Platelet-immune cell interactions in inflammation and cancer

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The more I learn, the more I realize

how much I don’t know.

Albert Einstein

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1 Summary

Platelets are well-known for their role in hemostasis and only more recently, they have also been acknowledged for their immune regulatory roles. Despite being small sized and anucleated, they can respond to inflammatory stimuli, secrete a plethora of different inflammatory mediators and interact extensively with other immune cells to initiate and maintain immune responses. In addition to their function in inflammatory conditions, platelets are also involved in cancer development and metastasis. They can not only induce tumor growth, angiogenesis and the survival of circulating tumor cells (CTCs) but also interact with tumor cells directly, which yields "tumor educated" platelets. In this work, I present two independent but complimentary stories that describe novel functions of platelets in driving IL-1 inflammation during innate immune responses and in the regulation of immune checkpoints in cancer.

Multimeric signaling platforms, called inflammasomes, are an intracellular pattern recognition receptor (PRR) that can sense many different activators upon pathogen invasion, including bacterial toxins. Although a variety of different inflammasomes and a multitude of activators and activating mechanisms have been discovered over the last decade, most of these studies were conducted *in vitro* monocultures of macrophages and monocytes and the regulatory and networking effects of other immune cells were largely neglected. Therefore, the interaction of innate immune cells with platelets during inflammasome activation was investigated in the first part of this work.

My results show for the first time that platelets enhance NLPR3 inflammasome responses in bone-marrow derived macrophages (BMDMs), human monocyte derived macrophages (hMDMs) and human neutrophils, and are crucial for cytokine secretion from NLRP3 activated human monocytes. Platelets alone do not express NLRP3 inflammasome components or secrete IL-1β, excluding a role for platelet-derived NLPR3 or IL-1 cytokines in this effect. Further, the main findings of this study reveal that platelets regulate NLRP3 activation in hMDMs by enhancing NLRP3 and IL-1β transcription, increasing caspase-1 activity and promoting the assembly of ASC specks. This effect is independent of platelet-derived COX1/2 or LOX lipid mediators, nucleosides (ADP and ATP), nucleic acids and platelet α-granule derived factors, but instead mediated by a platelet-derived, soluble and heat sensitive factor that is partly dependent on extracellular calcium. Finally, RNA sequencing analysis identified genome wide transcriptional changes in hMDMs in the presence of platelets or platelet

supernatant pointing to an involvement of metabolic and cancer pathways. Together with a proteomic analysis of platelet supernatant, this study offers an array of new possible candidates that could be involved in the platelet-mediated NLPR3 regulation of hMDMs. Altogether, these results establish platelets as important regulators of the IL-1 inflammatory response in innate immune cells and more detailed studies will be necessary to elucidate the underlying molecular mechanisms.

Since platelets also play important roles in the development of cancer, the potential of platelets as new therapeutic targets in Head and Neck Squamous Cell Carcinoma (HNSCC) patients was investigated in the second part of this work. Despite the unprecedented and durable effects of checkpoint inhibition therapy in HNSCC patients, in total only a small fraction of patients responded favorably and the general mortality rates in this cancer still remain very high. So far, there are no easily accessible biomarkers that could identify suitable patients and predict their response to therapy.

In this work, I show for the first time that platelets and PBMCs from HNSCC and lung cancer patients and smokers express the immune checkpoint protein Programmed cell death ligand 1 (PD-L1). This expression was independent of the disease stage, the occurrence of metastasis or the incidence of the tumor. Further analyses demonstrated that PD-L1 expression on platelets was decreased in response to immunotherapy with the anti-PD-L1 antibody atezolizumab, but reconstituted after 20 days. These findings and the enhanced PD-L1 expression on platelets from smokers highlight the potential of platelets as easily accessible biomarkers for the detection of early cancer development and as predictor of therapeutic success.

2 Introduction

2.1 The immune system

The immune system evolved over millions of years as a host defense mechanism. Its main function is to distinguish self from non-self to combat foreign bacteria, viruses and other pathogens. The immune system comprises two lines of defense: the evolutionarily ancient innate immune response and the relatively new adaptive immune response[1]. Both cooperate to generate a fully functional immune response against pathogens, with innate immunity acting rapidly as an initial, non-specific response and adaptive immunity mounting a more specific and effective response[2].

Physical and chemical barriers, such as the skin, mucosal layers and gastric acid form the first line of innate immune defense against invading pathogens[2]. Once these barriers are breached, pathogens encounter humoral defense factors, such as the complement system, and innate immune cells. Innate immune cells include phagocytes (macrophages, neutrophils, dendritic cells (DCs)), granulocytes (mast cells, eosinophils, basophils) and natural killer (NK) cells[1,2]. They are activated upon pathogen encounter and either clear the infection directly or activate the adaptive immune response, which relies on B and T lymphocytes expressing somatically recombinant and clonally selected receptors that are antigen-specific[1,2]. This thesis is focused on the innate branch of immunity investigating the interaction of platelets with different innate immune cells in the scope of inflammatory responses and cancer.

2.1.1 The innate immune system and pattern recognition receptors

The innate immune system acts as the first line of defense against pathogens. Its main functions are to sense and recognize invading pathogens, initiate a host immune response, activate adaptive immune responses and induce tissue repair to return to homeostasis after the elimination of the threat[3]. To sense invading microorganisms, innate immune cells employ invariant pattern recognition receptors (PRRs) that recognize highly conserved structures specific for different classes of pathogens, termed pathogen-associated molecular patterns (PAMPs)[4]. Similarly, PRRs can also recognize damage-associated molecular patterns (DAMPS), which are endogenous factors released from damaged cells. PRRs are either present on the plasma membrane, in the cell's cytoplasm or on intracellular membranes. PAMP recognition by PRRs initiates intracellular signaling cascades leading to the transcription of genes that encode proinflammatory cytokines, chemokines and antimicrobial proteins[4].

Proinflammatory cytokines amplify the immune response by recruiting more innate immune cells to the site of infection. This is part of the process known as inflammation. The recruited immune cells use different mechanisms to fight a pathogen, such as phagocytosis, release of toxic molecules including reactive oxygen species (ROS), or pathogen degradation through proteases[3]. After a successful clearance of the invading pathogen, the inflammatory response is resolved in the resolution and repair phase. If the inflammatory response fails to eliminate the pathogen, however, the second line of immune defense takes over and adaptive immune cells come to the aid[5].

Immune cells dispose of five structurally distinct families of PRRs: Nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), Toll-like receptors (TLRs), AIM2-like receptors (ALRs), C-type lectin receptors (CLRs) and retinoic acid inducible gene I (RIG-I)-like receptors (RLRs)[6]. TLRs and CLRs are found on membranes (plasma or endosomal membranes) and recognize extracellular or endocytosed pathogens molecules. In contrast, RLRs, ALRs and NLRs are present in the cell cytoplasm and sense intracellular pathogens[6].

2.1.2 Key innate immune cells and their functions

Most innate immune cells descend from myeloid stem cells. Key innate immune cells derived from the common myeloid progenitors are granulocytes (neutrophils, monocytes, macrophages, eosinophils, basophils), mast cells, DCs and megakaryocytes producing platelets. All of these cells express PRRs to detect invading pathogens and initiate an immune response[2].

Monocytes and macrophages belong to the family of professional phagocytes detecting and clearing pathogens and dead cells. Besides phagocytosis, they can also secrete proinflammatory cytokines and ROS and activate adaptive immune cells through antigen presentation on their cell surface[7]. Under homeostatic conditions, monocytes are short-lived cells, which circulate in the bloodstream before they undergo apoptosis spontaneously. Monocyte populations are heterogenous and are classified according to their expression of antigenic markers into three groups: classical monocytes ($CD14^{++}CD16^{-}$), intermediate monocytes ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$)[8]. Most monocytes (approx. 90%) belong to the classical monocyte population ($CD14^{++}CD16^{-}$)[7]. These classes of monocytes vary in their cytokine secretion profiles and their ability to differentiate into DCs or macrophages[9]. Classical monocytes are mostly phagocytes without displaying inflammatory

features, whereas non-classical monocytes show inflammatory characteristics and present antigens[10].

Monocytes can differentiate into macrophages in response to specific differentiation factors. For instance, granulocyte-macrophage colony-stimulating factor (GM-CSF) polarizes human blood monocytes to an M1 phenotype whereas macrophage colony-stimulating factor (M-CSF) leads to differentiation into M2 macrophages[11]. M1 and M2 macrophages elicit distinct cytokine expression profiles and immune responses, with M1 macrophages producing high levels of proinflammatory cytokines and reactive nitrogen intermediates and ROS, and promoting T helper cell responses. In contrast, M2 macrophages are mostly important for tumor progression, anti-parasitic immunity and tissue remodeling[12], and display an anti-inflammatory phenotype[13]. Macrophages have a long life-span and populate nearly every tissue in the body making them essential in the initial immune response[7].

There are different types of circulating granulocytes, with neutrophils being the most abundant (70 %)[14]. Neutrophils are recruited to the inflammatory site in response to inflammatory molecules and cytokines secreted by tissue-resident cells, like macrophages.

At the infection site, neutrophils are efficient in killing pathogens through various mechanisms such as phagocytosis, degranulation or formation of neutrophil extracellular traps (NETs)[15]. PRRs on resident or tissue infiltrating immune cells recognize invading microbes which leads to their phagocytosis. Then, neutrophil granule content with antimicrobial proteins and proteolytic enzymes together with ROS leads to intracellular killing of phagocytosed pathogens[16]. Neutrophils can also degranulate and release a plethora of cytokines, antimicrobial peptides and enzymes which aids in the killing of extracellular pathogens. This process is lethal for the neutrophils[15,16].

Platelets are small, anucleate cells that are derived from megakaryocytes in the bone marrow or the lung[17]. After red blood cells, they are the second most abundant cell type in the blood and were recently shown to play important immunological roles in addition to their well-acknowledged role in blood clotting. Through their expression of PRRs and other receptors, platelets can interact with both immune cells and pathogens[18]. Platelets also store cytokines, antimicrobial peptides or proinflammatory mediators in different types of granules, which are released upon activation and lead to the degradation of pathogens and the recruitment of immune cells[18]. The role of platelets in hemostasis and inflammation is discussed in more detail in chapter 2.2.

2.1.3 Metabolic regulation in homeostasis and inflammation

The cell's energy homeostasis is maintained through a network of biochemical reactions called metabolism. Metabolic pathways provide key products needed for cell growth and survival. The metabolic state of immune cells is closely linked to their function, with different immune cells having different metabolic needs according to their immunological tasks. Although there are many different metabolic pathways with diverse end products, they are all closely linked through their dependency on shared nutrients[19,20].

Under homeostatic conditions, cells need sufficient nutrients and oxygen to function. Resting cells mainly produce energy in the form of Adenosine triphosphate (ATP) by aerobic metabolism[20]. When glucose uptake is low, cells can generate ATP either through substrate phosphorylation during glycolysis or through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation involving the electron transport chain[20].

Glycolysis starts with the uptake of extracellular glucose, which is processed in the cytosol to yields pyruvate. While glycolysis is a rather inefficient pathway for the generation of ATP, it also produces the reducing agent nicotinamide adenine dinucleotide (NADH) and provides biosynthetic intermediates for the synthesis of amino acids, fatty acids and ribose for nucleotides, thereby supporting anabolic growth of cells[19]. Hence, glycolysis is dominantly used by proliferating cells.

In comparison, the TCA cycle and oxidative phosphorylation constitute a highly efficient way of ATP production mostly used by cells which are long-lived and have high energy demands[19]. Many different nutrients fuel the TCA, such as glucose-derived pyruvate, fatty acids or glutamate[19]. The reducing agents NADH and Flavin adenine dinucleotide ($FADH_2$) are two major products of the TCA, which provide electrons for the electron transport chain, thereby promoting oxidative phosphorylation and ATP production[19,20]. This form of energy production is used by most cells in the resting state.

During inflammation, tissue resident cells are activated through engagement of their PRRs which induces the recruitment, differentiation and proliferation of immune cells. Additionally, tissue infiltrating immune cells have to fulfill new effector functions such as phagocytosis and cytokine release. During this time, nutrients and oxygen can become scarce making it necessary for immune cells to adapt metabolically[20].

When proinflammatory M1 macrophages are activated they rely mainly on glycolysis, while mitochondrial oxidative phosphorylation is reduced and the TCA cycle is inhibited[21-23]. Due to the reduced dependence on the TCA cycle, accumulating TCA intermediates, including citrate

and succinate, can be transported out of the mitochondria into the cytosol[23]. In the cytosol, citrate can function as a substrate for the generation of proinflammatory and antimicrobial molecules, and for the production of fatty acids, which are important for membrane biogenesis[19,20]. After its translocation, TCA cycle-derived succinate is involved in macrophage cytokine production. Through inhibition of prolyl hydroxylases, succinate activates the transcriptional regulator hypoxia-inducible factor 1a (HIF-1α), which induces the expression of inflammatory proteins, including pro-IL-1β[24].

In contrast, the more anti-inflammatory M2 macrophages utilize mitochondrial biogenesis and fatty acid oxidation to fuel the TCA cycle and oxidative phosphorylation. They are mainly involved in tissue regeneration and the resolution of inflammation, having no urgent need for fast ATP production. Thus, they only show low rates of glycolysis[19,20].

Other myeloid cells, such as neutrophils and DCs, mainly follow the same metabolic processes as described for macrophages. They primarily shift to aerobic glycolysis and low rates of oxidative phosphorylation during an inflammatory reaction[20]. Activated platelets also show a glycolytic phenotype but similarly preserve mitochondrial functioning[25]. Metabolism in platelets is further described in section 2.2.4.

2.1.4 Toll-like receptors (TLRs)

TLRs were the first PRRs discovered in the mid-1990s[26]. They are also the best-characterized PRR family and can recognize both intracellular and extracellular pathogens. Until now, 10 different functional human (TLR 1-10) and 12 murine (TLR 1-9, 11-13) TLRs have been described[27]. TLRs are divided into two groups, based on their cellular localization and their specific ligands. The first group of human TLRs (TLR1, 2, 4, 5, 6, 10) is located on the plasma membrane and mainly senses microbial proteins, lipoproteins and lipids. The other second group (TLR3, 7, 8, 9) are exclusively present in the intracellular vesicles, such as endosomes, lysosomes and the endoplasmic reticulum (ER), and recognize nucleic acids[27,28].

TLRs are composed of N-terminal leucine-rich repeats (LRRs), a transmembrane region and cytosolic Toll-IL-1 receptor (TIR) homology domain, activating downstream signaling pathways[27]. Each of the different TLRs recognizes specific PAMPs, including double-stranded RNA (TLR3), lipoproteins (TLR1, 2, 6), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5), single-stranded RNA (TLR 7 and 8), and DNA (TLR9) (Fig. 2.1). Currently, there is no ligand identified yet for TLR10[4,27]. Upon sensing of these PAMPs, TLRs form homo- or heterodimers and initiate a ligand specific immune response culminating in transcriptional regulation[4]. They

signal through recruitment of different TIR domain-containing adaptor proteins: myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor protein-inducing IFN-β (TRIF), TIR-associated protein (TIRAP) and TRIF-related adaptor molecule (TRAM)[4] (Fig. 2.1).

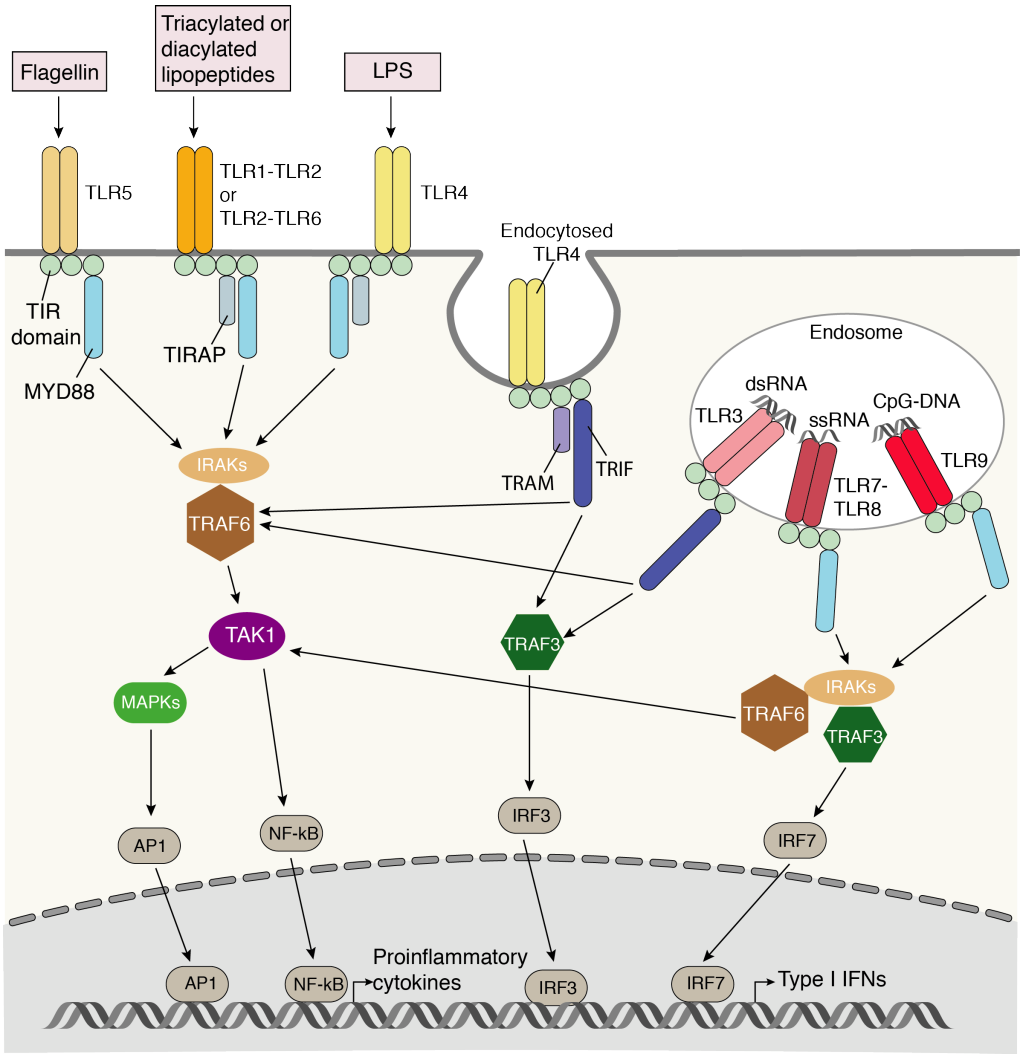

Figure 2.1 Human TLR activation and downstream signalling. Schematic and simplistic representation of TLR activation and subsequent signalling in human TLRs. TLR activation leads to the recruitment of the adaptors MyD88, TRIF, TIRAP and TRAM. Different signalling cascades culminate into the activation of the transcription factors AP1, NF-κB, or IRF. The figure is inspired by O'Neill et al.[26].

All TLRs except TLR3 utilize the adaptor protein MyD88, which leads to mitogen activated protein (MAP) kinase and nuclear factor kappa B (NF-κB) activation and expression of proinflammatory cytokines, such as IL-6, TNFα and IL-12[27,29]. TLR3 and TLR4 recruit the alternative adaptor TRIF to induce NF-κB and IRF3 activation, and induction of type I interferon (IFN) production[27]. TLR4 is the only TLR that can signal through both the MyD88-

dependent and TRIF-dependent pathways, depending on its cellular localization. It recruits MyD88 through the adaptor TIRAP upon LPS engagement on the cell surface activating NF-κB and MAP kinases and early production of proinflammatory cytokines. After endocytosis, TLR4 forms a complex with the adaptor proteins TRAM and TRIF inducing type I IFN through IRF3. Additionally, NF-κB activation through TRAF6 and TAK1 leads to late induction of proinflammatory cytokines[4,27].

2.1.5 NOD-like receptors (NLRs) and inflammasomes

The NLR family is important for host physiology and innate immunity through their intracellular detection of PAMPs and DAMPs[30]. NLR orthologues are present across phylogenetically distinct species. Plants, for instance, evolved resistance (R) genes, which mediate pathogen detection and innate immune response. This structural and functional convergence highlights the vital role that NLRs play in the innate immune system[30,31].

There are 22 human NLRs. Their role is not restricted to immunity but extends to embryonic development and reproduction[30], and dysregulation of NLRs is linked to cancer, autoimmune, and metabolic diseases[32]. Most NLRs are composed of three domains: an N-terminal protein-protein interaction domain, a central NOD (or also known as NACHT) domain necessary for oligomerization, and a C-terminal leucine-rich repeat (LRRs) involved in ligand recognition. In the absence of ligand, LRRs are proposed to be in a conformation that hides their N-terminal domain and represses NLR signaling[30,33,34]. Mammalian NLRs are classified into five sub-families based on their N-terminal domain: NLRA (NLR family and acidic domain containing), NLRB or neuronal apoptosis inhibitor proteins (NAIPS) (NLR family and baculovirus inhibitor of apoptosis protein repeat (BIR) containing), NLRCs (NLR family and CARD domain containing), NLRPs (NLR family and pyrin domain containing) and NLRX (NLR family and CARD-related X effector domain containing)[33,35].

Upon ligand recognition and NLR activation, the LRR domain changes its conformation, which exposes its N-terminal domain and leads to its interaction with downstream signaling adaptors to form an oligomeric complex[30]. These NLR platforms recruit and activate the protease caspase-1[30], which can proteolytically cleave the pro-forms of IL-1β and IL-18, leading to their maturation into active cytokines and their subsequent release[36]. Additionally, caspase-1 induces an inflammatory form of cell death through cleavage of gasdermin D, called pyroptosis[32].

These multimeric NLR signaling platforms that recruit caspase-1 and mediate proinflammatory cytokine secretion are called inflammasomes. So far, NLRP1[37], NLRP3[38], NLRP6[39], NLRP7[40], NLRP9b[41], NLPR12[42] and NLRC4[43] were described to form inflammasomes among the NLR family. The NLRP1 inflammasome assembles upon sensing lethal toxin produced by *Bacillus anthracis*, for instance, whereas NLRC4 inflammasome assembles in response to the rod and needle subunits of bacterial type 3 secretion systems (T3SSs, PrgI) detection by NAIP. NLRP3 is unique in its ability to be activated by structurally diverse stimuli and not only by bacterial components. Potent activators are, for example, extracellular ATP, the ionophore nigericin, different crystals such as silica and several pathogens[32].

Besides these NLR family members, the proteins absent in melanoma 2 (AIM2) and pyrin have also been described as inflammasome-forming sensors, whereas retinoic acid-inducible gene I (RIG-I) and interferon-γ-inducible protein 16 (IFI16) have only been shown to induce caspase-1 activation[32].

2.1.6 The NLRP3 inflammasome in health and disease

NLRP3 is one of the best-studied and characterized inflammasomes to date. It was first discovered through its gain of function mutations in cryopyrin-associated periodic fever syndromes (CAPS), such as Muckle-Wells syndrome (MWS), familial cold autoinflammatory syndrome (FCAS) and Neonatal Onset Multisystem Inflammatory Disease (NOMID)[44]. Because of the gain of function mutations in NLRP3, these diseases are characterized by an overproduction of IL-1β and IL-18, leading to fever and severe tissue-specific inflammation. Since its discovery, NLRP3 has been associated with a variety of other inherited and acquired inflammatory diseases, such as gout, atherosclerosis, inflammatory bowel disease and type 1 and type 2 diabetes[45]. Due to this strong inflammatory potential, NLRP3 has become an appealing drug target. So far, different pharmacological inhibitors of the NLRP3 pathway are developed with cytokine release inhibitory drug 3 (CRID3, MCC950, or CP-456773) being the best-studied[45]. The small-molecule CRID3 is highly specific for the NLRP3 inflammasome and inhibits NLRP3 activation by all known stimuli without affecting NLRC4 or AIM2 activation[46,47]. However, the exact mechanism of action remains unknown.

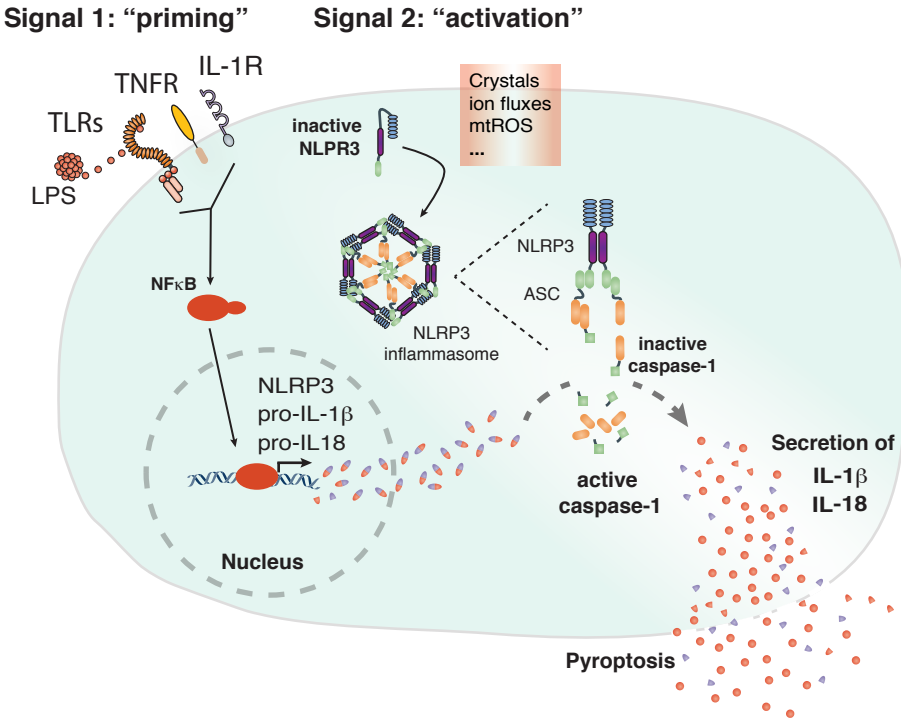

Figure 2.2 NLRP3 activation and signalling. Schematic and simplistic representation of NLRP3 activation and subsequent signalling. In a first "priming step" stimulation of different receptors, such as TLRs, TNF receptor (TNFR) and the IL-1 receptor (IL-1R), lead to the activation of NF-κB and subsequent transcription of NLRP3 and other pro-inflammatory genes. In the second "activation step", different PAMPs and DAMPs initiate NLRP3 assembly and subsequent activation of caspase-1, inducing IL-1β and IL-18 maturation and release.

NLRP3 is a member of the NLRP family and comprised of an amino-terminal PYRIN domain, a carboxyterminal LRR domain, and a central nucleotide-binding NACHT domain with ATPase activity. Activation of NLRP3 requires two steps in most cell types (Fig. 2.2). Because NLRP3 expression in the basal state is not sufficient for inflammasome activation in myeloid cells, the first 'priming' step (signal 1) is needed. During this step, NF-κB is activated and induces transcription of NLRP3 and other proinflammatory genes including pro-IL-1β (Fig. 2.2). In *in vitro* inflammasome assays, the TLR4 agonist LPS is frequently used as a priming stimulus. During priming various other mechanisms are regulated, such as NLRP3 translation, which can be inhibited by microRNAs, post-translational NLRP3 modifications to keep NLRP3 in an inactive conformation, and the activity of metabolic pathways[45,48]. When macrophages constitutively express high levels of NLPR3, priming is unnecessary and the inflammasome can be activated without prior stimulation, highlighting the transcriptional aspect of priming[49].

In the second, 'activation' step (signal 2), recognition of PAMPs and DAMPs initiates NLRP3 inflammasome assembly, leading to the recruitment of the adaptor protein ASC and caspase-1. Once caspase-1 is activated, it leads to IL-1β and IL-18 maturation and secretion, and causes pyroptotic cell death[48] (Fig. 2.2). NLRP3 responds to a variety of structurally different activation stimuli, rendering a direct interaction with each of them unlikely. It seems more likely that NLRP3 senses a common event triggered by all of the different activators. However, neither a common factor nor mechanism activating NLRP3 have been identified yet despite intense research. Several different models and mechanisms were proposed[49]. The exposure to pore-forming or ionophore bacterial toxins (e.g. nigericin) and ion fluxes such as potassium and chloride efflux, sodium influx and calcium signaling were proposed to be critical for NLRP3 activation[45]. Most NLRP3 agonists also cause mitochondrial ROS production, which is another proposed trigger of NLRP3 activation[48]. Additionally, mitochondrial dysfunction, oxidized mitochondrial DNA, post-translational modifications of NLPR3, and lysosomal disruption after internalization of particulate matter are suggested to promote NLRP3 activation[45,48]. Taken together, a variety of different activators but no unified mechanism for NLRP3 activation has been described so far.

2.1.7 Communication between innate immune cells

To combat pathogen invasion, innate immune cells do not only use intracellular mechanisms but they also need to cooperate with each other to provide a coordinated, efficient immune response. For this purpose they utilize a plethora of different extracellular mediators such as cytokines, chemokines, growth factors, eicosanoids, peptides and complement. In recent years, different studies also suggested that infected or dying cells can transfer PAMPs and host signaling molecules to uninfected bystander cells, facilitating their activation, thereby sustaining and amplifying the innate immune response[50,51]. In the following, the most important extracellular mediators to this study will be described shortly.

Cytokines are key extracellular signaling molecules and consist of a group polypeptides/glycoproteins produced by different immune cells. After their release they can either act on their production site in an autocrine fashion, on nearby cells in paracrine action or on distant cells in an endocrine manner[52]. Different cytokine types have been described, including interferons (IFNs), interleukins (ILs), chemokines and tumor necrosis factor (TNF)[52]. The functions of cytokines are diverse and depend on the source of the cytokine and its target cells. Key proinflammatory cytokines produced upon microbial challenge include IL-1, IL-6,

TNFα and IFNs in the case of a virus infection. IL-12 and IL-10 constitute examples of anti-inflammatory cytokines[50].

Chemokines are a specific type of cytokines that are predominantly produced to recruit leukocytes to the inflammatory site in a process called chemotaxis. Due to the expression of specific chemokines, receptors and adhesion molecules distinct leukocyte subsets migrate to specific inflamed or injured tissues. Chemokines are mostly small proteins (8-12 kDa) that contain three to four conserved cysteine residues, with the majority of chemokines belonging to the CXC or C-C subfamilies. They bind and signal through G protein-coupled receptors (GPCRs)[50].

Eicosanoids are reactive lipid species that are produced by the enzymatic or non-enzymatic oxidation of arachidonic acid, the main membrane phospholipid component in cells[53,54]. Enzymatic peroxidation of arachidonic acids via lipoxygenase (LOX) or cyclooxygenase (COX) yields prostaglandins (PGs) (including PGE_2, prostacyclin (PGI_2), PGF_2 and thromboxane A2 (TXA_2)) or leukotrienes and lipoxins respectively. Prostaglandins and leukotrienes mediate inflammatory responses through binding to their respective receptors on immune cells. PGE_2 is one of the most prominent and abundant PG in the body and is involved in the generation of inflammation, increasing arterial dilation and blood flow and inducing pain by acting on peripheral sensory neurons[55].

More recently, it was shown that infected or dying host cells not only release cytokines but also PAMPs and other host derived signaling molecules (DAMPs) to uninfected neighboring cells. Thereby immune cells can maintain and amplify innate immune responses even if they are unable to produce cytokines anymore. For instance, nucleic acids such as viral RNA can be transferred from the cytoplasm of virally infected cells to uninfected bystander cells via extracellular vesicles called exosomes[51]. Cells can also release host PRR proteins such as ASC specks. These specks, formed after the activation of most inflammasomes, are released during pyroptosis and remain active extracellularly, leading to inflammation upon encountering their substrates pro-IL-1β and pro-IL-18 in the extracellular environment. Additionally, they can be phagocytosed by neighboring immune cells and lead to subsequent inflammasome activation, thereby spreading inflammation in an paracrine manner[51,56,57].

Thus, communication between innate immune cells by signaling molecules constitutes an important way to maintain and amplify inflammation and to extinguish it after the immune response is complete. Extending beyond the production of cytokines and chemokines, it also

involves transfer of PAMPs and host PRRs to neighboring cells via direct or indirect mechanisms, which allows immune cells to battle pathogen defense mechanisms.

2.1.8 The IL-1 family in inflammation

The IL-1 family of cytokines are particularly interesting. Some members of this family are closely linked to inflammation through their pyrogenic effects and their unbalanced production is associated with several autoimmune, infectious and auto-inflammatory diseases such as atherosclerosis[58]. The IL-1 family comprises 11 members with IL-1β being the strongest pyrogen in the human body[59]. They signal through the IL-1 receptor (IL-1R) family receptors, which are expressed by nearly all cells in the human body, allowing IL-1 cytokines to influence a wide range of immunological responses[60]. For instance, IL-1 cytokines modulate the innate immune response by inducing fever and leading to increased leukocyte migration. They also stimulate neutrophil and macrophage effector functions and prolong their lifespan during an immune response. Additionally, IL-1 cytokines modulate adaptive immune responses as IL-1β can specifically stimulate T cells to drive their differentiation into T helper cells[50,61]. IL-1α and IL-1β are the most prominent and researched members of the IL-1 family. Although they are encoded by different genes, they have similar biological properties and bind to the same receptor (IL-1R1)[61].

The IL-1α precursor is constitutively expressed in many cells and can be released upon cell death by necrosis. However, IL-1α is rarely detected in the circulation as it is mostly contained in apoptotic bodies. IL-1α can also be membrane bound and is found in many cell types, especially on activated monocytes and B lymphocytes. As the IL-1α precursor is constitutively expressed in many cell types, it does not need proteolytic maturation to be biologically active and can be rapidly released upon pathogen detection, thereby mediating early phases of inflammation[60,61].

By contrast, pro-IL-1β is only produced in response to proinflammatory stimuli. The IL-1β precursor is cleaved by caspase-1 after inflammasome activation and released into the circulation as described in section 2.1.6. Elevated IL-1β secretion is linked to auto-inflammatory diseases, tumor angiogenesis and metastatic spread, thus playing an important role in many different diseases[61].

In recent years, different therapeutics targeting IL-1 signaling or the IL-1 receptor have been developed. So far, there are three different IL-1 inhibitors approved for therapeutic use: canakinumab, an IL-1β neutralizing antibody, anakinra, an IL-1 receptor antagonist, and

rilonacept, a soluble IL-1 decoy receptor[45]. The recent results from the Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS) showed that treatment with canakinumab significantly reduced the incidence of atherosclerosis, arthritis and gout, all of which are NLRP3-related diseases. The CANTOS study also demonstrated that IL-1β blockage leads to a reduced cancer fatality, incident lung cancer and fatal lung cancer[45,62]. This study together with other recent findings highlighted the role of IL-1 cytokines in many common inflammatory and metabolic diseases and also linked IL-1β signaling to cancer development.

2.2 Platelets as immune factors

2.2.1 Platelet development and characteristics

Human platelets are small (2-5 μm in diameter), anucleate cells derived from megakaryocytes in the bone marrow and lung[17,63]. They are the second most abundant cell type in the body, after erythrocytes, with about one trillion platelets circulating in the blood of human adults (150,000 to 400,000 platelets per microliter). Because the lifespan of platelets is only between 7 and 10 days, approximately 100 billion new platelets are produced every day to ensure homeostasis[63,64]. Senescent platelets can be degraded in different ways. In immune thrombocytopenia patients, for instance, platelets are cleared through binding of autoantibodies targeting them for clearance by macrophages[65]. Platelet glycoproteins usually contain complex carbohydrate modifications including different glycans, which are often capped with sialic acids. Platelet senescence leads to desialylation of these membrane glycoprotein, which is another mediator of platelet clearance and leads to degradation of platelets by hepatocytes via the Ashwell-Morell receptor[65,66].

Platelets are not confined to mammals and platelet-like cells are also found in lower vertebrates and invertebrates. In contrast to anucleate platelets in mammals, both haemocytes in invertebrates and thrombocytes in lower vertebrates contain a nucleus. Haemocytes are structurally and functionally more similar to macrophages, performing both immune and hemostatic actions[64]. In lower vertebrates, thrombocytes are already more specialized for hemostatic functions. Thus, hemostatic and immune functions of platelets diverged during evolution with new cell types emerging for specific immune functions possibly explaining, why human platelets still play a role in innate immunity[64].

Platelets were long thought to be produced by megakaryocytes in the bone marrow. However, work from Lefrancais et al. identified the lung as primary site of platelet biogenesis[17]. In the lung and bone marrow, megakaryocytes produce platelets by forming long cytoplasmic extensions, called proplatelets, by rearranging their cytoplasm. These elongated proplatelets extend into blood vessels through junctions in the endothelial lining and release thousands of platelets and platelet microparticles into the blood stream[64].

Resting platelets display a circular discoid shape, which is maintained by specialized cytoskeleton and can withstand the high shear forces in the blood circulation. The platelet membrane contains different cell surface receptors and shows regular invaginations leading to the open canalicular system (OCS)[63]. This complex network of membrane tubes provides

platelets with a much greater surface area and allows them to take up proteins and molecules from their surroundings. Additionally, platelets also contain an internal membrane system called dense tubular system (DTS), which stores calcium and enzymes that support platelet activation[63]. Although platelets do not have a nucleus, other cell organelles, such as mitochondria, secretory granules and glycosomes, are present. Upon detection of tissue damage, DAMPs or PAMPs, platelets get activated and undergo a drastic shape change from a smooth discoid to a spiny sphere shape with lamellipodial and filopodial extensions[63,64].

Platelets can store proteins and biologically active molecules in specific granule types: α-granules, dense granules and lysosomes[63]. The most abundant granules are α-granules, with approximately 40-80 granules per platelet[64]. They store more than 300 proteins which platelets secrete upon activation, including platelet adhesion, aggregation, and coagulation factors required for platelet functions in hemostasis and thrombosis, such as von Willebrand factor (vWF) and thrombospondin[64]. In addition, α-granules contain proteins and peptides related to specific immune cell recruitment and activation, highlighting the role of platelets in the immune response. Examples include P-selectin, which is transferred to the platelet membrane upon activation, and different chemokines such as CXCL4, CXCL7, RANTES (also known as CCL5)[63,64]. Dense granules are approximately 10-fold less abundant than α-granules and are also released upon platelet activation. These granules predominantly store cations (calcium and magnesium), nucleotides (ATP and Adenosine diphosphate (ADP)) and serotonin[63]. Lysosomes, the third kind of granules found in platelets, mainly contain lysosomal-associated membrane proteins (CD63) and proteases such as cathepsins and carboxypeptides[63].

2.2.2 The role of platelets in hemostasis and inflammation

2.2.2.1 Platelets in hemostasis

Platelets are well-known for their role in hemostasis and blood coagulation. Upon vascular damage, platelets adhere to the site of injury through binding of platelet glycoprotein (GP)Ibα (GPIbα) to vWF on the subendothelial matrix[67]. Subsequently, a stable platelet adhesion is established by binding of several integrins to their ligands on the vessel wall, such as integrin αIIbβ3 to fibrinogen[67]. Through the activation of coagulation pathways at the site of injury, the serine protease thrombin is generated and binds to GPIbα or to protease-activated receptors (PARs) on platelets, activating them. Activated platelets release their granular content, including different cytokines and ADP, and metabolize arachidonic acid using COX-1 and

thromboxane synthase to create TXA_2[63,68]. Binding of ADP and TXA_2 to their respective receptors on platelets leads to autocrine platelet activation[63]. Through the recruitment and activation of further platelets a thrombus is formed. Additionally, platelets recruit and activate leukocytes either through direct contact or indirectly through cytokines and platelet-derived microvesicles (PMVs) to combat emerging infections[8]. Together, this complex cooperation ensures rapid and reliable damage control, blood clotting and ultimately maintains hemostasis.

2.2.2.2 Platelets as immune cells

Work in the last decade has shown that besides sensing vascular damage, platelets also respond to a variety of pathogens and inflammatory triggers and play important roles in the innate immune response. Platelets are among the first cells recruited to the site of infection and express a plethora of different membrane receptors, including TLRs, to detect PAMPs and DAMPs, which lead to platelet activation[69,70]. TLR4 is the most abundant TLR expressed on platelets and has been shown to be functional since engagement of this receptor by LPS is linked to severe thrombocytopenia in murine models[71,72]. Platelets mediate the response to pathogens either via direct pathogen binding or indirectly, by recruiting and interacting with immune cells at the site of infection[64] (Fig. 2.3).

Platelets can directly bind and destroy invading pathogens. To kill pathogens, they can rapidly release anti-microbial mediators from their α-granules, such as thrombocidins which are lethal to a wide range of bacteria[64]. Besides the release of soluble mediators, platelets can also directly interact with bacteria, aggregate around them and 'trap' them for elimination by phagocytes[70,73]. Gaertner et al. showed that platelets can actively migrate to sites of injury or inflammation and act as cellular scavengers, using their adhesion receptors to scan the vascular surface for invading pathogens[74]. They collect and bundle potential invaders, thereby increasing the activity of professional phagocytes, such as neutrophils[74].

Another way by which platelets enhance the innate immune response is through interaction with the complement system. The complement system consists of a large number of soluble and membrane-bound glycoproteins that respond to surface targets on pathogens and can interact with each other to induce a series of inflammatory responses[75]. Platelets and the complement system can activate each other reciprocally[75]. Activated platelets have been shown to stimulate the complement system in various ways, including the phosphorylation of complement factors, the release of ATP and protein kinases. Additionally, platelets secrete

platelet activating factor (PAF) enhancing phagocytosis of complement-bound erythrocytes by monocytes[63].

