Platelet-derived transcription factors as modulators of NFκB-dependent inflammatory responses in human monocytes

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From the Institute of Innate Immunity

All we have to decide is what to do with the time that is given us.

— Gandalf, The Fellowship of the Ring (J.R.R. Tolkien)

dedicated to my family

A portion of the work presented in this thesis has been published in the following original research articles:

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List of abbreviations

ADP	Adenosine diphosphate
ALRs	AIM2-like receptors
ANOVA	Analysis of variance
APCs	Antigen-presenting cells
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BMDM	Bone Marrow-Derived Macrophage
BM	Bone marrow
BP	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
CD40L	CD40 ligand
CLRs	C-type lectin receptors
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTB	CellTiter-Blue®
DAMPs	Danger-associated molecular patterns
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
EVs	Extracellular vesicles
FasL	Fas ligand
FCS	Fetal calf serum
FcR	Fc receptor
GP	Glycoprotein
h	Hours
HTRF	Homogeneous time-resolved fluorescence
HLA	Human leukocyte antigen
IKK	IκB kinase
IL	Interleukin
IRF	Interferon regulatory factor
ITP	Idiopathic thrombocytopenia
IκB	Inhibitor of κB
KO	Knockout
LDH	Lactate dehydrogenase
LDS	Lithium dodecyl sulfate
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LRRs	Leucine-rich repeats
MAPK	Mitogen-activated protein kinase
MDCSs	Myeloid-derived suppressor cells
miRNAs	MicroRNAs

MKs	Megakaryocytes
MPAs	Monocyte-platelet aggregates
min	Minutes
ms	Milliseconds
NEMO	NFκB essential modulator
NGS	Next-generation sequencing
NIK	NFκB-inducing kinase
NLRP3	NOD-, LRR-, and pyrin domain-containing protein 3
NLRs	NOD-like receptors
NFκB	Nuclear factor kappa B
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PDMo	Platelet-depleted monocytes
PDP-L1	Programmed death-ligand 1
PGE_1	Prostaglandin E1
PF4	Platelet factor 4
Plt	Platelet
Plts	Platelets
PLAs	Platelet-leukocyte aggregates
PPRs	Pattern recognition receptors
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PRRs	Pattern recognition receptors
PS	Platelet supernatant
PCR	Polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RLRs	RIG-I-like receptors
rpm	Rounds per minute
RNP	Ribonucleoprotein
RT	Room temperature
sec	Seconds
SILAC	Stable isotope labeling by amino acids in cell culture
StdMo	Standard monocytes
Tfh	T follicular helper cells
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TPO	Thrombopoietin
vWF	von Willebrand factor
WASP	Wiskott-Aldrich syndrome protein
WT	Wild-type

1. Introduction

1.1. The immune system

The immune system is an omnipresent, evolutionarily conserved surveillance network that enables the organism to detect and respond to threats rapidly and effectively. Over millions of years, it has evolved into a highly specialized host defense system. Its primary function is to distinguish between self and non-self in order to protect the organism against pathogenic threats such as bacteria, viruses, fungi, or malignant cells (Janeway & Medzhitov, 2002; Medzhitov, 2008). In addition, it plays a critical role in maintaining tissue homeostasis. As one of the most complex defense systems in biology, the immune system relies on the finely coordinated interaction of cells, tissues, soluble mediators, and molecular signaling pathways to initiate a coherent and effective immune response (Medzhitov, 2007a; Paludan et al., 2021). It is functionally divided into two interconnected branches, the innate and the adaptive immune system. Innate immunity represents an ancient and rapidly acting defense mechanism that relies on germline-encoded sensors to detect conserved molecular patterns on pathogens via pattern recognition receptors (PRRs). In contrast, adaptive immunity develops more slowly and is based on the recognition of specific antigens by somatically recombined receptors on B and T lymphocytes. It confers immunological memory, enabling a faster and more efficient response upon re-exposure to the same pathogen (Akira et al., 2006; Iwasaki & Medzhitov, 2010; Medzhitov, 2007a; Paludan et al., 2021).

These two branches operate in close coordination. While adaptive immunity ensures the targeted and long-lasting elimination of specific threats, innate immunity constitutes the first layer of defense and plays a pivotal role in initiating adaptive responses. The initial barrier consists of physical and chemical defense layers such as the skin, mucosal surfaces, and gastric acid. Once breached, pathogens encounter humoral components like the complement system and cellular components of innate immunity. These include phagocytes such as macrophages, monocytes and neutrophils, as well as granulocytes and natural killer cells. Upon recognition of microbial structures, these cells become activated, eliminate the intruder directly, or process and present antigens to adaptive immune cells, thereby promoting antigen-specific responses (Iwasaki & Medzhitov, 2015; Medzhitov, 2007b, 2008; Nicholson, 2016; Paludan et al., 2021).

The immune system’s extraordinary ability to respond to a wide range of potential threats while avoiding damage to self relies on a finely tuned balance between activation and regulation.

1.2. Pattern recognition and innate immune signaling

A hallmark of the innate immune system, is its capacity to recognize conserved microbial structures known as pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs) released from damaged or stressed cells. Recognition of these signals is mediated by a diverse set of PRRs that trigger intracellular signaling cascades, orchestrating inflammation, pathogen elimination, and the initiation of adaptive immune responses (Akira et al., 2006; Medzhitov, 2007b; Rakoff-Nahoum & Medzhitov, 2009).

PRRs are grouped into five main families: Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), and AIM2-like receptors (ALRs). These families differ in subcellular localization, ligand specificity, and downstream signaling. TLRs and CLRs are membrane-associated and located either at the plasma membrane or within endosomes. Among them, TLRs are the most extensively studied and are classified based on their localization (Medzhitov, 2007b; Rakoff-Nahoum & Medzhitov, 2009). TLR1, 2, 4, 5, 6, and 10 are expressed on the cell surface and recognize extracellular microbial components such as lipopolysaccharides (LPS), which are detected by TLR4. In contrast, intracellular TLRs such as TLR3, 7, 8, and 9, as well as the mouse-specific TLR11, 12, and 13, are localized to the endoplasmic reticulum, endosomes, or lysosomes and detect nucleic acids that become accessible after microbial degradation (Akira et al., 2006; Brubaker et al., 2015). Other cytosolic PRRs include NLRs, RLRs, and ALRs. NLRs sense bacterial peptidoglycans, such as NOD1 and NOD2. ALRs like AIM2 bind double-stranded DNA, while RLRs detect viral RNA and initiate type I interferon responses (Brubaker et al., 2015).

Ligand binding to PRRs induces conformational changes that facilitate the recruitment of adaptor proteins and the activation of downstream signaling pathways, including nuclear factor kappa B (NFκB), mitogen-activated protein kinase (MAPK), and interferon regulatory factor (IRF) cascades. These pathways lead to the transcription of pro-inflammatory genes and the secretion of cytokines such as tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β), as well as chemokines and

antimicrobial peptides. While TNFα is secreted via the classical pathway, IL-1β requires proteolytic cleavage by caspase-1, which is typically activated within a multiprotein inflammasome complex (Baldwin, 1996; Cui et al., 2014; Mantovani et al., 2019).

Inflammasomes are assembled by intracellular PRRs such as NLRP3 or AIM2 in response to specific stimuli, including ATP, cytosolic DNA, or pore-forming toxins such as Nigericin. These complexes consist of a sensor protein (NLR or ALR), the adaptor ASC, and caspase-1. Once assembled, they cleave pro-IL-1β and pro-IL-18 and Gasdermin D into their active forms. Cleaved Gasdermin D promotes pore formation in membranes allowing the release of IL-1β and IL-18 along with other DAMPs. Once in the extracellular space, these cytokines amplify the immune response and promote inflammation, characterized by immune cell recruitment, increased vascular permeability, and initiation of tissue repair processes (Akbal et al., 2022).

When innate mechanisms are insufficient to eliminate the threat, antigen-presenting cells (APCs) such as dendritic cells (DCs), monocytes and macrophages serve as a bridge to adaptive immunity. However, dysregulated PRR signaling can lead to pathological inflammation. Chronic activation of TLRs has been implicated in autoimmune diseases, and gain-of-function mutations in NLRs are the underlying cause of several monogenic autoinflammatory syndromes. Excessive inflammasome activation also contributes to metabolic and neurodegenerative diseases, while PRR hyperactivation, particularly of TLR4, plays a central role in septic shock (Cosson et al., 2024; Farrugia & Baron, 2017; Lorenz et al., 2002; Voet et al., 2019).

Current research has revealed that PRRs also functions beyond pathogen recognition. Intracellular processes such as autophagy, metabolic sensing, and non-canonical complement activity, collectively referred to as the complosome, modulate PRR signaling and help maintain immune homeostasis (Deretic et al., 2013; O'Neill et al., 2016; West & Kemper, 2023). Disruption of these regulatory pathways can lead to spontaneous immune activation or persistent inflammation, highlighting the importance of precise immunological control (Deretic et al., 2013; Kawai & Akira, 2010; Medzhitov & Horng, 2009; O'Neill et al., 2016; West & Kemper, 2023).

Given their central role in host defense and immune regulation, PRRs represent highly promising therapeutic targets in the context of infectious diseases, autoimmunity, cancer immunotherapy, and inflammatory disorders.

1.3. The role of monocytes in innate immune defense

Among the key cellular effectors activated downstream of PRR-dependent signaling pathways are monocytes. As highly plastic mononuclear phagocytes derived from hematopoietic stem cells in the bone marrow, monocytes play a central role in immune surveillance, inflammation, and tissue homeostasis. Upon activation by PRRs and local cytokines, monocytes are rapidly mobilized from the bloodstream to sites of infection or injury, where they contribute to pathogen elimination, coordinate immune responses, and support tissue repair (Ginhoux et al., 2021; Ziegler-Heitbrock, 2015).

Human monocytes constitute approximately 10% of circulating leukocytes and are now recognized as a functionally distinct and highly adaptable immune cell population. Although they were traditionally considered as precursors of macrophages and DCs, fate-mapping and single-cell studies have shown that many tissue-resident macrophages originate from embryonic progenitors and maintain themselves independently of circulating monocytes. Consequently, monocytes are increasingly acknowledged as autonomous effectors with specialized immunological functions (Ginhoux & Jung, 2014; Ziegler-Heitbrock, 2015; Ziegler-Heitbrock et al., 2010).

Based on the expression of CD14 and CD16, human monocytes are subdivided into three major subsets. Classical monocytes ($CD14^{high}CD16^{-}$) comprise the majority of the population, accounting for 80-90%. They express high levels of C-C chemokine receptor type 2 (CCR2) and exhibit strong phagocytic activity as well as robust secretion of pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6. Intermediate monocytes ($CD14^{high}CD16^{+}$) make up about 2-5% of monocytes. They are potent producers of cytokines and show enhanced antigen presentation capacity, often being enriched in chronic inflammatory and neoplastic conditions including rheumatoid arthritis, asthma, and colorectal cancer. Non-classical monocytes ($CD14^{+}CD16^{high}$) account for 2-10% of the monocyte pool. They are characterized by high expression of CX3CR1 and patrol the endothelium under homeostatic conditions. In addition to their roles in phagocytosis and tissue repair, they contribute to antiviral defense. Although they produce lower levels of cytokines compared to classical monocytes (Ginhoux et al., 2021; Ginhoux & Jung, 2014; Kapellos et al., 2019).

This subset classification reflects distinct functional profiles. However, recent multiomics studies have revealed additional heterogeneity within the monocyte population, identifying novel subsets with unique expression signatures and

transcriptional programs. These findings suggest that monocyte identity exists along a functional continuum rather than as fixed subsets (Villani et al., 2017; B. Zhang et al., 2022; Zhao et al., 2020).

Upon tissue infiltration, monocytes undergo context-dependent differentiation into macrophages or monocyte-derived DCs. This differentiation process is highly plastic and regulated by local environmental cues. Transcription factors such as IRF1, ZNF366, and PU.1 play central roles in shaping the monocyte fate. This transition often occurs within the first 24 hours of tissue entry and enables monocytes to adapt rapidly to local immune or regenerative demands (Ginhoux et al., 2021; Ginhoux & Jung, 2014).

Monocytes are also critically involved in various pathological processes. In early stages of liver fibrosis for example, monocytes are recruited to fibrotic tissue where they secrete inflammatory cytokines that drive the inflammatory response. At later stages, they can differentiate into macrophages, which produce matrix metalloproteinases, contributing to collagen degradation and fibrosis resolution (C.-C. Gao et al., 2022). In contrast, in atherosclerosis, monocytes infiltrate the intimal layer of arterial walls, where they internalize oxidized low-density lipoproteins (LDL) and release cytokines that recruit additional leukocytes, promoting chronic inflammation and plaque development. This positive feedback loop may be involved in processes contributing to cardiovascular dysfunction or disease (Woollard & Geissmann, 2010).

In the tumor microenvironment, monocytes may exert both anti-tumor and pro-tumor activities. They can differentiate into immunosuppressive myeloid-derived suppressor cells (MDSCs), which support regulatory T cells and inhibit anti-tumor immunity (Sica & Strauss, 2017). Moreover, in systemic inflammatory conditions such as sepsis or trauma, monocyte function can become impaired. Downregulation of HLA-DR expression, for example, is associated with reduced antigen-presenting capacity of monocytes and increased host susceptibility to infections (Joshi et al., 2023).

Overall, monocytes are not merely transitional precursors but represent a functionally independent and essential component of the innate immune system. Their rapid mobilization, high functional plasticity and ability to adapt their differentiation programs enable them to participate in a wide spectrum of immunological processes under physiological and pathological conditions.

1.4. Platelets in hemostasis and thrombopoiesis

In addition to monocytes, platelets represent another critical cellular component involved in innate immune responses and inflammatory processes. Traditionally known for their role in hemostasis and thrombosis, platelets are small, anucleate cytoplasmic fragments that originate primarily from megakaryocytes (MKs) in the bone marrow. They circulate in high numbers in human blood (150 000-400 000/μL), with a diameter of approximately 2-4 μm and a lifespan of five to nine days (Michelson et al., 2019; Tyagi et al., 2022). Following vascular injury, platelets are rapidly recruited to sites of endothelial disruption, where they initiate thrombus formation through a tightly regulated sequence of adhesion, activation, and aggregation. Binding to subendothelial von Willebrand factor (vWF) via the glycoprotein Ib/V/IX (GP) complex and to collagen via GPVI and integrin α2β1 triggers intracellular signaling cascades. These events induce shape change, degranulation, and the release of secondary mediators such as ADP, serotonin, and thromboxane A2, which amplify platelet recruitment and promote stabilization of the thrombus through fibrinogen-mediated crosslinking via integrin αIIbβ3 (GPIIb/IIIa also known as CD41/CD61) (Michelson et al., 2019; Sangkuhl et al., 2011).

Platelet biogenesis occurs through thrombopoiesis, in which mature MKs extend cytoplasmic protrusions, known as pro-platelets, into the bone marrow sinusoids. Interestingly, studies in mice have also shown the lungs to be an additional site of thrombopoiesis (Lefrançais et al., 2017). The generation of platelets is driven by cytoskeletal remodeling involving β1-tubulin-mediated microtubule organization, with each MK producing thousands of platelets. Thrombopoietin (TPO) acts through the c-Mpl receptor to regulate MK differentiation and platelet shedding. The lifespan of circulating platelets is determined by a balance of pro- and anti-apoptotic factors, including Bcl-xL and Bax/Bak. In addition, the exposure of phosphatidylserine or the desialylation of surface glycoproteins serves as clearance signals for aged or damaged platelets, which are removed by hepatocytes or splenic macrophages (Michelson et al., 2019; van der Meijden & Heemskerk, 2019).

1.5. Platelets as innate immune effectors

In recent decades, the understanding of platelet function has expanded considerably. It is now well established that platelets serve not only as mediators of vascular integrity,

but also as active participants in immune regulation (Marcoux et al., 2021; Morrell et al., 2019; Scherlinger et al., 2023; Semple et al., 2011; Semple & Freedman, 2010; Thomas & Storey, 2015; Tyagi et al., 2022; van der Meijden & Heemskerk, 2019). Platelets express a wide range of immunologically relevant receptors, including TLRs, integrins, and Fc receptors, enabling them to sense PAMPS and DAMPs. Their three major granule types, alpha, dense and lysosomal, store numerous immune mediators. Alpha granules contain cytokines, chemokines such as CCL5 and CXCL12, co-stimulatory molecules including CD40 ligand (CD40L) and CD62P and various growth factors. Dense granules are enriched in small signaling molecules such as calcium, ADP, ATP and serotonin, while lysosomal granules store degradative enzymes that further contribute to immune modulation (Michelson et al., 2019; Thomas & Storey, 2015; Tyagi et al., 2022).

Platelets interact closely with innate immune cells, particularly neutrophils and monocytes. Through P-selectin and integrin-dependent adhesion, they form platelet-leukocyte aggregates (PLAs), which influence leukocyte migration, cytokine production, and effector function (Kral et al., 2016; Ribeiro et al., 2019). In addition, platelet-derived extracellular vesicles (EVs) transport proteins, lipids, and nucleic acids to immune cells, enhancing NLRP3-dependent inflammasome priming in macrophages and cytokine responses in monocytes (Gasecka et al., 2019; Hawwari & Rossnagel et al., 2024; Puhm et al., 2021; Vats et al., 2020; Vidal-Gómez et al., 2025). Platelets also support neutrophil recruitment to sites of inflammation, promote transendothelial migration, and facilitate the release of neutrophil extracellular traps (NETs) (Freedman & Loscalzo, 2002; Hawwari & Rossnagel et al., 2024; Z. Li & Smyth, 2019; Rolfes et al., 2020).

1.6. Platelets in adaptive immunity and inflammatory disease

Besides the above-mentioned roles of platelets in innate immune responses, platelets also contribute to adaptive immunity by influencing T and B cell responses. Upon activation, platelets upregulate CD40L, which engages CD40 on APCs, thereby enhancing T cell priming (Elzey, Sprague, et al., 2005; Iannacone, 2016; C. Yan et al., 2023). Platelets can also recognize and trap bacteria into clusters, limiting their dissemination and facilitating their clearance by immune cells (Marcoux et al., 2021; Semple et al., 2011). Moreover, platelets promote T cell differentiation and cytokine production both through direct cell-cell interactions and indirectly by releasing

chemokines such as platelet factor 4 (PF4, also known as CXCL4) and CCL5. Additionally, they express immunomodulatory molecules including Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL), IL-7 and the above mentioned CD40L, all of which are critical for the regulation of adaptive immune responses (R. Ahmad et al., 2001; Crist et al., 2004; Gerdes et al., 2011; C. Yan et al., 2023). Furthermore, platelets can express or acquire immune checkpoint molecules such as PD-L1, which directly inhibit the function of $CD4^+$ and $CD8^+$ T cells. This mechanism has been observed not only in cancer patients but also in platelets from individuals with COVID-19 (Elzey et al., 2003; Hinterleitner et al., 2021; Paletta et al., 2022; Polasky et al., 2020; C. Yan et al., 2023). In contrast to T cells, the role of platelets in B cell regulation is less well understood. However, several studies have shown that platelets interact with B cells through the CD40-CD40L axis, promote their activation, and enhance antibody production by peripheral blood B cells (Cognasse et al., 2007; Elzey et al., 2003; Elzey, Grant, et al., 2005; C. Yan et al., 2023).

Clinically, platelet dysfunction or altered numbers have been associated with various pathologies and inflammatory diseases. Enhanced platelet reactivity and increased formation of monocyte-platelet aggregates (MPAs) have been reported in cardiovascular diseases, diabetes, chronic inflammation, and sepsis (Fogerty & Kuter, 2024; Freedman & Loscalzo, 2002; Greaves & Pula, 2025; Nurden et al., 2020; Rolling et al., 2023). In severe COVID-19, elevated MPA levels correlate with higher mortality (Hottz et al., 2020; Jakobs et al., 2022; Schrottmaier et al., 2021). Conversely, depletion of platelets or blockade of their interaction with leukocytes, such as through anti-P-selectin antibodies, has shown protective effects in models of acute lung injury (D. R. S. Ghosh et al., 2019; Liu et al., 2020; Zarbock et al., 2006). In contrast, in other settings such as liver cirrhosis, platelet transfusion has been shown to improve liver function and platelets may exert protective effects during septic shock (Luo et al., 2017; Maruyama et al., 2013; Xiang et al., 2013).

Taken together, platelets are not merely passive participants in coagulation but are multifunctional effector cells of the immune system. Their ability to rapidly sense and respond to vascular injury, to modulate immune cell function and to participate in both innate and adaptive immunity underscores their pivotal role in maintaining vascular homeostasis and orchestrating complex immunological processes.

1.7. Extracellular vesicle-mediated platelet crosstalk with monocytes and other immune cells

Beyond their fundamental roles in hemostasis and thrombosis, platelets actively engage in complex intercellular communication networks that influence immune cell function and inflammatory responses. This communication occurs through multiple parallel routes: the release of soluble mediators, direct cell-cell interactions, and most notably, through the production of EVs, which serve as potent vehicles of bioactive cargo and immune modulation (Gasecka et al., 2019; Morrell et al., 2014; Scherlinger et al., 2023).

Among the various modes of platelet-mediated communication, platelet-derived EVs have emerged as potent mediators of systemic intercellular signaling. In general, EVs found in the blood, are small, heterogeneous membrane-bound structures released predominantly from platelets, although a proportion may also originate from MKs. Most circulating EVs (~80%) in human blood express the platelet marker CD41, yet the exact contribution of platelet versus MK origin remains a subject of ongoing debate (Berckmans et al., 2001; Flaumenhaft et al., 2009; George et al., 1982; Horstman & Ahn, 1999; Italiano Jr et al., 2010; Joop et al., 2001; Mause & Weber, 2010; Taus et al., 2019). Platelet-derived EVs encompass multiple subpopulations, including microvesicles (100-300 nm), exosomes (40-100 nm), and mitochondria-containing vesicles. These platelet-derived EVs carry a diverse array of immunologically relevant cargo, such as cytokines, chemokines, transcription factors, microRNAs (miRNAs), and in some cases even intact mitochondria (Pelletier et al., 2023; Puhm et al., 2021; Yeh et al., 2025). The molecular composition of platelet-derived EVs is closely linked to the activation state of the parent platelet, allowing for targeted modulation of recipient cell behavior and immune function (Diamant et al., 2004; French et al., 2020). Platelet-derived EVs are capable of delivering immunomodulatory miRNAs such as miR-126, miR-223-3p, and miR-320b to monocytes and macrophages. These miRNAs suppress TLR4-driven pro-inflammatory signaling, promote IL-10 production, and steer monocytes toward an anti-inflammatory phenotype (Kaudewitz et al., 2016; Nagalla et al., 2011; Tyagi et al., 2022). Furthermore, platelet-derived EVs transport functional membrane receptors such as GPIbα, which are incorporated into the membrane of recipient monocytes, thereby enhancing their ability to bind vWF and increasing their adhesion under shear stress. In inflammatory conditions, this receptor

transfer can facilitate monocyte recruitment to activated endothelia, contributing to the progression of vascular inflammation and atherosclerosis (Chimen et al., 2019; Z. Han et al., 2023; He & Wu, 2023).

In addition to modulating monocyte phenotype and adhesion capacity, platelet-derived EVs play a pivotal role in shaping monocyte metabolism and antigen-presenting function. The transfer of respiratory-competent mitochondria via platelet-derived EVs has been shown to enhance oxidative phosphorylation in monocytes, thereby increasing their survival in metabolically demanding environments such as the hypoxic tumor niche (Chaudhary et al., 2023; Pelletier et al., 2023; Puhm et al., 2021). Platelet-derived EVs also deliver angiogenic factors like VEGF-A and FGF2, which promote monocyte-driven tissue repair and neovascularization during resolution of inflammation or ischemic injury (Chaudhary et al., 2023; Voron et al., 2014).

Importantly, platelet-derived EVs contribute to inflammasome regulation. Studies have demonstrated that platelet-derived releasates and vesicles enhance NLRP3 inflammasome priming in monocytes and macrophages, thereby amplifying IL-1β-dependent immune responses (Hawwari & Rossnagel et al., 2024; Rolfes et al., 2020). In pathologies such as sepsis, systemic lupus erythematosus and cancer, platelet-derived EVs act as critical immunoregulatory agents that shape monocyte behavior toward either immunosuppressive or pro-inflammatory states, depending on the context and cargo composition (Fendl et al., 2021; Jiang et al., 2023; Lou & Cai, 2025).

In parallel to vesicle-mediated communication, platelets engage monocytes via direct receptor-ligand interactions. P-selectin on activated platelets binds to PSGL-1 on monocytes, triggering calcium flux, NFκB activation and downstream cytokine production. These adhesive interactions further facilitate the transfer of platelet-derived proteins, stabilize monocyte-platelet aggregates and link thrombosis to inflammation (P. Han et al., 2020; Rolling et al., 2023). Interestingly, our group demonstrated that classical monocytes isolated from patients with idiopathic thrombocytopenia (ITP), a heterogeneous autoimmune disorder characterized by platelet counts below 10^5/µL and an increased risk of bleeding, exhibited markedly reduced responsiveness to pro-inflammatory stimuli. However, the addition of heterologous platelets restored their immune responsiveness, allowing the monocytes to react similarly to healthy monocytes (Hawwari & Rossnagel et al., 2024).

Taken together, these findings highlight platelets as central orchestrators of immune cell programming, with platelet-derived EVs acting as potent conveyors of signaling

cues that fine-tune monocyte function in health and disease. Among the intracellular pathways modulated during this crosstalk, the NFκB signaling axis emerged as a pivotal regulator integrating inflammatory signals and shaping monocyte responses.

1.8. Structural and functional overview of the NFκB signaling cascade

Through the release of platelet-derived EVs, platelets act as powerful modulators of monocyte function, influencing their inflammatory programming, antigen-presenting capacity, and transcriptional landscape (Hawwari & Rossnagel et al., 2024; Kral et al., 2016; Puhm et al., 2021; R. Weiss et al., 2018). These interactions reflect a broader principle in innate immunity, where extracellular stimuli must be translated into rapid and context-specific gene expression program (S. Chen et al., 2023; Foulem et al., 2025; Puhm et al., 2021). Central to this transcriptional control is the NFκB signaling pathway, which integrates a wide variety of immune and inflammatory signals to orchestrate cellular responses across diverse physiological and pathological conditions. The NFκB pathway serves as a central transcriptional hub activated downstream of PRRs, cytokines or by antigen recognition. It enables immune and non-immune cells to mount rapid responses to infection, cellular stress and tissue damage (Guo et al., 2024; Vallabhapurapu & Karin, 2009; Yu et al., 2020). Given its pivotal role in regulating immune homeostasis, inflammation, and cell survival, NFκB has been extensively studied and is widely recognized as a promising therapeutic target in chronic inflammatory, autoimmune and malignant diseases (Bakrim et al., 2025; Guo et al., 2024; Hawwari & Rossnagel et al., 2024; Kumar et al., 2024).

1.8.1. Molecular organization of the NFκB system

The mammalian NFκB family comprises five transcription factors: RELA (p65), RELB, c-REL, NFκB1 (p105 and p50), and NFκB2 (p100 and p52). All family members share a conserved N-terminal REL homology domain that mediates DNA binding, dimerization, and interaction with inhibitor of κB (IκB) proteins. RELA, RELB, and c-REL also contain C-terminal transactivation domains that promote transcriptional activation of target genes (Figure 1). In contrast, p50 and p52 lack such domains and function as transcriptional repressors unless dimerized with activating partners or coactivators such as Bcl-3. Interestingly, these five subunits can form up to 15 distinct homo- and heterodimers, underscoring the structural versatility and cell-type-specific

functions of the NFκB system. p50 and p52 are generated through the proteolytic processing of their precursor proteins p105 and p100. Whereas p105 is constitutively processed into p50, the conversion of p100 to p52 is tightly regulated and primarily occurs during non-canonical NFκB activation (Guo et al., 2024; V. F.-S. Shih et al., 2011; Vallabhapurapu & Karin, 2009; Q. Zhang et al., 2017).

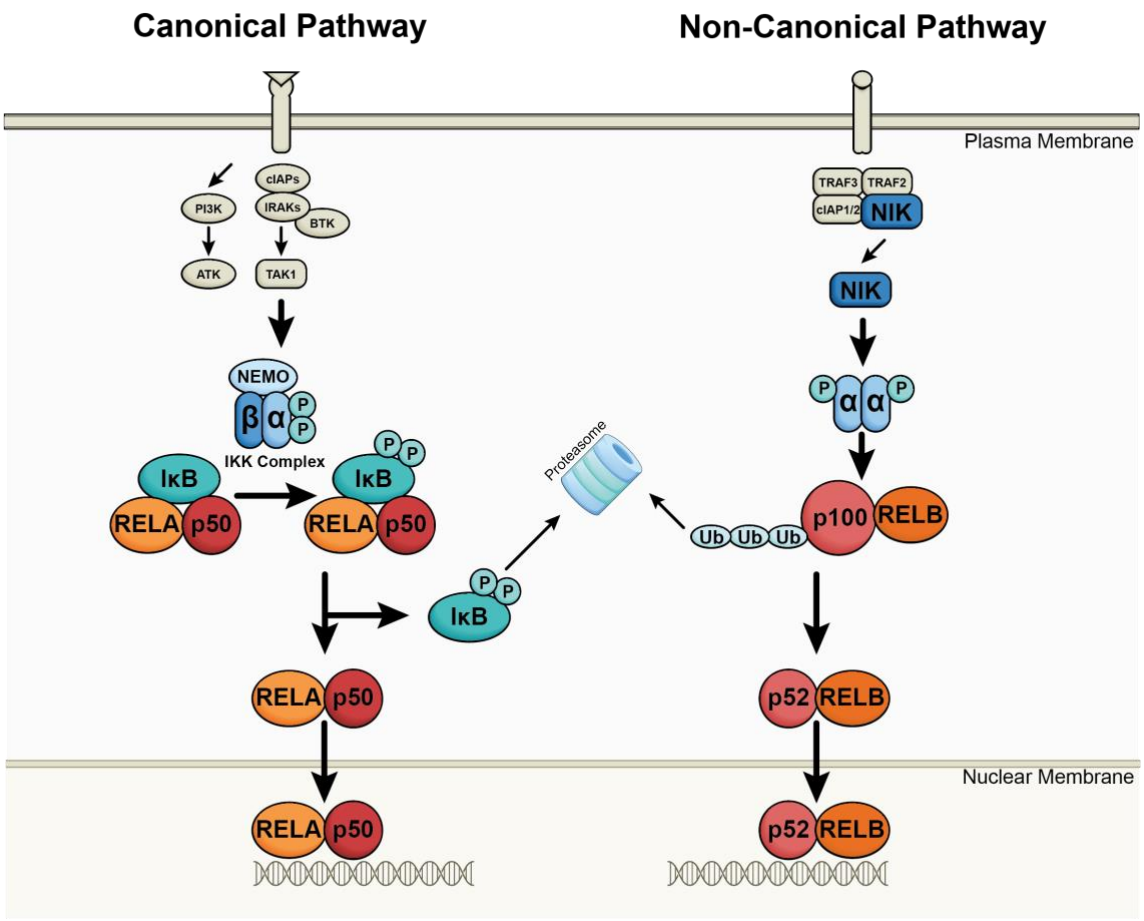


Figure 1: Overview of canonical and non-canonical NFκB signaling. Schematic overview of canonical (left) and non-canonical (right) NFκB signaling pathways. Canonical activation involves IKK complex-mediated phosphorylation and degradation of IκB, releasing the RELA/p50 dimer to translocate into the nucleus. Non-canonical signaling involves NIK-mediated processing of p100 into p52, enabling nuclear translocation of RELB/p52 complexes. The illustration was made with the help of Salie Maasewerd.

