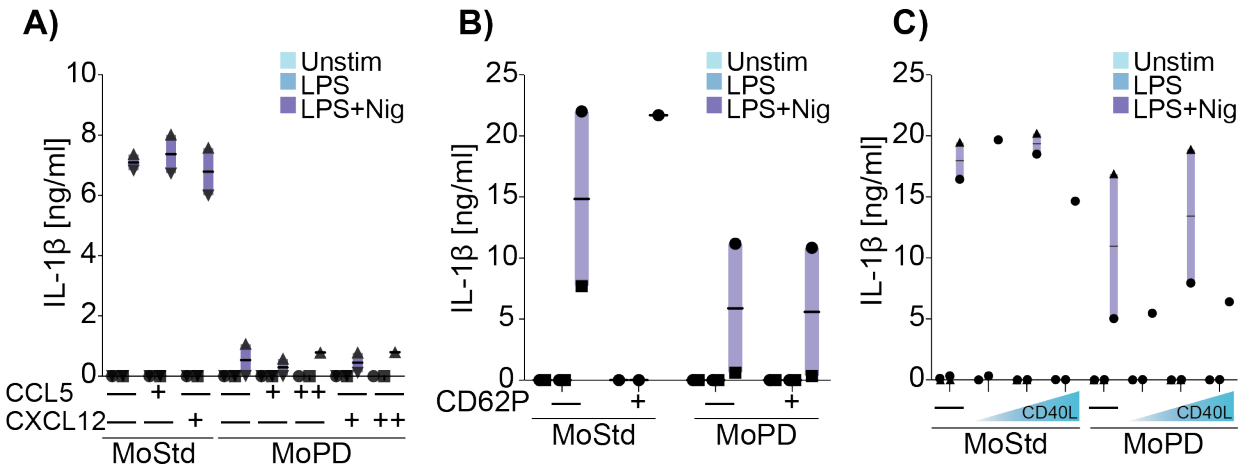

Figure 20: Recombinant human CCL5, CXCL12, CD62P or CD40L do not reconstitute the platelet-induced boost of monocytic cytokine secretion. (**A-C**) HTRF data showing

levels of IL-1β released from standard monocytes (MoStd) or platelet-depleted monocytes (MoPD). Cells were pre-treated with (**A**) CCL5 [300 pg/ml (+) or 600 pg/ml (++)], CXCL12 [2 ng/ml (+) or 4 ng/ml (++)], (**B**) CD62P [40 ng/ml] or (**C**) CD40L [10, 50 or 100 ng/ml]. For inflammasome activation, cells were stimulated with LPS [2 ng/ml] for 3 h followed by nigericin [10 µM] for 1.5 h. Floating bars show max/min values and mean. Each symbol represents one blood donor of n=2 donors.

Sialic acid-binding immunoglobulin-type lectins (Siglecs) have been reported to act as immune checkpoints mediating phagocytosis, adhesion and cell-cell interactions and signaling in leukocytes (Crocker et al., 2007; Lubbers et al., 2018). It has been shown that the cross-linking of Siglec-7 on monocytes acts as a co-stimulatory signal exclusively on monocytes, licensing their capability to produce cytokines, thus inducing a pro-inflammatory state (Varchetta et al., 2012). Therefore, I decided to test whether the cross-linking of Siglec-7 on monocytes would have similar effects as the addition of platelets, resulting in an enhanced cytokine secretion. I used an activating antibody directed against Siglec-7. First, 96-well plates were coated overnight with human Siglec-7 antibody (R&D) or monoclonal mouse IgG_{2B} control antibody. Cells were seeded in the pre-coated plates and stimulated with either LPS [2 ng/ml] for 4.5 h or with LPS for 3 h followed by nigericin [10 µM] for 1.5 h. Supernatants were collected and cytokine levels were measured. In agreement with previous data, monocytes showed a disrupted secretion of IL-1β and IL-6 after platelet depletion, which was rescued when autologous platelets were replenished. However, the co-stimulation of MoPD with plate-bound anti-Siglec-7 antibody did not rescue cytokine secretion (Figure 21).

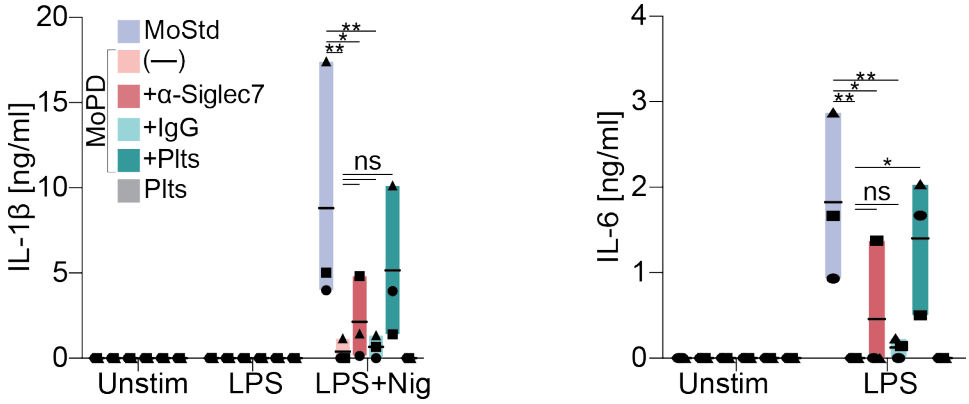

Figure 21: Siglec-7 cross-linking on primary human monocytes. HTRF data show the measured cytokine concentrations. MoPD were either co-incubated with platelets or with plate-bound anti-Siglec-7 [10 µg/ml] antibody or an IgG control. Cells were then stimulated

with LPS [2 ng/ml] for 4.5 h or for 3 h followed by 1.5 h nigericin [10 µM]. Floating bars display the max/min values with indication of the mean. Each symbol represents one of n=3 blood donors. Two-way ANOVA with Tukey's multiple comparison test and 95% confidence interval were used. P-value < 0.05 (*). < 0.01 (**) and < 0.001 (***).

These findings suggest that recombinant human CCL5, CXCL12, CD40L CD62P or the engagement of Siglec-7 are not involved in the platelets-induced boosting effect of cytokine secretion in primary human monocytes.

3.6.3 Platelets or platelet-derived particles require a direct interaction with monocytes to boost their cytokine response

To gain a better understanding of how platelets and monocytes communicate, I aimed to study the axis and mechanism of this intercellular cross-talk. Intercellular communication can occur in several ways. Cells can communicate via soluble factors, extracellular vesicles and particles or physical cell-cell contact (Armingol et al., 2021). To determine the nature of the heterotypic interaction between platelets and monocytes, I used a transwell assay. As before, MoStd, MoPD or Plts alone were seeded into the wells. Then, two reconstitution groups were generated: i) Plts were added directly to MoPD into the wells, or ii) Plts were added into the insert ($Plts^{insert}$) of the transwell system, leading to their physical separation from MoPD in the well by a 0.4 µm membrane (sharing only the medium). Cells were stimulated with LPS [2 ng/ml] for 4.5 h or with 3 h LPS [2 ng/ml] followed by 1.5 h of nigericin. Supernatants were collected and IL-1β levels were measured. In line with previous data, platelet depletion resulted in a decreased IL-1β secretion in monocytes. Importantly, IL-1β production was only restored when Plts were added into the well directly to MoPD, but not when Plts were separated from monocytes by a 0.4 µm membrane, suggesting a direct physical interaction (Figure 22A).

To expand on these data by a complementary approach, I tested the transwell assay results using platelet-conditioned cell-free supernatants. These experiments were performed together with Agnieszka Demczuk at the Institute of Innate Immunity Bonn. To this end, Plts ($1x10^8$) were incubated in 1 ml of RPMI either under basal conditions ($Plts^{Sups}$) or stimulated with LPS [2 ng/ml] ($Plts^{LPS\text{-}Sups}$) for 3 h at 37 °C. Platelet

suspensions were centrifuged at 3,000 x g for 10 min at room temperature (RT) and supernatants were collected. Importantly, MoPD that were treated with $Plts^{LPS\text{-}Sups}$ followed by LPS priming received consequently a double-dose of LPS. Hence, a platelet-free medium containing LPS (LPS^{CM}) was used as a control. Then, MoPD were co-incubated with $Plts^{Sups}$, $Plts^{LPS\text{-}Sups}$, $Plts^{CM}$ or Plts. Finally, cells were stimulated with LPS or LPS+Nig as described before and supernatants were collected. As observed before, platelet depletion abolished cytokine secretion, whereas the replenishment with autologous platelets reconstituted it (Figure 22B). Unexpectedly, although the results of the transwell assay showed that platelets and monocytes require direct interaction, MoPD co-incubated with Plt-conditioned media enhanced IL-1β secretion; however, to a lower extent compared to MoPD+Plts. Although the LPS carryover control (MoPD+LPS^{CM}) induced a minor level of cytokine secretion in the MoPD, this was negligible. Especially, because no differences were observed between the co-incubation of MoPD with $Plts^{Sup}$ and $Plts^{LPS\text{-}Sup}$, indicating that the platelets' boosting effect is constitutively present and does not require induction by LPS (Figure 22B).

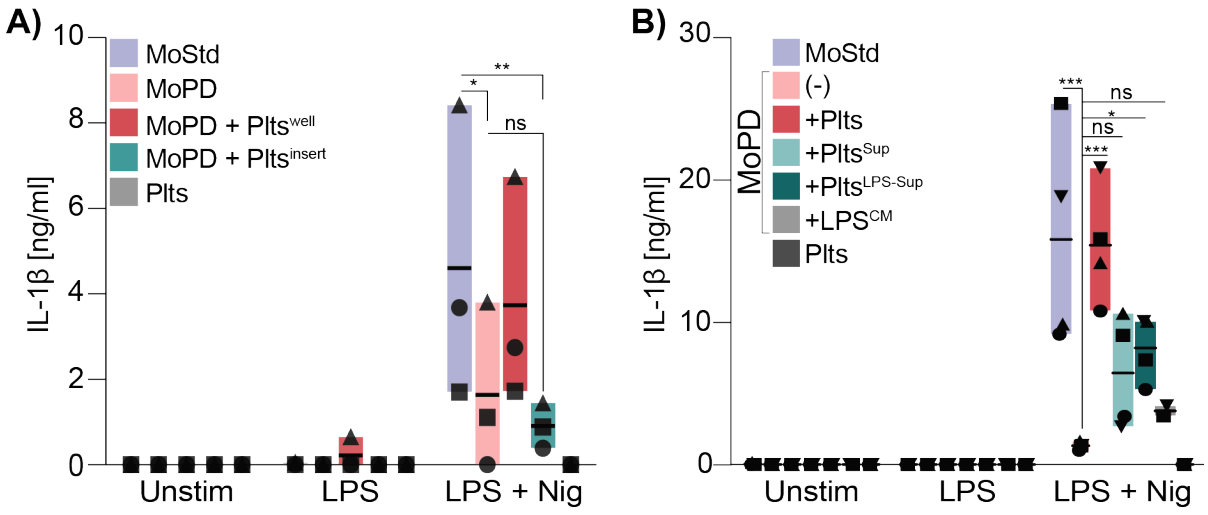

Figure 22: Platelet's boosting effect on monocyte cytokine secretion requires direct interaction between platelets and monocytes. (**A**) In a transwell assay, platelets were added to MoPD either directly into the well (MoPD+$Plts^{Well}$) or into the insert (MoPD+$Plts^{Insert}$) separated by a 0.4 µm membrane. (**B**) Platelet-conditioned supernatants were generated by incubation of $1x10^8$ Plts in 1 ml RPMI for 3 h under either unstimulated ($Plts^{Sup}$) conditions or stimulated with 2 ng/ml LPS ($Plts^{LPS\text{-}Sup}$). Additional LPS-conditioned medium was produced by the addition of 2 ng/ml LPS to cell-free RPMI (LPS^{CM}). Supernatants or platelets were added to MoPD and IL-1β secretion was assessed after LPS + Nig stimulation using HTRF. Floating bars display max/min values and the mean. Each symbol represents one of n=3-4 blood donors. Two-way ANOVA with

Tukey's multiple comparison test and 95% confidence interval were used. P-value < 0.05 (*). < 0.01 (**) and < 0.001 (***).

In conclusion, these data imply that the cytokine boost induced by platelets requires a direct interaction with monocytes and exclude the involvement of a humoral factor. The unexpected observation that platelet-derived supernatants partially induced a rescue of monocytic cytokine secretion suggests an additional axis of communication, possibly involving not only platelets, but also platelet-derived particle or EVs larger than 0.4 µm.

3.6.4 A role for phagocytosis in the effect of platelets on monocytes?

As my previous findings showed that the cytokine boosting effect induced by platelets might involve platelet-derived particles or EVs, I proceeded to test whether the required direct interaction between platelets and monocytes occurs via contact at the surface of monocytes or whether platelets or platelet-derived particles are engulfed. To test this, experiments were carried out by Agnieszka Demczuk at the Institute of Innate Immunity, Bonn. Two inhibitors of β-Actin polymerization, and consequently, phagocytosis were used: cytochalasin D (CytoD) and latrunculin B (Lat-B). Cells were treated with CytoD [50 µM], Lat-B [2 µM], 0.2-0.25% dimethyl sulfoxide (DMSO – vehicle control) or left untreated. Then, cells were stimulated with LPS or LPS+Nig and cytokine levels were assessed. As expected, platelet depletion abolished the secretion of IL-1β and TNFα in monocytes, which was restored when platelets were reconstituted. Interestingly, the replenishment of MoPD with platelets did not rescue the cytokine secretion of MoPD pre-treated with CytoD or Lat-B (Figure 23A-B). Furthermore, while CytoD abolished the rescue effect only in the reconstitution group MoPD+Plts (Figure 23A), Lat-B generally decreased cytokine production in all groups, irrespective of the presence of platelets (Figure 23B). This would indicate that Lat-B may have off-target effects impacting the priming, independently of the platelet effect. These findings suggest that phagocytosis may be involved in the mechanism of platelet-induced cytokine secretion enhancement in primary human monocytes.

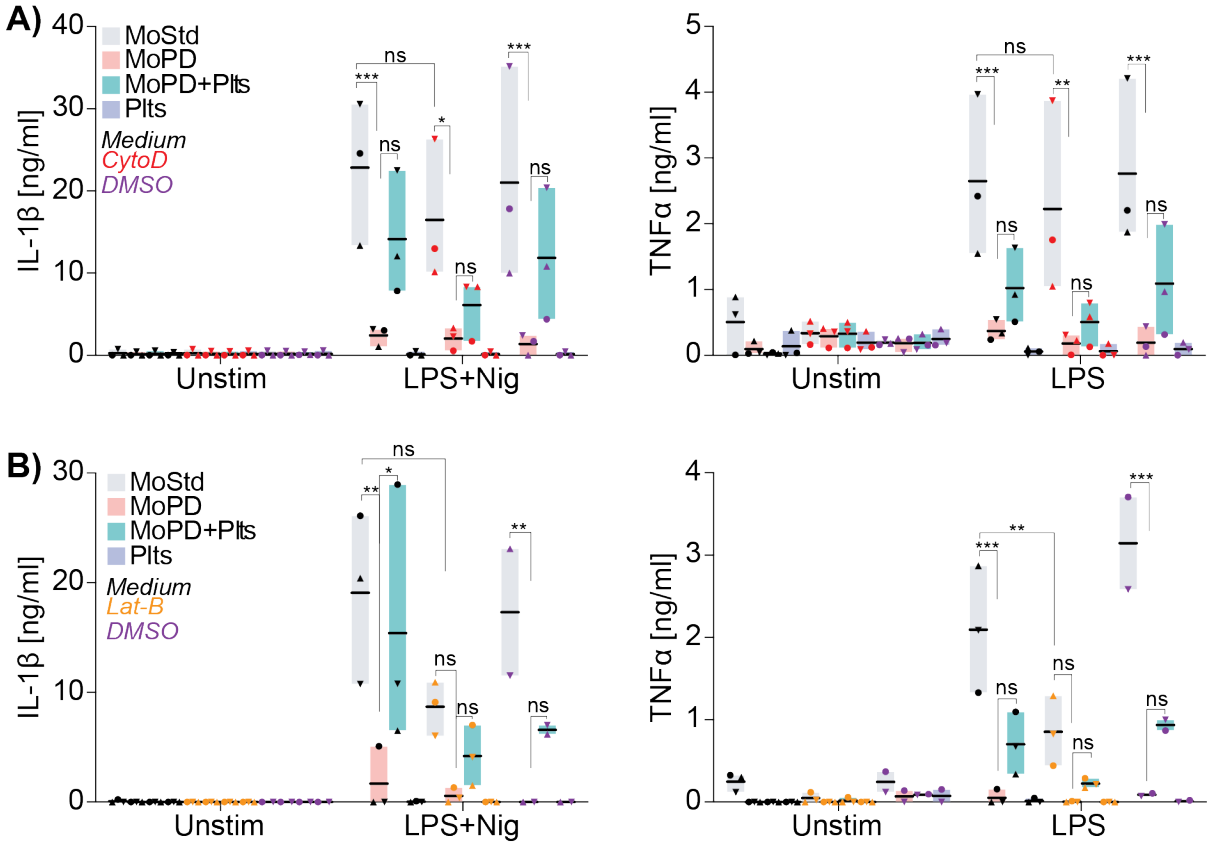

Figure 23: Phagocytosis is required for the platelet-induced cytokine secretion boost in primary human monocytes. HTRF data display cytokine levels of standard monocytes (MoStd), platelet-depleted monocytes (MoPD) or MoPD reconstituted with platelets (MoPD+Plts) after the inhibition of phagocytosis with (**A**) cytochalasin D (CytoD; red) [50 µM] or (**B**) latrunculin-B (Lat-B; orange) [2 µM]. For cytokine secretion, cells were stimulated with either LPS [2 ng/ml] for 4.5 h or LPS for 3 h followed by nigericin [10 µM] for 1.5 h. Floating bars show the max/min values and mean. Each symbol represents one of n=3 blood donors. Two-way ANOVA with Tukey's multiple comparison test and 95% confidence interval were used. P-value < 0.05 (*). < 0.01 (**) and < 0.001 (***). These experiments were carried out by Agnieszka Demczuk at the Institute of Innate Immunity Bonn.

To corroborate these findings, I next imaged standard monocytes using confocal microscopy. MoStd were stimulated with LPS [2 ng/ml] for 3 h at 37 °C. After fixation, cells were stained with anti-human CD41 (platelet marker) and anti-human CD14 (monocyte marker) antibodies and Hoechst 34580 (nuclear counterstain), before imaging on a TCS SP5 confocal microscope (Leica). In n=4 biological replicates, confocal imaging failed to show platelets engulfed by monocytes (Figure 24A). Nevertheless, I quantified the number of platelets per monocyte using the Fiji (ImageJ) software. Here, each monocyte was counted and the number of interacting platelets per monocyte was quantified. I observed

a trend of an increase in the number of platelets per monocyte following LPS stimulation. However, statistical analysis did not reach significance (Figure 24B).

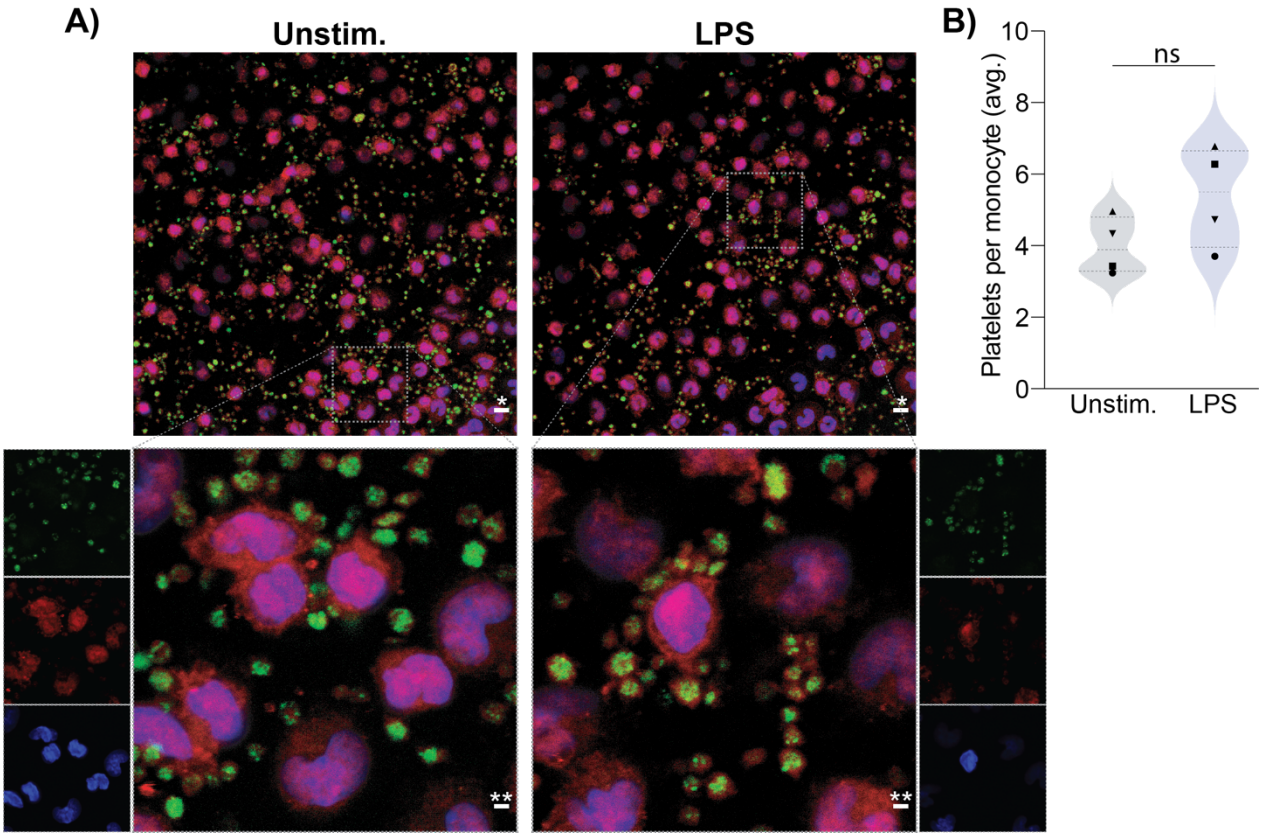

Figure 24: Monocytes do not phagocytose platelets, but LPS increases the formation of monocyte-platelet aggregates. (**A**) Confocal imaging of isolated standard monocytes under unstimulated conditions or after treatment with LPS [2 ng/ml] for 3 h. $CD14^+$ Monocytes (red), $CD41a^+$ platelets (green) and nuclei (Hoechst 34580, magenta). *Scale bar: 10 µm, **scale bar: 2 µm. (**B**) Quantification of platelets per monocytes before and after LPS stimulation. Violin plot displays the median and quartiles. Each symbol represents one of n=4 blood donors. Statistical analysis was performed with a parametric two-tailed paired T-test. P-value < 0.05 (*). < 0.01 (**) and < 0.001 (***).