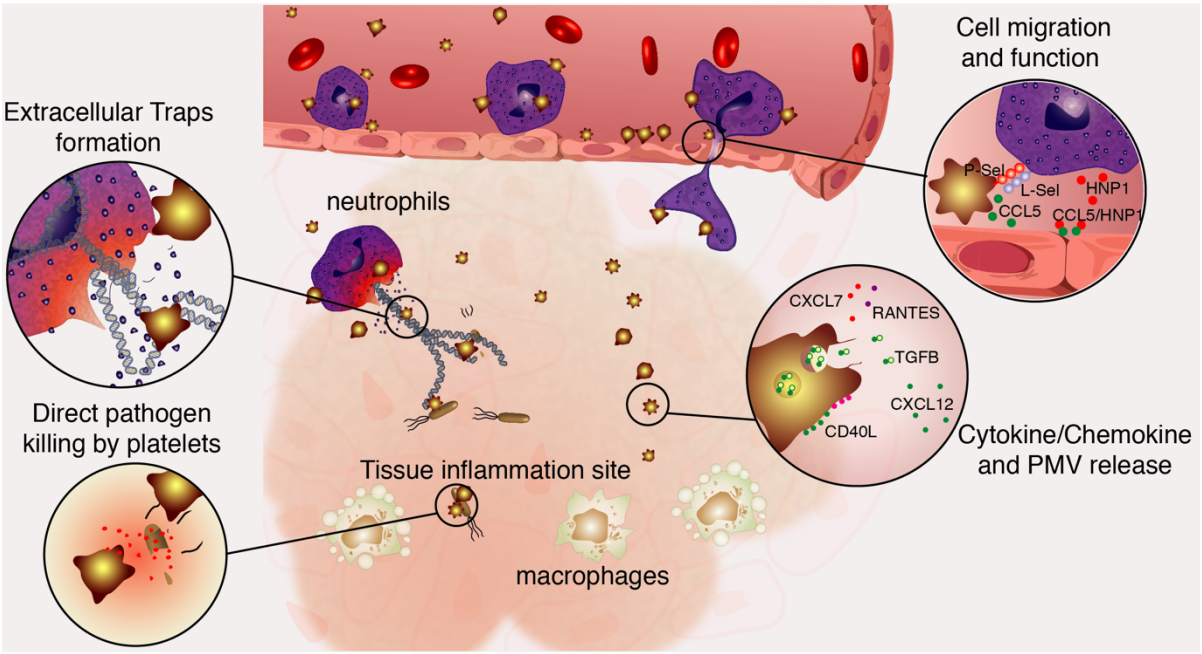

Figure 2.3 Platelet interaction with immune cells and invading pathogens. Schematic representation of platelet function in innate immune responses. Platelets can either directly interact with pathogens and kill them, interact with immune cells and regulate their immune functions or secrete platelet-derived microvesicles (PMVs) and a plethora of different factors themselves.

Next to direct interaction with pathogens and the complement system, platelets also recruit leukocytes, interact with them and initiate and amplify innate immune responses. Despite lacking a nucleus, platelets carry proteins produced in megakaryocytes, which are stored in their granules or are expressed on their membrane. Platelets also possess mitochondrial DNA (mtDNA), a broad array of microRNAs and messenger (m)RNAs and have the ability to synthesize proteins upon activation[63,76-80]. Denis et al. showed, for instance, that activated platelets splice and translate IL-1β mRNA into protein[80] This is possible, because anucleate platelets possess a functional spliceosome, including small nuclear RNAs, endogenous pre-mRNAs and splicing proteins. LPS stimulation or clustering of FcαR1 receptors on platelets induces splicing of IL-1β pre-mRNA into mature mRNA, which can be translated into protein subsequently[80].

The majority of this newly synthesized pro-IL-1β is retained in the cell[81], although platelets have also been reported to secrete IL-1β after different stimulations, promoting and initiating inflammatory responses in leukocytes[82-85]. In patients with dengue, Hottz et al. not only

observed increased expression of IL-1β but also showed NLRP3 inflammasome assembly and caspase-1 activation in platelets for the first time[86]. They describe that IL-1β is mostly released in microvesicles after ROS induced NLPR3 activation[86]. Since then, two other groups have reported NLRP3 activation in platelets in the context of thrombus formation and sickle cell disease[87,88]. In line with these findings, Tunjungputri et al. investigated a cohort of 500 Caucasian healthy individuals and determined correlations of IL-1β levels with platelet numbers and reactivity[89]. They found a positive association of platelet counts with IL-1β plasma concentrations and platelet degranulation and activation was linked to IL-1β and IL-6 production[89]. Altogether, these studies highlight the importance of the inter-relationship between platelets and IL-1β mediated inflammation.

Besides IL-1β, proteomic analysis revealed the ability of platelets to secrete more than 300 different proteins after thrombin activation[90]. Platelets release a variety of chemokines from α-granules after platelet activation, including Macrophage Inflammatory Protein (MIP) 1α, a monocyte chemotactic protein, RANTES, which recruits and activates leukocytes, and platelet factor 4 (PF4) and β-thromboglobulin, which recruit neutrophils[63]. Additionally, platelets secrete different growth factors such as platelet derived growth factor (PDGF) and they are major sources of transforming growth factor-β (TGF-β) in the human body, which has potent physiological and pathological effects on a variety of immune cells[63,91]. Platelets can also form a bridge between the innate and adaptive immune response by peripheral B cell activation through soluble CD40L (sCD40L, also known as CD154) and RANTES secretion, or the induction of a productive T cell response through sCD40L secretion[63,64].

Platelets do not only use the secretion of soluble cytokines for communication with innate immune cells or the endothelium but they can also enclose cytokines or other molecules in bioactive microvesicles before their release into the extracellular environment. PMVs constitute the most abundant cell-derived microvesicles in the human body and show high heterogeneity in size and content[92]. Small PMVs (100-500 nm) are mostly enriched with proteins from α-granules, whereas larger PMVs mostly contain lipid mediators and mitochondrial proteins[92]. Increased PMV counts have been linked to a variety of diseases, such as rheumatoid arthritis, gout and cancer. PMVs act similarly to platelets and contain different, disease specific factors, for instance IL-1 family cytokines in inflammatory arthritis or angiogenic factors and lipids in cancer patients[92].

In addition to cytokine and PMV secretion, platelets interact directly with leukocytes in the circulation and tissue, forming platelet-leukocyte aggregates (PLAs). Activated platelets can

recruit leukocytes and aid their extravasation out of the blood vessel into the tissue at sites of injury and inflammation[93,94]. During inflammation, platelets rapidly adhere to the inflamed microvasculature and capture leukocytes through interaction with different leukocyte receptors, such as CD40 and P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes. This induces conformational changes in leukocyte surface integrins and promotes successive extravasation out of the blood vessel into the inflamed tissue[94].

The interaction of platelets with leukocytes also induces proinflammatory cytokine expression in leukocytes. For instance, platelet P-selectin binding to PSGL-1 and the release of RANTES by platelets induce monocytes to express different proinflammatory cytokines such as monocyte chemotactic protein 1 (MCP-1), TNFα, (pro-)IL-1β and IL-6. Likewise, platelets interact with DCs via CD40L-CD40 leading to enhanced IFNα release[8].

Platelet interaction with leukocytes such as neutrophils and macrophages also induces extracellular trap formation during inflammatory responses leading to bacterial clearance[8,95]. Additionally, platelets can regulate leukocyte oxidative burst by modulating ROS and myeloperoxidase (MPO) release from leukocytes. Platelet-derived sCD40L was shown to induce ROS production from neutrophils and platelet-derived HMGB1 induces MPO translocation to their cell membrane[8]. Platelet-leukocyte interactions are further characterized in section 2.2.3.

Besides the proinflammatory effects, platelets can also dampen or restrict inflammation through release of different factors. The chemokine CXCL4, for instance, does not only regulate proinflammatory actions but also down-regulates chemotactic receptors in monocytes, inhibiting monocyte migration[8]. Besides activating the adaptive immune system, sCD40L released by activated platelets also causes anti-inflammatory effects. In monocytes it enhances IL-10 expression while downregulating IL-6 and TNFα expression[8]. Thus, platelet function is a delicately balanced system with both pro- and anti-inflammatory effects on immune cells either inducing or restricting inflammation if necessary[8].

2.2.3 Interaction of platelets with innate immune cells

The previous section described interaction between platelets and different leukocytes to mediate immune responses against invading pathogens. The interactions with the most relevant innate immune cells to this thesis, monocytes, macrophages and neutrophils, will be described here in more detail.

2.2.3.1 Platelet-monocyte interactions

Platelets influence the immune responses of monocytes in various ways. Monocytes can rapidly bind to activated platelets in the circulation, displaying P-selectin, and adhere to them for prolonged times[94,96]. This leads to the formation of platelet-monocyte aggregates (PMAs) which activate monocytes and ultimately leads to increased CD40, PSGL-1, C-C chemokine receptor 2 and Integrin alpha M expression[8]. Expression of these proteins enhances further PMA formation and recruits more monocytes to the endothelium. Initial platelet P-selectin binding with monocyte PSGL-1 is stabilized by CD40L-MAC-1 (also known as CD11b/CD18 receptor) interactions and by intercellular adhesion molecule 1 (ICAM-1) binding to platelet-bound fibrinogen[8,94]. Recently, Alard et al. demonstrated that heteromers of platelet-derived RANTES and neutrophil-derived human neutrophil peptide 1 (HNP1) also play an important role in monocyte adhesion during inflammation[97]. Additionally, platelets secrete chemokines such as CXCL7, RANTES or macrophage migration inhibiting factor (MIF) which induce monocyte arrest at the sites of inflammation. Platelet MIF and stromal cell-derived growth factor 1 secretion (SDF-1, also known as CXCL12) induce further recruitment of monocytes through chemotaxis. Besides this, platelets also boost the destruction of phagocytosed pathogens in monocytes by inducing endogenous oxidative burst through ROS production[8].
In general, platelets preferably bind to $CD16^+$ inflammatory monocytes. Activated platelets have been shown induce CD16 expression in monocytes by TGF-β secretion, thereby eliciting a proinflammatory phenotype[8,98]. Yet, platelet interaction with monocytes can not only induce a new monocyte phenotype but also promote monocyte differentiation into macrophages, for instance through platelet-derived SDF-1[99].
Platelet-monocyte interactions and PMAs have been associated with different diseases, such as rheumatoid arthritis, Dengue infection and atherosclerosis[100-102]. Patients with rheumatoid arthritis showed increased platelet activation and PMA formation compared to healthy individuals[100]. Hottz et al. also observed enhanced levels of PMAs in the blood from Dengue patients, indicating their role in this disease[101]. In atherosclerosis, the formation of PMAs induces expression of COX-2 in monocytes via P-selectin leading to eicosanoid production and subsequent onset of inflammation[102]. Also platelet secretion of proinflammatory mediators such as RANTES, CXCL4 and CXCL7 contribute to monocyte migration to the inflammatory site, inducing further progression of atherosclerosis[103].

2.2.3.2 Platelet-macrophage interactions

While platelet interaction with monocytes has been studied extensively, our understanding of platelet interaction with differentiated macrophages remains more limited. Still, some important features of platelet-macrophage interactions have been discovered in the last years. Scull et al. showed that macrophages phagocytose autologous, activated platelets leading to enhanced proinflammatory cytokine secretion, such as TNFα, IL-6 and IL-23[104]. Macrophages can also interact with microRNA containing PMVs leading to their subsequent internalization. PMVs deliver functional microRNAs to macrophages, thereby regulating their gene expression[105]. These changes lead to differential cytokine secretion and increased phagocytic activity of macrophages[105]. In the liver, resident macrophages (Kupffer cells) interact with platelets to increase clearance of invading bacteria such as *Bacillus cereus* or *Staphylococcus aureus* (MRSA)[70]. In hemostasis, platelets transiently interact with Kupffer cells in the liver via binding of platelet GPIbα (CD42b) to vWF constitutively expressed on Kupffer cells. Once Kupffer cells capture pathogens, this binding intensifies and allows platelets to encapsulate the invading bacteria and help to facilitate its clearance[70]. More recently, Ali et al. demonstrated in a mouse model that activated platelets also enhance phagocytosis of MRSA by peritoneal macrophages in a contact independent manner[106].

Similar to neutrophils, macrophages can release their chromatin fibers and form extracellular traps during acute kidney injury, called macrophage extracellular traps (METs)[95]. Okubo et al. reported that during rhabdomyolysis, a syndrome induced through skeletal muscle injury, heme activates platelets leading to increased MET production through intracellular ROS production and histone citrullination in macrophages[95]. Thus, platelets sense heme from injured muscles and act as a scaffold for macrophages to form METs and induce inflammation[95].

Besides these proinflammatory effects, platelet-macrophage interactions can also induce anti-inflammatory responses. For instance, platelets promote IL10 and dampen TNFα secretion from human macrophages or monocytes through the production of prostaglandin E2 (PGE_2)[107]. Thereby, macrophages and monocytes can restrict excessive immune responses induced by activated platelets.

2.2.3.1 Platelet-neutrophil interactions

The interaction of platelets with neutrophils during immune responses is well established and has been studied extensively over the last decade.

Binding of platelets to neutrophils leads to the formation of platelet-neutrophil complexes (PNCs) and has multiple effects, including enhanced adhesion to the endothelium, increased ROS production and NET formation[108]. However, PNCs have also been associated with multiple diseases, such as asthma, rheumatoid arthritis and sepsis, highlighting their role in immune responses. Platelet-neutrophil interaction is mainly mediated through platelet P-selectin binding to its receptor PSGL-1 on neutrophils. Sreeramkumar et al. showed that neutrophils scan for the presence of activated platelets in activated venules using PSGL-1[93]. Binding to platelets results in polarization of receptor distribution in neutrophils and induces neutrophil rolling and migration[93]. In different studies, depletion of P-selectin or platelets has been shown to inhibit recruitment of neutrophils to the inflammatory site, showing that P-selectin and platelets are vital for neutrophil recruitment[108]. As described before, platelets can also act as pathfinders for leukocytes, more specifically for neutrophils and monocytes. During inflammation, platelets bind to distinct sites in venular microvessels and capture circulating neutrophils through CD40-CD40L interactions[94]. Additionally, both adherent neutrophils and platelets recruit monocytes to these sites of extravasation in the microvasculature. Subsequently, platelet P-selectin binds to PSGL-1 on neutrophils and monocytes inducing conformational changes in their surface integrins, promoting their successive extravasation out of the blood vessel into the inflamed tissue[94].

Like macrophages, neutrophils can form extracellular traps, where chromatin and granular proteins are released into the extracellular space, forming fibers that can immobilize and kill bacteria. Platelets are critical for NET formation during inflammatory responses[108]. TLR4 activation through PAMP detection in the blood causes platelets to adhere to neutrophils either via P-selectin - PSGL-1, platelet GPIbα - vWF or neutrophil lymphocyte-function-associated-antigen-1 (LFA-1) - CD40L interactions and induce NET formation[8,73,108]. This has been shown to occur in sepsis, where LPS stimulated platelets adhere to neutrophils and stimulate them to produce NETs to trap free bacteria[73]. Platelets also induce NET formation and bacterial clearance through secretion of PMVs or soluble factors, including TXA_2, CXCL4, high-mobility group box 1 protein (HMGB1) or β-defensin[8,108]. β-defensin is an antimicrobial peptide that not only inhibits bacterial growth directly but also induces NET formation[108].

In general, a strong connection exists between the activation state of neutrophils and their interaction with platelets. In comparison to free neutrophils, neutrophils in PNCs display an increased activation state with enhanced CD11b expression, ROS production and phagocytic

activity. Interaction with resting, unstimulated platelets restricted neutrophil activation, indicating that platelet and neutrophil activation are closely interconnected[108].

However, platelet interaction with neutrophils can also induce an anti-inflammatory phenotype as described for other leukocytes. Upon PNC formation, lipoxin A4 is generated, which inhibits neutrophil adhesion and extravasation to the inflammatory site[8]. Thus, neutrophil-platelet interactions play important roles in the generation of innate immune response in response to invading pathogens.

2.2.4 Platelets in metabolism

Platelets are involved in many energy-demanding processes like thrombosis or innate immune responses. During hemostasis, platelets utilize both oxidative phosphorylation and glycolysis to produce energy from glucose in the form of ATP. Upon platelet activation and aggregation, platelets increase glycolytic metabolism, similar to the Warburg effect described for other immune cells like monocytes, macrophages and dendritic cells[25,109]. In contrast, platelets also retain robust oxidative phosphorylation, thereby maintaining their metabolic plasticity[109]. Next to glucose, resting or thrombin activated platelets are also capable of generating ATP from fatty acids and glutamine[110,111]

Recently, Aibibula et al. confirmed and refined these findings, showing that resting platelets can freely switch between oxidative phosphorylation and glycolysis using either glucose or fatty acids[25]. Upon activation, when platelet energy demands increase drastically, rapid uptake of exogenous glucose is promoted. This leads to the promotion of a predominantly glycolytic phenotype in activated platelets but mitochondrial oxygen consumption is also preserved[25]. Thus, Aibibula et al. described a high metabolic plasticity in platelets, especially in nutrient-limiting conditions, where platelets can either use glucose, fatty acids or glycogen independently to facilitate activation[25]. This metabolic flexibility might aid platelets to fulfill their divergent roles in hemostasis and immunity, and function well in a variety of different environments with different nutrient availabilities. For instance, the availability of nutrients and oxygen may be very restricted within the thrombus, whereas oxygen and nutrients are more accessible in the bloodstream or in certain tissues[25].

2.2.5 The role of platelets in cancer

Besides their function in hemostasis and inflammation, platelets also play a well-recognized role in cancer. High platelet counts and platelet-lymphocyte ratios have been associated with a poor prognosis in many cancer types[112], and cancer patients have an enhanced risk for

thrombosis, being one of the biggest contributing factor for mortality[113]. Besides their contribution to thrombosis, activated platelets have been connected to tumor growth, angiogenesis, survival of circulating tumor cells (CTCs), tumor cell arrest and metastasis, highlighting the important role of platelets in cancer[114].

Activated platelets release a multitude of factors and PMVs that contribute to the modulation of the tumor environment. Examples include pro-angiogenic growth factors, such as vascular endothelial growth factors (VEGF) and fibroblast growth factors (FGFs) or chemokines that recruit immune cells to the tumor. Recruited leukocytes remodel the extracellular matrix and contribute to angiogenesis[112]. PMVs not only store pro-angiogenic factors or proinflammatory cytokines, but also contribute to receptor transfer to other immune and tumor cells through membrane fusion. This interaction can be bidirectional as recent reports have described tumor educated platelets (TEPs)[115]. TEPs have altered transcriptomic profiles and could be used as liquid biopsies to predict tumor occurrence and location[116]

Apart from this, platelets promote tumor metastasis using different mechanisms. The first crucial step for metastasis is the survival of CTCs in the circulation as they have to withstand shear stress and anti-cancer immune cell responses of NK cells[114]. Platelets rapidly sense and bind CTCs in the blood, become activated and form platelet-CTC aggregates, via platelet P-selectin and Fibrinogen receptor binding to their respective ligand on tumor cells[114]. Thereby, platelets protect CTCs from shear stress and help evade immune surveillance while simultaneously producing growth factors to support CTC survival. Besides shielding CTCs from immune cells, activated platelets suppress the immune action against CTCs. Activated platelets secrete TGF-β, which inhibits granule mobilization, cytokine secretion and cytotoxicity of NK cells[114,117].

Platelets are also involved in CTC extravasation and invasion of the tissue at secondary sites. Following platelet-induced arrest of CTCs at the vascular wall, platelets secrete ATP from dense granules, which interacts with endothelial receptors to open the endothelial barriers. Subsequently, CTCs transmigrate into the tissue and form metastatic foci[117].

Thus, platelets significantly promote cancer progression, angiogenesis and formation of distant metastases. Inhibition of platelet activity using non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, rofecoxib and sulindac, has been shown to reduce cancer incidences[115]. Aspirin inhibits platelet activation through irreversible acetylation of COX-1, thereby blocking arachidonate metabolism[118]. Despite some side effects, it showed promising effects in reducing cancer progression and is suitable for long-term use[115]. Thus, based on these

and other results from clinical studies, targeting platelets seems to be sensible therapeutic strategy[115].

2.3 Head and neck squamous cell carcinoma (HNSCC)

2.3.1 Occurrence and development of HNSCC

Cancer is a deadly disease, in which the activation of oncogenes and/or the deactivation of tumor suppressor genes leads to uncontrolled cell growth and the gain of metastatic properties[75]. HNSCC is the sixth most abundant type of cancer with 600.000 new cases per year worldwide[119]. Its occurrence is classically associated with smoking and excessive alcohol consumption. More recently, it has been shown that infection with high-risk human papillomaviruses also promotes development of HNSCC and accounts for a rising proportion of tumors, especially in the Western world[120].

HNSCC tumors develop from the epithelial cells that line the mucosal surfaces of the food passages and upper airways. Due to the complex and diverse anatomical structures in these areas of the body, HNSCC is a very heterogenous disease[120]. The mortality rates in this cancer are high, with a 5 year survival rate of approximately 50% for all stages combined[121]. Despite improvements in HNSCC therapy, this rate has not improved over the past decades mainly due the advanced tumor and metastatic state of the patients at diagnosis, the general lack of personalized treatment approaches and missing knowledge to reliably predict cancer therapy outcomes[120,122]. Because molecular, not visible mutations precede clinical symptoms, many HNSCC cases remain undiagnosed until an advanced, irreversible cancer state. For late-stage tumors, treatment becomes difficult and inefficient[122]. So far, only tissue samples are used for diagnostics, which require biopsies or needle aspirations causing high costs and discomfort in patients. Molecular biomarkers such as cancer specific RNA, DNA, proteins or CTCs, could be used as diagnostics to assess the disease state in body fluids in a less-invasive way. Recently, Kulasinghe et al. showed, for instance, that CTCs in HNSCC patients could be used as diagnostic markers, with CTCs predicting poorer outcome in patients[123]. Although this and other studies prove the feasibility of liquid biopsies, so far no biomarker is used in clinical settings. Especially, the high heterogeneity of tumors, tumor sites, molecular mechanisms and causes of cancer (viral infection or alcohol/tobacco) have obstructed the development and implementation of reliable biomarkers[122].

2.3.2 New therapies in HNSCC

Besides surgery, traditional HNSCC treatment consists of radiation and chemotherapy. In the last decade, however, different new approaches to a more personalized treatment of HNSCC

have been developed and tested such as monoclonal antibodies as immune checkpoint inhibitors[119].

The approval of the monoclonal antibody targeting epidermal growth factor receptor (EGFR) for HNSCC in 2006 marked the beginning of targeted HNSCC therapy. EGFR is activated to a higher extent in HNSCC patients in comparison to healthy controls, leading to tumor promotion and a more aggressive disease progression[119]. As radiation therapy further induces EGFR expression, this monoclonal antibody is mostly used in combination with radiation therapy increasing overall and progression-free survival in HNSCC patients. So far, however, we lack biomarkers that predict the patient response[119].

Checkpoint inhibitor therapy uses monoclonal antibodies to block immune checkpoints, thereby enhancing the immune response to cancer cells. Immune checkpoints are key regulatory interactions between cells that lead to stimulatory or inhibitory actions. In homeostasis, inhibitory immune checkpoints prevent aberrant or chronic immune responses, however cancer cells can hijack these pathways to protect themselves. By expressing ligands or receptors that lead to inhibition of T cell responses, they dampen the anti-cancer immune responses. Checkpoint inhibition therapies block these hijacked inhibitory pathways and release the molecular brakes of anti-tumor T cell responses[124].

Currently, there are several different checkpoint inhibition therapies available for different cancers targeting the molecules cytotoxic T lymphocyte-associated protein 4 (CTLA4), programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1), yielding durable clinical results[124]. So far, only the monoclonal antibodies against PD-1 (pembrolizumab or nivolumab) were approved for treatment of HNSCC patients[119]. These antibodies target PD-1, which is expressed by T cells and which interacts with PD-L1 expressed on tumor cells. Uncoupling this inhibitory interaction releases the molecular brakes from T cells leading to an effective anti-tumor response[124]. Although treatment with monoclonal antibodies against PD-1 showed unprecedented and long lasting positive effects for some patients, in total only 10-20% of HNSCC patients responded to this checkpoint inhibition therapy[119,120]. Diverse responses to these therapies are not uncommon as several factors determine the success of treatment, including the expression of PD-L1 on cancer cells[125], the quality of T cells[126] and the mutational rate of cancer cells[127].

Besides anti-PD-1 therapy, anti-CTLA-4 and anti-PD-L1 therapy delivered promising results in other cancers. Monoclonal antibodies targeting CTLA-4, such as ipilimumab, that inhibit regulatory T cell suppression and promotes T cell effector functions, greatly improved the

overall survival of melanoma patients. Currently, this therapy is under investigation for several other cancer types[124]. Next to anti-PD-1 antibodies, targeting PD-L1 with immune checkpoint inhibitors constitutes a way to uncouple the inhibitory PD-1-PD-L1 interaction between T cells and cancer cells. For previously treated non-small cell lung cancer (NSCLC) patients, therapy with monoclonal antibodies targeting PD-L1, such as atezolizumab, proved superior to chemotherapy, with higher overall survival rates[128]. Currently, there are various different monoclonal antibodies against PD-L1 being tested in the clinics in different types of cancers, however, so far not yet in HNSCC[124].

Besides targeting co-inhibitory receptors, other therapeutic approaches are also researched which promote stimulatory proteins. For instance, TLRs can be stimulated by agonists leading to an innate immune response and host defense mechanisms. Another line of research investigates the impact of modifying the tumor microenvironment to activate antitumor responses. Although all of these different therapeutic approaches are promising and some already showed unprecedented results in HNSCC patients, they are only targeting a fraction of cancer types and cancer patients. So far, reliable biomarkers that identify patients that are most likely to respond and benefit from certain immunotherapies are still missing[119].

2.4 Research objectives

Inflammasomes are multimeric signaling platforms that have been studied extensively since their discovery over a decade ago, leading to the discovery of different inflammasomes and a multitude of different activators and activating mechanism[36]. However, most of these studies were conducted *in vitro* in monocultures of macrophages or monocytes, thereby disregarding the regulatory, networking and synergistic effects of other immune cells *in vivo*.

Platelets are the second-most abundant cell-type in the human blood. They are well-known for their role in hemostasis but have also been acknowledged for their role as immune regulatory cells more recently. Despite their anucleate status and their small size, platelets can respond to a variety of inflammatory stimuli, secrete a plethora of different inflammatory mediators and interact extensively with other immune cells to initiate and maintain immune responses[18]. Badrnya et al. showed that platelets promote phenotypic changes in monocytes and induce monocyte extravasation into the tissue[129], while Scull et al. demonstrated that LPS stimulated macrophages phagocytose activated platelets leading to enhanced cytokine secretion[104]. More recently, different studies also described the assembly of functional NLRP3 inflammasomes in platelets from patients with Dengue and sickle cell disease[86-88]. However, so far the involvement of platelets or platelet-derived inflammasomes in the inflammatory response of leukocytes has not been investigated yet.

Therefore, the interaction of platelets with different innate immune cells during inflammasome activation and its effect on their inflammatory properties was investigated. As such, the specific aims for the first part of this thesis were:

i) To investigate the effect of platelet-leukocyte interactions during NLRP3 or NLRC4 inflammasome activation
ii) To understand the significance of platelet inflammasome activation or platelet-derived inflammasome components in this interaction
iii) To elucidate the mechanism of platelet-leukocyte interactions during inflammasome activation

Besides their function in hemostasis and inflammation, platelets are also well-known for their role in cancer development and metastasis. Not only are high platelet counts associated with a poor prognosis in many cancers, activated platelets have also been described to induce tumor growth, angiogenesis and the survival of CTCs[114]. More recently, Best et al. showed that platelets can also be educated by the tumor, yielding altered transcriptomic profiles, and could be used as liquid biopsies to predict the tumor occurrence in patients[116,130].

In the cancer field, checkpoint inhibition therapies antibodies have proved to be a promising treatment for different cancer types[124]. In HNSCC, the use of monoclonal antibodies targeting the PD-L1/PD-1 immune checkpoints has shown unprecedented and durable effects and immunotherapy is now employed besides classical cancer therapy[119]. However, in total only 10-20% of HNSCC patients responded to this checkpoint inhibition therapy and in general the mortality rates in this cancer still remain very high, with only every second patients surviving longer than 5 years post diagnosis[119,120]. There are diverse reasons for this low response in HNSCC patients, including the expression of PD-L1 on cancer cells[125], the quality of T cells[126] and the mutational rate of cancer cells[127]. So far, no easily accessible biomarker has been discovered to identify eligible patients that would benefit most from these immunotherapies.
Since platelets play a prominent role in cancer development and can be educated by the tumor, the potential of platelets as new therapeutic target in HNSCC patients was investigated in the second part of this thesis. In detail, the specific aims of this study were:

i) To understand if platelets express checkpoint inhibition proteins in HNSCC patients
ii) To investigate the influence of platelets on immunotherapy in patients

3 Materials and Methods

This section is separated into two parts. Section 3.1 lists the materials used for this work including for example cell lines, mice, antibodies, chemicals, buffers and primers, whereas the experimental procedures are described in section 3.2.

3.1 Materials

3.1.1 Consumables

384-well microplate, small volume, flat bottom, white	Greiner Bio-One (Kremsmünster, Austria)
384-well PCR plates	Sarstedt (Nürnbrecht, Germany)
5 ml polystyrene flow cytometry tubes	Sarstedt (Nürnbrecht, Germany)
8-well IBIDI µ-slide	Ibidi (Martinsried, Germany)
Bottle vacuum filter system, pore size 0.2 µm	Corning (Corning, NY, USA)
Cell scrapers	Sarstedt (Nürnbrecht, Germany)
Centrifuge tubes (15 ml/ 50 ml)	Greiner Bio-One (Kremsmünster, Austria)
EASYstrainerTM 70µm cell strainer, sterile	Greiner Bio-One (Kremsmünster, Austria)
Eppendorf safe-lock tubes (1.5 ml/ 2 ml)	Eppendorf (Hamburg, Germany)
HTS Transwell 96-well plate, TC treated, 0.4 µm Polycarbonate Membrane	Corning (Corning, NY, USA)
Low-binding Durapore PVDF membrane 0.5 µm	Merck (Darmstadt, Germany)
Nunclon™ Delta Surface 6-well plates	ThermoFisher Scientific (Waltham, MA, USA)
NuPAGE Novex 4-12% Bis-Tris Gel 1.5 mm, 15 Well	ThermoFisher Scientific (Waltham, MA, USA)
Opti-Seal Optical Disposable Adhesive	Bioplastic (Landgraaf, The Netherlands)
S-Monovette® 9NC	Sarstedt (Nürnbrecht, Germany)

S-Monovette® K3EDTA	Sarstedt (Nürnbrecht, Germany)
Safety-Multifly® 20G 200 mmlang	Sarstedt (Nürnbrecht, Germany)
Seahorse XFe96 FluxPaks	Agilent Technologies (Santa Clara, CA, USA)
Single-use syringes 10 ml	Braun (Melsungen, Germany)
Sterile filter tips (1 ml, 200 µl, 20 µl)	Mettler Toledo (Columbus, OH, USA)
Tissue culture flasks (25/ 75/ 175 mm^2)	Sarstedt (Nürnbrecht, Germany)
Tissue culture plates (6-well/ 96-well), sterile, flat bottom, with lid	Sarstedt (Nürnbrecht, Germany)
Tissue culture plates (96-well), sterile, U bottom	Greiner Bio-One (Kremsmünster, Austria)
Whatman® cellulose chromatography paper, 3 mm CHR	Whatman (Maidstone, United Kingdom)

3.1.2 Chemicals and reagents

(R)-(+)- Etomoxir sodium salt	Tocris Bioscience (Bristol, United Kingdom)
2-Deoxyglucose (2-DG)	Sigma-Aldrich (Taufkirchen, Germany)
2-mercaptoethanol	Sigma-Aldrich (Taufkirchen, Germany)
Acetylsalicylic acid	Sigma-Aldrich (Taufkirchen, Germany)
Acid-citrate-dextrose (ACD)	Sigma-Aldrich (Taufkirchen, Germany)
Adenosine 5-diphosphate sodium salt (ADP)	Sigma-Aldrich (Taufkirchen, Germany)
Adenosine 5-triphosphate disodium salt (ATP)	Sigma-Aldrich (Taufkirchen, Germany)
Anthrax Protective Antigen (PA), recombinant from Bacillus anthracis	List Biological Laboratories (Campbell, CA, USA)
Antimycin A	Sigma-Aldrich (Taufkirchen, Germany)
Apyrase from potatoes, High Activity, ATPase > 600 units/mg protein	Sigma-Aldrich (Taufkirchen, Germany)
BAPTA AM	Enzo Life Sciences Inc. (Farmingdale, NY, USA)
BAPTA, Tetrasodium salt	Santa Cruz Biotechnology (Dallas, TX, USA)

Benzonase® Nuclease	Sigma-Aldrich (Taufkirchen, Germany)
Bovine Serum Albumin (BSA)	Sigma-Aldrich (Taufkirchen, Germany)
BPTES	Selleck Chemicals (Houston, TX, USA)
Brain-derived neutrotrophic factor (BDNF)	Tocris Bioscience (Bristol, United Kingdom)
Calcium chloride	Sigma- Aldrich (Taufkirchen, Germany)
Chloroform	Merck (Darmstadt, Germany
cOmplete EDTA-free protease inhibitor Cocktail Tablets	Sigma-Aldrich (Taufkirchen, Germany)
CRID3 (CP-456773-02, MCCP90)	Pfizer (New York City, NY, USA)
Cutasept® F Haut-Desinfiziens	Hartmann (Heidenheim an der Benz, Germany)
Cytochalasin D	Sigma-Aldrich (Taufkirchen, Germany)
D-(+)-Glucose powder	Sigma-Aldrich (Taufkirchen, Germany)
D-(+)-Glucose solution	Sigma-Aldrich (Taufkirchen, Germany)
Di-Sodium hydrogen phosphate (Na_2HPO_4)	Sigma- Aldrich (Taufkirchen, Germany)
Dimethyl sulfoxide (DMSO), cell culture grade	PAN-Biotech GmbH (Aidenbach, Germany)
dNTP Mix (10 nM each)	ThermoFisher Scientific (Waltham, MA, USA)
Epidermal growth factor (EGF)	Sigma-Aldrich (Taufkirchen, Germany)
Ethanol (EtOH) 99% absolute	AppliChem (Darmstadt, Germany)
FcR Blocking Reagent, human	Miltenyi Biotec (Bergisch Gladbach, Germany)
FcR Blocking Reagent, mouse	Miltenyi Biotec (Bergisch Gladbach, Germany)
Ficoll® Paque PLUS endotoxin tested	Sigma-Aldrich (Taufkirchen, Germany)
Fluoro-carbonyl cyanide phenylhydrazone (FCCP)	Tocris Bioscience (Bristol, United Kingdom)
Gibco™ RANTES recombinant human protein	ThermoFisher Scientific (Waltham, MA, USA)
Glycerol	Merck (Darmstadt, Germany)
Hoechst 34580	ThermoFisher Scientific (Waltham, MA, USA)
Human Met-RANTES trifluoroacetate salt	Bachem Holding AG (Bubendorf BL, Switzerland)

Invitrogen™ Alexa Fluor™ 647 Antibody labeling kit	ThermoFisher Scientific (Waltham, MA, USA)
Invitrogen™ Dithiothreitol (DTT)	ThermoFisher Scientific (Waltham, MA, USA)
Invitrogen™ eBioscience™ DRAQ5™	ThermoFisher Scientific (Waltham, MA, USA)
Invitrogen™ Nigericin, free acid	ThermoFisher Scientific (Waltham, MA, USA)
Invitrogen™ OneComp eBeads™ Compensation Beads	ThermoFisher Scientific (Waltham, MA, USA)
Invitrogen™ SuperScript® III reverse transcriptase	ThermoFisher Scientific (Waltham, MA, USA)
Invitrogen™ Wheat Germ Agglutinin (WGA) Alexa Fluor 555	ThermoFisher Scientific (Waltham, MA, USA)
LPS-EB Ultrapure from E.coli O111:B4	InvivoGen (Toulouse, France)
MACS CD14 monocyte isolation	Miltenyi Biotec (Bergisch Gladbach, Germany)
Magnesium chloride ($MgCl_2$) solution	Sigma- Aldrich (Taufkirchen, Germany)
Methanol	Merck (Darmstadt, Germany)
Oligo(dT)18 primer	ThermoFisher Scientific (Waltham, MA, USA)
Oligomycin	Tocris Bioscience (Bristol, United Kingdom)
PAF-AH human	Sigma-Aldrich (Taufkirchen, Germany)
PageRuler™ Plus Prestained Protein Ladder, 10 to 250kDa	ThermoFisher Scientific (Waltham, MA, USA)
Pam3SCK4	InvivoGen (Toulouse, France)
PDGF-BB	Biolegend (San Diego, CA, USA)
PhosSTOP Easypack Phasphatase Inhibitor Cocktail Tablets	Sigma-Aldrich (Taufkirchen, Germany)
Piperazine-1,4-bis(2ethanesulfonic acid) (PIPES)	Sigma- Aldrich (Taufkirchen, Germany)
Poly-L-Lysine solution	Sigma-Aldrich (Taufkirchen, Germany)
Potassium chloride (KCl)	Merck (Darmstadt, Germany)
LFn-PrgI fusion protein	Gift from Prof. Matthias Geyer and Dr. David Fußhöller (Institute of Structural Biology, University of Bonn, Germany)
Prostaglandin E1	Sigma-Aldrich (Taufkirchen, Germany)
R848 (Resiquimod)	InvivoGen (Toulouse, France)

Recombinant human CD40L / CD154, soluble	Enzo Life Sciences Inc. (Farmingdale, NY, USA)
Recombinant human GRO-alpha / MGSA (CXCL1)	PeproTech (Rocky Hills, NJ, USA)
Recombinant human NAP-1 (CXCL7)	PeproTech (Rocky Hills, NJ, USA)
Recombinant human P-selectin	R&D systems (Minneapolis, MN, USA)
Recombinant human PF4 (CXCL4)	PeproTech (Rocky Hills, NJ, USA)
Recombinant human PLGF-1 (PIGF-1)	ThermoFisher Scientific (Waltham, MA, USA)
Recombinant human SDF-alpha (CXCL12)	PeproTech (Rocky Hills, NJ, USA)
Recombinant human VEGF	ThermoFisher Scientific (Waltham, MA, USA)
Rotenone	Sigma-Aldrich (Taufkirchen, Germany)
Sodium chloride (NaCl)	Sigma- Aldrich (Taufkirchen, Germany)
Sodium deoxycholate	Sigma- Aldrich (Taufkirchen, Germany)
Sodium dodecyl sulfate (SDS)	Sigma- Aldrich (Taufkirchen, Germany)
Sodium hydroxide (NaOH)	Sigma- Aldrich (Taufkirchen, Germany)
TAK242 (Resatorvid)	InvivoGen (Toulouse, France)
Thrombin from human plasma	Sigma-Aldrich (Taufkirchen, Germany)
Triton X-100	Carl Roth (Karlsruhe, Germany)
Tween® 20	Sigma-Aldrich (Taufkirchen, Germany)
UK5099	Biomol GmbH (Hamburg, Germany)
Zileuton	Tocris Bioscience (Bristol, United Kingdom)

3.1.3 Buffers and solutions

3.1.3.1 Western Blot

NuPAGE® LDS sample Buffer, 4x	ThermoFisher Scientific (Waltham, MA, USA)
NuPAGE® MOPS SDS Running Buffer, 20x	ThermoFisher Scientific (Waltham, MA, USA)
NuPAGE® Sample Reducing Agent, 10x	ThermoFisher Scientific (Waltham, MA, USA)
Permeabilization buffer	PBS with 2% (v/v) FCS and 0.5% (v/v) Triton X-100

Pierce® Tris-Glycine buffer, 10x	ThermoFisher Scientific (Waltham, MA, USA)
RIPA lysis buffer	1x RIPA, 1x PhosSTOP cocktail, 1x cOmplete protease inhibitor
RIPA, 2x	20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate in distilled water. The buffer is adjusted to pH 7.4 and sterile filtered.
TBS-T 0.1%	1x TBS; 0.1% (v/v) Tween® 20
Transfer buffer	1x Tris-Glycine buffer, 15% (v/v) methanol
Tris Buffered Saline (TBS), 20x	ThermoFisher Scientific (Waltham, MA, USA)

3.1.3.2 Tissue culture

Calcium free medium	125 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM Glucose, 10 mM HEPES, adjust to pH 7.4 with 1N NaOH
Complete DMEM	10% (v/v) FCS and 1% (v/v) Penicillin-Streptomycin (10.000 U/ml) in DMEM
Complete RPMI	10% (v/v) FCS and 1% (v/v) Penicillin-Streptomycin (10.000 U/ml) and 1% (v/v) Glutamax in RPMI
Flow cytometry buffer	PBS with 2% (v/v) FCS
PIPES/saline/glucose (PSG) buffer	5 mM PIPES, 145 mM NaCl, 4 mM KCl, 50 µM Na_2HPO_4, 1 mM $MgCl_2$ $6H_2O$, 5.5 mM Glucose
Trypan blue solution (0.4%)	Sigma-Aldrich (Taufkirchen, Germany)
Trypsin-EDTA (0.05%)	ThermoFisher Scientific (Waltham, MA, USA)

3.1.3.3 Others

Formaldehyde solution 37 wt % in H_2O, 10-15% methanol as stabilizer	Sigma-Aldrich (Taufkirchen, Germany)
Invitrogen™ UltraPure™ 0.5M EDTA, pH 8.0	ThermoFisher Scientific (Waltham, MA, USA)
Nuclease-free water	ThermoFisher Scientific (Waltham, MA, USA)

Phosphate buffered saline (PBS), 10x	PAN-Biotech GmbH (Aidenbach, Germany)
Rnase-free water	Qiagen (Hilden, Germany)
Tris-HCl, pH 7.4 (1M)	Sigma- Aldrich (Taufkirchen, Germany)

3.1.4 Cell culture and supplements

Dulbecco's Modified Eagle Medium (DMEM), high glucose	ThermoFisher Scientific (Waltham, MA, USA)
Fetal calf serum (FCS)	ThermoFisher Scientific (Waltham, MA, USA)
Gibco™ Dulbecco's Phosphate-buffered saline (DPBS)	ThermoFisher Scientific (Waltham, MA, USA)
Gibco™ GlutaMAX™ (100X)	ThermoFisher Scientific (Waltham, MA, USA)
Gibco™ Penicillin-Streptomycin (10.000 U/ml)	ThermoFisher Scientific (Waltham, MA, USA)
Gibco™ RPMI 1640 Medium	ThermoFisher Scientific (Waltham, MA, USA)
Gibco™ Sodium pyruvate 100 mM	ThermoFisher Scientific (Waltham, MA, USA)
L929	Produced by Gudrun Engels (Institute of Innate Immunity, University Hospital Bonn)
Recombinant human (rh) GM-CSF	Immunotools (Friesoythe, Germany)
RPMI 1640 Medium, non-buffered, glucose-free, with L-glutamine and sodium bicarbonate	Sigma- Aldrich (Taufkirchen, Germany)

3.1.5 Commercial reagent sets (kits)

Caspase-Glo® 1 Inflammasome Assay	Promega (Fitchburg, WI, USA)
Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex™ Panel 1	ThermoFisher Scientific (Waltham, MA, USA)
EasySep™ Direct Human Neutrophil Isolation Kit	STEMCELL Technologies (Vancouver, BC, Canada)
EasySep™ Human Monocyte Isolation Kit	STEMCELL Technologies (Vancouver, BC, Canada)
HTRF® Human TNF alpha kit	Cisbio (Codolet, France)
HTRF® Human IL-1 beta kit	Cisbio (Codolet, France)

Immuno-Oncology Checkpoint 14-Plex Human ProcartaPlex™ Panel 1	ThermoFisher Scientific (Waltham, MA, USA)
Maxima™ SYBR Green/ROX qPCR Master Mix	ThermoFisher Scientific (Waltham, MA, USA)
Pierce™ BCA Protein Assay Kit	ThermoFisher Scientific (Waltham, MA, USA)
Pierce™ LDH cytotoxicity assay kit	ThermoFisher Scientific (Waltham, MA, USA)
RNase-Free DNase Set	Qiagen (Hilden, Germany)
RNeasy® Mini Kit, Part 1	Qiagen (Hilden, Germany)

3.1.6 Antibodies

Antibodies used in this work are listed in tables 3.1 and 3.2 according to their applications.