1.8.2. Canonical NFκB signaling pathway

The canonical NFκB pathway is the principal mode of activation in most cell types and is typically triggered by pro-inflammatory cytokines such as TNFα and IL-1β or by microbial ligands recognized by TLRs. Signal transduction is initiated by the IκB kinase complex, which consists of the catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ, also known as NFκB essential modulator (NEMO). Upon receptor engagement, upstream kinases such as TAK1 activate IKKβ, which phosphorylates the inhibitory protein IκBα. This modification targets IκBα for proteasomal degradation, allowing NFκB dimers such as p65 and p50 to translocate into the nucleus and initiate transcription of pro-inflammatory cytokines, chemokines, and survival factors (Guo et al., 2024; V. F.-S. Shih et al., 2011; Q. Zhang et al., 2017).

1.8.3. Non-Canonical NFκB signaling pathway

The non-canonical pathway is activated by a limited subset of TNF superfamily ligands, including CD40L, BAFF and lymphotoxin beta. This pathway is critical for lymphoid organogenesis, B-cell maturation, and long-term immune regulation. It operates independently of IKKγ and instead relies on IKKα homodimers and NFκB-inducing kinase (NIK). In resting cells, NIK is continuously degraded by a complex that includes TRAF2, TRAF3 and cellular inhibitors of apoptosis. Upon stimulation, degradation is halted, leading to NIK accumulation, activation of IKKα, and phosphorylation of p100. This results in the generation of p52 and nuclear translocation of RELB-containing dimers that drive expression of genes involved in immune architecture and function (Guo et al., 2024; S. Sun, 2012; S.-C. Sun, 2017).

1.8.4. The role of IκB proteins and the IKK complex

The NFκB signaling pathway is tightly regulated by a balance of inhibitory and activating mechanisms that control its temporal and spatial dynamics. Under basal conditions, IκB proteins, including IκBα, IκBβ, and IκBε, as well as the precursor proteins p105 and p100, retain NFκB dimers in the cytoplasm, thereby preventing spurious activation. Among these, IκBα is rapidly degraded upon stimulation but is also promptly re-synthesized as part of a negative feedback loop that serves to constrain signal duration. In contrast, IκBε is induced with delayed kinetics and modulates the magnitude and persistence of NFκB activation during prolonged stimulation (V. F.-S.

Shih et al., 2011; S.-C. Sun, 2017; Q. Zhang et al., 2017). Upstream signal integration is mediated by the IκB kinase (IKK) complex, which serves as a central activation hub. IKKβ, a key catalytic subunit, contains a kinase domain, a ubiquitin-like domain, and a scaffold dimerization domain that facilitate dimer formation, catalytic function and substrate specificity. In its resting state, IKKβ exists in a closed conformation, but upon stimulation, the complex undergoes structural rearrangements that enable oligomerization and autophosphorylation. The regulatory subunit NEMO is essential for sensing polyubiquitin chains on upstream adaptor proteins and for coordinating the activation of IKK subunits (V. F.-S. Shih et al., 2011; S.-C. Sun, 2017; Vallabhapurapu & Karin, 2009; Q. Zhang et al., 2017).

1.8.5. Functional roles and therapeutic implications of the NFκB pathway

NFκB governs the transcription of over 200 genes involved in inflammation, innate and adaptive immunity, cell cycle progression, and apoptosis. In monocytes and macrophages, it promotes inflammatory activation, cytokine release and inflammasome priming. In lymphocytes, NFκB supports antigen receptor signaling, proliferation, and long-term survival (Guo et al., 2024; V. F.-S. Shih et al., 2011; Yu et al., 2020; Q. Zhang et al., 2017). Beyond its role in immunity, the pathway contributes to epithelial barrier maintenance, tissue regeneration, and responses to metabolic and genotoxic stress. Dysregulated NFκB signaling is implicated in a wide range of diseases, including autoimmune disorders, chronic inflammation and cancer (Guo et al., 2024; Q. Zhang et al., 2017). Therapeutic strategies targeting various components of the pathway, such as proteasome inhibitors or selective IKK or NIK inhibitors, are under active investigation. However, preserving physiological immune responses while mitigating pathological signaling remains a central challenge (Guo et al., 2024; Yu et al., 2020). A deeper understanding of the context-specific regulation of NFκB is essential for the rational design of future targeted therapies.

2. Aims

Platelets are traditionally known for their central role in hemostasis and coagulation. However, accumulating evidence highlights their additional function as key modulators of immune responses. As immunologically active cells, platelets influence various leukocyte populations and promote the production and release of pro-inflammatory cytokines. Among these interactions, the crosstalk between platelets and monocytes has gained increasing attention due to its relevance in both physiological immune surveillance and inflammatory disease contexts.

Classical $CD14^+$ monocytes represent the predominant subset of circulating monocytes in human blood and play a critical role in host defense by releasing inflammatory mediators such as IL-1β and TNFα. These cytokines are central to inflammatory responses but must be tightly regulated to prevent pathological outcomes such as cytokine storms or immune paralysis, as observed in conditions like sepsis or viral infections. Previous studies, including our own, have demonstrated that platelets are essential for efficient cytokine production by monocytes, independent of classical co-stimulatory mechanisms. Depletion of platelets from peripheral blood monocytes resulted in significantly reduced monocyte cytokine secretion and altered gene expression following PRR stimulation. This effect was also observed in clinical samples from patients with ITP. Notably, supplementation with platelets was sufficient to restore monocyte responsiveness in ITP patients. Despite these insights, the molecular mechanisms by which platelets influence monocyte function remain incompletely understood. In particular, it is unclear whether this communication is contact-dependent or mediated indirectly by molecular components, such as platelet-derived EVs, and how this intercellular communication shapes downstream signaling pathways and transcriptional responses.

The aim of this thesis was to investigate the mechanisms by which platelets regulate monocyte immune function and to define the extent and nature of this interaction. Specifically, the following objectives were addressed:

1. Validate the previously observed functional dependency of primary human monocytes on platelets using *ex vivo* models with platelet-depleted cells.
2. Identify candidate proteins transferred from platelets to monocytes using mass-spec proteomics and biochemical approaches.

3. Functionally confirm the role of transferred proteins using genetically modified monocyte cell lines lacking key transcriptional regulators.
4. Characterize the molecular mechanisms underlying intercellular protein transfer, including vesicle-mediated communication.
5. Dissect the role of the NFκB signaling pathway in platelets and monocytes and assess how its pharmacological or genetic inhibition affects monocyte activation.
6. Establish protocols for mouse monocyte and platelet isolation and investigate if platelet-monocyte interactions are conserved in the mouse system.
7. Utilize genetically engineered mouse models to further explore the signaling pathways and functional relevance of specific platelet-derived factors.
8. Develop and apply cross-species experimental systems to investigate platelet-monocyte communication.

3. Material and Methods

3.1. Materials

3.1.1. Experimental models

Table 1: Stable cell lines.

Product	Company
THP-1 (ATCC TIB-202)	ATCC
MEG-1 (ATCC CRL-2021)	ATCC

Table 2: Mice.

Strain	Company
C57BL/6J wild-type	Charles River
C57BL/6J PF4-tdTomato	Charles River
Flip-R26-mT-mG	III Bonn (AG Wachten)

3.1.2. Tissue culture reagents, chemicals, commercial assays, antibodies buffers and media

Table 3: Tissue culture reagents.

Name	Source
Dulbecco's Modified Eagle's Medium (DMEM), with 4.5 g/L glucose, 2 mM L-glutamine, phenol red	Gibco
Dulbecco's Phosphate-Buffered Saline (PBS) (1x)	Gibco
Dialyzed fetal calf serum	III Bonn (AG Meissner)
Fetal calf serum (FCS)	Thermo Fisher Scientific
Hanks' Balanced Salt Solution (HBSS)	Gibco
L929-conditioned medium (BMDM generation)	III Bonn
OptiMEM	Gibco
Penicillin-Streptomycin (10 000 U/mL)	Thermo Fisher Scientific
Poly-L-lysine (0.1% aqueous solution)	Sigma
RPMI 1640 (1x) with L-glutamine	Gibco

SILAC RPMI 1640 Flex medium, without glucose, phenol red, L-arginine, L-glutamine, L-lysine	Gibco
TrypLE Express (1x) Enzyme	Thermo Fisher Scientific
Trypsin/Lys-C mix	Promega

Table 4: Chemicals and commercial assays.

Name	**Source**
4x Laemmli Sample Buffer	Bio-Rad
10x PBS	Pan Biotech
10x Tris/Glycine/SDS	Thermo Fisher Scientific
20x MOPS Buffer	Life Technologies
20x Tris-Buffered Saline (TBS) (400 mM Tris, 3 M NaCl, pH 7.4)	Santa Cruz Biotechnology
4-12% NuPAGE Bis-Tris gel	Life Technologies
16% Formaldehyde, methanol-free	Life Technologies
12-230 kDa Jess or Wes Separation Module, 25 capillary cartridges	ProteinSimple
[$^{13}C_6{}^{15}N_4$] L-arginine	Silantes
[$^{13}C_6{}^{15}N_2$] L-lysine	Silantes
Agarose	Biozym
Alt-R® CRIPRS-Cas9 tracrRNA	IDT
Alt-R™ S.p. Cas9 Nuclease V3	IDT
Benzonase nuclease	Sigma-Aldrich
Bode Cutasept® F Hautantiseptikum	Hartmann
Bovine serum albumin (BSA)	Thermo Fisher Scientific
Bond-Breaker TCEP	Thermo Fisher Scientific
CellTiter-Blue™ cell viability assay	Promega
Chloroacetamide (CAA)	Sigma-Aldrich
cOmpleteTM EDTA-free protease inhibitor cocktail tablets	Roche Diagnostics

CyQUANT™ LDH Cytotoxicity Assay	Thermo Fisher Scientific
CD11b MicroBeads, human and mouse	Miltenyi Biotec
Dimethylsulfoxide (DMSO), cell culture grade	PanReac AppliChem
Ethanol absolute (molecular biology grade)	Thermo Fisher Scientific
Ethylenediaminetetraacetic acid (EDTA) (0.5 M, pH 8.0)	Life Technologies
FcR blocking reagent, human	Miltenyi Biotec
FcR blocking reagent, mouse	Miltenyi Biotec
Formaldehyde (16% aqueous solution, methanol-free)	Thermo Fisher Scientific
Ficoll® Paque PLUS	GE Healthcare
Goat serum	Life Technologies
Human IL-1β kit (HTRF®)	Cisbio
Human TNFα kit (HTRF®)	Cisbio
LentiArrayTM Human Whole Genome CRISPR Library	Invitrogen
Methanol	Roth
Mouse IL-1β kit (HTRF®)	Cisbio
NEBNext High-Fidelity 2X PCR Master Mix	New England Biolabs
NuPAGE LDS sample buffer (4x)	Life Technologies
NuPAGE sample reducing agent (10x)	Life Technologies
PageRuler™ Plus prestained protein ladder	Thermo Fisher Scientific
PhosSTOP easypack phosphatase inhibitor cocktail tablets	Roche
Poly-L-lysine	Sigma-Aldrich
Pierce™ 660 nm Protein Assay	Thermo Fisher Scientific
Pierce™ bicinchoninic acid (BCA) Protein-Assay	Thermo Fisher Scientific
PVDF membrane Immobilon	FL Milipore
QIAquick Gel Extraction Kit	QIAGEN
QIAquick PCR Purification Kit	QIAGEN
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
Styrol-divinylbenzene Reversed Phase Sulfonate (SDB-RPS)	Affinisep

Immobilon-FL polyvinylidene difluoride (PVDF) membrane	Millipore
Triton X-100	Carl Roth
Tris HCl pH 7.4 / 8.0	Carl Roth
Isopropanol	Carl Roth
Thrombin from human plasma	Sigma Aldrich
Tween 20	Carl Roth
UltraComp eBeads™ compensation beads	Invitrogen

Table 5: Buffers and media.

Name	Composition
1x TBS	100 mL 10x TBS (final: 1x), water to 1 L
1x TBS-T	100 mL 10x TBS (final: 1x), 1 mL Tween 20 (final: 0.01x), water to 1 L
Blocking buffer	3% (v/v) BSA in 1x TBS
Binding buffer	3% (v/v) BSA in 1x TBS-T
Buffer A	0.1% (v/v) formic acid in LC-MS-grade water
Buffer B	80% (v/v) acetonitrile, 0.1% (v/v) formic acid in LC-MS-grade water
Buffer R	2% (v/v) acetonitrile, 0.1% (v/v) formic acid in LC-MS-grade water
Buffer X	5% (v/v) ammonia, 80% (v/v) acetonitrile in LC-MS-grade water
Complete DMEM	10% (v/v) FCS, 1% (v/v) Penicillin-Strepomycin
Complete RIPA	5 ml RIPA buffer (2x), 3.4 ml H_2O, 400 µl c0mplete™ EDTA-free protease inhibitor cocktail (50x), 1 ml PhosSTOP™ easypack phosphatase inhibitor cocktail (10x)

DMEM for BMDM differentiation	Complete DMEM + 20% (v/v) L929
FACS buffer	PBS + 2% (v/v) FCS
Lysis buffer	0.4 mg/mL proteinase K, 2 mM $CaCl_2$, 6 mM $MgCl_2$, 2 mM EDTA, 2% (v/v) Triton X-100, 20 mM Tris, pH 7.5
RPMI for THP-1 an MEG-01 cell culture (complete RPMI)	10% (v/v) FCS, 1% (v/v) Penicillin-Strepomycin, 1x (v/v) Glutamax, 1x (v/v) HEPES, 1x (v/v) sodium pyruvate
SILAC RPMI flex medium	10% (v/v) dialyzed FCS, 1% (v/v) Penicillin-Strepomycin, Glutamax, 1x (v/v) HEPES, 1x (v/v) sodium pyruvate, 4.5 g/L D-glucose, 42 g/L [$^{13}C_6{}^{15}N_4$] L-arginine, 73 g/L [$^{13}C_6{}^{15}N_2$] L-lysine
Radioimmunoprecipitation assay (RIPA) lysis 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
Transfer buffer	100 mL 10x Tris-glycine (final: 1x), 150 mL methanol (final: 6.7x), water to 1 L

Table 6: Low-molecular-weight inhibitors and activators of biological processes.

Name	**Source**	**Activity**
(-)-DHMEQ	MedChemExpress (MCE®)	NFκB inhibitor (RelA + RelB inhibitor)
Lipopolysaccharide (LPS)	Invivogen	TLR4 agonist
Nigericin	Thermo Fisher Scientific	NLRP3 activator
Pam3CysK4	InvivoGen	TLR1/2 agonist
ACHP	MCE	NFκB inhibitor (IKKα and IKKβ inhibitor)
B022	MCE	NFκB inhibitor (NIK inhibitor)
IKK16	MCE	NFκB inhibitor (IKKα and IKKβ inhibitor)

TPCA-1	MCE	NFκB inhibitor (IKKβ inhibitor)
XT2	MCE	NFκB inhibitor (NIK inhibitor)

Table 7: Primary and secondary antibodies for Western blot and WES.

Name	Source	Dilution
Anti-Cre recombinase	Invitrogen	1:1000
Anti-Mouse Detection Module for Jess/Wes	Biotechne	-
Anti-Rabbit Detection Module for Jess/Wes	Biotechne	-
Human β-actin (mouse)	LI-COR	1:1000
Rat IRDye 680 (goat)	LI-COR	1:20 000
Mouse IRDye 800 (donkey)	LI-COR	1:20 000
NFκB1 p50/105 (mouse)	Invitrogen	1:50
NFκB2 p52/100 (rabbit)	Cell signaling	1:50
NFκB p65 (L8F6)	Cell signaling	1:50

Table 8: Human blocking and staining antibodies for flow cytometry.

Name	Company	Clone	Dilution
CD41a FITC	eBioscience	HIP8	1:50
CD14 APC	eBioscience	61D3	1:200
CD45 PE	eBioscience	2D1	1:200
CD62P APC	eBioscience	AK-4	1:50
FcR blocking reagent	Miltenyi Biotec	-	1:10

Table 9: Mouse blocking and staining antibodies for flow cytometry.

Name	Company	Clone	Dilution
CD45 FITC	BioLegend	30-F11	1:200
CD41a APC	BioLegend	MWReg30	1:50
CD11b PE	eBioscience	M1/70	1:200

Ly6C eFluor450	Invitrogen	HK1.4	1:200
FcR blocking reagent	Miltenyi Biotec	-	1:10

3.1.3. Laboratory plastics, equipment and software

Table 10: Laboratory equipment.

Device	**Company**
0.1-2 µL, 2-20 µL, 20-200 µL, 100-1000 µL pipettes	Mettler-Toledo/Rainin
2-20 µL, 20-200 µL 12-channel pipette	Mettler-Toledo/Rainin
4 °C fridge	Liebherr
-20 °C freezer	Liebherr
-80 °C freezer	Thermo Fisher Scientific
-150 °C freezer	Sanyo Biomedical
Centrifuges	Eppendorf
Epoch Microplate Spectrophotometer	BioTek
Flow cytometer Attune NxT	Thermo Fisher Scientific
Flow cytometer BD Canto	BD Bioscience
Gene Pulser Xcell™ Electroporation System	Bio-Rad
Heatblock Thermomixer	Eppendorf
Incubator for tissue culture	Sanyo Biomedical
LI-COR Odyssey® Infrared Imaging System	LI-COR Biosciences
MidiMACS™ separator	Miltenyi Biotec
Mini-PROTEAN Tetra Vertical Electrophoresis Cell	Bio-Rad
MiSeq Sequencing System	Illumina
Mr. Frosty Freezing Container	VWR
NanoDrop spectrophotometer	Thermo Fisher Scientific
Orbitrap Exploris 480 mass spectrometer	Thermo Fisher Scientific
SpectraMax i3 Multi-Mode Microplate Reader	Molecular Devices
Vanquish Neo chromatographic system	Thermo Fisher Scientific
Vi-CELL BLU Cell Analyzer	Beckman Coulter
Wide-field fluorescent microscope Zeiss Observer.Z1	Carl Zeiss Jena

Table 11: Laboratory plastics.

Product	Company
20 µL, 200 µL, 1000 µL filtered and unfiltered tips	Mettler-Toledo/Rainin
0.45 µm filters	BD Bioscience
70 µm cell strainer	Greiner Bio-One
10 cm, 15 cm tissue culture-treated dishes	Greiner Bio-One
14 mL tubes for bacterial cultures	Braun Melsungen
15 mL, 50 ml tubes (Falcon-type)	Eppendorf
5 mL polystyrene flow cytometry tubes	Sarstedt
5 mL, 10 mL, 25 mL serological pipettes	Greiner Bio-One
6-well, 12-well, 24-well plates, tissue culture-treated	Sarstedt/Greiner Bio-One
96-well plates, flat-bottom, tissue culture-treated	Sarstedt/Greiner Bio-One
96-well plates, U-bottom, tissue culture-treated	Sarstedt/Greiner Bio-One
96-well plates, V-bottom, tissue culture-treated	Sarstedt/Greiner Bio-One
384-well microplates, small-volume, white (for HTRF)	Greiner Bio-One
Amicon® Ultra Centrifugal Filter, 100 kDa MWCO	Milipore/Merck
Cell scrapers	Labomedic
Cryotubes 2 mL	Sarstedt
Liquid reservoirs, 50 mL	VWR
Liquid reservoirs, 12-channel	Carl Roth
MACS® Cell Separation Columns	Miltenyi Biotec
Needles, 20G (for bone flushing)	B. Braun
Needles (Butterfly-needle), 20G, 21G (for peripheral blood collection from humans and cardiac blood collection from mice)	B. Braun
Opti-Seal optical disposable adhesive	Falcon
PhenoPlate™ Microplates, 96-well, flat-bottom, tissue culture treated, black	Revvity
S-Monovette® Citrate 3.2 %, (LxØ): 92 x 15 mm	Sarstedt
S-Monovette® K3 EDTA, (LxØ): 92 x 16 mm	Sarstedt
S-Monovette® Serum, (LxØ): 92 x 16 mm	Sarstedt

Safety-Multifly® needle, 20G x 3/4", 200 mm	Sarstedt
T-25, T-75, T-175 tissue culture flasks, tissue culture-treated	Perkin Elmer
Whatman® cellulose chromatography paper, 3 mm CHR	Whatman

Table 12: Software.

Name	Version	Company
Adobe Illustrator	29.5	Adobe
Browsergenome.org	1.1	Schmid-Burgk et al., 2015
Compass for Simple Western	4.0.0	Protein Simple
Fiji (ImageJ)	2.0.0	Java
GraphPad Prism	10.5.0	GraphPad Software, LLC
MaxQuant software suite	2.0.3.0	MaxQuant
Mendeley	2.135.0	Mendeley Ltd.
Microsoft Excel	16.78.3	Microsoft
Microsoft PowerPoint	16.78.3	Microsoft
Microsoft Word	16.78.3	Microsoft
NTA software	3.1	Malvern Panalytical
OutKnocker.org	2.0 Beta	Schmid-Burgk et al., 2014
Perseus software suite	1.6.15	MaxQuant
SoftMax Pro	6.3.0	Molecular Devices
ZEN Pro	2.3	Zeiss

3.2. Methods

3.2.1. Study subjects

Primary human cells were isolated from whole blood collected from healthy volunteers who provided written informed consent, in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the University of Bonn (Protocol No. 282/17).

3.2.2. Human monocyte isolation

Peripheral venous blood was collected from healthy volunteers in S-Monovette® K3EDTA. Whole blood was diluted 1:3 in phosphate-buffered saline (PBS) and layered over Ficoll® Paque PLUS for density gradient centrifugation (700 x g, 20 minutes (min), acceleration 1, no brake, room temperature (RT)). The peripheral blood mononuclear cell (PBMC) layer was collected, washed with PBS, and used for monocyte isolation. Primary human monocytes were isolated from PBMCs by negative selection using the EasySep™ Human Monocyte Isolation Kit, following the manufacturer's protocol. PBMCs were resuspended in cold PBS containing 2% fetal calf serum (FCS) and 1 mM EDTA, and incubated with the Monocyte Isolation Cocktail (50 µL/mL) in 5 mL polystyrene tubes. To obtain platelet-depleted monocytes (PDMo), an additional Platelet Removal Cocktail (50 µL/mL) was added. After 5 min of incubation at RT, EasySep™ D Magnetic Particles were added and incubated for another 5 min. Cells were separated using an EasySep™ Magnet, and both standard monocytes (StdMo) and PDMo were collected. Monocyte purity was assessed by flow cytometry (Method 3.2.12).

3.2.3. Human platelet isolation

Human platelets were isolated from venous blood collected from healthy volunteers in S-Monovette® 9NC tubes, following procedures described previously (Alard et al., 2015, Hawwari & Rossnagel et al., 2024) with modifications. Whole blood was centrifuged at 340 x g for 15 min (acceleration 1, no brake) at RT to obtain platelet-rich plasma (PRP), which was carefully collected and transferred to a new 15 mL tube containing 200 nM Prostaglandin E_1 (PGE_1) to prevent platelet aggregation. PRP was mixed 1:1 with supplement-free pre-warmed PBS and centrifuged at 240 x g for 10 min to pellet leukocyte contaminants (acceleration 1, brake 1). The resulting platelet-

containing supernatant was transferred to a new tube containing 200 nM PGE_1 and centrifuged at 700 x g for 15 min to pellet the platelets. The platelet pellet was then washed once with pre-warmed PBS containing 200 nM PGE_1 and resuspended in supplement-free pre-warmed RPMI medium. Subsequently, platelet counts were determined using a Casy cell counter, and the platelet concentration was adjusted to $2x10^8$ platelets/mL. Platelet purity and activation status were assessed by flow cytometry (Method 3.2.12).

3.2.4. Human serum isolation

Peripheral blood was collected via venipuncture into serum collection tubes. The tubes were left undisturbed at RT for 30 min to allow coagulation. Subsequently, the contents of all tubes were pooled into a 50 mL Falcon tube and centrifuged at 1500 x g for 10 min at RT. The supernatant was transferred to a new Falcon tube and centrifuged again at 3000 x g for 10 min at RT to ensure complete removal of residual cells and debris. The resulting supernatant was transferred to a fresh tube, and residual cell content was assessed using a CASY cell counter to confirm the absence of cells. Only cell-free serum was used for subsequent experiments, prepared in serial dilutions ranging from 100% to 6.25%, using supplement-free RPMI as the diluent in 100 µl total volume.

3.2.5. Human plasma isolation

Peripheral blood was collected via venipuncture into EDTA tubes to prevent coagulation. The contents were pooled into a 50 mL Falcon tube and centrifuged at 1500 x g for 10 min at RT. The resulting PRP was transferred to a new Falcon tube and centrifuged at 3000 x g for 10 min at RT to pellet all cells and cellular debris. A small volume of PRP was retained for subsequent cell count analysis using a CASY cell counter. The supernatant, representing platelet-poor plasma (PPP), was transferred to a new tube. Both PRP and PPP samples were analyzed using the CASY system to confirm the absence of cells. Only cell-free PPP was used for downstream experiments, diluted in supplement-free RPMI to final dilutions ranging from 100% to 6.25% in 100 µl total volume.

3.2.6. Mice

C57BL/6J wild type, PF4-Cre-tdTomato and Flip-R26-mT-mG mice were obtained from Charles River Laboratories or from working groups at the Institute of Innate Immunity (Bonn) and housed in the House for Experimental Therapy (HET) facility at the University Hospital of Bonn under standard specific pathogen-free (SPF) conditions.

3.2.7. Mouse blood monocyte isolation

Mice were euthanized by CO_2 inhalation and death was confirmed by the absence of the hindlimb reflex. Cardiac blood was collected from the still-beating heart via intracardiac puncture using 21G needles and syringes pre-coated with sodium citrate. Blood from three to four mice was pooled into EDTA-containing tubes (1.5-2 mL per tube) and diluted with 5-6 mL PBS in 15 mL conical tubes. The diluted blood was layered over 4.5 mL Ficoll® Paque PLUS and centrifuged at 850 x g for 20 min at 22 °C (acceleration 1, no brake). The PBMC layer was carefully collected, filtered through a 40 µm nylon mesh, and diluted to 15 mL with PBS. Cells were pelleted by centrifugation at 550 x g for 10 min at 22 °C. Mouse monocytes were isolated using CD11b MicroBeads following the manufacturer's instructions, with modified centrifugation steps at 550 x g instead of 300 x g. Briefly, the cell pellet was resuspended in 90 µL of MACS buffer per 10^7 total cells and 10 µL of CD11b MicroBeads were added per 10^7 cells. After 15 min of incubation at 4 °C, cells were washed with 1-2 mL MACS buffer per 10^7 cells and centrifuged. Up to 10^8 cells were resuspended in 500 µL MACS buffer and applied to MACS separation columns. The columns were washed three times with 500 µL MACS buffer and $CD11b^+$ monocytes were eluted. Final monocyte suspensions were adjusted to $2x10^6$ cells/mL. Quality control and immunophenotyping was assessed by flow cytometry (Method 3.2.12).

3.2.8. Bone marrow-derived macrophage isolation and differentiation

Bone marrow (BM) was isolated from age-matched C57BL/6J Flip-R26-mT-mG mice (6-24 weeks old). Following euthanasia, femurs and tibias were carefully dissected from the hind limbs and briefly washed in PBS, followed by a short disinfection in 70% ethanol. Bone marrow was flushed from the bones using a syringe with approximately 10 mL of ice-cold PBS and filtered through a 70 µm cell strainer to obtain a single-cell

suspension. Cells were pelleted by centrifugation at 340 x g for 5 min at 4 °C. The resulting cell pellet was either cryopreserved in FCS supplemented with 10% DMSO and stored at -150 °C, or directly resuspended in complete DMEM supplemented with 20% L929-conditioned medium to support macrophage differentiation. Cells were seeded in 10-cm tissue culture dishes at a density corresponding to two dishes per mouse leg. Differentiation into bone marrow-derived macrophages (BMDM) was carried out over six days in a humidified incubator at 37 °C and 5% CO_2. After differentiation, adherent BMDMs were washed once with 10 mL PBS, detached using a cell scraper in 10 mL cold PBS, and collected by centrifugation at 340 x g for 5 min at 4 °C. Cells were then resuspended in complete DMEM, and the cell density was determined using a VI-CELL BLU automated cell counter or manually using a Neubauer hemocytometer. The final cell suspension was adjusted to a concentration of $1x10^6$ cells/mL. For experiments, BMDMs were seeded in 96-well plates at a density of $1x10^5$ cells per well. Cells were rested for 16-18 h at 37 °C and 5% CO_2 before stimulation.

3.2.9. Mouse platelet isolation

Cardiac blood was collected as mentioned above. Blood from three to four mice was pooled into citrate-containing tubes and diluted 1:2 with pre-warmed Hanks' Balanced Salt Solution (HBSS). Samples were centrifuged at 250 x g for 10 min at 22 °C (acceleration 1, no brake) to obtain PRP, which was carefully transferred to a new tube and treated with 100 nM PGE_1 to prevent activation. Platelets were then pelleted by centrifugation at 1250 x g for 20 min at 22 °C (acceleration 1, no brake), and resuspended in supplement-free RPMI. The final platelet concentration was adjusted to $2x10^8$ platelets/mL. Platelet purity was assessed by flow cytometry (Method 3.2.12).

3.2.10. Generation of platelet supernatants

Platelet supernatants were prepared from suspensions of $1x10^8$ platelets resuspended in supplement-free RPMI. Platelets were either left untreated or stimulated with LPS (2 ng/mL) for 3 h or Thrombin (1 U/mL) for 30 min at 37 °C. Following incubation, platelets were pelleted by centrifugation at 700 x g for 10 min (acceleration 1, no brake). The resulting supernatant was transferred to a new tube and further clarified by centrifugation at 3000 x g for 10 min. The final platelet supernatants were collected

and used for downstream stimulations. To characterize vesicle content, aliquots were analyzed using the NanoSight NS300 nanoparticle tracking system (Method 3.2.12).

3.2.11. Generation of vesicle-free platelet supernatant

For generation of vesicle-free platelet supernatant, platelets were isolated and platelet supernatants prepared as described above. Once cell-free, the cell-free supernatant was centrifuged again at 3000 x g for 10 minutes and subsequently sterile-filtered through a 0.2 µm membrane filter to remove residual particles and cell debris. The filtered supernatant was then subjected to ultrafiltration using 100 kDa molecular weight cutoff Amicon® Ultra-50 centrifugal filter units, following the manufacturer's protocol. The resulting flow-through, representing vesicle-free platelet supernatant, was validated using the NanoSight NS300 system to confirm the absence of vesicles (Method 3.2.12).

3.2.12. Flow cytometry for characterization of isolated human and mouse monocytes and platelets

Flow cytometric analyses were performed using an Attune NxT or a BD FACS Canto to assess the purity of isolated primary human and mouse cells, as well as the activation status of human and mouse platelets. For immune cell analysis, cells were transferred to 96-well V-bottom plates, washed and maintained in FACS buffer (PBS supplemented with 2% FCS). Cells were incubated with Fc receptor (FcR) Blocking Reagent for 20 min at 4 °C, followed by washing with FACS buffer. Subsequently, cells were stained with fluorescently-labeled monoclonal antibodies for 30 min at 4 °C in the dark. After staining, cells were washed three times with FACS buffer and immediately analyzed by flow cytometry. To evaluate the purity of isolated primary human monocytes, cells were stained with fluorochrome-conjugated monoclonal antibodies targeting CD14, CD45 and CD41a. CD14 and CD45 served as markers for monocytes and leukocytes, respectively, while CD41a was used to assess platelet contamination and to distinguish between StdMo and PDMo. For mouse monocyte characterization, cells were stained with antibodies against CD45, CD11b, Ly6C and CD41a to assess leukocyte identity, monocyte subsets, and platelet contamination. For platelet analysis, isolated platelets were seeded into 96-well U-bottom plates and incubated with FcR Blocking Reagent for 15 min at 37 °C in a humidified atmosphere with 5% CO_2. To evaluate the activation status of the platelets, cells were either left unstimulated or

stimulated with 1 U/mL human thrombin for 30 min at 37 °C in a 5% CO_2 atmosphere (to serve as a positive control for activation). Platelets were then stained with fluorescently-labeled monoclonal antibodies for 30 min at RT in the dark. Samples were directly analyzed using the aforementioned flow cytometers. Isolated platelets were stained with CD41a as a platelet-specific marker, CD62P assess the activation status, and CD45 to detect leukocyte contamination. All cells were analyzed using FlowJo.