In summary, although no engulfed platelets were visualized, these findings demonstrate that the phagocytic machinery is required for the platelet-induced boost in cytokine secretion by monocytes. These observations, together with previous results with Plt-derived supernatants inducing partial rescue of cytokine secretion (section 3.6.3), reinforce the scenario in which platelet-derived particles or EVs might be engulfed by monocytes, but would be too small for visualization by microscopy because of methodological limitations.

3.6.5 Integrins, GPIIb/IIIa and sialic acids are not involved in the platelet-mediated activating signal in primary human monocytes

Previous findings implicate the importance of the close proximity between platelets/platelet-derived particle and monocytes. Therefore, I next addressed the role of adhesion and signaling molecules expressed by platelets. For intercellular interactions, platelets are known to express a variety of glycoproteins, integrins, and their cell surface is rich in sialic acid residues. These molecules are not only crucial for the functionality of platelets, but they are also important mediators of adhesion, interaction and activation of other cells (Crook, 1991; Spangenberg et al., 1993). To address whether this class of molecules is involved in the pro-inflammatory effect of platelets on monocytes, I used several inhibitors of these proteins. The hypothesis was that the inhibition of key interaction partners for formation of MPAs would disrupt the rescue of cytokine secretion when MoPD are replenished with autologous platelets. To this end, cells expressing the target molecule were treated with an inhibitor of the target molecule prior to co-incubation of Plts and MoPD. The following cell-cell interaction inhibitors were tested: arginine-glycine-aspartate tripeptides (RGD) [50 µM] to mask the binding sites on of integrins (Ruoslahti, 1996), anti-CD11b antibody [10 µg/ml] to inhibit the MAC-1 complex (CD11b/CD18) (Wolf et al., 2018), GR 144053 trihydrochloride [10 µM] to inhibit the interactions mediated by glycoprotein GPIIb/IIIa (Eldred et al., 1994), and neuraminidase from *Arthrobacter ureafaciens* (sialidase) [100 mU/ml] to desialylate platelets. However, none of these inhibitors affected the platelet-mediated rescue of IL-6, TNFα (not shown) and IL-1β secretion (Figure 25A-C).

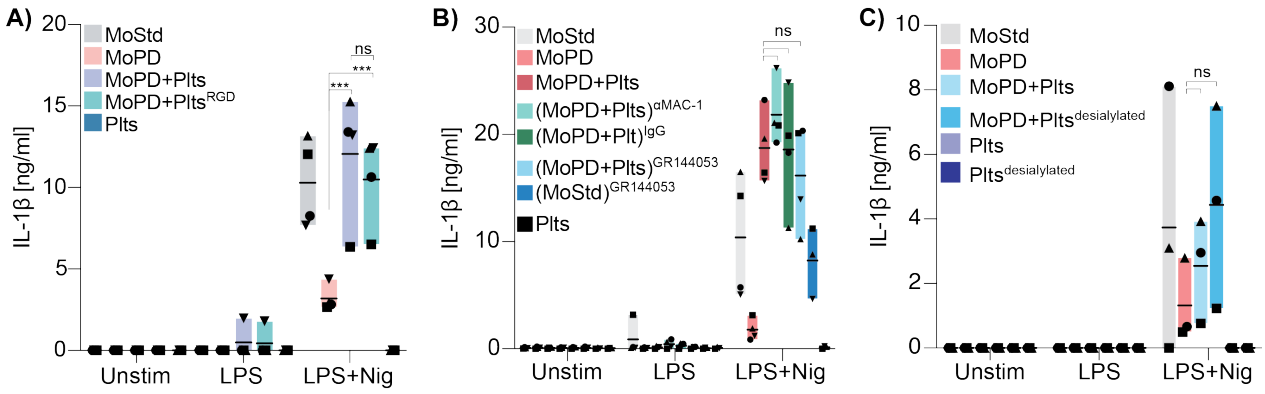

Figure 25: Platelets interact with monocytes independently of integrins, GPIIb/IIIa or sialic acids. (**A-C**) HTRF showing the secretion levels of IL-1β and IL-6 after the treatment with different inhibitors. Cells were treated with (**A**) RGD [50 µM] to inhibit cell-cell interactions mediated by integrins, (**B**) anti-CD11b antibody [20 µg/ml] to inhibit MAC-1 (CD11b/CD18) or GR 144053 trihydrochloride [10 µM] to inhibit GPIIb/IIIa (**C**) with neuraminidase (sialidase) [100 mU/ml] from *Arthrobacter ureafaciens* to enzymatically remove sialic acids from the platelet surface. For stimulation, cells were treated with 2 ng/ml LPS for 3 h followed by 1.5 h of 10 µM nigericin or for 4.5 h with LPS alone. Floating bars represent the max/min values with indication to the mean. Each symbol represents of n=3-4 blood donors. Two-way ANOVA with Tukey's multiple comparison test and 95% confidence interval were used. P-value < 0.05 (*). < 0.01 (**) and < 0.001 (***).

Together, these data show that the platelets interaction with monocytes is independent of CD11b, GPIIb/IIIa and that sialic acids do not play a role in the platelet-mediated cytokine secretion boost.

3.7 Platelets augment NF-κB activity in monocytes and are a carrier of p65

The NanoString data have shown that platelet-depleted monocytes upregulate NF-κB suppressor genes (section 3.3.3) and the kinase activity assays suggested general alterations in signal transduction (section 3.5), including in the NF-κB pathway. Furthermore, previous findings implicated the synergistic role of platelets in TLR-induced cytokine secretion. Therefore, I decided to investigate whether platelets influence the activity of NF-κB. To do so, I used the THP-1 Dual™ NF-κB-secreted alkaline phosphatase (SEAP) and IRF-Lucia luciferase reporter cell line (Invivogen). These cells express SEAP reporter gene driven by NF-κB consensus transcriptional response elements as well as Lucia luciferase reporter gene under the control of interferon-stimulated response elements (Invivogen). Using the QUANTI-Blue™ reagent, the activity of SEAP can be quantified in the cell supernatants, thus representing the activity of NF-κB. THP-1 Dual™ cells were co-incubated with increasing numbers of platelets (THP-1:platelet ratios of 1:10, 1:20, 1:50 and 1:100) and then stimulated with Pam3CysK4 [100 ng/ml] for 16 h. Supernatants were then collected and incubated with the QUANTI-Blue™ reagent. THP-1 Dual™ stimulated with Pam3CysK4 showed a 1.3-fold higher NF-kB-SEAP signal compared to the unstimulated condition. Interestingly, the addition of platelets enhanced the NF-kB-SEAP signal in a dose-dependent manner. Specifically, THP-1 Dual™ cells co-

incubated with platelets in ratio of 1:100 displayed a 2.5-fold induction of NF-κB activity. This corresponded to a 1.9-fold stronger NF-κB activation than it was observed in THP-1 Dual™ stimulated with Pam3CysK4 without platelets (Figure 26A).

To validate these data in primary human monocytes, I used the HTRF® Phospho-NF-κB (Ser536) cellular Kit to measure the levels of phosphorylated p65 (p-p65) NF-κB subunit in cell lysates. To do so, MoStd, MoPD, MoPD+Plts and platelets were seeded and stimulated with LPS [2 ng/ml] for 2, 5, 10, 15 and 30 min. Cells were then lysed and the p-p65 signals were measured following the manufacturer's instructions. Monocytes displayed an increasing NF-κB activity in the first 5 min after LPS stimulation. MoStd displayed a higher p-p65 signal than MoPD, whereas the addition of platelets (MoPD+Plts) increased the signal to an intermediate signal intensity (Figure 26B). Surprisingly, platelets alone displayed a p-p65 level that was comparable to MoPD+Plts, indicating that platelets contribute a major part of the p-p65 signal measured in MoStd and MoPD+Plts (Figure 26B). However, although the detected signal was above that of the negative control, it was still very low when compared to the positive control included with the kit. Hence, these results must be considered with caution due to the generally low detected signal and a possible additive effect derived from platelets.

Consequently, I decided to assess NF-κB activation in primary human monocytes using western blotting. Furthermore, this time, after isolation of MoStd, MoPD and Plts, cells were first rested for 45 m at 37 °C followed by 15 min at 4 °C to lower the background of phosphorylation activities. Next, cells were stimulated with LPS [2 ng/ml] for 5, 15, 30 and 60 min. Cells were then lysed with 16.25 µl complete RIPA containing Benzonase to generate whole-well lysates. These whole-well lysates were subjected to SDS-PAGE and western blotting. The membrane was probed for phospho-p65 NF-κB subunit (Ser536) and total p65. The results show that LPS treatment increased the levels of p-p65 over time (Figure 26C). Interestingly, the levels of p65, both total and phosphorylated, were lower in MoPD than in MoStd. Surprisingly, platelets showed a high expression of p65 and a high p65 phosphorylation, confirming the previous observation from the HTRF® Phospho-NF-κB (p65-Ser536) cellular kit. Of note, the high p65 phosphorylation in platelets was not further induced by LPS stimulation (Figure 26C). Nonetheless, the interpretation of these

data are challenging due to the masking signal, resulting additive effect when platelets are present.

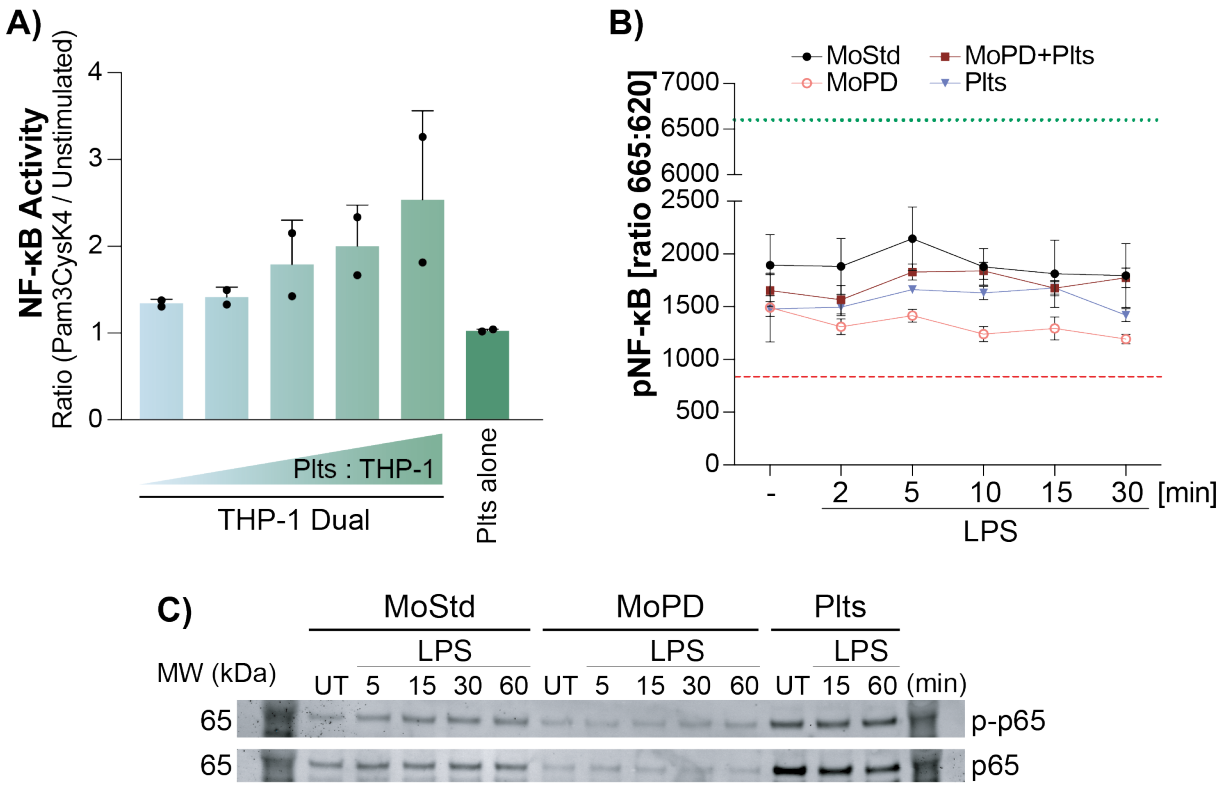

Figure 26: Platelets enhance NF-κB activity in monocytes. (**A**) Measurement of NF-κB-driven secreted alkaline phosphatase (SEAP) in THP-1 Dual™ cells co-incubated with an increasing ratio of platelets (1:10, 1:20, 1:50 and 1:100). Cells were stimulated with Pam3CysK4 [100 ng/ml] for 16 h. Collected supernatants were co-incubated with QUANTI-Blue™ to measure the NF-κB-SEAP activity. Bars display the mean and standard deviation. Each symbol represents one of n=2 experiments (**B**) Measurement of p65 NF-κB subunit phosphorylation using the HTRF® Phospho-NF-κB (Ser536) cellular kit. Cells were stimulated with LPS [2 ng/ml] for 2, 5, 10, 15 and 30 min and then lysed. NF-κB activity was measured in cell lysates. Dotted lines display the signal threshold of the positive (green) and negative (red) controls supplied by the kit. Each symbol represents the average value of n=3 experiments with indication to the standard deviation (**C**) Western blot displaying phosphorylated NF-κB (p-p65) and total NF-κB (p65) in lysates of MoStd, MoPD and platelets after stimulation with LPS [2 ng/ml] for 5, 15, 30 and 60 min.

These data suggest that platelets enhance NF-κB activity in monocytic cells. Moreover, platelets express components of the NF-κB pathway despite the absence of a nucleus and have constitutively phosphorylated p65.

3.8 Platelets or platelet-derived particles are carriers p38 MAPK

Previous data have shown that platelets express high levels of active NF-κB components despite the lack of a nucleus. Furthermore, the kinase activity assay did not only reveal that platelets have very high kinase activity, but it also indicated changes in monocyte signal transduction upon platelet depletion, including in the NF-κB and p38 MAPK pathways. Hence, I asked whether platelets or platelet-derived particles could serve as vehicles delivering these regulatory factors to monocytes. To test this, I first assessed the presence of p65 NF-κB subunit and p38 in platelet-derived supernatants. If platelets communicate with monocytes via vehicles, such as EVs, their cargo should be detectable in cell-free supernatants. I isolated platelets from fresh blood and incubated them in supplement-free RPMI either with LPS [2 ng/ml] or untreated for 3 h. Then, I centrifuged the platelet suspensions either at 500 x g for 5 min, 3,000 x g for 10 min, 10,000 x g for 20 min or full speed (FS) at 20,817 x g for 30 min. I collected supernatants and precipitated the proteins using methanol and chloroform. For each centrifugation run, the pellet was directly lysed in pre-warmed Laemmli buffer. Samples were used for western-blot to detect NF-κB p65 and β-actin. The p65 NF-κB subunit was strongly expressed in the platelet cell lysates, but was barely detectable in the supernatants, suggesting that p65 is mainly located inside platelets (Figure 27A). Interestingly, p38 was present in both, platelet cell lysates and platelet-derived supernatants. In the platelet-derived supernatants, p38 signal decreased with higher centrifugations speed. Importantly, it was still detectable in the supernatants following centrifugation at speeds, where platelets would be pelleted. In the same line, although the supernatants were platelet-free, they still contained β-actin, indicating the presence of cellular particles/vesicles (Figure 27A-B).

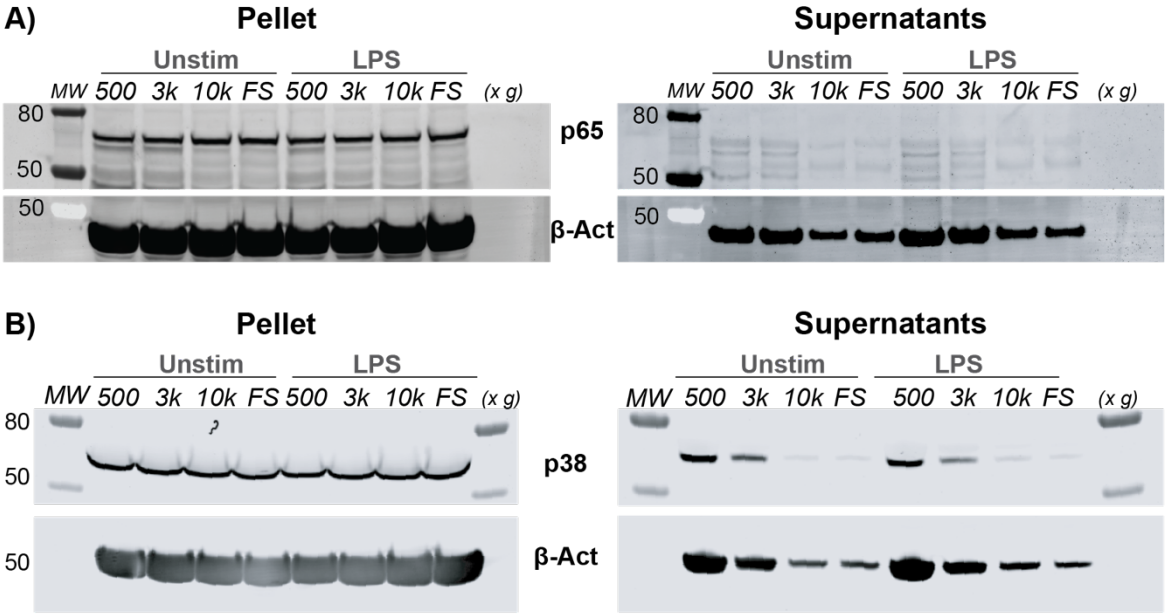

Figure 27: Platelets are carriers of p65 NF-κB and p38 MAPK. (**A-B**) Western blot of (**A**) p65 NF-κB subunit and (**B**) p38 MAPK in platelet's cell lysates and platelet-conditioned supernatants. Platelets were incubated in RPMI for 3 h, untreated or stimulated with LPS [2 ng/ml]. Cell suspensions were centrifuged at 500 x g for 5 min, 3,000 x g for 10 min, 10,000 x g for 20 min or 20,817 x g for 30 min. Supernatants were collected for protein precipitation and cell pellets were lysed in Laemmli for western blotting.

These data indicate that platelets express p65 NF-κB subunit and p38 MAPK. While the presence of p65 NF-κB subunit in the supernatants remains unclear, p38 MAPK is detected at a high level in the supernatants. The detected β-actin in the supernatants indicates the presence of non-cellular particles, i.e. platelet-derived particles or extracellular vesicles, which require other measurements to be validated.

3.9 Platelets require active NF-κB and p38 pathways to license the cytokine secretion in primary human monocytes

Previous data have shown that platelets express components of the NF-κB pathway and the p38 MAPK pathway, which might play a role in the licensing of monocytic cytokine secretion. To address whether NF-κB or p38 MAPK pathways are involved, I took advantage of the IκBa-inhibitor BAY 11-7082 to inhibit NF-κB activation and SB203580 to specifically inhibit p38 phosphorylation (Badger et al., 1998; Melisi & Chiao, 2007; Mori et al., 2002; Shi et al., 2015). Here, platelets were left untreated (Plts) or were treated with

either 50 µM BAY 11-7082 (Plts^{B}), 20 µM SB203580 (Plts^{S}) or 0.1% DMSO (Plts^{D}) for 20 min. The inhibitors were then washed out with PBS and the different groups of pre-treated platelets were co-incubated with MoPD. Cells were stimulated with LPS [2 ng/ml] for 4.5 h or 3 h LPS followed by 1.5 h of nigericin [10 µM]. An additional control group was generated to control for a possible carry-over of BAY 11-7082 or SB203580: MoStd were directly treated with these inhibitors throughout the stimulation assay. Finally, supernatants were collected and cytokine concentrations were measured. In line with previous data, platelet depletion abolished cytokine secretion and was rescued when platelets were replenished. However, when the activation of NF-κB or p38 was inhibited in platelets, they were no longer able to rescue the cytokine secretion in monocytes (Figure 28). Furthermore, monocytes treated with BAY 11-7082 throughout the experiment showed an abrogated cytokine levels, whereas SB203580 did not affect the cytokine secretion. This highlights the importance of platelets' p38 in the rescue of cytokine secretion in monocytes (Figure 28). Importantly, CTB data indicated that cell viability was not affected by any carry-over effects of the tested inhibitors (not shown). Importantly, platelets displayed a decreased degranulation when treated with BAY 11-7082. Platelet degranulation was determined by measuring the exposure of CD62P on the cell surface after stimulation with thrombin [1 unit/ml] for 30 min. FACS data showed that the treatment with BAY 11-7082 abolished the ability of platelets to respond to activation by thrombin (Figure S6).