Table 3.1 List of antibodies for flow cytometric and microscopic analysis

Antibody	Company	Clone	Dilution
Anti-human CD14-APC	ThermoFisher Scientific	61D3	1:5
Anti-human CD41a FITC	ThermoFisher Scientific (Waltham, MA, USA)	HIP8	1:5
Anti-human CD45 PE	ThermoFisher Scientific	2D1	1:5
Anti-human/mouse CD62p (P-selectin) APC	ThermoFisher Scientific	Psel.KO.2.3	1:5
Anti-mouse CD41 eFluor450	ThermoFisher Scientific	eBioMWReg30	1:5
Anti-mouse CD45 PE	ThermoFisher Scientific	30-F11	1:5
Anti-mouse Ly6G-AF488	ThermoFisher Scientific	RB6-8C5	1:5
Anti-mouse Ly6G-APC	ThermoFisher Scientific	1A8-ly6g	1:5
Mouse IgG1 K Iso Control APC	ThermoFisher Scientific	P3.6.2.8.1	1:5
Mouse IgG1 K Iso Control FITC	ThermoFisher Scientific	P3.6.2.8.1	1:5
Mouse IgG1 K Iso Control PE	ThermoFisher Scientific	P3.6.2.8.1	1:5
Rat Anti-mouse CD14-FITC	Biolegend (San Diego, CA, USA)	Sa14-2	1:5
Rat IgG1 K Iso Control eFluor450	ThermoFisher Scientific	eBRG1	1:5
Rat IgG2a K Iso Control APC	ThermoFisher Scientific	eBR2a	1:5

Rat IgG2b, K Iso Control PE	ThermoFisher Scientific	eB149/10H5	1:5

Table 3.2 List of antibodies used for western blot analysis

Antibody	Company	Clone	Dilution
Anti-mouse IRDye 680	Li-Cor Biosciences	-	1:25000
Anti-NLRP3/NALP3, mAb mouse	Adipogen International (Liestal, Switzerland)	Cryo-2	1:2000
Anti-rabbit IRDye 800	Li-Cor Biosciences	-	1:25000
beta-Actin rabbit Antibody	Li-Cor Biosciences (Lincoln, NE, USA)	-	1:5000
Human IL-1 beta/IL-1F2 Antibody, goat	R&D systems (Minneapolis, MN, USA)	-	1:1000
Purified anti-human CD42b Antibody, mouse	Biolegend (San Diego, CA, USA)	HIP1	1:200

Table 3.3 List of other antibodies used in this work

Antibody	Company	Clone	Dilution
Anti human NAP2 (CXCL7)	PeproTech (Rocky Hills, NJ, USA)	-	-
Human Anti CXCL12 / SDF-1α	R&D systems (Minneapolis, MN, USA)	-	-
Normal Goat IgG control	R&D systems	-	-
Purified anti-ASC (TMS-1) Antibody	Biolegend (San Diego, CA, USA)	HASC-71	-
Purified Mouse IgG, k Isotype	Biolegend (San Diego, CA, USA)	MG1-45	-

Purified anti-ASC (TMS-1) and the respective isotype control (purified mouse IgG) were directly stained with the Invitrogen™ Alexa Fluor™ 647 Antibody labeling kit according to the manufacturer's instructions (ThermoFisher Scientific) to yield directly labelled anti-ASC-647 and mouse-IgG-647 (stock: 0.5 mg/ml).

3.1.7 Primers for quantitative PCR

Primers used for quantitative real-time PCR (qPCR) were designed to amplify 100 to 150 base pairs of the target gene and to cross exon boundaries to avoid amplification of genomic DNA. Primers (desalted) were obtained from Metabion (Martinsried) and resuspended in nuclease-free water to get a concentration of 100 µM.

Table 3.4 List of qPCR primers used for the amplification of human genes

Gene name	Forward primer (5' to 3')	Reverse primer (5' – 3')
ACTB	fw ccaccatgtaccctggcatt	rv cggagtacttgcgctcagga
CASP1	fw acaacccagctatgcccaca	rv gtgcggcttgacttgtccat
CD14	fw gagctcagaggttcggaaga	rv cttcatcgtccagctcacaa
GP1BA	fw ctgctctttgcctctgtggt	rv ctccaggtgtgtggtttgtg
IL1B	fw tgggcagactcaaattccagct	rv ctgtacctgtcctgcgtgttga
NLRP3	fw tcggagacaaggggatcaaa	rv agcagcagtgtgacgtgagg
PF4	fw ctgaagaagatggggacctg	rv gtggctatcagttgggcagt
PYCARD	fw gagctcaccgctaacgtgct	rv actgaggaggggcctggat

3.1.8 Cells

3.1.8.1 Immortalized macrophage cell line

The NLRP3 overexpressing immortalized mouse macrophage (inflammasome reporter iMacs) cell line was generated from immortalized NLRP3-deficient murine macrophages, that were reconstituted with NLRP3-FLAG and transduced with ASC-mCerulean in our institute as described by Stutz and colleagues[131].

3.1.8.2 Primary BMDMs

Primary BMDMs were generated as described in section 3.2.4.2. Unless stated otherwise, bone marrow cells were isolated from WT C57BL/6 mice and BMDMs were generated using L929 supernatants.

3.1.9 Mice

Wild-type C57BL/6 mice, the inflammasome reporter mice ASC-mCitrine Tg (B6.Cg-Gt(ROSA)26Sortm1.1(CAG-Pycard/mCitrine*,-CD2*)Dtg/J), the *Il1r1*$^{-/-}$ (B6.129S7-

Il1r1tm1Imx/J) and the *Il18r1*$^{-/-}$(B6.129P2-Il18r1tm1Aki/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The *Nbeal2*$^{-/-}$ mice were kindly provided by Prof. Bernhard Nieswandt (University of Würzburg, Würzburg, Germany). The ASC mCherry knock in mice were generated by Prof. Paul Herzog (Monash University, Australia) and a gift to our institute.

3.2 Methods

3.2.1 Cell culture conditions

All cell lines and primary cells were cultured at 37°C with 5% CO_2 in a humidified atmosphere. Cell culture work was always performed in tissue culture hoods using pyrogen-free and sterile consumables and reagents.

Nlrp3$^{-/-}$ immortalized murine macrophages overexpressing mouse Nlrp3 and human ASC-mCerulean (Inflammasome reporter iMacs) were cultured as monolayers in DMEM supplemented with 10% (v/v) FCS and 1% (v/v) Penicillin-Streptomycin (10.000 U/ml) (complete DMEM) in cell culture flasks. Depending on the actual growth of the cells, the cells were passaged every second or third day. For detachment, the cells were first washed with phosphate-buffered saline (PBS) before they were incubated with trypsin-EDTA for 3 minutes at 37°C and neutralized in complete DMEM afterwards. Cells are centrifuged (350 x g, 5 minutes) before the pellet is resuspended in fresh complete DMEM for counting. Approximately 3 x 10^6 cells are seeded in 14 ml in a T75 tissue culture flask.

A Neubauer cell counting chamber was used to determine the cell number manually. To this end, cells were diluted appropriately in trypan blue solution, which selectively colors dead cells. Using a light microscope, only the unstained cells were counted inside the four big outer squares of the Neubauer chamber and the cell number was calculated:

$$\frac{Cell\ number}{ml} = \frac{Cell\ count\ in\ all\ 4\ squares}{4} * dilution\ factor * 10^4$$

3.2.2 Generation of human primary macrophages (hMDMs)

Buffy coats from healthy donors were obtained according to protocols accepted by the institutional review board at the University of Bonn (local ethics votes Lfd. Nr. 075/14). Primary human macrophages were generated through differentiation of $CD14^+$monocytes in medium complemented with 500 U/mL rhGM-CSF for 3 days. In brief, human peripheral blood mononuclear cells (PBMCs) were generated from buffy coats of healthy donors by density gradient centrifugation in Ficoll® Paque PLUS (700 x g, 20 minutes). PBMCs were incubated at 4°C with magnetic microbeads conjugated to monoclonal anti-human CD14 antibodies according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). Thereby, $CD14^+$monocytes were magnetically labeled and then isolated using a MACS column (Miltenyi Biotech, Bergisch Gladbach, Germany) placed in a magnetic field. $CD14^+$monocytes were cultivated in complete RPMI (10% (v/v) FCS and 1% (v/v) Penicillin-

Streptomycin (10.000 U/ml) and 1% (v/v) Glutamax in RPMI) complemented with 1% Sodium Pyruvate and 500 U/mL rhGM-CSF at a concentration of $2x10^6$/mL in 6-well plates to generate monocyte-derived macrophages. Macrophages were harvested at day 3 by scraping and pelleted by centrifugation (350 x g, 5 minutes). The cells were seeded in 100 µl at a concentration of 1 x 10^5 /well in complete RPMI medium complemented with 1% Sodium Pyruvate and 125 U/mL rhGM-CSF in 96-well flat-bottom plates and incubated overnight for experiments on the next day.

3.2.3 Human blood cell isolations

Peripheral blood was obtained by venipuncture of healthy volunteers after signature of informed consent, and approval of the study by the Ethics Committee of the University of Bonn (Protocol# 282/17), and in accordance with the Declaration of Helsinki.
For the study of platelets in cancer in collaboration with the Prof. Barbara Wollenberg's group in Lübeck (Universitätsklinikum Schleswig-Holstein, Germany), the ethics committee of the University of Lübeck (Az16-278/2017) approved the study[132]. All enrolled patients signed an informed written consent after being educated about the study and the use of their blood samples. In this study, blood from healthy donors (n=8), healthy smokers (n=12), head and neck squamous cell carcinoma (HNSCC) patients (HNSCC stage I: 4; stage II: 2; stage III: 7; stage IV: 17) and lung cancer patients (stage IV: 4) was collected. Patient selection was random in the time from July to December 2017[132].

3.2.3.1 Neutrophil isolation from human blood

Venous blood was collected in S-Monovette®K3EDTA tubes and neutrophils were isolated using the EasySep™Direct Human Neutrophil Isolation Kit according to the manufacturer instructions (STEMCELL Technologies, Vancouver, BC, Canada). In brief, whole blood was incubated with the Neutrophil Isolation Cocktail and RapidSpheres™ (5 minutes) and diluted with neutrophil isolation buffer. After incubation in the EasySep™ Magnet (STEMCELL Technologies, Vancouver, BC, Canada) (5 minutes), the enriched cell suspension was poured into a new tube and incubated again with RapidSpheres™ (5 minutes), followed by a second, and third round of magnetic separation. The isolated neutrophils were pelleted by centrifugation (350 x g, 5 minutes) and resuspended in complete RPMI medium. Cells were seeded in 100 µl at a concentration of 1 x 10^5/well in a 96-well round-bottom plate for immediate use. The purity of the purified neutrophils was assessed by flow cytometry.

Neutrophil experiments were performed by Salie Maasewerd (Institute of Innate Immunity, University Hospital Bonn), a Bachelor student under my supervision.

3.2.3.2 Platelet isolation from human blood

Human platelets were isolated as previously described by Alard et al. with slight modifications optimized by myself (Fig. 3.1)[97].

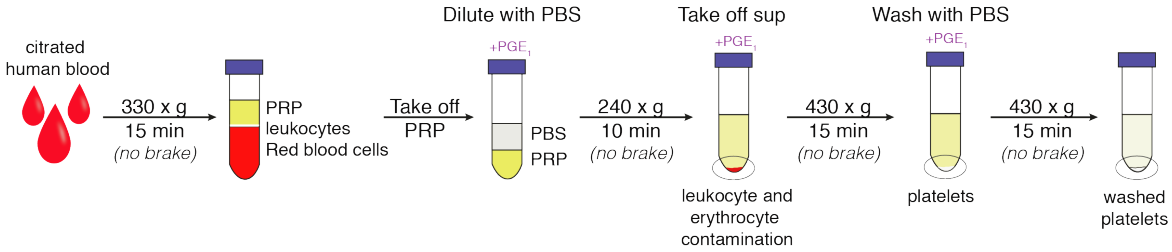

Figure 3.1 Schematic representation of platelet isolation protocol from citrated human blood. The protocol was adapted from Alard et al. [97]. PRP: Platelet-rich plasma, PGE_1: Prostaglandin E_1

In brief, venous blood was drawn into S-Monovette®9NC collection tubes. The blood was centrifuged (330 x g, 15 minutes) without brake to obtain platelet-rich plasma (PRP). All following centrifugation steps were performed without brake and in the presence of 200 nM PGE_1 to inhibit platelet activation. PRP was transferred to a new tube and diluted 1:1 with PBS and centrifuged (240 x g, 10 minutes) to reduce leukocyte contamination. Platelets were pelleted by centrifugation (430 x g, 15 minutes) and washed once with 10 ml PBS. After centrifugation (430 x g, 15 minutes) platelets were resuspended in RPMI medium to a concentration of 5 x 10^7/ml unless otherwise indicated or lysed in RIPA lysis buffer for protein assessment. Purity and viability of the prepared platelets were assessed by flow cytometry.

3.2.3.3 PBMC isolation for cancer study

PBMCs were isolated from the same blood as platelets. After taking off PRP the remaining blood was layered on Ficoll® Paque PLUS and the PBMC fraction was taken off after density gradient centrifugation (700 x g, 20 minutes). PBMCs were washed twice afterwards with PBS, before they were either used for flow cytometric analysis or lysed in RIPA lysis buffer.

3.2.3.4 Monocyte isolation from human blood

Venous blood was collected in S-Monovette®K3EDTA tubes and PBMCs were obtained by density gradient centrifugation in Ficoll® Paque PLUS (700 x g, 20 minutes). Monocytes were

isolated from PBMCs using the EasySep™ Human Monocyte Isolation Kit according to the manufacturer instructions (STEMCELL Technologies, Vancouver, BC, Canada). In brief, PBMCs were washed twice with PBS complemented with 2% FCS and 1mM EDTA before they were incubated with the supplied monocyte isolation cocktail and in case of platelet depletion with the platelet removal cocktail for 5 minutes each. Magnetic beads were added to this suspension for another 5 minutes before magnetic separation in a EasySep™ Magnet. After 2.5 minutes, the enriched suspension was poured into a new tube. The isolated monocytes were centrifuged (350 x g, 5 minutes) and resuspended in RPMI medium. Cells were seeded in 100 µl at a concentration of $1x10^5$/well in a 96-well flat-bottom plate for immediate use. Monocyte enrichment and platelet depletion was assessed by flow cytometry.

3.2.4 Mice

Mice were housed under standard conditions at 22°C and a 12 h light-dark cycle with free access to food and water until they were used for experiments. Maximilian Rothe (Institute of Innate Immunity, University Hospital Bonn) helped with animal work in this thesis and performed blood drawing from anaesthetized mice.

3.2.4.1 Platelet isolation from murine blood

Blood was drawn by puncturing the vena facialis of anaesthetized WT or Nbeal2 KO mice. Blood from mice of the same genotype were pooled in a sterile 5 mL polystyrene tube containing one-sixth blood volume of pre-warmed acid-citrate-dextrose solution (ACD). PRP was prepared by centrifugation (330 x g, 5 minutes) without brake. All following centrifugation steps were performed without brake and in the presence of PGE_1. PRP was transferred to a new tube and diluted in twice as much volume of PSG buffer with the final concentration of 1.5 µM PGE_1. The suspension was centrifuged (240 x g, 10 minutes) to reduce leukocyte and erythrocyte contamination. The supernatant was transferred into a tube with PGE_1 in a final concentration of 0.7 µM in PSG buffer. The platelets were pelleted by centrifugation (1000 x g, 5 minutes) and washed once with 1.5 µM PGE_1 in PSG buffer. The washed platelets were resuspended in DMEM to a concentration of 5 x 10^6/ml unless otherwise indicated. Purity and viability of the prepared platelets were assessed by flow cytometry.

3.2.4.2 Generation of murine bone marrow derived macrophages (BMDMs)

WT, *Il1r1* KO or *Il18r1* KO mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Femur and tibia from hind limbs were removed and the bones were briefly disinfected with 70% ethanol. The bone marrow cavity was flushed with PBS and the cell suspension was filtered through a 70 µm cell strainer before centrifugation (400 x g, 5 minutes). Cells were resuspended in complete DMEM supplemented with 20% L929 supernatant and cultured for 6 days in T175 tissue culture flasks to differentiate into macrophages (BMDMs). On day six, cells were harvested using cold PBS containing 5 mM EDTA and 2% FCS and scraping. After centrifugation (350 x g, 5 minutes), the BMDM were seeded in 100 µl at a concentration of 1 x 10^5/well in complete DMEM with 20% L929 supernatant in flat-bottom 96-well plates and incubated overnight for experiments on the next day. Experiments with *Il1r1* KO mice and the addition of platelets to BMDMs were performed by Lisa Böttcher (Institute of Innate Immunity, University Hospital Bonn) as indicated.

3.2.5 Flow cytometry

3.2.5.1 Purity assessment

Samples of isolated human and murine platelets, PBMCs and standard or platelet-depleted monocytes were analyzed for purity and/or platelet pre-activation and platelet viability after each experiment.

Isolated murine or human platelets were activated with 0.5 or 0.1 U/mL thrombin respectively for 30 min at 37°C to assess platelet activatability. Before staining, all cells were blocked with 1:10 mouse or human FcR blocking reagent for 10 minutes at room temperature (RT). The platelet samples were stained with fluorochrome-conjugated monoclonal anti-mouse or anti-human Ig antibodies against CD41 (platelet marker), CD62p (activation marker) and CD45 (leukocyte and thus contamination marker) (1:5 dilution each) for 30 minutes in the dark at RT (table 3.1).

Isolated standard or platelet-depleted monocytes were stained with fluorochrome-conjugated anti-human Ig antibodies against CD14 (monocyte marker) and CD41 (platelet marker) (1:5 dilution each) to assess monocyte enrichment and platelet depletion after the isolation (table 3.1).

All cells were washed and resuspended in flow cytometry buffer before flow cytometry analysis. Compensation beads (OneComp eBeads) and isotype controls were stained in the

same way. Flow cytometry was performed with a MacsQuant® Analyzer10 or MacsQuant® VYB (both, Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed using FlowJo vX10.1.16 (Tree Star, Ashland, USA). For flow cytometry performed in Lübeck, a FACS Canto A flow cytometer (BD Biosciences, Heidelberg, Germany) was used and the results were analyzed using FACS Diva software 6.0 (BD Biosciences, Heidelberg, Germany) and FlowJo software. The gating strategy was based on doublet discrimination and isotype-matched control antibodies or unstained controls.

3.2.5.2 Whole bone marrow cell staining

WT, ASC-mCitine or ASC-mCherry reporter mice were anesthetized with isoflurane and sacrificed by cervical dislocation. As described before femur and tibia from hind limbs were removed and the bones were briefly disinfected with 70% ethanol. The bone marrow cavity was flushed with PBS and the cell suspension was filtered through a 70 µm cell strainer before centrifugation (400 x g, 5 minutes). Cells were resuspended in complete DMEM and blocked with 1:10 mouse FcR blocking reagent for 10 minutes at RT before staining. Afterwards, fluorochrome-conjugated anti-mouse Ig antibodies against CD41 (platelet marker), Ly6G (neutrophil marker), CD14 (monocyte marker) or CD45 (leukocyte marker) (1:5 dilution each) were added to the samples for 30 minutes in the dark at RT. All cells were washed and resuspended in flow cytometry buffer before flow cytometry analysis. Compensation beads (OneComp eBeads) and isotype controls were prepared in the same way.
Flow cytometry was performed MacsQuant® VYB and analyzed using FlowJo. The gating strategy was based on doublet discrimination and isotype-matched control antibodies or unstained controls.

3.2.6 Generation of human platelet supernatants

Human platelets were isolated from peripheral blood as described before (section 3.2.3.2). The platelet suspension was adjusted to a concentration of $5x10^7$/ml in RPMI afterwards. Platelets were transferred to Eppendorf tubes (1 ml each) and stimulated with 200 ng/ml LPS, 1 U/ml Thrombin or were left untreated. After three hours of incubation at 37°C, platelets were pelleted by centrifugation (3000 x g, 10 minutes). Then, the supernatant was transferred into new Eppendorf tubes without disturbing the platelet pellet and frozen at -80°C until further use.

3.2.7 Inflammasome stimulation assays

BMDMs and hMDMs were seeded at 1 x 10^5/well, iMacs at 0.5 x10^5/well into flat bottom 96-well plates one day prior to the experiment. BMDMs were cultured in DMEM supplemented with 20% L929, hMDMs in complete RPMI medium complemented with 1% Sodium Pyruvate and 125 U/mL rhGM-CSF and iMacs in complete DMEM. Monocytes and Neutrophils were seeded at 1 x 10^5/well into flat bottom 96-well plates after their isolation on the day of the experiment. Before the start of the stimulation, plates were centrifuged (500 x g, 5 minutes) and the supernatant was replaced by 100 µl serum-free DMEM (for BMDMs and iMacs) or RPMI (for human neutrophils, monocytes and hMDMs) as medium control or by human or murine platelet suspensions (5 x 10^7/ml) as indicated.

3.2.7.1 NLRP3 stimulation assay

For NLRP3 stimulation, cells were primed with 200 ng/mL LPS (TLR 4 agonist) for 3 hours and activated with 10 µM Nigericin or 5 mM ATP for 90 minutes at 37ºC unless otherwise indicated. R848 (TLR7/8 agonist) and Pam3CSK4 (TLR1/2 agonist) were used in some experiments for priming at a concentration of 10 µM and 1 µg/ml respectively for the same time period as LPS. Human monocytes were primed with 2 ng/ml before being activated with 10 µM Nigericin for 90 min at 37ºC.
In some experiments, the NLRP3 inhibitor CRID3 was used at 2.5 µM. Cells were treated with CRID3 30 minutes before addition of Nigericin.
After centrifugation (500 x g, 5 minutes) cell-free supernatants were collected and frozen at -20°C until cytokine measurement by HTRF® or ProcartaPlex™ multiplex immunoassays.

3.2.7.2 NLRC4 stimulation assay

For NLRC4 stimulation, cells were primed with 200 ng/mL LPS for 3 hours before addition of 2 µg/mL LFn-PrgI and 0.5 µg/mL PA for 2 hours at 37°C. After stimulation, cells were centrifuged (500 x g, 5 minutes) and supernatants were collected to measure cytokine levels by HTRF®.

3.2.8 Cytochalasin D treatment

To inhibit actin polymerization, hMDMs were treated with Cytochalasin D. HMDMs were treated with 5 µM Cytochalasin D for 30 minutes prior to the start of the inflammasome assay. Afterwards cells were centrifuged (500 x g, 5 minutes) and either platelets or RPMI medium

was added to hMDMs as described in section 3.2.7. Additionally, hMDMs were also seeded for confocal analysis at a concentration of 2 x 10^5/well in 8-well IBIDI µ-slide and the cells were treated the same way.
Afterwards the NLRP3 inflammasome was stimulated with LPS and Nigericin as described in section 3.2.7.1. After the inflammasome assay, cells were centrifuged (500 x g, 5 minutes) and the supernatants were transferred to a new 96-well plate for further HTRF® analysis.
Cells were stained for confocal analysis after the assay with fluorescently labelled antibody against CD41 (1:5 dilution), the membrane dye WGA Alexa Fluor 555 (5 µg/ml) and the DNA binding dye draq5 (1:5000) in PBS for 20 minutes in the dark at RT. Afterwards, cells were washed twice and fixed with 4% paraformaldehyde for 10 minutes on ice. After washing cells with PBS twice, they were imaged using a Leica TCS SP5 SMD confocal microscope (Leica Microsystems, Wetzlar, Germany). Images were acquired by Prof. Bernardo Franklin (Institute of Innate Immunity, University Hospital Bonn) using a 63X objective, with a numerical aperture of 1.2, and analyzed using Volocity 6.01 software (PerkinElmer, Waltham, U.S.A.).

3.2.9 Transwell assay

HMDMs were seeded at a concentration of 1 x 10^5/100 µl in HTS Transwell 96-well plates on day prior to the experiment. The transwell plates contained pores of 0.4 µm to inhibit platelet migration into the lower chamber but allow free movement of smaller, secreted molecules. Platelets were added to the upper wells at a concentration of 6.25 x 10^7 in 80 µl. A platelet only and a medium control was included in the assay to control for molecules secreted by either platelets or macrophages alone. After addition of platelets, the NLRP3 inflammasome assay was carried out as described before (section 3.2.7.1) and cell-free supernatants were analyzed by HTRF® afterwards.

3.2.10 Compound addition to hMDMs

Recombinant human proteins were added to hMDMs in PRMI before the start of the NLRP3 inflammasome assay. The following concentrations of recombinant human proteins were added: rhCD40L (10 ng/ml), rhCXCL12 (1 ng/ml), rhRANTES (300 pg/ml), rhPF4 (50 ng/ml), BDNF (5 ng/ml), rhPDGF-BB (500 pg/ml), rhP-selectin (40 ng/ml), rhCXCL1 (100 pg/ml), rhEGF (200 pg/ml), rhVEGF-α (200 pg/ml), rhPIGF (80 pg/ml), rhCXCL7 (1 ng/ml, 10 ng/ml, 100 ng/ml, 1 µg/ml).

Similarly, different concentrations of ADP (0.3 µM, 3 µM or 30 µM) or calcium chloride (100-1200 µM as indicated) were added directly to hMDMs to test their involvement in NLRP3 inflammasome regulation. ADP was added to hMDMs in RPMI, but calcium addition was performed in calcium-free medium, so that no external calcium was present.
After addition of the described compounds, hMDMs were stimulated with LPS and Nigericin as described before in section 3.2.7.1 and cell-free supernatants were analyzed by HTRF® afterwards.

3.2.11 Addition of inhibitors and antibodies to hMDMs

To inhibit TLR4 signaling, the small-molecule inhibitor Resatorvid (TAK242) was used. TAK242 was added to hMDMs at a concentration of 0.5 µg/ml 5 minutes before the start of the inflammasome assay. Cells were then stimulated with the TLR7/8 agonist R848 for priming as described before (section 3.2.7.1.)
To block the cytokine CXCL12, an antibody against the human CXCL12 (anti-CXCL12) or the matching IgG isotype control were added at 10 µg/ml to the hMDMs cultured alone or in the presence of platelets before the NLRP3 inflammasome assay (table 3.3) . Similarly, an antibody against CXCL7 was added to hMDMs cultured with or without platelets before the inflammasome assay at a concentration of either 4 and 20 µg/ml as indicated.
To inhibit RANTES signaling via its receptor CCR5, hMDMs were incubated with the 10 nM of the CCR5 agonist met-RANTES for 1.5 hours prior to platelet addition and the start of the inflammasome assay. Treatment with met-RANTES was shown to internalize CCR5 similar to RANTES addition[133].
The COX1/2 and LOX inhibition experiments were carried out by Dr. Lucas Ribeiro (Institute of Innate Immunity, University Bonn). Platelets were incubated with 100 µM of either acetylsalicylic acid (aspirin), or zileuton for 60 min at 37ºC. The treated platelets were centrifuged (430 x g, 15 minutes) and added to hMDMs before the start of the inflammasome assay. Also 100 µM of either aspirin or zileuton was added to hMDMs directly as a control.
Then, stimulation of hMDMs with LPS and Nigericin after inhibitor and platelet addition was performed as described before (section 3.2.7.1) and cell-free supernatants were analyzed by HTRF® afterwards.

3.2.12 Calcium chelation experiment

The calcium chelator BAPTA or the cell-permeable calcium chelator BAPTA AM were used to examine extracellular and intracellular calcium involvement respectively. hMDMs were

seeded in RPMI medium (for BAPTA AM treatment) or in calcium-free medium (for BAPTA treatment) with or without platelets as described before (section 3.2.7). Cells were primed with 200 ng/ml LPS for 3 hours before the addition of either 0.5 mM BAPTA or 5 µM BAPTA AM. Then, cells were stimulated with 10 µM Nigericin for NLRP3 Inflammasome activation. Cell-free supernatants were analyzed afterwards by HTRF® for cytokine secretion.

3.2.13 Atezolizumab treatment plan

For the cancer study in collaboration with Prof. Barbara Wollenbergs group, four lung cancer patients were treated according to new treatment standards with atezolizumab (trade name Tecentriq). Atezolizumab is a fully humanized, monoclonal antibody with IgG1 isotype against protein programmed cell death-ligand 1 (PD-L1). Patients received 1200 mg i.v. flat dose of atezolizumab every three weeks. Patient recruitment and treatment with atezolizumab was solely performed by Prof. Barbara Wollenberg and her colleagues in Lübeck. No relevant side effects were recorded and the four patients tolerated the clinical therapy very well. IL-1β[132].

3.2.14 Cytokine measurements

3.2.14.1 HTRF®

Commercially available HTRF® (homogeneous time resolved fluorescence) kits were used to quantify human or murine cytokine levels (IL-1β and TNFα) in cell-free cell culture supernatants. The HTRF® kits were used according to the manufacturer's instructions.
In brief, HTRF® assays detect cytokines using FRET (Fluorescence Resonance Energy Transfer) technology in a sandwich assay format. Two different, specific antibodies against the respective cytokine were labelled with FRET dyes: either Cryptate (donor) or XL (acceptor). When labelled antibodies bind to the respective cytokine and get into close proximity, excitation of the donor through a light source elicits a FRET towards the acceptor. Then, the acceptor emits a fluorescent signal at 665 nm (Cisbio, Product Information). For the measurement of IL-6, IL-1β and TNFα levels, the respective antibodies were mixed according to the manufacturer's instructions and pipetted into a 384-shallow well, polypropylene microplate together with the cell-free supernatant. The plate was incubated at RT in the dark for different, cytokine specific time periods according to the manufacturer's instructions. Afterwards, the FRET signal was measured using a SpectraMax® i3 plate reader (Molecular Devices, Sunnyvale, CA, USA). The signal intensity is proportional to the respective

cytokine's concentration and can be used to calculate the concentration of cytokine in the supernatant.

3.2.14.2 ProcartaPlex™ multiplex immunoassay

ProcartaPlex™ multiplex cytokine arrays were used for the detection of an array of human cytokines in cell-free supernatants or cell lysates. ProcartaPlex™ multiplex assays quantify multiple cytokine targets in a single sample. The bead-based assay uses the principles of a sandwich ELISA together with the Luminex® xMAP® technology and is measured on a MAGPIX instrument (Merck, Darmstadt, Germany). The differently dyed, magnetic beads are coated with target-specific antibodies for a quantitative analysis of multiple cytokines in one sample (ThermoFisher Scientific, Product information).
The Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex™ Panel 1 and Immuno-Oncology Checkpoint 14-Plex Human ProcartaPlex™ Panel 1 were used according to the manufacturer's instructions. The Immuno-Oncology Checkpoint 14-Plex was performed on whole cell lysates instead of cell-free supernatants. Therefore, slight modifications to the protocol were necessary. Here, 200 µl of sample and standard were loaded onto the 96-well plate before overnight incubation at 4°C. To this end, the standard stock vial was diluted in 900 µl and a 1:2 dilution series was employed. Results were analyzed with GraphPad Prism Software Version 7.0f (GraphPad Software, San Diego, USA).

3.2.15 Lactate dehydrogenase (LDH) assay

To determine the extracellular lactate dehydrogenase (LDH) in cell-free supernatants, the Pierce LDH cytotoxicity assay was performed. LDH is a cytosolic enzyme that it is released from cells when their cell membrane integrity is compromised in any way. Therefore it can be used as a biomarker for cellular cytotoxicity and cytolysis. The concentration of LDH in cell-free supernatants is quantified by enzymatic reactions, in which LDH catalyzes lactate to pyruvate conversion, which leads to NAD+ reduction into NADH. The enzyme diaphorase then uses NADH to reduce tetrazolium salt into formazon, that can be measured at 490 nm using the Spectramax i3 (ThermoFisher User Guide: Pierce LDH Cytotoxicity Assay). The LDH assays was performed according to the manufacturer's instructions. Control hMDMs cultured at the same conditions as the samples were lysed with the provided Lysis buffer (10x) and served as positive control for maximal LDH release.

3.2.16 Caspase-1 activity assay

Caspase-Glo® 1 Inflammasome Assay was used to selectively measure caspase-1 activity in cell-free supernatants and it was carried out according to the manufacturer's instructions (Promega,Fitchburg, WI, USA). The kit consists of a luminogenic caspase-1 substrate, Z-WEHD-aminoluciferin, in a lytic reagent that enables the detection of catalytically active caspase-1.

Caspase-Glo buffer and Z-WEHD substrate were mixed at RT before the assay. The buffer (25 µl) was added to the same amount of cell-free supernatant in an opaque, white 96-well plate. After mixing and incubation of the plate in the dark at RT, the luminescence signal was read after one hour using a SpectraMax® i3 plate reader.

3.2.17 Reverse transcription quantitative real-time PCR

Reverse transcription quantitative real-time PCR was used to quantify the amount of messenger RNA (mRNA) in a sample. Macrophages, platelets or macrophage-platelet co-cultures were seeded in flat-bottom 96-well plates and stimulated as described in 3.2.7 or as indicated.

3.2.17.1 RNA preparation

After the incubation time, plates were centrifuged (500 x g, 5 minutes) and placed on ice. The supernatants were taken off and 100 µl Buffer RLT (RNeasy® Mini Kit, Part 1) supplemented with 1% (v/v) β-mercaptoethanol was added directly to each well. The supernatants were transferred into new round-bottom 96-well plates and frozen at -80°C for further cytokine analysis. RLT cell lysates were transferred into 1.5 ml tubes and frozen at -80°C until RNA isolation.

Total RNA containing small RNAs was purified using the RNeasy® Mini Kit with additional on-column DNase digestion according to the manufacturer's instructions (Qiagen). Absorbance at 260 nm and 280 nm, indicating the protein contamination, and at 230 nm, indicating the contamination with organic compounds, was measured to determine RNA concentration and purity. Absorbance was quantified using a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

3.2.17.2 cDNA synthesis

Approximately, 0.5 – 1 µg of isolated RNA from each sample was synthesized into complementary DNA (cDNA) by reverse transcription PCR (RT-PCR). The RT-PCR reaction

mix contained RNA (0.5 – 1 µg), 0.1 µl (20 U) SuperScript III reverse transcriptase with its associated first-strand buffer, 5 µM Oligo(dT)$_{18}$ primer, 0.5 mM dNTPs and 5 mM DTT in a final volume of 20 µl. Additionally, a reaction mix without reverse transcriptase was prepared to control for genomic DNA contamination. First, the correct amount of RNA was diluted in nuclease-free water and mixed with 5 µM Oligo(dT)$_{18}$ primers. After heating at 65°C for 5 minutes, the mixture was incubated on ice for 1 minute, before SuperScript III reverse transcriptase with associated first-strand buffer, 0.5 mM dNTPs and 5 mM DTT were added. The RT-PCR reaction was conducted at 50°C for 50 minutes and inactivated at 80°C for 5 minutes afterwards. The cDNA was stored at -20°C until further use.

3.2.17.3 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed with the fluorescent, DNA intercalating dye Maxima SYBR® Green. The dye's fluorescence intensity was used to monitor the double-stranded DNA amplification.
For the qPCR reaction, cDNA was mixed with 0.4 µM gene specific primers (table 3.4), 10X Maxima SYBR® Green and nuclease free water in a final volume of 10 µl. Additionally, a no template control was prepared to control for DNA contaminations in the qRT-PCR reagents. The qRT-PCR was performed in 384-well format on a QuantStudio 6 Flex RT PCR machine (Life Technologies, Carlsbad, CA, USA) for 40 cycles (50°C for 2 minutes and 95°C for 10 minutes, 40 cycles of 95°C, 15 seconds; 60°C, 1 minute) followed by a melt curve analysis for off-target products. The relative mRNA expression to the housekeeping gene actin was calculated using the $\Delta\Delta C_T$ method (Applied Biosystems, Foster City, CA, USA).

3.2.18 Western blot analysis

Western Blot analysis was used to determine protein expression. This method was carried out by Dr. Lucas Ribeiro or myself as indicated. For NLRP3 activation analysis in macrophages or macrophage-platelet co-cultures, 3 x 10^6/well human macrophages were seeded in 6-well plates with or without addition of platelets (5 x 10^7/ml). The NLRP3 inflammasome was activated in the cells as described before in section 3.2.7.1.

3.2.18.1 Sample preparation

After centrifugation (500 x g, 5 minutes), the supernatant was collected for protein precipitation and 100 µl RIPA lysis buffer was added to the cells. RIPA lysis buffer was freshly prepared

from a 2X stock solution supplemented with 1X cOmplete protease inhibitor cocktail and 1X PhosSTOP cocktail. The cells were incubated on ice for 10 minutes, before they were scraped off and the samples were transferred to 1.5 ml tubes. The whole procedure was performed on ice. Centrifugation of the samples (10000 x g, 10 minutes, 4°C) pulled down the heavy weight DNA afterwards. Supernatants were transferred into new 1.5 ml tubes and stored at -80°C until further use.

3.2.18.2 Protein precipitation from supernatant

To precipitate proteins from the collected supernatants, 500 µl methanol and 125 µl chloroform were mixed with 500 µl supernatant. After vortexing, the mixture was centrifuged (13000 x g, 3 minutes) and the upper-most methanol/water layer was taken off, without disturbing the subjacent protein pellet. Then, 500 µl methanol were added and the mixture was vortexed and centrifuged (13000 x g, 3 minutes) again. Afterwards, the supernatant was discarded and the pellet was dried and resuspendend in RIPA lysis buffer or directly in the final sample buffer including a mixture of LDS buffer (stock: 4X) and Reducing agent (10X).

3.2.18.3 Protein quantification

To quantify protein concentrations, a bicinchoninic acid assay (BCA) is performed using the BCA protein assay kit according to the manufacturer's instructions. The absorbance was read at 562 nm using the Spectramax i3 and the concentrations were determined using a bovine serum albumin (BSA) standard curve.

3.2.18.4 SDS-page gel electrophoresis

The samples were diluted with RIPA lysis buffer in order to have equal protein concentrations (30-50 µg). Additionally, LDS buffer (stock: 4X) and Reducing agent (10X) were added and the samples were heated for 10 minutes at 90°C for denaturation and cooled down before loading onto pre-cast 4 - 12% Bis-Tris gels. To evaluate protein size, PageRuler Plus Prestained protein ladder was also loaded onto the gel as a size standard, ranging from 10 to 250 kDa. The prepared gel was run using MOPS running buffer at 150V for approximately 1.30 hours

3.2.18.5 Western blotting

After SDS-page gel electrophoresis, proteins were transferred to a PVDF membrane, which was activated with methanol. Using a semi-wet transfer set (XCell II Blot Module, Novex Life

Technologies, Carlsbad, CA, USA), the protein transmission took place at 30V for 1.30 hours. The membranes were blocked afterwards with 3% BSA in Tris buffered Saline (TBS) for at least one hour before overnight incubation at 4°C with the specific primary antibody (section 3.1.6) in 1% BSA in TBS with Tween® (TBS-T). Membranes were washed three times in TBS-T the following day prior to addition of the appropriate secondary antibody (section 3.1.6) diluted 1:25000 in 1% BSA in TBS-T in the dark for 1 hour. After two washes with TBS-T and one wash in TBS, the reactivity of the antibodies was detected by measuring the infrared fluorescent signals using an Odyssey Imager (Li-Cor Biosciences, Lincoln, NE, USA).