3.2.13. Vesicle characterization and quality control

Aliquots from each experimental condition involving vesicle enrichment or depletion were analyzed using the NanoSight NS300 instrument to assess vesicle content and ensure quality control. Each sample was diluted 1:10 in PBS (final volume: 1 mL) and loaded into the analysis chamber. Each sample was recorded in triplicate for 60 seconds (sec) and analyzed using the NTA software. To prevent cross-contamination, the chamber was rinsed twice with PBS between each sample. A background control using PBS alone was recorded at the beginning of each measurement session.

3.2.14. Cell lines

All cell lines used in this study were maintained under standard culture conditions at 37 °C and 5% CO_2. The human monocytic cell line THP-1 (ATCC TIB-202) was cultured in non-tissue culture-treated flasks using RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 1% Penicillin/Streptomycin, 1% HEPES, and 1% GlutaMAX, defined from now on as complete RPMI. Cells were maintained at densities between $2x10^5$ and $5x10^5$ cells/mL and used up to passage 15. The human megakaryocytic cell line MEG-1 (ATCC CRL-2021) was cultured in tissue culture-treated flasks also with complete RPMI 1640 medium. MEG-1 cells were maintained at densities between $1x10^5$ and $3x10^5$ cells/mL and used up to passage 20. For the SILAC experiments, MEG-01 cells were cultured in SILAC RPMI flex medium, supplemented with 10% heat-inactivated and dialyzed FCS (from AG Meissner), 1% Penicillin/Streptomycin, 1% HEPES, 1% GlutaMAX and further supplemented with 4.5 g/L D-glucose, 42 g/L [$^{13}C_6{}^{15}N_4$] L-arginine, 73 g/L [$^{13}C_6{}^{15}N_2$] L-lysine.

3.2.15. Freezing and thawing of cell lines

Frozen cell stocks were prepared from early-passage cultures. Approximately 3-5x10^6 cells were resuspended in 1 mL of ice-cold freezing medium consisting of 90% FCS and 10% DMSO. The suspension was aliquoted into pre-labeled cryovials, which were then placed in a Mr. Frosty™ freezing container and stored at −80 °C overnight to ensure controlled-rate freezing. The following day, cryovials were transferred to −150 °C for long-term storage. For recovery, cryovials were thawed in a 37 °C water bath, and the contents were immediately diluted with pre-warmed complete medium and transferred to culture flasks for cultivation.

3.2.16. CRISPR-Cas9-mediated genome editing of THP-1 cells

Genome editing of THP-1 monocytes was performed using the CRISPR-Cas9 system according to established protocols (Schmid-Burgk et al., 2014), with assistance from Marius Jentsch (Prof. Schmid-Burgk's lab) and Salie Maasewerd. Briefly, 0.5 nmol of Alt-R CRISPR custom crRNAs targeting early exons were mixed with 0.5 nmol tracrRNA in nuclease-free Duplex Buffer and incubated at 95 °C for 5 min, followed by 10 min at RT to allow formation of the guide RNA duplex. Subsequently, 25 µg of Alt-R *S. pyogenes* Cas9 Nuclease V3 was added and incubated for an additional 10 min at RT to generate the ribonucleoprotein (RNP) complex. In parallel, 2.5x10^6 THP-1 cells were washed once with PBS, resuspended in 250 µL OptiMEM and combined with the gRNA-Cas9 RNP complex. After 20 min of incubation at RT, electroporation was carried out using the Gene Pulser Xcell™ Electroporation System under the following conditions: 250 V, 950 µF, exponential decay mode. Cells were immediately transferred to a 6-well plate containing 3 mL of warm complete RPMI medium and cultured under standard conditions (37 °C, 5% CO_2). To generate monoclonal THP-1 knockout (KO) cell lines, cells were harvested 72 h post-electroporation and seeded by limiting dilution at 0.5 cells per well in 100 µL of complete RPMI medium in U-bottom 96-well plates. Four plates were seeded per target. After 3-4 weeks, wells were screened microscopically to identify viable single-cell-derived colonies. For genotyping, 5 µL of cells from each growing clone were lysed in 5 µL of lysis buffer (0.4 mg/mL proteinase K, 2 mM $CaCl_2$, 6 mM $MgCl_2$, 2 mM EDTA, 2% Triton X-100, 20 mM Tris, pH 7.5). Lysates were incubated at 65 °C for 10 min and 95 °C for 15 min. 1 µL of the lysate was used in a PCR reaction containing 3 µL NEBNext PCR

MasterMix, 1 µL water, and 1 µL target-specific forward and reverse primers (0.5 µM final concentration). PCR was performed using the manufacturer's protocol (annealing temperature: 60 °C, elongation: 20 secs, 20 cycles). A second-round PCR using barcode primers unique to each clone was performed with 1 µL of the first reaction. PCR products were pooled and separated on a 2% pre-cast E-Gel EX agarose gel using the E-Gel Precast Gel Electrophoresis System. DNA bands (~450 bp) were excised and purified using the QIAquick Gel Extraction Kit, followed by further purification using the QIAquick PCR Purification Kit. DNA was eluted in 30 µL water and quantified with a NanoDrop spectrophotometer. Amplicons were sequenced on an Illumina MiSeq system according to the manufacturer's protocol. FASTQ sequencing files were analyzed using the online tool Outknocker.org to determine genome editing efficiency (Schmid-Burgk et al., 2014). Up to 96 clones were screened per gene, and a maximum of 10 verified KO clones per target were selected for downstream functional validation. Everything was executed under the supervision and help of Marius Jentzsch and Salie Maasewerd.

3.2.17. Stimulation assays

Primary monocytes, THP-1 cells, or BMDMs were seeded at a density of $1x10^5$ cells per well into 96-well F-bottom plates. Platelets were either cultured alone or co-cultured with PDMo, THP-1 cells, or BMDMs at ratios ranging from 1:100 (corresponding to $1x10^7$ platelets per well) to 1:10. For experiments involving platelet supernatants, 50 µL of supernatant was added to 1 $x10^5$ cells resuspended in 50 µL of medium. In plasma or serum stimulation assays, titrations were performed using volumes ranging from 100 µL to 6.25 µL (corresponding to 100% to 6.25%), diluted in supplement-free RPMI. Platelets, serum, and plasma alone were included as individual controls. For inflammasome activation assays, cells were first primed with TLR agonists for 3 h at 37 °C and 5% CO_2. Specifically, Pam3CysK4 (1 µg/mL for THP-1 cells) was used to activate TLR1/2, while LPS was used to activate TLR4 (2 ng/mL for human cells; 1 µg/mL for mouse cells). After priming, cells were stimulated with the NLRP3 inflammasome activator Nigericin (10 µM for both human and mouse cells) for 1.5 h. For NFκB inhibition experiments, either monocytes or platelets were pre-treated with one of six NFκB pathway inhibitors: ACHP, IKK16, TPCA-1, XT2, B022 or (-)-DHMEQ. Cells were incubated with inhibitor concentrations ranging from 0 to 50 µM for 16-18 h, followed by PBS washing before priming and activation steps. The solvent

used to prepare the inhibitors served as the vehicle control at its highest concentration. After stimulation, cell-free supernatants were typically collected for cytokine and LDH measurement (Method 3.2.18), while the cells were used for cell viability assays (Method 3.2.19), cell lysis (Method 3.2.20), or microscopy (Method 3.2.25).

3.2.18. Cytokine measurement

Cytokine concentrations in cell culture supernatants were measured using two complementary approaches. For quantification of individual cytokines such as TNFα and IL-1β, a homogeneous time-resolved fluorescence (HTRF) assay was performed using HTRF® kits according to the manufacturer's protocol. In a white 384-well microplate, 12 µL of (diluted) sample or standard was mixed with 1.5 µL each of donor and acceptor antibodies. Plates were centrifuged at 1000 x g for 1 min and incubated at 4 °C for 16 h. Fluorescence signals were measured at 620 nm (donor) and 665 nm (acceptor) using a SpectraMax i3 plate reader.

3.2.19. Cell viability assays

Pyroptotic cell death was assessed by quantifying lactate dehydrogenase (LDH) released into cell culture supernatants using the CyQUANT™ LDH Cytotoxicity Assay according to the manufacturer’s instructions. For accurate measurement, freshly collected supernatants were used. In a clear 384-well flat-bottom plate, 12.5 µL of supernatant was mixed with 12.5 µL of LDH assay reagent. Plates were centrifuged at 1000 x g for 1 min and incubated for 30 min at 37 °C in the dark. To assess overall cell viability, both the LDH assay and the CellTiter-Blue® (CTB) Cell Viability Assay were applied. After stimulation, supernatants were collected for LDH analysis, while the remaining cells were incubated with CTB reagent for 60-120 min at 37 °C and 5% CO_2. In both assays, cells treated with Triton X-100 served as a 100% cell death control. All measurements were conducted using the SpectraMax i3 plate reader.

3.2.20. Cell lysis

Cells were placed on ice and washed once with ice-cold PBS to remove residual medium. Lysis was performed directly on the plate using a cell scraper in 50-100 µL per well of ice-cold radioimmunoprecipitation assay (RIPA) buffer supplemented with cOmplete™ EDTA-free protease inhibitor cocktail (Table 5). Cells were incubated in

lysis buffer for 15 min on ice to ensure complete lysis, after which the lysates were transferred to 1.5 mL microcentrifuge tubes. Samples were centrifuged at 1000 x g for 15 min at 4 °C to remove cell debris and nuclei. The resulting supernatants, containing the cytoplasmic and nuclei-free protein fractions, were transferred to fresh 1.5 mL tubes and stored at -80 °C until further processing.

3.2.21. Protein concentration measurement

Protein concentrations were determined using the bicinchoninic acid (BCA) assay according to the manufacturer's instructions. Bovine serum albumin (BSA) standards and diluted protein samples were pipetted in duplicates into a 96-well microplate. BCA working reagent was added to each well, and the plate was incubated at 37 °C for 30 min. Absorbance was measured at 562 nm using a microplate reader. Protein concentrations of unknown samples were calculated by interpolation from the BSA standard curve.

3.2.22. Capillary western immunoassay (WES)

Protein expression was analyzed using the Wes capillary-based immunoassay system. Protein concentrations were first determined using the BCA protein assay and samples were prepared at a concentration of 5 µg/µL. Whole-cell lysates or supernatants were diluted in complete RIPA buffer and mixed at a 4:1 ratio with 5x Fluorescent Master Mix, which contained fluorescently labeled standards, 200 mM DTT and sample buffer. The samples were then denatured at 95 °C for 5 min. After denaturation, the prepared samples, biotinylated molecular weight ladder (applied to the first capillary as a reference), blocking reagent, primary antibodies (all diluted 1:50), HRP-conjugated secondary antibodies, and chemiluminescent substrate were loaded into a designated microplate pre-filled with running buffer. Each capillary operates independently and uses internal fluorescent standards to normalize separation distances. The plate was placed into the Wes system, where automated electrophoretic separation and immunodetection were performed. Chemiluminescent signals were detected and quantified using Compass for Simple Western software, which provided molecular weight estimation and signal intensity for target proteins.

3.2.23. Western Blot: SDS-PAGE and immunoblotting

For Western blot analysis, 20 µg of total protein per sample were mixed with 4x lithium dodecyl sulfate (LDS) sample buffer and 10x reducing agent, followed by denaturation at 95 °C for 10 min. Samples were either stored at -20 °C or immediately used for electrophoresis. Proteins were separated on 4-12% NuPAGE™ Bis-Tris gradient gels in MOPS running buffer under denaturing and reducing conditions. Electrophoresis was performed at 75 V for 10 min and then at 150 V for 1 h. A PageRuler Plus prestained protein ladder (3 µL) was loaded as a molecular weight marker. Following separation, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes in transfer buffer (Table 5) at 32 V for 1 h. Membranes were blocked in blocking buffer (Table 5) for at least 1 h at RT with gentle agitation and then incubated overnight at 4 °C with primary antibodies diluted in binding buffer. The next day, membranes were washed three times with 1x Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and incubated for 2 h at RT with fluorescently labeled secondary antibodies. After two further washes in TBS-T and one final wash in 1x TBS, membranes were imaged using the LI-COR Odyssey infrared imaging system. Antibodies were used at the following dilutions: anti-Cre 1:1000, anti-β-actin 1:1000, and secondary antibodies (anti-rat IRDye 680 and anti-mouse IRDye 800) 1:20 000.

3.2.24. Coating of microscopy plates

To enhance cell adhesion for microscopy experiments, 96-well F-bottom plates with black walls were coated in-house with poly-L-lysine. A volume of 75 µL per well of a 0.01% aqueous poly-L-lysine solution was added and the plates were incubated at room temperature for 30 to 90 min. After incubation, the coating solution was removed, and the plates were centrifuged upside-down at 1000 x g for 5 min at RT to dry.

3.2.25. Widefield fluorescence microscopy

Following cell stimulation and fixation with formaldehyde in black 96-well F-bottom plates, samples were stored at 4 °C and imaged within one week. Imaging was performed using a Zeiss Observer.Z1 widefield fluorescence microscope (Microscopy Core Facility of the Medical Faculty, University of Bonn) equipped with either a 20x LD Plan-Neofluar objective (numerical aperture 0.4) or a 20x Plan-Apochromat objective (numerical aperture 0.8). The microscope was operated using Zeiss Zen Pro 2.3

software. The lower and upper limits of the lookup table were linearly adjusted to optimize visualization, and all images from a given experiment were processed using the same parameters. Images were exported as TIFF files using Zeiss Zen Lite software. No nonlinear modifications were applied, and any additional adjustments for figure preparation were performed using Microsoft PowerPoint or Adobe Illustrator.

3.2.26. SILAC-based quantitative proteomics and sample preparation for Liquid chromatography-mass spectrometry (LC-MS)

Mass spectrometry-based proteomics combined with stable isotope labeling by amino acids in cell culture (SILAC) was used to quantify protein expression changes with the assistance of Dr. Sebastian Kallabis and Anushka Kudaliyanage. The human megakaryocytic cell line MEG-01 was cultured in SILAC RPMI 1640 Flex medium lacking D-glucose, L-arginine, L-lysine, and phenol red, supplemented with 10% dialyzed FCS (AG Meissner). For labeling, "heavy" isotopes of [$^{13}C_6{}^{15}N_4$] L-arginine and [$^{13}C_6{}^{15}N_2$] L-lysine were added (for exact supplementation see Table 5). Cells were maintained for at least five passages to ensure incorporation of the heavy amino acids, and labeling efficiency (>95%) was confirmed by LC-MS. For experiments, SILAC-labeled MEG-01 cells were washed twice with PBS and resuspended in supplement-free RPMI at a density of $1x10^6$ cells/mL. Cells were incubated for 3 h at 37 °C, after which the conditioned medium was harvested. Supernatants were cleared first by centrifugation at 170 x g for 7 min and then at 3000 x g for 10 min to remove cellular debris. The cell-free supernatant (100 µL per well) was transferred to $1x10^5$ primary human monocytes in 96-well plates. Monocytes were then either left untreated or stimulated with LPS (2 ng/mL), or sequentially with LPS (2 ng/mL) and Nigericin (10 µM), as described in method section 3.2.17. After stimulation, plates were centrifuged at 500 x g for 5 min. A 60 µL aliquot of each supernatant was used for IL-1β and TNFα quantification by HTRF, contingent on cytokine responsiveness. The remaining supernatant was discarded and monocytes were washed twice with PBS. Cells were then lysed in 50 µl of 1% sodium deoxycholate (SDC) in 100 mM Tris-HCl (pH 8.5), supplemented with Benzonase (7.5 U/mL), and incubated at RT for 30 min. Protein disulfide bonds were reduced and alkylated by incubating lysates at 95 °C for 10 min in the presence of 55 mM Bond-Breaker TCEP and 10 mM chloroacetamide (CAA). Protein concentrations were measured using the Pierce 660 nm Protein Assay and 20 µg of total protein was used for enzymatic digestion. Trypsin/Lys-C mix was

added at a 1:25 enzyme-to-protein ratio, and digestion was performed overnight at 37 °C with shaking at 800 round per minute (rpm). The next day, digestion was stopped by adding stop buffer (1% formic acid in isopropanol) in a 5:1 buffer-to-sample volume ratio. Samples were centrifuged at maximum speed for 5 min, and peptides were desalted using in-house-made StageTips filled with Styrol-divinylbenzene Reversed Phase Sulfonate (SDB-RPS) made by AG Meissner and according to the protocol of Rappsilber and colleagues (Rappsilber et al., 2007). StageTips were activated with 20 µL methanol, washed with 20 µL of buffer B (80% acetonitrile, 0.1% formic acid) and equilibrated twice with 20 µL of buffer A (0.1% formic acid). Peptides were loaded, washed once with 100 µL of buffer A and twice with 100 µL of buffer B, and eluted with 60 µL of buffer X (5% ammonia, 80% acetonitrile). Centrifugation was performed at ~800 x g for 20 sec at each step. Eluted peptides were vacuum-dried and resuspended in buffer R (2% acetonitrile, 0.1% formic acid). Peptide concentration was determined, and 150 ng of peptide per sample was injected for LC-MS analysis.

3.2.27. LC-MS analysis

Proteomics samples were analyzed using a data-dependent acquisition (DDA) mode on a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system consisting of a Vanquish Neo chromatographic system coupled to an Orbitrap Exploris 480 mass spectrometer. Peptides were separated using an in-house packed analytical column (30 cm length) (AG Meissner) containing 1.9 µm ReproSil-Pur 120 C18-AQ material (Dr. Maisch). Separation was achieved using a binary gradient system: buffer A (0.1% formic acid in LC-MS-grade water) and buffer B (80% acetonitrile, 0.1% formic acid in LC-MS-grade water), with a constant flow rate of 350 nL/min over a 120 min gradient. The gradient program increased buffer B linearly from 6% to 32% over 92 min, followed by an increase to 55% over 14 min. Buffer B was then raised to 95% over 4 min and the column was washed at 95% buffer B for an additional 10 min to remove any residual peptides. Eluting peptides were on-line transferred to the Orbitrap mass spectrometer and ionized by nano-electrospray ionization, using a constant spray voltage of 2.4 kV. Precursor MS scans were performed at a resolution of 60 000, with an AGC target of 300%, a maximum injection time of 25 ms, and a scan range of 350-1750 m/z. The top 20 precursor ions with a minimum intensity of $2x10^4$ were selected for fragmentation and subsequent MS/MS analysis. Fragmentation MS/MS scans were acquired at a resolution of 15 000, with an AGC target of 100% and a

maximum injection time of 22 milliseconds (ms). The quadrupole isolation window was set to 1.4 m/z, and ions were fragmented by higher-energy collisional dissociation (HCD) at 30% collision energy. Selected precursor ions were dynamically excluded from MS/MS analysis for 30 sec. The resulting mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository, with the dataset identifier PXD052113 (Vizcaíno et al., 2014). Everything was executed under the supervision and help of Dr. Sebastian Kallabis and Anushka Kudaliyanage.

3.2.28. Statistical analysis of mass spectrometry proteomics data

Mass spectrometric raw data were processed using the MaxQuant software suite (version 2.0.3.0, MaxQuant website) and its integrated Andromeda scoring algorithm (Cox et al., 2011). The SwissProt human proteome was used as the reference sequence database for peptide identification (downloaded on 15.02.2023). Lys-8 and Arg-10 were employed as heavy isotope labels, with a maximum of five modifications allowed per peptide. Modifications included fixed cysteine carbamidomethylation, variable methionine oxidation, and variable N-terminal acetylation. Trypsin/P was selected as the protease, with a maximum of two missed cleavages per peptide and a minimum peptide length of seven amino acids. The "Match between Runs" feature was enabled using the default settings. Peptide and protein group identifications were filtered at a false discovery rate (FDR) of < 1%, using the unique + razor peptides strategy for protein quantification and intensity assignment. Statistical analysis was performed using the Perseus software suite (version 1.6.15) (Tyanova et al., 2016). SILAC heavy-to-light (H/L) protein ratios were log2-transformed and normalized by median subtraction. Protein groups were retained for further analysis if identified in at least two replicates of a given condition. Missing values were imputed by randomly drawing values from a normal distribution, downshifted by 1.8 standard deviations with a broad distribution (0.3 standard deviations).

Significantly altered SILAC H/L ratios in the LPS-stimulated condition were identified using paired Welch’s t-test, with permutation-based FDR correction (significance cutoff: FDR < 0.1, S0 = 0.1). Gene ontology (GO) terms or UniProt keywords that were significantly enriched or depleted were identified using 1D annotation enrichment analysis (significance cutoff: Benjamini-Hochberg FDR < 0.1). All statistical analysis were conducted by Dr. Sebastian Kallabis.

3.2.29. Statistical analysis

Statistical analyses were conducted using GraphPad Prism Version 10.5.0. Unless specified otherwise, all graphs represent pooled data from a minimum of two independent experiments (biological replicates), each performed in duplicates (technical replicates). Prior to statistical testing, normality and lognormality tests were performed to determine the appropriate parametric or nonparametric tests. For experiments involving multiple groups (e.g., StdMo, PDMo, PDMo + Ptls, and Plts, stimulated vs. unstimulated), P values were calculated using either one-way or two-way ANOVA, followed by Tukey's, Sidak's, or other multiple comparison tests as appropriate. Additional statistical details, including specific tests and significance thresholds, are provided in the corresponding figure legends.

3.2.30. Data presentation and text editing

Unless otherwise stated in the figure legends, all graphs are presented as floating bars indicating the mean as well as minimum and maximum values. Data are derived from pooled results of at least two independent experiments (biological replicates), each performed in technical triplicates, using monocytes or platelets from different donors. Each symbol represents the average of three technical replicates per donor or experiment, particularly in the case of cell line-based assays. Distinct symbols (e.g., ●, ■, ▼) are used to denote individual donors or experimental replicates, allowing for the visualization of inter-donor or inter-experimental variability. For spelling and grammar correction, AI-based tools including ChatGPT, Perplexity, and DeepL were used to support the writing process.

4. Results

4.1. Platelets are closely associated with circulating human monocytes

Monocytes are a subset of circulating leukocytes that play essential roles in maintaining immune homeostasis, responding to pathogen invasion, and orchestrating inflammatory processes. They contribute to host defense by recognizing and eliminating microbial threats and by shaping downstream immune responses. In peripheral blood, monocytes are in continuous interaction with platelets, which have been shown to modulate monocyte activation and function. Consistent with previous studies (Bhattacharjee et al., 2018; P. Han et al., 2020; Hawwari & Rossnagel et al., 2024; Rolfes et al., 2020), and in particular with the work of Ibrahim Hawwari (Hawwari, 2023), monocytes isolated using standard magnetic separation methods often contain a significant number of platelets. This thesis investigates the interaction between platelets and monocytes and explores how platelets influence monocyte immune responses under homeostatic and inflammatory conditions.

4.2. Isolation and characterization of human monocytes and platelets by flow cytometry

To obtain purified human monocytes and platelets for downstream analyses, peripheral blood from healthy donors was processed using a sequential isolation protocol (Figure 2A). PBMCs were first isolated by density gradient centrifugation using Ficoll-Paque®. Classical monocytes were then purified by magnetic negative selection using a monocyte isolation kit, yielding StdMo. To further reduce platelet contamination, an additional platelet-depletion cocktail was applied, resulting in PDMo. Platelets were isolated independently from PRP through differential centrifugation.

To verify the identity and purity of the isolated cell populations and to ensure consistency with previous findings, including those reported by Ibrahim Hawwari (Hawwari, 2023), flow cytometric analyses were performed in each experiment (Figure 2B). StdMo preparations contained three main populations: $CD14^+$ monocytes, $CD14^+CD41a^+$ MPAs, and free $CD41a^+$ platelets. As expected, platelet depletion using a specific antibody cocktail effectively reduced the proportion of free platelets and from now on called PDMo. Notably, the MPA population remained largely unchanged. Additional staining for CD45 and CD14 showed low levels of leukocyte contamination

in both StdMo and PDMo preparations, indicating that the main effect of platelet depletion was the removal of free platelets and the relative increase in monocyte purity. Isolated platelet preparations were confirmed to be highly pure and showed minimal leukocyte contamination. Platelet activation, assessed by CD62P expression, indicated that most platelets remained in a resting state (Figure 2C).

These results confirm the successful and reproducible isolation of phenotypically defined populations of human monocytes and platelets, providing a robust platform for downstream functional assays.

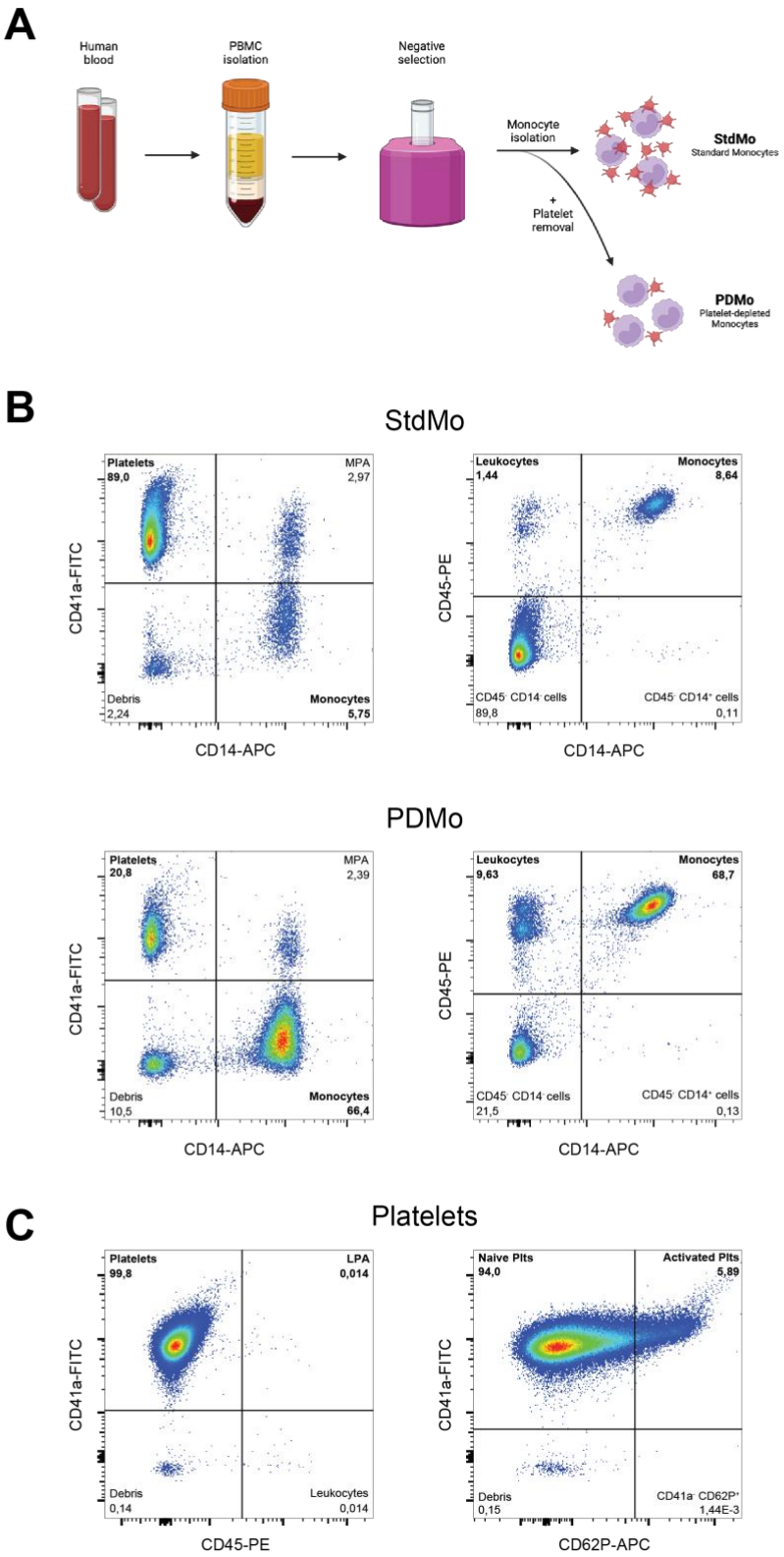


Figure 2: Isolation method, purity check and characterization of human monocytes and platelets by flow cytometry. (**A**) Schematic workflow illustrating isolation procedures for PBMCs and platelets. Whole blood underwent density

gradient centrifugation using Ficoll-Paque®, resulting in PBMC separation from plasma and red blood cells. Monocytes were isolated via magnetic cell sorting with a classical monocyte-negative selection kit (STEMCELL®), yielding standard monocytes (StdMo) and platelet-depleted monocytes (PDMo) upon addition of a platelet-removal cocktail. Platelets were purified from platelet-rich plasma (PRP) through sequential centrifugation. (**B + C**) Representative flow cytometry with represented donors with dot plots illustrating isolated cell population purity. Monocytes were defined as $CD14^+$ cells, platelets as $CD41a^+$ cells, and platelet activation was assessed by CD62P expression. (**B**) Monocyte purity was indicated by $CD14^+CD41a^-$ cells, monocyte-platelet aggregates (MPAs) as $CD14^+CD41a^+$, and platelets as $CD14^-CD41a^+$. Leukocyte contamination was identified by $CD45^+CD14^-$ in monocytes and $CD41a^+CD45^+$ in platelets. (**C**) Platelet activation status was classified as $CD41a^+CD62P^-$ (naïve) or $CD41a^+CD62P^+$ (activated). The data are based and referenced to previous work of our working group (Hawwari & Rossnagel et al., 2024; Rolfes et al., 2020), with substantial work carried out by Verena Rolfes, Lucas Ribeiro and Ibrahim Hawwari. The data and graphs shown here were independently generated by me for this thesis and support the findings of the previously mentioned studies. The schematic workflow was created using BioRender®.

4.3. Platelets enhance pro-inflammatory cytokine secretion of human monocytes

To investigate the functional role of platelets (Plts) in modulating monocyte-driven inflammation, PDMo were cultured alone or in co-cultures with platelets at a defined ratio (1:100, monocyte:platelet ratio), and the results were compared to StdMo preparations. Cells were primed with LPS, a TLR4 ligand, followed by activation of the NLRP3 inflammasome using Nigericin (Figure 3A).

Cytokine levels of IL-1β and TNFα in the cell-free supernatants were measured and compared between the conditions. Upon stimulation with LPS and Nigericin, StdMo secreted significantly higher levels of IL-1β compared to PDMo (Figure 3B). Supplementation of PDMo with autologous or heterologous platelets restored IL-1β secretion to levels comparable to StdMo, indicating that platelets play an essential role in enhancing inflammasome-dependent IL-1β production. Platelets alone did not secrete measurable amounts of IL-1β under these conditions ruling them out as sources of this cytokine in the assays.

Similarly, TNFα secretion was markedly reduced in PDMo compared to StdMo following LPS priming. Co-culture of PDMo with platelets significantly restored TNFα production. Again, platelet-only controls did not contribute to TNFα release, confirming that the observed effects were dependent on monocyte-platelet interactions rather than direct cytokine production by platelets.