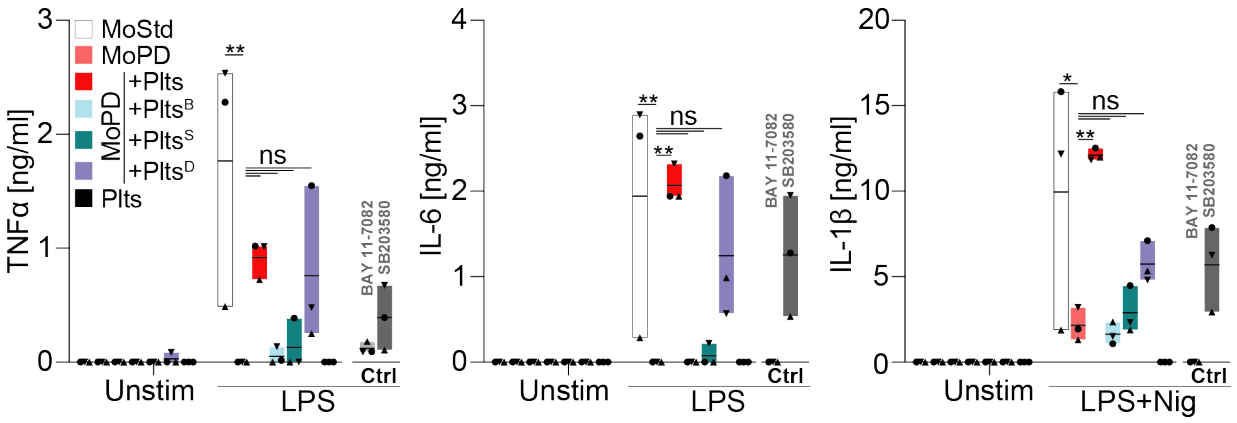

Figure 28: Inhibition of NF-κB and p38 in platelets disrupts their ability to license the monocytic cytokine secretion. HTRF measurement of TNFα, IL-6 and IL-1β. Platelets were pre-treated with BAY 11-7082 (Plts^{B}), SB203580 (Plts^{S}), DMSO (Plts^{D}) or left untreated (Plts). Inhibitors were washed out and platelets were added to MoPD. Cells were

stimulated with LPS [2 ng/ml] for either 4.5 h or 3 h followed by 1.5 h nigericin [10 µM]. Control groups (Ctrl) displays MoStd that were continuously treated with the inhibitors displaying 100% capacity of the inhibitors throughout the stimulation assay. Floating bars display max/min values with an indication to the mean. Each symbol represents one of n=3 blood donors. Two-way ANOVA with Tukey's multiple comparison test and 95% confidence interval were used. P-value < 0.05 (*). < 0.01 (**) and < 0.001 (***).

Together, these data suggest that platelet-intrinsic p38 MAPK are required for the licensing of cytokine production in primary human monocytes. In contrast to p38, inhibition of NF-κB in platelets generally disrupts their functionality.

3.10 Platelet addition bypasses p38 inhibition in monocytes and rescues their cytokine secretion

Previous results highlighted that the rescue of monocytic cytokine secretion requires active NF-κB and p38 MAPK pathways in platelets. However, whether these pathways are crucial for platelet functionality only or it they exert intercellular modulations remained to be explored. Hence, I assessed the impact of naïve untreated platelets on the cytokine secretion of standard monocytes pre-inhibited with BAY 11-7082 or SB203580. MoStd were first treated with the inhibitors for 30 min. Then, these were washed out and MoStd were co-incubated with increasing numbers of untreated platelets. Finally, cells were stimulated as previously described with LPS or LPS+Nig. HTRF results show that MoStd secreted high levels of IL-1β, IL-6 and TNFα and after stimulation. As expected, the inhibition of NF-κB and p38 disrupted the secretion of these cytokines. Surprisingly, the addition of naïve platelets rescued the abolished cytokine secretion caused by p38 inhibition and boosted cytokine levels in a dose-dependent manner. In contrast, platelets did not rescue cytokine secretion when the NF-κB pathway was inhibited in monocytes (Figure 29). On that note, monocytes treated with BAY 11-7082 displayed marked levels of cell death (Figure S7).

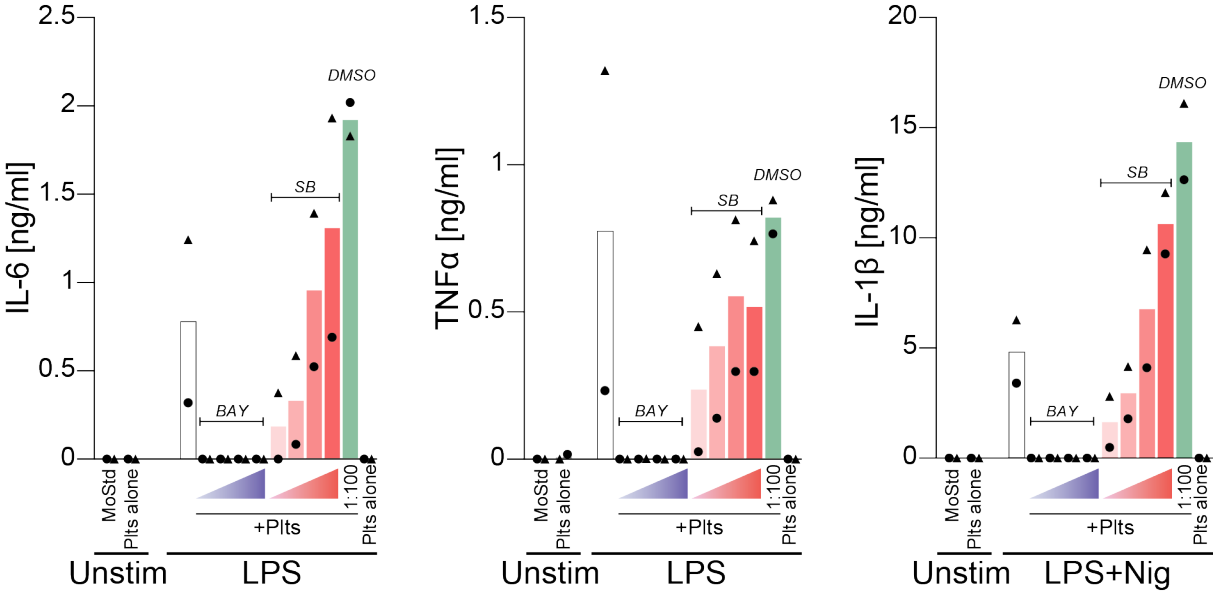

Figure 29: Platelets addition bypasses p38 inhibition in primary human monocytes. HTRF measurement of IL-6, TNFα and IL-1β in standard monocytes. MoStd were treated with BAY 11-7082, SB203580 or DMSO for 30 min and the inhibitors were then washed out. Naïve platelets were added to MoStd at an increasing monocyte:platelet ratio (1:5, 1:50, 1:100) and cells were stimulated with LPS or LPS and nigericin (LPS+Nig). Each symbol represents one of n=2 blood donors.

These results put p38 in a spotlight as a second signaling axis involved in cytokine secretion in primary human monocytes, next to the well described NF-κB pathway. Platelets restored the disrupted cytokine secretion induced by p38 inhibition, suggesting that platelets are able to bypass p38 inhibition and rescue the capacity of primary human monocytes to secrete cytokines.

3.11 The licensing of cytokine secretion in monocytes is dependent on platelet-derived p38 signaling

P38 MAPK is an important pathway for cytokine responses in monocytes (Lee et al., 1994). So far, my results show that p38 MAPK in platelets is important to induce cytokine production rescue in primary human monocytes. Moreover, platelet addition was able to bypass the p38 inhibition in monocytes and rescue the p38-dependent cytokine secretion. To corroborate the role of p38 MAPK in the context of platelets acting as an enhancer of the monocytic immune response, I performed experiments in which p38 was inhibited in

either one or both cell populations. To this end, four different conditions were generated: i. MoPD+Plts: untreated MoPD co-incubated with untreated Plts, ii. MoPD+Plts^{S}: untreated MoPD co-incubated with Plts pre-treated with SB203580, iii. MoPD^{S}+Plts: MoPD pre-treated with SB203580 and co-incubated with untreated Plts, and iv. MoPD^{S}+Plts^{S}: MoPD pre-treated with SB203580 co-incubated with Plts also pre-treated with SB203580. Cells were stimulated as previously described with LPS or LPS+Nig and the secreted cytokine levels were measured. In line with previous observations, platelet depletion resulted in the loss of cytokine secretion, which was rescued when untreated platelets were reconstituted. As shown above, p38 inhibition in platelets abolished their ability to rescue cytokine secretion in MoPD (MoPD+Plts^{S}). When untreated platelets were added to MoPD pre-treated with p38 inhibitor (MoPD^{S}+Plts), platelets rescued the monocytic cytokine levels. Confirming the hypothesis that p38 signaling plays a central role, no rescue of cytokine secretion was observed when p38 was inhibited in both cell populations (MoPD^{S}+Plts^{S}) (Figure 30).

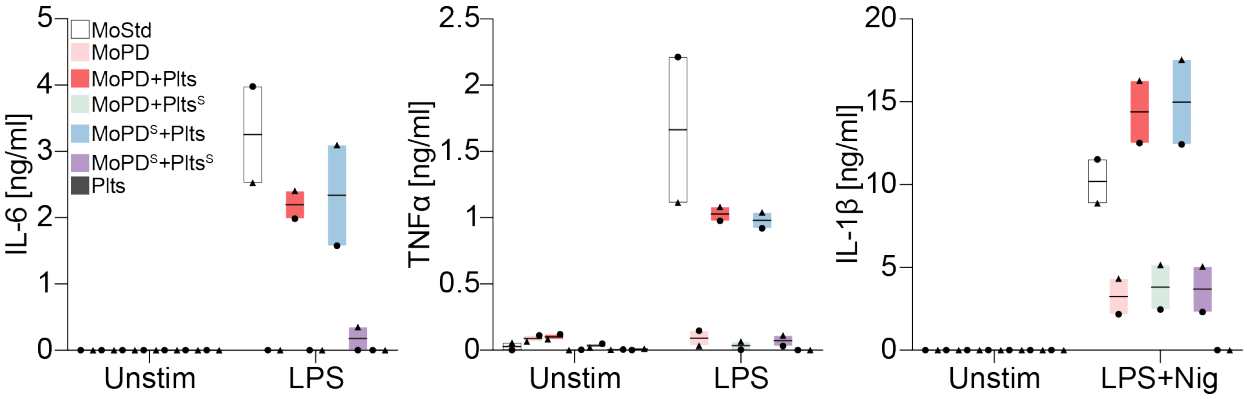

Figure 30: p38 MAPK inhibition in platelets and monocytes. HTRF measurement of IL-6, TNFα and IL-1β. MoPD and Plts were reciprocally or simultaneously inhibited with SB203580 resulting in: i. untreated MoPD co-incubated with untreated Plts (MoPD+Plts), ii. untreated MoPD co-incubated with Plts pre-treated with SB203580 (MoPD+Plts^{S}) iii. MoPD pre-treated with SB203580 and co-incubated with untreated Plts (MoPD^{S}+Plts), and iv. MoPD pre-treated with SB203580 co-incubated with Plts pre-treated with SB203580 (MoPD^{S}+Plts^{S}). Cells were treated with either LPS [2 ng/ml] for 3 h or LPS for 3h followed by nigericin stimulation for 1.5 h (LPS+Nig). Floating bars display max/min values and the mean. Each symbol represents one of n=2 blood donors.

Taken together, these results suggest that p38 MAPK plays a central role in the communication between platelets and monocytes. Platelets intrinsically require an active

p38 pathway to license the cytokine production in monocytes. Thus, the platelet-induced licensing of monocytic cytokine response is dependent on the p38 pathway.

4. Discussion

Over the last decades, platelets have been increasingly described beyond their role in hemostasis and coagulation. Platelets have important functions in immune responses and host defense regulation. They are not only able to directly trap pathogens leading to their clearance, but they also guide and assist leukocytes during an immune response (Ali et al., 2015; Morrell et al., 2014). Increasing evidence shows that platelets interact and form aggregates with circulating blood monocytes (Fu et al., 2021). As a consequence, platelets “steer” the phenotype of monocytes, thus governing the monocyte-driven immune responses. However, the interaction between monocytes and platelets was only described in the specific context of disease, such as COVID-19, cardiovascular or liver-associated diseases (Allen et al., 2019; D'Mello et al., 2017; Hottz et al., 2020). How platelets broadly affect and shape the function of primary human monocytes and which mechanisms underlie this interaction, was yet to be elucidated. In my PhD thesis, I provide an insight into how platelets shape the function of monocytes and which possible platelet-related mechanisms are responsible for the regulation of monocyte-driven immune responses. These findings unravel novel aspects of how platelets control monocyte functions and thus highlight the importance of platelets in the immune system and host defense.

4.1 Platelets license the pro-inflammatory response of primary human monocytes

For a functional immune system and efficient host defense, cellular communication and coordination between leukocytes are fundamental. Leukocytes can communicate by secreting a variety of soluble mediators, such as cytokines, as well as by direct cell-cell contact. Upon cellular contact, the ligation of surface molecules induces signal transduction, shaping the immunological phenotype of the recipient cell (Jin et al., 2021; Li et al., 2000; Mantovani & Dejana, 1989; Walters et al., 2021). In my thesis, I show that platelets and monocytes are constantly in a tight contact. This suggested that these two cell populations are constantly in a close heterotypic interaction. Indeed, previous studies have shown platelet aggregation and the formation of monocyte-platelet complexes in the blood, which was increased in several pathologies (Allen et al., 2019; Ashman et al., 2003;

D'Mello et al., 2017; Mandal et al., 1993). Severe COVID-19 patients display an increased platelet activation and MPAs (Hottz et al., 2020). Furthermore, the formation of MPAs was shown to be enhanced in the circulation in cardiovascular diseases, and serves as a hallmark of platelet activation and monocyte-driven inflammation in peripheral artery disease (Allen et al., 2019; Pluta et al., 2022).

While investigating the significance and consequences of the interaction between monocytes and platelets on the monocytic immune response, one of the major discoveries in my thesis was the relation between the presence of platelets and the monocytic capacity to produce cytokines. Platelet depletion induced monocytic immune paralysis, which was manifested in a disrupted cytokine secretion. The re-addition of autologous platelets reversed this dysfunction and rescued monocyte cytokine secretion. Most importantly, these observations were not biased or artifactual due to a specific isolation method. While the positive isolation (Miltenyi) targeted $CD14^+$ monocytes, the negative selection (STEMCELL) targeted all cells, including platelets, but $CD14^+$ monocytes. Hereby, the positive isolation with MACS (Miltenyi) yielded a pure monocyte population with drastically reduced platelet contaminants, which correlated with low responsiveness to TLR stimulation (section 3.1.3). Also in this setup, the addition of platelets restored the cytokine secretion. These observations are in line with the results from Bhattacharjee et al. (2017); however, with a different conclusion (Bhattacharjee et al., 2017). Although the report from Bhattacharjee et al. (2017) has shown that positive magnetic sorting results in functionally impaired primary human monocytes, which was accompanied by a decreased secretion of IL-8 (CXCL8), RANTES (CCL5) and transforming growth factor β1 (TGF-β1), the authors did not consider the role of platelets. This appears to be an unfortunate omission since two classical platelet-derived cytokines (CCL5 and TGF-β1) were decreased in monocytes isolated in this study. Instead, Bhattacharjee et al. (2017) suggested that the observed lack of monocyte responsiveness could be due to the ligation of CD14 by the CD14-beads, which the authors posited might block the CD14 interaction with LPS (Bhattacharjee et al., 2017). In support of this theory, it was shown that an anti-CD14 antibody induces the internalization of TLR4 leading to attenuated responsiveness of human monocytes to LPS (Kim & Kim, 2014). Nevertheless, my data show that the addition of platelets to positively isolated monocytes restores their function and cytokine

secretion. This emphasizes that the lack of monocyte responsiveness is in fact a result of platelet depletion and thus distinct from the inhibition of CD14 and TLR4 signaling. Furthermore, the stimulation of platelet-depleted monocytes with other TLR ligands also showed the ablation of the monocytes' cytokine response (section 3.2.1). Together, my findings indicate that monocytes enter the state of immune paralysis in the absence of platelets, regardless of the method of cell isolation. Notably, the MACS (Miltenyi) kit for monocyte isolation is applied worldwide. My observation that such isolated monocytes are not responsive to inflammatory stimulation raises questions about the impact of the findings obtained with the use of the MACS kit collected in many previous studies. Thus, the results acquired from studies using positively isolated $CD14^+$ monocytes might only describe "one side of the coin", neglecting the impact of the absence of platelets.

In agreement with the literature, my data highlight the importance of platelets in the modulation of pro-inflammatory responses. So far, platelets were described to guide leukocytes to the sites of inflammation (Rossaint et al., 2018; Zuchtriegel et al., 2016). Indeed, platelets assist the diapedesis of neutrophils and their response NETs (Clark et al., 2007; Pitchford et al., 2017). Furthermore, our group had previously shown that platelets regulate the NLRP3-driven immune response in macrophages, highlighting their importance in IL-1 immune responses (Rolfes et al., 2020). Moreover, some studies show that the immune function of monocytes stands in a relationship with platelets' activities. For instance, platelets were reported to activate mouse monocytes during a *Leishmania major* infection. Here, activated platelets secrete platelet-derived growth factor leading to the release of CCL2 by bystander cells, which in turn selectively recruits $Ly6C^+$ monocytes to the site of infection (Goncalves et al., 2011). Furthermore, activated platelets can directly interact with circulating classical $CD14^+$ human monocytes under inflammatory conditions, form MPAs and consequently induce a pro-inflammatory phenotype by polarizing the monocytes towards a $CD14^+CD16^+$ state (Passacquale et al., 2011). Indeed, monocytes exhibit an enhanced pro-inflammatory activity upon aggregation with platelets in sepsis, rheumatoid arthritis, hemolytic-disorders-paroxysmal nocturnal hemoglobinuria, sickle cell disease, and liver disease associated sickness behavior (D'Mello et al., 2017; Fu et al., 2021; Rong et al., 2014; Singhal et al., 2017). My findings are in line with these earlier observations and thus confirm the findings of previous studies.

Nevertheless, my data add a novel layer of platelet depletion-induced immune paralysis in human monocytes, which had not been described so far. Hereby, platelets act as “helper” and facilitate the monocytic activity and immune response, which could be potentially indicating yet unexplored possible analogy to the role of helper T cells in the innate immune system. Most of the studies in the human system were either performed using platelet addition approaches or described pathological conditions with an enhanced monocyte-platelet aggregation. In contrast, my work presents a novel aspect of the importance of platelets, whereby they act as a tonic “signal” to license and maintain the monocytic activity and enable the initiation of cytokine response.

4.2 Thrombocytopenia-induced immune paralysis in human monocytes

A few studies have shown that thrombopenic patients exhibit an elevated frequency of non-classical and intermediate monocytes, accompanied by increased levels of TNFα and IL-1β in the plasma as well as hyperactivation of platelet-reactive T and B cells (Yang et al., 2017; Yazdanbakhsh, 2016). However, most of the publications focus on the causes of ITP and on which leukocyte subpopulations are involved in the induction of this pathology (Liu et al., 2013). There are only few studies suggesting an inversely proportional correlation between platelet counts and the risk of infections, indicating that patients with chronic ITP are more susceptible to viruses, fungi, and other infections (Ekstrand et al., 2016; Qu et al., 2018). A population-based cohort study has shown a higher mortality rate of adults with ITP as a result of cardiovascular diseases or infections (Frederiksen et al., 2014). Yet, less is known about the consequences of ITP on host defense mechanisms during infections. In my thesis, I provide a novel insight into how primary thrombocytopenia impacts the monocytic functionality in ITP patients. Monocytes from ITP patients showed an abolished secretion of IL-1β, IL-6 and TNFα in comparison to monocytes from healthy donors. Furthermore, cytokine levels secreted by monocytes from ITP patients and those of platelet-depleted monocytes from healthy donors were comparably low. These data suggested that patients with thrombocytopenia have monocytes with a defective immune response comparable to the immune paralysis observed after platelet depletion *ex vivo*. Over the course of investigating the functionality of primary human monocytes from ITP patients, I found that heterologous healthy platelets

were able to restore the abolished immune response and rescue the monocytic cytokine secretion that was absent due to thrombocytopenia. This *ex vivo* approach could be translated to clinical settings where the transfusion of heterologous platelets could potentially help ITP patients to respond to infections. Interestingly, the immune paralysis induced by the low platelet counts was specific to monocytes. Indeed, cytokine levels in *ex vivo* whole blood assays showed no differences between healthy and ITP people upon TLR stimulation. This would be due to the cellular heterogeneity of whole blood resulting in compensatory cytokine secretion by other, platelet-independent leukocytes. In other words, ITP patients might not be broadly immunologically paralyzed, but rather might exhibit abolished immune responses specifically driven by monocytes. So far, the lack of monocyte responsiveness was extensively described in the context of trauma and surgery, in patients with an acute-on-chronic liver failure, or in association with cirrhosis patients with endotoxemia (Haupt et al., 1998; Lin et al., 2007; Wasmuth et al., 2005). Yet, to my best knowledge, the relationship between thrombocytopenia and immune paralysis, and how ITP in particular impacts the function of circulating human monocytes remains elusive. Thus, my data suggest a novel aspect of monocyte immune paralysis as one of the consequences of ITP. Patients with ITP would be a vulnerable cohort with a higher risk of secondary or nosocomial infections that require monocyte-driven immune response for efficient pathogen clearance and host defense. These data highlight the significance of platelets in immune responses and underline their clinical relevance in monocyte-driven host defense specifically in patients with thrombocytopenia.

4.3 The impact of platelets on monocyte-derived cells

As platelets exert pivotal roles in monocyte-driven immune responses, they can be expected to have a broader impact, also affecting the fate and function of monocyte-derived macrophages and DCs. DCs and macrophages can derive from blood monocytes and are involved in host defense mechanisms (Geissmann et al., 2010). DCs play key roles in antigen presentation and provide a link between the innate and the adaptive arms of the immune system, while macrophages are professional phagocytic cells responsible for pathogen clearance as well as antigen presentation (Lipscomb & Masten, 2002; Wynn et al., 2013). Although my data only focus on primary human monocytes, I conjecture that

platelets might as well affect the fate of monocyte-derived macrophages and DCs and their immune responses. Indeed, differentiation of monocytes towards DCs in the presence of platelets results in an enhanced expression of HLA-DR and co-stimulatory molecules, which in turn induces a stronger T cell proliferation (Nguyen et al., 2008). Similarly, platelets steer the differentiation of monocytes and induce their polarization towards $CD163^+$ macrophages, which are associated with plaque progression, and foam cells (Chatterjee et al., 2015; Guo et al., 2018). Collectively, these observations indicate that the effect of the interaction between platelets and monocytes is broader than simply governing monocyte-driven immune responses. Thrombopenic patients might have DCs with a defective antigen-presentation, which in turn would lead to an attenuated T cell activation and thus affect the adaptive immune response. The boosting role of platelets in antigen-presentation by DCs was described, however it has not yet been addressed in the literature in the context of ITP (Han et al., 2020; Nishat et al., 2018). In contrast, thrombocytopenia might be protective in the context of atherosclerosis, as macrophage polarization towards foam cells and the formation of an atherosclerotic plaque is supported by platelets (Bobryshev, 2006; Chatterjee et al., 2015). Hence, platelets might shape the outcome of monocyte-driven immune diseases, as well as regulate pathological mechanisms in monocyte-derived cells.