3.2.19 Proteomics

3.2.19.1 Sample preparation

Platelet supernatants for secretome analysis of human platelets were generated as described before (section 3.2.6). After platelet supernatants were generated, a 1x cOmplete protease inhibitor cocktail was added to the sample to inhibit protein degradation. Then samples were frozen at -80°C and sent to the CECAD/CMMC Proteomics Core Facility in Cologne (University Cologne, Germany) for proteomic analysis.

3.2.19.2 Mass spectrometry coupled with liquid chromatography

All samples were analyzed by the CECAD/CMMC Proteomics Core Facility on a Q Exactive Plus Orbitrap (Thermo Scientific) mass spectrometer that was coupled to an EASY-nLC (Thermo Scientific). In short, Peptides were loaded in 0.1% formic acid in water onto an in-house packed analytical column (50 cm ó 75 µm I.D., filled with 2.7 µm Poroshell EC120 C18, Agilent). Peptides were chromatographically separated at a constant flow rate of 250 nl/minute with the following gradient: 3-4% solvent B (0.1% formic acid in 80 % acetonitrile) within 1 minute, 4-27% solvent B within 119 minute, 27-50% solvent B within 19 minutes, 50-95% solvent B within 1 minutes, followed by washing and equilibration of the column. The mass spectrometer was operated in data-dependent acquisition mode.

3.2.19.3 Data processing and statistical analysis

All mass spectrometric raw data were by the CECAD/CMMC Proteomics Core Facility processed using Maxquant (version 1.5.3.8) with default parameters. Shortly, MS2 spectra were analyzed against the Uniprot HUMAN.fasta (downloaded at: 16.6.2017) database, including a list of common contaminants. False discovery rates on protein and PSM level were

estimated by the target-decoy approach to 1% FDR for both. The minimal peptide length was determined to be 7 amino acids and carbamidomethylation at cysteine residues was considered as a fixed modification. Oxidation and acetylation were included as variable modifications. For the analysis, the match-between runs option was enabled. Label-free quantification (LFQ) was activated using default settings.

3.2.20 RNA-sequencing analysis

3.2.20.1 Sample preparation

To analyze the changes in gene profile of hMDMs with or without platelets after inflammasome stimulation, I seeded 1 x 10^5 hMDMs/well in 96-well plates as described before (section 3.2.7). Human platelets were isolated on the day of the experiment from peripheral blood as described before (section 3.2.3.2) and added to hMDMs at a concentration of 5x10^7/ml. Cells then were primed with 200 ng/mL LPS for 3 hours and activated with 10 µM Nigericin for 90 minutes. After centrifugation (500 x g, 5 minutes), supernatants were transferred to new 96-well plates and frozen at -20°C until HTRF® analysis for cytokine secretion. The cells were kept on ice and directly lysed with 100 µl RLT buffer per well. RNA was isolated as described before (section 3.2.15.1). RNA concentration and quality was controlled by using a NanoDrop spectrophotometer. In total hMDMs and platelets from four donors were isolated and co-cultured for RNA sequencing analysis.

3.2.20.2 RNA sequencing

Library preparation and RNA sequencing was performed by André Heimbach and colleagues from the NGS core facility (Institute of Human Genetics, University Hospital Bonn). The library generation was conducted according to the manufacturer's guidelines with an input of 100 ng total RNA using Quant™ Seq's 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen). In short, reverse transcription was performed with oligo dT priming (first strand) and random priming (second strand). After a magnetic bead-based purification step, the libraries were amplified using 15 PCR cycles. The libraries were sequenced on a HiSeq 2500 using HiSeq v4 with a read length of 1 x 50 base pairs. On overage 20 million raw reads were generated per sample.

3.2.20.3 Data processing and statistical analysis

The analysis of the RNA sequencing dataset was performed by Dr. Susanne V. Schmidt (Institute of Innate Immunity, University Hospital Bonn). She aligned the raw fastq-files against the human genome hg19 using STAR v2.5.3a with modified default options using BySJout for filtering, 20 as maximum read mapping, allowing 999 as maximum mismatches and 0.6 as mismatch mapped ratio in PartekFlow. The transcript quantification on gene level was performed in PartekFlow with the E/M Algorithm against the hg19 RefSeq Transcript database version 2018-11-01. This resulted in a read count table containing 18,234 genes, which was exported for normalization using DESeq2 in R v3.5. The normalized gene expression values were into the Partek® Genomics Suite v7.18.0402 (Partek Incorporated, St. Louis, MO, USA). Read counts <1 were floored to 1. The technical variation derived by different sequencing runs was removed as batch effect. Prof. Bernardo Franklin and myself performed ANOVA analysis between different test groups to identify differentially expressed genes (DEGs) which showed at least a fold-change in expression of |2| with a relaxed p value of < 0.01. The least mean profile of DEGs was analyzed using a One-way ANOVA with $p < 0.01$. Partek® pathway™ (Partek Incorporated, St. Louis, MO, USA) analysis was used to analyze DEGs for enriched pathways. Prof. Bernardo Franklin generated figures using R.

3.2.21 Imaging

3.2.21.1 Confocal Laser Scanning microscopy of whole bone marrow cells

Platelets and immune cells were prepared and stained with fluorescent antibodies against CD41 and CD45 as described in section 3.2.5.2. Additionally, cells were also stained with the DNA binding dye draq5 (1:2000 dilution) for 30 minutes in the dark at RT. After fixation with 4% paraformaldehyde for 10 minutes on ice, cells were washed with PBS twice. Samples were imaged with a Leica TCS SP5 SMD confocal system (Leica, Wetzlar, Germany). Images were acquired by Prof. Bernardo Franklin and myself using a 63X objective, with a numerical aperture of 1.2, and analyzed using Volocity 6.01 software.

3.2.21.2 ASC specking assay

To examine ASC specking, hMDMs were generated as described in section 3.2.2 and seeded at a concentration of 2×10^5/well in 8-well IBIDI µ-slide one day prior to the experiment.

Freshly isolated platelets were added to hMDMs on the day of the experiment at a concentration of 5 x 10^7/ml. Cells were stimulated with 200 ng/ml LPS for 3 hours and the NLRP3 inflammasome was activated with 10 µM Nigericin for 45 minutes. Afterwards, the plate was centrifuged (500 x g, 5 minutes) and the cells were fixed with 4% paraformaldehyde in PBS for 10 minutes on ice. After two washes with PBS, all cells were blocked with 1:10 human FcR blocking reagent for 10 minutes at RT. Then cells were stained with directly labelled anti-ASC-647 or the same amount of directly labelled IgG1 control (1:25 dilution each from respective stock: 0.5 mg/ml) and incubated overnight at 4°C in the dark (section 3.1.6). On the next day, cells were washed twice with permeabilization buffer (500 x g, 5 minutes) before staining with the DNA dye Hoechst (1:3000 dilution) in PBS for 10 minutes at RT in the dark. After two subsequent washes with PBS, cells were resuspended in PBS and imaged using a Observer.Z1 epifluorescence microscope, 20× objective (dry, PlanApochromat, NA 0.8; ZEISS, Oberkochen, Germany), Axiocam 506 mono, and ZEN Blue software (ZEISS, Oberkochen, Germany). Representative images were taken using the a Leica TCS SP5 SMD confocal system.

For ASC speck counting, the image analyzer software CellProfiler 3.0 (Open source software: https://cellprofiler.org) was employed. To analyze ASC specks in the images, a pipeline was constructed to convert the fluorescent images into binary ones. Through this conversion, the nuclei and ASC specks were identified and quantified. In short, the corrected illumination was calculated first, before it was applied and nuclei were identified. ASC specks were identified using the EnhanceFeature tool. The obtained data was exported to an Excel sheet and arranged according to the treatment of the samples and analyzed using GraphPad Prism Version 7.0f as indicated.

3.2.21.3 Two-photon microscopy of cytospins

Cytospin preparation for analysis of PBMCs by Two-photon microscopy (TPEF) was entirely performed by Prof. Barbara Wollenbergs group in Lübeck[132]. To this end, 100 µl PBMC cell suspension was added to cytospins. After centrifugation (800 x g, 4 minutes), cytospins were air-dried in the dark overnight. For fixation and permeabilization, slides were incubated with 20% acetone, before addition of the specific primary antibodies (anti-CD41, anti-PD-L1) or IgG matched isotype controls and overnight incubation at 4 °C. The next day, cytospins were washed with PBS and the secondary antibodies (goat anti-rabbit Cy3, goat anti-rabbit FITC) were added for 45 minutes. Afterwards, the slides were washed three times in PBS, before

nuclei were stained with 1 µg/ml DAPI. Finally, after three more washes with PBS, the samples were embedded in Fluoromount G and imaged using PTEF.

3.2.22 Seahorse extracellular flux assay

3.2.22.1 Sample preparation

Seahorse extracellular flux assays were mostly performed by the rotational Master student Friederike S. Gorki (Institute of Innate Immunity, University Hospital Bonn), which I supervised. Monocytes were isolated and differentiated into hMDMs as described in section 3.2.2. HMDMs were seeded at a concentration of $1x10^6$/ml in Poly-L-Lysine coated XF96 cell culture microplates one day prior to the experiments. On the day of the experiment, fresh platelets were isolated as described in section 3.2.3.2 and platelet supernatants were generated as described in section 3.2.6. Platelets (5×10^7) or platelet supernatants were added to the hMDMs and stimulated with 200 ng/ml LPS for 3 hours at 37°C 5% CO_2. Then, cells were subjected to extracellular flux analysis (EFA).

For inhibitor experiments, hMDMs were co-cultured with platelets for 2 hours before they were used for EFA. BPTES (10 µM), UK5099 (10 µM), etomoxir (3 µM) or medium were injected via the sensor cartridge. At the same time, cells were also co-cultured in the presence of UK5099 and 200 ng/ml LPS for 3 hours in common 96-well plates. Afterwards the NLPR3 inflammasome was activated with 10 µM Nigericin for 1.5 hours. Supernatants were collected after centrifugation (500 x g, 5 minutes) and frozen until HTRF® analysis.

3.2.22.2 Seahorse extracellular flux assay

Both, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a Seahorse Bioscience 96-well XF extracellular flux analyzer (Agilent Technologies, Santa Clara, CA, USA). Glycolytic stress tests were conducted in basal XF medium (non-buffered, glucose-free RPMI-1640 with 2 mM glutamine) while mitochondrial stress tests were performed in basal XF medium supplemented with 11 mM glucose and 1 mM sodium pyruvate.

ECAR was measured under basal conditions (5 cycles, 6 minutes each) in a glycolytic stress test in response to 10 mM glucose (substrate to glycolysis), 1 µM oligomycin (ATP-Synthase inhibitor) and 0.1 M 2-Deoxyglucose (2-DG) (Fig. 3.2a). OCR was measured in a mitochondrial stress test under basal conditions (5 cycles, 6 minutes each) in response to 1 µM oligomycin, 1.5 µM fluoro-carbonyl cyanide phenylhydrazone (FCCP, mitochondrial

uncoupler) and 0.5 µM rotenone together with 0.5 µM antimycin A (inhibitors of complex 1 and III of the electron transport chain) (Fig. 3.2b). In experiments with metabolic inhibitors, OCR was analyzed in basal conditions after injection of either 10 µM BPTES, 50 µM UK5099, 3 µM etomoxir or a medium control. Afterwards, injection of the inhibitors the mitochondrial stress test was performed in response to 1 µM oligomycin, 1.5 µM FCCP and 0.5 µM rotenone together with 0.5 µM antimycin. After each injection, three cycles of mixing and measuring followed (6 minutes each). To calculate the spare respiratory capacity (SRC) and the glycolysis rate, the following equations were employed:

$SRC = OCR_{FCCP} - OCR_{basal}$

$Glycolysis\ rate = ECAR_{glucose} - ECAR_{basal}$

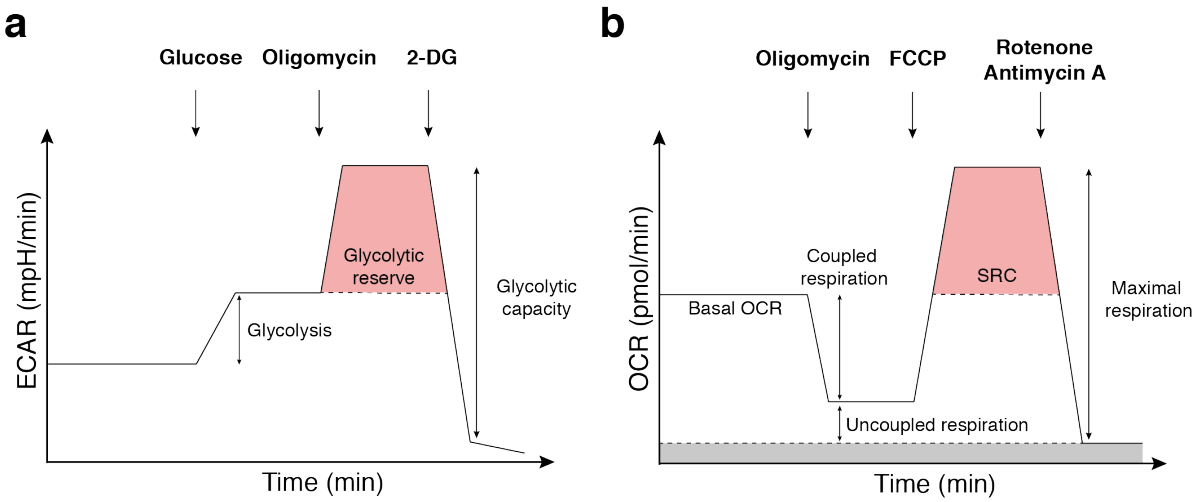

Figure 3.2 Schematic representation of the glycolytic stress test and the "Mitostress test". (**a**) The glycolytic capacity was assessed by measuring the extracellular acidification rate (ECAR) after the injection of glucose, Oligomycin and 2-Deoxyglucose (2-DG). (b) The mitochondrial function was assessed by measuring the oxygen consumption rate (OCR) after injection of Oligomycin, FCCP and Rotenone together with Antimycin A. SRC = Spare respiratory capacity.

3.2.23 Statistical analysis

All statistical analyses were performed using GraphPad Prism Version 7.0f. Data is represented as symbols, where each symbol symbolizes an individual donor. Floating bars with mean and minimum to maximum values are shown and represent pooled data from independent experiments as indicated in the figures.

In section 4.2, the mean and standard deviation (SD) is depicted for three or less donors. For four or more biological replicates, the mean and standard error (SEM) are shown. The differences between groups of more than three donors was determined by using the appropriate parametric (T-test), or non-parametric tests (1-way ANOVA, Tukey's multiple comparisons

test) as indicated in the figures after testing for Gaussian distribution (normality tests): $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

4 Results

In this thesis, I have investigated the role of platelets in the context of inflammatory responses and in HNSCC cancer. Therefore, I show the results of my findings in two parts, with the first part focusing on how human and murine platelets regulate inflammasome activation in innate immune cells (section 4.1) and the second part discussing the role of platelets in cancer (section 4.2).

4.1 Platelets regulate NLPR3 inflammasome activation in innate immune cells

Inflammasomes as signaling platforms have been extensively studied since their discovery over a decade ago. However, the majority of *in vitro* studies was conducted in monocultures of macrophages or monocytes. While this is an adequate approach to elucidate the molecular mechanism underlying an inflammasome response, it neglects the networking, synergistic and regulatory effects with other immune cells *in vivo*. Thus, understanding the effects of immune cell interactions on inflammasome responses *in vitro* could help to understand the causes of IL-1 cytokine variations *in vivo*. As many autoimmune and autoinflammatory diseases are caused by malfunctioning inflammasomes, this knowledge will help to understand how and why certain individuals are more susceptible to auto-immune diseases than others.
Since platelets are the second-most abundant cell type in the human blood and frequently interact with immune cells, I hypothesized that platelets influence the inflammasome response of encountered immune cells.

4.1.1 Platelets amplify the NLRP3 inflammasome response of immune cells

One of the most intensely studied inflammasomes is the NLRP3 inflammasome, which primarily assembles in myeloid cells, such as macrophages, monocytes and neutrophils. Activation of NLRP3 inflammasome is controlled at the transcriptional as well as the post-translational level and requires two distinct signals: the first priming signal and a second activation trigger[134]. The assembled NLRP3 inflammasome recruits and activates caspase-1 and gasdermin D, leading to pyroptosis and the maturation and release of the proinflammatory cytokines IL-1β and IL-18[32].

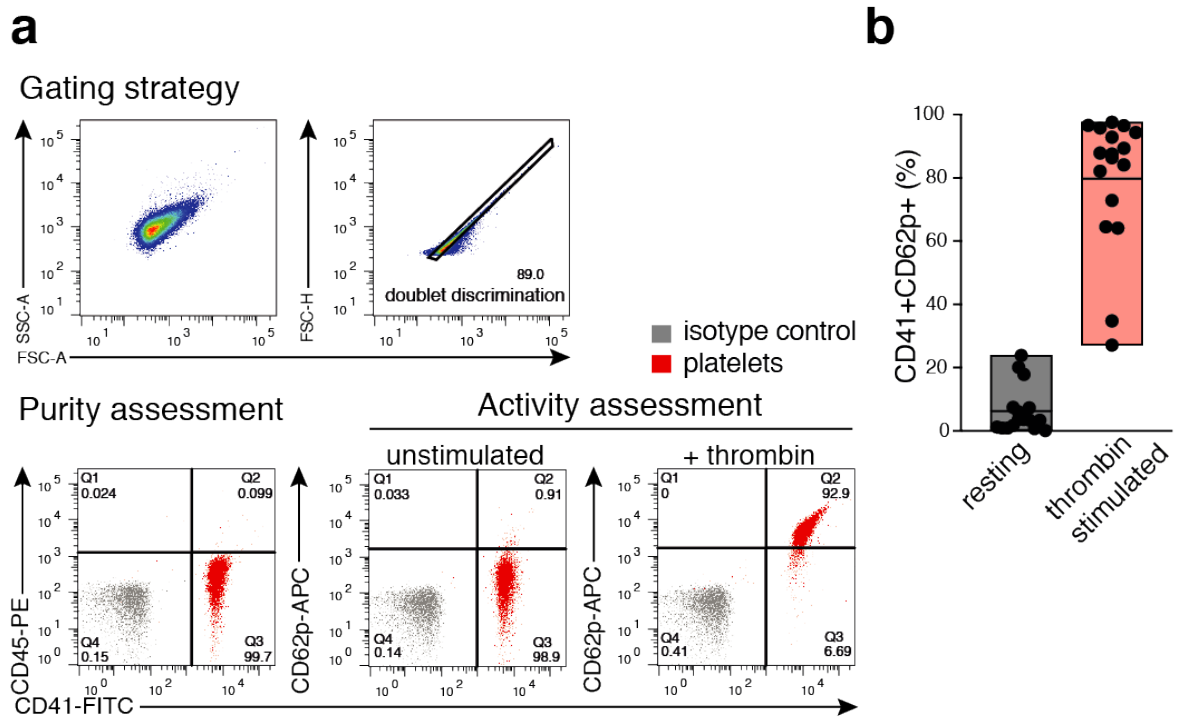

Figure 4.1 Isolated human platelets are pure and show low P-selectin expression. (**a**) Representative image of flow cytometry analysis of platelet purity and activation status. Surface expression of CD41 (platelet marker), CD45 (leukocyte marker) and CD62p (P-selectin, platelet activation marker) was assessed in freshly isolated platelets. The gating strategy included doublet discrimination and matching isotype controls were used as negative controls. (**b**) Floating bars showing CD62p expression on unstimulated (resting) or thrombin activated platelets (CD41+CD62+) from several independent experiments (n=17). Floating bars show mean and minimum to maximum values. Each symbol represents a different donor.

To investigate the interaction of platelets with immune cells in the context of inflammasome activation, I first optimized a suitable isolation protocol for platelets from human blood based on the protocol used by Alard et al. [97], which is described in section 3.2.3.2 (Fig. 3.1). I assessed platelet purity, viability and activation state after each platelet purification conducted in this work by flow cytometry (Fig. 4.1). To confirm purity, I examined expression of the leukocyte marker CD45 in isolated platelets, which are CD45 negative cells. The activation state and viability of platelets were determined by measuring surface expression of P-selectin (CD62p) before and after stimulation with thrombin. P-selectin is stored in α- granules (inside the cell) in resting platelets and transported to the plasma membrane only upon activation with thrombin[135].

Figure 4.1 shows that all preparations of isolated platelets used in this work are highly pure (99%) and express low levels of P-selectin in resting state (Fig. 4.1a-b). Upon stimulation with thrombin, isolated platelets upregulated P-selectin on the cell membrane, indicating their responsiveness to stimulation and thus viability (Fig. 4.1b).

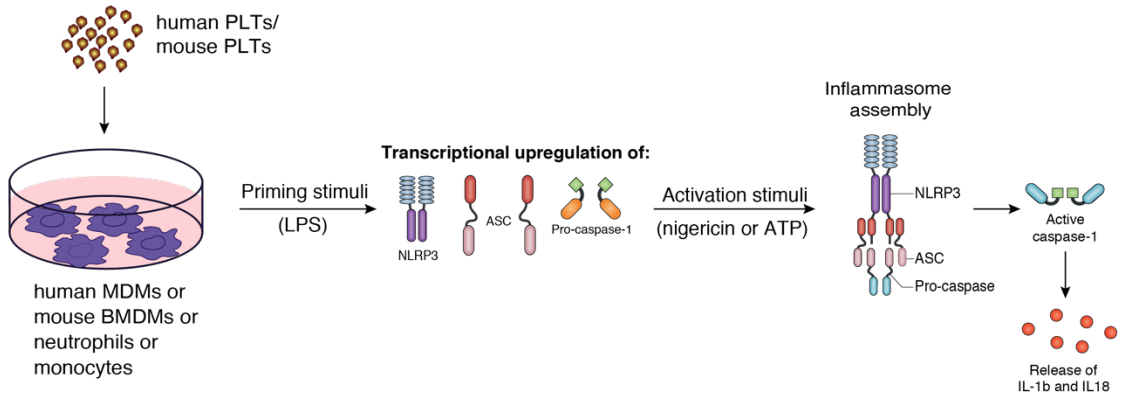

Figure 4.2 Schematic representation of experimental setup for inflammasome activation. Murine or human platelets (PLTs) are added to human monocyte derived macrophages (MDMs), mouse bone marrow derived macrophages (BMDMs), human neutrophils or human monocytes. Co-cultures were activated with LPS and nigericin or ATP, leading to IL-1β and IL-18 release.

Having established a suitable platelet isolation protocol, I next investigated whether platelet-immune cell interactions influence the inflammasome response of leukocytes (Fig. 4.2). For this purpose, increasing numbers of platelets were co-cultured with Human monocyte-derived macrophages (hMDMs) (Fig. 4.3a), murine bone marrow derived macrophages (BMDMs) (Fig. 4.3b), human neutrophils (Fig. 4.3c) or human monocytes (Fig. 4.3c). Co-cultured cells were either untreated, or primed with LPS, followed by activation with either ATP or the ionophore nigericin, which are canonical NLRP3 inflammasome stimuli (Fig. 4.2). Afterwards, I assessed IL-1β and TNFα release by HTRF® in cell-free supernatants. Platelets cultured alone served as a control to assess their ability to secrete cytokines. The experiments involving the addition of platelets to BMDMs were carried out by Lisa Böttcher (Institute of Innate Immunity, University Bonn) in the course of her Master thesis work. Salie Maasewerd (Institute of Innate Immunity, University Bonn) performed experiments involving the addition of platelets to neutrophils in the course of her Bachelor thesis work, which I supervised.

Addition of platelets amplified IL-1β production after inflammasome stimulation in hMDMs, BMDMs and human neutrophils in a concentration dependent manner (Fig. 4.3a-c), whereas IL1β secretion was not altered in human monocytes after platelet addition (Fig. 4.3d). Regardless of the presence of platelets, LPS stimulation alone was not sufficient to induce IL-1β secretion in any of these cells. In contrast, TNFα secretion was decreased after platelet addition to BMDMs, human neutrophils and monocytes, whereas TNFα secretion was not changed in hMDMs (Fig. 4.3a-d). Importantly, platelets alone did not secrete any IL-1β or TNFα, indicating that they are not the source of additional IL-1β measured in the co-cultures.

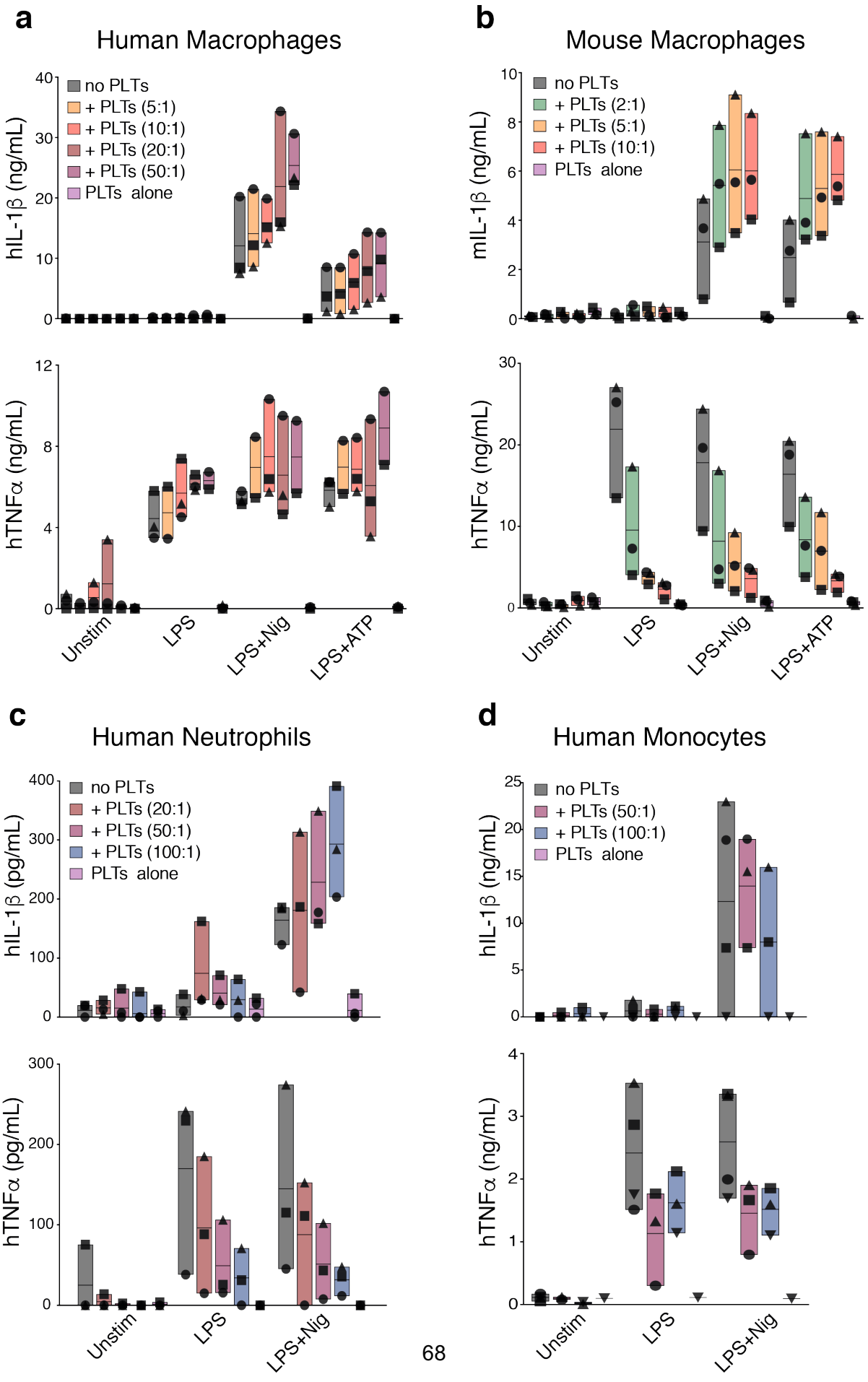
a
Human Macrophages
no PLTs
+ PLTs (5:1)
+ PLTs (10:1)
+ PLTs (20:1)
+ PLTs (50:1)
PLTs alone
hIL-1β (ng/mL)
hTNFα (ng/mL)
Unstim
LPS
LPS+Nig
LPS+ATP
b
Mouse Macrophages
no PLTs
+ PLTs (2:1)
+ PLTs (5:1)
+ PLTs (10:1)
PLTs alone
mIL-1β (ng/mL)
hTNFα (ng/mL)
Unstim
LPS
LPS+Nig
LPS+ATP
c
Human Neutrophils
no PLTs
+ PLTs (20:1)
+ PLTs (50:1)
+ PLTs (100:1)
PLTs alone
hIL-1β (pg/mL)
hTNFα (pg/mL)
Unstim
LPS
LPS+Nig
d
Human Monocytes
no PLTs
+ PLTs (50:1)
+ PLTs (100:1)
PLTs alone
hIL-1β (ng/mL)
hTNFα (ng/mL)
Unstim
LPS
LPS+Nig

Figure 4.3 Platelets amplify inflammasome-driven IL-1β secretion by immune cells. (a) HTRF® measurements of IL-1β and TNFα in cell-free supernatants of unstimulated (Unstim), LPS-primed (200 ng/ml, 3 hours), or LPS primed and nigericin (10 μM, 90 minutes) or ATP (5 mM, 90 minutes) activated human monocyte derived macrophages (hMDMs). Cells were cultivated alone (No PLTs) or in the presence of increasing ratios of platelets as indicated (+PLTs). Platelets cultivated alone served as control (PLTs alone). Floating bars (with mean and minimum to maximum values) show pooled data from three independent experiments with platelets and macrophages from different donors. (**b**) HTRF® measurements of murine IL-1β and TNFα in cell-free supernatants from wild-type BMDMs. Cells were treated as described in a. These experiments were carried out by Lisa Böttcher (Institute of Innate Immunity, University Bonn). Floating bars (with mean and minimum to maximum values) represent pooled data from three independent experiments with platelets and BMDMs from different mice. (**c**) HTRF® measurement of IL-1β and TNFα in cell-free supernatants of human neutrophils or (**d**) human CD14+ isolated monocytes stimulated as in **a**. The addition of platelets to neutrophils was performed by Salie Maasewerd (Institute of Innate Immunity, University Bonn). Floating bars (with mean and minimum to maximum values) represent pooled data from three independent experiments with platelets and neutrophils or monocytes from three different donors. Each symbol in this figure represents the average of technical triplicates from different donors.

These results show that platelets influence IL-1β and TNFα production in immune cells in a concentration dependent manner after NLRP3 stimulation.

As addition of platelets to hMDMs increased their IL-1β but not TNFα secretion after inflammasome activation, I was interested whether the secretion of other inflammation-related factors was also affected. To address this, I performed a multiplex cytokine assay to measure 45 cytokines, chemokines or growth factors in cell-free supernatants of unstimulated or inflammasome activated hMDMs cultured with or without platelets (Fig. 4.4).

The results showed a changed cytokine/chemokine signature of inflammasome stimulated hMDMs when co-cultured with platelets. In line with the earlier finding, the secretion of all three members of the IL-1 family, IL-1β, IL-1α and IL-18, was increased in inflammasome activated hMDM co-cultured with platelets. Interestingly, SDF-1α, PIGF-1, VEGFα, RANTES, PDGF-BB and EGF were also upregulated, whereas IL-5, MIP 1α, IL-9, MCP1 and IL-22 were downregulated (Fig.4.5). As before, platelets in monoculture served as a control and, except for low production of RANTES, did not accumulate the investigated cytokines beyond background level.

Taken together, these results show the importance of platelet-immune cell interactions during inflammasome activation, with platelets selectively boosting inflammasome-derived IL-1 cytokines and other proinflammatory cytokines/chemokines in hMDMs and neutrophils.

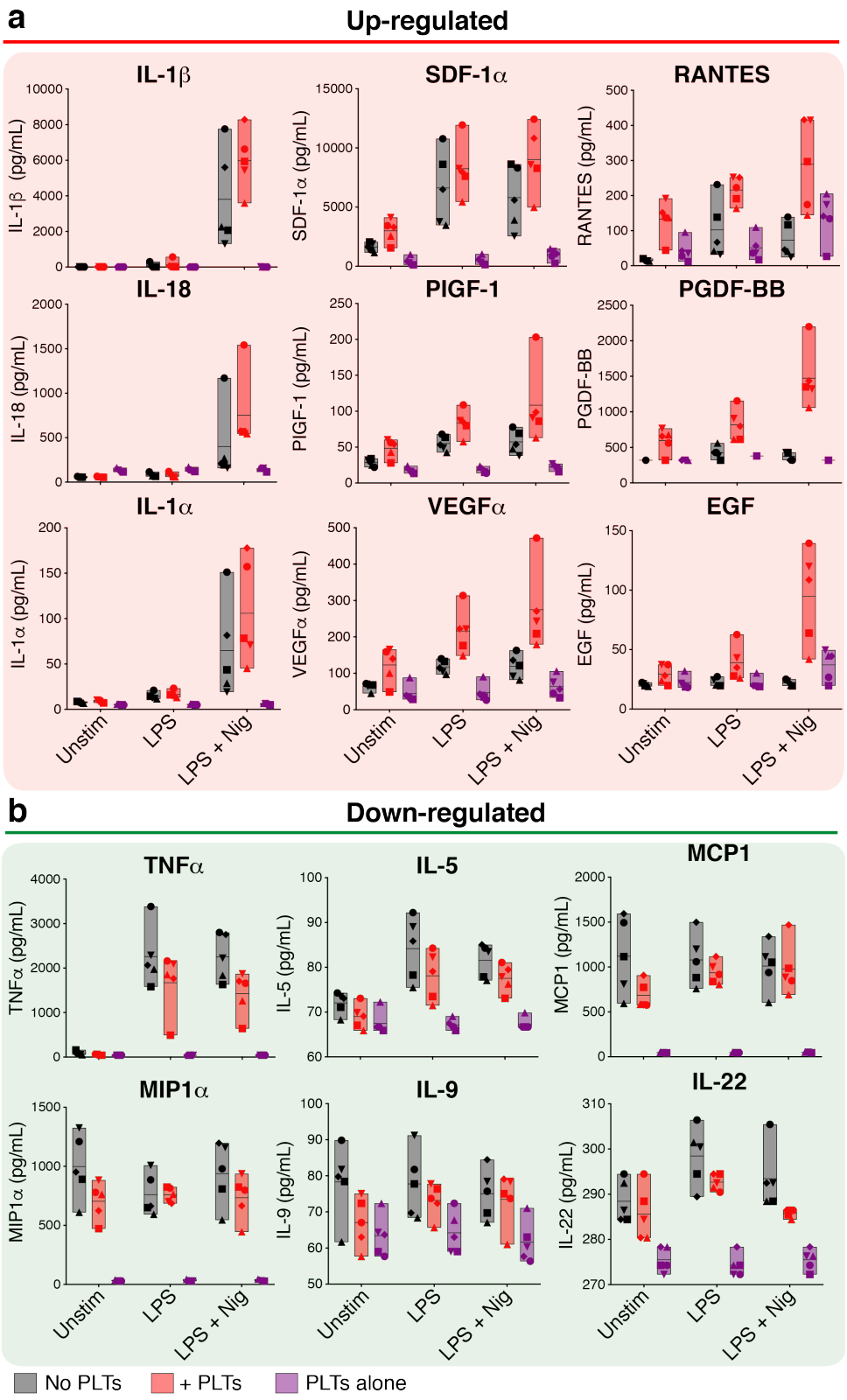

Figure 4.4 Platelet addition induces a cytokine signature change in NLRP3 activated hMDMs. (**a**) Cytokines and growth factors upregulated and (**b**) downregulated in cell-free supernatants of unstimulated (Unstim), LPS-primed (200 ng/ml, 3 hours), or LPS primed and nigericin (10 µM, 90 minutes) activated hMDMs. Cells were either cultivated alone (No PLTs), or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs). Platelets cultivated alone served as control (PLTs alone). Cytokines and growth factors were measured using a 45-Plex Human ProcartaPlex™. Floating bars (with mean and minimum to maximum values) show pooled data from five independent experiments with platelets and macrophages from different donors. Each symbol in this figure represents the average of technical triplicates from different donors.

4.1.2 Platelets are critical for the production of IL-1β from human monocytes

Since the addition of platelets to human monocytes did not alter their IL-1β secretion after NLRP3 stimulation, in contrast to hMDMs and BMDMs (Fig. 4.3a-d), I argued that monocyte signaling might differ from that of macrophages because monocytes are surrounded by platelets in steady state. Thus, further addition of platelets might not impact the interaction between these two cell types.

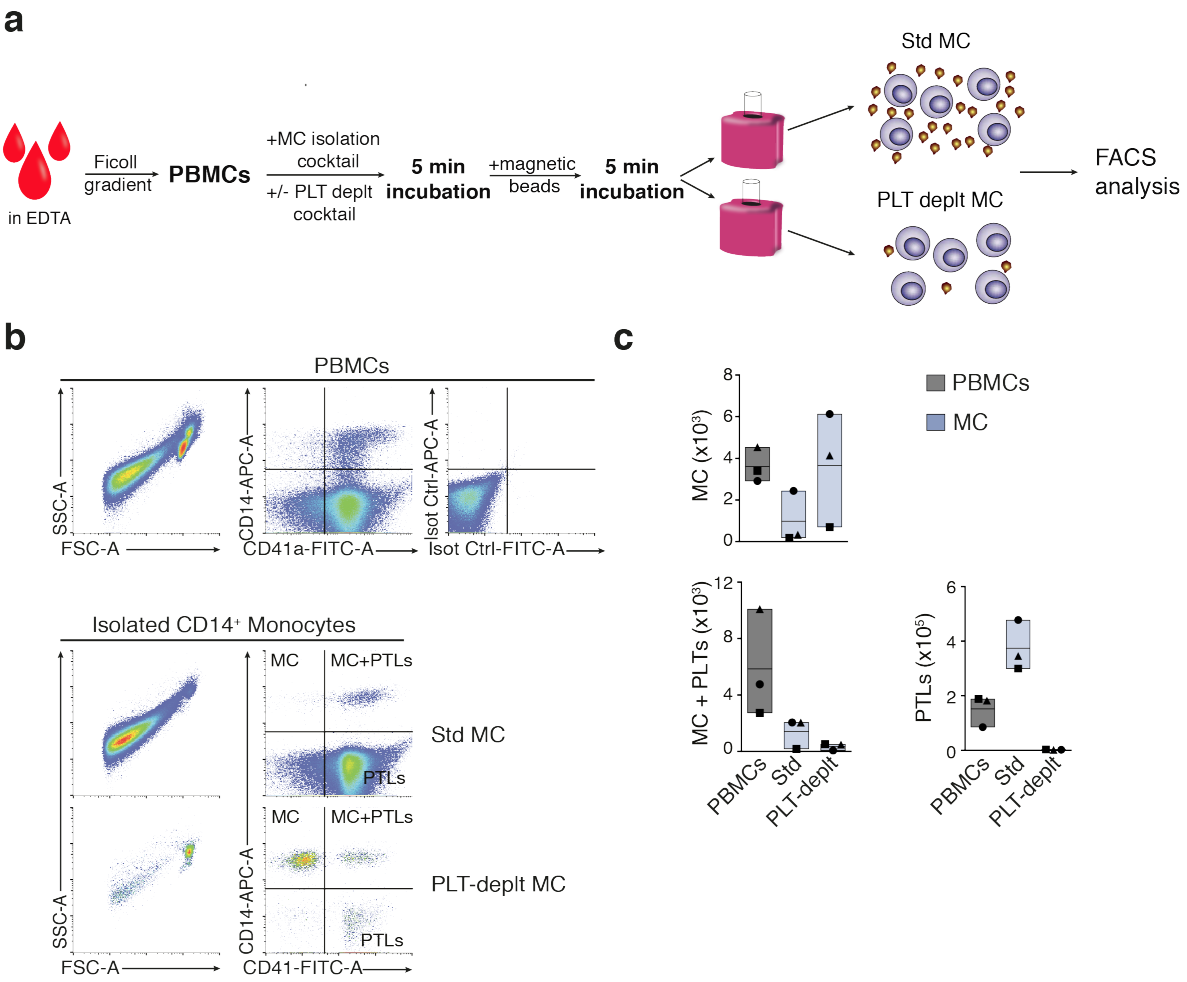

Figure 4.5 Platelet depletion of human monocytes leads to reduced platelet numbers and monocyte-platelet aggregates. (**a**) Schematic representation of human primary CD14$^+$ monocytes (MC) isolation from human blood collected in EDTA. Monocytes were isolated using the conventional magnetic EasySep^{TM}Human Monocyte Isolation Kit with (PLT deplt) or without (Std) addition of the supplied platelet-depletion cocktail. (**b**) Flow cytometric analysis of human PBMCs or CD14$^+$ isolated human monocytes with or without platelet depletion. Surface expression of CD41 (platelet marker) and CD14 (monocyte marker) was assessed in all cell populations. Matching isotype controls were used for setting the gates. (**c**) Flow cytometric-based quantification of PBMCs and isolated conventional or platelet depleted CD14$^+$ monocytes, showing the frequency of contaminating platelets (CD41$^+$, CD14$^-$), platelet and monocyte aggregates (CD41$^+$, CD14$^+$) and enriched CD14$^+$ monocytes (CD41$^-$, CD14$^+$). Floating bars (with mean and minimum to maximum values) are shown from pooled data from three independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

To test this hypothesis, I performed platelet depletion experiments to assess the effect platelets have on monocytes. I isolated CD14^{+} monocytes from human peripheral blood using the commercially available STEMCELL Technologies^{TM} isolation kit, with or without the addition of a platelet removal cocktail (Fig.4.5a). To confirm purity and the efficiency of the removal of platelets, I examined the expression of the monocyte marker CD14 and the platelet marker CD41 in isolated CD14^{+} monocytes by flow cytometry (Fig. 4.5b). Indeed, addition of the platelet depletion cocktail reduced the number of platelets (PLTs: CD41+, CD14-) and platelet-monocyte aggregates (MC+PLTs: CD41+,CD14+) and enriched the platelet-free monocyte fraction (MC: CD41-CD14+) in comparison to standard CD14^{+} monocytes (Std MC) (Fig. 4.5b-c).