To better illustrate the relevant effects, an alternative data visualization was used (Figure 3C). Experimental conditions that did not result in measurable cytokine

responses, such as the unstimulated control or the LPS control during the NLRP3 activation step, are not represented. Only conditions that were observed to induce cytokine production were included. For TNFα, this referred to LPS stimulation (referred as LPS), and for IL-1β, to the combination of LPS and Nigericin (referred as LPS + Nig). Unless otherwise specified, cytokine levels in the excluded conditions were below the detection limit. This focused approach enables more effective visualization within each experimental group by concentrating only on the relevant conditions (Figure 3B+C).

These findings are consistent with our previous work and the doctoral thesis of Ibrahim Hawwari (Hawwari, 2023; Hawwari & Rossnagel et al., 2024), and confirm that platelets enhance the inflammatory capacity of monocytes by promoting the secretion of IL-1β and TNFα.

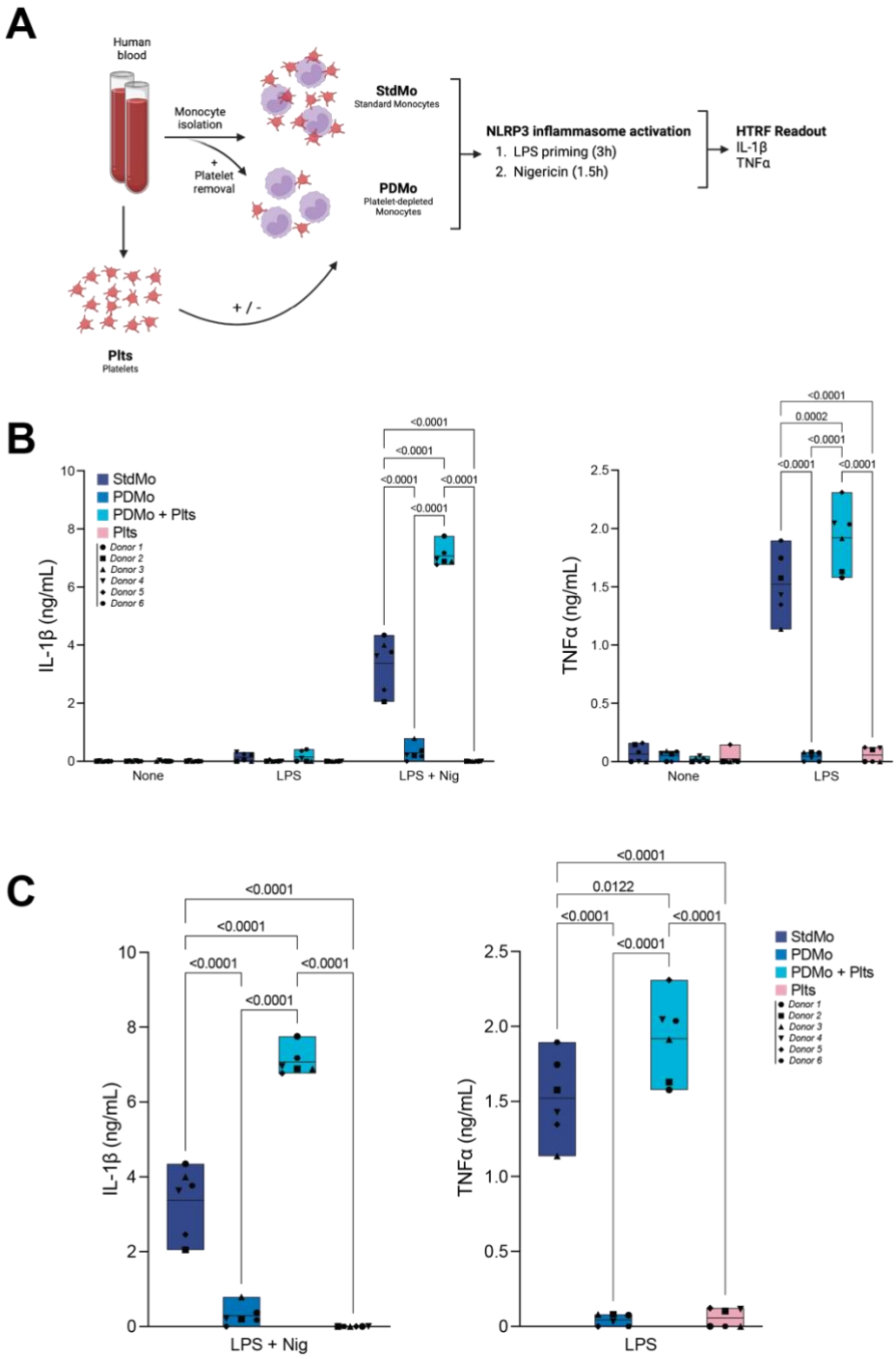


Figure 3: Platelets enhance pro-inflammatory response of human monocytes by boosting cytokine secretion. (**A**) Experimental design: Primary human monocytes were isolated via negative selection, generating StdMo or PDMo. Separately isolated platelets (Plts) were co-cultured with PDMo (1:100, PDMo:platelets ratio). Monocytes were primed with LPS (2 ng/mL, 3 h), followed by inflammasome activation with Nigericin (Nig) (10 µM, 1.5 h). (**B**) Cytokine secretion (IL-1β, TNFα) quantified via HTRF in cell-free supernatants of StdMo (dark blue), PDMo (blue), and PDMo with autologous or heterologous Plts (light blue), post-stimulation (LPS ± Nigericin). Plts alone (pink) served as control. Data presented as floating bars representing minimum-to-maximum values with mean (black line) with individual symbols representing

independent donors (n = 6). Statistical analysis: two-way ANOVA with Tukey's multiple comparisons. Significant P-values indicated. (**C**) Alternative visualization with focus on the LPS+Nig (IL-1β) and LPS (TNFα) experimental conditions: floating bars representing minimum-to-maximum values with mean (black line). Statistical significance determined by one-way ANOVA with Tukey's multiple comparisons. The data are based and reference to our paper and the doctoral dissertation of I. Hawwari (Hawwari, 2023; Hawwari & Rossnagel et al., 2024), with substantial work carried out by Ibrahim Hawwari. The data and graphs shown here were done and created by me. The schematic workflow was created using BioRender®.

4.4. Platelet-derived factors are transferred to monocytes enhancing their pro-inflammatory capacity

As previous evidence only indirectly suggested that platelets release soluble factors taken up by monocytes, we employed an unbiased proteomic approach in collaboration with the group of Prof. Dr. Felix Meissner and the support of Dr. Sebastian Kallabis and Anushka Kudaliyanage. This approach combined mass spectrometry with SILAC (Ong et al., 2003; Ong & Mann, 2006). The human megakaryocytic cell line MEG-01 releases platelet-like particles into the culture medium and was therefore used to identify candidate platelet-derived proteins potentially involved in modulating monocyte responses. To enable protein tracing, I cultured MEG-01 cells over multiple passages in medium supplemented with [$^{13}C^{15}N$]-labeled L-lysine and L-arginine (referred to as "heavy amino acids") (Figure 4A), resulting in incorporation efficiencies exceeding 95%. Under these conditions, newly synthesized proteins are isotope-labeled and can be distinguished from endogenous monocyte-derived proteins by a characteristic mass shift during MS analysis.

To determine whether MEG-01-derived supernatants exert similar effects as primary platelets and whether the SILAC labeling influences MEG-01 secretome function, I co-incubated cell-free supernatants from SILAC-labeled MEG-01 cultures (SILAC Sup) with PDMo, followed by LPS priming and Nigericin stimulation. As shown in Figure 4B, reconstitution with either isolated platelets or SILAC Sup fully restored IL-1β production of PDMo, indicating that the response was due to intrinsic properties of the secreted proteins and also not affected by isotope labeling.

After co-incubating PDMo with SILAC supernatant, I stringently washed the cells to remove non-specific surface interactions and then lysed them to identify the proteins transferred to the monocytes. MS of the lysates (Figure 4C) revealed 33 "heavy"-labeled proteins enriched in LPS-stimulated PDMo, confirming their origin from MEG-01 cells. These included transcriptional regulators and signaling molecules such as

NFκB2 (p100/p52), ANP32B, THOC1, ZFP36, EEF2A, and PAIP1, as well as components of the MAPK/ERK signaling cascade. Notably, five of the transferred proteins had transmembrane-associated functions, suggesting active uptake or vesicle-mediated delivery.

Volcano plot analysis (Figure 4 I.) confirmed significant enrichment of platelet-derived proteins under LPS stimulation, with NFκB2 among the most enriched (Figure 4 III.). Furthermore, principal component analysis (PCA) revealed distinct clustering of H/L protein ratios driven by stimulation conditions (Figure 4 II.). These data provide the first direct evidence that platelets release regulatory proteins that are taken up by monocytes and contribute to their pro-inflammatory response.

In summary, these findings demonstrate that platelets or platelet-like cells secrete immunomodulatory soluble factors that enhance inflammasome activation in human monocytes and actively transfer functional regulatory proteins, including components of the NFκB signaling pathway, into recipient monocytes.

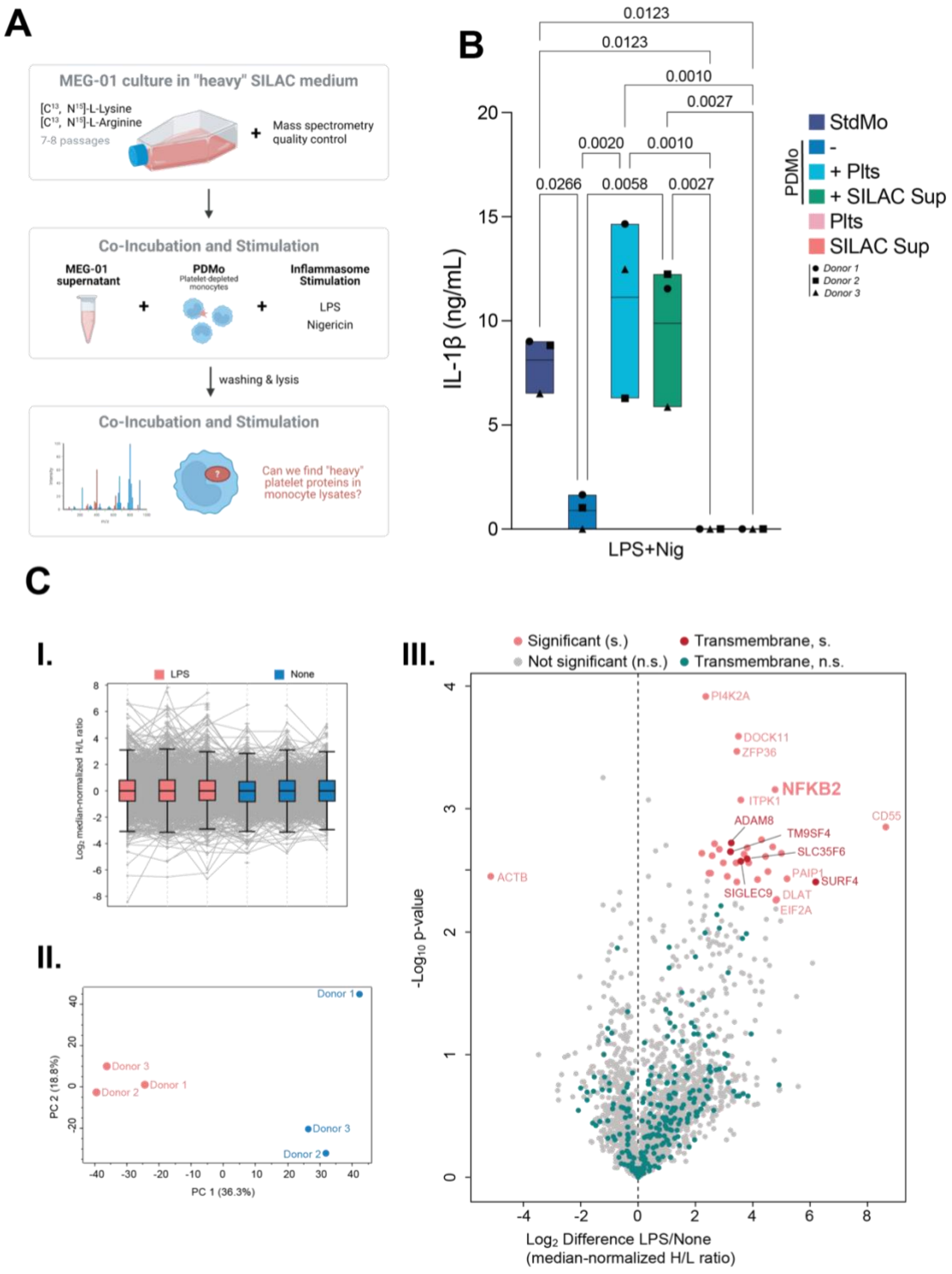


Figure 4: Enrichment of platelet-derived NFκB2 in MEG-01 via SILAC. (**A**) Experimental design: MEG-01 cells cultured SILAC medium (H) with heavy isotopes (L-lysine, L-arginine). After 7-8 passages, isotope incorporation (>95%) was confirmed by MS. Cell-free supernatants from normal and heavy MEG-01 cultures were applied to PDMo, which were then primed with LPS (2 ng/mL, 3 h) and activated with Nigericin (10 µM, 1.5 h). Monocytes were washed, lysed and analyzed via MS. (**B**) Quality control via IL-1β secretion assay confirming MEG-01 supernatant efficacy without SILAC medium-induced artifacts. Statistical analysis: one-way ANOVA with Tukey's

multiple comparisons. Individual symbols represent independent donors (n = 3). (**C**) SILAC-MS proteomic analysis: (**I.**) Log2-transformed heavy-to-light protein ratios normalized by median substitution. (**II.**) Principal component analysis (PCA) reveals condition-specific proteomic profiles. Unstimulated conditions are shown in blue, while LPS-stimulated conditions are shown in red. (**III.**) Volcano plot identifying significant enrichment of NFκB2 and 32 other proteins post-LPS stimulation; one protein decreased. Functional enrichment analysis (Benjamini-Hochberg false discovery rate (FDR) = 0.1) highlighted "transmembrane" proteins. Statistical evaluation by paired Welch's t-test with permutation-based FDR correction (FDR = 0.1, S0 = 0.1, n = 3). Significant hits are shown in light red, non-significant hits in grey. Significant transmembrane proteins are shown in dark red and non-significant transmembrane proteins are labeled in grey. Analysis was performed by Dr. Sebastian Kallabis. The schematic workflow was created using BioRender®.

4.5. Generation and validation of THP-1 knockouts lacking NFκB subunits

To investigate the functional relevance of the canonical and non-canonical NFκB pathways in human monocytes, I generated THP-1 cell lines with targeted deletions of key NFκB subunits with the help of Salie Maasewerd and Marius Jentzsch (Figure 5A). The aim was to establish a cellular context in which any functional NFκB protein could only be supplied by platelets, thereby enabling selective tracing of platelet-derived NFκB components. This approach was guided by SILAC-based proteomic analysis, which identified NFκB2 as a candidate platelet-derived protein potentially involved in monocyte modulation. NFκB signaling is mediated through two distinct pathways: the canonical and the non-canonical branch. To dissect their respective contributions, I used CRISPR/Cas9 to delete NFκB2, a central component of the non-canonical pathway. Since this intervention selectively disrupts non-canonical signaling, I also deleted RELA, a key subunit of the canonical NFκB pathway. The canonical pathway relies on IKK complex-mediated degradation of IκB inhibitors, allowing nuclear translocation of RELA/p50 dimers. In contrast, the non-canonical pathway is activated through NIK-dependent processing of NFκB2 (p100) into p52, which then heterodimerizes with RELB and translocates to the nucleus (Figure 1).

To allow for complete disruption of both NFκB signaling arms, I also generated a double knockout (KO) line deficient in both RELA and NFκB2. In total, three THP-1 KO lines were generated: $RELA^{-/-}$, $NF\kappa B2^{-/-}$, and $RELA^{-/-}NF\kappa B2^{-/-}$ (Figure 5A). For each condition, I isolated multiple independent single-cell clones to ensure reproducibility and knockout efficiency.

In collaboration with Salie Maasewerd, I designed and ordered the Guide RNA. Marius Jentzsch from the laboratory of Prof. Dr. Jonathan Schmid-Burgk and I performed the

CRISPR/Cas9-mediated gene editing and validated the genetic deletions using next-generation sequencing (NGS). We first verified the knockout efficiency by NGS, after which I selected and expanded the most robust clones for further experiments.
Additionally, I performed knockout validation at protein level prior to each experiment using capillary-based Western blotting (WES), targeting the respective protein products (Figure 5B). As expected, RELA-deficient clones showed complete loss of p65 expression, while NFκB2-deficient clones lacked detectable p100 protein. In the double knockout clones, neither p65 nor p100 was detectable, confirming the successful simultaneous disruption of both genes. Wild-type (WT) THP-1 cells served as positive controls and showed strong expression of both NFκB subunits.
These knockout cell lines provide a powerful model system to dissect the individual and combined functions of RELA and NFκB2 in NFκB signaling and inflammasome regulation in human monocytes.

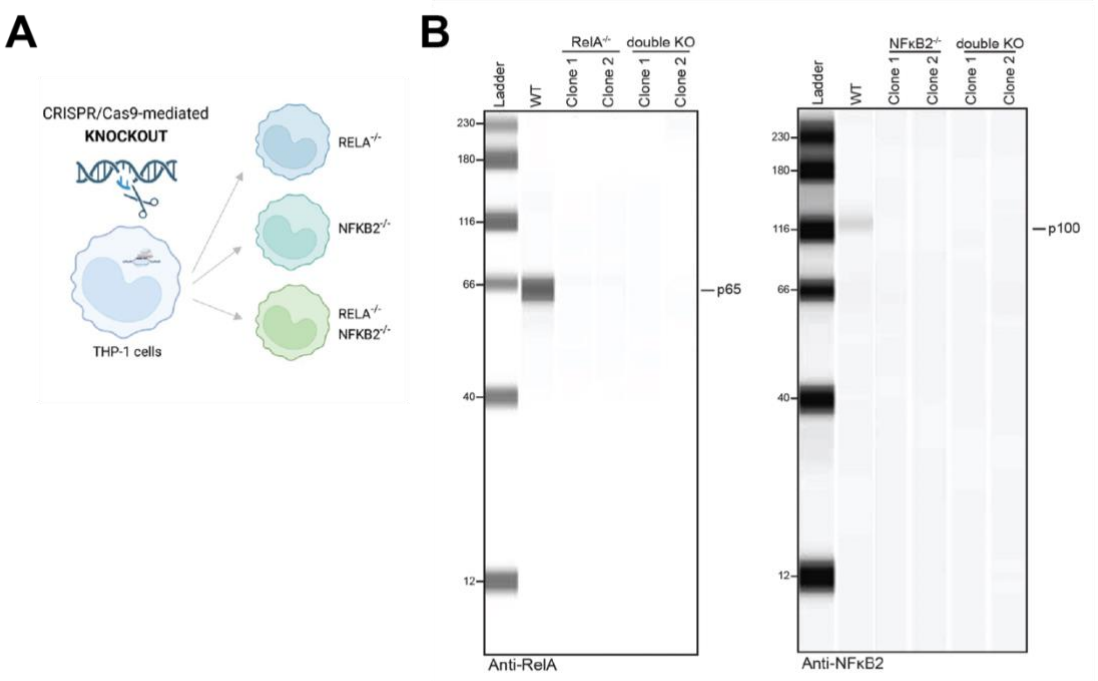


Figure 5: CRISPR/Cas9-mediated knockout of canonical and non-canonical NFκB pathway components in THP-1 cells. (**A**) CRISPR/Cas9 knockout strategy targeting RELA (p65), NFκB2 (p100), or both (RELA/NFκB2) in THP-1 monocytes. (**B**) Validation by capillary-based immunodetection (WES): absence of p65 (Anti-RELA) and p100/52 (Anti-NFκB2) in single ($RELA^{-/-}$, $NFκB2^{-/-}$) and double knockout (double KO) clones. Two representative clones per knockout presented. The schematic workflow was created using BioRender®.

4.6. Platelets partially rescue the impaired pro-inflammatory response of RELA- and NFκB2-deficient monocytes

To directly assess whether components of the canonical and non-canonical NFκB signaling pathways are required for platelet-mediated enhancement of monocyte activation, and whether these transcription factors are indeed involved, I stimulated WT THP-1 cells as well as knockout lines deficient in RELA (RELA$^{-/-}$), NFκB2 (NFκB2$^{-/-}$), or both (RELA$^{-/-}$NFκB2$^{-/-}$) with the TLR1/2 agonist Pam3CysK4, followed by the NLRP3 inflammasome activator Nigericin. IL-1β secretion was measured in the cell-free supernatants.

As shown in Figure 6A, stimulation of WT cells induced robust IL-1β secretion. This response was significantly reduced in both RELA$^{-/-}$ and NFκB2$^{-/-}$ clones and was almost completely abolished in RELA$^{-/-}$NFκB2$^{-/-}$ double knockout cells (double KO). This confirms that both RELA and NFκB2 are required for full inflammasome activation in this context.

To investigate whether platelets can enhance pro-inflammatory activity in WT cells, I co-cultured THP-1 monocytes with increasing concentrations of platelets (1:20, 1:50, 1:100 THP-1:platelet ratio) and stimulated those as described above. As shown in Supplement Figure 1, platelet supplementation enhanced IL-1β secretion upon Nigericin stimulation, in a ratio-dependent manner, with the strongest effect observed at the 1:100 ratio. No IL-1β secretion was detected in unstimulated conditions or with Pam3CysK4 priming alone, irrespective of platelets. These controls were included to demonstrate that both the parental and clonal THP-1 cells react in a manner consistent with primary human monocytes.

Next, I tested whether platelets could restore IL-1β production in NFκB-deficient clones (Figure 6B). In RELA$^{-/-}$ and NFκB2$^{-/-}$ cells, platelet supplementation partially restored IL-1β secretion in a ratio-dependent manner to a level comparable to basal cytokine levels of WT cells without platelet addition. Notably, NFκB2$^{-/-}$ clones showed a particularly strong response when I added platelets, reaching even higher IL-1β levels than the unstimulated WT baseline, suggesting partial functional compensation through platelet-derived factors. In contrast, double KO cells showed only minimal restoration of IL-1β secretion upon platelet addition. Although low, this residual increase is notable, as both arms of the NFκB pathway are completely disabled in these cells, yet platelet-derived factors were still able to modestly restore the response

of these monocytes. This further supports the notion that both RELA and NFκB2 are essential for the full platelet-mediated enhancement of inflammasome activation.

In summary, these findings demonstrate that platelets enhance IL-1β production in monocytes via both the canonical (RELA) and non-canonical (NFκB2) NFκB pathways with molecules likely arising from the platelets and that both arms of the signaling cascade are required to mediate the full extent of monocyte inflammasome activation.

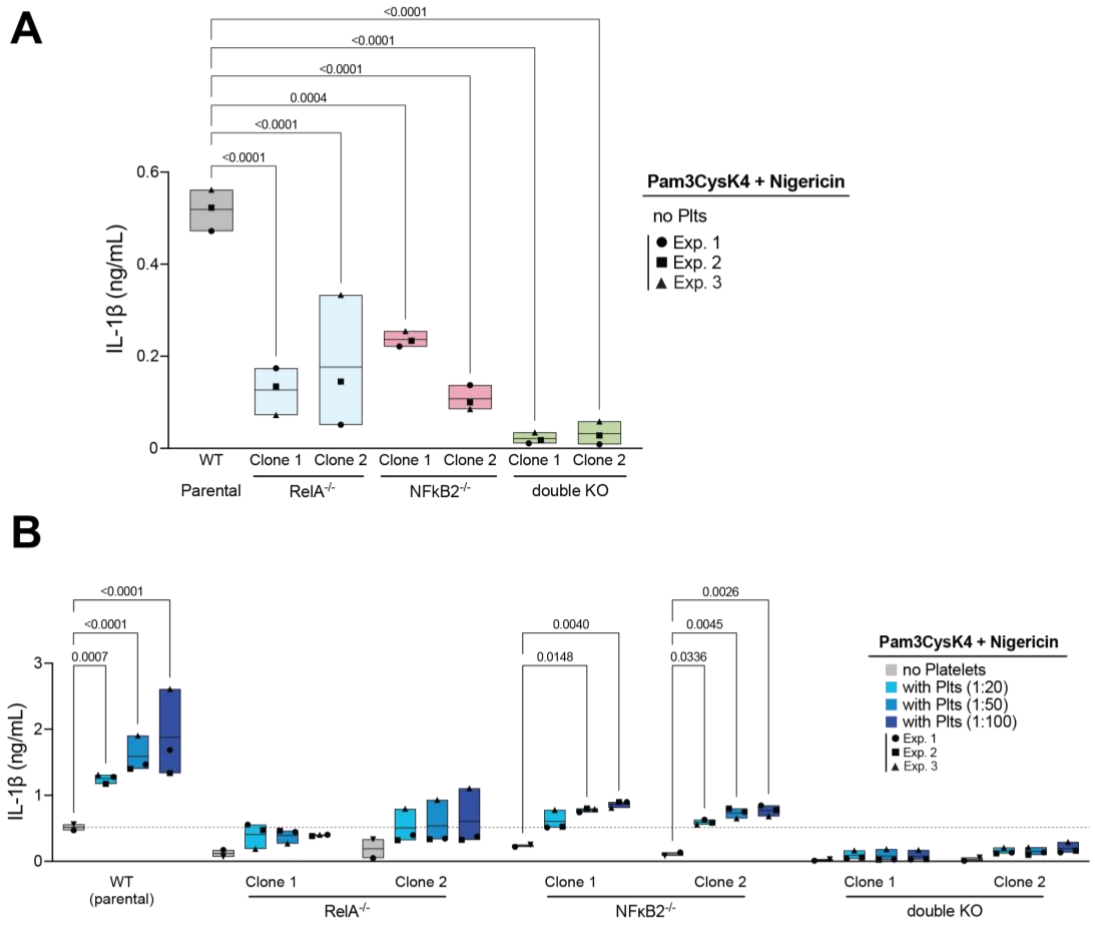


Figure 6: Functional validation of canonical and non-canonical NFκB knockout THP-1 cells and platelet-mediated inflammasome activation. (**A**) IL-1β concentrations in the supernatants of RELA$^{-/-}$, NFκB2$^{-/-}$, and RELA$^{-/-}$NFκB2$^{-/-}$ double knockouts (double KO) THP-1 clones versus wild-type (WT), following Pam3CysK4 (1 µg/mL, 3 h) priming and Nigericin (10 µM, 1.5 h) stimulation. Floating bars display max/min values with indication to the mean (black bands). P values were calculated using one-way ANOVA, Tukey's multiple comparison test compared to the parental WT THP-1 cells are indicated in the figure. Each symbol represents one independent experiment (n = 3). (**B**) IL-1β concentrations in the supernatants of stimulated WT, NFκB2$^{-/-}$, RELA$^{-/-}$, and double KO THP-1 clones co-cultured with platelets (ratios 1:20, 1:50, 1:100). Cells were stimulated with Pam3CysK4 (1 µg/mL, 3 h) and Nigericin (10 µM, 1.5 h). Floating bars display max/min values with indication to the mean (black

bands) with mean of parental WT THP-1 cells without platelet addition for basal IL-1 β secretion capability. P values were calculated using two-way ANOVA, Tukey's multiple comparison test compared to the control group (no Plts) and are indicated in the figure. Each symbol represents one experiment and platelet donor. Data is pooled from three independent experiments.

4.7. Platelets constitutively secrete processed NFκB2 in vesicles independent of pro-inflammatory stimulation

To determine whether platelet activation through LPS stimulation contributes to their ability to enhance monocyte responses and to rule out the possibility that LPS exposure directly activates platelets, I isolated human platelets and incubated them with LPS (2 ng/mL, 3 h) or without stimulation. Following incubation, I collected the platelet supernatant (PS) (Figure 7A). All resulting fractions (intact platelets and PS) from unstimulated and LPS-stimulated platelets were subsequently transferred to PDMo, followed by inflammasome priming with LPS and activation with Nigericin.

First, I measured total protein concentrations in platelet (Plt) lysates and PS fractions to determine whether LPS stimulation affects overall protein secretion (Figure 7B). As expected, Plt lysates contained the highest protein levels, while the PS fraction had lower protein content. Notably, LPS treatment did not significantly alter total protein concentrations in any fraction, indicating that platelet activation via LPS does not increase general protein release and is therefore unlikely to be the cause of enhanced monocyte activation.

Next, I assessed the functional impact of each platelet-derived fraction on monocyte cytokine production. As shown in Figure 7C, co-incubation of PDMo with either LPS-stimulated or unstimulated platelets or PS resulted in comparable levels of IL-1β and TNFα secretion following LPS and Nigericin stimulation, while PDMo alone were significantly impaired in their cytokine secretion as described before. The modest enhancement observed with PS and vesicles was independent of prior LPS stimulation of the platelets, suggesting that platelet-derived boosting of monocyte activation occurs constitutively and does not require an external inflammatory trigger.

To better understand the behavior of the SILAC-identified candidate NFκB2 in this context, I performed WES using an antibody specific to NFκB2 (p100/p52) on the same samples (Figure 7D). Interestingly, I found that platelets process NFκB2 from its inactive p100 precursor into the active p52 form independent of LPS stimulation. While p100 remained detectable in Plt lysates, the processed p52 form was exclusively

enriched in the PS fractions, indicating that NFκB2 processing occurs within platelets and the active form is subsequently released into the extracellular space.

These findings demonstrate that, despite lacking a nucleus, platelets harbor transcription factors such as NFκB2 and are capable of processing and secreting them in their active form. Specifically, they constitutively convert NFκB2 (p100) into p52 and secrete them, independent of inflammatory stimulation. This supports a model in which platelets promote monocyte activation through NFκB-dependent pathways via contact-independent mechanisms.