4.4 Platelets shape the transcriptional landscape of monocytes

The findings presented in my thesis suggest that platelets play a central role in shaping the function of monocytes. Investigating the transcriptional alterations in monocytes revealed new aspects of regulation governed by platelets. Indeed, I have shown that platelet-depleted monocytes are transcriptionally nonresponsive to LPS. While standard monocytes up- and downregulated multiple genes upon LPS stimulation, platelet-depleted monocytes were unable to do so and did not acquire the LPS-induced gene signature. Importantly, this effect was reversible since the re-addition of platelets to platelet-depleted monocytes restored their LPS-induced transcriptional response. In other words, the “unstimulated” gene signature persists in platelet-depleted monocytes despite LPS stimulation. Hence, the transcriptional response triggered by LPS in monocytes is

dependent on platelets which control the magnitude of the transcriptional response to stimuli.

Platelets did not just steer the transcriptional response of primary human monocytes to LPS; their presence also shaped the monocyte transcriptomic signature at the baseline. Indeed, platelet depletion alone resulted in a transcriptional reprogramming of primary human monocytes. Rolfes et al. (2020) have shown that platelets can control the gene expression in human macrophages. The addition of platelet-derived supernatants upregulated the transcription of *NLRP3* and *IL1B* in human monocyte-derived macrophages without the need of any additional stimulation (Rolfes et al., 2020). On the one hand, my data show that platelet depletion upregulated several genes which are described to be negative regulators of the immune responses. For example, *PPAR-γ* was upregulated and has been described to suppress pro-inflammatory cytokine production in monocytes (Jiang et al., 1998; Mendes et al., 2021). Moreover, *ATF3* and *SPRY2* were also upregulated upon platelet depletion. ATF3 is a negative regulator of NF-κB and SPRY2 is a suppressor of ERK and FGF2 signaling (Glienke et al., 2000; Kwon et al., 2015; Tsavachidou et al., 2004). Both ATF3 and SPRY2 are known to have immune-inhibitory functions and they suppress the pro-inflammatory immune responses (De Nardo et al., 2014; Zhang et al., 2015). On the other hand, platelet depletion led to the downregulation of gene sets known for their involvement in several pro-inflammatory processes such as *BTK*, *CD14*, *MAPK14* and *RAB3D*. Both CD14 and BTK are pro-inflammatory key players in TLR-induced signaling in monocytes. While CD14 binds to LPS and subsequently induces TLR4 internalization and signaling, BTK interacts intracellularly with MyD88, IRAK1 and TLR4 (Jefferies et al., 2003; Weber et al., 2017; Zanoni et al., 2011). Mitogen-activated protein kinase 14 (also known as p38α) is a kinase involved in several immune processes and display, next to NF-κB, an additional signaling axis to induce cytokine responses (Herlaar & Brown, 1999; Yang et al., 2014). Lastly, Rab GTPases, like RAB3D, are key regulators of the membrane trafficking machinery with important roles in the fusion of organelles and extracellular vesicles. They are also involved in signal transduction during immune responses (Prashar et al., 2017; Su & Zheng, 2021). Hence in the absence of platelets, monocytes acquire an anti-inflammatory transcriptomic signature by promoting anti-inflammatory genes and downregulating pro-

inflammatory genes. Therefore, platelets act as an “ON switch” signal to maintain monocytes in a state equipped to transcriptionally respond to pro-inflammatory stimuli.

4.5 Platelets and immune responses in the murine system

In the course of this study, I also characterized the impact of platelets on monocytes in the mouse system. First, I observed that the results from the mouse experiments were generally different than the ones obtained in the human system. While platelet depletion abolished the immune response in the human system, this trend appeared to be reversed in the murine system (section 3.4). This was manifested by enhanced levels of LPS-induced cytokines in the plasma of thrombopenic mice in comparison to mock-treated mice (section 3.4.1). Platelets thus seem rather to hold the magnitude of mouse cytokine responses in check. Platelets could be a deciding factor in models of sepsis. Indeed, platelets protect mice from septic shock as thrombopenic mice show exacerbated levels of IL-6 and TNFα. In these settings, transfusion of platelets reduced mortality caused by the severity of sepsis (Xiang et al., 2013). Nevertheless, platelets are still crucial in the induction of immune responses, host defence against secondary infections, pathogen clearance and the maintenance of pro-inflammatory equilibrium (Loria et al., 2013; Ribeiro et al., 2019; Wuescher et al., 2015).

An additional difference between the mouse and human system was observed in the RNA-Seq data. Indeed, in human monocytes, I have shown that platelets control the magnitude and generally the occurrence of the transcriptional response to LPS (section 3.3.2). Yet, in mouse, the transcriptional response itself was not ablated in the absence of platelets (section 3.4.2), which stands in stark contrast to MoPD in the human system (section 3.3.2). Importantly, monocytes from both mock and thrombopenic mice induced the expression of pro- and anti-inflammatory genes to a similar extent upon LPS-treatment (section 8, Figure S5). This suggests that the presence of platelets does not regulate the expression magnitude of pro-inflammatory genes, e.g. chemokines and other cytokines, as observed in the human system. However, monocytes from thrombopenic mice showed 3.2-fold more LPS-induced DEGs than monocytes from the mock-treated group. Among them were genes uniquely expressed in platelet-depleted mice. These results indicate

that the differences in the gene expression profiles between monocytes from mock-treated and platelet-depleted mice rather concern the classes of the altered genes and the gene sets uniquely expressed in the monocytes from thrombopenic mice. Gene ontology analysis showed that these monocytes from LPS-challenged thrombopenic animals predominantly displayed changes in gene sets controlling mechanisms of cellular proliferation and homeostasis such as DNA replication, cell division, cell cycle and chromosomal segregation. In contrast, monocytes from mock-treated mice showed an enrichment in genes controlling mechanisms of innate immune responses and inflammatory host defense. Hence, *in vivo* platelet depletion polarizes mouse monocytes towards a state of cellular maintenance and proliferation, rather than towards inflammation and host defense. Although monocytes are considered as non-proliferative cells and only few monocytic subpopulations have proliferative properties (Lari et al., 2009). A monocyte thus has to “commit” to induce a program of either pro-inflammatory immune response or proliferation/cell homeostasis. Whether monocytes acquire a “refractory” state downregulating pro-inflammatory transcriptional responses remains to be investigated. Nevertheless, commonly expressed genes were similarly expressed in both mouse groups and the protein concentration of circulating cytokines such as IL-1β, IL-6 and TNFα were also similar between these groups (section 3.4.1). Hence, although monocytes from thrombopenic mice showed important transcriptional alterations (unique DEGs upon LPS treatment and gene profiles of proliferation and cell maintenance), I speculate that these transcriptional alterations in the mouse system are not associated with the inflammatory context studied in my thesis. Moreover, as already the transient platelet depletion *in vivo* acts as a trigger inducing transcriptional reprogramming in monocytes, a chronic thrombocytopenia could have long-term genomic consequences. Indeed, Christ et al. (2018) have shown in the context of trained immunity that western diet triggers irreversible transcriptional and epigenomic reprogramming of myeloid progenitor cells. These cells showed higher proliferation and augmented innate immune responses despite the elimination of the trigger (Christ et al., 2018). Hence, my findings prompt new questions about the long-term effects, such as the proliferative potential of monocytes from thrombopenic mice and the fate of their cellular progeny, i.e. monocyte-derived macrophages and DCs, which could be further investigated.

Considering the transcriptomic changes in mouse monocytes in the absence of LPS, comparing the platelet-depleted and mock-treated animals, only few DEGs were altered upon platelet depletion. Among them, platelet-specific genes showed lower levels, confirming the elimination of platelets. Notably, although the samples subjected to sequencing contained sorted monocytes of high purity, the detection of platelet-related genes suggests that these monocytes were complexed with platelets in the circulation. GO and KEGG analysis suggested that several biological processes and pathways are altered in monocytes due to the absence of platelets. Monocytes showed an altered signal transduction, changes in sensing of external stimuli and in immune responses. Furthermore, changes in hemostasis, coagulation and the complement system were top hits, which was not surprising due to the close relation between the complement and coagulation systems. Interestingly, platelet depletion by itself led to alterations in transcripts of chemokine signaling pathway, PI3K-Akt as well as Rap1 pathways. The latter two pathways involve the activation of p38, which mediates the paralysis of chemotaxis in monocytes upon TLR stimulation (Yi et al., 2012).

Taken together, platelets play a role in the immune responses in the murine system. However, they may act through different mechanisms and shape the cytokine responses differently than is the case in the human system. In mice, platelets play a protective role in controlling the extent of the LPS-induced cytokine response. Moreover, the ablation of platelets led to unique alterations in the expression of gene sets involved in cellular maintenance and proliferation, indicating that platelets may play broader roles beyond the context of the immune system. Lastly, the findings on the role of platelets cannot be generalized from the murine and human system as there are considerable differences between the species (Lin et al., 2014). Moreover, the presence of bystander cells in the mouse *in vivo* approaches adds another layer of complexity.

4.6 Platelet-depleted monocytes have altered signal transduction

The data presented in this thesis revealed that platelets steer the immune responses of monocytes on several levels. Indeed, platelet depletion induced immune paralysis, transcriptional reprogramming, changed kinase activities, and disrupted cytokine

secretion in monocytes. There were several hints that p38 and NF-κB signaling, in particular, are altered upon platelet depletion. The NanoString data showed that platelet depletion by itself induces a spontaneous upregulation of NF-κB suppressor genes, whereas the expression of *MAPK14* (p38α) is decreased (section 3.3.3). Furthermore, RNA-sequencing showed that monocytes from thrombopenic mice exhibit alterations in several pathways, including the PI3K-Akt pathway, which also involves p38 MAPK (section 3.4.4, Figure 16). Remarkably, profiling of kinase activity of PTKs and STKs in primary human monocytes unraveled global changes in kinase activities upon platelet depletion. Here, p38 MAPK and p38 MAPK-related kinases showed a decreased activity upon platelet depletion. Similarly, platelet depletion reduced the activity of NF-κB-activating kinases, such as BTK. Indeed, THP-1 Dual cells showed stronger NF-κB activity upon co-incubation with platelets. Collectively, my data suggest that the NF-κB and p38 MAPK signaling pathways are differentially regulated by platelets. Signal transduction of pathways required for pro-inflammatory responses seem to strongly depend on the presence of platelets. Interestingly, Rolfes et al. (2020) have also shown that platelets are crucial for the priming event of the NLRP3 inflammasome in macrophages, indicating their involvement in the TLR-induced signal transduction (Rolfes et al., 2020). Furthermore, it has been reported that the interaction between platelets and monocytes induces phosphorylation of the Akt kinase in monocytes (Stephen et al., 2013). GO and KEGG analysis highlighted that platelet-depleted monocytes show changes in the expression of genes involved in sensing of extracellular stimuli and signal transduction. This could explain the results in the NanoString mRNA expression analysis, where platelet-depleted monocytes cluster and persist in the “unstimulated” region of the PCA plot, regardless of the treatment conditions (with or without LPS). Thus, platelets appear to regulate kinase activities in monocytes and therefore the activation of intracellular signaling cascades that determine the cellular responses.

4.7 Direct communication between platelets and monocytes

Cellular communication is one of the essential mechanisms of the immune system to transfer information and alert against invading pathogens to ensure the onset of a host defense. Platelets can release several cytokines and pro-inflammatory mediators, thus

steering other leukocytes' activation status and immune responses (Ali et al., 2015). It was shown that platelets can communicate with macrophages via soluble factors and consequently boost their NLRP3-driven immune response (Rolfes et al., 2020). As platelets express an arsenal of immune modulatory molecules, the involvement of platelet-derived stimulatory cytokines and ligands in the communication between monocytes and platelets were experimentally tested in my thesis. It was reported that platelets can regulate monocytes through the secretion of RANTES (CCL5) and CXCL12 (Chatterjee et al., 2015; von Hundelshausen et al., 2005). Furthermore, platelets are known to express CD40L and activate leukocytes via the CD40L-CD40 interaction (Cognasse et al., 2022). Moreover, platelets upregulate surface CD62P upon activation and degranulation, which can interact with PSGL-1 on monocytes. In turn, this augments their activation by enhancing the nuclear translocation of NF-κB and thus their cytokine production (Weyrich et al., 1995). Surprisingly, none of the recombinant human CD40L, CXCL12, CD62P or CCL5 were able to rescue the abolished cytokine secretion in primary human platelet-depleted monocytes (section 3.6.2). Furthermore, targeting GPIIb/IIIa and integrins to disrupt the interaction between platelets and monocytes excluded the involvement of these surface glycoproteins and adhesion molecules in the MPAs formation (section 3.6.5; Figure 25A-B). This was surprising since GPIIb/IIIa is highly expressed by platelets and was shown to intrinsically regulate the function of platelets as well as their interaction with surface molecules of other leukocytes, such as CD11b and other integrins resulting in their activation (Fullard, 2004; Li et al., 2000; Spangenberg et al., 1993; van Gils et al., 2009). Additionally, it has been shown that sialic acid residues fulfil regulatory roles in platelets and platelet-modulated immune responses (Crocker et al., 2007; Crook, 1991; Lubbers et al., 2018). Interestingly, it was described that the crosslinking of sialic acid-binding Ig-like lectin 7 on monocytes induces a pronounced boost in their cytokine secretion (Varchetta et al., 2012). Therefore, I tested whether sialic acid residues exposed on the platelet surface and Siglec-7 on monocytes are involved in the platelet-induced cytokine-boosting effect. However, Siglec-7 and sialic acid residues were not involved in this phenomenon (section 3.6.2, Figure 21; section 3.6.5, Figure 25C). It was unexpected that none of these well described co-stimulatory molecules and mediators of cell activation and inflammation were involved in the platelet-monocyte interaction and platelet-induced cytokine boost. It remains possible that the interaction between platelets and monocytes

is dependent on not just one of these molecules, but rather a combination of several ligands and receptors. Furthermore, the usage of recombinant proteins *in vitro* might not fully resemble the physiological conditions *in vivo*, such as posttranscriptional modifications, glycosylation of receptors and ligands. Finally, the engagement of surface molecules might require a higher density of ligands interacting to a receptor, e.g. ligands embedded and concentrated in lipid rafts, to induce signal transduction. This situation might not be recapitulated by *ex vivo* and *in vitro* approaches in the present work.

The mechanisms of the heterotypic interaction between platelets and leukocytes were reported in different studies, suggesting a direct cell-cell contact or soluble factors to be involved (Cerletti et al., 2010; Rolfes et al., 2020; Rossaint et al., 2018). Even though the transwell assay data implied that monocytes require direct contact with platelets for restoration of their cytokine secretion (section 3.6.3), thus excluding soluble mediators, platelet-derived supernatants also induced a partial rescue of cytokine secretion (section 3.6.3). These findings may, at first glance, seem to be in conflict; however, platelet-derived supernatants may not necessarily only contain soluble proteins. Western blot analysis of cell-free platelet-derived supernatants obtained following a centrifugation at 3,000 x g has shown the presence of the housekeeping protein beta-actin, suggesting that cellular fragments, but not whole platelets (which are pelleted at lower g-forces), may be present in this fluid. These might be platelet-derived particles, e.g. vesicles, which can be larger than the 0.4 µm pore size of the transwell membrane. These platelet-derived particles might contain immunomodulatory molecules that determine the functionality of the recipient cells. Interestingly, platelets were characterized as one of the major producers of vesicles in the blood, a phenomenon that was initially termed as “platelet-dust” (Alberro et al., 2021; Chargaff & West, 1946; Wolf, 1967). These extracellular vesicles reach the cytosol of recipient cells via different uptake mechanisms, including phagocytosis (Mulcahy et al., 2014). Although my microscopy data did not show the engulfment of platelets by monocytes, the inhibition of phagocytosis disrupted the platelet-induced rescue of cytokine secretion by monocytes (section 3.6.4). This suggested that the phagocytic machinery is involved in the platelet-induced cytokine boost. In turn, this brings platelet-derived particles or vesicles into focus again, as these could be phagocytosed by monocytes; however, could not be visualized due to resolution limitations. Taken together,

these data exclude the involvement of soluble factors, such as small molecules, soluble metabolites and proteins. Since platelet-derived supernatants partially restore the cytokine secretion in monocytes, I speculate that the interaction between platelets and monocytes requires both physical cell-cell interaction and the delivery of immunomodulatory cargo via platelet-derived vesicles.

4.8 P38 MAPK pathway plays a central role in the platelet-induced cytokine boost in primary human monocytes

Cytokine responses can be mediated by several signaling pathways, among them NF-κB and p38 MAPK. NF-κB activation occurs upon TLR activation, whereby downstream signaling molecules facilitate a cascade of phosphorylation events resulting in the nuclear translocation of NF-κB heterodimers, e.g. p50 and p65. In the nucleus, phosphorylated p65 induces the transcription of pro-inflammatory target genes (Liu et al., 2017). P38 MAPKs play regulatory roles in pro- and anti-inflammatory responses. P38 can activate several transcription factors, such as NF-κB, to induce cytokine production, control the expression of cytokine receptors and the stability of mRNA transcripts. Thus, p38 steers the direction of an immune response (Bachstetter & Van Eldik, 2010; Canovas & Nebreda, 2021).

Several findings in my thesis imply that the platelet-induced rescue of cytokine responses in monocytes involves the p38 MAPK and NF-κB pathways. Here, THP-1 Dual cells have shown higher NF-κB activation in the presence of platelets (section 3.7). Furthermore, western blot data show high expression levels of NF-κB and p38 in platelets (section 3.8), and NanoString data highlighted the spontaneous upregulation of NF-κB-suppressor genes and downregulation of p38α in monocytes upon platelet depletion (section 3.3.3). Thus, platelets might either control these two signaling modules *in trans*, or might provide monocytes with transcription factors and kinases. In fact, kinase activity profiling using the PamGene technology revealed that platelets contain a variety of highly active kinases, even in the absence of stimulation. Platelets might resemble “cellular packages” containing a variety of functional signaling molecules as well as transcription factors, despite the lack of a nucleus. Indeed, it was reported that platelets express several

transcription factors and kinases important in cell-intrinsic regulation, independent of *de novo* transcription (Lannan et al., 2015). In these cells, the p65 NF-κB subunit is required for activation and aggregation of platelets (Beaulieu & Freedman, 2009). Furthermore, p38 is required for intrinsic signaling in platelets and can also control the activity of NF-κB (Lannan et al., 2015; Lu et al., 2012; Saha et al., 2007).

Since platelets are anucleate cells, the detection of p38 and p65 NF-κB in platelet cell lysates and supernatants raised the question of whether these two proteins could also influence the activity of monocytes upon platelet-monocyte interaction, in addition to their platelet-intrinsic activity. Here, I showed that platelets require functional NF-κB and p38 to induce the rescue of cytokine responses in monocytes (section 3.9). Indeed, the disrupted cytokine secretion was not rescued in platelet-depleted monocytes when co-incubated with platelets pre-treated with NF-κB or p38 inhibitors. This suggests that NF-κB and p38, or their downstream effectors, play a key role in the platelet-induced cytokine rescue in monocytes. Platelets might require NF-κB or p38 as parts of an activation cascade, at which end the “platelet boosting factor” is induced. Alternatively, platelets might directly transfer p65 and/or p38 to monocytes and thus steer their signaling. Since my western blot data show that the expression of NF-κB and p38 was detected not only in platelet lysates but also in cell-free supernatants, which could contain platelet-derived vesicles and particles, platelets may package and transfer p38, possibly NF-κB, and other signaling molecules to monocytes via vesicular cargo.

When NF-κB and p38 were inhibited in monocytes, the LPS-induced cytokine secretion was abolished. In contrast to p38, the inhibition of NF-κB was cytotoxic and cell viability was low, making data interpretation difficult. Interestingly, platelets were able to bypass p38 inhibition in monocytes and rescue their cytokine secretion. This either suggests that platelets indeed provide monocytes with a functional p38 protein or that they supply monocytes with p38-downstream signals essential for the cytokine response. Indeed, when p38 was inhibited in both platelets and monocytes, no cytokine rescue was observed. Thus, the communication between platelets and monocytes is mediated through the p38 MAPK signaling pathway.

Mechanistically, how platelets overcome p38 inhibition in monocytes and consequently induce the cytokine boost, requires more investigation. The concept of intercellular transfer of intracellular organelles, transcription factors and signaling molecules emerged over the last decade. It has been described as a unique mechanism of communication between cells, occurring via transfer of proteins or organelles. For example, cells can share mitochondria via membranous tubular protrusions, dendrites, an extrusion of uncoated mitochondria and uptake by the recipient cells, or via macrovesicles (Liu et al., 2021). Platelets were shown to transfer CXCR4, arachidonic acid and transcription factors such as PPAR-γ via platelet-derived vesicles (Lannan et al., 2015). Moreover, platelets can release and provide other cells with cell-free extracellular mitochondria or embedded in platelet-derived particle and steer the inflammatory responses (Boudreau et al., 2014). Interestingly, it was shown that platelets can be engulfed by monocytes and consequently polarize their differentiation towards pro-inflammatory macrophages (Singhal et al., 2018). Therefore, intercellular transfer is a possible mechanism by which platelets pass on intact p38 protein or p38-dependent downstream molecules to monocytes via platelet-derived fragments/vesicles, in addition to the physical cell-cell contact. Platelets could provide monocytes with these active signaling molecules directly or they may feed into signaling events downstream of the p38 pathway in monocytes, resulting in the transcription of pro-inflammatory cytokines.

4.9 Conclusion

Platelets are mainly known for their regulatory roles in blood coagulation and hemostasis. However, the role of platelets in immune responses has gained increasing recognition over the last decades. Here, I provide an insight into the immune modulatory function of platelets, acting as “helper platelets” facilitating the initiation of immune responses in monocytes. I show, for the first time, that monocyte-driven immune responses are dependent on platelets. My findings shed light on a novel aspect of platelet-mediated monocyte immune regulation and focus on the thrombocytopenia-induced immune paralysis of human monocytes.