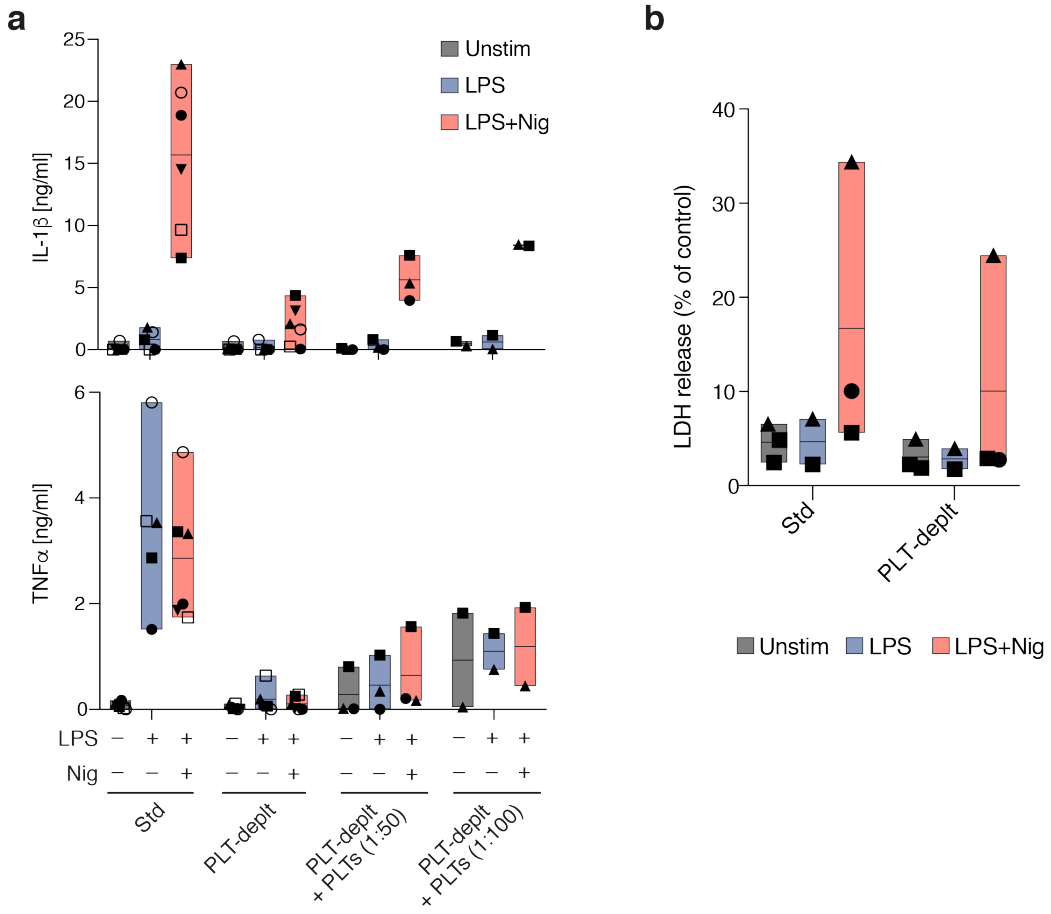

Figure 4.6 Platelets are critical for cytokine production from NLRP3 activated human monocytes. (**a**) HTRF measurements of IL-1β and TNFα in cell-free supernatants of unstimulated (Unstim), LPS-primed (2 ng/ml, 3 hours), or LPS primed and nigericin (10 µM, 90 minutes) activated CD14^{+} monocytes cultured with or without platelets. Monocytes were isolated using conventional magnetic EasySep^{TM}Human Monocyte Isolation Kit with (PLT deplt) or without (Std) addition of the supplied platelet-depletion cocktail. Freshly isolated autologous platelets were also added back to PLT-deplt monocytes in a 100:1 platelet to monocyte ratio. Floating bars (with mean and minimum to maximum values) are shown from pooled data from three to six independent experiments. (**b**) Measurement of LDH release in cell-free supernatants of Std and PLT-deplt monocytes stimulated as described in **a**. Floating bars (with mean and minimum to maximum values) are shown from pooled data from three independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

To address whether platelet depletion influences the $CD14^+$ monocyte inflammasome response, I assessed IL-1β secretion after NLRP3 inflammasome activation of standard and platelet-depleted $CD14^+$ monocytes by HTRF®. Strikingly, depletion of platelets impaired the ability of $CD14^+$ monocytes to produce IL-1β and TNFα upon NLPR3 inflammasome activation. Consistent with this, replenishing platelet depleted $CD14^+$ monocytes with growing concentrations of autologous platelets partially restored IL-1β and TNFα secretion (Fig. 4.6a), indicating that platelet-depleted $CD14^+$ monocytes are still viable and able to produce cytokines. This was also confirmed by a cytotoxicity assay measuring lactate dehydrogenase (LDH) release, which is a marker for cellular toxicity and cytolysis. Monocyte LDH release after platelet removal was similar to standard $CD14^+$ monocytes during NLRP3 activation, suggesting that monocyte viability was unchanged after using the platelet-depletion cocktail (Fig. 4.6b).

From these results, I conclude that platelets are critical for human $CD14^+$ monocytes to reach their full inflammasome activation capacity and IL-1β secretion.

4.1.3 The platelet mediated IL-1β amplification by hMDMs does not involve platelet-derived-IL-1 or IL-18 signaling

Platelets have been proposed to contain and release IL-1 cytokines on their own, such as IL-18[136], IL-1α and IL-1β[82-85]. As shown in Fig. 4.3, IL-1 cytokines were not detected in monocultures of platelets. To exclude the possibility that the increased IL-1 measured in co-cultures of hMDMs and platelets is due to platelet production of IL-1 cytokines, I co-cultured wild-type platelets with BMDMs isolated from mice with a genetic deficiency of the IL-1R (*Il1r1*$^{-/-}$), IL-18 receptor (*Il18r1*$^{-/-}$), or WT mice as control. The NLRP3 inflammasome was activated using LPS and nigericin.

Addition of WT platelets to IL-1R deficient BMDMs boosted IL-1β release after inflammasome stimulation to the same degree as in WT BMDMs (Fig. 4.7a). Similarly, addition of WT platelets boosted the inflammasome response of IL-18 receptor deficient BMDMs like in WT BMDMs (Fig.4.7b). Again, platelets cultured alone did not secrete any IL-1β above background level (Fig. 4.7).

These results generally exclude a role of platelet-derived IL-1α, IL-1β or IL-18 in the regulation of the platelet-mediated IL-1β amplification effect in macrophages after NLRP3 inflammasome stimulation.

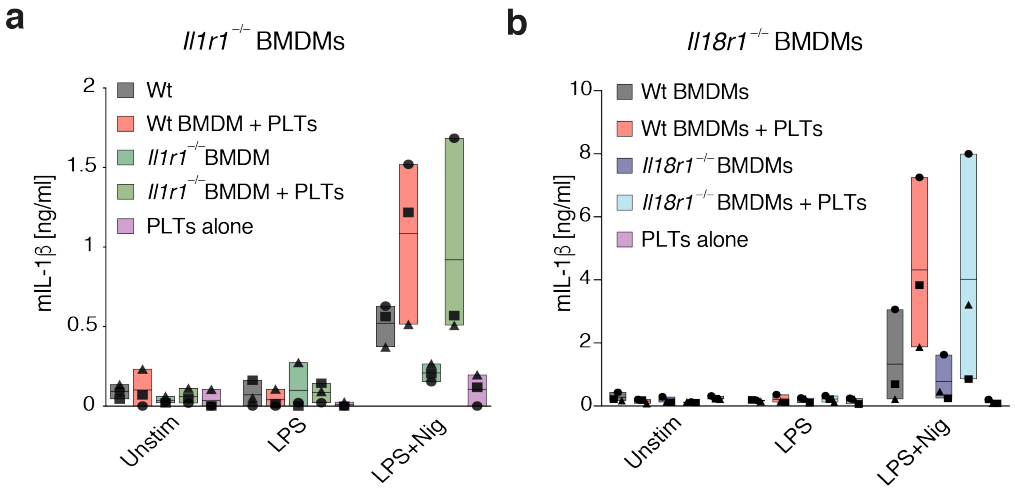

Figure 4.7 The platelet mediated IL-1β boost in NLRP3 activated hMDMs is independent of IL-1 and IL-18 cytokines in platelets. (**a**) HTRF® measurement of mouse IL-1β in cell-free supernatants of wild-type (Wt) or IL-1R deficient (*Il1r1*$^{-/-}$) or (**b**) IL-18R deficient (*Il18r1*$^{-/-}$) BMDMs cultivated alone, or in the presence of platelets (+PLTs, 5:1 ratio PLTs to BMDMs) from wild-type mice. Cells were primed with LPS (200 ng/ml, 3 hours) and activated with nigericin (10 µM, 90 minutes). Platelets cultured alone served as a control. Floating bars (with mean and minimum to maximum values) are shown from pooled data from three independent experiments. Each symbol represents the average of technical triplicates from different mice. The experiments in **a** were performed by Lisa Böttcher.

4.1.4 Platelets do not express NLRP3 inflammasome components or IL-1β cytokines

In recent years, the expression and assembly of NLRP3 in human platelets was reported, showing an involvement in the pathologies of Dengue fever[86] and sickle cell disease[87]. The assembly of the NLRP3 inflammasome in platelets could influence my observations, particularly in the context of IL-1 cytokine production. To determine whether platelets express and assemble a NLRP3 inflammasome, I employed transgenic (Tg) ASC-mCitrine or ASC-mCherry knock in mice to examine ASC expression in platelets (Fig. 4.8).

I stained total bone marrow cells from ASC-mCitrine Tg or ASC-mCherry knock in mice for total leukocytes (CD45), monocytes (CD14), neutrophils (Ly6G) and platelets (CD41) using fluorescently labelled antibodies. ASC expression was assessed in each of those cell populations using flow cytometry (Fig. 4.8b-c). Additionally, I also examined the inflammasome assembly in leukocytes and platelets by assessing the formation of ASC specks after NLRP3 inflammasome stimulation (LPS priming and nigericin activation) in total bone marrow cell extracts from ASC-mCitrine Tg mice by confocal microscopy (Fig. 4.8a).

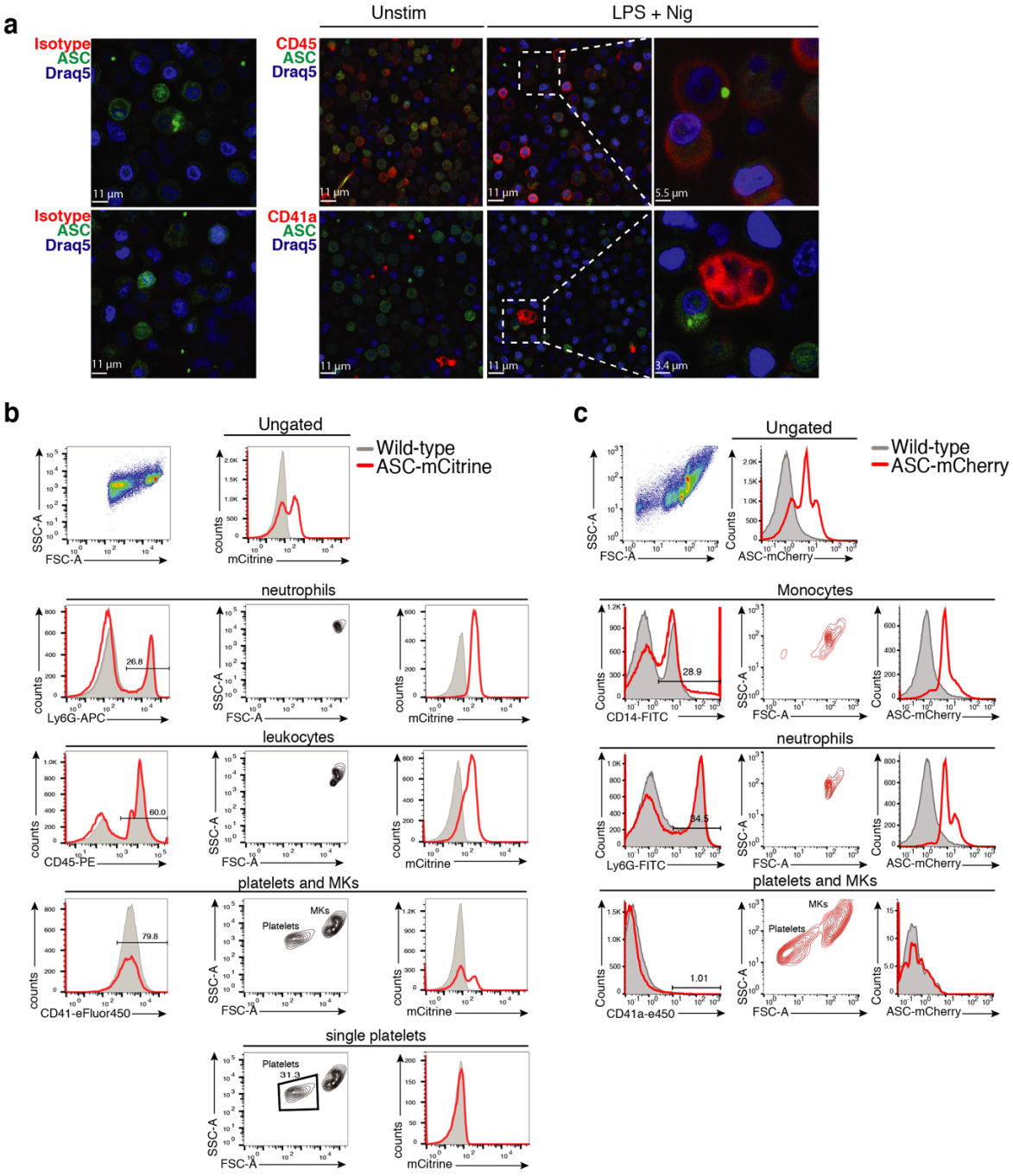

Figure 4.8 Platelets from inflammasome reporter mice do not express ASC. (**a**) Representative confocal microscopy of total bone marrow cells from transgenic ASC-mCitrine mice, comparing ASC expression (green) in unstimulated (Unstim) or LPS (200 ng/ml, 3 hours) and nigericin (Nig, 10 µM, 90 minutes) treated leukocyte ($CD45^+$) and platelets ($CD41a^+$). Cells were also stained with matching isotype controls and Draq5 was used to stain DNA (blue). The size of the scale bar is indicated in the figure. (**b**) Flow cytometric analysis of total bone marrow cells from wild-type (depicted in grey) or transgenic ASC-mCitrine mice (depiced as red line) stained with markers for neutrophils ($Ly6G^+$), leukocytes ($CD45^+$) and platelets and megakaryocytes (MKs) ($CD41^+$). ASC-expression was analysed in each of the respective cell types and wild-type cells served as a negative control. The data is representative for two independent experiments. (**c**) Flow cytometric analysis of total bone marrow cells from wild-type or ASC-mCherry knock in mice stained with markers for monocytes ($CD14^+$), neutrophils ($Ly6G^+$) and platelets and megakaryocytes (MKs) ($CD41^+$). ASC expression was analysed as described in **b**. The data is representative for two independent experiments.

Confocal imaging and flow cytometric analysis showed a clear visualization of ASC-mCitrine in neutrophils and total leukocytes in bone marrow cells from ASC-mCitrine Tg mice. In contrast, no co-localization of ASC-mCitrine and the platelet marker (CD41) was observed (Fig. 4.8a-b). These results were mirrored in bone marrow cells from ASC-mCherry knock in mice. No ASC-mCherry expression was observed in platelets or megakaryocytes by flow cytometry (Fig. 4.8c). In addition, ASC speck formation was detected in leukocytes in total bone marrow cells after NLRP3 inflammasome activation using confocal microscopy, as described before[137,138]. As expected, ASC specks formed in leukocytes ($CD45^+$) but not in platelets or megakaryocytes ($CD41a^+$) (Fig. 4.8a).

Taken together, these results indicate that murine platelets and megakaryocytes do not express ASC and therefore cannot assemble the NLRP3 inflammasome, in contrast to murine leukocytes.

To confirm these results in human cells, the expression of IL-1β, the inflammasome molecules NLRP3, ASC, and caspase-1 was assessed in unstimulated human platelets and PBMCs from healthy donors at the mRNA level by qPCR (Fig. 4.9a). NLRP3 and IL-1β protein expression was also validated by Western blot (Fig. 4.9b), and IL-1β and TNFα secretion was analyzed by HTRF® (Fig. 4.9c). The expression of PF4 and GPIbα on the mRNA or CD42b at the protein level were assessed as controls, as they are well-known platelet-specific proteins. The purity of isolated platelets was confirmed prior to each analysis as described before (Fig. 4.1) and through monitoring of CD14 expression by qPCR (Fig. 4.9a) to assure minimal leukocyte contamination, which could confound results especially on the mRNA level.

In line with the ASC expression data in murine platelets, no ASC, IL-1β, NLRP3, or caspase-1 expression was observed in human platelets at the mRNA level although these factors were expressed in PBMCs from the same donors (Fig. 4.9a). This was confirmed at the protein level for NLRP3 and IL-1β (Fig. 4.9b). NLRP3 and IL-1β expression was observed in human PBMCs by Western blot and upregulated upon inflammasome activation with LPS and ATP (Fig. 4.9b). Importantly, the platelet controls PF4 and GPIbα (qPCR) or CD42b (Western blot) were expressed in the respective assays, indicating that the analysis was functional for platelets (Fig. 4.9a-b). Likewise, human platelets did not secrete mature IL-1β or TNFα upon inflammasome activation or after stimulation with the established platelet activators thrombin, ADP or platelet activating factor (PAF) for 18 hours. In contrast, PBMCs from the same donor did show IL-1β secretion after inflammasome activation or TNFα secretion after LPS stimulation as expected (Fig.4.9c).

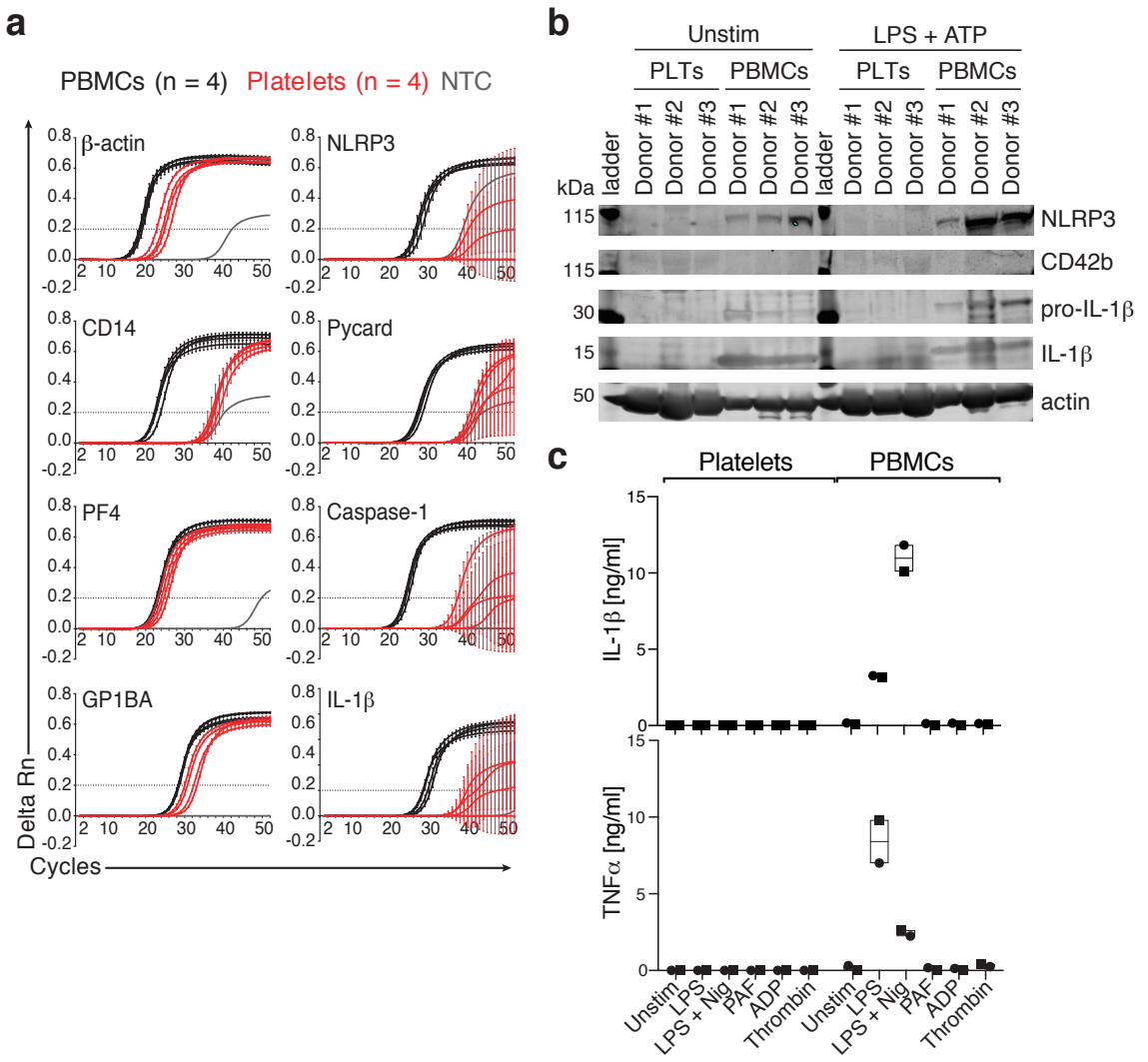

Figure 4.9 No expression of NLRP3, ASC, casapase-1 or IL-1β in platelets. (**a**) Quantitative real-time PCR (qPCR) analysis using of the inflammasome components NLRP3, Pycard (ASC), caspase-1 and IL-1β in PBMCs (depicted as black lines) and purified human platelets (depicted as red lines) from four different donors (n=4). Expression of platelet glycoprotein Ib alpha chain (GPIbα) and platelet factor 4 (PF4) served as controls for platelets. CD14 expression served as monocyte contamination marker and β-actin was used as house-keeping gene control. NTC: no template control. (**b**) Western blotting for NLRP3, CD42b, IL-1β and β-actin in lysates of unstimulated (Unstim) or LPS (200 ng/ml, 3 hours) and ATP (5 mM, 90 minutes) activated human PBMCs and platelets (PLTs) from three different donors. (**c**) HTRF® measurement of IL-1β and TNFα in cell-free supernatants from human platelets or PBCMs stimulated with LPS (200 ng/ml, 18 hours), LPS + nigericin (LPS+Nig, 200 ng/ml LPS, 3 hours + 10 µM nigericin, 90 minutes), platelet activating factor (PAF, 1 nM, 18 hours), ADP (20 µM, 18 hours), thrombin (1 U/ml, 18 hours). Floating bars (with mean and minimum to maximum values) are shown from pooled data from two independent experiments. Each symbol represents the average of technical triplicates from different donors (n=2).

Conclusively, these results demonstrate that human platelets from healthy donors do not express IL-1β or the inflammasome molecule NLRP3 and are unable to secrete IL-1β or TNFα after either LPS (alone), inflammasome stimulation or specific platelet activation. This is in contrast to the previously published reports[83,86,87] and indicates that inflammasome activation and assembly in platelets is no relevant factor in my experimental setup.

4.1.5 The platelet-mediated IL-1β amplification in inflammasome activated hMDMs requires NLRP3 activation

NLRP3 and NLRC4 both contain a central NACHT domain and carboxy terminal LRRs, and were shown to activate caspase-1 leading to processing of pro-IL-1β through the formation of distinct inflammasomes[134].

Given that platelets boost the NLRP3 inflammasome response in hMDMs, I wondered whether they also influence other inflammasomes, such as the NLRC4. To test this, I activated the NLRC4 inflammasome by LFn-PrgI fusion protein treatment of LPS-primed hMDMs cultured with or without platelets and measured IL-1β secretion. The fusion protein was composed of an amino terminal domain of anthrax lethal toxin (LFn) in frame with the T3SS needle protein of *S.typhimurium* (PrgI) and was delivered to the cell's cytosol using the anthrax protective antigen (PA) delivery system[32]. I observed robust IL-1β secretion after NLRC4 inflammasome stimulation with PA and PrgI after LPS priming in hMDMs (Fig. 4.10a). Addition of platelets to hMDMs elevated this IL-1β secretion slightly while TNFα secretion did not change (Fig. 4.10a), suggesting that platelets also boost the NLRC4 inflammasome to some extent.

To further validate this finding, I tested whether the platelet-mediated IL-1β amplification was dependent on the expression of NLRP3. To test this, I added the NLRP3 inhibitor cytokine release inhibitory drug 3 (CRID3) to the co-cultures of hMDMs and platelets after LPS priming but before inflammasome activation with Nigericin (Fig. 4.10b) or Prgi and PA (Fig. S1). CRID3 has been shown to be a potent NLRP3 but not NLRC4 inflammasome inhibitor, by preventing ASC oligomerization [46,47].

HTRF® analysis of IL-1β release showed that the platelet-mediated IL-1β boost by hMDMs requires a functional NLRP3. No IL-1β secretion was detected in hMDMs cultured with or without platelets after CRID3 treatment (Fig. 4.10b). Both, the NLRP3 independent TNFα secretion and NLRC4 activation were unchanged after CRID3 treatment. These results indicate that CRID3 was specific for NLRP3 and the cells were still functional after inhibitor treatment (Fig. 4.10b, S1).

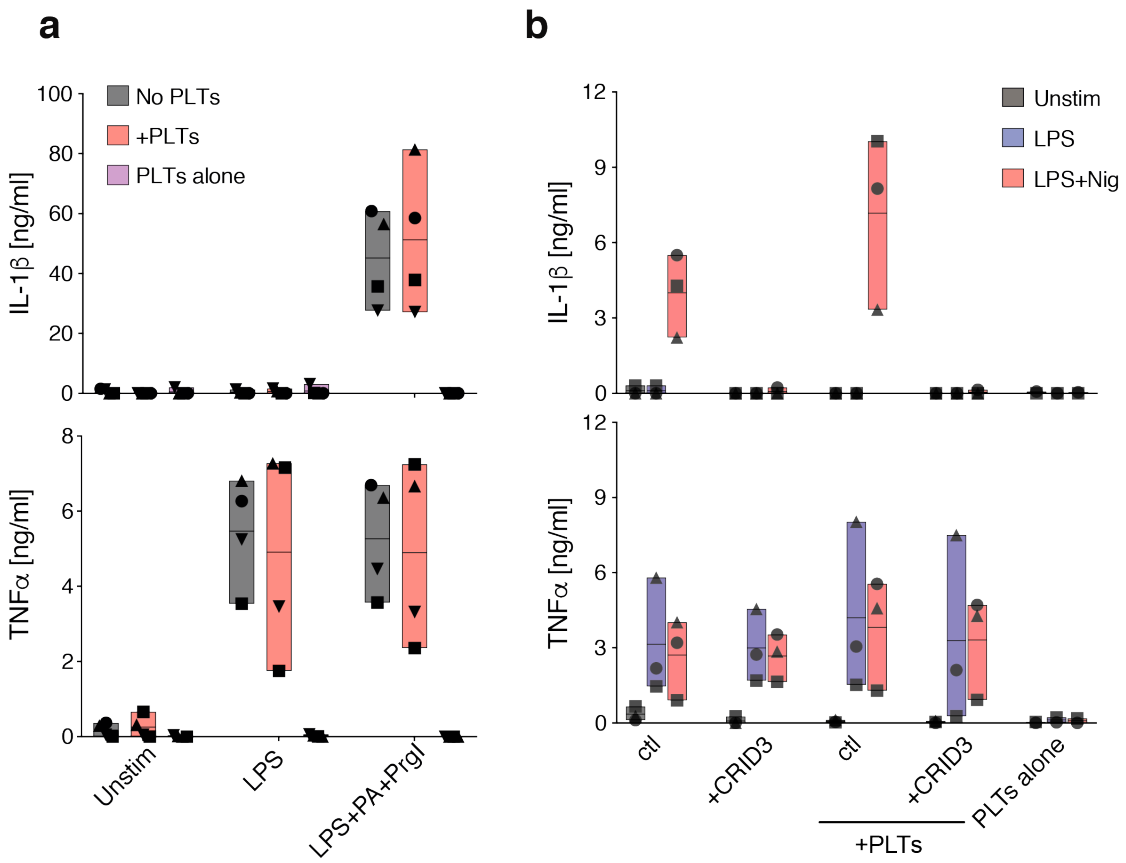

Figure 4.10 Platelets amplify the NLRC4 response in hMDMs and require functional NLRP3 to induce IL-1β amplification in NLRP3 activated hMDMs (**a**) HTRF® measurement of IL-1β and TNFα in cell-free supernatants of unstimulated (Unstim), LPS stimulated hMDMs (200 ng/ml, 3 hours), or LPS primed and PrgI and PA (2 µg/ml and 0.5 µg/ml respectively, 2 hours) treated hMDMs. Cells were cultivated alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs). (**b**) HTRF® measurement of IL-1β and TNFα in cell-free supernatants of unstimulated (Unstim), LPS stimulated hMDMs (200 ng/ml, 3 hours), or LPS primed and nigericin (Nig, 10 µM, 90 minutes) activated hMDMs. Cells were cultured as in **a.** The NLRP3 inhibitor CRID3 (2.5 µM) was added to the macrophage-platelet co-culture 30 minutes prior nigericin stimulation. Floating bars (with mean and minimum to maximum values) are shown from pooled data from four (**a**) or three (**b**) independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

Taken together, these results show that the platelet-mediated IL-1β boosting effect in hMDMs is dependent on a functional NLRP3 and indicate that platelets do not enhance IL-1β secretion through NLRP3 independent mechanism. Also, platelets enhance IL-1β secretion in NLRC4 activated hMDMs, although only to a small degree. Further experiments will be necessary to determine the impact of this effect.

4.1.6 The platelet-mediated IL-1β amplification in inflammasome activated macrophages is contact-independent

For a long time, macrophage function has been linked to the phagocytosis of cells and cellular debris at the inflammatory site or during wound healing[139].

Macrophages can ingest aged platelets as a clearance mechanism[140] and phagocytosis of activated platelets enhances LPS-induced cytokine secretion from macrophage[104]. These reports suggest that the platelet-mediated IL-1β amplification in inflammasome-activated macrophages could be caused by phagocytosis of platelets.

To verify this hypothesis, I employed confocal microscopy to visualize hMDM co-cultured with human platelets after inflammasome activation (Fig. 4.11a). Half of the hMDMs were pre-treated with cytochalasin D, a potent actin polymerization inhibitor, to prevent phagocytosis of platelets. Cells were primed with LPS and activated with nigericin for NLRP3 inflammasome assembly. Afterwards they were fixed and stained with fluorescently labeled wheat germ agglutinin (WGA) to visualize the plasma membrane and with fluorescent antibodies against CD41 to stain the platelets.

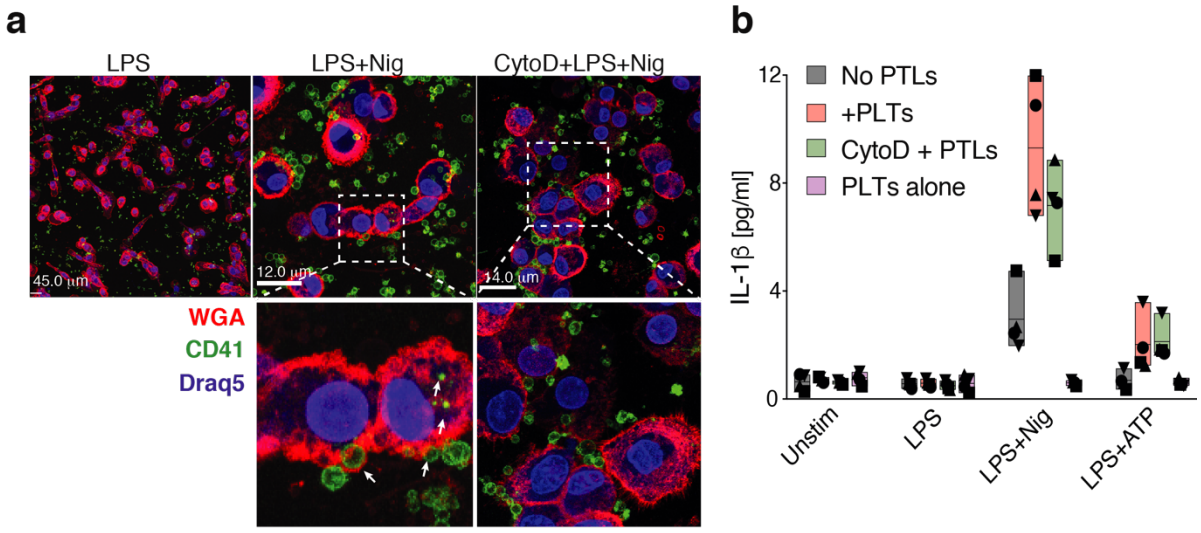

Figure 4.11 Platelet-mediated cytokine amplification is independent of macrophage phagocytosis. (**a**) Confocal imaging of hMDMs that were co-cultured with platelets (50:1 ratio PLTs to hMDMs). HMDMs were pre-treated or not with Cytochalasin D (CytoD, 50 µM, 30 minutes) before platelet addition. Co-cultures were primed with LPS (200 ng/ml, 3 hours) and activated with nigericin (Nig, 10 µM, 90 minutes). Nuclei were stained with Draq5 (blue), the plasma membrane with WGA (red) and platelets with anti-CD41-FITC labelled antibodies (green). The size of the scale bar is indicated in the figure. (**b**) HTRF® measurement of IL-1β in cell-free supernatants of hMDMs cultured and treated as in **a**. Additionally, cells were also activated with ATP (5 mM, 90 minutes) after LPS priming. Floating bars (with mean and minimum to maximum values) are shown from pooled data from four independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

Confocal microscopy revealed that hMDMs phagocytosed platelets in co-culture after NLRP3 inflammasome activation (Fig. 4.11a). This phagocytosis was blocked by pre-treatment of hMDMs with cytochalasin D, so that no platelets (CD41+ cells) were visible inside of hMDMs anymore (Fig. 4.11a). To understand whether this phagocytosis of platelets by hMDMs leads to the observed IL-1β amplification after inflammasome activation, I used the same

experimental setup to quantify IL-1β cytokine secretion in cell-free supernatants by HTRF®. Interestingly, platelets still amplified IL-1β secretion from inflammasome activated hMDMs even after pre-treatment with cytochalasin D (Fig. 4.11b), although it clearly blocked phagocytosis (Fig. 4.11a). However, the IL-1β amplification was decreased by approximately 20 % in hMDMs pre-treated with cytochalasin D in comparison to untreated hMDMs (Fig. 4.11b). Thus, the observed platelet-dependent IL-1β amplification in hMDMs is not predominantly phagocytosis dependent.

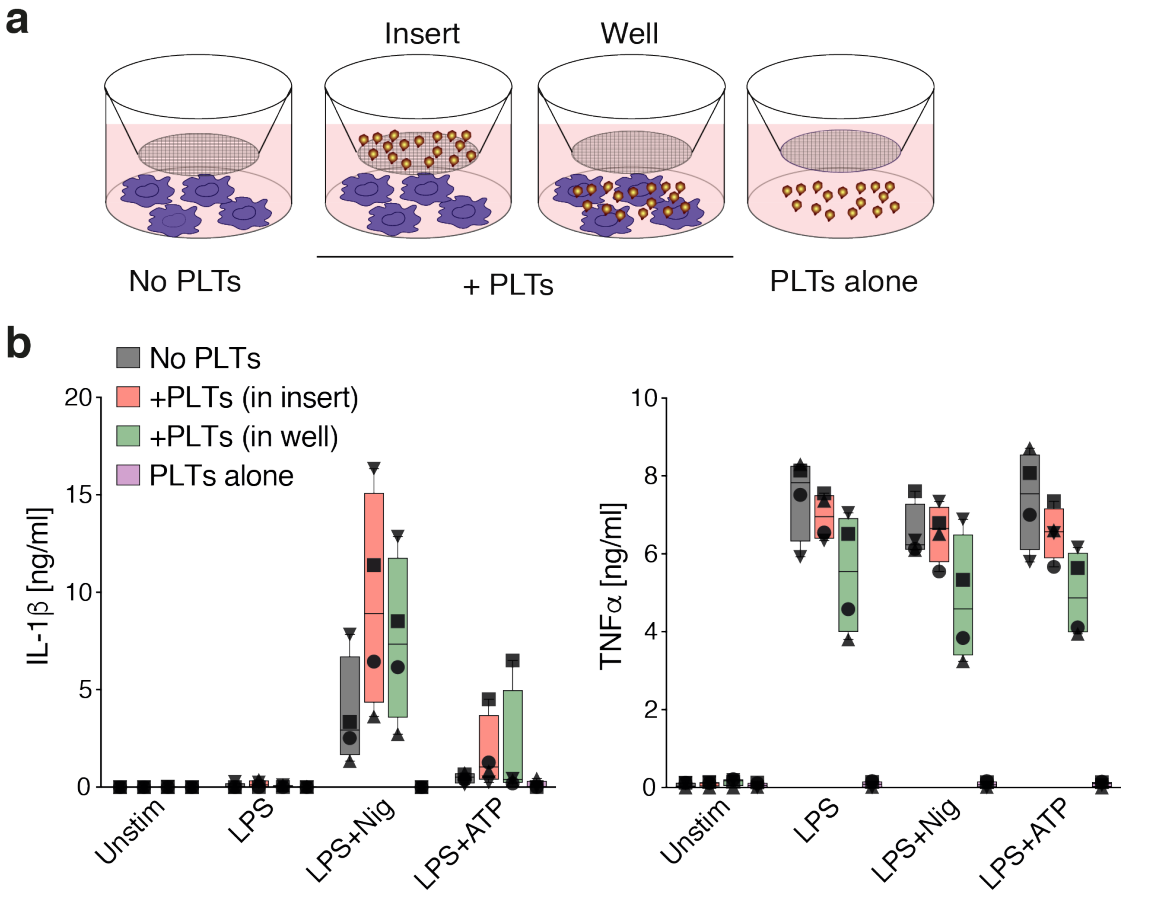

Figure 4.12 Platelet-mediated IL-1β amplification from NLRP3 activated hMDMs is contact-independent. (**a**) Schematics of the transwell system with hMDMs seeded in the lower wells either cultured alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs) in the well or in the insert, separated by a 0.4 µm pore. Platelets cultured alone (PLTs alone) served as a control. (**b**) HTRF® measurement of IL-1β and TNFα levels in cell-free supernatants of cell cultured as shown in **a**. Cells were either left unstimulated (Unstim), or were primed with LPS (200 ng/ml, 3 hours), or LPS primed and nigericin activated (Nig, 10 µM, 90 minutes). Floating bars (with mean and minimum to maximum values) are shown from pooled data from four independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

Next, I wanted to understand better to what extent physical contact between platelets and hMDMs is necessary for induction of the IL-1β boosting effect. To test this, I employed a transwell system with platelets and hMDMs either seeded alone in single cultures, in direct co-

cultures or in transwell cultures separated by a 0.4 µm pore sized membrane (Fig. 4.12a) and activated the NLRP3 inflammasome (LPS + nigercin). To assure that platelets could not cross the 0.4 µm pore sized membrane, I checked the wells by light microscopy after each experiment. Cytokine secretion was measured in cell-free supernatants by HTRF®.

Platelets induced IL-1β amplification in hMDMs both in direct and transwell co-cultures after NLRP3 inflammasome activation (LPS + nigericin or LPS + ATP) (Fig. 4.12b). In contrast, TNFα release was only diminished when platelets interacted with hMDMs directly (Fig. 4.12b). These results indicate that cellular contact between platelets and hMDMs is not necessary to induce the IL-1β boosting effect and that soluble factors secreted by platelets are decisive.