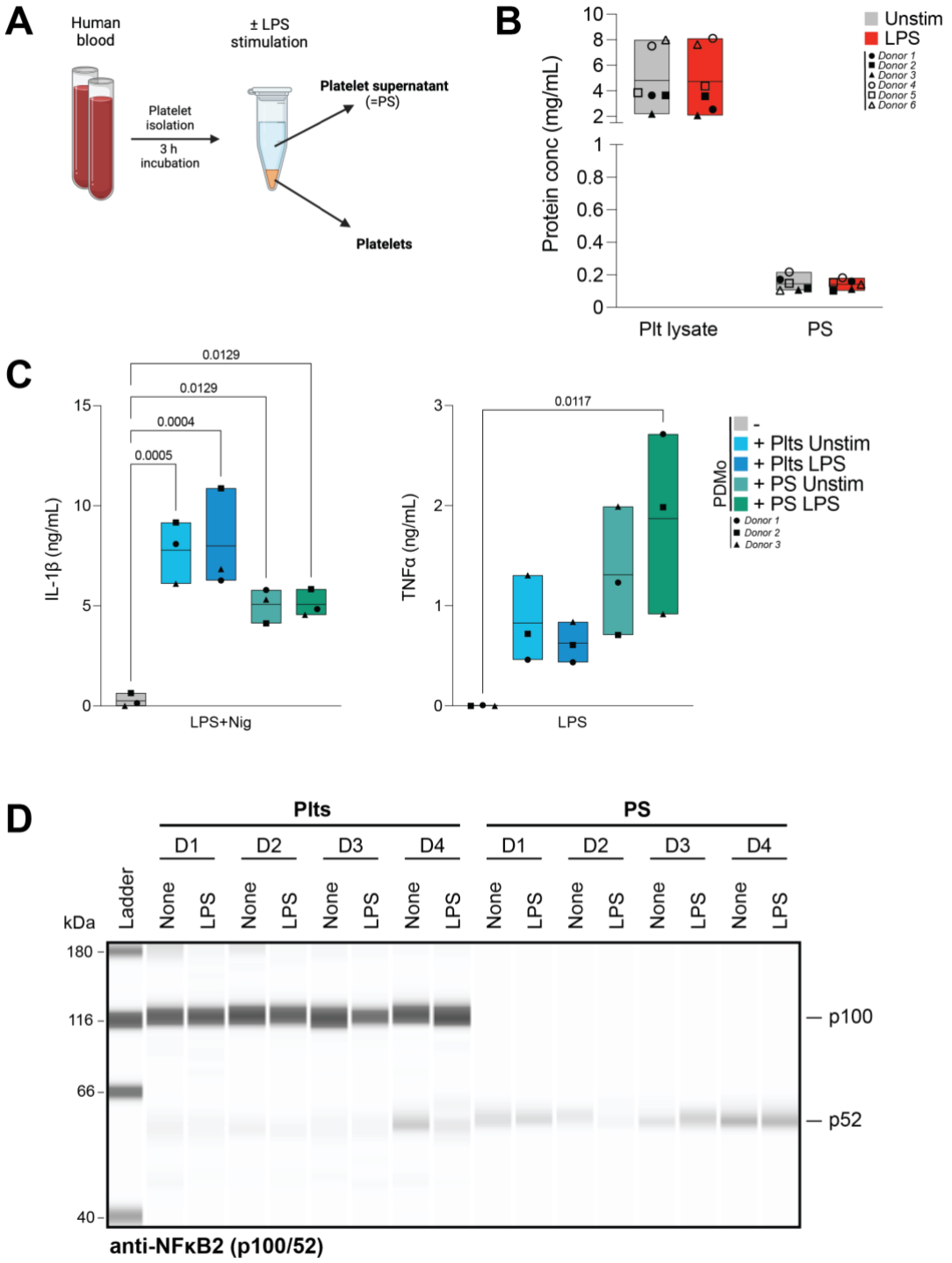


Figure 7: Platelet-derived vesicles support monocytes for pro-inflammatory response unrelated to pre-stimulation of platelets. (**A**) Experimental design: Platelets incubated ± LPS (2 ng/mL, 3 h) and then supernatants were separated from platelets. (**B**) Total protein quantified via bicinchoninic acid (BCA) assay in platelet (Plt) lysates and platelet supernatants (PS) ± LPS stimulation (2 ng/mL, 3 h). Symbols represent independent donors (n = 6). (**C**) IL-1β and TNFα levels in the cell-free supernatant of PDMo and PDMo supplemented with Plts or PS. Plts or PS were pre-treated with LPS (2 ng/mL, 3 h) before addition to PDMo. Afterwards, the conditions were stimulated with LPS (2 ng/mL, 3 h) followed by activation with Nigericin (10 µM, 1.5 h). Graphs with floating bars depict maximum/minimum values relative to mean.

P-values were calculated using one-way ANOVA, Tukey's multiple comparison test and are indicated in the figure. Each symbol represents one donor (n = 3). (**D**) NFκB2 (p100/p52) protein levels assessed via capillary electrophoresis immunoblotting (WES) in Plts and PS ± LPS stimulation (2 ng/mL, 3h) from four healthy subjects. The schematic workflow was created using BioRender®.

4.8. Platelet-derived vesicles functionally enhance monocyte cytokine responses

To investigate whether platelets release transcription factors such as NFκB2 in soluble form or packaged within vesicles, I established a vesicle depletion protocol. I subjected PS to ultrafiltration using a 100-kDa molecular weight cutoff filter to separate PS from vesicle-free fractions (Figure 8A).

To characterize the vesicle fractions and validate the vesicle depletion approach, I analyzed all PS using a nanoparticle tracking analysis (Figure 8B). Size distribution and particle concentration measurements confirmed a clear enrichment of vesicles in the >100-kDa PS fraction, whereas vesicles were nearly absent from the filtrate, indicating successful depletion.

Protein analysis of platelet lysates, PS, and vesicle-free PS (filtrate) from multiple donors further characterized these fractions (Figure 8C). As I observed before, the processed form of NFκB2 (p52), but not RELA (p65), was found exclusively in PS. In contrast, I detected both full-length NFκB2 (p100) and RELA in platelet lysates. Importantly, p52 was absent in the vesicle-free fraction, indicating that secretion of processed NFκB2 occurs specifically via vesicles. To explore the possibility that platelets could become activated during stimulation in co-culture with monocytes, I additionally tested platelets that were stimulated with Thrombin before the ultrafiltration. Of note, I observed no substantial differences between Thrombin-activated and non-activated platelets, suggesting that NFκB2 processing and vesicle-associated secretion occur independently of platelet activation.

Since I found NFκB2 enriched in vesicles, I next performed functional validation. For this, I co-incubated all previously analyzed fractions with PDMo, followed by LPS priming and Nigericin stimulation. As shown in Figure 8D, both intact platelets and PS significantly enhanced IL-1β and TNFα secretion. Varying the incubation times did not result in notable differences in this boosting effect. In contrast, vesicle-free fractions failed to induce cytokine production and yielded levels comparable to PDMo alone. These findings further support the conclusion that the immunomodulatory activity is mediated exclusively by vesicle-associated components.

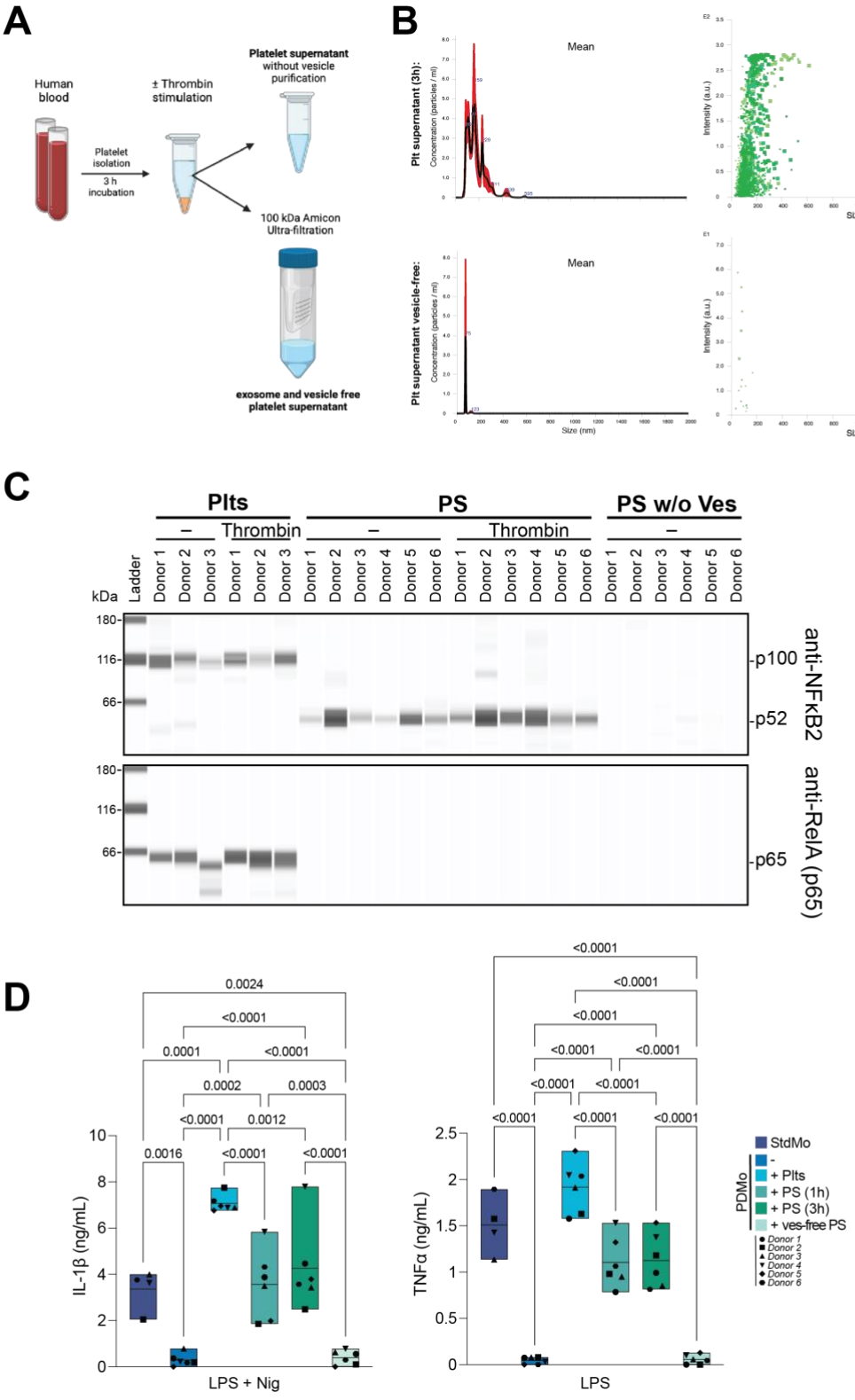


Figure 8: Vesicle-mediated transfer of NFκB signaling components from platelets to monocytes. (**A**) Experimental workflow. Platelets incubated ± Thrombin (1 U/mL, 0.5 h) and then supernatants were separated from platelets. PS was subsequently processed by 100 kDa ultrafiltration to generate PS and vesicle-free fractions. (**B**) Vesicle analysis of the different PS using NanoSight NS300. Each sample was diluted 1:10 with PBS and 1 ml total volume was used and recorded three

times for 60 seconds before analysis. On the left is the calculated mean and, on the right, each detected vesicle is shown on its on with its intensity. The Y-axis represents the concentration of particles detected per milliliter (left) or intensity per vesicle (right), and the X-axis shows the size (nm) of the vesicles/particles. Each graph represents one independent experiment or donor. (**C**) WES capillary electrophoresis and immunoblotting of NFκB2 on lysates, PS, and vesicle-free PS of purified Plts from six healthy volunteers. Platelets were left untreated or activated with Thrombin (1U/mL, 0.5 h). (**D**) Assessment of IL-1β and TNFα levels in the supernatants of StdMo, PDMo and PDMo that were replenished with Plts (PdMo + Plts), or unfractionated PS, which was either incubated for 1 h (+PS 1 h) or 3 h (+ PS 3 h) or releasates depleted of vesicles (+ ves-free PS) of the PS incubated with 3 h. Conditions were stimulated with LPS (2 ng/mL, 3 h) followed by activation with Nigericin (10 μM, 1.5 h). Graphs with floating bars show maximum/minimum values relative to the mean (black bands). P values were calculated using one-way ANOVA, Tukey's multiple comparison test, and are shown in the figure. Each symbol represents one donor (n = 6). The schematic workflow was created using BioRender®.

These results demonstrate that platelets actively process NFκB2 from its inactive p100 precursor to the bioactive p52 form and secrete it into vesicles. These vesicles can be functionally taken up by monocytes and enhance inflammasome-driven cytokine production. Importantly, this mechanism appears to be independent of stimulation or platelet activation, suggesting that it represents a constitutive, contact-independent function of platelets in modulating monocyte inflammatory responses.

4.9. Pharmacological inhibition of NFκB signaling in monocytes and platelets

My previous results demonstrate the ability of platelet-derived EVs enriched in NFκB signaling molecules to restore the impaired cytokine production in stimulated NFκB-knockout THP-1 monocytes. To further explore and validate the functional relevance of NFκB signaling in platelet-monocyte communication, I tested a series of small-molecule inhibitors targeting different nodes within the NFκB pathway (Figure 9A). This approach was particularly insightful given that NFκB2 was identified as a major hit in our SILAC proteomics approach (Figure 4C), while RELA deletion also resulted in impaired cytokine secretion, which could be restored through platelet supplementation (Figure 5). These findings suggest that both canonical and non-canonical NFκB signaling pathways are involved in the intercellular communication between platelets and monocytes.

To dissect this further, all inhibitors were first tested for their suppressive capacity in StdMo, identifying suitable concentrations that achieved pathway inhibition without inducing cytotoxicity or off-target effects (Figure 9B I.). Subsequently, I selected

concentrations to pre-treat isolated platelets, which were then washed and co-cultured with PDMo, to assess whether they retained the capacity to enhance monocyte responses (Figure 9B II.). For each experiment, I included untreated platelets from the same donors and vehicle (solvent) controls at the highest concentration as controls. Additionally, I performed cell viability assays on the co-cultures and treated platelets to rule out cytotoxic effects.

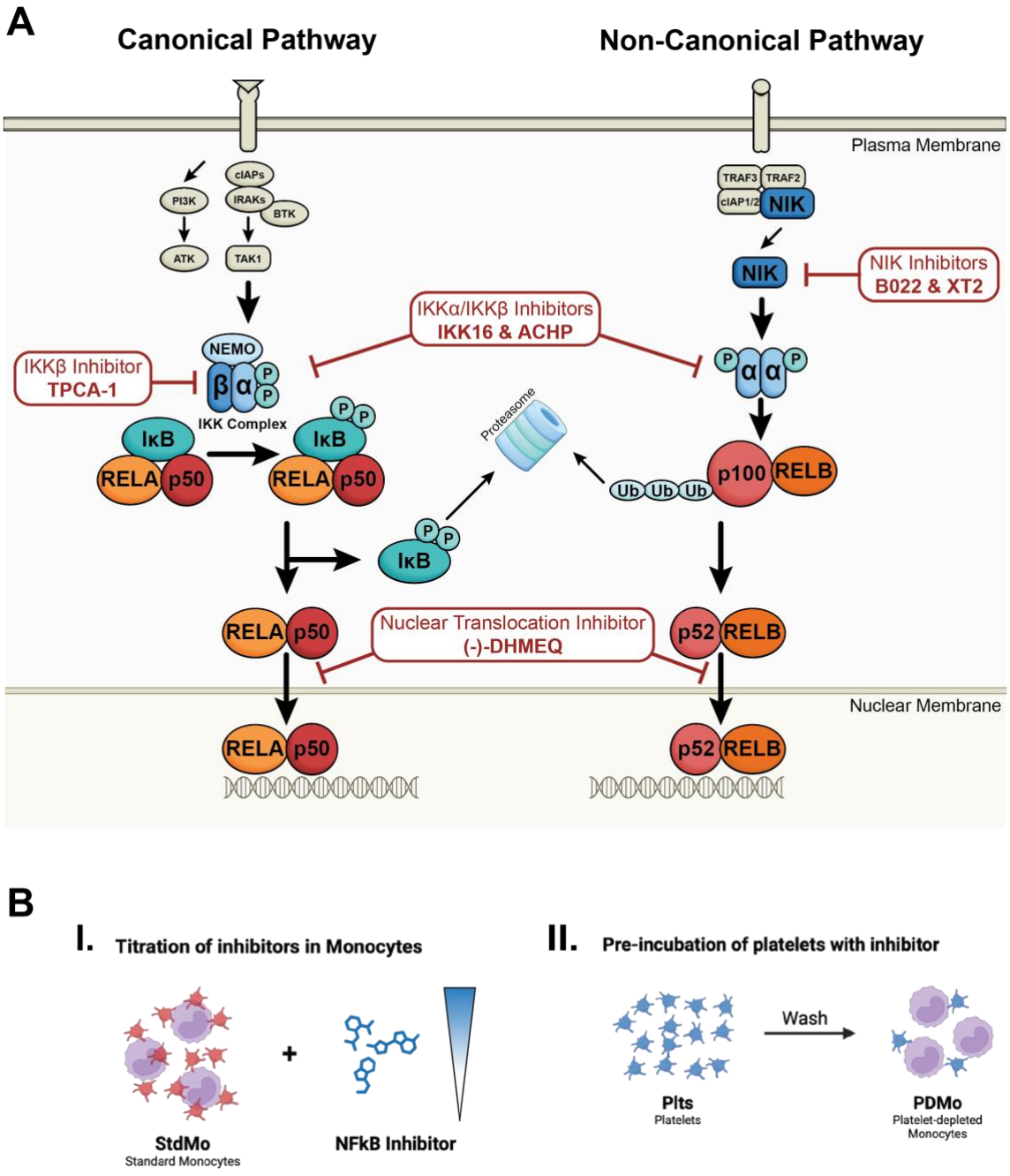


Figure 9: Experimental strategy to investigate the role of canonical and non-canonical NFκB signaling in monocyte-platelet crosstalk using pharmacological

inhibition. (**A**) Schematic representation of all the inhibitors and where they target the canonical and non-canonical NFκB signaling pathways. (**B**) Experimental design: (**I.**) Primary human monocytes were isolated from PBMCs via negative selection and treated with increasing concentrations of the inhibitors prior to stimulation. (**II.**) Platelets were pre-treated with pre-selected concentrations of the inhibitors and assessed for their capacity to restore cytokine secretion in PDMo. The schematic workflow was created using BioRender® and illustrated with the help of Salie Maasewerd.

I selected the small-molecule inhibitors ACHP and IKK16, which block both canonical and non-canonical NFκB signaling via IKKα and IKKβ inhibition, for detailed analysis. This allowed upstream inhibition of both RELA (p65) and NFκB2 (p100/p52) activation. In titration experiments, increasing concentrations of ACHP and IKK16 led to a pronounced and dose-dependent suppression of IL-1β and TNFα secretion in inflammasome-stimulated monocytes (Figure 10A, C). Inhibition of ACHP was already evident at 0.5 µM, with near-complete cytokine suppression at higher concentrations (5-10 µM) Similarly, IKK16 inhibition was detectable from 0.3 µM and nearly complete at 3-9 µM. DMSO vehicle controls had no effect, confirming the specificity of both inhibitors.

To assess further that canonical and non-canonical NFκB activity is required for platelet-mediated enhancement of monocyte activation, I co-cultured PDMo together with platelets pre-treated with either ACHP or IKK16. In both cases, the addition of platelets failed to restore both IL-1β and TNFα production in the presence of the inhibitors, indicating that platelet-derived signals rely on intact IKKβ-dependent NFκB signaling within (Figure 10B, C). This indicates that platelet-driven activation of monocytes depends on intact NFκB signaling, requiring functional IKKα and IKKβ activity in platelets.

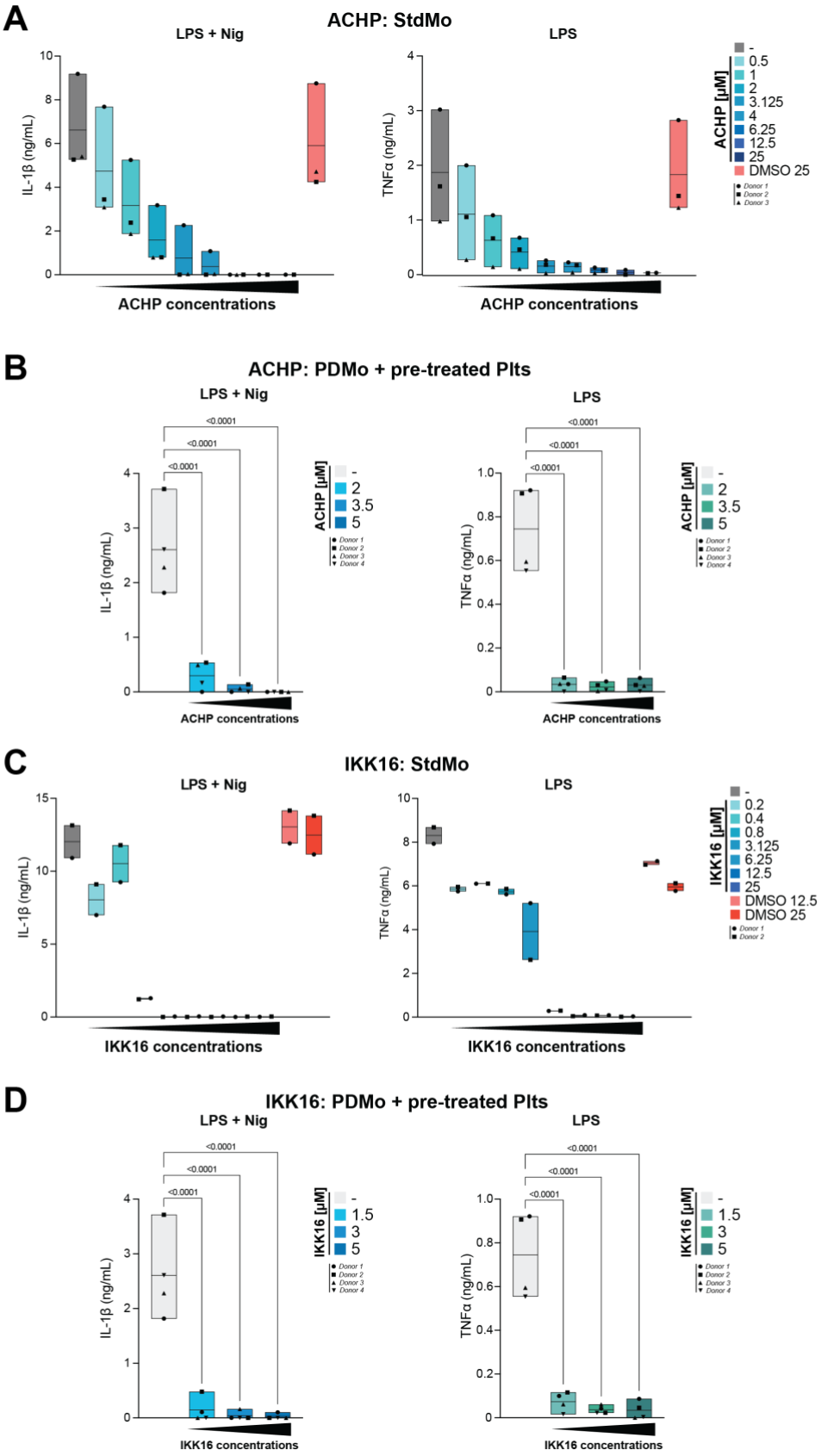


Figure 10: Pharmacological inhibition of canonical and non-canonical NFκB signaling by ACHP and IKK16 in monocytes and platelets. (**A**) Titration of ACHP (0.5-25 µM) and (**C**) IKK16 (0.2-25 µM) in StdMo. Monocytes were primed with LPS

(2 ng/mL, 3 h) and activated with Nigericin (10 µM, 1.5 h). IL-1β and TNFα levels in the supernatant were quantified using HTRF assay. DMSO was used as the solvent control. (**B + D**) Platelets were left untreated or pre-treated with ACHP or IKK16 concentrations determined in (**A + C**), washed, and incubated with PDMo. Cells were primed with LPS (2 ng/mL, 3 h) and stimulated with Nigericin (10 µM, 1.5 h), and IL-1β and TNFα secretion was measured by HTRF assay. Graphs with floating bars show maximum/minimum values relative to the mean (black bands). P values were calculated using one-way ANOVA, Tukey's multiple comparison test, and are shown in the figure. Each symbol represents one donor (n = 4).

To exclude cytotoxicity as a confounding factor, I performed LDH assays to indirectly assess cell viability in monocytes and platelets (Supplement Figure 2). The LDH assay detects the release of extracellular lactate dehydrogenase, which serves as a marker of pyroptotic cell death. No increase in LDH release was observed in platelets under untreated or LPS-stimulated conditions across all tested concentrations, indicating that pyroptosis did not occur in these settings. As expected, a marked increase in LDH release was observed in PDMo with untreated platelets following LPS plus Nigericin stimulation, consistent with inflammasome-induced pyroptosis (Supplement Figure 2A + B). Interestingly, when PDMo were co-incubated with pre-treated platelets, a reduced LDH release was detected, further supporting the observation that IL-1β secretion was diminished or entirely abrogated due to reduced inflammasome activation. To further assess cell viability, I used the CellTiter-Blue® Cell Viability Assay (CTB assay), which measures the metabolic activity of living cells through their ability to convert the redox dye resazurin into the fluorescent product resorufin (Supplement Figure 2). Across all concentrations tested, no reduction in metabolic activity was observed in the untreated or LPS-stimulated conditions, indicating that the compound was not cytotoxic. Only the LPS + Nigericin condition showed a decrease in viability, as expected due to inflammasome activation (Supplement Figure 2A + B).

Together, these findings confirm that canonical and non-canonical NFκB signaling are essential for cytokine responses in monocytes, including those enhanced by platelets, and demonstrate that pharmacological blockade of the NFκB pathway effectively suppresses IL-1β and TNFα secretion without impairing cell viability, inducing nonspecific cellular stress, or affecting platelet function.

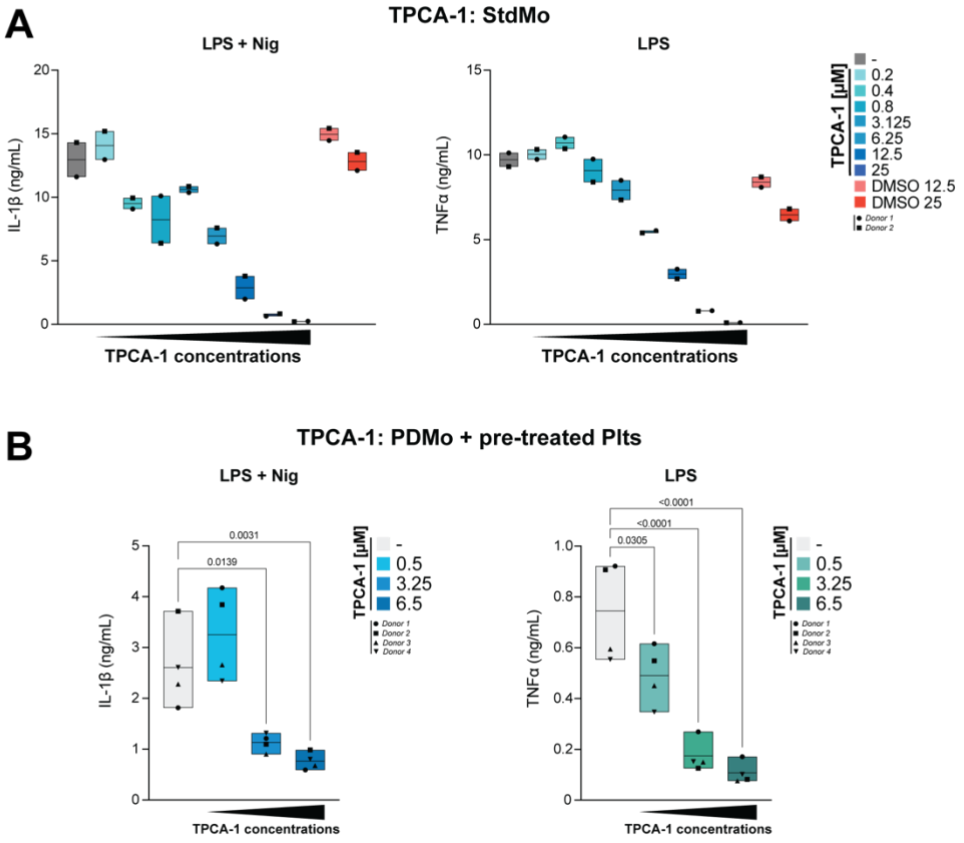


Figure 11: Pharmacological inhibition of canonical NFκB signaling by TPCA-1 in monocytes and platelets. (**A**) Titration of TCPA-1 (0.2-25 µM) in StdMo. Monocytes were primed with LPS (2 ng/mL, 3 h) and activated with Nigericin (10 µM, 1.5 h). IL-1β and TNFα levels in the supernatant were quantified using HTRF assay. DMSO was used as the solvent control. (**B**) Platelets were left untreated or pre-treated with concentrations determined in (**A**), washed, and incubated with PDMo. Cells were primed with LPS (2 ng/mL, 3 h) and stimulated with Nigericin (10 µM, 1.5 h), and IL-1β and TNFα secretion was measured by HTRF assay. Graphs with floating bars show maximum/minimum values relative to the mean (black bands). P values were calculated using one-way ANOVA, Tukey's multiple comparison test, and are shown in the figure. Each symbol represents one donor (n = 4).

Next, I used the IKKβ-selective inhibitor TPCA-1 to define the specific role of canonical NFκB signaling in monocyte cytokine responses and its enhancement by platelets. TPCA-1 inhibited NFκB1/RELA-dependent NFκB activation without interfering with the non-canonical axis.

In dose-response experiments, TPCA-1 induced a pronounced and concentration-dependent reduction in IL-1β and TNFα secretion by inflammasome-stimulated StdMo (Figure 11A). Cytokine release was inhibited starting at 0.5 µM, with near-complete suppression observed at concentrations between 5 and 10 µM. DMSO vehicle controls

had no effect, confirming that the observed inhibition was not due to cytotoxicity or nonspecific solvent effects. To determine whether canonical NFκB activity is required for the platelet-mediated enhancement of monocyte activation, I co-cultured PDMo together with platelets pre-treated with selected concentrations of TPCA-1. In the presence of the inhibitor, the addition of platelets failed to restore cytokine production, indicating that the canonical NFκB pathway also contributes to this process (Figure 11B). CTB and LDH assays (Supplement Figure 2C) confirmed stable metabolic activity and no increase in LDH release across all concentrations in both monocytes and platelets, excluding cytotoxic effects.

Collectively, these findings establish that canonical NFκB signaling is critical for both intrinsic and platelet-amplified cytokine responses in human monocytes. Selective pharmacological blockade of this pathway effectively suppressed IL-1β and TNFα production without compromising cell viability or platelet integrity.

To further dissect the role of the non-canonical NFκB pathway in monocyte activation and platelet-monocyte communication, I used two pharmacological inhibitors (XT2 and B022). These compounds target the kinase NIK, which is responsible for the processing of NFκB2 (p100) into its active form p52, and thereby prevents the nuclear translocation of RELB/p52 complexes. As shown in Figure 12A, treatment of inflammasome-stimulated StdMo with increasing concentrations of XT2 resulted in a dose-dependent suppression of TNFα secretion. This effect became evident at 10 µM and was nearly complete at 50 µM. In contrast, IL-1β secretion was only modestly affected, with a noticeable reduction observed only at the highest concentration. Notably, slight cytokine suppression was also observed in the DMSO vehicle control at high concentrations, suggesting potential solvent-related effects.

To determine whether platelet-mediated enhancement of monocyte activation also depends on NIK activity, I co-cultured PDMo together with platelets that were pre-treated with XT2. Similarly, to the monocyte inhibition, XT2 dose-dependently inhibited TNFα secretion, while IL-1β levels remained largely unchanged (Figure 12B). A clear suppressive effect was observed only at 50 µM, though this effect may be confounded by a similar reduction seen in the DMSO control.

Next, to confirm and extend these findings, I tested another NIK-targeting small molecule inhibitor B022. As shown in Figure 12C, B022 induced a modest, dose-dependent reduction in TNFα secretion in StdMo, with a similarly weak trend observed for IL-1β. Notably, reductions in cytokine secretion were again only apparent at higher

concentrations, and were also partially mirrored in DMSO-treated controls, suggesting limited specificity. Interestingly, co-cultures of PDMo with B022-pre-treated platelets (Figure 12D) again showed a significant reduction in TNFα production, while IL-1β levels remained unaffected.

For both XT2 and B022, CTB and LDH assays (Supplement Figure 2D + E) also revealed no evidence of cytotoxicity, with consistent cell viability observed at all tested concentrations.

In summary, these findings indicate that the non-canonical NFκB pathway contributes to platelet-mediated amplification of monocyte inflammatory responses, particularly TNFα production, while IL-1β appears to be regulated through distinct mechanisms.