My data show that the elimination of platelets results in monocytic immune paralysis, transcriptional alterations, changes in the NF-κB and p38 MAPK signaling pathways, and disrupted cytokine secretion. Platelets synergistically enhance the TLR-induced “priming” of monocytes and augment NF-κB activation and, consequently, cytokine secretion. Patients with idiopathic thrombocytopenia, as well as thrombopenic conditions recapitulated *ex vivo*, showed a disrupted monocytic cytokine secretion. Furthermore, monocytes show broad transcriptional alterations upon platelet depletion, highlighting that platelets shape the baseline gene expression profile in primary human monocytes. Moreover, platelets control the magnitude of the LPS-induced transcriptional response. Platelet-depleted monocytes are incapable of acquiring an LPS-induced gene signature and instead persist in an “unstimulated” state. Finally, platelet-depleted monocytes upregulate gene sets known to modulate anti-inflammatory processes, while the gene sets required for stimulus sensing, signal transduction and inflammation are downregulated. Thus, platelets act as a tonic signal to maintain monocytes “ready and equipped” to respond to microbial or sterile stimuli, and thus assist in the initiation of an immune response.

My findings suggest that monocytes require a direct interaction with platelets to fulfill their immune-boosting function, excluding the involvement of humoral factors. However, my data also suggest that monocytes and platelets require a two-step interaction: i) physical cell-cell contact for either juxtapositioning or signaling followed by ii) the consequent transfer of modulatory molecules, e.g. p38 or p38-downstream effectors, via platelet-derived vesicles to facilitate the pro-inflammatory immune response (Figure 31).

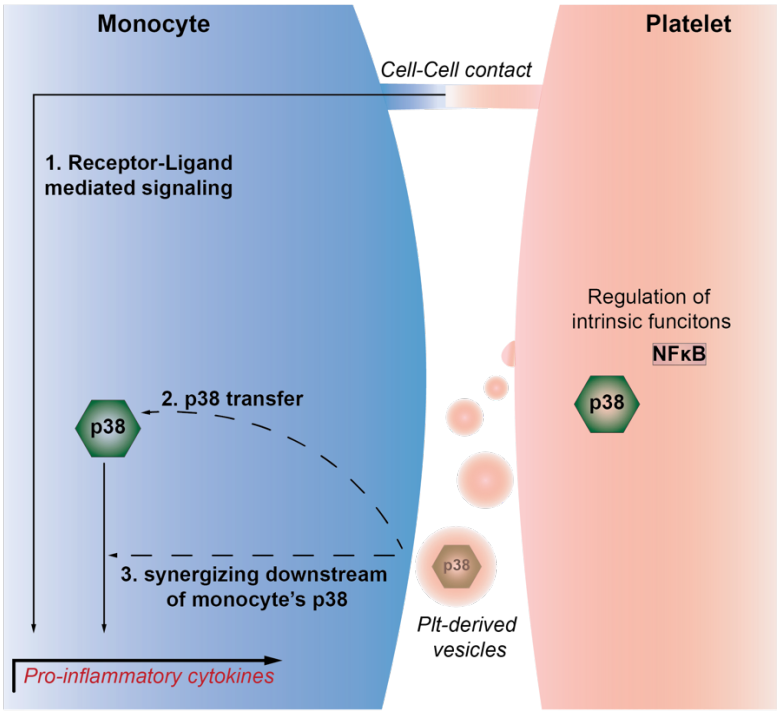

Figure 31: Possible mechanism of action in monocyte-platelet aggregates. A platelet (red) interacting with a monocyte (blue) via cell-cell contact. This interaction induces intracellular signaling by three possible mechanisms: 1. Receptor-Ligand binding, 2. donation of bioactive platelet-derived p38 transferred via platelet-derived vesicles, or 3. platelets synergize monocytic p38 signaling cascade by the transfer of other bioactive molecules, which activate p38-downstream effectors. The interaction between platelets and monocytes results in the licensing and induction of pro-inflammatory cytokine response in monocytes.

The findings in my thesis emphasize that platelets are indispensable for the monocytic immune functions. They act as a licenser of inflammatory responses and do not only define the magnitude of the immune response, but are also fundamental for the initiation of cytokine responses in monocytes, thus highlighting their importance for an efficient host defense.

4.10 Perspectives

In my thesis, I applied several methodologies to characterize the role of platelets in the monocyte-driven cytokine responses. My findings emphasize the central role of platelets in assisting and initiating the pro-inflammatory immune response in primary human

monocytes. Yet, there are several open questions that have to be further investigated or corroborated.

Here, I focused specifically on the immune responses of monocytes in the presence and absence of platelets. It would be interesting to further investigate how platelets shape the differentiation and the subsequent immune responses of monocyte-derived macrophages and DCs. Monocytes could be co-cultured with platelets and differentiated towards macrophages or DCs. Monocyte-derived cells would then be treated with different stimuli and activation cell-surface markers such as HLA-DR and CD80/86 as well as cytokine release could be assessed. Furthermore, it would be also interesting to investigate whether the platelet-effect is restricted to p38/NF-κB-related cytokine responses. Here, the activity of other pathways and the sensitivity to other stimuli could be also tested, e.g. to activate the JAK/STAT, Wnt or other signaling pathways. The role of platelets in other monocytic functions could also be investigated. Cell migration and recruitment could be assessed using chemotaxis assays, phagocytosis assays could be performed using beads or bacteria to measure the phagocytic capacity of monocytes, and proliferation assays such as carboxyfluorescein succinimidyl ester labeling and flow cytometry could be used to assess the proliferation capacity of monocytes in the presence or absence of platelets.

It would be interesting to determine whether all platelets or a specific platelet subtype is involved in the immune-boosting effect. There are hints implicating that platelets could be divided into subpopulations according to their function and expression of specific surface markers, e.g. phosphatidylserine for procoagulant platelets, GPIIb/IIIa for aggregatory platelets, CD62P and LAMP-1 exposing for secretory cells (Hamad et al., 2022; Sodergren & Ramstrom, 2018). Platelet subpopulations could be sorted according to these characteristics and co-incubated with platelet-depleted monocytes to assess whether a specific platelet subpopulation is responsible for the platelet-induced cytokine boost in primary human monocytes.

Furthermore, the involvement of platelet-derived vesicles in the regulation of primary human monocytes could be further studied. As platelets are one of the major vesicle-

shedding populations in the blood, the involvement of platelet-derived vesicles could represent an important axis of communication between platelets and monocytes. Over the course of such investigation, the presence of platelet-derived vesicles and particles in platelet supernatants could be assessed using a nanoparticle-tracking device. These supernatants could be processed and fractionated using differential centrifugation steps or size exclusion chromatography. Platelet-depleted monocytes could be co-incubated with each fraction, then stimulated and the cytokine levels could be measured. In addition, the cargo of such platelet-derived vesicles and particles could be defined using proteomic and metabolomic approaches, e.g. to determine the presence of immunomodulatory molecules, transcription factors or signaling molecules.

Lastly, the role of intercellular protein transfer between platelets and monocytes could be further studied using protein overexpression setups. In such scenario, MEG-01 and THP-1 cell lines could be used to model megakaryocytes-derived platelets and monocytes, respectively. MEG-01 is a megakaryocytic cell line, which is described to shed platelet-like particles (Takeuchi et al., 1998). Using a split-green fluorescent protein (GFP) system, MEG-01 and THP-1 could each be transfected or transduced with either GFP^{1-10} or GFP^{11}. Each GFP^{n} corresponds to one of the α-helixes responsible for the fluorescence of full-length GFP (Tamura et al., 2021). Upon co-incubation, the uptake of platelet-like particles from MEG-01 cells by THP-1 monocytes could be detected as GFP fluorescence is recovered from the dimerization of both split GFP parts. A more targeted experiment could deploy a cargo molecule of interest, e.g. p38, fused to GFP^{11} in the donor cells and assess its specific transfer to GFP^{1-10}-expressing THP-1 cells.

5. Abstract

Monocytes are circulating immune cells essential to host defense, orchestrating immune responses through the secretion of immunomodulatory mediators. While exuberant monocyte-driven immune responses have life-threatening consequences, monocytic immune paralysis, characterized by abolished cytokine responses, compromises host defense and increases vulnerability to opportunistic infections. Hence, elucidating the mechanisms underlying monocyte-driven cytokine responses are relevant in clinical settings. In the circulation, monocytes are constantly surrounded by a large number of platelets. Beside their roles in hemostasis and coagulation, platelets can influence the function of leukocytes and steer immune responses. However, how platelets impact monocyte-driven immune responses is poorly understood. My findings uncover an unprecedented layer of platelet-induced regulation of monocyte immune function. Data presented in this thesis demonstrate the fundamental role of platelets in licensing the pro-inflammatory cytokine response of primary human monocytes. In contrast to previous assumptions, my data show that monocytes are not autonomous in their pro-inflammatory cytokine response, and require the interaction with platelets to reach their full pro-inflammatory capacity. Platelet depletion impairs the monocytic immune function through transcriptional shut-down of pro-inflammatory genes, alterations in kinase activities, and the subsequent disruption of pro-inflammatory cytokine secretion. Notably, this state of paralysis is reversed when platelet-depleted monocytes are reconstituted with autologous platelets. Interestingly, monocytes from patients with idiopathic thrombocytopenia display an impaired capacity to produce IL-1β, IL-6 and TNFα. The addition of healthy platelets to monocytes from ITP patients ameliorates their cytokine responses. Mechanistically, I demonstrate that platelet depletion affects the NF-κB and p38 MAPK signaling pathways, identifying these as central components of the platelet-monocyte communication. Specifically, my data suggest that platelets license full monocyte cytokine responses via a two-step interaction: i) physical cell-cell contact and a subsequent ii) transfer of p38 or p38-downstream effectors via platelet-derived vesicles. Hence, the data in my thesis highlight platelets as a novel “ON” switch signal, facilitating the pro-inflammatory response of monocytes and maintaining them “ready and equipped” for an efficient host defense.

6. List of figures

7. Supplementary figures

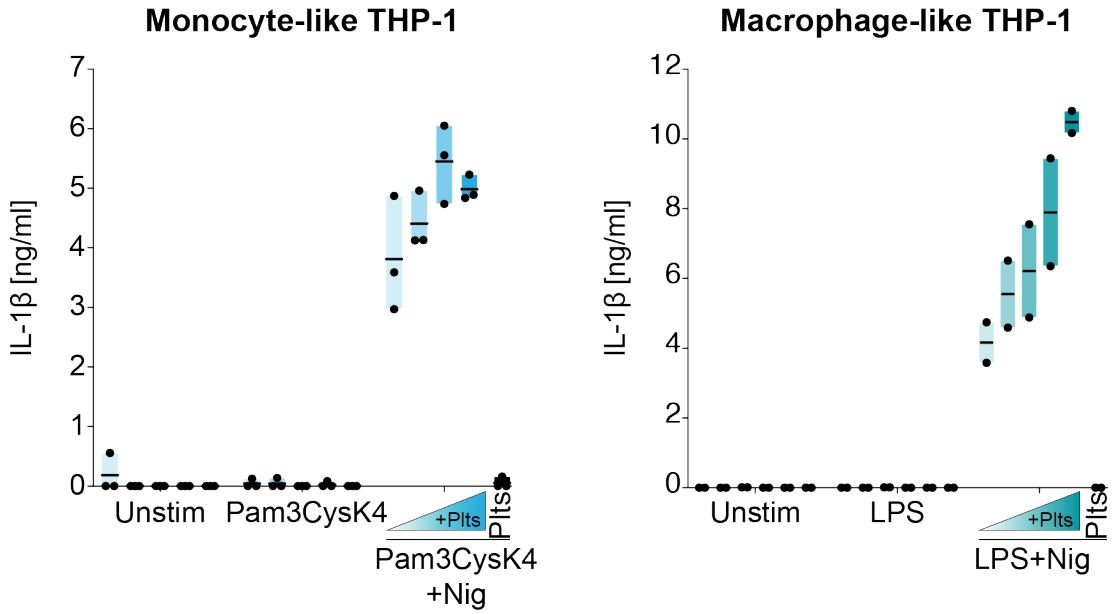

Figure S 1: Platelets boost IL-1β secretion in THP-1 cell line. HTRF data display secretion levels of IL-1β. Monocyte-like THP-1 and PMA-differentiated macrophage-like THP-1 were co-incubated with an increasing number of platelets. Cells were stimulated with Pam3CysK4 [1 µg/ml] or LPS [1 µg/ml] for 4.5 h or for 3 h followed by 1.5 h of nigericin [10 µM]. Floating bars show max/min values with indication to the mean. Each symbol represents one of n=2-4 experiments. My data of Macrophage-like THP-1 are shown in the published paper of Rolfes et al. (2020).

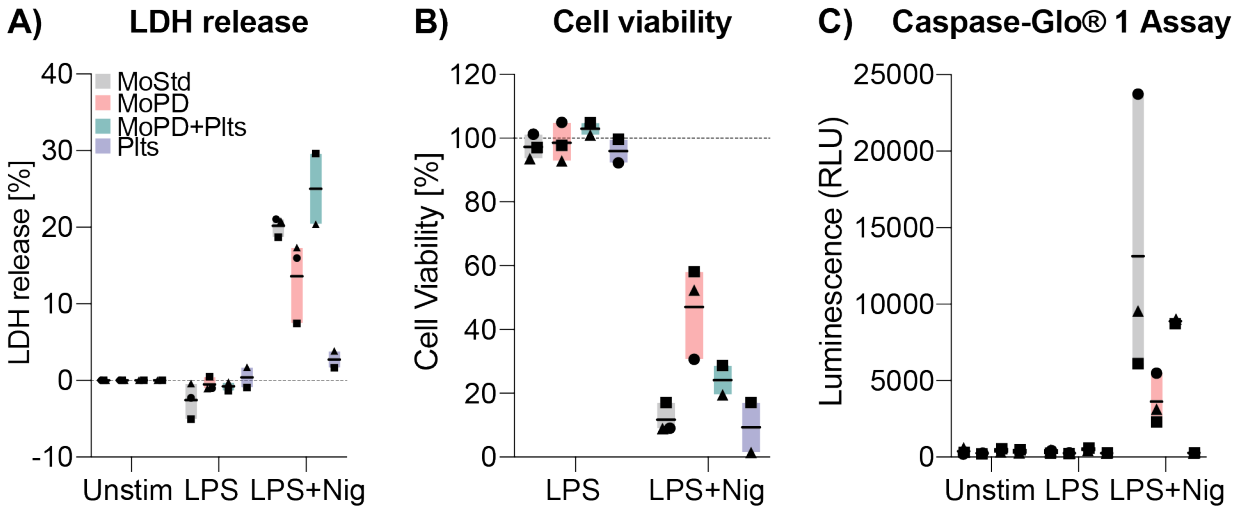

Figure S 2: Platelets rescue primary human monocytes from apoptosis. Cell viability assays display (**A**) LDH release or (**B**) Cell-Titer blue assay. (**C**) Caspase-Glo® 1 Assay shows Caspase-1 activity in collected supernatants after NLRP3 activation. Floating bars

display max/min values with indication to the mean. Each symbol represents one of n=2-3 blood donors.

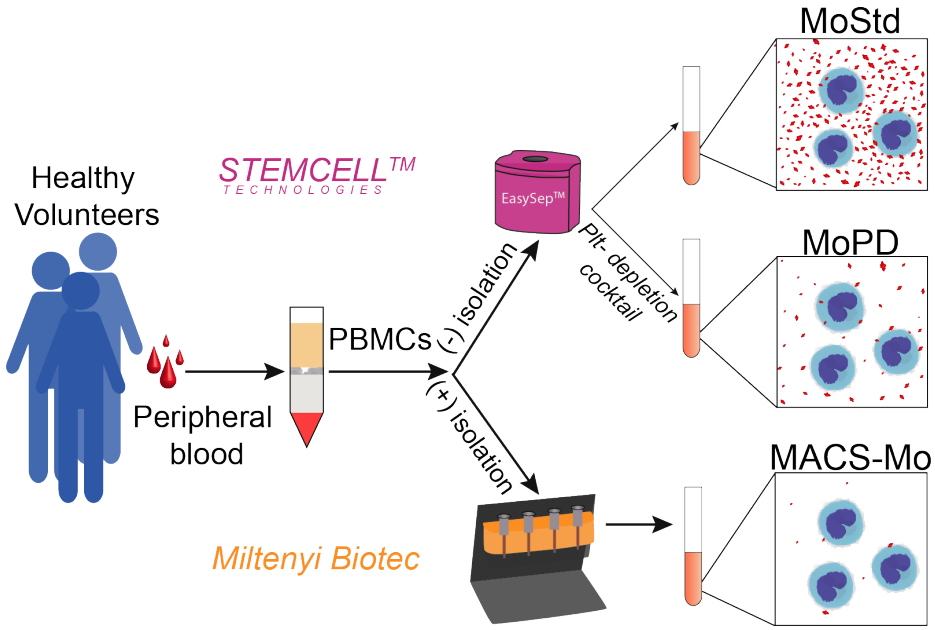

Figure S 3: Isolation of primary human monocytes. Schematic presentation of isolated primary human monocytes with STEMCELL Kit resulting in standard monocytes (MoStd) and platelet-depleted monocytes (MoPD) or using MACS Miltenyi Kit (MACS-Mo).

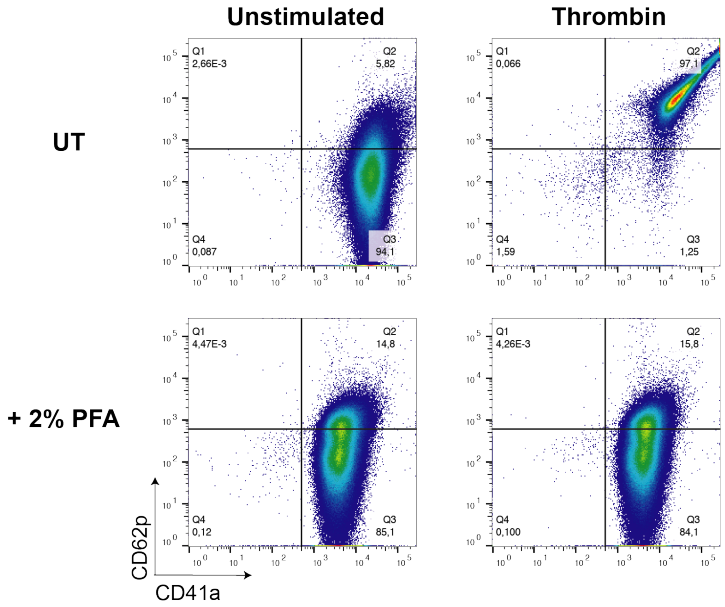

Figure S 4: Platelet degranulation assay. Representative flow cytometry analysis displays CD62P expression in untreated (UT) and PFA-fixed human platelets upon treatment with Thrombin for 30 min at 37 °C and 5% CO_2.

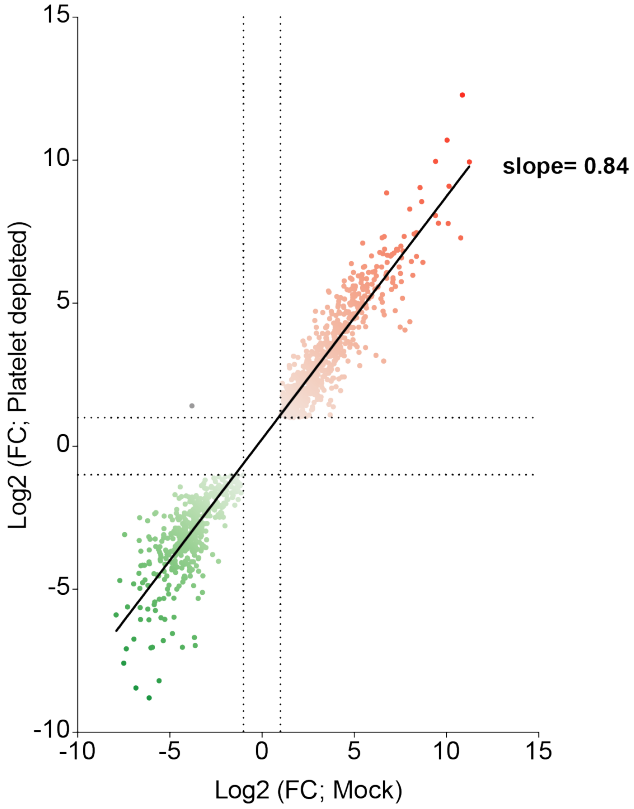

Figure S 5: Correlation of differentially expressed genes. Fold change (FC) values of significant DEGs, commonly expressed in platelet-depleted and mock-treated mice, were correlated. Linear regression displays a slope value of 0.84. Each dot represents the FC value of a commonly expressed gene in platelet-depleted mice (Y-axis) vs. mock-treated mice (X-axis) in n=3 experiments. Green: downregulated genes, red: upregulated genes.

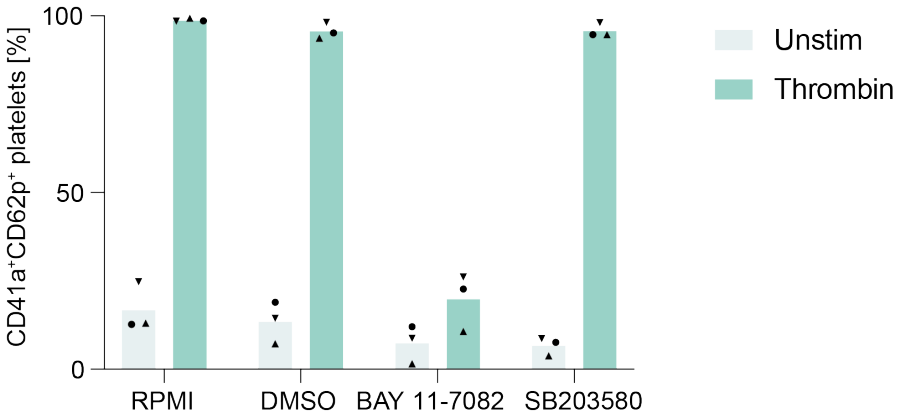

Figure S 6: BAY 11-7082 disrupts platelet degranulation. Quantification of platelet degranulation assay measured by flow cytometry. Platelets were pre-treated with DMSO, BAY 11-7082, SB203580 or were left untreated. Cells were stimulated with Thrombin for 30 min at 37 °C and 5% CO_2 and CD62P exposition was measured by FACS. Each symbol represents one of n=3 blood donors.