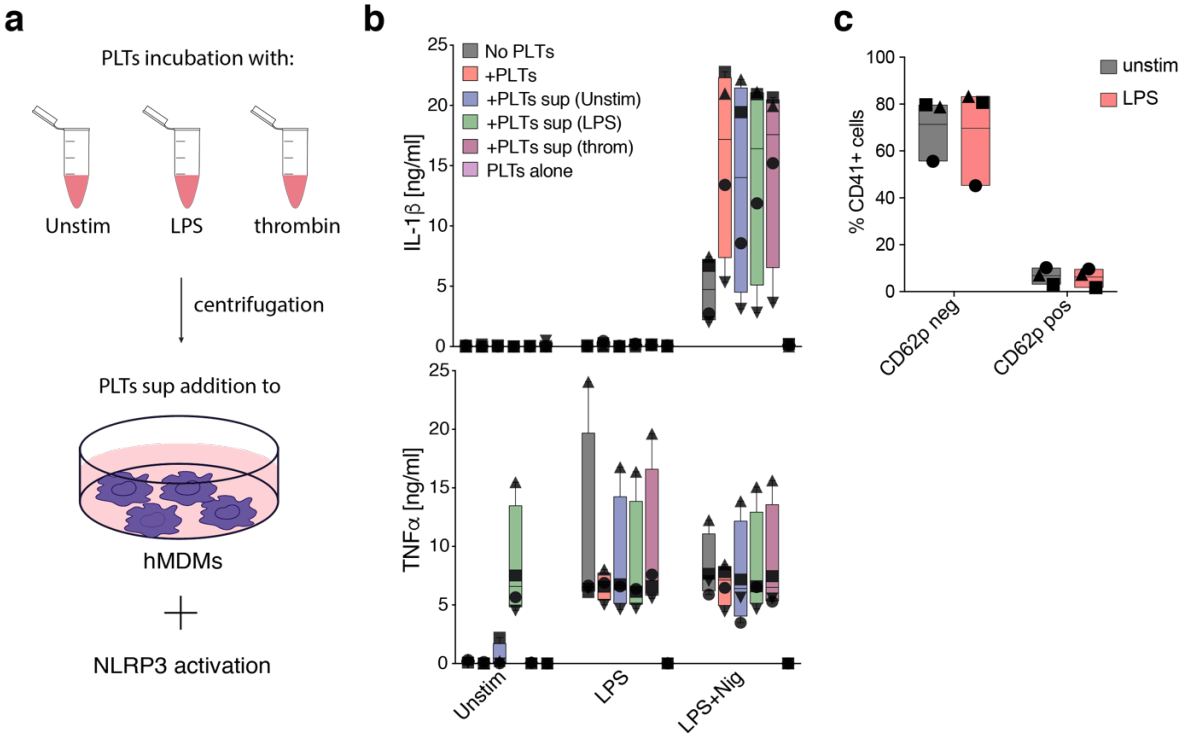

Figure 4.13 Platelet supernatant boosts IL-1β secretion from NLRP3 activated hMDMs. (**a**) Schematics of platelet supernatant generation and addition to hMDMs. Platelet supernatant (PLTs sup) was generated from unstimulated (Unstim) or LPS (200 ng/ml) or thrombin (0.1 U/ml) stimulated platelets (5 x 10^7/ml, for 3 hours). Platelet supernatant was added to hMDMs and cells were primed with LPS (200 ng/ml, 3 hours) and activated with nigericin (Nig, 10 µM, 90 minutes). (**b**) HTRF® measurement of IL-1β in cell-free supernatant of hMDMs cultured with platelets (+PLTs, 50:1 ratio PLTs to hMDMs) or with platelet supernatant generated as described in **a**. Floating bars (with mean and minimum to maximum values) are shown from pooled data from four independent experiments. (**c**) Flow cytometry analysis of the surface expression of CD41 (platelet marker) and CD62p (P-selectin, platelet activation marker) in platelets after supernatant generation. The gating strategy included doublet discrimination and matching isotype controls were used as negative controls. Floating bars (with mean and minimum to maximum values) are shown from pooled data from three independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

To interrogate the role of soluble, platelet-released factors, I generated platelet supernatants by incubating freshly isolated human platelets with LPS or thrombin for three hours. Supernatants from unstimulated platelets were prepared as a control. Afterwards, platelet supernatants instead of platelets were added to hMDMs before the start of the NLRP3 inflammasome assay (Fig. 4.13a). IL-1β secretion was quantified by HTRF® in cell-free supernatants as before (Fig. 4.13b). Additionally, platelets were analyzed for expression of the platelet activation marker P-selectin after platelet supernatant generation by flow cytometry to see whether LPS treatment caused their activation (Fig. 4.13c).

Surprisingly, I observed that supernatants from unstimulated platelets were as potent in inducing IL-1β amplification in hMDMs as freshly isolated platelets or as supernatants from LPS or thrombin activated platelets (Fig. 4.13b). TNFα release was similar to macrophages without platelets in all conditions, except when platelet supernatants from LPS stimulated platelets were added (+PLTs sups (LPS)). Here, LPS was already present in the platelet supernatant, leading to TNFα secretion from unstimulated hMDMs (Fig. 4.13b). Importantly, IL-1β secretion from hMDMs after inflammasome stimulation was unaffected by direct thrombin addition (Fig. S3). Thus, the effect observed after transfer of thrombin-activated platelet supernatants (+PLTs sup (throm)) was not caused by thrombin stimulation of hMDMs. Interestingly, no P-selectin upregulation was observed when I analyzed platelets after supernatant generation by flow cytometry, suggesting that no α-granule shedding took place during supernatant generation (Fig. 4.13c).

From these results I conclude that the platelet-mediated IL-1β amplification by inflammasome activated hMDMs is contact-independent and mediated by one or more soluble factors, most likely not secreted from platelet α-granules but instead released by platelets independent of their activation status. In the following sections, I will first investigate how platelets and platelet supernatant influence NLRP3 activation in hMDMs. Then, I will elucidate the nature of the soluble factor in platelet supernatant by verifying the role of α-granules and by testing the involvement of different well-known platelet-derived factors.

4.1.7 Platelets enable inflammasome amplification in hMDMs through transcriptional regulation of NLRP3 and IL-1β

Activation of the NLRP3 inflammasome requires two distinct steps in macrophages. First, a priming signal induces the transcription of IL-1β and NLRP3, before a second activation signal, such as K+ efflux and ATP, leads to NLRP3 inflammasome assembly, caspase-1

recruitment and maturation of IL-1 cytokines[36]. I wondered which level of NLRP3 inflammasome activation was affected in hMDMs by platelet addition: the transcriptional level during priming or the post-translational level during activation of the NLRP3 inflammasome.

To investigate if platelets influence the priming step, I co-cultured unprimed NLRP3 overexpressing immortalized mouse macrophages (iMacs) with human platelets and stimulated them with nigericin (Fig. 4.14a). Due to NLRP3 overexpression in these cells, formation of an NLRP3 inflammasome can be achieved without a priming step. Since the priming step would still be necessary to induce pro-IL-1β, I used a Caspase-Glo® 1 Inflammasome Assay instead to selectively measure caspase-1 activity as a readout for inflammasome activation in these cells. Additionally, I quantified IL-1β secretion by HTRF® after NLRP3 inflammasome activation (LPS + nigericin) to assure the functionality of NLRP3 overexpressing iMacs in this trans-species experiment and I observed a similar IL-1β amplification phenotype as in hMDMs (Fig.4.14b).

Although the co-culture of NLRP3 overexpressing iMacs with platelets showed the same IL-1β amplification effect as hMDMs (Fig.4.14b), addition of platelets to iMacs did not enhance the caspase-1 activity of these cells (Fig.4.14a). This suggests that platelets act during the priming stage of NLRP3 activation in hMDMs, enabling transcription of inflammasome components, which can then leads to IL-1β amplification. To confirm this hypothesis, I performed a qPCR analysis of hMDMs cultured with or without platelet supernatant and analyzed the expression of pro-IL-1β, NLRP3, ASC and pro-caspase-1 after LPS stimulation. Here, I used platelet supernatants instead of platelets to minimize possible RNA contamination by platelets.

Indeed, I observed that addition of platelet supernatant to hMDMs amplified their pro-IL-1β and NLRP3 expression (Fig. 4.14c). Surprisingly, even platelet supernatant from unstimulated platelets was as efficient as LPS priming to induce IL-1β and NLRP3 in hMDMs alone. The expression of pro-caspase-1 (CASP1) and ASC (Pycard) was not affected by addition of platelet supernatant (Fig. 4.14c). I therefore conclude that platelets regulate NLRP3 inflammasome activation at least partially on the transcriptional level by enhancing IL-1β and NLRP3 expression in hMDMs.

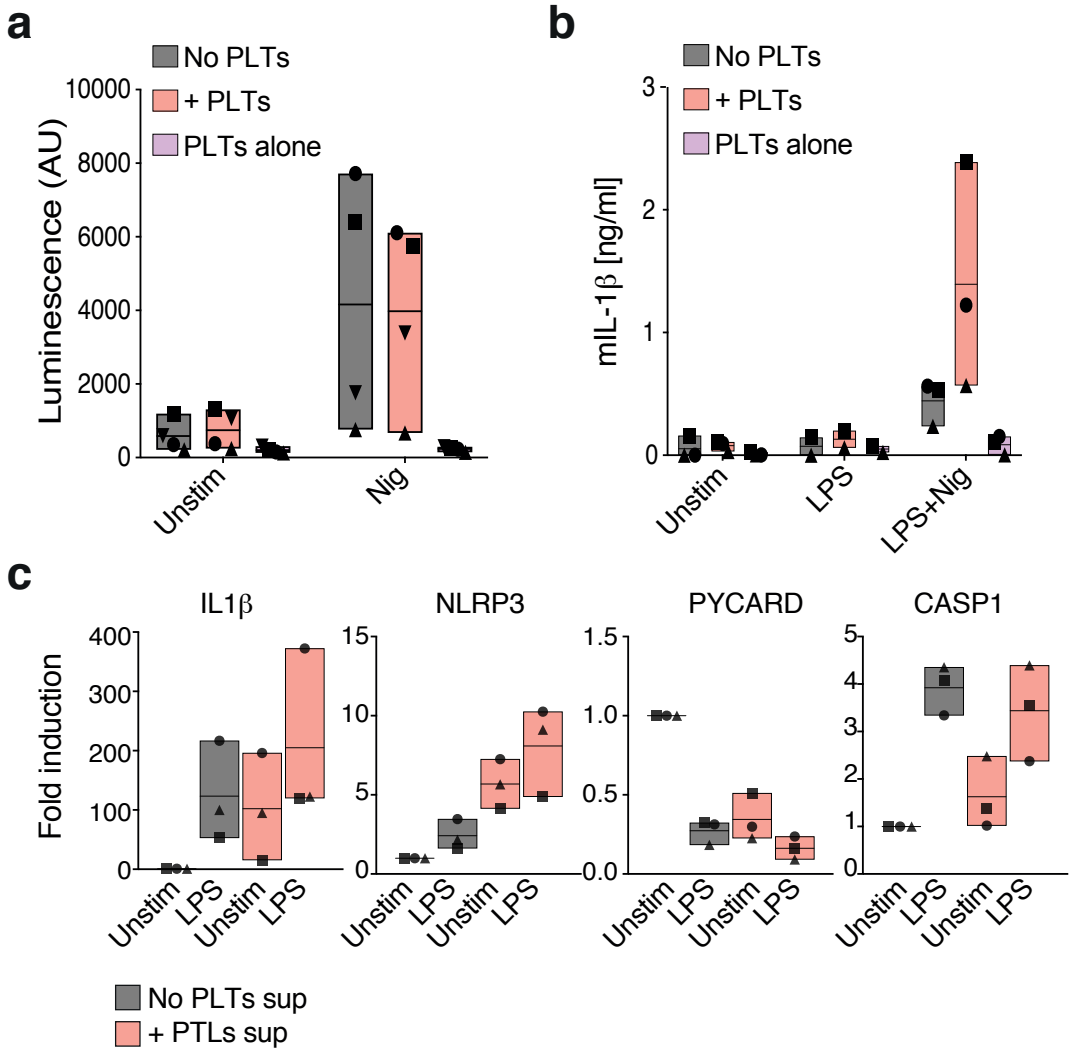

Figure 4.14 Platelets transcriptionally regulate NLPR3 activation in hMDMs. (**a**) Caspase-1 reporter luciferase activity of the luminogenic caspase-1 specific substrate, Z-WEHD-aminoluciferin in cell-free supernatants of NLRP3-overexpressing immortalized mouse macrophages. Cells were left unstimulated (Unstim), or were activated with nigericin (Nig, 10 µM, 90 minutes) without LPS priming. Cells were cultivated alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs). (**b**) HTRF® measurement of IL-1β and TNFα in cell-free supernatants of the same cells as in **a**. Cells were either left unstimulated (Unstim), primed with LPS (200 ng/ml, 3 hours) or LPS primed and activated with nigericin (Nig, 10 µM, 90 minutes). (**c**) QPCR analysis of the expression of the indicated genes in hMDMs cultured alone (No PLT sups) or in the presence of supernatant generated from unstimulated or LPS (200 ng/ml, 3 hours) stimulated platelets. Cells were left untreated or stimulated with LPS (200 ng/ml, 3 hours). Floating bars (with mean and minimum to maximum values) are shown from pooled data from four (**a**) or three (**b** and **c**) independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

Next, I wondered how this transcriptional up-regulation impacts the NLRP3 inflammasome activity. To this end, I first quantified the number of ASC specks formed after inflammasome activation in hMDMs after platelet addition by wide-field fluorescent microscopy. HMDMs cultured alone or co-cultured with platelets were fixed after inflammasome activation and stained with a fluorescently labelled anti-ASC antibody and the fluorescent dye Hoechst to visualize DNA (Fig. 4.15a-b).

The imaging showed formation of ASC specks after NLRP3 inflammasome activation in hMDMs, which was amplified after the addition of platelets (Fig. 4.15a). Quantification of the images showed a 50% increase in ASC speck formation in hMDMs co-cultured with platelets (Fig. 4.15b). HMDMs stained with isotype matched IgG controls as well as platelets cultured alone did not show any ASC specks (Fig. 4.15a).

In line with increased formation of ASC specks and enhanced caspase-1 activity, Dr. Lucas Ribeiro (Institute of Innate Immunity, University Bonn) visualized IL-1β maturation at the protein level by western blot and assessed caspase-1 activity by luminescence using the Caspase-Glo® 1 Inflammasome Assay. Dr. Lucas Ribeiro observed an increased amount of pro-IL-1β (31kDa) but also cleaved, mature IL-1β (17kDa) in cell-free supernatants from inflammasome activated hMDMs co-cultured with either platelets or unstimulated platelet supernatant (Fig. 4.12b, see bands at p31 kDa or p17 kDa). Pro-IL-1β was also detected after LPS priming in hMDMs, which might be due to cell-death caused by the high LPS dose used (200 ng/ml). Moreover, caspase-1 activation, measured by luminescence, was also increased in platelet-hMDM co-cultures after inflammasome activation (Fig. 4.15c). Consistent with earlier results, platelets alone did not show any IL-1β protein expression.

Altogether, these findings support the conclusion that platelets increase NLRP3 activation during priming through the enhanced transcription of NLRP3 and pro-IL-1β. This leads to increased inflammasome activity in hMDMs, evidenced by increased ASC speck formation, caspase-1 activation, and IL-1β maturation.

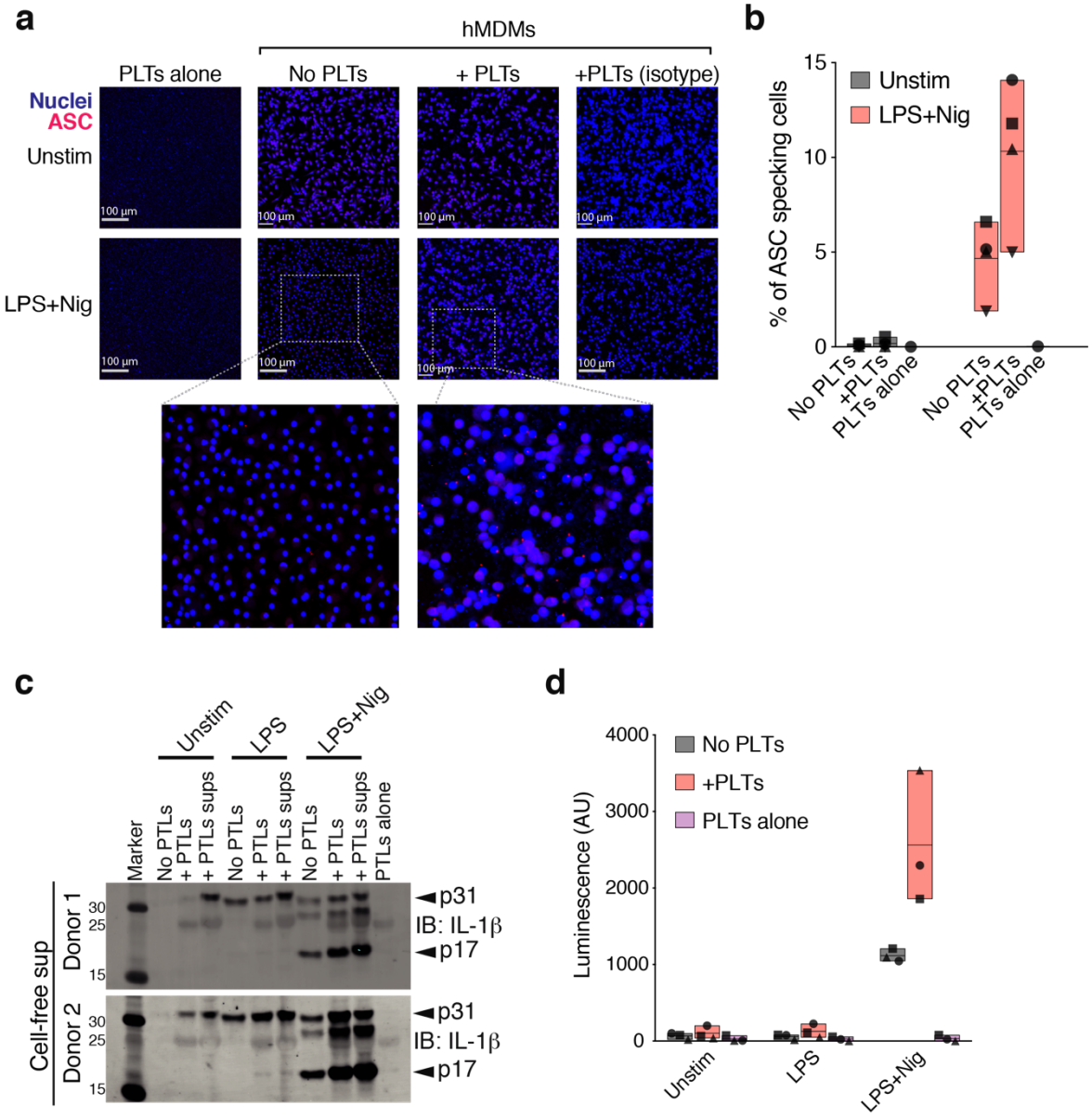

Figure 4.15 Platelets boost NLRP3 activation in hMDMs. (**a**) Confocal microscopy and (**b**) imaging quantification of ASC specks in LPS primed (200 ng/ml) and nigericin activated (10 µM, 45 minutes) hMDMs. Cells were either cultured alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs). Platelets alone (PLTs alone) served as a control. Floating bars (with mean and minimum to maximum values) are shown from pooled data from three independent experiments. (**c**) Immunoblotting for IL-1β in cell-free supernatants of unstimulated (Unstim), LPS primed (LPS 200 ng/ml, 3 hours) or LPS primed and nigericin activated (Nig, 10 µM, 90 minutes) hMDMs cultured alone (No PLTs), in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs) or in the presence of platelet supernatant (+PLTs sup). Data from two different donors are shown. (**d**) Caspase-1 reporter luciferase activity of the luminogenic caspase-1 specific substrate, Z-WEHD-aminoluciferin in cell-free supernatants of hMDMs cultered and treated as in **a**. Floating bars (with mean and minimum to maximum values) are shown from pooled data from three independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors. Experiments in **c** and **d** were carried out by Dr. Lucas Ribeiro (Institute of Innate Immunity, University Bonn).

4.1.8 The platelet-mediated regulation of NLRP3 priming in hMDMs is TLR4 independent

After observing that platelets license inflammasome amplification in hMDMs through transcriptional regulation of NLRP3 and IL-1β, I wondered whether this effect was dependent on LPS priming through TLR4. To this end, I primed hMDMs with the TLR2 (Pam3CSK4) or TLR7/8 (R848) agonists instead of LPS and examined whether platelet addition would still boost IL-1β after NLRP3 activation.

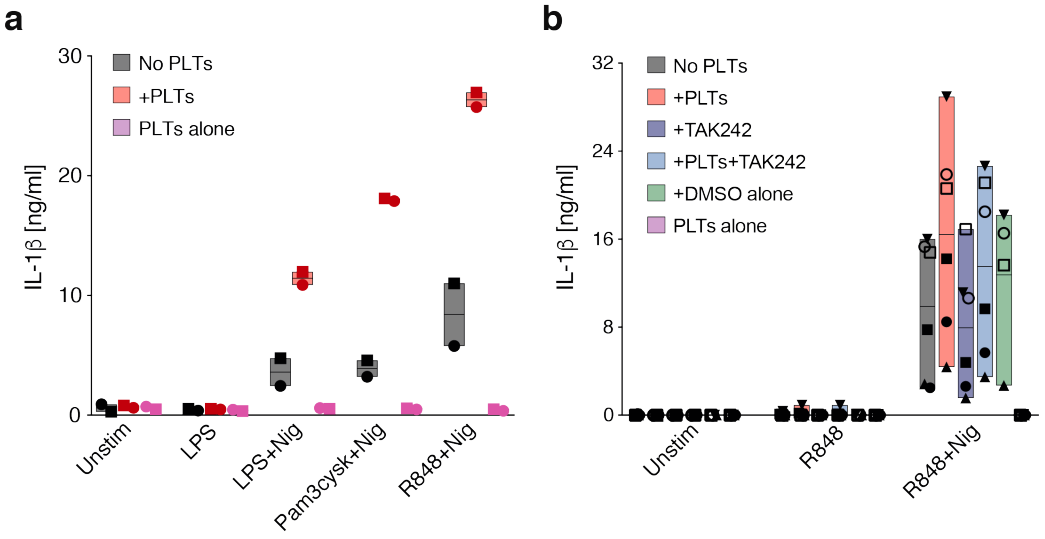

Figure 4.16 Platelet-mediated cytokine amplification in NLPR3 activated hMDMs is TLR4 independent. (**a**) HTRF® measurement of IL-1β in cell-free supernatants of unstimulated (Unstim), LPS stimulated hMDMs (200 ng/ml, 3 hours), or LPS or Pam3CSK4 (1 µg/ml, 3 hours) or R848 (10 µM, 3 hours) primed and nigericin activated (Nig, 10 µM, 90 minutes) hMDMs. Cells were cultivated alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs). Platelets alone (PLTs alone) served as a control. (**b**) HTRF measurements of IL-1β in cell-free supernatants of cells cultured as in **a**. Cells were left unstimulated (Unstim), primed with R848 (10 µM, 3 hours) or R848 primed and nigericin activated (Nig, 10 µM, 90 minutes). TAK242 (0.5 µg/ml) was added to the co-culture 5 minutes prior to the start of the assay. Floating bars (with mean and minimum to maximum values) are shown from pooled data from four (**a**) or four to six (**b**) independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

Interestingly, I still observed platelet-mediated IL-1β amplification after priming with the TLR2 agonist Pam3CSK4 or the TLR7/8 agonist R848, even to a higher degree in comparison with LPS priming (Fig. 4.16a). To confirm these findings, I also blocked TLR4 signaling in hMDMs with or without platelets using Resatorvid (TAK242), a small-molecule inhibitor of TLR4[141,142]. First, I examined IL-1β secretion after NLRP3 inflammasome activation with LPS priming to control TLR4 blocking by TAK242 treatment (Fig. S2). As expected, no IL-1β secretion was observed after TAK242 treatment in LPS primed hMDMs, whereas TLR7/8 priming with R848 induced solid IL-1β secretion on activation with nigericin (Fig. S2). Having

shown that TAK242 treatment efficiently blocked TLR4 activation, I next measured IL-1β secretion from hMDMs co-cultured with or without platelets after TLR7/8 priming and nigericin treatment (Fig. 4.16b). TAK242 treatment did not prevent IL-1β amplification in hMDM-platelet co-cultures (Fig. 4.16b), indicating that the platelet effect on hMDM is not restricted to TLR4 priming and not mediated by TLR4. Together with my previous results, I conclude that the platelet-mediated IL-1β amplification in hMDMs is independent of TLR4 and can also be observed after TLR2 or TLR7/8 priming before NLRP3 inflammasome activation.

4.1.9 The platelet-released factor is most likely α-granule independent

Having established that the platelet-mediated IL-1β amplification in NLRP3 activated hMDMs is contact-independent and mediated by a platelet-derived soluble factor(s), I wanted to verify the role of platelet α-granules in my experimental setting next. Platelet α-granules are the most abundant form of platelet granules. They mainly contain soluble proteins, which are involved in inflammation, hemostasis and wound healing[143]. A lack of α-granules and their contents is associated with the so-called gray platelet syndrome (GPS), a rare bleeding disorder in which the neurobeachin-like2 (NBEAL2) gene is mutated. *Nbeal2*-deficient mice (*Nbeal2*$^{-/-}$) display defective α-granule biogenesis in megakaryocytes and no α-granules in platelets, thus mirroring the characteristics of human GPS[18].

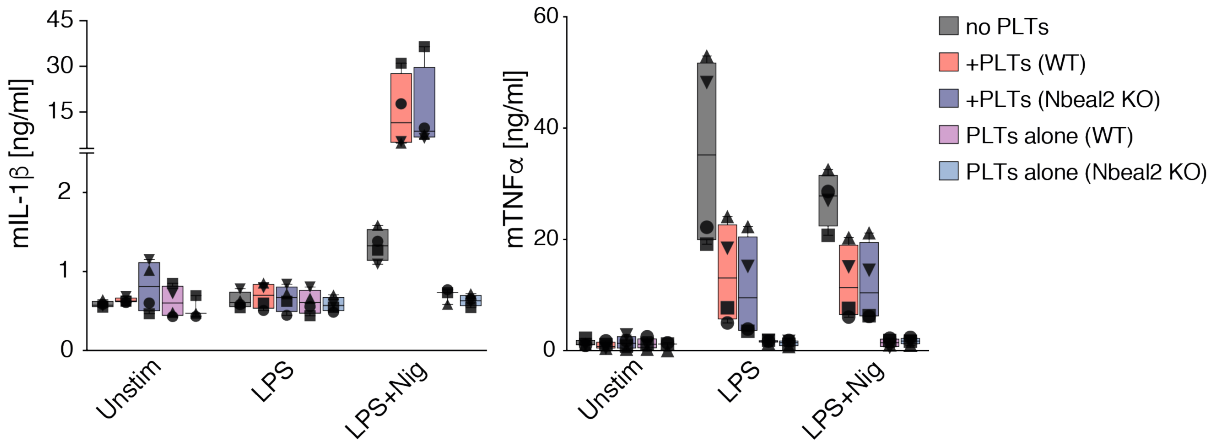

Figure 4.17 Platelets from *Nbeal2* KO mice still boost IL-1β secretion from NLRP3 activated BMDMs. HTRF® measurement of mouse IL-1β and TNFα in cell free supernatant of wild-type (WT) BMDMs cultivated alone or in the presence of platelets (+PLTs, 5:1 ratio PLTs to BMDMs) from wild-type (WT) or *Nbeal2* knockout mice (Nbeal2 KO). Cells were primed with LPS (200 ng/ml, 3 hours) and activated with nigericin (10 µM, 90 minutes). Platelets cultured alone served as a control. Floating bars (with mean and minimum to maximum values) are shown from pooled data from four independent experiments. Each symbol represents the average of technical triplicates from different mice.

To investigate the origin of the soluble factor or factors that amplify the macrophage NLRP3 inflammasome response, I added platelets from *Nbeal2*$^{-/-}$ mice to wild-type BMDMs. Interestingly, I found that α-granule deficient platelets still boosted the IL-1β response of inflammasome activated BMDMs, while inhibiting TNFα secretion, (Fig. 4.17) which is in line with results shown in Fig. 4.3. These results also confirm previous observations, in which platelets that lacked P-selectin expression after LPS stimulation (Fig.4.13c) were still capable of amplifying macrophage IL-1β response, and suggest that the soluble, platelet-derived factor is not stored in α-granules.

Supporting this finding, the most common platelet-derived cytokines and cytokines found in the multiplex analysis (Fig. 4.4), which are stored in platelet α-granules, were excluded to play role in the platelet-mediated IL-1β amplification in hMDMs using different experimental approaches.

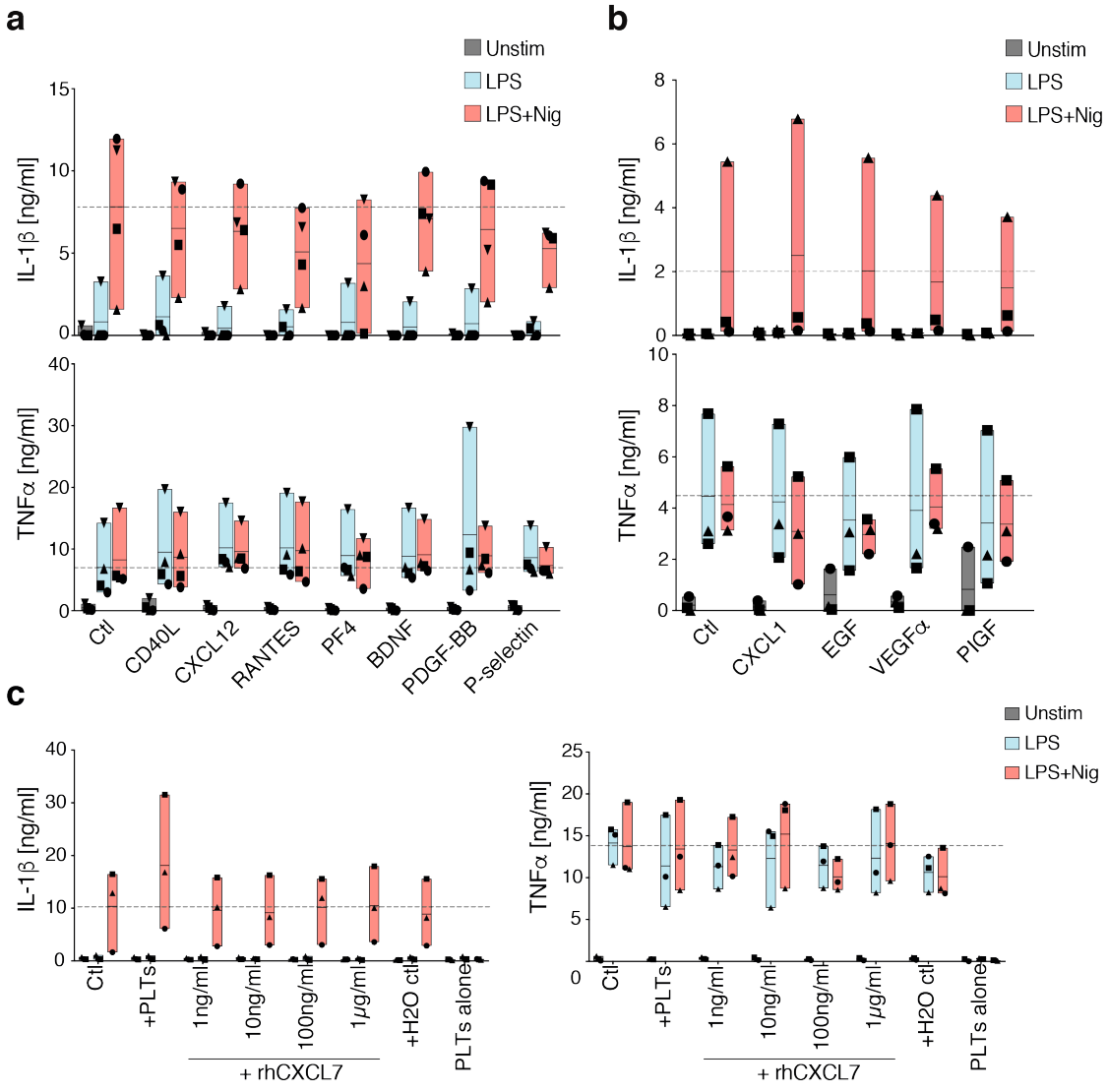

Figure 4.18 Recombinant human platelet proteins do not increase IL-1β secretion by NLRP3 activated hMDMs. (**a**) HTRF® measurement of IL-1β and TNFα in cell-free supernatants of unstimulated (Unstim), LPS stimulated hMDMs (200 ng/ml, 3 hours), or LPS primed and nigericin (Nig, 10 μM, 90 minutes) activated hMDMs. The following recombinant human proteins were added to hMDMs before the assay: CD40L (10 ng/ml), CXCL12 (1 ng/ml), RANTES (300 pg/ml), PF4 (50 ng/ml), brain-derived neurotrophic factor (BDNF) (5 ng/ml), PDGF-BB (500 pg/ml), P-selectin (40 ng/ml), (**b**) CXCL1 (100 pg/ml), EGF (200 pg/ml), VEGFα (200 pg/ml), PIGF (80 pg/ml) or (**c**) different concentrations of CXCL7 (1 ng/ml, 10 ng/ml, 100 ng/ml, 1 μg/ml). In **c** hMDMs were also co-cultured with platelets (+PLTs, 50:1 ratio PLTs to hMDMs). Platelets alone (PLTs alone) served as a control. Experiments in **a** and **b** were carried out by Nathalia Rosero (Institute of Innate Immunity, University Bonn).Floating bars (with mean and minimum to maximum values) are shown from pooled data from three independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

I started by adding recombinant human proteins of CD40L, SDF-1α (CXCL12), RANTES, PF4, brain-derived neurotrophic factor (BDNF), PDGF-BB, P-selectin, GROα, EGF, VEGFα, PIGF-1, and CXCL7 directly to hMDMs to examine whether they could increase IL-1β secretion in hMDMs after NLRP3 inflammasome stimulation (Fi. 4.18a-c). The concentrations of recombinant proteins to be used in the experiment were either based on the amount of platelet-secreted cytokine found in my multiplex analysis (Fig.4.4), or they were determined either by literature search or by testing different concentrations. However, none of the recombinant proteins tested was able to boost IL-1β secretion in comparison to NLRP3 activated hMDMs (Fig. 4.18a-c). Also, TNFα secretion was not affected by the addition of recombinant proteins (Fig.4.18a-c).

Further, I employed blocking antibodies against CXCL7 or SDF-1α (CXCL12) and an inhibitor of RANTES (met-RANTES) [133] (Fig. 4.19a-b). CXCL7 is the most abundant platelet-secreted cytokine and known for its regulation of neutrophil recruitment[108,144]. The cytokine SDF-1α regulates monocyte function and differentiation[99] and RANTES deposition by platelets was shown to trigger monocyte arrest during inflammation[145]. Because of either their sheer abundance or their well-known effect on monocytes, I considered these platelet-derived cytokines as candidates for further testing. To this end, I added the blocking antibodies (CXCL7, CXCL12) or an IgG matched isotype control to cultures of macrophages with or without platelets before the start of the NLRP3 inflammasome assay. To inhibit RANTES signaling, hMDMs were pre-treated with the CCR5 inhibitor (met-RANTES) for 90 minutes prior to platelet addition and the start of the NLRP3 inflammasome assay. As met-RANTES leads to over 60 % internalization of the RANTES receptor CCR5 receptor after 90 minutes[133], I assumed that an effect would be visible after platelet addition if RANTES was involved.

Neither the CXCL7/12 blocking antibodies nor the RANTES signaling inhibitor diminished IL-1β amplification in hMDM-platelet co-cultures (Fig. 4.19a-b), suggesting that CXCL7, SDF-1α (CXCL12) and RANTES are not the platelet-derived soluble factors responsible for

IL-1β amplification. Together, these findings provide evidence that this platelet-derived amplification factor is most likely not α-granule derived.

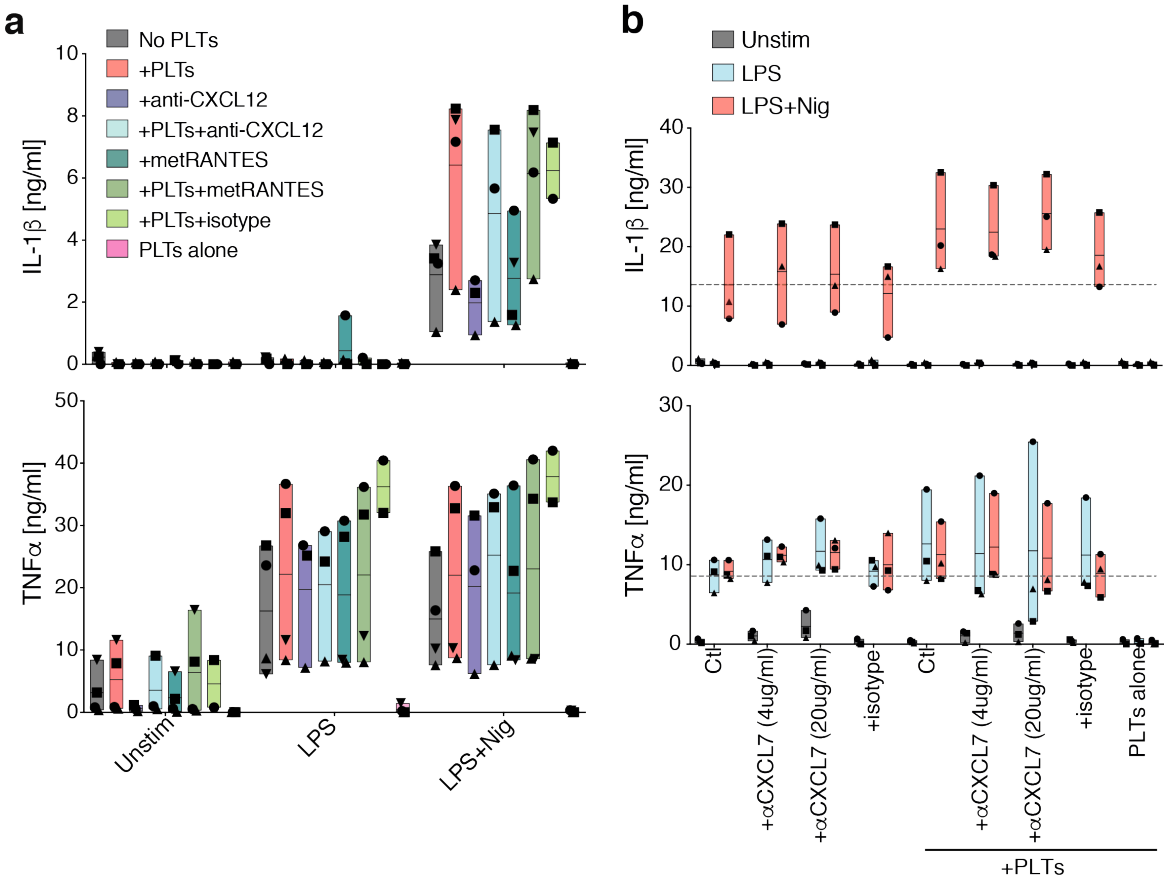

Figure 4.19 Blocking of CXCL12, RANTES or CXCL7 did not inhibit platelet-mediated IL-1β amplification by NLRP3 activated hMDMs. (**a**) HTRF® measurement of IL-1β and TNFα in cell-free supernatants of unstimulated (Unstim), LPS stimulated hMDMs (200 ng/ml, 3 hours), or LPS primed and nigericin (Nig, 10 µM, 90 minutes) activated hMDMs. Cells were cultivated alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs) as indicated. To block CCR5, hMDMs were pre-incubated with met-RANTES (10 ng/ml, 90 minutes) before the start of the assay. The antibodies against CXCL12 (10 µg/ml) or (**b**) CXCL7 (4 or 20 µg/ml as indicated) were added directly to the co-cultured cells before the start of the assay. Matching IgG isotype controls were added at the same concentrations as the respective blocking antibodies. Platelets alone (PLTs alone) served as a control. Floating bars (with mean and minimum to maximum values) are shown from pooled data from two to four independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

4.1.10 The IL-1β amplification in hMDMs is partly dependent on calcium but independent of ATP or ADP

Considering that the platelet-derived IL-1β amplification factor is most likely not α-granule derived and that platelet secretory effector molecules are mainly stored in either α- or dense granules, I hypothesized that platelet dense granule content could contain the soluble factor

inducing IL-1β amplification in NLRP3 activated hMDMs. In comparison to α-granules, dense granules contain much fewer molecules, mainly ADP, ATP, calcium and serotonin[146].