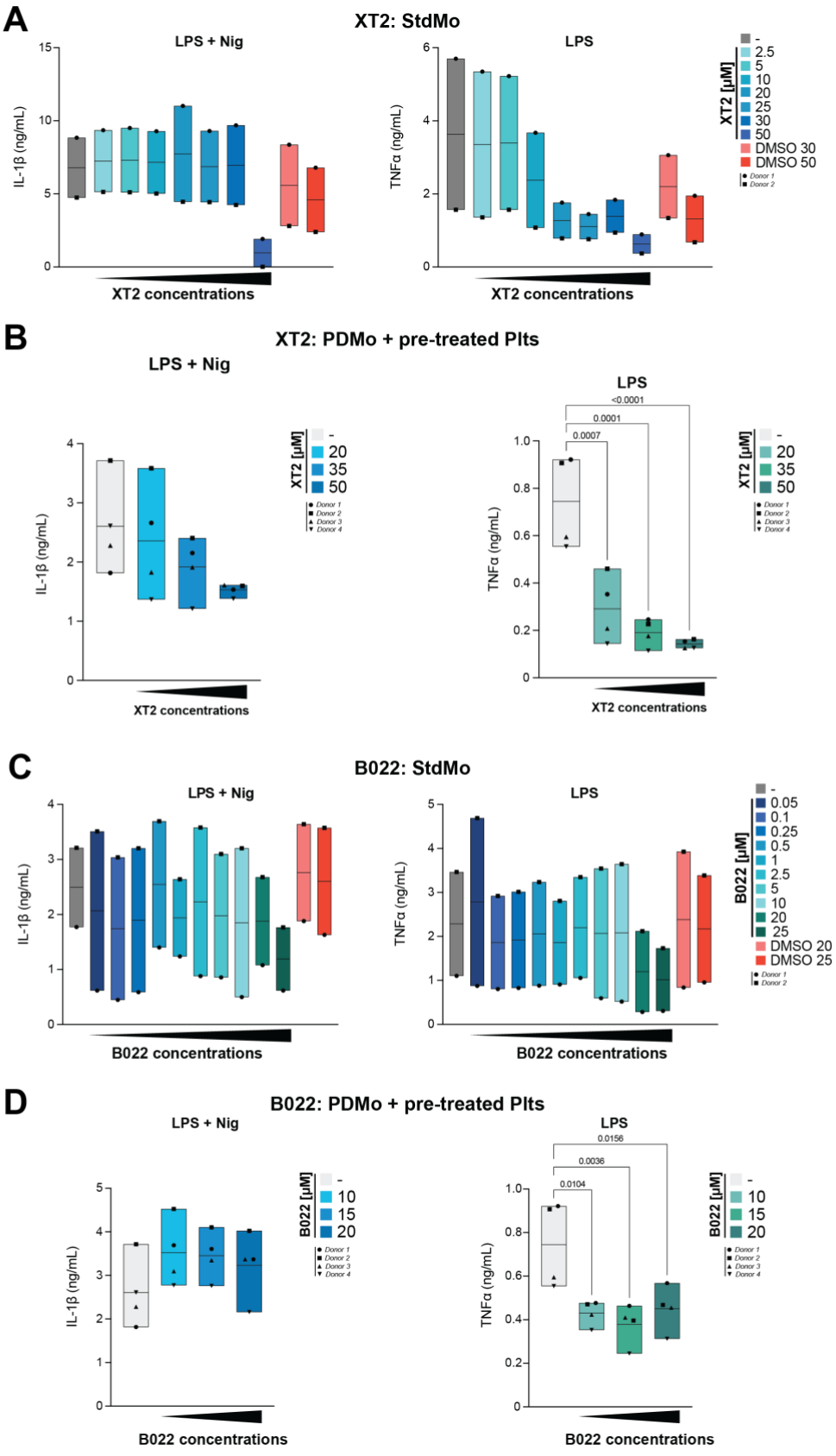


Figure 12: Pharmacological inhibition of non-canonical NFκB signaling by XT2 and B022 in monocytes and platelets. (**A**) Titration of XT2 (2.5-50 µM) and (**C**) IKK16 (0.05-25 µM) in StdMo. Monocytes were primed with LPS (2 ng/mL, 3 h) and

activated with Nigericin (10 µM, 1.5 h). IL-1β and TNFα levels in the supernatant were quantified using HTRF assay. DMSO was used as the solvent control. (**B + D**) Platelets were left untreated or pre-treated with XT2 or B022 concentrations determined in (**A + C**), washed, and incubated with PDMo. Cells were primed with LPS (2 ng/mL, 3 h) and stimulated with Nigericin (10 µM, 1.5 h), and IL-1β and TNFα secretion was measured by HTRF assay. Graphs with floating bars show maximum/minimum values relative to the mean (black bands). P values were calculated using one-way ANOVA, Tukey's multiple comparison test, and are shown in the figure. Each symbol represents one donor (n = 4).

Finally, I used the inhibitor (-)-DHMEQ to investigate the role of nuclear translocation of NFκB subunits in monocyte activation. This compound specifically blocks the nuclear import of the large NFκB subunits RELA and RELB. (-)-DHMEQ treatment induced a concentration-dependent reduction in both IL-1β and TNFα secretion from pre-treated and inflammasome-stimulated StdMo. IL-1β production was already markedly reduced at concentrations as low as 1 µM, while TNFα levels dropped substantially starting at 5 µM and were nearly completely suppressed at 10-12.5 µM (Figure 13A). Again, DMSO vehicle controls showed no inhibitory effect. To more precisely determine whether nuclear translocation of these NFκB subunits is also required for platelet-mediated enhancement of monocyte responses, I co-cultured PDMo with platelets pre-treated with (-)-DHMEQ. Interestingly, no clear dose-dependent inhibition of IL-1β was observed, and only a minor trend of TNFα reduction was detected (Figure 13B).

Moreover, I ruled out cytotoxic effects of (-)-DHMEQ on monocytes and platelets by performing CTB and LDH assays. The CTB assay demonstrated consistent metabolic activity across all tested (-)-DHMEQ concentrations, and the LDH assay revealed no increase in extracellular enzyme release, indicating the absence of cytotoxic or lytic effects (Supplement Figure 2F).

These findings suggest that nuclear translocation of the major NFκB subunits is critical for proper cytokine responses in monocytes themselves, but appears to be dispensable for platelet-mediated signaling to monocytes.

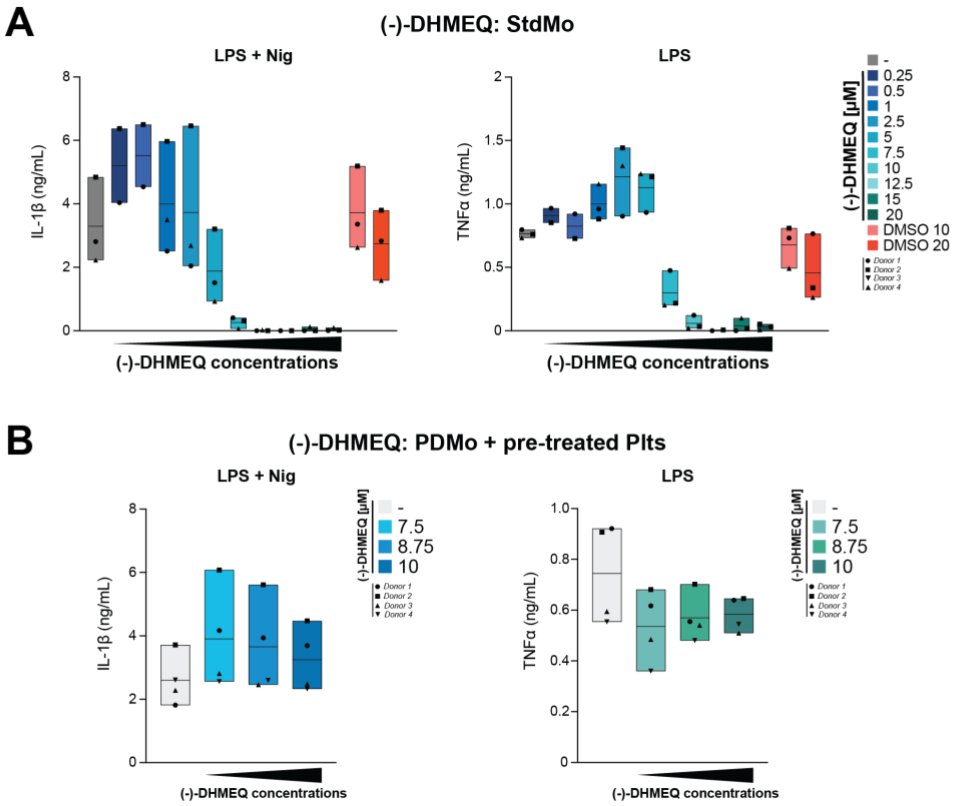


Figure 13: Pharmacological inhibition of REL protein nuclear translocation by (-)-DHMEQ in monocytes and platelets. (**A**) Titration of (-)-DHMEQ (0.25-20 μM) in StdMo. Monocytes were primed with LPS (2 ng/mL, 3 h) and activated with Nigericin (10 μM, 1.5 h). IL-1β and TNFα levels in the supernatant were quantified using HTRF assay. DMSO was used as the solvent control. (**B**) Platelets were left untreated or pre-treated with (-)-DHMEQ concentrations determined in (**A**), washed, and incubated with PDMo. Cells were primed with LPS (2 ng/mL, 3 h) and stimulated with Nigericin (10 μM, 1.5 h), and IL-1β and TNFα secretion was measured by HTRF assay. Graphs with floating bars show maximum/minimum values relative to the mean (black bands). P values were calculated using one-way ANOVA, Tukey's multiple comparison test, and are shown in the figure. Each symbol represents one donor (n = 4).

The collective results from my pharmacological inhibition experiments provide compelling evidence that both the canonical and non-canonical NFκB signaling pathways play integral roles in mediating monocyte inflammatory responses, as well as in the amplification of these responses by platelets, as seen with the inhibitors ACHP and IKK16. Selective blockade of IKKβ, using TPCA-1, robustly suppressed IL-1β and TNFα production in monocytes, including in co-culture settings with platelets, highlighting the essential contribution of the canonical NFκB arm (NFκB1/RELA). In contrast, inhibition of the non-canonical pathway through NIK blockade with XT2 or B022 had more selective and partial effects, particularly on TNFα secretion. While XT2

and B022 significantly reduced TNFα levels, IL-1β production was only modestly affected, and in some conditions, remained largely intact. These findings suggest that the non-canonical pathway (NFκB2/RELB) contributes to monocyte activation and platelet-mediated amplification, but its influence may be more prominent in the regulation of specific cytokines or under specific contexts. Interestingly, downstream blockade of NFκB nuclear translocation using (-)-DHMEQ, which targets both canonical and non-canonical NFκB subunits (RELA and RELB), suppressed monocyte cytokine responses effectively, yet did not abrogate platelet-driven enhancement. These findings are in line with the ability of platelets to reconstitute cytokine production in stimulated RELA and NFκB2 knockout THP-1 monocytes and indicates that platelet-mediated boosting of monocyte responses might involve alternative mechanisms of action or downstream signaling bypassing classical nuclear NFκB translocation. Moreover, this aligns with my previous data that demonstrate while platelets express RELA, they do not secrete it in vesicles, supporting the idea that platelet-monocyte communication relies on a distinct, possibly non-transcriptional mode of NFκB modulation (Figure 8C).

Taken together, these findings support a model in which both canonical and non-canonical NFκB pathways contribute to monocyte activation, but with potentially divergent or complementary roles in platelet-driven immunomodulation. The canonical pathway appears indispensable for both basal and platelet-driven cytokine production, whereas the non-canonical pathway may fine-tune responses such as TNFα secretion. The precise hierarchy, interplay, and potential redundancy between these pathways in the context of intercellular communication remain to be fully elucidated. Further work dissecting pathway crosstalk and compartmentalized signaling will be essential to clarify how platelets dynamically shape innate immune responses through NFκB-dependent mechanisms.

4.10. Systemic extracellular distribution of platelet-derived NFκB transcription factors via plasma and serum

Next, I considered whether the observation that platelets process the transcription factor NFκB2 into its active form, package it into platelet-derived EVs and secrete it in a way that enhances the pro-inflammatory response of monocytes, might imply a similar effect from human cell-free serum and plasma. This idea is supported by reports in the literature indicating that up to 80% of platelet-derived EVs in the

bloodstream originate from platelets (Berckmans et al., 2001; Horstman & Ahn, 1999; Italiano Jr et al., 2010; Joop et al., 2001; Taus et al., 2019).

Taking into account the inhibitor data, the findings suggest that both the non-canonical and the canonical NFκB pathway contribute to platelet-monocyte communication. Since the canonical pathway effect does not appear to rely on the large REL subunits (such as RELA or RELB), I extended the analysis only to the small subunit of the canonical pathway, NFκB1 (p105/p50). The aim was to investigate whether it is also present in platelets and whether it follows a similar pattern of processing and secretion as observed for NFκB2.

To directly address both canonical and non-canonical pathways, I examined whether processed NFκB1 and NFκB2 subunits could also be detected in circulating blood compartments, specifically in platelets, PS, PPP and serum (Figure 14A). Next, I first prepared PRP by centrifuging whole blood once and quantified the number of cells as a reference for comparison with PPP and serum. To obtain cell-free PPP, I repeatedly centrifuged the sample and transferred the supernatant, then quantified the remaining cells; from this point on, I refer to PPP simply as plasma. Although serum is largely cell-free after coagulation, I additionally centrifuged it to remove any residual cellular components and then quantified the remaining cells (Figure 14B).

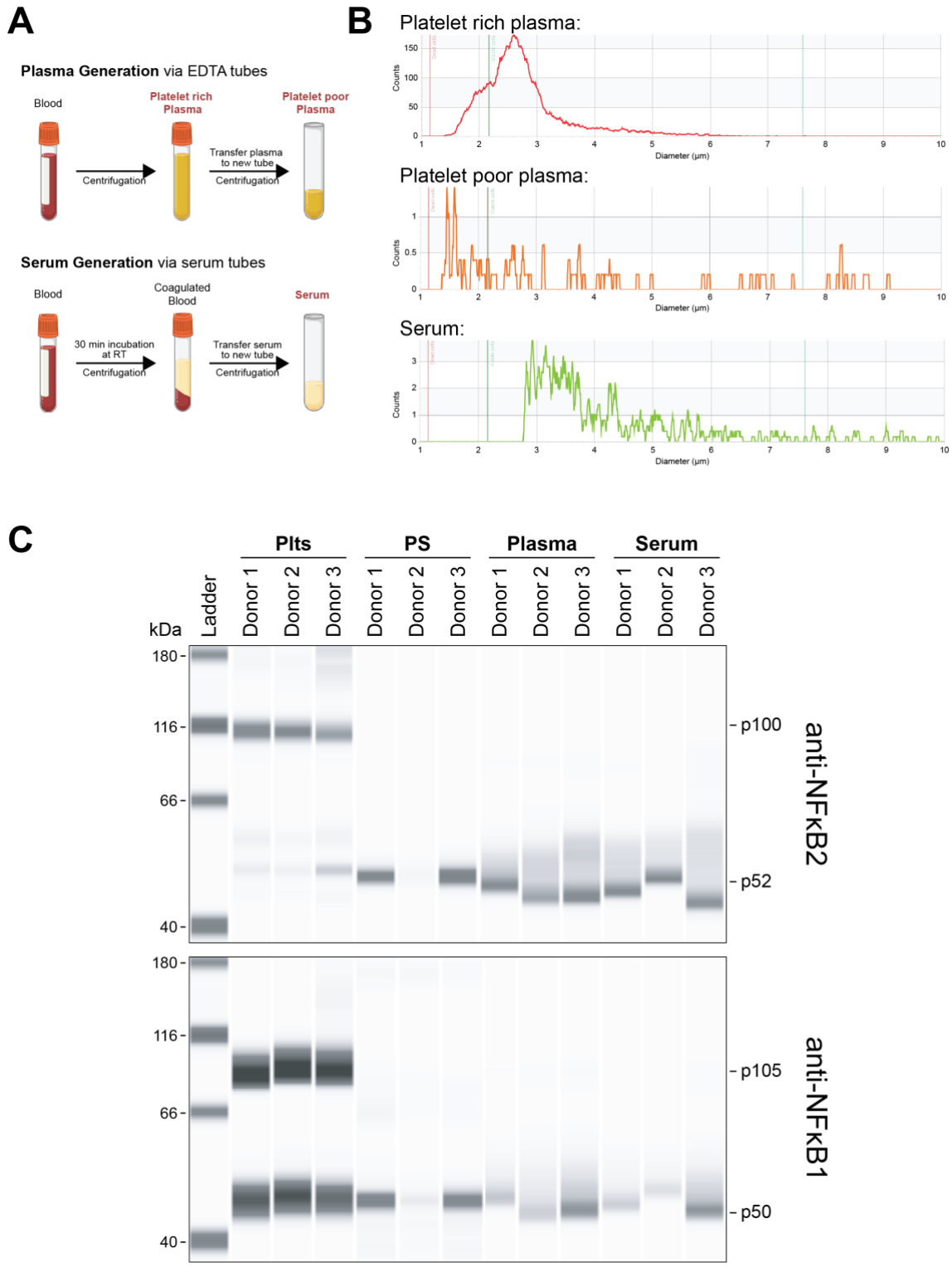


Figure 14: Comparison of NFκB subunit content in platelets, PS, plasma and serum samples. (**A**) Schematic workflow for the preparation of platelet-rich (PRP) and platelet-poor plasma (PPP), and serum from whole blood. PPP was obtained by centrifugation of blood collected in EDTA tubes at 1300 x g for 10 min. To further ensure that there are no cells, a second centrifugation step was executed. Serum was prepared from whole blood collected in serum tubes by clotting at RT for 30 min, followed by two times centrifugation at 1300 x g for 10 min. (**B**) CASY automated cell quantification of PRP, PPP, and serum. For each sample 5 µl were diluted with 10 ml

of CASY tone solution. (**C**) WES protein analysis of the small NFκB subunits of PRP, PPP, and serum samples. Blots were probed with antibodies against NFκB2 (detecting p100 and p52) and NFκB1 (detecting p105 and p50). Samples included isolated Plts, PS, plasma, and serum from 3 individual donors. The schematic workflow was created using BioRender®.

To determine the distribution of the canonical and non-canonical NFκB subunits in these blood-derived fractions, I performed WES using antibodies against NFκB2 (p100/p52) and NFκB1 (p105/p50) (Figure 14C). In platelet lysates, the precursor proteins p100 and p105 were readily detectable. In contrast, their active, processed forms, p52 and p50, were predominantly found in the corresponding supernatants, as well as in plasma and serum. Notably, p52 was consistently detected in plasma and serum from all donors, suggesting that processed NFκB2 subunits are not only released from isolated platelets *in vitro*, but also circulate systemically *in vivo*. A similar pattern was observed for NFκB1, with the active p50 subunit strongly present in extracellular fractions. These results are consistent with previous observations from the inhibitor experiments (Figure 11), supporting the idea that canonical NFκB components are also secreted.

In conclusion, these findings show that both canonical and non-canonical NFκB subunits are released by platelets in their bioactive forms and are present in plasma and serum. This supports a model in which platelets modulate immune responses through a contact-independent mechanism, delivering transcriptional regulators such as p50 and p52 into the circulation and thereby contributing to systemic inflammatory signaling.

To further evaluate whether the platelet-derived soluble factors previously identified in isolated platelet supernatants and vesicles are also functionally active in physiologically circulating blood compartments, I examined the pro-inflammatory potential of human plasma and serum on monocytes. This idea was further supported by WES data, which showed that both NFκB2 and NFκB1 were detectable in their bioactive forms, p52 and p50, respectively, in both plasma and serum. These findings aligned with the patterns previously observed in isolated platelet supernatants (Figure 8C).

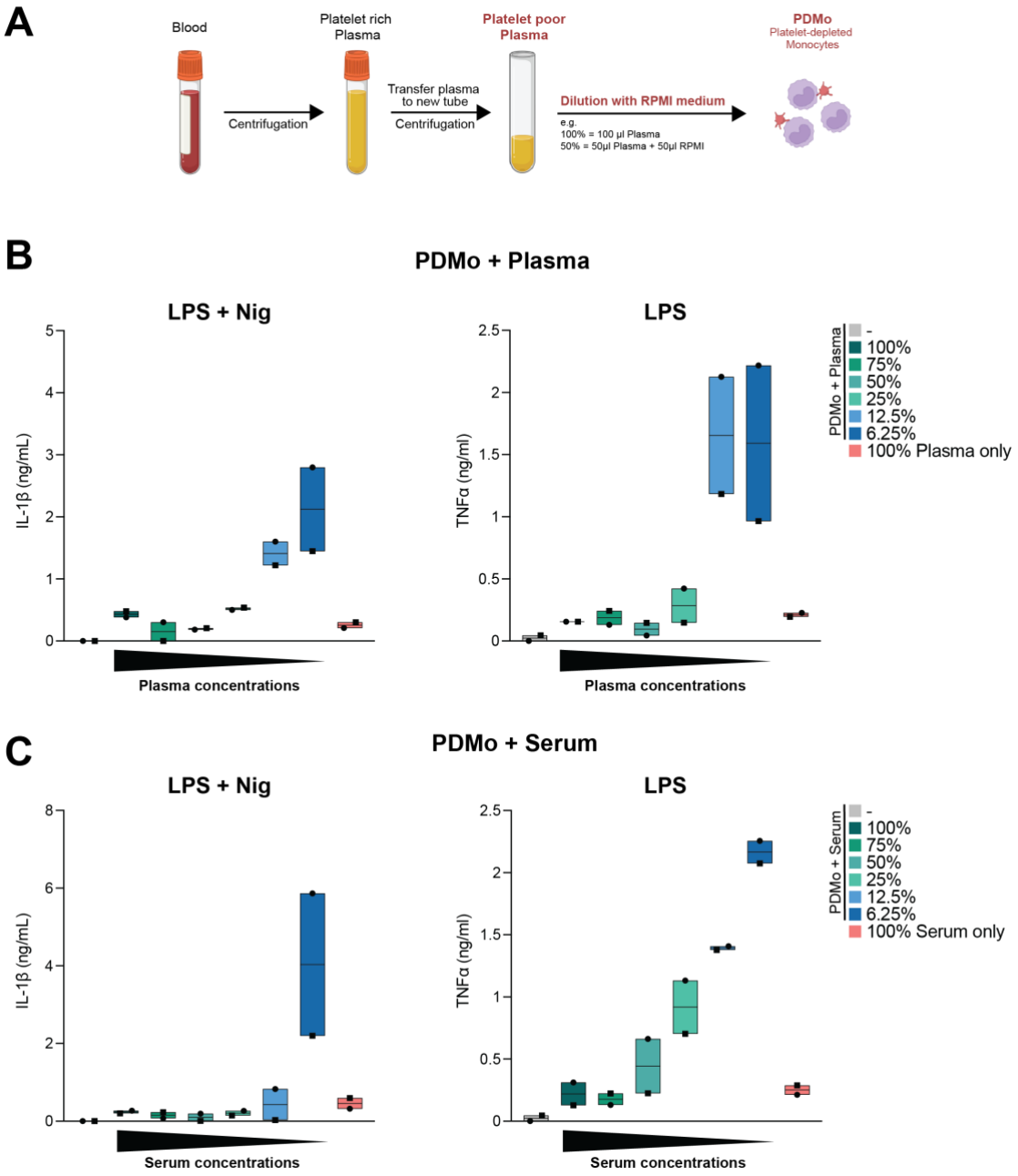


Figure 15: Effect of plasma and serum dilution on cytokine secretion by PDMo. (**A**) Experimental workflow: Plasma was obtained by centrifugation of blood collected in EDTA tubes at 1300 x g for 10 min. To further ensure that there are no cells, a second centrifugation step was executed. Monocytes were isolated from human PBMCs using negative selection, and residual platelets were removed to generate PDMo. PDMo were cultured in varying dilutions of plasma or serum. Plasma or serum only were used as controls. (**B**) Cytokine secretion by PDMo cultured in diluted plasma. Cells were primed with LPS (2 ng/mL, 3 h) and activated with Nigericin (10 µM, 1.5 h). IL-1β (left) and TNFα (right) concentrations in cell-free supernatants were measured using HTRF assay. (**C**) Cytokine secretion by PDMo cultured in diluted serum. Cells were primed and stimulated as in (**B**), and IL-1β (left) and TNFα (right) levels were quantified by HTRF assay. The schematic workflow was created using BioRender®.

For this experiment, I incubated PDMo with increasing dilutions of human plasma or serum, ranging from 12.5-100%, and either primed with LPS alone or activated with LPS and Nigericin (Figure 15A).

Remarkably, the addition of plasma and serum led to a significant increase in IL-1β and TNFα secretion. This effect was dose-dependent, but followed an unexpected inverse pattern. The strongest cytokine responses were observed at the lowest plasma or serum concentrations, particularly at 6.25% (Figure 15B + C). This applied to both IL-1β and TNFα. It was notable that neither undiluted plasma nor serum substantially enhanced cytokine production. An increase in cytokine levels became evident only at intermediate dilutions, specifically between 25% and 12.5%. These results indicate that while plasma and serum do contain functionally active mediators released by platelets, higher concentrations may include non-specific proteins or even inhibitory components that interfere with the boosting effect. Control conditions in which I incubated plasma or serum without monocytes did not result in any cytokine release, confirming that the effects observed were not due to residual cytokines or technical artifacts.

In summary, these findings demonstrate that physiological blood components such as plasma and serum contain soluble mediators, most likely released from platelets, which enhance monocyte inflammatory responses in a concentration-dependent manner. This supports the idea that platelets contribute to systemic immune regulation by releasing factors that influence monocyte activation independently of direct cell contact. Taken together, the results suggest that platelets continuously release vesicle-associated transcription factors into the bloodstream, rather than doing so exclusively in response to activation. This interpretation is further supported by the findings from LPS and Thrombin stimulation experiments (Figure 7 + Figure 8). This may reflect a mechanism by which platelets help maintain an immunological niche, providing circulating immune cells with essential cofactors to support appropriate inflammatory responses.

4.11. Mouse platelets enhance inflammasome activation in monocytes, demonstrating evolutionary conservation of platelet-monocyte communication

To investigate whether the platelet-mediated enhancement of inflammasome activation in monocytes observed in human cells is evolutionarily conserved across species, I evaluated this phenomenon in a mouse model. If conserved, this would

enable the use of various mouse lines to further dissect the mechanisms of platelet-monocyte communication.

For this purpose, I established a protocol that enabled the isolation of monocytes from mouse blood. Cardiac blood was collected from healthy mice, followed by PBMC isolation using a Ficoll® gradient and subsequent positive selection for $CD11b^+$ cells. To verify that the enriched population indeed consisted predominantly of monocytes, I further analyzed the cells by flow cytometry using the mouse monocyte-specific marker Ly6C in combination with CD11b. Interestingly, platelets make up approximately 50% of the cell population in the isolate, despite the use of a positive selection protocol. This was confirmed by a flow cytometry-based gating strategy that distinguished $CD45^+Ly6C^+$ monocytes and $CD41^+$ platelets (Figure 16A). Initial singlet and size gating allowed the exclusion of debris and aggregates, followed by clear separation of leukocytes and platelets based on CD45 expression. Monocytes were identified as $Ly6C^+CD11b^+$ and platelets as $CD41^+$ cells.

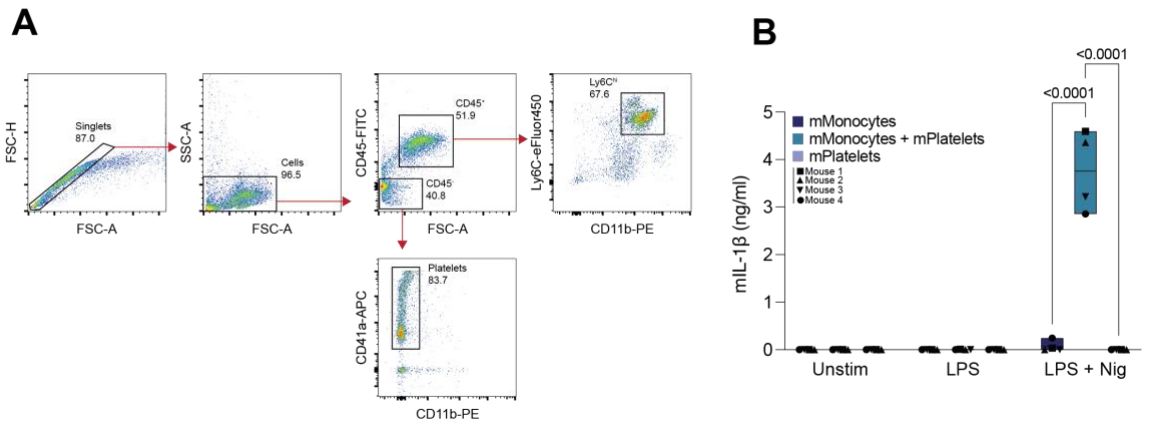


Figure 16: Isolation and functional stimulation of mouse blood monocytes with or without platelets. (**A**) Representative flow cytometry staining of blood monocytes from naive C57BL/6 mice for identification und purity confirmation. Mouse blood was isolated from the heart, and PBMCs were subsequently separated using a Ficoll® gradient. Positive selection was performed using CD11b, and cells were stained for CD45, Ly6C, CD11b, and CD41a to confirm monocyte purity from the isolated blood. As depicted, doublets were excluded followed by gating on cell morphology. All leukocytes were identified based on their CD45 expression and separated from smaller events. The smaller $CD45^-$ events were also further identified as platelets as they are almost exclusively positive for CD41a. The leukocytes were further identified as $CD11b^+Ly6C^+$ blood monocytes. The presented FACS plots show one representative sample from multiple experiments. (**B**) IL-1β levels in the cell-free supernatants of mouse blood monocytes alone or supplemented with mouse Plts and of mouse Plts alone. Cells were stimulated with LPS (1 µg/mL, 3 h) followed by

activation with Nigericin (10 µM, 1.5 h). Graphs with floating bars depict maximum/minimum values relative to the mean (black bands). P values were calculated using two-way ANOVA, Tukey's multiple comparison test, and are indicated in the figure. Each symbol represents a biological replicate.

For functional validation, I cultured the isolated mouse monocytes either alone or in co-culture with isolated mouse platelets and stimulated them either with LPS or LPS + Nigericin for inflammasome activation. As shown in Figure 16B, co-culture with mouse platelets significantly enhanced IL-1β secretion, thereby recapitulating the platelet-mediated boost previously observed in human monocytes (Figure 3). Notably, monocytes cultured alone behaved similarly to human PDMo with almost no cytokine response, which can be attributed to the relatively low platelet content in the positive selection protocol, compared to negative selection without additional platelet deletion. This observation is consistent with findings previously reported by our group (Hawwari, 2023; Hawwari & Rossnagel et al., 2024).

These results demonstrate that platelets also enhance inflammasome responses in mouse monocytes, indicating that this mechanism of platelet-monocyte communication is conserved across species. This establishes the functional relevance of platelet-derived signals in innate immune regulation and paves the way for future mechanistic studies using genetically modified mouse models.

4.12. Functional testing of platelet-derived protein transfer using a Cre-reporter mouse model

To further investigate the potential *in vivo* contribution of platelet-derived protein transfer to monocytes, I employed a conditional Flip-reporter mouse model. The Flip-R26-mT-mG mouse (courtesy of AG Wachten) carries a globally expressed construct inserted into the Rosa26 safe-harbor locus, where a tdTomato cassette followed by a stop codon precedes a downstream GFP gene. In this system, all cells fluoresce red (tdTomato) unless exposed to Cre-recombinase, which removes the tdTomato and stop cassette and induces a permanent switch to GFP expression (Figure 17A). When Cre enters the nucleus, this switch from red to green provides a visual readout of successful Cre-mediated recombination.

The central hypothesis was that platelets from PF4-Cre mice, which express Cre recombinase under control of the PF4 promoter and thus restrict expression to MKs and platelets, might release Cre protein in vesicles. If taken up by and delivered to the

nucleus, this would result in a color switch from red to green in the Flip-reporter cells. This would provide functional proof that platelet-derived proteins can not only be internalized but also trafficked to the nucleus of recipient immune cells where they remain active. WT platelets from mice of the same C57BL/6 background served as negative controls, while a recombinant nuclear-localized Cre protein (TAT-Cre) was used as a positive control due to its known ability to enter mammalian cells and induce recombination.