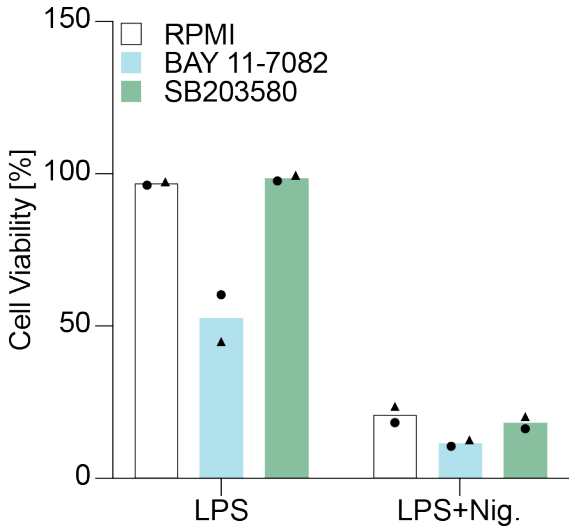

Figure S 7: Cell viability assay of standard monocytes. Cell-Titer blue displays cell viability of MoStd. Cells pre-treated with BAY 11-7082 or SB203580 were stimulated with LPS for 4.5 h or for 3 h followed by 1.5 h nigericin. Cells were incubated in Cell-Titer blue buffer and cell viability was measured. Values were normalized on the untreated, unstimulated condition of MoStd. Each symbol represents one of n=2 blood donors.

8. References

Adak, S., Chowdhury, S., & Bhattacharyya, M. (2008). Dynamic and electrokinetic behavior of erythrocyte membrane in diabetes mellitus and diabetic cardiovascular disease. *Biochim Biophys Acta*, *1780*(2), 108-115. https://doi.org/10.1016/j.bbagen.2007.10.013

Alard, J. E., Ortega-Gomez, A., Wichapong, K., Bongiovanni, D., Horckmans, M., Megens, R. T., Leoni, G., Ferraro, B., Rossaint, J., Paulin, N., Ng, J., Ippel, H., Suylen, D., Hinkel, R., Blanchet, X., Gaillard, F., D'Amico, M., von Hundelshausen, P., Zarbock, A., . . . Soehnlein, O. (2015). Recruitment of classical monocytes can be inhibited by disturbing heteromers of neutrophil HNP1 and platelet CCL5. *Sci Transl Med*, *7*(317), 317ra196. https://doi.org/10.1126/scitranslmed.aad5330

Alberro, A., Iparraguirre, L., Fernandes, A., & Otaegui, D. (2021). Extracellular Vesicles in Blood: Sources, Effects, and Applications. *Int J Mol Sci*, *22*(15). https://doi.org/10.3390/ijms22158163

Ali, R. A., Wuescher, L. M., & Worth, R. G. (2015). Platelets: essential components of the immune system. *Curr Trends Immunol*, *16*, 65-78. https://www.ncbi.nlm.nih.gov/pubmed/27818580

Allen, N., Barrett, T. J., Guo, Y., Nardi, M., Ramkhelawon, B., Rockman, C. B., Hochman, J. S., & Berger, J. S. (2019). Circulating monocyte-platelet aggregates are a robust marker of platelet activity in cardiovascular disease. *Atherosclerosis*, *282*, 11-18. https://doi.org/10.1016/j.atherosclerosis.2018.12.029

Armingol, E., Officer, A., Harismendy, O., & Lewis, N. E. (2021). Deciphering cell-cell interactions and communication from gene expression. *Nat Rev Genet*, *22*(2), 71-88. https://doi.org/10.1038/s41576-020-00292-x

Arnold, D. M., Nazi, I., Toltl, L. J., Ross, C., Ivetic, N., Smith, J. W., Liu, Y., & Kelton, J. G. (2015). Antibody binding to megakaryocytes in vivo in patients with immune thrombocytopenia. *Eur J Haematol*, *95*(6), 532-537. https://doi.org/10.1111/ejh.12528

Ashman, N., Macey, M. G., Fan, S. L., Azam, U., & Yaqoob, M. M. (2003). Increased platelet-monocyte aggregates and cardiovascular disease in end-stage renal failure patients. *Nephrol Dial Transplant*, *18*(10), 2088-2096. https://doi.org/10.1093/ndt/gfg348

Awad, F., Assrawi, E., Jumeau, C., Georgin-Lavialle, S., Cobret, L., Duquesnoy, P., Piterboth, W., Thomas, L., Stankovic-Stojanovic, K., Louvrier, C., Giurgea, I., Grateau, G., Amselem, S., & Karabina, S. A. (2017). Impact of human monocyte and macrophage polarization on NLR expression and NLRP3 inflammasome activation. *PLoS One*, *12*(4), e0175336. https://doi.org/10.1371/journal.pone.0175336

Bachstetter, A. D., & Van Eldik, L. J. (2010). The p38 MAP Kinase Family as Regulators of Proinflammatory Cytokine Production in Degenerative Diseases of the CNS. *Aging Dis*, *1*(3), 199-211. https://www.ncbi.nlm.nih.gov/pubmed/22720195

Badger, A. M., Cook, M. N., Lark, M. W., Newman-Tarr, T. M., Swift, B. A., Nelson, A. H., Barone, F. C., & Kumar, S. (1998). SB 203580 inhibits p38 mitogen-activated protein kinase, nitric oxide production, and inducible nitric oxide synthase in bovine cartilage-derived chondrocytes. *J Immunol*, *161*(1), 467-473. https://www.ncbi.nlm.nih.gov/pubmed/9647257

Beaulieu, L. M., & Freedman, J. E. (2009). NFkappaB regulation of platelet function: no nucleus, no genes, no problem? *J Thromb Haemost*, *7*(8), 1329-1332. https://doi.org/10.1111/j.1538-7836.2009.03505.x

Berenson, L. S., Yang, J., Sleckman, B. P., Murphy, T. L., & Murphy, K. M. (2006). Selective requirement of p38alpha MAPK in cytokine-dependent, but not antigen receptor-dependent, Th1 responses. *J Immunol*, *176*(8), 4616-4621. https://doi.org/10.4049/jimmunol.176.8.4616

Bhattacharjee, J., Das, B., Mishra, A., Sahay, P., & Upadhyay, P. (2017). Monocytes isolated by positive and negative magnetic sorting techniques show different molecular characteristics and immunophenotypic behaviour. *F1000Res*, *6*, 2045. https://doi.org/10.12688/f1000research.12802.3

Bhattacharya, S., Qian, J., Tzimas, C., Baker, D. P., Koumenis, C., Diehl, J. A., & Fuchs, S. Y. (2011). Role of p38 protein kinase in the ligand-independent ubiquitination and down-regulation of the IFNAR1 chain of type I interferon receptor. *J Biol Chem*, *286*(25), 22069-22076. https://doi.org/10.1074/jbc.M111.238766

Bobryshev, Y. V. (2006). Monocyte recruitment and foam cell formation in atherosclerosis. *Micron*, *37*(3), 208-222. https://doi.org/10.1016/j.micron.2005.10.007

Boilard, E., Nigrovic, P. A., Larabee, K., Watts, G. F., Coblyn, J. S., Weinblatt, M. E., Massarotti, E. M., Remold-O'Donnell, E., Farndale, R. W., Ware, J., & Lee, D. M. (2010). Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science*, *327*(5965), 580-583. https://doi.org/10.1126/science.1181928

Bollig, F., Winzen, R., Gaestel, M., Kostka, S., Resch, K., & Holtmann, H. (2003). Affinity purification of ARE-binding proteins identifies polyA-binding protein 1 as a potential substrate in MK2-induced mRNA stabilization. *Biochem Biophys Res Commun*, *301*(3), 665-670. https://doi.org/10.1016/s0006-291x(03)00015-9

Boudreau, L. H., Duchez, A. C., Cloutier, N., Soulet, D., Martin, N., Bollinger, J., Pare, A., Rousseau, M., Naika, G. S., Levesque, T., Laflamme, C., Marcoux, G., Lambeau, G., Farndale, R. W., Pouliot, M., Hamzeh-Cognasse, H., Cognasse, F., Garraud, O., Nigrovic, P. A., . . . Boilard, E. (2014). Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. *Blood*, *124*(14), 2173-2183. https://doi.org/10.1182/blood-2014-05-573543

Brandt, E., Petersen, F., Ludwig, A., Ehlert, J. E., Bock, L., & Flad, H. D. (2000). The beta-thromboglobulins and platelet factor 4: blood platelet-derived CXC chemokines with divergent roles in early neutrophil regulation. *J Leukoc Biol*, *67*(4), 471-478. https://doi.org/10.1002/jlb.67.4.471

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., & Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science*, *303*(5663), 1532-1535. https://doi.org/10.1126/science.1092385

Broz, P., & Dixit, V. M. (2016). Inflammasomes: mechanism of assembly, regulation and signalling. *Nat Rev Immunol*, *16*(7), 407-420. https://doi.org/10.1038/nri.2016.58

Canovas, B., Igea, A., Sartori, A. A., Gomis, R. R., Paull, T. T., Isoda, M., Perez-Montoyo, H., Serra, V., Gonzalez-Suarez, E., Stracker, T. H., & Nebreda, A. R. (2018). Targeting p38alpha Increases DNA Damage, Chromosome Instability, and the Anti-tumoral Response to Taxanes in Breast Cancer Cells. *Cancer Cell*, *33*(6), 1094-1110 e1098. https://doi.org/10.1016/j.ccell.2018.04.010

Canovas, B., & Nebreda, A. R. (2021). Diversity and versatility of p38 kinase signalling in health and disease. *Nat Rev Mol Cell Biol*, *22*(5), 346-366. https://doi.org/10.1038/s41580-020-00322-w

Cerletti, C., de Gaetano, G., & Lorenzet, R. (2010). Platelet - leukocyte interactions: multiple links between inflammation, blood coagulation and vascular risk. *Mediterr J Hematol Infect Dis*, *2*(3), e2010023. https://doi.org/10.4084/MJHID.2010.023

Chabaud-Riou, M., & Firestein, G. S. (2004). Expression and activation of mitogen-activated protein kinase kinases-3 and -6 in rheumatoid arthritis. *Am J Pathol*, *164*(1), 177-184. https://doi.org/10.1016/S0002-9440(10)63108-2

Chaplin, D. D. (2010). Overview of the immune response. *J Allergy Clin Immunol*, *125*(2 Suppl 2), S3-23. https://doi.org/10.1016/j.jaci.2009.12.980

Chargaff, E., & West, R. (1946). The biological significance of the thromboplastic protein of blood. *J Biol Chem*, *166*(1), 189-197. https://www.ncbi.nlm.nih.gov/pubmed/20273687

Chatterjee, M., von Ungern-Sternberg, S. N., Seizer, P., Schlegel, F., Buttcher, M., Sindhu, N. A., Muller, S., Mack, A., & Gawaz, M. (2015). Platelet-derived CXCL12 regulates monocyte function, survival, differentiation into macrophages and foam cells through differential involvement of CXCR4-CXCR7. *Cell Death Dis*, *6*, e1989. https://doi.org/10.1038/cddis.2015.233

Christ, A., Gunther, P., Lauterbach, M. A. R., Duewell, P., Biswas, D., Pelka, K., Scholz, C. J., Oosting, M., Haendler, K., Bassler, K., Klee, K., Schulte-Schrepping, J., Ulas, T., Moorlag, S., Kumar, V., Park, M. H., Joosten, L. A. B., Groh, L. A., Riksen, N. P., . . . Latz, E. (2018). Western Diet Triggers NLRP3-Dependent Innate Immune Reprogramming. *Cell*, *172*(1-2), 162-175 e114. https://doi.org/10.1016/j.cell.2017.12.013

Clark, S. R., Ma, A. C., Tavener, S. A., McDonald, B., Goodarzi, Z., Kelly, M. M., Patel, K. D., Chakrabarti, S., McAvoy, E., Sinclair, G. D., Keys, E. M., Allen-Vercoe, E., Devinney, R., Doig, C. J., Green, F. H., & Kubes, P. (2007). Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med*, *13*(4), 463-469. https://doi.org/10.1038/nm1565

Cognasse, F., Duchez, A. C., Audoux, E., Ebermeyer, T., Arthaud, C. A., Prier, A., Eyraud, M. A., Mismetti, P., Garraud, O., Bertoletti, L., & Hamzeh-Cognasse, H. (2022). Platelets as Key Factors in Inflammation: Focus on CD40L/CD40. *Front Immunol*, *13*, 825892. https://doi.org/10.3389/fimmu.2022.825892

Crocker, P. R., Paulson, J. C., & Varki, A. (2007). Siglecs and their roles in the immune system. *Nat Rev Immunol*, *7*(4), 255-266. https://doi.org/10.1038/nri2056

Crook, M. (1991). Sialic Acid: its importance to platelet function in health and disease. *Platelets*, *2*(1), 1-10. https://doi.org/10.3109/09537109109005496

Cuenda, A., & Rousseau, S. (2007). p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim Biophys Acta*, *1773*(8), 1358-1375. https://doi.org/10.1016/j.bbamcr.2007.03.010

Cui, J., Chen, Y., Wang, H. Y., & Wang, R. F. (2014). Mechanisms and pathways of innate immune activation and regulation in health and cancer. *Hum Vaccin Immunother*, *10*(11), 3270-3285. https://doi.org/10.4161/21645515.2014.979640

Czapiga, M., Kirk, A. D., & Lekstrom-Himes, J. (2004). Platelets deliver costimulatory signals to antigen-presenting cells: a potential bridge between injury and immune activation. *Exp Hematol*, *32*(2), 135-139. https://doi.org/10.1016/j.exphem.2003.11.004

D'Mello, C., Almishri, W., Liu, H., & Swain, M. G. (2017). Interactions Between Platelets and Inflammatory Monocytes Affect Sickness Behavior in Mice With Liver Inflammation. *Gastroenterology*, *153*(5), 1416-1428 e1412. https://doi.org/10.1053/j.gastro.2017.08.011

Daly, M. E. (2011). Determinants of platelet count in humans. *Haematologica*, *96*(1), 10-13. https://doi.org/10.3324/haematol.2010.035287

Dasgupta, S. K., Abdel-Monem, H., Niravath, P., Le, A., Bellera, R. V., Langlois, K., Nagata, S., Rumbaut, R. E., & Thiagarajan, P. (2009). Lactadherin and clearance of platelet-derived microvesicles. *Blood*, *113*(6), 1332-1339. https://doi.org/10.1182/blood-2008-07-167148

De Nardo, D., Labzin, L. I., Kono, H., Seki, R., Schmidt, S. V., Beyer, M., Xu, D., Zimmer, S., Lahrmann, C., Schildberg, F. A., Vogelhuber, J., Kraut, M., Ulas, T., Kerksiek, A., Krebs, W., Bode, N., Grebe, A., Fitzgerald, M. L., Hernandez, N. J., . . . Latz, E. (2014). High-density lipoprotein mediates anti-inflammatory reprogramming of macrophages via the transcriptional regulator ATF3. *Nat Immunol*, *15*(2), 152-160. https://doi.org/10.1038/ni.2784

de Souza, V. C. A., Pereira, T. A., Teixeira, V. W., Carvalho, H., de Castro, M., D'Assuncao C, G., de Barros, A. F., Carvalho, C. L., de Lorena, V. M. B., Costa, V. M. A., Teixeira, A. A. C., Figueiredo, R., & de Oliveira, S. A. (2017). Bone marrow-derived monocyte infusion improves hepatic fibrosis by decreasing osteopontin, TGF-beta1, IL-13 and oxidative stress. *World J Gastroenterol*, *23*(28), 5146-5157. https://doi.org/10.3748/wjg.v23.i28.5146

Debrincat, M. A., Pleines, I., Lebois, M., Lane, R. M., Holmes, M. L., Corbin, J., Vandenberg, C. J., Alexander, W. S., Ng, A. P., Strasser, A., Bouillet, P., Sola-Visner, M., Kile, B. T., & Josefsson, E. C. (2015). BCL-2 is dispensable for thrombopoiesis and platelet survival. *Cell Death Dis*, *6*, e1721. https://doi.org/10.1038/cddis.2015.97

Dejardin, E., Droin, N. M., Delhase, M., Haas, E., Cao, Y., Makris, C., Li, Z. W., Karin, M., Ware, C. F., & Green, D. R. (2002). The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways. *Immunity*, *17*(4), 525-535. https://doi.org/10.1016/s1074-7613(02)00423-5

Diacovo, T. G., Roth, S. J., Buccola, J. M., Bainton, D. F., & Springer, T. A. (1996). Neutrophil rolling, arrest, and transmigration across activated, surface-adherent platelets via sequential action of P-selectin and the beta 2-integrin CD11b/CD18. *Blood*, *88*(1), 146-157. https://www.ncbi.nlm.nih.gov/pubmed/8704169

Ekstrand, C., Linder, M., Cherif, H., Kieler, H., & Bahmanyar, S. (2016). Increased susceptibility to infections before the diagnosis of immune thrombocytopenia. *J Thromb Haemost*, *14*(4), 807-814. https://doi.org/10.1111/jth.13267

Eldred, C. D., Evans, B., Hindley, S., Judkins, B. D., Kelly, H. A., Kitchin, J., Lumley, P., Porter, B., Ross, B. C., Smith, K. J., & et al. (1994). Orally active non-peptide fibrinogen receptor (GpIIb/IIIa) antagonists: identification of 4-[4-[4-(aminoiminomethyl)phenyl]-1-piperazinyl]-1-piperidineacetic acid as a long-acting, broad-spectrum antithrombotic agent. *J Med Chem*, *37*(23), 3882-3885. https://doi.org/10.1021/jm00049a006

Elzey, B. D., Tian, J., Jensen, R. J., Swanson, A. K., Lees, J. R., Lentz, S. R., Stein, C. S., Nieswandt, B., Wang, Y., Davidson, B. L., & Ratliff, T. L. (2003). Platelet-mediated modulation of adaptive immunity. A communication link between innate

and adaptive immune compartments. *Immunity*, *19*(1), 9-19. https://doi.org/10.1016/s1074-7613(03)00177-8

Finsterbusch, M., Schrottmaier, W. C., Kral-Pointner, J. B., Salzmann, M., & Assinger, A. (2018). Measuring and interpreting platelet-leukocyte aggregates. *Platelets*, *29*(7), 677-685. https://doi.org/10.1080/09537104.2018.1430358

Frederiksen, H., Maegbaek, M. L., & Norgaard, M. (2014). Twenty-year mortality of adult patients with primary immune thrombocytopenia: a Danish population-based cohort study. *Br J Haematol*, *166*(2), 260-267. https://doi.org/10.1111/bjh.12869

Fu, G., Deng, M., Neal, M. D., Billiar, T. R., & Scott, M. J. (2021). Platelet-Monocyte Aggregates: Understanding Mechanisms and Functions in Sepsis. *Shock*, *55*(2), 156-166. https://doi.org/10.1097/SHK.0000000000001619

Fullard, J. F. (2004). The role of the platelet glycoprotein IIb/IIIa in thrombosis and haemostasis. *Curr Pharm Des*, *10*(14), 1567-1576. https://doi.org/10.2174/1381612043384682

Gabrilovich, D. I., & Nagaraj, S. (2009). Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*, *9*(3), 162-174. https://doi.org/10.1038/nri2506

Gaertner, F., Ahmad, Z., Rosenberger, G., Fan, S., Nicolai, L., Busch, B., Yavuz, G., Luckner, M., Ishikawa-Ankerhold, H., Hennel, R., Benechet, A., Lorenz, M., Chandraratne, S., Schubert, I., Helmer, S., Striednig, B., Stark, K., Janko, M., Bottcher, R. T., . . . Massberg, S. (2017). Migrating Platelets Are Mechano-scavengers that Collect and Bundle Bacteria. *Cell*, *171*(6), 1368-1382 e1323. https://doi.org/10.1016/j.cell.2017.11.001

Ge, S. X., Jung, D., & Yao, R. (2020). ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics*, *36*(8), 2628-2629. https://doi.org/10.1093/bioinformatics/btz931

Geissmann, F., Manz, M. G., Jung, S., Sieweke, M. H., Merad, M., & Ley, K. (2010). Development of monocytes, macrophages, and dendritic cells. *Science*, *327*(5966), 656-661. https://doi.org/10.1126/science.1178331

Ghoshal, K., & Bhattacharyya, M. (2014). Overview of platelet physiology: its hemostatic and nonhemostatic role in disease pathogenesis. *ScientificWorldJournal*, *2014*, 781857. https://doi.org/10.1155/2014/781857

Ginhoux, F., & Guilliams, M. (2016). Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity*, *44*(3), 439-449. https://doi.org/10.1016/j.immuni.2016.02.024

Ginhoux, F., & Jung, S. (2014). Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat Rev Immunol*, *14*(6), 392-404. https://doi.org/10.1038/nri3671

Glienke, J., Fenten, G., Seemann, M., Sturz, A., & Thierauch, K. H. (2000). Human SPRY2 inhibits FGF2 signalling by a secreted factor. *Mech Dev*, *96*(1), 91-99. https://doi.org/10.1016/s0925-4773(00)00378-6

Goncalves, R., Zhang, X., Cohen, H., Debrabant, A., & Mosser, D. M. (2011). Platelet activation attracts a subpopulation of effector monocytes to sites of Leishmania major infection. *J Exp Med*, *208*(6), 1253-1265. https://doi.org/10.1084/jem.20101751

Grommes, J., Alard, J. E., Drechsler, M., Wantha, S., Morgelin, M., Kuebler, W. M., Jacobs, M., von Hundelshausen, P., Markart, P., Wygrecka, M., Preissner, K. T., Hackeng, T. M., Koenen, R. R., Weber, C., & Soehnlein, O. (2012). Disruption of

platelet-derived chemokine heteromers prevents neutrophil extravasation in acute lung injury. *Am J Respir Crit Care Med*, *185*(6), 628-636. https://doi.org/10.1164/rccm.201108-1533OC

Guilliams, M., Mildner, A., & Yona, S. (2018). Developmental and Functional Heterogeneity of Monocytes. *Immunity*, *49*(4), 595-613. https://doi.org/10.1016/j.immuni.2018.10.005

Guo, L., Akahori, H., Harari, E., Smith, S. L., Polavarapu, R., Karmali, V., Otsuka, F., Gannon, R. L., Braumann, R. E., Dickinson, M. H., Gupta, A., Jenkins, A. L., Lipinski, M. J., Kim, J., Chhour, P., de Vries, P. S., Jinnouchi, H., Kutys, R., Mori, H., . . . Finn, A. V. (2018). CD163+ macrophages promote angiogenesis and vascular permeability accompanied by inflammation in atherosclerosis. *J Clin Invest*, *128*(3), 1106-1124. https://doi.org/10.1172/JCI93025