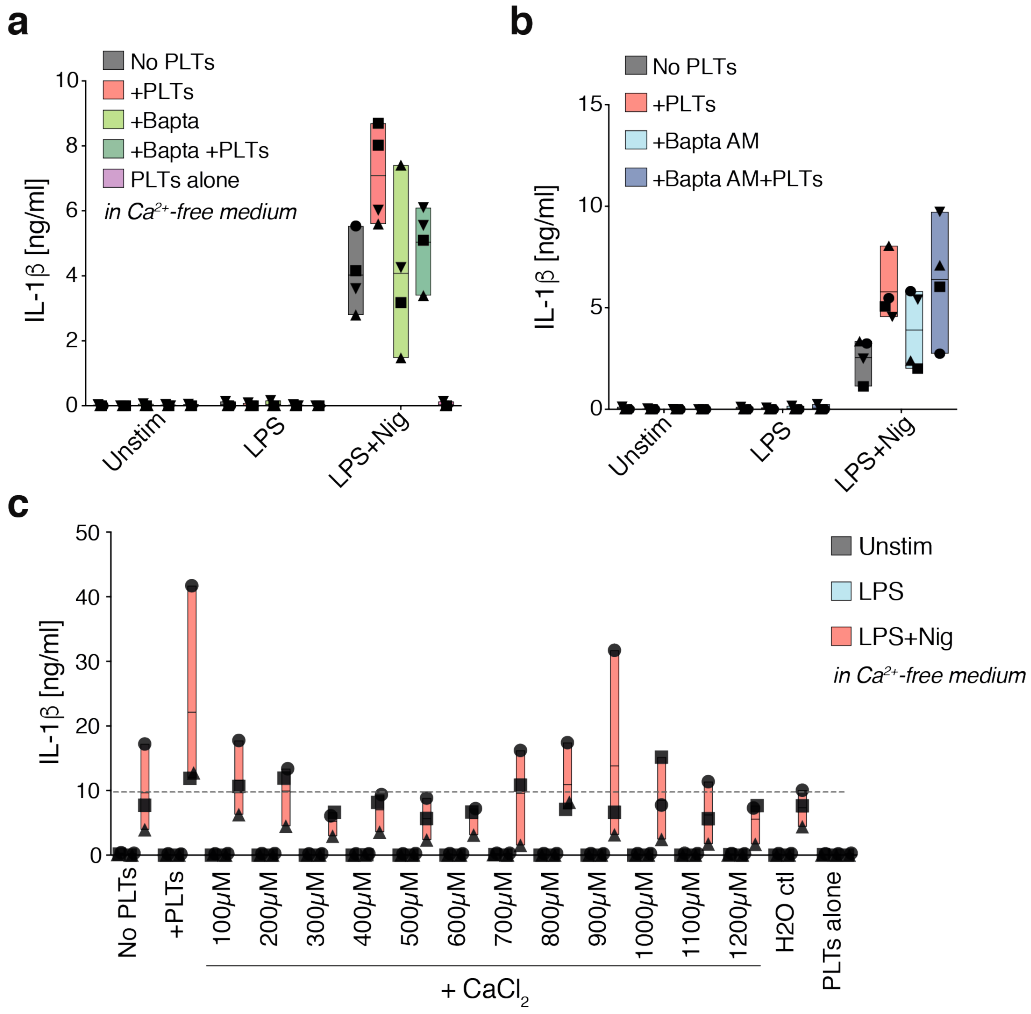

Figure 4.20 The platelet-mediated regulation of NLRP3 in hMDMs is partly dependent on extracellular calcium. (**a**) HTRF® measurement of IL-1β in cell-free supernatants of unstimulated (Unstim), LPS stimulated hMDMs (200 ng/ml, 3 hours), or LPS primed and nigericin (Nig, 10 µM, 90 minutes) activated hMDMs. Cells were cultivated alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs) as indicated. HMDMs alone or in the presence of platelets were treated with Bapta (0.5 mM) in calcium free medium (Ca^{2+}-free medium) or (**b**) with Bapta AM (5 µM) in normal RPMI medium before activation with nigericin as indicated. (**c**) HTRF® measurement of IL-1β in cell-free supernatants of hMDMs cultured and stimulated as described in **a**. Different concentrations of calcium chloride ($CaCl_2$) were added to hMDMs in Ca^{2+}-free medium before the start of the inflammasome stimulation. Floating bars (with mean and minimum to maximum values) are shown from pooled data from four (**a** and **b**) or three (**c**) independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

First, I investigated whether calcium was involved in the platelet-mediated effect in hMDMs. To this end, the calcium chelator BAPTA (Fig.4.20a) and the cell-permeant calcium chelator BAPTA AM (Fig. 4.20b) were used to examine extracellular and intracellular calcium involvement, respectively. I added the calcium chelators to macrophages cultured alone or with

platelets either in calcium-free medium (BAPTA) or in normal RPMI medium (BAPTA AM) after LPS priming and before NLRP3 activation.

BAPTA (Fig.4.20a) but not BAPTA AM (Fig. 4.20b) treatment led to reduced IL-1β amplification from hMDMs after NLRP inflammasome activation by approximately 30% in comparison to untreated cells. This indicates the involvement of extracellular calcium during the activation stage of inflammasome activation.

To further validate this finding, I added different concentrations of calcium chloride directly to hMDMs (Fig. 4.20c) in calcium-free medium instead of platelets. Surprisingly, calcium addition alone did not yield higher IL-1β secretion from inflammasome-activated hMDMs, whereas direct platelet addition to hMDMs in calcium-free medium reproduced the IL-1β amplification observed before (Fig. 4.20c).

These results suggest, that extracellular calcium is required but not sufficient to boost the IL-1β response in inflammasome-activated hMDMs by platelets, but could indirectly influence the effect, for instance through calcium sensitive proteins.

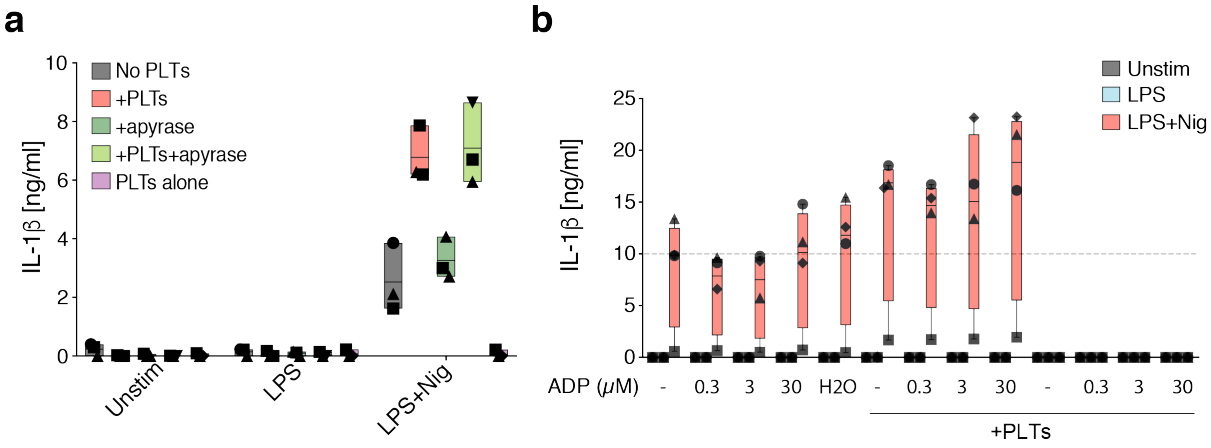

Figure 4.21 ADP and ATP are not involved in the platelet-mediated NLRP3 regulation of hMDMs. (**a**) HTRF® measurement of IL-1β in cell-free supernatants of unstimulated (Unstim), LPS stimulated hMDMs (200 ng/ml, 3 hours), or LPS primed and nigericin (Nig, 10 μM, 90 minutes) activated hMDMs. Cells were cultivated alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs) as indicated. HMDMs alone or in the presence of platelets were treated with apyrase (0.5 U/ml) before the start of the inflammasome assay. (**b**) HTRF® measurement of IL-1β in cell-free supernatants of hMDMs cultivated and stimulated as described in **a**. Different concentrations of ADP (0.3, 3, 30 μM) or a water control at the same dilution were added to hMDMs alone or in co-culture with platelets. Floating bars (with mean and minimum to maximum values) are shown from pooled data from three independent experiments in **a** and **b**. Each symbol in this figure represents the average of technical triplicates from different donors.

Next, I examined the dense granule resident molecules ATP or ADP. To this end, I added the ATPase apyrase to hMDMs cultured with or without platelets before the start of the inflammasome assay to hydrolyze both nucleoside triphosphates and diphosphates (Fig. 4.21). Interestingly, apyrase treatment had no effect on the platelet-mediated IL-1β amplification in

inflammasome-activated hMDMs in comparison to non-treated controls (Fig. 4.21a), excluding a role for ATP and ADP. Direct addition of ADP to hMDMs instead of platelets or together with platelets supported this finding. No difference in IL-1β production by inflammasome activated hMDMs with or without ADP treatment was observed (Fig. 4.21b). Direct addition of ATP to hMDMs was not possible, because ATP itself is an activator of the NLRP3 inflammasome via the P2X7 receptor[147,148].

Together, these findings provide evidence that the platelet-mediated IL-1β amplification in hMDMs is independent of ATP and ATP, but at least partially dependent on calcium.

4.1.11 The IL-1β amplification in hMDMs is independent of lipid mediators synthesized by LOX1 and COX1/2, and nucleic acids, but likely mediated by protein factors

My results suggest that a soluble, platelet-derived factor is involved in the regulation of inflammasome activity in hMDMs. As activated platelets are also important sources of inflammatory lipid mediators, I decided to test this further. During platelet activation, arachidonic acid derived from phospholipids can be oxidized by LOX and COX to form prostaglandins and other eicosanoid mediators[68]. The COX1/2 derived mediator prostaglandin E_2 (PGE_2) has been shown to boosts LPS-induced pro-IL-1β transcription and increases IL-1β release after ATP stimulation[149], while inhibiting TNFα production in BMDMs[149,150]. Additionally, Linke et al. recently linked PGE_2 secreted by activated platelets to decreased TNFα secretion from human monocytes and BMDMs[107]. Since these findings are similar to my results, they suggest the involvement of platelet-derived lipid mediators in the observed platelet-mediated IL-1β amplification of inflammasome activated hMDMs.

To investigate this hypothesis, freshly isolated, human platelets were pre-treated with either the COX1/2 inhibitor aspirin or the LOX inhibitor zileuton, before their addition to hMDMs and inflammasome activation (Fig. 4.22a) by Dr. Ribeiro. Interestingly, platelet pre-treatment with aspirin or zileuton did not affect the platelet-mediated IL-1β amplification by hMDMs (Fig. 4.22b), indicating that COX1/2 and LOX-induced lipid mediators do not play a role in the effect I observed.

As activated platelets have also been shown to release respiratory-competent mitochondria, which lead to the hydrolysis of inflammatory mediators such as mitochondrial DNA that promote leukocyte activation[76], I wondered whether mtDNA could be involved in the observed IL-1β amplification in hMDMs instead.

To test this, I added the nuclease BenzonaseR to hMDMs cultured with or without platelets to assess the involvement of nucleic acids. However, similar to lipid mediator inhibition, extracellular DNA digestion did not prevent IL-1β amplification in inflammasome activated hMDMs (Fig. 4.22c), indicating that the observed platelet-mediated effect is independent of nucleic acids.

These results together with the earlier findings, support the conclusion that the platelet-mediated effect is elicited by a soluble factor which is most likely not α-granule derived, not a COX1/2 or LOX derived lipid mediator, not ATP, ADP or a nucleic acids, but partly dependent on calcium. However, the molecular nature of the platelet-derived factor remains elusive after these experiments.

Finally, I performed heat inactivation experiments of platelet supernatants to determine if the platelet-derived factor was sensitive to heat and, thus, likely to be a protein. Unstimulated or LPS stimulated platelet supernatants were either heat inactivated at 80°C for 30 minutes or not, prior to their addition to hMDMs and the inflammasome assay. Strikingly, heat inactivation abolished the platelet-mediated IL-1β amplification in hMDMs completely (Fig. 4.22d), showing that the factor is indeed heat sensitive and likely of protein nature.

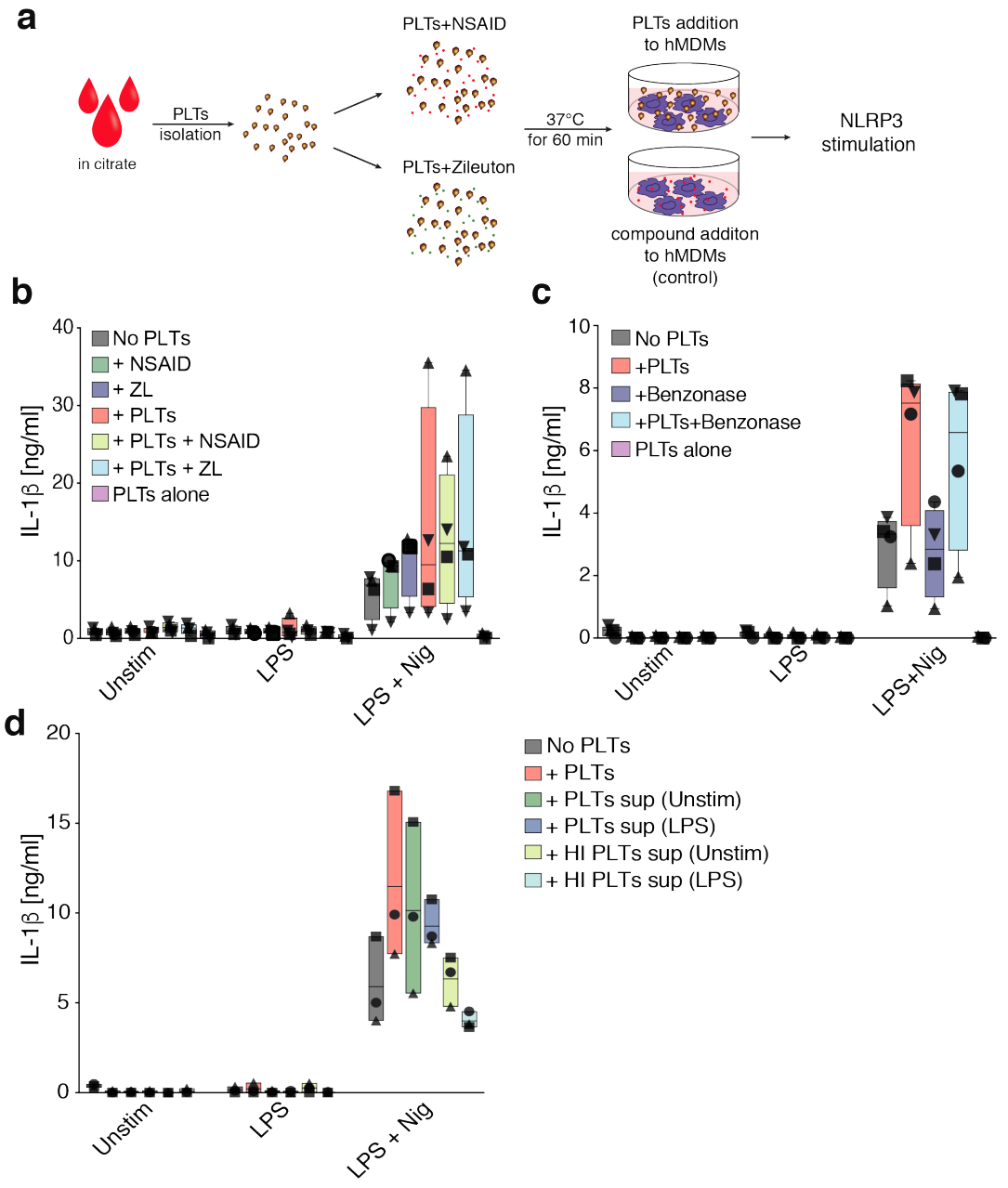

Figure 4.22 The platelet-mediated NLRP3 regulation in hMDMs is independent of COX1/2 or LOX derived lipid mediators, nucleic acids but of protein nature. (**a**) Schematic representation of the experimental setup with COX1/2 inhibitor (aspirin) or LOX inhibitor (zileuton) pre-treatment of platelets (PLTs). (**b**) HTRF® measurement of IL-1β in cell-free supernatants of unstimulated (Unstim), LPS stimulated hMDMs (200 ng/ml, 3 hours), or LPS primed and nigericin (Nig, 10 µM, 90 minutes) activated hMDMs. Cells were cultivated alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs), which were either pretreated with aspirin or zileuton (100 µM each, 60 minutes) or left untreated, as depicted in **a**. Aspirin and zileuton were also added to hMDMs directly as control. (**c**) HTRF® measurement of IL-1β in cell-free supernatants of hMDMs cultured alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs), which were stimulated as in **b**. HMDMs alone or in the presence of platelets were treated with Benzonase (1:20000 dilution) before the start of the inflammasome assay. (**d**) HTRF® measurement of IL-1β in cell-free supernatants of hMDMs cultivated alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs), platelet supernatant (+PLTs sup) or heat inactivated platelet supernatant (+HI PLTs sup, 80°C, 30 minutes) from unstimulated or LPS stimulated platelets. Cells were activated as in **b**. Floating bars (with mean and minimum to maximum values) are shown from pooled data from four (**b** and **c**) or three (**d**) independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors. The experiments in **b** were performed by Dr. Ribeiro (Institute of Innate Immunity, University Bonn).

4.1.12 RNA sequencing analysis identifies genome wide transcriptional changes in hMDMs upon platelet addition

So far, I found that platelet addition to hMDMs selectively boosts IL-1 cytokine and other proinflammatory cytokine/chemokine secretion after NLRP3 inflammasome activation (Fig. 4.3 and 4.4). Additionally, I showed that the putative factor is soluble, heat sensitive and that the IL-1β amplification effect is partly calcium dependent. Further, none of the different known platelet molecules I tested could be linked to the IL-1β amplification effect in inflammasome activated hMDMs.

To gain a better understanding of the scope of the changes in the hMDMs gene expression profile after platelet addition, I performed 3' mRNA sequencing experiments at the NGS core facility (Institute of Human Genetics, University Hospital Bonn).

HMDMs from four donors were either cultured alone, with freshly isolated human platelets from four different donors or with unstimulated platelet supernatants from two different donors and treated with LPS for 3 hours. Platelets supernatant addition served as control to account for platelet-released and platelet-derived RNA involvement. Cells were lysed directly after the assay and whole RNA was isolated. RNA sequencing analysis was then performed by André Heimbach and colleagues from the NGS core facility and the data was analyzed by myself using Partek®, but also independently by Dr. Susanne V. Schmidt (Institute of Innate Immunity, University Hospital Bonn) and my supervisor Prof. Bernardo Franklin (Institute of Innate Immunity, University Hospital Bonn).

Principal component analysis (PCA) showed clustering of different biological replicates according to their treatment (Fig. 4.23a), indicating that the variance in the data set is mostly attributed to treatment rather than to the biological replicates.

As expected, platelet addition to hMDMs caused numerous transcriptional changes. To visualize these transcriptional changes, I first analyzed differentially expressed genes (DEG) between unstimulated or LPS stimulated hMDMs with or without platelets (Fig. 4.23b). Both in unstimulated as well as LPS stimulated hMDMs, expression of a plethora of genes was significantly changed upon platelet addition: 657 DEGs for unstimulated and 890 DEGs for LPS stimulated hMDMs. One example is PF4, which is a platelet marker, indicating the presence of platelets in the co-culture (Fig. 4.23b).

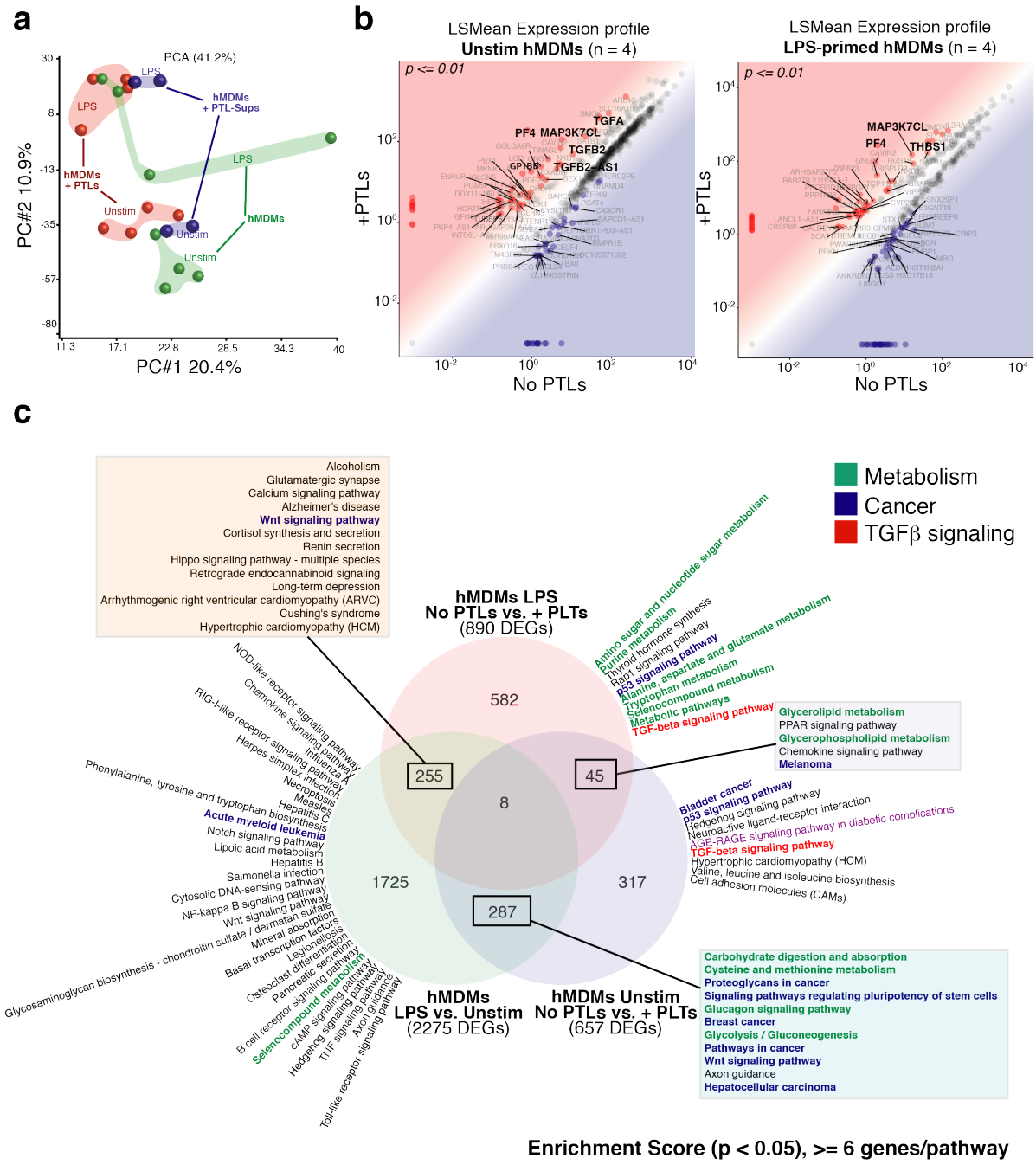

Figure 4.23 Platelets induce genome wide transcriptional changes in hMDMs. (a) Principal component analysis (PCA) of PC1 vs PC2 (18234 genes), including the following groups: hMDMs (n=4), hMDMs with platelets (+PLTs, 50:1 ratio PLTs to hMDMs, n=4) or hMDMs with platelet supernatant (+PLT sups, n=2). Cells were either unstimulated or stimulated with LPS (200 ng/ml, 3 hours). (**b**) Least square (LS) mean profile of differentially (One-way Anova $P < 0.01$) expressed genes (DEGs) in unstimulated or LPS stimulated (200 ng/ml, 3 hours) hMDMs with (+PLTs) or without PLTs (No PLTs). DEGs which showed at least an expression fold-change of ± 2 were identified by One-way ANOVA (P-value < 0.01) between the different test groups. (**c**) Venn diagram comparing the DEGs from differentially stimulated hMDMs with or without platelets from **b**. DEGs were analyzed for enriched pathways using Partek® pathway™ analysis (p-value < 0.05, with restriction of at least 6 genes/pathway). The PCA in **a** and the figures in **b** were generated by Prof. Bernardo Franklin using Partek and R respectively.

Then, I compared the DEGs from differently stimulated hMDMs (+/- platelets) with each other using a Venn diagram to find specifically changed DEGs for each treatment and after platelet

addition (Fig. 4.23c). Finally, I utilized Partek® pathway™ analysis to understand which pathways these DEGs belonged to (Fig. 4.23c).

I observed that platelet addition to hMDMs (Unstim or LPS) regulated pathways of three major groups: TGF-β signaling, metabolism and cancer associated pathways (Fig. 4.23c). Metabolism associated pathways included purine metabolism or amino sugar and nucleotides sugar metabolism for LPS stimulated hMDMs (+/- platelets) or cysteine and methionine metabolism for unstimulated hMDMs (+/- platelets). These pathways were not differentially regulated by LPS stimulation of hMDMs without platelets (Fig. 4.23c). Bladder and melanoma as well as the p53 signaling pathway were some of the most upregulated cancer associated pathways after platelet addition to hMDMs (Unstim or LPS) and not upregulated in hMDMs alone.

To understand which part of these results are associated specifically with platelet secreted factors (and not with platelet presence per se), I performed the same analyses for the addition of platelet supernatants to hMDMs (Fig. 4.24a-b). Compared to the addition of platelets, less DEGs were observed after platelet supernatant addition to hMDMs (Unstim or LPS): 154 DEGs for unstimulated hMDMs and 399 DEGs for LPS stimulated hMDMs (Fig.4.24a). This suggests that either platelet presence itself regulates more genes in macrophages than their secretome or that platelets are the source of some of the DEGs.

Utilizing Partek® pathway™ analysis, I observed that platelet supernatant addition to hMDMs (Unstim or LPS) mainly regulated DEGs associated to two pathways: cancer associated and metabolism associated pathways (Fig.4.24b). TGF-β signaling pathways were not among the highest regulated pathways after platelet supernatant addition in contrast to platelet addition. Cancer associated pathways regulated in hMDMs by both the addition of platelets or platelet supernatant include Wnt signaling pathway, proteoglycans in cancer and hepatocellular carcinoma. Examples of metabolic pathways associated with both platelet and platelet supernatant addition to hMDMs are amino sugar and nucleotide sugar metabolism and purine metabolism.

Taken together, RNA sequencing analysis showed an enrichment of genes associated with metabolism and cancer associated pathways after both platelet and platelet supernatant addition to unstimulated or LPS stimulated hMDMs. Available literature supports a functional interconnection between these pathways, which needs to be further validated and is discussed in section 5.1.

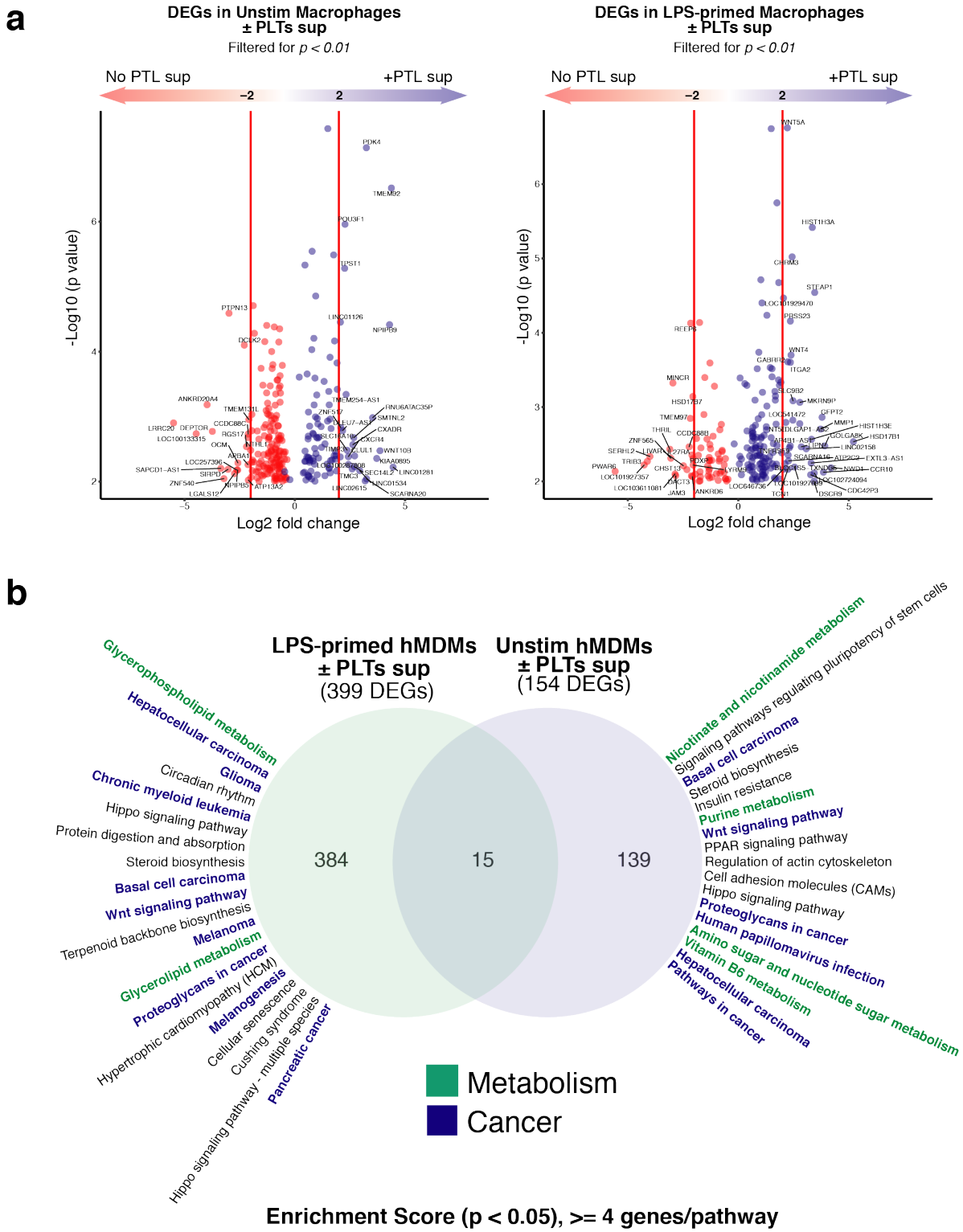

Figure 4.24 Platelet supernatant induces genome-wide transcriptional changes in hMDMs. (a) Differentially expressed genes (DEGs, One-way Anova P < 0.01) in unstimulated or LPS stimulated (200 ng/ml, 3 hours) hMDMs with (+PLTs sup) or without PLT supernatant (No PLT sup). DEGs which showed at least an expression fold-change of ± 2 were identified by One-way ANOVA (P-value < 0.01) the different test groups. (**b**) Venn diagram comparing the DEGs from differentially stimulated hMDMs with or without platelet supernatant from **a**. DEGs were analyzed for enriched pathways using Partek® pathway™ analysis (p-value < 0.05, with at least 4 genes/pathway). The figures in **a** and **b** were generated by Prof. Bernardo Franklin using R and Partek/Illustrator respectively.

4.1.13 Proteomic analysis of platelet supernatants complements transcriptomics results

I showed that platelet or platelet supernatant addition to hMDMs leads to genome-wide transcriptional changes, with enrichment of genes associated with two major pathways: metabolism and cancer associated pathways. Although different platelet secretome analyses were conducted during the last decade[79,90], I decided to analyze the proteomic secretome of unstimulated and LPS stimulated human platelets used in this study. Knowing which proteins are secreted by platelets in my experimental setup will complement the RNA sequencing results and help me to validate them.

I generated platelet supernatants by incubating unstimulated or LPS stimulated platelets from four different donors for three hours and harvested the supernatants. Mass spectrometric analysis of these samples was performed by the CECAD/CMMC Proteomics Core Facility in Cologne (University Cologne, Germany). The core facility quantified proteins using the label-free quantification (LFQ) method. LFQ quantifies the concentration of proteins in two or more biological samples without using stable isotope labeling[151].

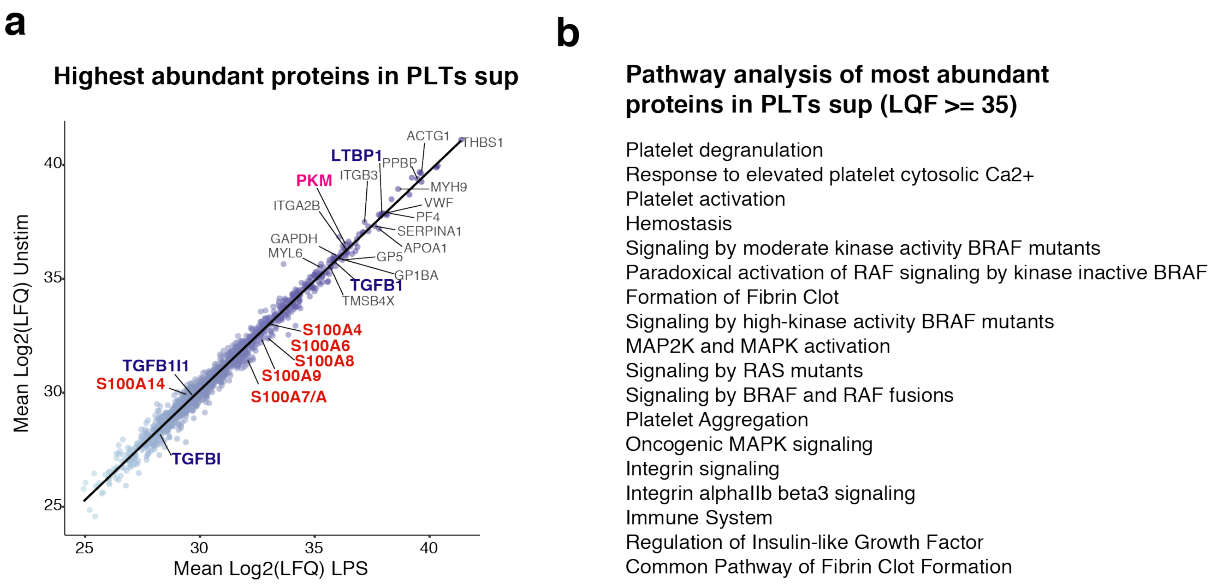

Figure 4.25 Proteomic analysis of platelet supernatant shows that platelets secrete more than 600 different proteins. (**a**) Mean log2 intensity (LFQ) of the consistently identified (in 4 out of 4 samples) proteins in the proteomics of platelet supernatant from unstimulated vs LPS-stimulated (200 ng/ml, 3 hours) platelets. Proteomic quantification of results using the label-free quantification (LFQ) method was performed by the CECAD/CMMC Proteomic Core Facility. Proteomic analysis was performed by Prof. Bernardo Franklin using R. (**b**) Partek® pathway™ analysis (p-value < 0.05, with at least 4 proteins/pathway) of the most abundant proteins with an LFQ >= 35. The figure was generated by Prof. Bernardo Franklin using R

The proteomics analysis revealed the presence of a total of 689 proteins in both unstimulated and LPS stimulated platelet supernatant from all four donors. All of these values had an LFQ higher than 25. Plotting the mean LFQ of LPS stimulated platelet supernatants against unstimulated supernatants showed a high correlation with few differently expressed proteins depending on the treatment (Fig. 4.25a). This is in line with my earlier results, which showed that LPS stimulated platelets after supernatant generation lack P-selectin expression similar to unstimulated platelets (Fig. 4.13c). This indicates that both unstimulated and LPS stimulated platelets produced platelet supernatant containing similar factors. A pathway analysis of the most abundant proteins (LFQ >35) showed that most proteins were associated with platelet degranulation and activation pathways or associated with hemostasis (Fig. 4.25b). Examples of hemostasis associated proteins found in the proteomics analysis are for instance thrombospondin 1, vWF or PF4 (Fig. 4.25a). Also α-granule derived proteins associated with platelet activation were found in the data set, such as different proteins associated with TGF-β signaling: LTBP1, TGFB1, TGFB1I1, TGFBI (Fig. 4.25a). These results validate and support one part of my transcriptomics results, where TGF-β signaling was one of the main pathways associated with platelet addition (Fig.4.23c) and indicate that platelets indeed secrete TGF-β in this experimental setup.

Surprisingly, the pathway analysis of the most abundant proteins in both unstimulated or LPS stimulated platelet supernatants suggested platelet degranulation and activation. This is in contrast to earlier findings, where I show that the activation marker P-selectin is not upregulated after platelet isolation before the beginning of the experiment (Fig. 4.1). These results suggest that even unstimulated platelets used for supernatant generation might be activated differentially, without α-granule content release and subsequent P-selectin transfer to the platelet plasma membrane.

Another group of highly abundant proteins found were S100 proteins. I found six of the 25 described S100 proteins in my platelet proteomics analysis[152] (Fig. 4.25). They are small, calcium binding proteins known to regulate different intra- and/or extracellular functions, such as calcium homeostasis, transcriptional regulation and release of cytokines[153].

Finally, I also found pyruvate kinase PKM to be highly abundant in platelet supernatants (Fig. 4.25). The presence of pyruvate kinase PKM, a glycolytic enzyme, in platelet supernatant could point to their role in metabolic processes, although the function of extracellular glycolytic enzymes has not been described yet. Different metabolic pathways were enriched after both

platelet and platelet supernatant addition to hMDMs in my transcriptomic data (Fig. 4.23c), constituting another link between my proteomics and transcriptomics analysis.
Altogether, proteomic analysis of platelet supernatant revealed more than 600 highly abundant proteins in unstimulated and LPS stimulated platelet supernatants. As expected, I found proteins associated with hemostatic pathways, but also with TGF-β, RAGE, and metabolic signaling in the proteomic analysis, complementing and validating some of my transcriptomic findings. Surprisingly, pathway analysis of the most abundant proteins revealed that platelets were activated during supernatant generation even without any further stimulation. This remains to be validated and is discussed further in the section 5.1.

4.1.14 Platelets increase maximal oxygen consumption of hMDMs

To understand better to what extent metabolic pathways are involved in the platelet-mediated effect on hMDMs, I examined the effect of platelets or platelet supernatant on hMDM metabolism using an extracellular flux analyzer together with Friederike S. Gorki (Institute of Innate Immunity, University Hospital Bonn), a student I supervised. An extracellular flux analyzer assesses mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis, the two major energy generating pathways of cells, through measuring the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) respectively over time[154].
To this end, freshly isolated, non-activated human platelets or platelet supernatant were added to hMDMs prior to LPS stimulation. To measure the cell's basal respiration, ATP-coupled respiration, proton leak or maximal respiration, a mitochondrial stress test was performed. It featured the sequential addition of compounds targeting the electron transport chain: oligomycin, an ATP synthase inhibitor, the mitochondrial uncoupler FCCP, and a combination of rotenone, a complex I inhibitor, and antimycin A, a complex III inhibitor (Fig. 3.2). To get more information about glycolysis, the cell's glycolytic capacity, and its glycolytic reserve, glucose, oligomycin, and 2-Desoxy-D-glucose (2-DG), a glucose analog and inhibitor of hexokinase, were injected sequentially in a glycolytic stress test[154] (Fig. 3.2).
Unstimulated and LPS-stimulated hMDMs cultured with platelets showed an increased maximal respiration but no changes in basal OCR (Fig. 4.26a-b) when subjected to the mitochondrial stress test. Interestingly, I observed no change in maximal respiration when platelet supernatants were added. Platelets cultured alone showed very low OCR in all conditions, although they were responsive to the injected compounds (Fig. 4.26a-b). Analyzing maximal respiration revealed an increased spare respiratory capacity (SRC) of hMDMs co-

cultured with platelets, which could not be explained by an additive effect of platelet and hMDM SRC (Fig. 4.26c). Upon LPS stimulation, SRC was higher in all conditions but showed the same increase after platelet addition to hMDMs. In contrast, platelet addition did not enhance the glycolytic rate of unstimulated or LPS stimulated hMDMs (Fig. 4.26d). The slight elevation of glycolysis in platelet - hMDM co-cultures could be explained by considerable levels of glycolysis even in unstimulated platelet samples (Fig. 4.26d).

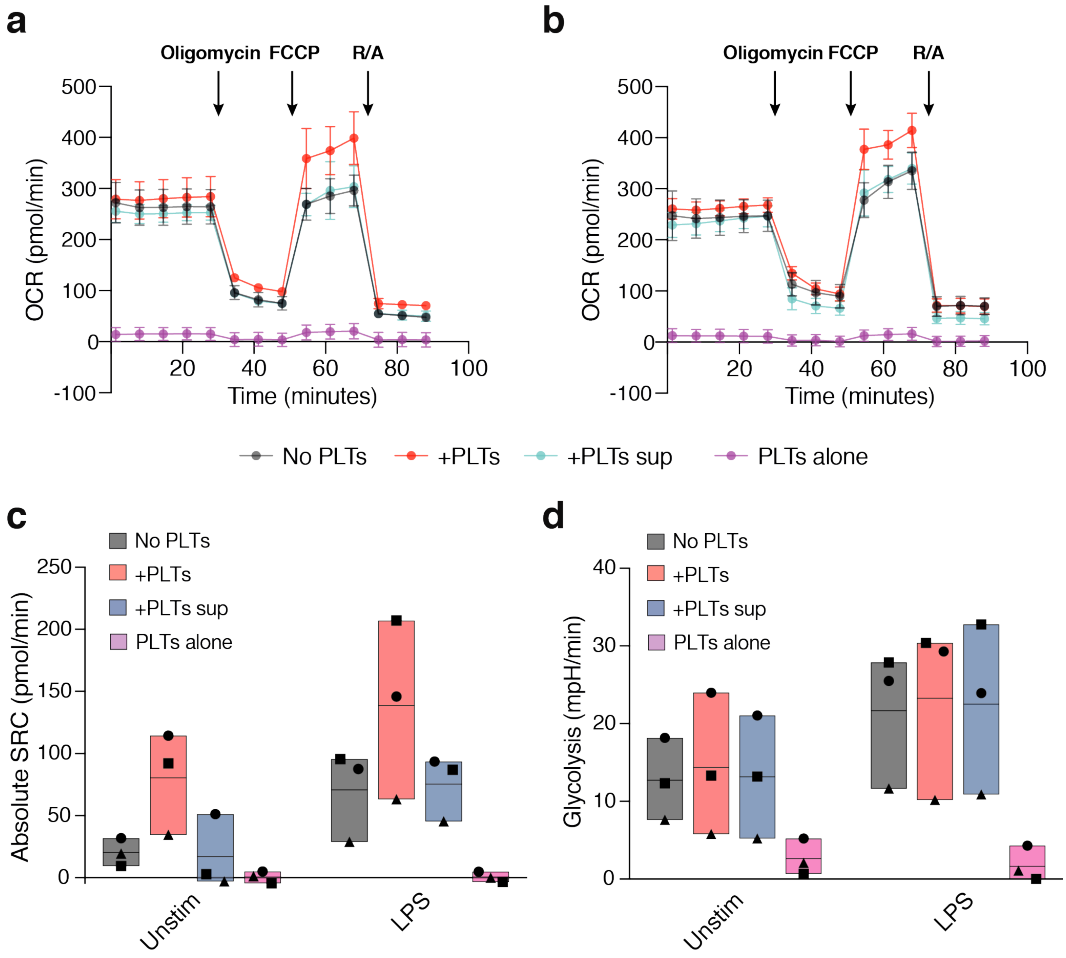

Figure 4.26 Platelets increase maximal consumption rate of hMDMs. (**a**) Representative Seahorse graph showing the oxygen consumption rate (OCR) hMDMs cultivated alone (No PLTs), in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs) or platelet supernatant (+PLTs sup) in response to a Mitostress test: Oligomycin (1 µM), FCCP (1.5 µM) and Rotenone (0.5 µM) + Antimycin (0.5 µM) (R/A). Cells were co-cultured for 3 hours in the presence of medium as a control or (**b**) LPS (200 ng/ml) before analysis in a Mitostress test using a Seahorse Extracellular Flux Analyzer. Platelets alone (PLTs alone) served as a control. (**c**) Calculated spare respiratory capacity (SRC) was calculated from data in **a** and **b**. (**d**) Basal glycolysis rate of cells cultivated as in **a** in response to a Glycolytic stress test: Glucose (10 µM), Oligomycin (1 µM) and 2-Deoxyglucose (2-DG, 0.1 M). Basal glycolysis rate was calculated after glucose injection. Floating bars (with mean and minimum to maximum values) are shown from pooled data from three (**c** and **d**) independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors. The experiments in this figure were performed by Friederike S. Gorki (Institute of Innate Immunity, University Bonn).