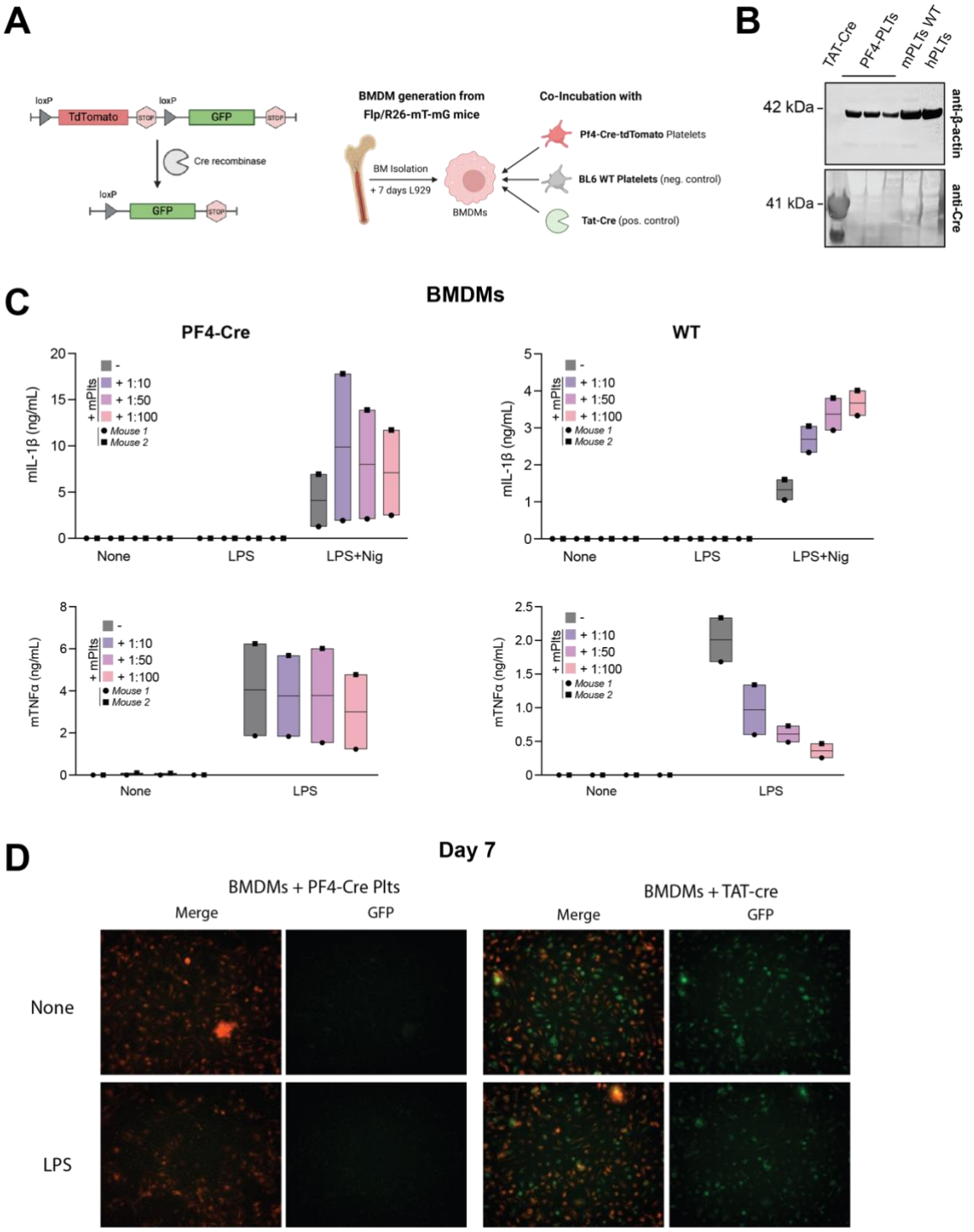


Figure 17: Functional contribution of platelet-derived factors in conditional reporter mice using Flip/R26-mT/mG system and PF4-Cre platelets or TAT-Cre. (**A**) Experimental workflow and schematic representation of the mT/mG reporter system: In the presence of Cre recombinase, tdTomato (mT) is excised and replaced by GFP (mG), allowing lineage tracing of platelet-derived Cre activity in recipient cells. After 1 week bone marrow was isolated from Flp/R26-mT-mG mice and differentiated to BMDMs. of differentiation on the day of the experiment, platelets were isolated from PF4-Cre or WT C57BL/6 mice. Washed platelets were then added to BMDMs. The co-cultures were subjected to stimulation with LPS (1 µg/mL, 3 h) ± Nigericin (10 µM, 1.5

h). Supernatants were collected for cytokine quantification and cells were used for widefield microscopy. The recombinant protein TAT-Cre was used as a positive control. (**B**) Western blot analysis of platelet and BMDM lysates for validation of the Flip/R26-mT/mG system and Cre-dependent recombination. Lysates were probed with anti-Cre antibodies with the expected band size at 41 kDa. (**C**) IL-1β and TNFα levels in the CFS of BMDMs co-cultured with increasing numbers of platelets ($1x10^6$, $2.5x10^6$, or $1x10^7$) from PF4-Cre (left) or WT (right) mice. Cytokine concentrations were measured by HTRF assay. Cells were stimulated with LPS (1 µg/mL, 3 h) followed by activation with Nigericin (10 µM, 1.5 h). Graphs with floating bars depict maximum/minimum values relative to the mean (black bands). Each symbol represents a mouse. (**D**) Representative widefield fluorescence microscopy images of Flip/R26-mT/mG BMDMs co-cultured with platelets from PF4-Cre or WT mice. tdTomato and GFP expression was visualized to assess Cre recombinase activity before and after stimulation with or without LPS. Images show merged and GFP-specific fluorescence channels. The schematic workflow was created using BioRender®.

Based on published data from our group, it has already been shown that platelets support macrophages in their pro-inflammatory response (Rolfes et al., 2020). Due to the difficulty of isolating sufficient numbers of monocytes from mouse blood and the fact that these cells differentiate within a few days and can no longer be considered real monocytes, I chose an alternative approach using BMDMs. BMDMs remained stable for the observation period of up to seven days. Therefore, I differentiated macrophages from the bone marrow of Flip-R26-mT-mG mice. Next, I isolated platelets from PF4-Cre and WT mice and co-cultured them with BMDMs at different ratios (1:10, 1:50, 1:100). I stimulated the cultures with LPS and Nigericin to activate the inflammasome. Supernatants were used for functional cytokine measurements by HTRF, and also imaged daily over the course of seven days to track a potential red-to-green fluorescence switch in BMDMs (Figure 17A). Functionally, WT platelets induced a dose-dependent increase in IL-1β secretion from BMDMs, whereas PF4-Cre platelets showed only a minor and variable effect. TNFα secretion, on the other hand, decreased with increasing platelet concentration, consistent with previous findings from our group (Rolfes et al., 2020).

To test for Cre transfer, I examined the cultures for GFP expression by microscopy (Figure 17D). I could not observe any GFP signal in BMDMs co-cultured with PF4-Cre platelets, even after seven days, indicating the absence of a functional Cre protein transfer. As expected, neither BMDMs alone nor the ones co-incubated with WT platelets resulted in any color switch. In contrast, the TAT-Cre positive control induced a robust red-to-green switch in more than half of the BMDMs by day seven, confirming

that the system was technically capable of detecting Cre activity. Western blot analysis (Figure 17B) supported these findings. I could not detect any Cre protein in platelet lysates from PF4-Cre mice. Only the recombinant TAT-Cre protein showed a detectable Cre band. β-actin was present in all samples except the TAT-Cre lane, verifying sample integrity, but raising doubts about whether Cre was ever present in the platelet samples at functionally relevant levels.

These results demonstrate that while platelets functionally enhance IL-1β secretion from macrophages, a direct protein transfer of Cre recombinase from PF4-Cre platelets to macrophages, resulting in nuclear recombination, is not detectable under the tested conditions.

4.13. Functional modulation of monocyte responses by platelets in a cross-species setting

As a final approach to gain deeper insight into the mechanisms of platelet-to-monocyte communication, I explored whether this process could be studied across species. Specifically, I tested the capacity of mouse and human platelets to modulate the inflammatory response of both mouse and human myeloid cells. The rationale behind this approach was that such a cross-species system might allow the detection of human proteins within mouse cells or vice versa. This could help identify a broader set of proteins involved in platelet-driven immune activation, providing a more comprehensive understanding of the molecular mechanisms that govern platelet-to-immune cell communication.

First, human PDMo were incubated with mouse platelets (mPlts) and inflammasome-stimulated (Figure 18A). Mouse platelets significantly enhanced human IL-1β secretion in human PDMo. This effect was comparable to that induced by human platelets (hPlts), indicating that mPlts possess conserved immunomodulatory mechanisms that can act on human target cells. Conversely, mouse BMDMs were treated with either hPlts or human PS (hPS) and similarly inflammasome-stimulated (Figure 18B). In this setting, both human platelet products robustly enhanced IL-1β secretion by mouse BMDMs, confirming the bidirectional compatibility and functional conservation of the platelet-monocyte communication axis across species.

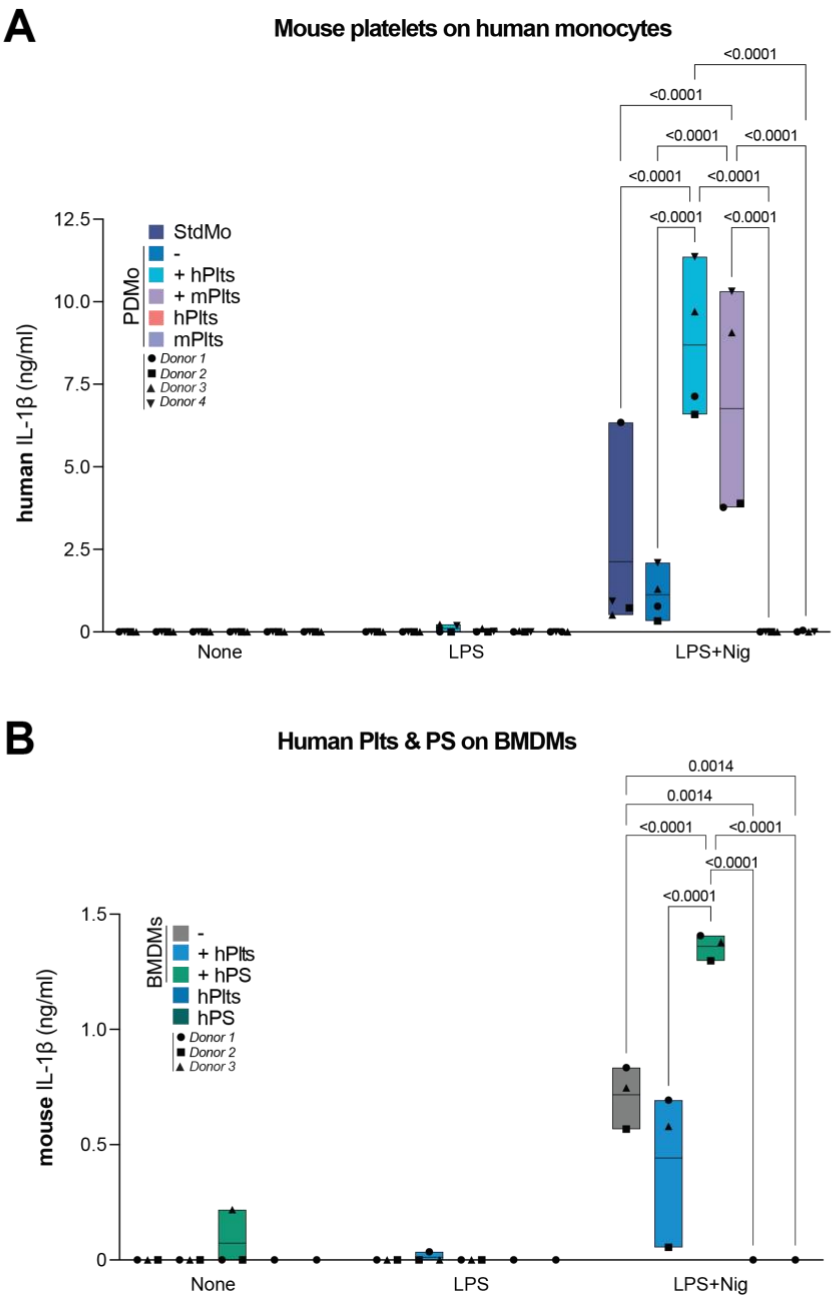


Figure 18: Cross-species platelet-monocyte interactions in BMDMs. (**A**) Human monocytes were stimulated with LPS (2 ng/mL, 3 h) and subsequently activated with Nigericin (10 µM, 1.5 h) in the presence or absence of mouse platelets. Platelets were added at increasing ratios (1:5, 1:10, 1:20, 1:40 relative to monocytes). Human IL-1β levels in the supernatant were measured using HTRF assay. (**B**) Mouse BMDMs were cultured alone or with human platelets or human PS. Cells were stimulated with LPS (1 µg/mL, 3 h) and activated with Nigericin (10 µM, 1.5 h). Mouse IL-1β concentrations in the supernatant were determined via HTRF assay. Data presented as floating bars representing minimum-to-maximum values with mean (black line) with individual symbols representing independent donors (n = 3). Statistical analysis: two-way ANOVA with Tukey's multiple comparisons. Significant P-values indicated.

4.14. Summary of results

My thesis aimed to dissect the role of platelets in modulating monocyte inflammatory responses, with a focus on NFκB signaling and contact-independent communication mechanisms. Through a series of mechanistic, pharmacological, and cross-species experiments, I provide converging evidence that platelets are active modulators of monocyte and macrophage function through the secretion of vesicle-associated signaling mediators.

First, I began by demonstrating that human platelets process NFκB2 into its bioactive form p52 and secrete it via platelet-derived EVs. These vesicles significantly boosted IL-1β and TNFα production upon LPS and Nigericin stimulation. This boost was abrogated in vesicle-depleted preparations, indicating that the vesicular compartment is essential for the observed pro-inflammatory modulation (Figure 8).

The use of pharmacological inhibitors of canonical and non-canonical NFκB pathways (ACHP, IKK16, TPCA-1, XT2, B022, and (-)-DHMEQ) revealed that both arms of the pathway contribute to cytokine production. Canonical NFκB signaling via IKKβ was critical for both basal and platelet-enhanced IL-1β and TNFα responses in monocytes, without inducing cytotoxicity (Figure 11). Non-canonical pathway inhibition via NIK blockade partially suppressed TNFα production but had less impact on IL-1β, suggesting a differential and possibly synergistic role for both pathways in platelet-mediated monocyte activation (Figure 12). Blocking further downstream at the nuclear translocation of NFκB subunits with (-)-DHMEQ strongly suppressed cytokine production in isolated monocytes, but not in co-cultures with pre-treated platelets, further supporting the hypothesis that platelets can bypass typical monocyte intracellular regulatory checkpoints via direct transfer of active signaling components (Figure 13). Using WES capillary electrophoresis, I detected processed NFκB2 (p52) and NFκB1 (p50) in platelet supernatants, plasma, and serum (Figure 14). The active subunits were not only present *in vitro* in vesicle fractions but also in circulating blood compartments, indicating a systemic and potentially constitutive release by platelets.

To assess the functional impact of these components in a physiologically relevant context, I incubated monocytes with increasing concentrations of human plasma and serum (Figure 15). Surprisingly, low dilutions (12.5% and 25%) induced the highest cytokine responses, whereas pure plasma or serum had little effect, possibly due to inhibitory factors or protein overload. This non-linear response further emphasized the

complexity of blood-derived modulators and their regulatory balance. Next, I translated my findings into the mouse system. Isolated mouse monocytes showed increased IL-1β secretion when co-cultured with mouse platelets, mimicking the human system (Figure 16). This conserved boost supported the physiological relevance of the platelet-monocyte axis. To investigate whether platelets can deliver functional protein cargo directly to monocytes, I employed a conditional Flip reporter mouse model in combination with PF4-Cre-expressing platelets. Despite robust Cre activity in recombinant controls, I could not observe any GFP switching in BMDMs co-cultured with PF4-Cre platelets, and no Cre protein was detectable in these platelets, indicating that functional protein transfer of Cre could not be confirmed in this experimental setup (Figure 17). Finally, I examined the species-transcending capacity of platelet-derived vesicles. Mouse platelets enhanced IL-1β production in human monocytes, and vice versa human platelets and their supernatants boosted mouse BMDMs (Figure 18).
Collectively, my results support a model in which platelets contribute to innate immune regulation via the release of vesicle-derived NFκB subunits and potentially other yet-to-be-identified factors. These vesicles are capable of modulating monocyte responses across species, operating via a contact-independent, transcription factor-mediated mechanism that leverages both canonical and non-canonical NFκB signaling pathways.

5. Discussion

5.1. Platelets as active regulators of immune responses

Historically, platelets have been predominantly recognized for their central roles in hemostasis and thrombosis. However, an expanding body of evidence highlights their function as multifaceted immune modulators involved in both innate and adaptive immunity (Marcoux et al., 2021; Michelson et al., 2019; Semple et al., 2011; Semple & Freedman, 2010). Platelets exert immunoregulatory effects through direct interactions with leukocytes, including monocytes, neutrophils, and lymphocytes, as well as through the release of immunologically active soluble factors and platelet-derived EVs (Lou & Cai, 2025; Morrell et al., 2014; Semple et al., 2011; Semple & Freedman, 2010; Thomas & Storey, 2015). Although platelet-monocyte aggregates have been extensively investigated in various disease contexts across inflammatory and autoimmune disorders, including cardiovascular disorders, systemic lupus erythematosus and infections, the precise molecular mechanisms governing these interactions remain incompletely understood (Assinger et al., 2019; Fendl et al., 2021; Hottz et al., 2020; Jiang et al., 2023; McFadyen & Peter, 2020; Rolling et al., 2023). As previously shown by our group in the context of platelet-macrophage communication, platelets can enhance immune responses (Rolfes et al., 2020). Building on this foundation, we observed a similar effect in monocytes in a subsequent study (Hawwari & Rossnagel et al., 2024), based on the excellent groundwork of Ibrahim Hawwari (Hawwari, 2023). My thesis specifically addresses this gap by elucidating novel vesicle-mediated interactions between platelets and monocytes, thereby contributing to a more comprehensive understanding of platelet-mediated immune regulation.

As part of this work, I demonstrated that platelets support monocyte function through a contact-independent mechanism involving vesicles that deliver, among other cargo, transcriptional regulators such as NFκB2. These platelet-derived EVs enhance monocyte responsiveness to pathogen-associated stimuli. Furthermore, I was able to detect these vesicles in both serum and plasma. Moreover, I also showed that the same mechanism is operational in mouse systems, suggesting that the underlying process is evolutionarily conserved, and functional across species. These findings offer new insights into how platelets modulate immune responses and suggest a potential role in establishing functional immune niches within the circulation.

5.2. Vesicle-mediated transfer of transcriptional regulators

In this work, I demonstrated that human platelets constitutively secrete EVs carrying processed subunits of NFκB (Figure 8). Quantitative SILAC-based proteomics revealed that platelet-derived NFκB2 (p52) and other regulatory proteins become enriched in human monocytes following platelet exposure, providing evidence for active intercellular transfer of transcriptional components. This finding, along with evidence from other studies, challenges the classical view that transcription factor activation is strictly cell-autonomous and reveals an additional layer of redundancy within immune signaling pathways (French et al., 2020; Hawwari & Rossnagel et al., 2024; Rolfes et al., 2020; S. Sun, 2012; S.-C. Sun, 2017; Yuan et al., 2023; Y. Zhang et al., 2024; Zhou et al., 2018). Research by Zhang and colleagues, demonstrated that the transcription factor FOXM1 mediates the loading of chromatin DNA fragments into EVs by interacting with LC3 in the nucleus. These FOXM1-associated DNA fragments are translocated to the cytoplasm and released via an autophagy-related mechanism, providing a mechanistic basis for the intercellular transfer of transcription factors and their regulatory elements (Y. Zhang et al., 2024). Supporting this concept, Zhou et al. showed that retinal progenitor cells release EVs containing transcripts of transcription factors such as Pax6, Hes1, and Sox2. Using a Cre-loxP recombination system, they provided functional evidence that these vesicles are taken up by recipient cells and induce gene expression, thereby demonstrating the functional transfer of transcriptional regulators between cells (Y. Zhang et al., 2024).

The mechanism identified here is consistent with recent studies indicating that platelets serve as cellular reservoirs of key transcriptional regulators, including components of both the canonical and non-canonical NFκB pathways. These factors are not only functionally active, but are also selectively packaged into vesicles and delivered to recipient immune cells. The observation that vesicle release occurs constitutively and independently of classical platelet activation signals, such as Thrombin or LPS, suggests that platelets continuously influence immune cell function through a mechanism that we described as “transcriptional licensing“ of monocytes (Hawwari & Rossnagel et al., 2024; Rolfes et al., 2020). It is particularly noteworthy that studies have reported that the release of platelet-derived components, including EVs, is typically dependent on the activation state of the platelets or induced by external stimuli (Burzynski et al., 2019; Ebeyer-Masotta et al., 2022; Gasecka et al.,

2019; Lou & Cai, 2025; Puhm et al., 2021; Suades et al., 2022; Zarà et al., 2022). Such stimulation leads to granule release and is generally considered a trigger-dependent process. In contrast, my data show that the secretion of platelet-derived EVs can occur independently of these classical activation signals (Figure 7 + Figure 8). This suggests the existence of a previously unrecognized, constitutive mechanism of platelet communication.

My findings further support the notion that the majority of EVs circulating in human blood originate from platelets rather than MKs (Flaumenhaft et al., 2009; Foulem et al., 2025; George et al., 1982; Mause & Weber, 2010; Ponomareva et al., 2017; Yuan et al., 2023). This is underscored by the observation that even a short incubation with isolated platelets, as brief as one hour, was sufficient to restore inflammatory cytokine production in PDMo (Figure 8D). Fractionation experiments confirmed that the active p52 subunit of NFκB2 predominated in vesicle fractions of PS and was absent in vesicle-depleted PS (Figure 8C). This strongly supports vesicles as the main delivery mechanism for platelet-derived transcriptional regulators. My data further suggest that platelet vesicle production is tightly regulated and enables selective loading of specific immunomodulatory factors. Interestingly, further evidence that the immune-regulatory functions of platelets extend beyond the circulation comes from studies using platelet-specific PTEN knockout mice. In these mice, dysregulated T cell activation, excessive accumulation of T follicular helper cells (Tfh), and increased platelet aggregates in lymph nodes were observed. PTEN-deficient platelets are hyperactive and produce elevated levels of cytokines that promote Tfh differentiation via the PDK1-mTORC2-AKT-SNAP23 signaling axis. These platelets also display increased interactions with $CD4^+$ T cells and facilitate their differentiation into Tfh cells. These findings highlight the importance of PTEN in maintaining immune homeostasis through the regulation of platelet activity and underscore the relevance of platelet-derived immune signals in modulating adaptive immune responses, not only in the blood but also in the lymph nodes (X. Chen et al., 2022).

Together, these observations support the concept that platelets contribute to the establishment of a distinct immunological environment. This environment is shaped by the continuous release of platelet-derived EVs and other bioactive mediators and appears to regulate the functional state and differentiation of circulating immune cells. This is further supported by evidence showing that monocytes exhibit a preferential association with platelet-derived EVs, suggesting a selective and functionally relevant

interaction that may influence monocyte identity and responsiveness (Fendl et al., 2018; R. Weiss et al., 2018). It is plausible that this platelet-mediated environment maintains monocytes in a precursor-like state while in circulation, and that the loss of this environment, upon tissue entry, facilitates their differentiation into macrophages. To test this hypothesis further, comparative analyses of the differentiation kinetics of StdMo and PDMo using transcriptomic profiling at multiple time points would be informative. A prolonged monocytic transcriptional identity in StdMo compared to a more rapid differentiation trajectory in PDMo would support the notion that platelet-derived EVs help maintain monocyte identity in the bloodstream, with their absence acting as a trigger for further differentiation in peripheral tissues.

5.3. Functional compensation in monocytes with deficient NFκB signaling

Using CRISPR/Cas9-engineered THP-1 monocyte cell lines deficient in either the canonical NFκB subunit RELA (p65) or the non-canonical component NFκB2 (p52), I demonstrated that supplementation with platelets could partially restore IL-1β production. This observation provides evidence for a previously unrecognized form of functional compensation, in which platelet-derived transcription factors substitute for missing endogenous immune regulators in monocytes. The ability of platelets to restore cytokine responses in signaling-deficient monocytes highlights a new level of redundancy within the innate immune system. This mechanism aligns with the broader immunological principle that essential immune functions are safeguarded through overlapping and compensatory pathways to maintain effective host defense (Broz et al., 2010; El-Brolosy & Stainier, 2017; Hawwari & Rossnagel et al., 2024; Nish & Medzhitov, 2011). To further investigate the robustness of this compensatory effect, it would be valuable to examine whether human plasma or serum alone can rescue IL-1β production in NFκB-deficient THP-1 cells. Furthermore, it would be interesting to delete all EVs by ultra-filtration in serum and plasma and see if this leads to a loss of the compensator effect on PDMo but also on the NFκB-deficient THP-1 cells. Such experiments could help determine whether circulating platelet-derived EVs are sufficient to mediate these effects in the absence of freshly isolated platelets. Positive results would strengthen the argument that platelet-derived EVs are the principal agents responsible for this form of functional rescue. My findings hold particular clinical relevance for patients with ITP, a condition marked by abnormally low platelet counts and impaired monocyte responses. Previous studies have shown that monocytes from

individuals with ITP produce reduced levels of inflammatory cytokines, which may contribute to their heightened susceptibility to infection (Audia et al., 2017, 2021; Hawwari & Rossnagel et al., 2024). Future experiments could investigate whether plasma or serum from patients with immune ITP similarly fail to restore cytokine responses in platelet-depleted or NFκB-deficient monocytes, and conversely, whether plasma or serum from healthy donors can rescue monocyte responses in ITP patients. In addition, quantifying EVs levels in ITP patient plasma or serum could help determine whether reduced rescue capacity is due to diminished vesicle abundance and further help to clarify whether platelets or MKs are the primary *in vivo* source of these immunomodulatory vesicles (Flaumenhaft et al., 2009; George et al., 1982; Mause & Weber, 2010; Ponomareva et al., 2017).

These findings open new avenues for therapeutic intervention. Strategies involving platelet supplementation or engineered platelet-derived EVs could potentially restore immune competence in patients with thrombocytopenia or genetically impaired innate immune signaling. Such approaches may be particularly beneficial in clinical contexts where immune function is compromised due to inherited defects or acquired platelet deficiencies, including autoimmune diseases, hematological malignancies, and chemotherapy-induced immunosuppression (J. Chen et al., 2024; Q. Li et al., 2021; Luo et al., 2017; Xiang et al., 2013).

5.4. Distinct contributions of canonical and non-canonical NFκB pathways

The results of my pharmacological inhibition experiments provide important mechanistic insights into the distinct roles of the canonical and non-canonical NFκB pathways in platelet-mediated regulation of monocyte cytokine responses. Using selective inhibitors targeting key components of these pathways, I assessed their respective contributions to the regulation of inflammatory cytokines (Figure 10-13).

The mammalian NFκB family consists of five subunits: NFκB1 (p105/p50), NFκB2 (p100/p52), RELA (p65), RELB and c-Rel. All of these share a conserved REL homology domain that enables them to form a variety of homo- and heterodimers, resulting in 15 theoretically possible combinations (Ben-Neriah & Karin, 2011; Guo et al., 2024; Hoffmann et al., 2006; V. F.-S. Shih et al., 2011; S. Sun, 2012; Vallabhapurapu & Karin, 2009). These dimers are normally held inactive in the cytoplasm through binding to IκB proteins. Among the various dimeric forms, the p65/p50 heterodimer is the most abundant and widely studied (S. Ahmad et al., 2022;

Yu et al., 2020). Inhibitors such as ACHP and IKK16 target the IκB kinase complex and thereby block NFκB activation by preventing phosphorylation and subsequent degradation of IκB. These inhibitors are not highly selective and affect both IKKα and IKKβ, making them effective at suppressing both canonical and non-canonical NFκB signaling (Murata et al., 2004; Sanda et al., 2005; Waelchli et al., 2006). Treatment of monocyte cultures with NFκB pathway inhibitors significantly reduced IL-1β and TNFα production, indicating involvement of both canonical and non-canonical signaling. The selective IKKβ inhibitor TPCA-1 confirmed the central role of the canonical pathway (Figure 11). Although SILAC proteomics did not detect canonical subunits transferred from platelets to monocytes (Figure 4), functional inhibition data and reduced cytokine levels in RELA-deficient THP-1 clones, which were partially rescued by platelet supplementation, support their contribution to platelet-monocyte communication. (Figure 6B). These findings indicate that canonical signaling plays a major role in platelet-monocyte communication and highlight functional redundancy between canonical and non-canonical pathways. In contrast, inhibition of NIK, the central kinase in the non-canonical NFκB pathway, via XT2 and B022, selectively reduced TNFα production without affecting IL-1β (Figure 12). This finding suggests a more specialized, context-dependent role for the non-canonical pathway in regulating inflammatory cytokines, particularly TNFα (S. Ahmad et al., 2022; Hou et al., 2018; V. F.-S. Shih et al., 2011; S. Sun, 2012; S.-C. Sun, 2017).

To further explore the interplay between canonical and non-canonical signaling in this intercellular communication, it would be valuable to examine whether pre-treating platelets with the same inhibitors used in monocytes would diminish their ability to rescue cytokine production in THP-1 knockout clones. Previous attempts to address this question using BAY 11-7082, a broad IKK inhibitor, yielded unexpected results (Hawwari & Rossnagel et al., 2024; Rauert-Wunderlich et al., 2013). Rather than impairing cytokine rescue, BAY 11-7082-treated platelets exhibited an even stronger capacity to restore cytokine production in RELA-deficient THP-1 cells compared to untreated platelets. This surprising outcome suggests that platelet-derived NFκB signaling may be dispensable or compensated for in THP-1 cells (Hawwari & Rossnagel et al., 2024).

These results imply that THP-1 monocytes are less dependent on a specific NFκB pathway than primary monocytes. Even when both canonical and non-canonical pathways were blocked in platelets using BAY 11-7082, cytokine production was not

impaired in RELA- or NFκB2-deficient THP-1 clones. This observation underscores the complexity of NFκB signaling and highlights the possibility that alternative NFκB pathways or non-canonical dimers may compensate for loss of classical signaling (S. Ahmad et al., 2022; Guo et al., 2024; Rauert-Wunderlich et al., 2013). Studies show that various subunits can form alternative dimers, such as p50 with c-REL or p50 with RELB, which may activate distinct transcriptional programs (Guo et al., 2024; Hou et al., 2018; Vallabhapurapu & Karin, 2009). The complexity of NFκB signaling is further illustrated by studies showing cell type-specific regulation. In DCs, for example, RELB can promote activation through canonical signaling via RELB-p50 dimers, illustrating non-traditional interactions among pathway components (V. F. S. Shih et al., 2012). It is therefore plausible that THP-1 cells employ different NFκB signaling mechanisms than primary monocytes, contributing to the observed differences in pathway dependency and redundancy.