Hamad, M. A., Krauel, K., Schanze, N., Gauchel, N., Stachon, P., Nuehrenberg, T., Zurek, M., & Duerschmied, D. (2022). Platelet Subtypes in Inflammatory Settings. *Front Cardiovasc Med*, *9*, 823549. https://doi.org/10.3389/fcvm.2022.823549

Han, P., Hanlon, D., Arshad, N., Lee, J. S., Tatsuno, K., Robinson, E., Filler, R., Sobolev, O., Cote, C., Rivera-Molina, F., Toomre, D., Fahmy, T., & Edelson, R. (2020). Platelet P-selectin initiates cross-presentation and dendritic cell differentiation in blood monocytes. *Sci Adv*, *6*(11), eaaz1580. https://doi.org/10.1126/sciadv.aaz1580

Haupt, W., Riese, J., Mehler, C., Weber, K., Zowe, M., & Hohenberger, W. (1998). Monocyte function before and after surgical trauma. *Dig Surg*, *15*(2), 102-104. https://doi.org/10.1159/000018601

Herlaar, E., & Brown, Z. (1999). p38 MAPK signalling cascades in inflammatory disease. *Mol Med Today*, *5*(10), 439-447. https://doi.org/10.1016/s1357-4310(99)01544-0

Hottz, E. D., Azevedo-Quintanilha, I. G., Palhinha, L., Teixeira, L., Barreto, E. A., Pao, C. R. R., Righy, C., Franco, S., Souza, T. M. L., Kurtz, P., Bozza, F. A., & Bozza, P. T. (2020). Platelet activation and platelet-monocyte aggregate formation trigger tissue factor expression in patients with severe COVID-19. *Blood*, *136*(11), 1330-1341. https://doi.org/10.1182/blood.2020007252

Huang, B., Pan, P. Y., Li, Q., Sato, A. I., Levy, D. E., Bromberg, J., Divino, C. M., & Chen, S. H. (2006). Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res*, *66*(2), 1123-1131. https://doi.org/10.1158/0008-5472.CAN-05-1299

Hwaiz, R., Rahman, M., Syk, I., Zhang, E., & Thorlacius, H. (2015). Rac1-dependent secretion of platelet-derived CCL5 regulates neutrophil recruitment via activation of alveolar macrophages in septic lung injury. *J Leukoc Biol*, *97*(5), 975-984. https://doi.org/10.1189/jlb.4A1214-603R

Italiano, J. E., Jr., Mairuhu, A. T., & Flaumenhaft, R. (2010). Clinical relevance of microparticles from platelets and megakaryocytes. *Curr Opin Hematol*, *17*(6), 578-584. https://doi.org/10.1097/MOH.0b013e32833e77ee

Jefferies, C. A., Doyle, S., Brunner, C., Dunne, A., Brint, E., Wietek, C., Walch, E., Wirth, T., & O'Neill, L. A. (2003). Bruton's tyrosine kinase is a Toll/interleukin-1 receptor domain-binding protein that participates in nuclear factor kappaB activation by Toll-like receptor 4. *J Biol Chem*, *278*(28), 26258-26264. https://doi.org/10.1074/jbc.M301484200

Jiang, C., Ting, A. T., & Seed, B. (1998). PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature*, *391*(6662), 82-86. https://doi.org/10.1038/34184

Jin, S., Guerrero-Juarez, C. F., Zhang, L., Chang, I., Ramos, R., Kuan, C. H., Myung, P., Plikus, M. V., & Nie, Q. (2021). Inference and analysis of cell-cell communication using CellChat. *Nat Commun*, *12*(1), 1088. https://doi.org/10.1038/s41467-021-21246-9

Kanehisa, M., Furumichi, M., Sato, Y., Ishiguro-Watanabe, M., & Tanabe, M. (2021). KEGG: integrating viruses and cellular organisms. *Nucleic Acids Res*, *49*(D1), D545-D551. https://doi.org/10.1093/nar/gkaa970

Kapellos, T. S., Bonaguro, L., Gemund, I., Reusch, N., Saglam, A., Hinkley, E. R., & Schultze, J. L. (2019). Human Monocyte Subsets and Phenotypes in Major Chronic Inflammatory Diseases. *Front Immunol*, *10*, 2035. https://doi.org/10.3389/fimmu.2019.02035

Karin, M., & Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol*, *18*, 621-663. https://doi.org/10.1146/annurev.immunol.18.1.621

Karlmark, K. R., Tacke, F., & Dunay, I. R. (2012). Monocytes in health and disease - Minireview. *Eur J Microbiol Immunol (Bp)*, *2*(2), 97-102. https://doi.org/10.1556/EuJMI.2.2012.2.1

Karlmark, K. R., Weiskirchen, R., Zimmermann, H. W., Gassler, N., Ginhoux, F., Weber, C., Merad, M., Luedde, T., Trautwein, C., & Tacke, F. (2009). Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology*, *50*(1), 261-274. https://doi.org/10.1002/hep.22950

Katlinski, K. V., Gui, J., Katlinskaya, Y. V., Ortiz, A., Chakraborty, R., Bhattacharya, S., Carbone, C. J., Beiting, D. P., Girondo, M. A., Peck, A. R., Pure, E., Chatterji, P., Rustgi, A. K., Diehl, J. A., Koumenis, C., Rui, H., & Fuchs, S. Y. (2017). Inactivation of Interferon Receptor Promotes the Establishment of Immune Privileged Tumor Microenvironment. *Cancer Cell*, *31*(2), 194-207. https://doi.org/10.1016/j.ccell.2017.01.004

Kelly, P. N., White, M. J., Goschnick, M. W., Fairfax, K. A., Tarlinton, D. M., Kinkel, S. A., Bouillet, P., Adams, J. M., Kile, B. T., & Strasser, A. (2010). Individual and overlapping roles of BH3-only proteins Bim and Bad in apoptosis of lymphocytes and platelets and in suppression of thymic lymphoma development. *Cell Death Differ*, *17*(10), 1655-1664. https://doi.org/10.1038/cdd.2010.43

Kim, D., & Kim, J. Y. (2014). Anti-CD14 antibody reduces LPS responsiveness via TLR4 internalization in human monocytes. *Mol Immunol*, *57*(2), 210-215. https://doi.org/10.1016/j.molimm.2013.09.009

Kodama, T., Takehara, T., Hikita, H., Shimizu, S., Shigekawa, M., Li, W., Miyagi, T., Hosui, A., Tatsumi, T., Ishida, H., Kanto, T., Hiramatsu, N., Yin, X. M., & Hayashi, N. (2011). BH3-only activator proteins Bid and Bim are dispensable for Bak/Bax-dependent thrombocyte apoptosis induced by Bcl-xL deficiency: molecular requisites for the mitochondrial pathway to apoptosis in platelets. *J Biol Chem*, *286*(16), 13905-13913. https://doi.org/10.1074/jbc.M110.195370

Kral, J. B., Schrottmaier, W. C., Salzmann, M., & Assinger, A. (2016). Platelet Interaction with Innate Immune Cells. *Transfus Med Hemother*, *43*(2), 78-88. https://doi.org/10.1159/000444807

Kuma, Y., Sabio, G., Bain, J., Shpiro, N., Marquez, R., & Cuenda, A. (2005). BIRB796 inhibits all p38 MAPK isoforms in vitro and in vivo. *J Biol Chem*, *280*(20), 19472-19479. https://doi.org/10.1074/jbc.M414221200

Kwon, J. W., Kwon, H. K., Shin, H. J., Choi, Y. M., Anwar, M. A., & Choi, S. (2015). Activating transcription factor 3 represses inflammatory responses by binding to the p65 subunit of NF-kappaB. *Sci Rep*, *5*, 14470. https://doi.org/10.1038/srep14470

Lannan, K. L., Sahler, J., Kim, N., Spinelli, S. L., Maggirwar, S. B., Garraud, O., Cognasse, F., Blumberg, N., & Phipps, R. P. (2015). Breaking the mold: transcription factors in the anucleate platelet and platelet-derived microparticles. *Front Immunol*, *6*, 48. https://doi.org/10.3389/fimmu.2015.00048

Lari, R., Kitchener, P. D., & Hamilton, J. A. (2009). The proliferative human monocyte subpopulation contains osteoclast precursors. *Arthritis Res Ther*, *11*(1), R23. https://doi.org/10.1186/ar2616

Lawrence, T. (2009). The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol*, *1*(6), a001651. https://doi.org/10.1101/cshperspect.a001651

Lee, J., Hong, F., Kwon, S., Kim, S. S., Kim, D. O., Kang, H. S., Lee, S. J., Ha, J., & Kim, S. S. (2002). Activation of p38 MAPK induces cell cycle arrest via inhibition of Raf/ERK pathway during muscle differentiation. *Biochem Biophys Res Commun*, *298*(5), 765-771. https://doi.org/10.1016/s0006-291x(02)02562-7

Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., Strickler, J. E., McLaughlin, M. M., Siemens, I. R., Fisher, S. M., Livi, G. P., White, J. R., Adams, J. L., & Young, P. R. (1994). A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*, *372*(6508), 739-746. https://doi.org/10.1038/372739a0

Lefrancais, E., Ortiz-Munoz, G., Caudrillier, A., Mallavia, B., Liu, F., Sayah, D. M., Thornton, E. E., Headley, M. B., David, T., Coughlin, S. R., Krummel, M. F., Leavitt, A. D., Passegue, E., & Looney, M. R. (2017). The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature*, *544*(7648), 105-109. https://doi.org/10.1038/nature21706

Levoux, J., Prola, A., Lafuste, P., Gervais, M., Chevallier, N., Koumaiha, Z., Kefi, K., Braud, L., Schmitt, A., Yacia, A., Schirmann, A., Hersant, B., Sid-Ahmed, M., Ben Larbi, S., Komrskova, K., Rohlena, J., Relaix, F., Neuzil, J., & Rodriguez, A. M. (2021). Platelets Facilitate the Wound-Healing Capability of Mesenchymal Stem Cells by Mitochondrial Transfer and Metabolic Reprogramming. *Cell Metab*, *33*(2), 283-299 e289. https://doi.org/10.1016/j.cmet.2020.12.006

Li, D., & Wu, M. (2021). Pattern recognition receptors in health and diseases. *Signal Transduct Target Ther*, *6*(1), 291. https://doi.org/10.1038/s41392-021-00687-0

Li, J., van der Wal, D. E., Zhu, G., Xu, M., Yougbare, I., Ma, L., Vadasz, B., Carrim, N., Grozovsky, R., Ruan, M., Zhu, L., Zeng, Q., Tao, L., Zhai, Z. M., Peng, J., Hou, M., Leytin, V., Freedman, J., Hoffmeister, K. M., & Ni, H. (2015). Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia. *Nat Commun*, *6*, 7737. https://doi.org/10.1038/ncomms8737

Li, N., Hu, H., Lindqvist, M., Wikstrom-Jonsson, E., Goodall, A. H., & Hjemdahl, P. (2000). Platelet-leukocyte cross talk in whole blood. *Arterioscler Thromb Vasc Biol*, *20*(12), 2702-2708. https://doi.org/10.1161/01.atv.20.12.2702

Li, W., Zhu, J., Dou, J., She, H., Tao, K., Xu, H., Yang, Q., & Mao, Z. (2017). Phosphorylation of LAMP2A by p38 MAPK couples ER stress to chaperone-mediated autophagy. *Nat Commun*, *8*(1), 1763. https://doi.org/10.1038/s41467-017-01609-x

Li, Y., Fu, J., Ling, Y., Yago, T., McDaniel, J. M., Song, J., Bai, X., Kondo, Y., Qin, Y., Hoover, C., McGee, S., Shao, B., Liu, Z., Sonon, R., Azadi, P., Marth, J. D., McEver, R. P., Ruan, C., & Xia, L. (2017). Sialylation on O-glycans protects platelets from clearance by liver Kupffer cells. *Proc Natl Acad Sci U S A*, *114*(31), 8360-8365. https://doi.org/10.1073/pnas.1707662114

Lin, C. Y., Tsai, I. F., Ho, Y. P., Huang, C. T., Lin, Y. C., Lin, C. J., Tseng, S. C., Lin, W. P., Chen, W. T., & Sheen, I. S. (2007). Endotoxemia contributes to the immune paralysis in patients with cirrhosis. *J Hepatol*, *46*(5), 816-826. https://doi.org/10.1016/j.jhep.2006.12.018

Lin, S., Lin, Y., Nery, J. R., Urich, M. A., Breschi, A., Davis, C. A., Dobin, A., Zaleski, C., Beer, M. A., Chapman, W. C., Gingeras, T. R., Ecker, J. R., & Snyder, M. P. (2014). Comparison of the transcriptional landscapes between human and mouse tissues. *Proc Natl Acad Sci U S A*, *111*(48), 17224-17229. https://doi.org/10.1073/pnas.1413624111

Lipscomb, M. F., & Masten, B. J. (2002). Dendritic cells: immune regulators in health and disease. *Physiol Rev*, *82*(1), 97-130. https://doi.org/10.1152/physrev.00023.2001

Liu, D., Gao, Y., Liu, J., Huang, Y., Yin, J., Feng, Y., Shi, L., Meloni, B. P., Zhang, C., Zheng, M., & Gao, J. (2021). Intercellular mitochondrial transfer as a means of tissue revitalization. *Signal Transduct Target Ther*, *6*(1), 65. https://doi.org/10.1038/s41392-020-00440-z

Liu, T., Zhang, L., Joo, D., & Sun, S. C. (2017). NF-kappaB signaling in inflammation. *Signal Transduct Target Ther*, *2*. https://doi.org/10.1038/sigtrans.2017.23

Liu, X., Hou, Y., & Peng, J. (2013). Advances in immunopathogenesis of adult immune thrombocytopenia. *Front Med*, *7*(4), 418-424. https://doi.org/10.1007/s11684-013-0297-8

Loria, G. D., Romagnoli, P. A., Moseley, N. B., Rucavado, A., & Altman, J. D. (2013). Platelets support a protective immune response to LCMV by preventing splenic necrosis. *Blood*, *121*(6), 940-950. https://doi.org/10.1182/blood-2011-08-376822

Lu, W. J., Lin, K. H., Hsu, M. J., Chou, D. S., Hsiao, G., & Sheu, J. R. (2012). Suppression of NF-kappaB signaling by andrographolide with a novel mechanism in human platelets: regulatory roles of the p38 MAPK-hydroxyl radical-ERK2 cascade. *Biochem Pharmacol*, *84*(7), 914-924. https://doi.org/10.1016/j.bcp.2012.06.030

Lubbers, J., Rodriguez, E., & van Kooyk, Y. (2018). Modulation of Immune Tolerance via Siglec-Sialic Acid Interactions. *Front Immunol*, *9*, 2807. https://doi.org/10.3389/fimmu.2018.02807

Luo, W., & Brouwer, C. (2013). Pathview: an R/Bioconductor package for pathway-based data integration and visualization. *Bioinformatics*, *29*(14), 1830-1831. https://doi.org/10.1093/bioinformatics/btt285

Ma, R., Xie, R., Yu, C., Si, Y., Wu, X., Zhao, L., Yao, Z., Fang, S., Chen, H., Novakovic, V., Gao, C., Kou, J., Bi, Y., Thatte, H. S., Yu, B., Yang, S., Zhou, J., & Shi, J. (2017). Phosphatidylserine-mediated platelet clearance by endothelium decreases platelet aggregates and procoagulant activity in sepsis. *Sci Rep*, *7*(1), 4978. https://doi.org/10.1038/s41598-017-04773-8

Mandal, S., Sarode, R., Dash, S., & Dash, R. J. (1993). Hyperaggregation of platelets detected by whole blood platelet aggregometry in newly diagnosed noninsulin-dependent diabetes mellitus. *Am J Clin Pathol*, *100*(2), 103-107. https://doi.org/10.1093/ajcp/100.2.103

Manning, G., Whyte, D. B., Martinez, R., Hunter, T., & Sudarsanam, S. (2002). The protein kinase complement of the human genome. *Science*, *298*(5600), 1912-1934. https://doi.org/10.1126/science.1075762

Mantovani, A., & Dejana, E. (1989). Cytokines as communication signals between leukocytes and endothelial cells. *Immunol Today*, *10*(11), 370-375. https://doi.org/10.1016/0167-5699(89)90270-3

Marcoux, G., Duchez, A. C., Rousseau, M., Levesque, T., Boudreau, L. H., Thibault, L., & Boilard, E. (2017). Microparticle and mitochondrial release during extended storage of different types of platelet concentrates. *Platelets*, *28*(3), 272-280. https://doi.org/10.1080/09537104.2016.1218455

Marshall, J. S., Warrington, R., Watson, W., & Kim, H. L. (2018). An introduction to immunology and immunopathology. *Allergy Asthma Clin Immunol*, *14*(Suppl 2), 49. https://doi.org/10.1186/s13223-018-0278-1

McFarland, J. (2002). Pathophysiology of platelet destruction in immune (idiopathic) thrombocytopenic purpura. *Blood Rev*, *16*(1), 1-2. https://doi.org/10.1054/blre.2001.0169

Mehta, J., & Mehta, P. (1979). Platelet function studies in heart disease. VI. Enhanced platelet aggregate formation activity in congestive heart failure: inhibition by sodium nitroprusside. *Circulation*, *60*(3), 497-503. https://doi.org/10.1161/01.cir.60.3.497

Melisi, D., & Chiao, P. J. (2007). NF-kappa B as a target for cancer therapy. *Expert Opin Ther Targets*, *11*(2), 133-144. https://doi.org/10.1517/14728222.11.2.133

Mellor, J. D., Brown, M. P., Irving, H. R., Zalcberg, J. R., & Dobrovic, A. (2013). A critical review of the role of Fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer. *J Hematol Oncol*, *6*, 1. https://doi.org/10.1186/1756-8722-6-1

Mendes, K., Schmidhofer, S., Minderjahn, J., Glatz, D., Kiesewetter, C., Raithel, J., Wimmer, J., Gebhard, C., & Rehli, M. (2021). The epigenetic pioneer EGR2 initiates DNA demethylation in differentiating monocytes at both stable and transient binding sites. *Nat Commun*, *12*(1), 1556. https://doi.org/10.1038/s41467-021-21661-y

Metz, K. S., Deoudes, E. M., Berginski, M. E., Jimenez-Ruiz, I., Aksoy, B. A., Hammerbacher, J., Gomez, S. M., & Phanstiel, D. H. (2018). Coral: Clear and Customizable Visualization of Human Kinome Data. *Cell Syst*, *7*(3), 347-350 e341. https://doi.org/10.1016/j.cels.2018.07.001

Mildner, A., & Jung, S. (2014). Development and function of dendritic cell subsets. *Immunity*, *40*(5), 642-656. https://doi.org/10.1016/j.immuni.2014.04.016

Mori, N., Yamada, Y., Ikeda, S., Yamasaki, Y., Tsukasaki, K., Tanaka, Y., Tomonaga, M., Yamamoto, N., & Fujii, M. (2002). Bay 11-7082 inhibits transcription factor NF-kappaB and induces apoptosis of HTLV-I-infected T-cell lines and primary adult T-cell leukemia cells. *Blood*, *100*(5), 1828-1834. https://doi.org/10.1182/blood-2002-01-0151

Morrell, C. N., Aggrey, A. A., Chapman, L. M., & Modjeski, K. L. (2014). Emerging roles for platelets as immune and inflammatory cells. *Blood*, *123*(18), 2759-2767. https://doi.org/10.1182/blood-2013-11-462432

Mulcahy, L. A., Pink, R. C., & Carter, D. R. (2014). Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles*, *3*. https://doi.org/10.3402/jev.v3.24641

Muller Kobold, A. C., Tulleken, J. E., Zijlstra, J. G., Sluiter, W., Hermans, J., Kallenberg, C. G., & Tervaert, J. W. (2000). Leukocyte activation in sepsis; correlations with disease state and mortality. *Intensive Care Med*, *26*(7), 883-892. https://doi.org/10.1007/s001340051277

Munnix, I. C., Cosemans, J. M., Auger, J. M., & Heemskerk, J. W. (2009). Platelet response heterogeneity in thrombus formation. *Thromb Haemost*, *102*(6), 1149-1156. https://doi.org/10.1160/TH09-05-0289

Nguyen, X. D., Muller-Berghaus, J., Kalsch, T., Schadendorf, D., Borggrefe, M., & Kluter, H. (2008). Differentiation of monocyte-derived dendritic cells under the influence of platelets. *Cytotherapy*, *10*(7), 720-729. https://doi.org/10.1080/14653240802378912

Nicholson, L. B. (2016). The immune system. *Essays Biochem*, *60*(3), 275-301. https://doi.org/10.1042/EBC20160017

Nishat, S., Wuescher, L. M., & Worth, R. G. (2018). Platelets Enhance Dendritic Cell Responses against Staphylococcus aureus through CD40-CD40L. *Infect Immun*, *86*(9). https://doi.org/10.1128/IAI.00186-18

Norris, P. A. A., Segel, G. B., Burack, W. R., Sachs, U. J., Lissenberg-Thunnissen, S. N., Vidarsson, G., Bayat, B., Cserti-Gazdewich, C. M., Callum, J., Lin, Y., Branch, D., Kapur, R., Semple, J. W., & Lazarus, A. H. (2021). FcgammaRI and FcgammaRIII on splenic macrophages mediate phagocytosis of anti-glycoprotein IIb/IIIa autoantibody-opsonized platelets in immune thrombocytopenia. *Haematologica*, *106*(1), 250-254. https://doi.org/10.3324/haematol.2020.248385

Novack, D. V., Yin, L., Hagen-Stapleton, A., Schreiber, R. D., Goeddel, D. V., Ross, F. P., & Teitelbaum, S. L. (2003). The IkappaB function of NF-kappaB2 p100 controls stimulated osteoclastogenesis. *J Exp Med*, *198*(5), 771-781. https://doi.org/10.1084/jem.20030116

Olson, C. M., Hedrick, M. N., Izadi, H., Bates, T. C., Olivera, E. R., & Anguita, J. (2007). p38 mitogen-activated protein kinase controls NF-kappaB transcriptional activation and tumor necrosis factor alpha production through RelA phosphorylation mediated by mitogen- and stress-activated protein kinase 1 in response to Borrelia burgdorferi antigens. *Infect Immun*, *75*(1), 270-277. https://doi.org/10.1128/IAI.01412-06