My findings are consistent with previous studies demonstrating that canonical and non-canonical NFκB pathways differentially regulate cytokine expression in various immune cell types (S. Sun, 2012; S.-C. Sun, 2017; Q. Zhang et al., 2017). Together, my data underscore the layered and highly context-specific nature of NFκB-mediated transcriptional regulation, with distinct signaling modules orchestrating complementary and sometimes overlapping cytokine programs. To further elucidate molecular events downstream of platelet-derived vesicle uptake, I used the NFκB inhibitor (-)-DHMEQ, which prevents nuclear translocation of REL family members (Quach et al., 2017) (Figure 13). This approach revealed that, although platelets contain the bigger subunits of NFκB such as RELA, these larger proteins are not packaged into EVs and are therefore not transferred to recipient monocytes (Figure 8C). In contrast, smaller subunits such as NFκB1 and NFκB2 were consistently detected within platelet-derived EVs and were functionally transferred to monocytes, where they contributed to transcriptional responses (Figure 14C). This finding marks a conceptual shift in our understanding of cell-to-cell communication. It suggests that platelet-derived EVs do not merely deliver pro-inflammatory cytokines, but instead serve as vehicles for the selective transfer of transcriptional regulators (Diamant et al., 2004; Ebeyer-Masotta et al., 2022; Foulem et al., 2025; Hawwari & Rossnagel et al., 2024; Mause & Weber, 2010; Rolfes et al., 2020). These processed subunits can form active transcription factor complexes with endogenously expressed partners in recipient cells. As a result, monocytes can initiate gene expression programs without the need for upstream

receptor stimulation or classical intracellular signaling cascades. The ability of platelet-derived vesicles to bypass classical activation checkpoints introduces a new dimension of immune regulation and challenges the traditional view that transcription factor activation must occur exclusively through cell-intrinsic mechanisms. Moreover, the selective packaging of small subunits of the NFκB pathway and the exclusion of large ones, suggests an evolutionarily refined system of immune modulation that prevents excessive systemic activation while still licensing a rapid and localized response (Hawwari & Rossnagel et al., 2024).

In summary, these findings position platelet-derived EVs as precise and versatile modulators of immune cell function. Through the selective delivery of NFκB subunits, they orchestrate immune responses in a manner that is both highly targeted and functionally redundant. This complexity emphasizes the need for further dissection of individual subunits and their combinations to fully understand the regulatory networks of NFκB signaling. Future experiments selectively inhibiting or knocking out specific NFκB components will be essential for clarifying the molecular underpinnings of this intercellular communication system.

5.5. Evolutionary conservation and cross-species compatibility

My cross-species experiments revealed a striking degree of evolutionary conservation in the mechanisms by which platelets modulate monocyte activation. Human platelets were able to enhance cytokine responses in mouse BMDMs, and reciprocally, mouse platelets significantly increased the inflammatory response in human monocytes (Figure 18). This bidirectional effect illustrates a deeply conserved capacity of platelets to modulate innate immune responses across species. It supports the concept that platelets possess a fundamental immunoregulatory function that likely evolved before the divergence of modern mammalian lineages and suggests that their role in immune modulation is an ancient and universal feature of vertebrate biology (Burzynski et al., 2019; Menter et al., 2022; Wu et al., 2019).

These findings also create an opportunity to use genetically engineered mouse models to further dissect the molecular basis of platelet-mediated immune regulation. For example, the PF4-Cre system could be employed to generate MK- or platelet-specific knockouts of individual NFκB components, such as NFκB1 or NFκB2. Platelets and platelet-derived EVs from these animals could then be tested for their ability to restore inflammatory responses in either human or mouse monocytes. Such experiments

would enable the precise identification of transcription factors involved in platelet-mediated immune licensing and help delineate the relative contributions of canonical and non-canonical NFκB signaling pathways. An alternative approach could involve the use of a genetically modified mouse strain that ubiquitously expresses an HA-tag, allowing for the tracking of all platelet-derived proteins transferred to recipient immune cells. Furthermore, these studies may uncover the involvement of additional signaling pathways, such as those mediated by MAPK (Hawwari, 2023; Kojok et al., 2019).

From an evolutionary standpoint, the existence of such conserved mechanisms suggests that platelet-mediated modulation of innate immunity originated in more primitive immune systems. In lower vertebrates, such as fish and amphibians, hemocytes perform combined roles in coagulation and immune defense (Menter et al., 2022). These cells are capable of detecting pathogens, initiating clot formation, and orchestrating inflammatory responses. In mammals, these functions have become partitioned across distinct cell types including MKs, platelets, and monocytes. Despite this specialization, certain critical intercellular communication pathways have remained preserved (Burzynski et al., 2019; Menter et al., 2022; Wu et al., 2019). It is plausible that these preserved interactions evolved as adaptive strategies to prevent inappropriate immune activation under homeostatic conditions, while still allowing for rapid and effective immune responses to pathogenic threats. This evolutionary continuity offers a compelling explanation for the presence of complex immune-regulatory capabilities in anucleate mammalian platelets. Rather than being inert fragments of MKs solely responsible for clot formation, platelets retain essential innate immune functions. These include the ability to recognize PAMPs, to release immune mediators such as cytokines and chemokines and to communicate with other immune cells through direct interactions or via platelet-derived EVs. These functional properties closely resemble those of ancestral hemocytes, suggesting that platelets have retained fundamental immune roles throughout evolution (Menter et al., 2022).

My findings support the hypothesis that platelets possess a conserved molecular machinery for post-translational modifications, selective cargo packaging and vesicular delivery of transcriptional regulators (Figure 8). From this perspective, platelets can be considered not only as guardians of vascular integrity but also as systemic immune modulators that help maintain inflammatory homeostasis (Morrell et al., 2014; Scherlinger et al., 2023; Semple et al., 2011). Interestingly, it has emphasized the co-evolution of coagulation and immune functions and highlighted the

molecular and functional convergence of these systems (Leslie, 2010). My data add further empirical support to this conceptual framework. The observation that monocytes require platelet-derived signals to reach their full inflammatory potential raises interesting evolutionary questions. Although the division of labor between hemostatic and immune cells has allowed for greater functional specialization in higher vertebrates, it has not eliminated their interdependence. Key communication pathways between platelets and monocytes have likely been preserved to maintain a balanced immune response. This balance may be necessary to avoid inappropriate activation while preserving the ability to mount a robust defense when required. However, it remains curious that such a high degree of redundancy has evolved, given that monocytes are capable of producing many of the same cytokines and transcription factors themselves (Ginhoux & Jung, 2014; Z. Han et al., 2023; Ziegler-Heitbrock et al., 2010). It is possible that this redundancy reflects a form of regulatory reinforcement, ensuring that immune responses are appropriately timed, spatially coordinated, and integrated with vascular surveillance.

Taken together, my findings underscore the importance of platelet-immune cell communication as an evolutionarily conserved mechanism that contributes to immune regulation across species. They also offer important insights into translational applications, including the potential use of engineered platelet-derived EVs or genetically modified platelets in immunotherapy. A deeper understanding of these ancient intercellular signaling pathways could help refine strategies to modulate inflammation in clinical settings without compromising protective immunity.

5.6. Clinical relevance of platelet-mediated immune regulation in inflammatory diseases

My findings additionally provide a mechanistic framework for understanding the heterogeneity of immune responses observed across a wide spectrum of inflammatory conditions. In states of thrombocytopenia, where platelet counts are low, the availability of platelet-derived immune mediators is likely diminished. This reduction can lead to a form of monocyte immunoparalysis, characterized by impaired cytokine production and downregulation of transcriptional programs involved in antimicrobial defense. As a result, patients with thrombocytopenia often exhibit increased susceptibility to infections and display compromised immune responses, even when monocyte counts remain within normal ranges. This phenomenon may help explain

clinical observations of immune dysfunction in conditions such as ITP and chemotherapy-induced thrombocytopenia (A. Gao et al., 2023; Hawwari & Rossnagel et al., 2024; Kuter, 2022; C. Li et al., 2024; Qu et al., 2018). In contrast, platelet activation strongly increases during heightened systemic inflammation, such as in cardiovascular disease, autoimmune disorders, or severe viral infections. This leads to augmented vesicle release and elevated transfer of transcriptional regulators from platelets to monocytes (Lebas et al., 2019; X. Li & Wang, 2023; Rolling et al., 2023; Yun et al., 2016). The resulting excessive signaling can shift monocytes toward a hyperinflammatory state, characterized by the production of large amounts of pro-inflammatory cytokines and increased expression of tissue factor and other pro-coagulant molecules. In this context, monocyte-platelet aggregates are more frequently detected and have been reported to be elevated across a variety of pathological conditions, including atherosclerosis, systemic lupus erythematosus, and COVID-19 (Bitsadze et al., 2025; J. Chen et al., 2024; Vanderbeke et al., 2021). These aggregates are not passive biomarkers but active contributors to disease progression. Their formation correlates with disease severity and promotes the emergence of monocytes with both pro-inflammatory and pro-thrombotic properties. This dual phenotype may underlie the increased risk of thrombo-inflammatory complications observed in many chronic and acute inflammatory disorders (Allen et al., 2019; Hottz et al., 2020; Oggero et al., 2021; Østerud, 2001; Rolling et al., 2023; L. Weiss et al., 2025; B. Zhang et al., 2022).

The elucidation of platelet-mediated immune regulation has significant therapeutic implications. Pharmacological strategies that selectively modulate platelet signaling pathways offer promising opportunities to influence immune responses while preserving essential hemostatic functions. For instance, inhibitors targeting the P2Y12 receptor, a central mediator of ADP-induced platelet activation, have demonstrated robust anti-inflammatory effects. These effects include reduced formation of monocyte-platelet aggregates and suppression of key inflammatory cytokines such as TNFα, IL-6, and CCL2. Importantly, these benefits can be achieved without impairing the ability of platelets to support hemostasis, making P2Y12 inhibition an attractive dual-action strategy in patients with cardiovascular or autoimmune diseases (Huang et al., 2021; Liverani et al., 2014; Mansour et al., 2020; Thomas et al., 2015; Tunjungputri et al., 2015). Other emerging therapeutic targets include GPVI, which mediates collagen-induced platelet activation (Billiald et al., n.d.; Jadoui et al., 2020;

Wichaiyo et al., 2022). Selective inhibition of GPVI may offer anti-thrombotic efficacy without increasing bleeding risk. In addition, Bruton's tyrosine kinase inhibitors represent a promising class of modulators. At low doses, these compounds can selectively disrupt CLEC-2-dependent signaling in platelets while sparing GPVI responses. This selective blockade allows for fine-tuning of platelet-driven inflammation while maintaining hemostatic balance (Dupont et al., 2025; Ungerer et al., 2011). Colchicine, a well-known anti-inflammatory drug with a favorable safety profile, has also been shown to inhibit platelet aggregation and reduce reactive oxygen species production by interfering with GPVI signaling at clinically relevant concentrations. Given its pleiotropic effects, colchicine may be repurposed to dampen platelet-driven inflammation in clinical contexts such as acute coronary syndromes or systemic autoimmune disease (Nicolson et al., 2020).

Together, these pharmacological strategies highlight the potential for targeting platelet-immune cell interactions to achieve therapeutic benefit in inflammatory disorders. By focusing on modulating platelet-mediated signaling rather than broadly suppressing the immune system, these approaches aim to restore immunological balance while minimizing adverse effects. The translational value of targeting platelet-monocyte communication lies in its capacity to influence both innate and adaptive immunity through a regulatory pathway that integrates hemostatic and inflammatory signals. This dual functionality makes it a promising focus for precision medicine approaches in chronic inflammatory and immune-mediated diseases.

5.7. Biomarker potential and reconsideration of immune communication paradigms

The continuous release of processed NFκB subunits by platelets into the circulation suggests a novel and promising approach for developing biomarkers to monitor immune system dysregulation. Among the most relevant candidates are platelet-derived EVs, which carry immunologically active molecules such as cytokines, transcription factors, and chemokines (Duchez et al., 2015; Kuravi et al., 2019; Vajen et al., n.d.; Yuan et al., 2023). These EVs are found in elevated concentrations in numerous autoimmune and inflammatory diseases and exhibit strong correlations with disease activity, cardiovascular risk, and inflammatory burden. As a result, circulating platelet-derived factors may serve as accessible and functionally relevant biomarkers that complement traditional inflammatory markers, including C-reactive protein and serum cytokine levels (Y. Chen et al., 2020; Duchez et al., 2015; X. Li & Wang, 2023).

A particularly noteworthy signaling pathway in this context is the CD40L and CD40 axis. Platelet-derived CD40L is a potent activator of endothelial cells and plays a central role in promoting PLA formation (Aoui et al., 2014; Cognasse et al., 2022; Henn et al., 1998). Activation of this signaling pathway contributes to inflammatory responses within the vasculature and has been implicated in the development and progression of atherosclerosis and systemic autoimmune disorders (Bosmans et al., 2021; Cognasse et al., 2022; Zirlik et al., 2007). Therapeutic modulation of CD40L and CD40 interactions has demonstrated promising effects in both preclinical models and early clinical trials. However, this signaling axis represents a regulatory double-edged sword. Excessive stimulation and complete inhibition of CD40L have both been associated with adverse outcomes, necessitating careful calibration and a nuanced approach in the design of therapies that target this pathway (Bosmans et al., 2021; Fadul et al., 2021; Singh et al., 2023). The findings presented in my thesis also call for a reconsideration of classical paradigms of immune cell communication. Traditional models emphasize cytokine secretion and cell-intrinsic activation of transcription factors in response to external stimuli such as PAMPs (Kawai et al., 1999; Medzhitov et al., 1997; Poltorak et al., 1998). In this framework, each immune cell functions largely independently, initiating intracellular signaling cascades that culminate in nuclear responses (Akira & Takeda, 2004; Janeway & Medzhitov, 2002). However, the discovery that transcriptional regulators can be directly transferred between cells via EVs suggests a previously underappreciated mode of communication. This mechanism implies that immune cells are not entirely autonomous but instead can incorporate extrinsic regulatory components from neighboring cells, such as platelets. This vesicle-mediated transfer mechanism significantly expands the current understanding of immune regulation by integrating trans-cellular signaling as a fundamental component of immune system architecture. The redundancy observed in the NFκB system, in which transcriptional activity can be restored in NFκB-deficient THP-1 monocytes by platelet-derived factors, illustrates the ability of the immune system to preserve functionality under conditions of genetic or environmental stress (Figure 6). Rather than relying solely on their intrinsic signaling machinery, monocytes can utilize regulatory elements provided by platelets to sustain essential immune functions such as cytokine production and inflammasome activation (G. Ghosh & Wang, 2021; Hawwari & Rossnagel et al., 2024; Mogensen, 2009).

These insights contribute to a growing body of literature suggesting that immune regulation operates through a more distributed and cooperative network than previously appreciated. Platelets emerge as central players in this distributed network, serving not only as mediators of hemostasis but also as active modulators of immune tone. Their ability to transfer transcriptional machinery positions them as key components in the orchestration of immune surveillance and homeostasis. This perspective opens new conceptual avenues for rethinking immune system function and for designing therapeutic interventions that strategically target intercellular signaling dynamics rather than focusing exclusively on intracellular pathways.

5.8. Translational potential and future perspectives in clinical application

The mechanistic insights obtained in my study provide a strong foundation for the development of targeted therapeutic strategies that modulate specific aspects of platelet-immune cell communication. Rather than employing broad immunosuppressive approaches, future therapies could focus on selectively influencing EV release, the intercellular transfer of transcription factors, or the activity of defined receptor-ligand interactions. These targeted strategies are well aligned with the principles of precision medicine and personalized immunotherapy, which aim to tailor treatment to individual variations in immune architecture and signaling dynamics (Cid et al., 2020; Isaacs & Burmester, 2020; Lee et al., 2025; Ma et al., 2021; Tian et al., 2025; M. Yan et al., 2024). Therapeutic applications may include the use of engineered platelet-derived EVs as delivery systems for immunomodulatory cargo targeted to specific leukocyte subsets. Platelet supplementation may offer therapeutic benefits in immunodeficiency syndromes associated with impaired monocyte function, defective inflammatory priming, or even cancer. For example, the immunological dysfunction in Wiskott-Aldrich syndrome results from mutations in the WAS gene, which encodes the Wiskott-Aldrich syndrome protein (WASP). WASP is critical for normal signaling in B cells and T cells (Buchbinder et al., 2014; Massaad et al., 2013). Since platelets also express WASP and display functional defects in patients with Wiskott-Aldrich syndrome, platelet supplementation could theoretically support both hemostatic and immunological functions. In addition, the selective inhibition of harmful platelet interactions with immune cells, as observed in thromboinflammatory conditions, represents a promising strategy for anti-inflammatory therapy (Mack et al., 2024; Potere et al., 2023; Sharma et al., 2022). Recent studies have also explored the

use of platelets as drug delivery vehicles in cancer treatment (Cai et al., 2025). These approaches take advantage of the natural tumor-targeting abilities of platelets. For instance, engineered platelets loaded internally with doxorubicin and modified externally with anti-PD-L1 antibodies have been shown to reduce tumor recurrence and metastasis after surgery in mouse models (Lu et al., 2022). Given the evolutionary conservation of platelet-mediated immune regulation, these strategies may be applicable across different mammalian species and thus hold significant translational potential (Q. Li et al., 2022; Tian et al., 2025).

Despite the promise of these approaches, several limitations must be considered. Although platelet-derived transcription factors were able to restore certain monocyte functions in experimental models with deficient NFκB signaling, the rescue effect remained partial and did not fully replicate the physiological baseline (Figure 6). This indicates that while immune signaling redundancy is a robust feature of the system, it does not always ensure complete functional equivalence. The extent of compensation may depend on the specific nature of the deficiency, the availability of cofactors, and the overall cellular signaling environment (Hawwari & Rossnagel et al., 2024). In addition, the therapeutic manipulation of platelet-immune communication carries inherent risks. Although anti-platelet therapies can effectively reduce platelet activation and aggregate formation, they are associated with an increased risk of bleeding, especially in patients with underlying hemostatic abnormalities (Serebruany et al., 2008). Similarly, immunomodulatory interventions must be carefully calibrated to avoid tipping the immune balance toward suppression or tolerance in contexts where immune competence is essential. These considerations highlight the need for cautious and rigorous pre-clinical evaluation in the development of platelet-targeted therapies.

In conclusion, while the findings of my work open up promising therapeutic possibilities, further research is required to fully understand the mechanistic underpinnings, safety considerations, and disease-specific applications of platelet-centered interventions. The next generation of immune therapies may well involve the harnessing of platelet-derived regulatory pathways, but such approaches must be implemented with precision and attention to context-specific immune dynamics.

5.9. Conclusion

My study provides further compelling evidence that platelets function as central regulators of inflammatory immune responses. Through the identification of a novel vesicle-mediated mechanism of intercellular communication, I demonstrated that platelets influence monocyte function by constitutively releasing EVs that contain functionally active transcriptional regulators, including processed NFκB subunits. This mode of communication allows platelets to modulate the transcriptional landscape and functional programming of recipient immune cells, thereby contributing to the orchestration of effective immune responses. The discovery that these vesicles enhance monocyte cytokine production, even in the absence of direct cell contact, reveals a mechanism of transcriptional licensing that expands our understanding of platelet biology. The conservation of this mechanism across species further supports its fundamental role in immune regulation. These findings contribute to a growing recognition that the functions of platelets extend well beyond their classical roles in hemostasis and thrombosis. By elucidating a molecular mechanism through which platelet-derived factors can restore cytokine production in NFκB-deficient THP-1 monocytes, my work also provides a new framework for understanding immune signaling redundancy and compensation. My data highlight the potential for platelet-derived EVs to act as therapeutic tools in immunodeficiency syndromes and inflammatory diseases. Integrating these insights into the broader context of immunological signaling networks enhances our understanding of how intercellular communication contributes to immune homeostasis and disease. My work not only advances our conceptual framework of platelet function, but could also lay the groundwork for novel therapeutic strategies aimed at selectively modulating immune responses through the targeted manipulation of platelet-immune cell interactions. These findings hold promise for the development of precision medicine approaches in the treatment of chronic inflammatory and immune-mediated disorders.

6. Abstract

Platelets have immunomodulatory roles beyond their classical function in hemostasis, for example regulating monocyte-driven inflammation. Classical $CD14^+$ monocytes dominate in human blood and mediate immune responses by producing pro-inflammatory cytokines such as IL-1β and TNFα. Tight regulation of these responses is crucial, as excessive activation leads to cytokine storms, whereas impaired activation causes immunoparalysis, commonly observed in sepsis or viral infections.

Previous research from my group demonstrated that platelets are required for optimal monocyte cytokine responses independently of classical co-stimulation. Platelet depletion reduces pro-inflammatory cytokine secretion and gene expression in activated monocytes, mirroring conditions like ITP. Supplementation with platelets restores inflammatory functions of monocytes, highlighting platelet-derived factors as critical regulators.

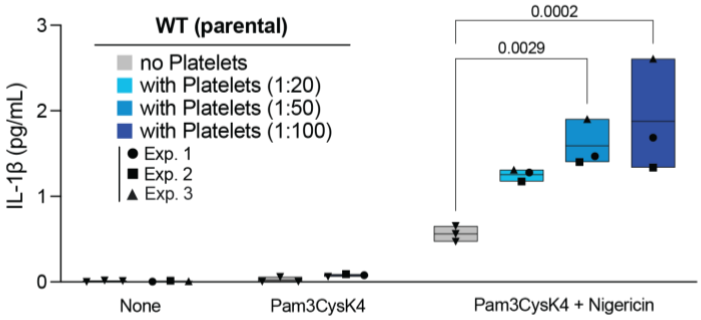
This thesis identifies a previously unrecognized mechanism in which platelets enhance monocyte cytokine production through extracellular vesicle (EV)-mediated transfer of NFκB pathway components. Particularly, SILAC-based proteomic analyses demonstrated that platelets transfer essential NFκB pathway components, such as the transcription factor NFκB2. This vesicle-mediated delivery occurs constitutively and independent of platelet activation or external stimulation. Using CRISPR-Cas9-generated NFκB-deficient THP-1 monocytes and pharmacological inhibitors, I confirmed that platelet-derived factors are essential for robust monocyte cytokine responses. Furthermore, I was able to show that platelet-derived EVs contain active NFκB components both *ex vivo* and in human serum and plasma, highlighting their physiological relevance. Comparative interspecies analyses indicate evolutionary conservation of platelet-monocyte interactions and EV-mediated transcription factor transfer between humans and mice.

Overall, this work defines a novel axis of immune regulation driven by platelet-derived EVs delivering bioactive transcriptional regulators. These insights enhance our understanding of platelets as key inflammatory regulators and suggest new therapeutic strategies for inflammatory and immunodeficiency disorders.

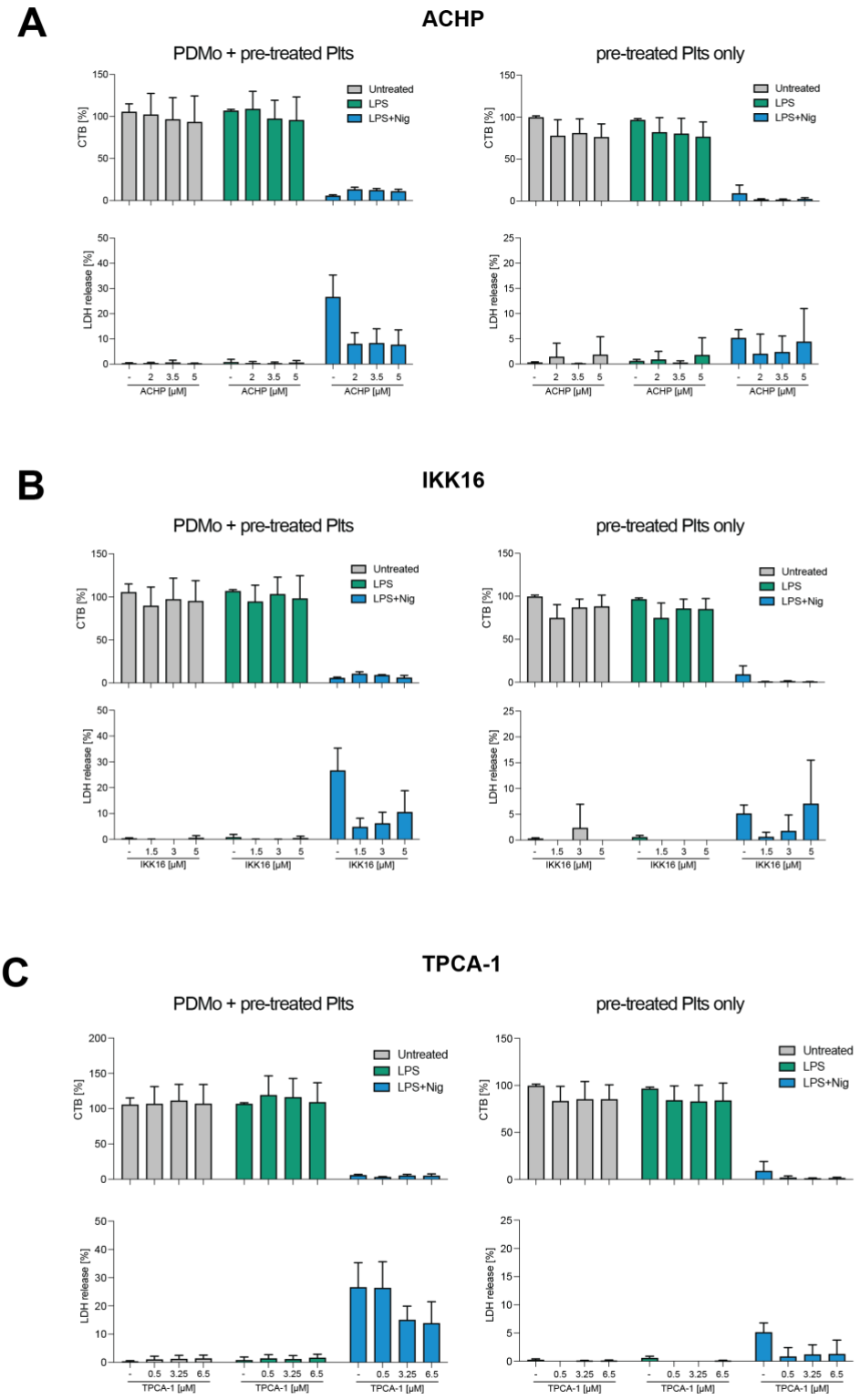
7. List of figures

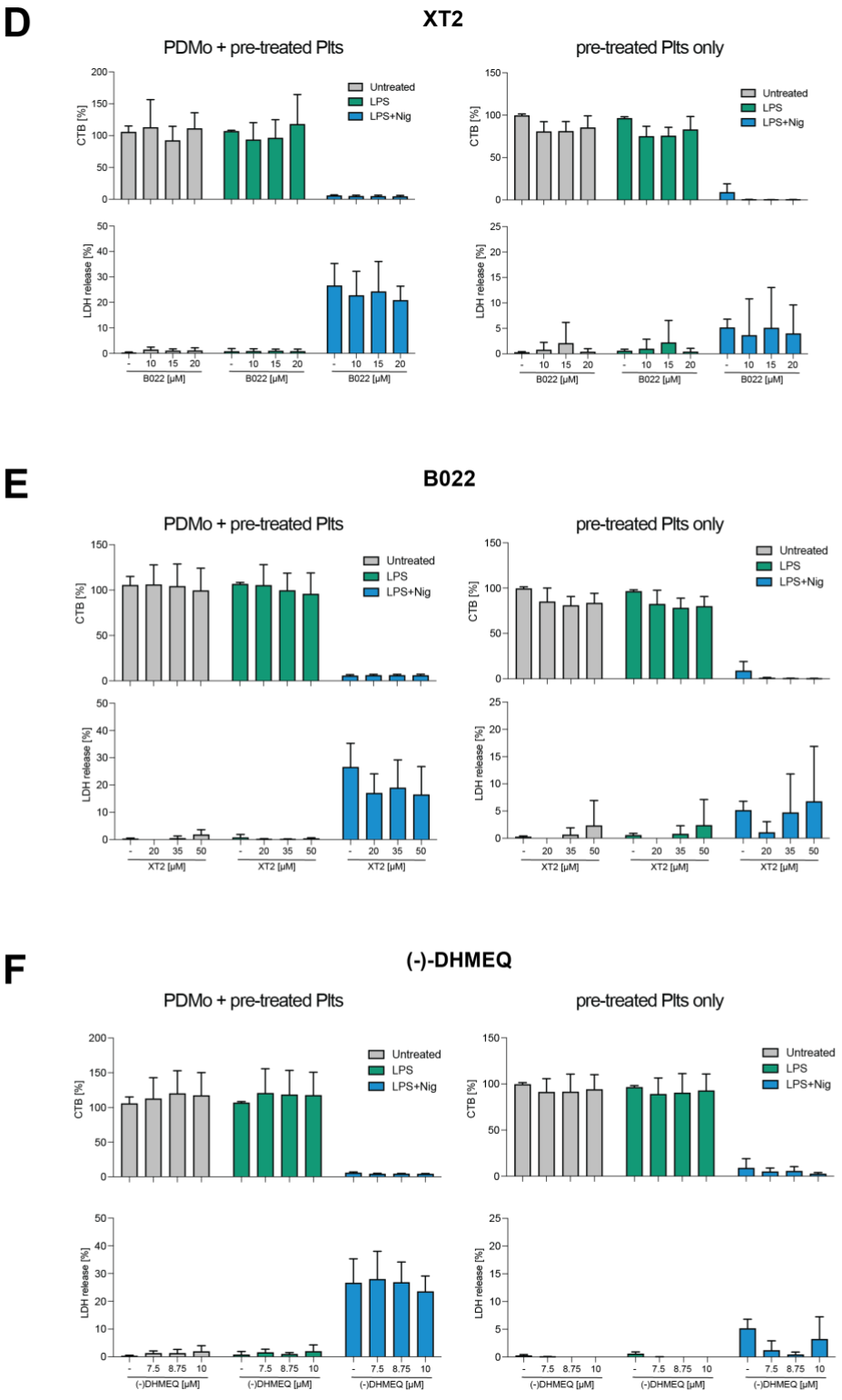
8. List of tables

9. Supplement



Supplement Figure 1: WT THP-1 monocytes supplemented with different amounts of platelets. IL-1β concentrations in the supernatants of stimulated parental WT THP-1 monocytes that were cultured alone or added with increasing ratios of freshly isolated platelets (1:20, 1:50, and 1:100). Cells were primed with Pam3CysK4 (1 µg/mL, 3 h) and activated with nigericin (10 µM, 1.5 h). Floating bars display max/min values with indication to the mean (black bands). P values were calculated using two-way ANOVA, Tukey's multiple comparison test compared to the control group (no Plts) and are indicated in the figure. Each symbol represents one donor. Data is pooled from three independent experiments.


A
ACHP
PDMo + pre-treated Plts
pre-treated Plts only
Untreated
LPS
LPS+Nig
CTB [%]
LDH release [%]
ACHP [µM]
B
IKK16
IKK16 [µM]
C
TPCA-1
TPCA-1 [µM]



Supplement Figure 2: Assessment of cell viability following pharmacological inhibition of the NFκB signaling pathway. (**A - F**) Platelets were pre-treated with various NFκB inhibitors (ACHP, IKK16, TPCA-1, XT2, B022 and (-)-DHMEQ) and

subsequently analyzed alone or in co-culture with platelet-depleted monocytes (PDMo) in different concentrations, ranging from 0 - 50 µM. Platelets were left untreated or pre-treated with the inhibitors, then washed and incubated with PDMo. Afterwards, the cells were primed with LPS (2 ng/mL, 3 h) and activated with Nigericin (10 µM, 1.5 h). When the stimulation was over, the cell viability was assessed using CellTiter-Blue® (CTB) and lactate dehydrogenase (LDH) assays to evaluate potential cytotoxic effects of the inhibitors on treated platelets and co-cultures. Data is pooled from four donors.

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