Paludan, S. R., Pradeu, T., Masters, S. L., & Mogensen, T. H. (2021). Constitutive immune mechanisms: mediators of host defence and immune regulation. *Nat Rev Immunol*, *21*(3), 137-150. https://doi.org/10.1038/s41577-020-0391-5

Passacquale, G., Vamadevan, P., Pereira, L., Hamid, C., Corrigall, V., & Ferro, A. (2011). Monocyte-platelet interaction induces a pro-inflammatory phenotype in circulating monocytes. *PLoS One*, *6*(10), e25595. https://doi.org/10.1371/journal.pone.0025595

Pavlos, N. J., Xu, J., Riedel, D., Yeoh, J. S., Teitelbaum, S. L., Papadimitriou, J. M., Jahn, R., Ross, F. P., & Zheng, M. H. (2005). Rab3D regulates a novel vesicular trafficking pathway that is required for osteoclastic bone resorption. *Mol Cell Biol*, *25*(12), 5253-5269. https://doi.org/10.1128/MCB.25.12.5253-5269.2005

Pitchford, S., Pan, D., & Welch, H. C. (2017). Platelets in neutrophil recruitment to sites of inflammation. *Curr Opin Hematol*, *24*(1), 23-31. https://doi.org/10.1097/MOH.0000000000000297

Pluta, K., Porebska, K., Urbanowicz, T., Gasecka, A., Olasinska-Wisniewska, A., Targonski, R., Krasinska, A., Filipiak, K. J., Jemielity, M., & Krasinski, Z. (2022). Platelet-Leucocyte Aggregates as Novel Biomarkers in Cardiovascular Diseases. *Biology (Basel)*, *11*(2). https://doi.org/10.3390/biology11020224

Prashar, A., Schnettger, L., Bernard, E. M., & Gutierrez, M. G. (2017). Rab GTPases in Immunity and Inflammation. *Front Cell Infect Microbiol*, *7*, 435. https://doi.org/10.3389/fcimb.2017.00435

Qu, M., Liu, Q., Zhao, H. G., Peng, J., Ni, H., Hou, M., & Jansen, A. J. G. (2018). Low platelet count as risk factor for infections in patients with primary immune thrombocytopenia: a retrospective evaluation. *Ann Hematol*, *97*(9), 1701-1706. https://doi.org/10.1007/s00277-018-3367-9

Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., & Davis, R. J. (1995). Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem*, *270*(13), 7420-7426. https://doi.org/10.1074/jbc.270.13.7420

Ratajczak, J., Wysoczynski, M., Hayek, F., Janowska-Wieczorek, A., & Ratajczak, M. Z. (2006). Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. *Leukemia*, *20*(9), 1487-1495. https://doi.org/10.1038/sj.leu.2404296

Renda, T., Baraldo, S., Pelaia, G., Bazzan, E., Turato, G., Papi, A., Maestrelli, P., Maselli, R., Vatrella, A., Fabbri, L. M., Zuin, R., Marsico, S. A., & Saetta, M. (2008). Increased activation of p38 MAPK in COPD. *Eur Respir J*, *31*(1), 62-69. https://doi.org/10.1183/09031936.00036707

Reustle, A., & Torzewski, M. (2018). Role of p38 MAPK in Atherosclerosis and Aortic Valve Sclerosis. *Int J Mol Sci*, *19*(12). https://doi.org/10.3390/ijms19123761

Ribeiro, L. S., Migliari Branco, L., & Franklin, B. S. (2019). Regulation of Innate Immune Responses by Platelets. *Front Immunol*, *10*, 1320. https://doi.org/10.3389/fimmu.2019.01320

Rolfes, V., Ribeiro, L. S., Hawwari, I., Bottcher, L., Rosero, N., Maasewerd, S., Santos, M. L. S., Prochnicki, T., Silva, C. M. S., Wanderley, C. W. S., Rothe, M., Schmidt, S. V., Stunden, H. J., Bertheloot, D., Rivas, M. N., Fontes, C. J., Carvalho, L. H., Cunha, F. Q., Latz, E., . . . Franklin, B. S. (2020). Platelets Fuel the Inflammasome Activation of Innate Immune Cells. *Cell Rep*, *31*(6), 107615. https://doi.org/10.1016/j.celrep.2020.107615

Rong, M. Y., Wang, C. H., Wu, Z. B., Zeng, W., Zheng, Z. H., Han, Q., Jia, J. F., Li, X. Y., & Zhu, P. (2014). Platelets induce a proinflammatory phenotype in monocytes via the CD147 pathway in rheumatoid arthritis. *Arthritis Res Ther*, *16*(6), 478. https://doi.org/10.1186/s13075-014-0478-0

Rossaint, J., Margraf, A., & Zarbock, A. (2018). Role of Platelets in Leukocyte Recruitment and Resolution of Inflammation. *Front Immunol*, *9*, 2712. https://doi.org/10.3389/fimmu.2018.02712

Ruoslahti, E. (1996). RGD and other recognition sequences for integrins. *Annu Rev Cell Dev Biol*, *12*, 697-715. https://doi.org/10.1146/annurev.cellbio.12.1.697

Saha, R. N., Jana, M., & Pahan, K. (2007). MAPK p38 regulates transcriptional activity of NF-kappaB in primary human astrocytes via acetylation of p65. *J Immunol*, *179*(10), 7101-7109. https://doi.org/10.4049/jimmunol.179.10.7101

Samson, M., Fraser, W., & Lebowitz, D. (2019). Treatments for Primary Immune Thrombocytopenia: A Review. *Cureus*, *11*(10), e5849. https://doi.org/10.7759/cureus.5849

Schett, G., Tohidast-Akrad, M., Smolen, J. S., Schmid, B. J., Steiner, C. W., Bitzan, P., Zenz, P., Redlich, K., Xu, Q., & Steiner, G. (2000). Activation, differential localization, and regulation of the stress-activated protein kinases, extracellular signal-regulated kinase, c-JUN N-terminal kinase, and p38 mitogen-activated protein kinase, in synovial tissue and cells in rheumatoid arthritis. *Arthritis Rheum*, *43*(11), 2501-2512. https://doi.org/10.1002/1529-0131(200011)43:11<2501::AID-ANR18>3.0.CO;2-K

Schroder, K., & Tschopp, J. (2010). The inflammasomes. *Cell*, *140*(6), 821-832. https://doi.org/10.1016/j.cell.2010.01.040

Senftleben, U., Cao, Y., Xiao, G., Greten, F. R., Krahn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S. C., & Karin, M. (2001). Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science*, *293*(5534), 1495-1499. https://doi.org/10.1126/science.1062677

Shi, Q., Cheng, L., Liu, Z., Hu, K., Ran, J., Ge, D., & Fu, J. (2015). The p38 MAPK inhibitor SB203580 differentially modulates LPS-induced interleukin 6 expression in macrophages. *Cent Eur J Immunol*, *40*(3), 276-282. https://doi.org/10.5114/ceji.2015.54586

Singh, A., Uzun, G., & Bakchoul, T. (2021). Primary Immune Thrombocytopenia: Novel Insights into Pathophysiology and Disease Management. *J Clin Med*, *10*(4). https://doi.org/10.3390/jcm10040789

Singhal, R., Chawla, S., Batra, H., Gupta, S., Ojha, A., Rathore, D. K., Seth, T., & Guchhait, P. (2018). Engulfment of Hb-activated platelets differentiates monocytes into pro-inflammatory macrophages in PNH patients. *Eur J Immunol*, *48*(8), 1285-1294. https://doi.org/10.1002/eji.201747449

Singhal, R., Chawla, S., Rathore, D. K., Bhasym, A., Annarapu, G. K., Sharma, V., Seth, T., & Guchhait, P. (2017). Development of pro-inflammatory phenotype in monocytes after engulfing Hb-activated platelets in hemolytic disorders. *Clin Immunol*, *175*, 133-142. https://doi.org/10.1016/j.clim.2016.12.007

Sodergren, A. L., & Ramstrom, S. (2018). Platelet subpopulations remain despite strong dual agonist stimulation and can be characterised using a novel six-colour flow cytometry protocol. *Sci Rep*, *8*(1), 1441. https://doi.org/10.1038/s41598-017-19126-8

Sorensen, A. L., Rumjantseva, V., Nayeb-Hashemi, S., Clausen, H., Hartwig, J. H., Wandall, H. H., & Hoffmeister, K. M. (2009). Role of sialic acid for platelet life span: exposure of beta-galactose results in the rapid clearance of platelets from the circulation by asialoglycoprotein receptor-expressing liver macrophages and hepatocytes. *Blood*, *114*(8), 1645-1654. https://doi.org/10.1182/blood-2009-01-199414

Spangenberg, P., Redlich, H., Bergmann, I., Losche, W., Gotzrath, M., & Kehrel, B. (1993). The platelet glycoprotein IIb/IIIa complex is involved in the adhesion of activated platelets to leukocytes. *Thromb Haemost*, *70*(3), 514-521. https://www.ncbi.nlm.nih.gov/pubmed/7505065

Sreeramkumar, V., Adrover, J. M., Ballesteros, I., Cuartero, M. I., Rossaint, J., Bilbao, I., Nacher, M., Pitaval, C., Radovanovic, I., Fukui, Y., McEver, R. P., Filippi, M. D., Lizasoain, I., Ruiz-Cabello, J., Zarbock, A., Moro, M. A., & Hidalgo, A. (2014). Neutrophils scan for activated platelets to initiate inflammation. *Science*, *346*(6214), 1234-1238. https://doi.org/10.1126/science.1256478

Stark, K. (2019). Platelet-neutrophil crosstalk and netosis. *Hemasphere*, *3*(Suppl). https://doi.org/10.1097/HS9.0000000000000231

Stasi, R., Pagano, A., Stipa, E., & Amadori, S. (2001). Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idiopathic thrombocytopenic purpura. *Blood*, *98*(4), 952-957. https://doi.org/10.1182/blood.v98.4.952

Stephen, J., Emerson, B., Fox, K. A., & Dransfield, I. (2013). The uncoupling of monocyte-platelet interactions from the induction of proinflammatory signaling in monocytes. *J Immunol*, *191*(11), 5677-5683. https://doi.org/10.4049/jimmunol.1301250

Su, C., & Zheng, C. (2021). When Rab GTPases meet innate immune signaling pathways. *Cytokine Growth Factor Rev*, *59*, 95-100. https://doi.org/10.1016/j.cytogfr.2021.01.002

Sun, R. J., & Shan, N. N. (2019). Megakaryocytic dysfunction in immune thrombocytopenia is linked to autophagy. *Cancer Cell Int*, *19*, 59. https://doi.org/10.1186/s12935-019-0779-0

Susanto, O., & Hickey, M. J. (2020). Using imaging to study inflammatory platelet-leukocyte interactions in vivo. *Platelets*, *31*(5), 610-617. https://doi.org/10.1080/09537104.2020.1718632

Sutterwala, F. S., Haasken, S., & Cassel, S. L. (2014). Mechanism of NLRP3 inflammasome activation. *Ann N Y Acad Sci*, *1319*, 82-95. https://doi.org/10.1111/nyas.12458

Swinkels, M., Rijkers, M., Voorberg, J., Vidarsson, G., Leebeek, F. W. G., & Jansen, A. J. G. (2018). Emerging Concepts in Immune Thrombocytopenia. *Front Immunol*, *9*, 880. https://doi.org/10.3389/fimmu.2018.00880

Takahashi, K., Murata, S., Fukunaga, K., & Ohkohchi, N. (2013). Human platelets inhibit liver fibrosis in severe combined immunodeficiency mice. *World J Gastroenterol*, *19*(32), 5250-5260. https://doi.org/10.3748/wjg.v19.i32.5250

Takeuchi, K., Satoh, M., Kuno, H., Yoshida, T., Kondo, H., & Takeuchi, M. (1998). Platelet-like particle formation in the human megakaryoblastic leukaemia cell lines, MEG-01 and MEG-01s. *Br J Haematol*, *100*(2), 436-444. https://doi.org/10.1046/j.1365-2141.1998.00576.x

Tamura, R., Jiang, F., Xie, J., & Kamiyama, D. (2021). Multiplexed labeling of cellular proteins with split fluorescent protein tags. *Commun Biol*, *4*(1), 257. https://doi.org/10.1038/s42003-021-01780-4

Thomas, G. D., Hamers, A. A. J., Nakao, C., Marcovecchio, P., Taylor, A. M., McSkimming, C., Nguyen, A. T., McNamara, C. A., & Hedrick, C. C. (2017). Human Blood Monocyte Subsets: A New Gating Strategy Defined Using Cell Surface Markers Identified by Mass Cytometry. *Arterioscler Thromb Vasc Biol*, *37*(8), 1548-1558. https://doi.org/10.1161/ATVBAHA.117.309145

Tomida, T., Takekawa, M., & Saito, H. (2015). Oscillation of p38 activity controls efficient pro-inflammatory gene expression. *Nat Commun*, *6*, 8350. https://doi.org/10.1038/ncomms9350

Tsavachidou, D., Coleman, M. L., Athanasiadis, G., Li, S., Licht, J. D., Olson, M. F., & Weber, B. L. (2004). SPRY2 is an inhibitor of the ras/extracellular signal-regulated

kinase pathway in melanocytes and melanoma cells with wild-type BRAF but not with the V599E mutant. *Cancer Res*, *64*(16), 5556-5559. https://doi.org/10.1158/0008-5472.CAN-04-1669

van Gils, J. M., Zwaginga, J. J., & Hordijk, P. L. (2009). Molecular and functional interactions among monocytes, platelets, and endothelial cells and their relevance for cardiovascular diseases. *J Leukoc Biol*, *85*(2), 195-204. https://doi.org/10.1189/jlb.0708400

Vandenberg, C. J., & Cory, S. (2013). ABT-199, a new Bcl-2-specific BH3 mimetic, has in vivo efficacy against aggressive Myc-driven mouse lymphomas without provoking thrombocytopenia. *Blood*, *121*(12), 2285-2288. https://doi.org/10.1182/blood-2013-01-475855

Varchetta, S., Brunetta, E., Roberto, A., Mikulak, J., Hudspeth, K. L., Mondelli, M. U., & Mavilio, D. (2012). Engagement of Siglec-7 receptor induces a pro-inflammatory response selectively in monocytes. *PLoS One*, *7*(9), e45821. https://doi.org/10.1371/journal.pone.0045821

von Hundelshausen, P., Koenen, R. R., Sack, M., Mause, S. F., Adriaens, W., Proudfoot, A. E., Hackeng, T. M., & Weber, C. (2005). Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium. *Blood*, *105*(3), 924-930. https://doi.org/10.1182/blood-2004-06-2475

Vrbensky, J. R., Nazy, I., Toltl, L. J., Ross, C., Ivetic, N., Smith, J. W., Kelton, J. G., & Arnold, D. M. (2018). Megakaryocyte apoptosis in immune thrombocytopenia. *Platelets*, *29*(7), 729-732. https://doi.org/10.1080/09537104.2018.1475637

Walters, N., Zhang, J., Rima, X. Y., Nguyen, L. T. H., Germain, R. N., Lammermann, T., & Reategui, E. (2021). Analyzing Inter-Leukocyte Communication and Migration In Vitro: Neutrophils Play an Essential Role in Monocyte Activation During Swarming. *Front Immunol*, *12*, 671546. https://doi.org/10.3389/fimmu.2021.671546

Wasmuth, H. E., Kunz, D., Yagmur, E., Timmer-Stranghoner, A., Vidacek, D., Siewert, E., Bach, J., Geier, A., Purucker, E. A., Gressner, A. M., Matern, S., & Lammert, F. (2005). Patients with acute on chronic liver failure display "sepsis-like" immune paralysis. *J Hepatol*, *42*(2), 195-201. https://doi.org/10.1016/j.jhep.2004.10.019

Weber, A. N. R., Bittner, Z., Liu, X., Dang, T. M., Radsak, M. P., & Brunner, C. (2017). Bruton's Tyrosine Kinase: An Emerging Key Player in Innate Immunity. *Front Immunol*, *8*, 1454. https://doi.org/10.3389/fimmu.2017.01454

Weyrich, A. S., Elstad, M. R., McEver, R. P., McIntyre, T. M., Moore, K. L., Morrissey, J. H., Prescott, S. M., & Zimmerman, G. A. (1996). Activated platelets signal chemokine synthesis by human monocytes. *J Clin Invest*, *97*(6), 1525-1534. https://doi.org/10.1172/JCI118575

Weyrich, A. S., McIntyre, T. M., McEver, R. P., Prescott, S. M., & Zimmerman, G. A. (1995). Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-alpha secretion. Signal integration and NF-kappa B translocation. *J Clin Invest*, *95*(5), 2297-2303. https://doi.org/10.1172/JCI117921

White, J. G. (2005). Platelets are covercytes, not phagocytes: uptake of bacteria involves channels of the open canalicular system. *Platelets*, *16*(2), 121-131. https://doi.org/10.1080/09537100400007390

Wolf, D., Anto-Michel, N., Blankenbach, H., Wiedemann, A., Buscher, K., Hohmann, J. D., Lim, B., Bauml, M., Marki, A., Mauler, M., Duerschmied, D., Fan, Z., Winkels, H., Sidler, D., Diehl, P., Zajonc, D. M., Hilgendorf, I., Stachon, P., Marchini, T., . . . Zirlik, A. (2018). A ligand-specific blockade of the integrin Mac-1 selectively targets

pathologic inflammation while maintaining protective host-defense. *Nat Commun*, *9*(1), 525. https://doi.org/10.1038/s41467-018-02896-8

Wolf, P. (1967). The nature and significance of platelet products in human plasma. *Br J Haematol*, *13*(3), 269-288. https://doi.org/10.1111/j.1365-2141.1967.tb08741.x

Wong, K. L., Tai, J. J., Wong, W. C., Han, H., Sem, X., Yeap, W. H., Kourilsky, P., & Wong, S. C. (2011). Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. *Blood*, *118*(5), e16-31. https://doi.org/10.1182/blood-2010-12-326355

Wuescher, L. M., Takashima, A., & Worth, R. G. (2015). A novel conditional platelet depletion mouse model reveals the importance of platelets in protection against Staphylococcus aureus bacteremia. *J Thromb Haemost*, *13*(2), 303-313. https://doi.org/10.1111/jth.12795

Wynn, T. A., Chawla, A., & Pollard, J. W. (2013). Macrophage biology in development, homeostasis and disease. *Nature*, *496*(7446), 445-455. https://doi.org/10.1038/nature12034

Xiang, B., Zhang, G., Guo, L., Li, X. A., Morris, A. J., Daugherty, A., Whiteheart, S. W., Smyth, S. S., & Li, Z. (2013). Platelets protect from septic shock by inhibiting macrophage-dependent inflammation via the cyclooxygenase 1 signalling pathway. *Nat Commun*, *4*, 2657. https://doi.org/10.1038/ncomms3657

Yang, Y., Kim, S. C., Yu, T., Yi, Y. S., Rhee, M. H., Sung, G. H., Yoo, B. C., & Cho, J. Y. (2014). Functional roles of p38 mitogen-activated protein kinase in macrophage-mediated inflammatory responses. *Mediators Inflamm*, *2014*, 352371. https://doi.org/10.1155/2014/352371

Yang, Y., Zhang, X., Zhang, D., Li, H., Ma, L., Xuan, M., Wang, H., & Yang, R. (2017). Abnormal Distribution and Function of Monocyte Subsets in Patients With Primary Immune Thrombocytopenia. *Clin Appl Thromb Hemost*, *23*(7), 786-792. https://doi.org/10.1177/1076029616652726

Yazdanbakhsh, K. (2016). Imbalanced immune homeostasis in immune thrombocytopenia. *Semin Hematol*, *53 Suppl 1*, S16-19. https://doi.org/10.1053/j.seminhematol.2016.04.006

Yi, L., Chandrasekaran, P., & Venkatesan, S. (2012). TLR signaling paralyzes monocyte chemotaxis through synergized effects of p38 MAPK and global Rap-1 activation. *PLoS One*, *7*(2), e30404. https://doi.org/10.1371/journal.pone.0030404

Zanoni, I., Ostuni, R., Marek, L. R., Barresi, S., Barbalat, R., Barton, G. M., Granucci, F., & Kagan, J. C. (2011). CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell*, *147*(4), 868-880. https://doi.org/10.1016/j.cell.2011.09.051

Zarbock, A., Singbartl, K., & Ley, K. (2006). Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. *J Clin Invest*, *116*(12), 3211-3219. https://doi.org/10.1172/JCI29499

Zhang, H., Nimmer, P. M., Tahir, S. K., Chen, J., Fryer, R. M., Hahn, K. R., Iciek, L. A., Morgan, S. J., Nasarre, M. C., Nelson, R., Preusser, L. C., Reinhart, G. A., Smith, M. L., Rosenberg, S. H., Elmore, S. W., & Tse, C. (2007). Bcl-2 family proteins are essential for platelet survival. *Cell Death Differ*, *14*(5), 943-951. https://doi.org/10.1038/sj.cdd.4402081

Zhang, W., Du, Z., Zhu, J., Yu, J., & Xu, Y. (2015). Sprouty2 suppresses the inflammatory responses in rheumatoid arthritis fibroblast-like synoviocytes through regulating the Raf/ERK and PTEN/AKT signals. *Mol Immunol*, *67*(2 Pt B), 532-539. https://doi.org/10.1016/j.molimm.2015.07.033

Ziegler-Heitbrock, L., & Hofer, T. P. (2013). Toward a refined definition of monocyte subsets. *Front Immunol*, *4*, 23. https://doi.org/10.3389/fimmu.2013.00023

Zinatizadeh, M. R., Schock, B., Chalbatani, G. M., Zarandi, P. K., Jalali, S. A., & Miri, S. R. (2021). The Nuclear Factor Kappa B (NF-kB) signaling in cancer development and immune diseases. *Genes Dis*, *8*(3), 287-297. https://doi.org/10.1016/j.gendis.2020.06.005

Zuchtriegel, G., Uhl, B., Puhr-Westerheide, D., Pornbacher, M., Lauber, K., Krombach, F., & Reichel, C. A. (2016). Platelets Guide Leukocytes to Their Sites of Extravasation. *PLoS Biol*, *14*(5), e1002459. https://doi.org/10.1371/journal.pbio.1002459