From these results I conclude, that platelets but not platelet supernatant increase the SRC of hMDMs independently of LPS stimulation. This is in line with my RNA sequencing data,

where I also observe more metabolic pathways being regulated in the presence of platelets than in the presence of platelet supernatant (Fig. 4.23a-c, 4.24a-b). In contrast, the glycolytic rate of hMDMs is not changed upon platelet or platelet supernatant addition in general.

To understand to what extent this enhanced SRC is fuel dependent, Friederike S. Gorki tested the influence of UK5099 on hMDMs cultured with or without platelets[155], which is an inhibitor of the mitochondrial pyruvate carrier (MPC), that transports pyruvate across the inner mitochondrial membrane[156,157]. As the platelet-mediated effect was LPS-independent and contact dependent, platelets were added to unstimulated hMDMs in the following experiments.

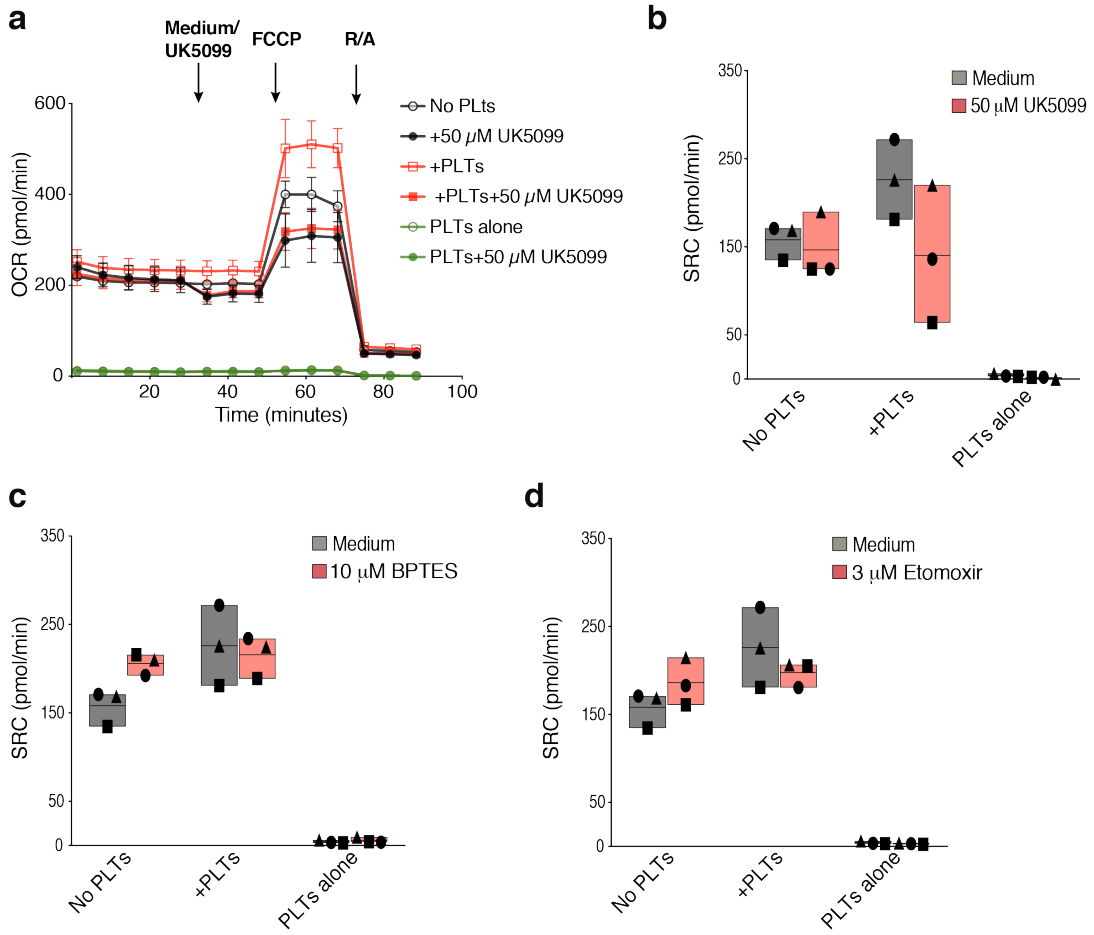

Figure 4.27 Inhibition of MPC partly inhibits platelet-mediated IL-1β amplification in NLRP3 activated hMDMs. (**a**) Representative Seahorse graph showing the oxygen consumption rate (OCR) of hMDMs cultivated alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs) in response to a modified Mitostress test: UK5099 (50 µM)/medium control, FCCP (1.5 µM) and Rotenone (0.5 µM) + Antimycin (0.5 µM) (R/A). Cells were in co-culture for 2 hours before analysis in the modified Mitostress test using a Seahorse Extracellular Flux Analyzer. Platelets alone (PLTs alone) served as a control. (**b**) Calculated spare respiratory capacity (SRC) was calculated from data in **a**. (**c**) SRC calculated from cells cultured as in a but treated with BPTES (10 µM) or (d) Etomoxir (3 µM) instead of UK5099. Floating bars (with mean and minimum to maximum values) are shown from pooled data from three (**b**, **c** and **d**) independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors. The experiments in this figure were performed by Friederike S. Gorki.

UK5099 treatment of hMDMs decreased both basal and maximal OCR in comparison to untreated controls. Strikingly, platelet addition to hMDMs did not increase OCR anymore after MPC inhibition by UK5099 treatment (Fig. 4.27a). Calculation of SRC confirmed this finding by showing similar SRC values for UK5099 treated hMDMs with or without platelets (Fig. 4.27b). Thus, inhibition of MPC abrogated the SRC boosting effect observed in platelet-hMDM co-cultures. Importantly, platelets cultured alone did not show any significant OCR (Fig. 4.26a-b) and can be neglected in this setting as described before.

Further experiments with the same set-up but using either the glutaminolysis inhibitor BPTES (Fig. 4.27c) or the fatty acid oxidation inhibitor Etomoxir (Fig. 4.27d) instead of UK5099 demonstrated that the platelet-mediated increase in SRC of hMDMs was not dependent on these fuels with the calculated SRCs being comparable to untreated controls.

I conclude that platelets increase SRC but not the glycolytic rate of unstimulated or LPS stimulated hMDMs. This effect was dependent on contact and on MPC function, but not on fatty acid oxidation or glutaminolysis.

4.1.15 MPC inhibition reduces platelet-mediated IL-1β increase in inflammasome activated hMDMs

The finding that the platelet-mediated effect on hMDM metabolism is contact dependent contrasts my earlier findings (Fig.4.15). I showed that the platelet-mediated boost of IL-1β secretion from inflammasome activated hMDMs is independent of contact and is also apparent after platelet supernatant addition. Assuming that mitochondrial activity is linked to overall cellular functions, this raised the question if these two findings are connected. To this end, I added different concentrations of the MPC inhibitor UK5099 to hMDMs cultured alone or in the presence of platelets and assessed their IL-1β secretion after NLRP3 inflammasome activation (Fig. 4.28).

I observed that the high concentration of UK5099 (50 μM) decreased IL-1β secretion, independently of platelets, while the lower concentration of UK5099 (10 μM) did not affect the IL-1β secretion by hMDMs (Fig. 4.28). This is in line with earlier findings from Laliberte et al. showing that UK5099 inhibits IL-1β release from inflammasome stimulated BMDMs[158]. However, both the higher and the lower concentration of UK5099 treatment also reduced the platelet-mediated boosting of IL-1β from hMDMs by approximately 20%.

This suggests a possible involvement of MPC function in the platelet-mediated IL-1β boosting effect in inflammasome activated hMDMs (Fig. 4.28). However, the biological significance of this observation and its mechanistic basis awaits further investigation.

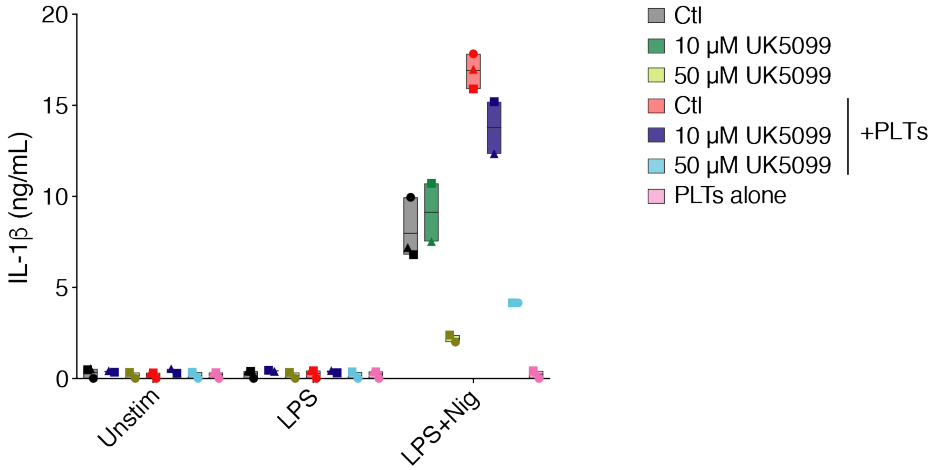

Figure 4.28 Inhibition of MPC partly inhibits platelet-mediated IL-1β amplification in NLRP3 activated hMDMs. HTRF® measurement of IL-1β in cell-free supernatants of unstimulated (Unstim), LPS stimulated hMDMs (200 ng/ml, 3 hours), or LPS primed and nigericin (Nig, 10 µM, 90 minutes) activated hMDMs. Cells were cultivated alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs) as indicated. HMDMs alone or in the presence of platelets were treated with the MPC inhibitor UK5099 (10 or 50 µM) before the start of the inflammasome assay. Floating bars (with mean and minimum to maximum values) are shown from pooled data from two to three independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

4.2 The role of platelets in cancer

Checkpoint inhibition therapies have recently emerged and become available for patients with many different cancers. Blocking immune checkpoints with monoclonal antibodies yielded unprecedented and durable clinical results in many cancers, including HNSCC[119,124]. While checkpoint inhibition therapies, such as PD-L1/PD-1 blockage, yielded promising results for some patients, it became clear that only a fraction of eligible patients with certain types of cancers respond to therapy. In HNSCC, only 10-20% of patients benefitted from immunotherapy and the mortality rates still remain very high[119,120]. The reasons for this are not fully understood and new and easily accessible biomarkers are needed to select suitable patients before immunotherapy and predict their responses.

Since platelets were shown to promote cancer development and become transcriptionally altered through interaction with tumor cells, they are promising targets for cancer biomarkers. Since Best et al. already showed their potential as liquid biopsies[116], I investigated the potential of platelets as new therapeutic targets in HNSCC together with Prof. Barbara Wollenberg's group in Lübeck (Universitätsklinikum Schlwesig-Holstein, Germany), collaborators on this project[132].

4.2.1 PBMCs show increased PD-L1 expression in HNSCC patients

Tumors that express PD-L1 have been shown to respond better to PD-1 or PD-L1 checkpoint inhibition therapy[159]. Although PD-L1 is expressed on tumor-infiltrating immune cells of HNSCC patients[160], expression of PD-L1 is still mostly assessed in tumor biopsies from patients. Less invasive, blood-based PD-L1 profiling and assessment of PD-L1 expression on immune cells in HNSCC patients has not been investigated extensively[132].

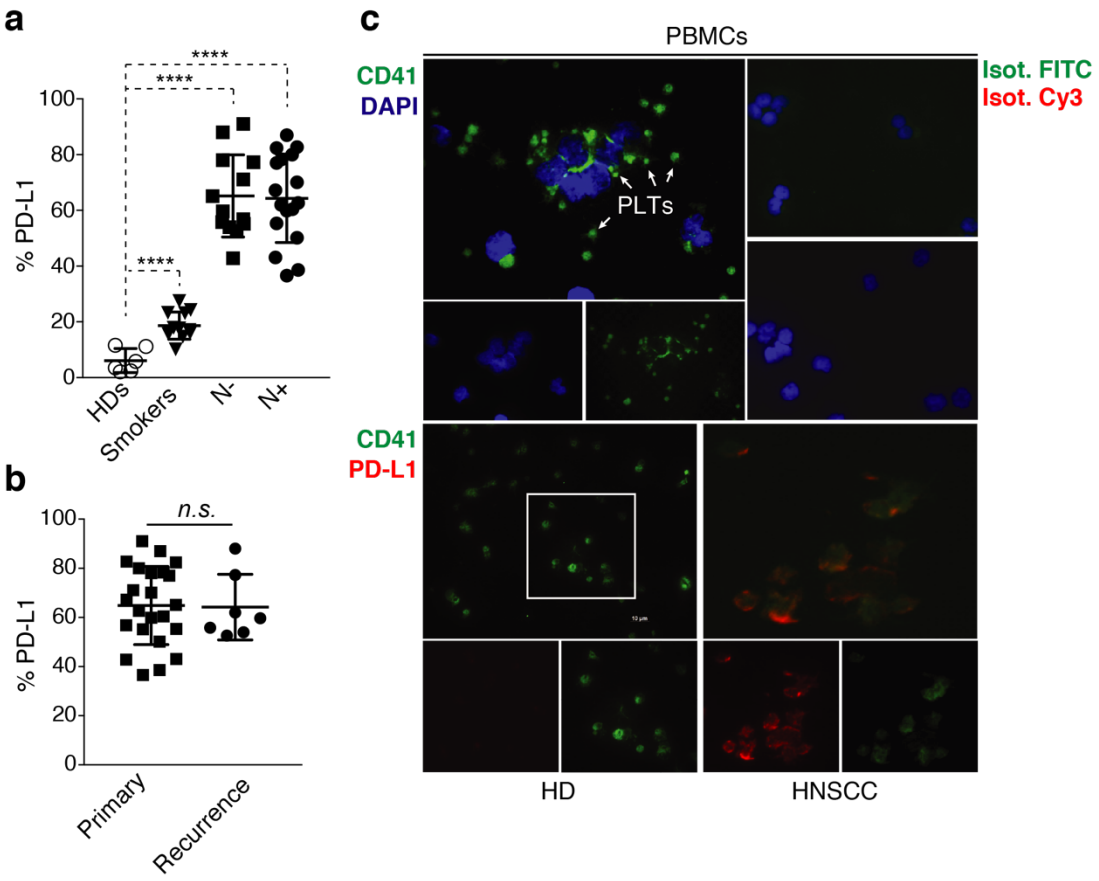

Figure 4.29 PD-L1 expression is upregulated in PBMCs and platelets from HNSCC patients. PD-L1 expression is upregulated in PBMCs and platelets from HNSCC patients. (**a**) Flow cytometric analysis of PD-L1 expression on PBMCs from healthy donors (HDs, n=6), smokers (n=12) and HNSCC patients with (N+, n=16) or without (N-, n=7) lymph node metastasis. Statistical analysis was performed using Ordinary one-way ANOVA, Tukey's multiple comparison test. **** $p < 0.0001$. (**b**) Further stratification of PD-L1 expression on N+ and N- HNSCC patients from **a** according to their tumor incidence as primary or recurrent cancer. Statistical analysis was carried out by using the unpaired *t* test. n.s. (non-significant) $p = 0.9230$. (**c**) Top panel: Two-photon excitation microscopy (TPEF) imaging of cytospin slides with PBMCs from a HNSCC patient. Surface expression of CD41 (platelet marker) was assessed. Nuclei were stained with DAPI. Bottom panel: TPEF imaging of cytospin slides with PBMCs from a healthy donor or a HNSCC patient. Surface expression of PD-L1 and CD41 was examined. The data is representative of 10 different experiments. Each symbol in this figure represents a different donor. PLTs = platelets. All experiments in this figure were carried out by Prof. Barbara Wollenberg's group. The figure is adapted from Rolfes et al.[132]

To confirm the earlier findings of PD-L1 expression on tumor infiltrating immune cells in HNSCC, PBMCs were isolated from either healthy donors, or HNSCC patients in different disease stages. Since smoking is the biggest risk factor for the development of HNSCC, smokers were also included in this study. Cigarette consumption is linked to increased interferon-gamma (IFN-g) production[161], which has been demonstrated to induce PD-L1 expression in immune cells[162]. Thus, PD-L1 expression in PBMCs from smokers was used to evaluate the predictive capacity of our results. Isolated PBMCs were stained with fluorescently labelled antibodies against PD-L1 and CD41 (platelet marker) for flow cytometric analysis (Fig. 4.29). HNSCC patients were grouped into metastatic (N+) or non-metastatic (N-) disease stages according to the occurrence of lymph node metastasis. Moreover, the incidence of the

tumor was assessed to be either primary or recurrent[132]. Flow cytometric analysis was performed by Prof. Barbara Wollenberg's group in Lübeck.

PD-L1 expression was increased in PBMCs from HNSCC patients in comparison to healthy donors, independent of their metastatic state (N- or N+) (Fig. 4.29a). This is in line with previous findings showing that PD-L1 is expressed on immune cells and might be a prognostic marker for HNSCC patients [160]. Interestingly, smokers had intermediate PD-L1 expression (Fig. 4.29a). Further analysis of PBMCs from HNSCC patients showed that the incidence of the tumor (either primary or recurrent tumors) did not have an effect on the amount of PD-L1 detected (Fig. 4.29b). I conclude from these data that neither the presence of metastasis nor the incidence of the tumor influence PD-L1 expression in PBMCs from HNSCC patients[132].

Next, our collaborators performed two-photon excitation fluorescence microscopy (TPEF) to associate PD-L1 expression in PBMCs from HNSCC patients to specific immune cells and understand if platelets are involved in the expression of this protein. Freshly isolated PBMCs from HNSCC patients or healthy donors were fixed on cytospin slides and stained with fluorescently labelled antibodies for platelets (CD41) and PD-L1 or with the respective isotype controls.

TPEF imaging showed large numbers of platelets present in the PBMC fraction. Interestingly, these platelets were PD-L1 positive in PBMCs from HNSCC patients but PD-L1 negative in PBMCs from healthy donors (Fig. 4.29c). These findings suggest that human platelets are among the immune cells expressing PD-L1 in HNSCC cancer patients[132].

4.2.2 PD-L1 expression is increased on isolated platelets from HNSCC patients

To investigate the expression of platelet PD-L1 (pPD-L1) in more detail, I isolated human platelets from peripheral blood of HNSCC patients or healthy donors using my optimized platelet isolation protocol (Fig. 3.1). Then, I analyzed PD-L1 abundance in platelets and PBMCs from the same donor using a luminex cytokine plex, while our collaborators in Lübeck conducted flow cytometric analysis (Fig. 4.30a).

To ensure platelet purity and exclude contamination by other cell types in this analysis, platelets were stained with platelet (CD41) and leukocyte markers (CD45) or matching isotype controls. Assessment by flow cytometry showed that platelets were pure, with very low proportion of contaminating leukocytes (Healthy donor: 0.25% CD41+CD45+ cells). Control

PBMC preparations, however, clearly showed leukocyte marker expression (Healthy donor: 14.7% CD45+ cells) [132] (Fig. 4.30b-c).

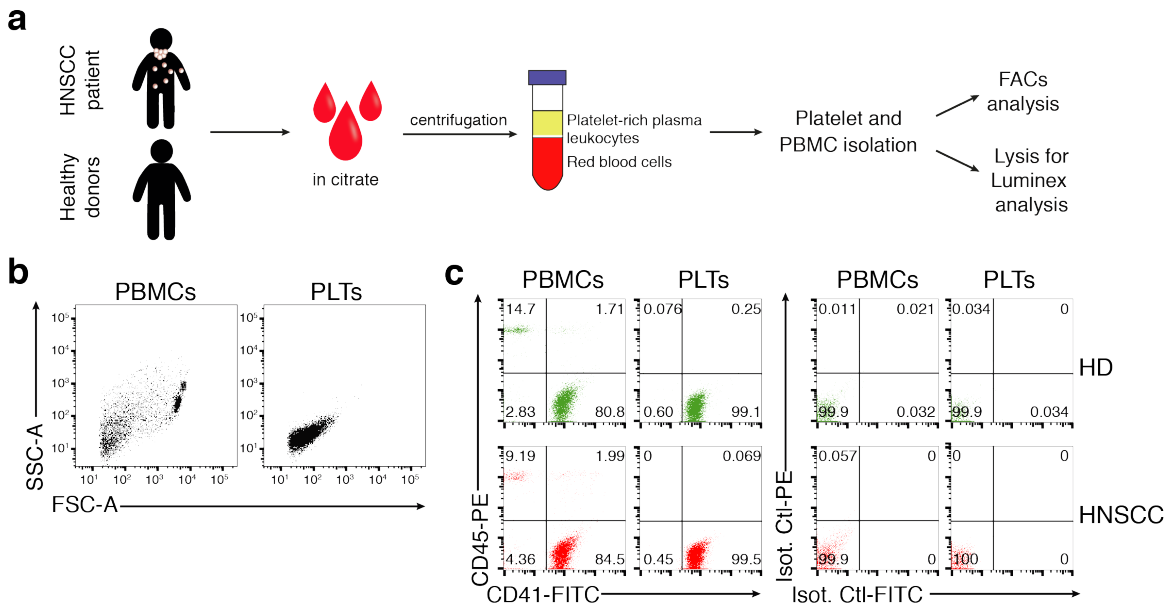

Figure 4.30 The isolated platelets from HNSCC and healthy donors are highly pure. (**a**) Schematic representation of the experimental setup of blood collection followed by PBMC and platelet isolation before further flow cytometric (FACs) or Luminex analysis. (**b**) Flow cytometric analysis of platelet purity showing the gating strategy in the form of scatter characteristics to assess platelet purity after isolation. (**c**) Surface expression of CD45 (leukocyte marker) and CD41 (platelet marker) was assessed in cells fromm **b** in comparison to staining with IgG matched isotype controls. The data shows one representative of a healthy donor (HD) and a HNSCC patient. The figure is adapted from Rolfes et al.[132].

Next, PD-L1 protein levels were assessed on the surface of freshly isolated human platelets from HNSCC patients and healthy donors (HDs) by flow cytometry. PD-L1 expression was significantly increased in platelets and PBMCs from HNSCC patients in comparison to healthy donors (Fig. 4.31a). Similar to the observations in PBMCs, PD-L1 expression in platelets was also not dependent on the metastatic status (Fig. 4.31b) or the incidence of the tumor (Fig. 4.31c). Interestingly, platelets isolated from smokers expressed PD-L1 to a similar degree as HNSCC patients (Fig. 4.31b). To confirm these findings, I measured PD-L1 expression in platelet or PBMC whole cell lysates using a luminex cytokine plex. Indeed, platelets and PBMCs from HNSCC patients showed significantly higher expression of PD-L1 in comparison to healthy donors[132] (Fig. 4.31d).

Together, these findings constitute the first report of PD-L1 expression in human platelets from HNSCC patients. PD-L1 expression in platelets is independent of the metastatic stage or incidence of the tumor. Similar trends in PD-L1 expression were observed in PBMCs.

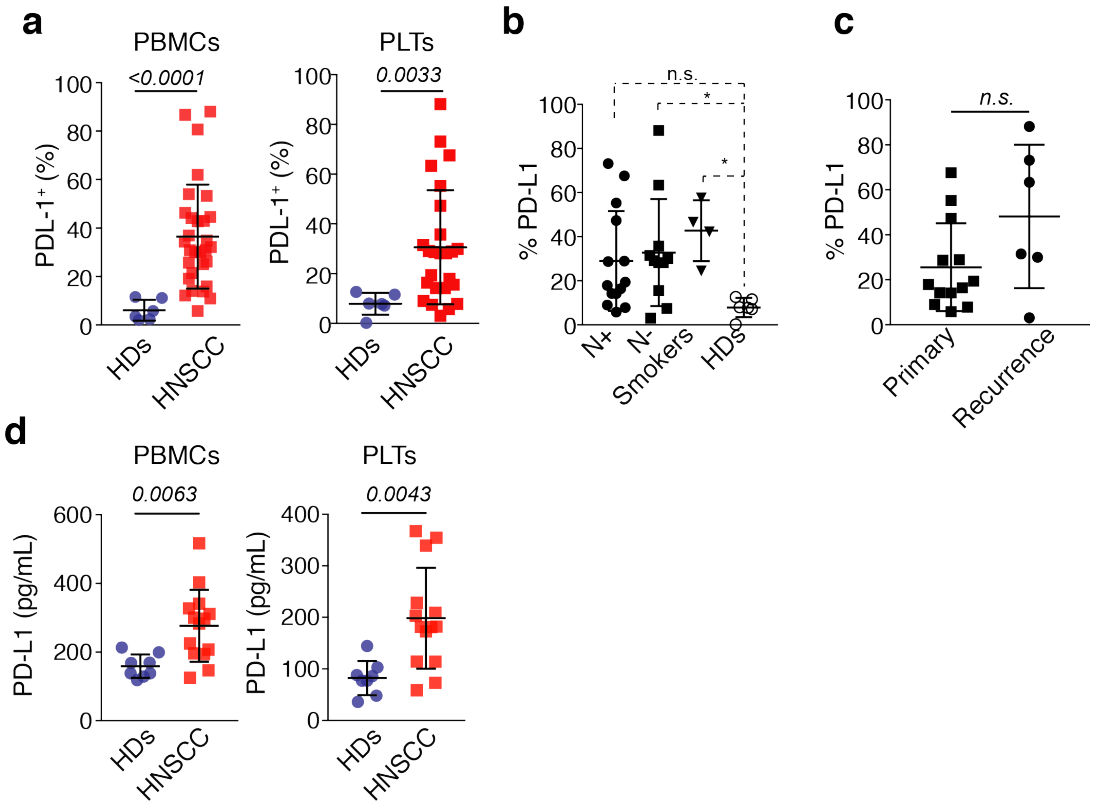

Figure 4.31 PD-L1 expression is upregulated in blood platelets from HNSCC patients. (**a**) Flow cytometric analysis of PD-L1 expression on human PBMCs and platelets (PLTs, PD-L1$^+$CD41$^+$) isolated from healthy donors (HDs, n=6, the same is in Fig. 29a) and HNSCC patients (n=37). Statistical analysis was performed using *t* test and p-values are as indicated. (**b**) Flow cytometric analysis of PD-L1 expression on platelets from healthy donors (HDs, n=6, same as in **a**), smokers (n=12) and HNSCC patients with (N+, n=16) or without (N-, n=7) lymph node metastasis. The statistical difference was calculated using Kruskal-Wallis, Dunn's multiple comparisons test. *p < 0.05. **(c)** Further stratification of N+ and N- HNSCC patients from **b** according to their tumor incidence, either being a primary or recurrent cancer. Graphs show the percentage of PD-L1 expression on platelets in relation to the tumor incidence. Statistical analysis was performed using the Mann-Whitney test with the p-values as indicated. (**d**) Analysis of PD-L1 levels in PBMCs and platelets isolated from healthy donors (HDs, n=8) and HNSCC patients (n=14) using a Luminex Cytokine Array. The statistical differences were calculated using an unpaired *t* test with p-values as indicated. The figure is adapted from Rolfes et al.[132]

4.2.3 pPD-L1 expression is affected by Atezolizumab treatment

Thrombocytopenia has been observed as a common side effect of checkpoint inhibitor therapy[163]. Therefore, platelets expressing PD-L1 could be a target for anti-PD-L1 therapies and lead to thrombocytopenia in cancer patients.

To test this, our collaborators in Lübeck assessed platelet number and PD-L1 expression in platelets in lung cancer patients before and after treatment with atezolizumab, an anti-PD-L1 antibody approved for clinical use. Patients were treated with a flat dose of 1200 mg atezolizumab once every three weeks. They were clinically monitored during this time, including assessment of whole blood cell counts and PD-L1 expression on platelets[132].

PD-L1 protein expression analysis showed significantly diminished PD-L1 expression in lung cancer platelets after seven days of atezolizumab treatment (Fig. 4.32a). The same trend was

also visible for PBMCs from the same patients (Fig. 4.32a). These findings were confirmed by flow cytometric analysis of PD-L1 expression, where PD-L1 expression dropped in platelets after one day of atezolizumab treatment (Fig. 4.32b). In contrast, this trend was not observed in PBMCs from the same donors. Whole blood cell and total platelet counts were unaffected by atezolizumab treatment (Fig. 4.32c). The fact that platelet counts are not changed suggests that atezolizumab might not target free but only complexed platelets, which are not assessed by clinical blood counters. After 21 days, before the start of the new round of atezolizumab treatments, PD-L1 expression on both PBMCs and platelets were fully reconstituted[132] (Fig. 4.32a-b).

Collectively, I conclude from these results, that anti-PD-L1 therapy with atezolizumab in lung cancer patients targets pPD-L1. The mechanism of anti-platelet activity and the reason why platelet counts are not affected by this treatment remains to be investigated.

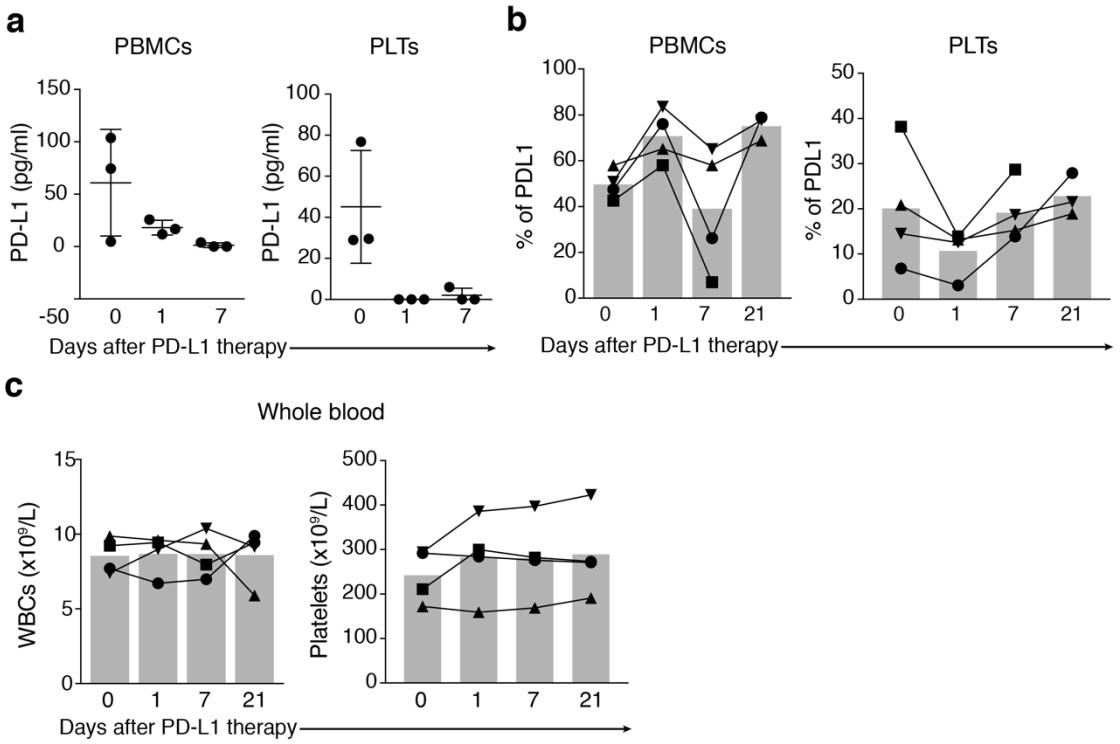

Figure 4.32 PD-L1 expression in platelets and PBMCs from lung cancer patients is affected by atezolizumab. (**a**) Analysis of PD-L1 levels in PBMCs (n=3) and platelets (PLTs, n=3) from lung cancer patients before (day 0) and at day 1 and 7 after therapy with 1200 mg of atezolizumab using a Luminex Cytokine Array. (**b**) Flow cytometric analysis of PD-L1 expression on PBMCs (n=4) and platelets (n=4) before (day 0) and several days after atezolizumab therapy (days 1, 7, 21) including the same donors as in **a**. (**c**) Total whole blood cell (WBCs) and platelet counts in the peripheral blood measured in same lung cancer patients treated with atezolizumab as in **b**. Each symbol in this figure represents a different donor. The figure is adapted from Rolfes et al.[132].

5 Discussion

Work in the last decades has shown that platelets contribute to processes beyond hemostasis. As their immunological effector functions become more evident, the role of platelets is being investigated in many different areas and diseases. In this thesis, I have examined the role of platelets in two different contexts: in inflammation and in HNSCC cancer. I will discuss my findings in two parts, with the first section focusing on how human and murine platelets regulate inflammasome activation in innate immune cells (section 5.1) and the second section discussing the discovery of PD-L1 on human platelets in HNSCC cancer patients (section 5.2).

5.1 Platelets regulate inflammasome activation in innate immune cells

Platelets are the second-most abundant cell type circulating in the human blood and have been shown to interact extensively with different immune cells. Work in the last decades has shown that besides being important for hemostasis, platelets also act as immune cells responding to a variety of different inflammatory triggers and interact extensively with different immune cells[108]. Interaction of platelets with monocytes, for instance, was shown to induce CD40, PSGL-1 and CD11b expression and leading to innate immune responses[129,164]. Furthermore, Scull et al. described that LPS stimulated macrophages phagocytose activated, autologous platelets inducing enhanced proinflammatory cytokine secretion[104]. However, they did not investigate the involvement of platelets in the inflammasome response of leukocytes. Recent findings showing the assembly of NLRP3 inflammasomes in platelets in the context of different diseases highlight the possible role of platelets in inflammasome responses[86-88]. Therefore, this study investigated the interaction of platelets with immune cells during inflammasome activation in more detail.

5.1.1 Platelets amplify NLRP3 inflammasome activation in leukocytes

As part of the results of this thesis, I have shown that platelets boost the IL-1β secretion of NLRP3 activated human and murine macrophages and neutrophils in a concentration dependent manner (Fig.4.3). Focusing on macrophages, I showed that platelets not only regulate IL-1β secretion but also change the cytokine signature of NLRP3 activated hMDMs more broadly. Next to IL-18 and IL-1α, two other members of the IL-1 family, also other chemokines (RANTES, SDF-1α) and growth factors (PIGF-1, VEGFα, , PDGF-BB, EGF)

were amplified (Fig.4.4). RANTES and SDF-1α are known for their chemotactic functions, recruiting lymphocytes and leukocytes[165,166]. PIGF-1, VEGFα and PDGF-BB were shown to play a role in vascular remodeling and blood vessel formation[167-170] and EGF induces cell differentiation and survival[171]. Thus, adding platelets to macrophages not only enhances proinflammatory cytokine secretion but also increases recruitment of immune cells and vascular remodeling. While the role of platelets in the recruitment of leukocytes to the inflammatory site has been described before[93,94], their involvement in the amplification of proinflammatory cytokines and angiogenic factors from NLRP3 activated hMDMs is novel and might offer new insights into the link between angiogenesis and inflammation in inflammatory diseases such as rheumatoid arthritis.

While enhanced proinflammatory cytokine secretion from macrophages but not neutrophils interacting with platelets has been reported before, this has not been done in context of inflammasome activation. So far, only Scull et al. described enhanced proinflammatory cytokine secretion, such as TNFα, IL-6 and IL-23, in LPS stimulated macrophages after interaction with autologous platelets[104]. In contrast to their findings, IL-6 and IL-23 were not differentially regulated by platelets in my results (Fig. 4.4) and might highlight the different molecular regulation of primed and NLPR3 activated cells by platelets. Importantly, I added heterologous (platelets from different donors) instead of autologous platelets to hMDMs in my experimental setting, which might also lead to different experimental outcomes. Similar to almost all nucleated cells, platelets also express Major Histocompatibility complex (MHC) Class 1 molecules on their surface[172,173]. MHC molecules detect foreign pathogens or substances in the body and display them on the cell surface for detection by T cells leading to an immune response. Heterologous platelets could be expressing different MHC molecules and thereby elicit an increased NLPR3 response in leukocytes. Different mechanistic studies in animal models indicate, however, that platelet MHC Class 1 molecules are not relevant physiologically and do not stimulate a T cell response but rather inhibit it[72,174]. Further experiments with autologous platelets will be necessary to clarify, whether they also elicit an IL-1β boost from NLPR3 activated hMDMs in my experimental setting.

Further, my results showed that platelets are critical for cytokine production by NLRP3 activated monocytes (Fig. 4.5). It is well-known that monocytes interact with platelets in various ways to promote monocyte adhesion and arrest[129,145], differentiation[98] and cytokine secretion[96,100,175]. Stephen et al. described that the induction of proinflammatory signaling in monocytes is dependent on both direct and indirect interactions with platelets, although the

complete mechanism still remains elusive[175]. Despite the strong focus on platelet-monocyte interactions in recent years, the requirement of platelet-monocyte interaction for proinflammatory IL-1β and TNFα secretion has not been described before.

Taken together, the data presented here show for the first time that platelets regulate NLRP3 inflammasome activation in leukocytes *in vitro*, indicating yet unappreciated roles for platelets in inflammatory diseases *in vivo*.

5.1.2 Platelets-mediated transcriptional regulation of NLRP3 in hMDMs

NLRP3 activation is known to require at least two steps: a first priming step, in which IL-1 and NLRP3 transcription is induced, and a second activating step leading to inflammasome assembly and IL-1 cytokine secretion[36]. My data shows that platelets regulate both, the priming and the activation step of NLRP3 activation in hMDMs, leading to enhanced NLRP3 and pro-IL-1β transcription, caspase-1 activity, IL-1β secretion and ASC speck formation (Fig. 4.14 and 4.15).

Different studies have shown that ASC specks remain active after their release from cells and can lead to subsequent inflammasome activation in neighboring immune cells[51,56,57]. My results suggest, that platelets do not only amplify the inflammatory response of hMDMs by enhanced proinflammatory cytokine production but also through direct activation of neighboring cells by enhanced ASC speck production and secretion. Thereby, platelets could spread inflammation and cell activation in a paracine way, leading to an even bigger amplification reaction than by proinflammatory cytokines alone. Further experiments will be necessary to show the relevance of this platelet-mediated amplification of ASC specks *in vivo* in the context of different inflammatory diseases, such as CAPS, gout or Atherosclerosis.

My investigation into the platelet-mediated NLRP3 regulation in hMDMs showed, that it was independent of TLR4 stimulation (Fig. 4.16). The fact that platelets still boosted IL-1β secretion from NLRP3 activated hMDMs irrespective of the TLR priming ligand could be explained by the following two scenarios. Platelets might exert an additive effect on the signaling cascade that is common to TLR1/2, TLR4 and TLR7/8. By acting on common downstream regulators of these TLRs, platelets could amplify the transcription of IL-1β and NLRP3. Alternatively, platelets could also act independent of these TLR signaling cascades, enhancing transcription and NLRP3 activation in a parallel, non-canonical pathway. Once the platelet-derived factor is identified, more targeted experiments will help to elucidate this mechanism.

Next to this, I observed an enhanced NLRC4 inflammasome response in hMDMs after platelet addition (Fig. 4.10a) and showed that the platelet-mediated NLRP3 regulation in hMDMs was dependent on functional NLRP3 (Fig. 4.10b).

Two recent studies showed that NLRP3 and NLRC4 can interact with each other during Salmonella infection, with NLRC4 recruiting NLRP3 and its adaptor ASC to activate caspase-1[176,177]. However, since NLRP3 inhibition by CRID3 did not affect the IL-1β amplification by NLRC4 activated hMDMs (Fig. S1), my results suggest that this response is independent of functional NLRP3. Since both NLRP3 and NLRC4 activation pathways are dependent on a first priming step to induce IL-1 transcription, platelets could amplify IL-1β secretion in inflammasome responses in general by enhancing the priming step and increasing IL-1 gene transcription. Enhanced pro-IL-1β production in hMDMs could then lead to a higher NLRP3 or NLRC4 inflammasome response with increased proinflammatory cytokine secretion. Testing the involvement of platelets in other inflammasome responses, such as AIM2, will show if this hypothesis holds true.

Altogether, my results demonstrate that platelets transcriptionally regulate NLRP3 inflammasome activation in hMDMs, independently of TLR4 priming. They also enhance NLRC4 inflammasome responses. The mechanisms underlying these processes remain elusive and further studies are necessary to get a full understanding of the molecular effects platelets cause in their interaction with hMDMs.

5.1.3 Platelets do not express inflammasome components or release IL-1β

Different studies have described platelets as produces of IL-1 cytokines with some of them reporting platelet-derived IL-1 activity indirectly in bioassays[82,85], while others observed IL-1 expression in platelets and showed IL-1 cytokine secretion upon activation[83,136,178,179]. More recently, human platelets have also been described to express and assemble NLRP3 inflammasomes in the context Dengue and sickle cell disease [86,87], linking IL-1 cytokine secretion in PMVs to NLRP3 inflammasome activation in platelets[86].

In contrast, using a series of different techniques with human and mouse cells, as well as inflammasome reporter mouse models, my results show that platelets do not express the inflammasome components NLRP3, ASC and caspase-1 or secrete IL-1β (Fig. 4.8 and 4.9).

One issue that needs to be considered in this context is the purity of platelets. Isolation of human platelets from whole blood is a laborious and intricate process, which is susceptible to

leukocyte contamination. Pillitteri et al. showed that IL-1β synthesis and secretion was not detectable in leukocyte-free platelet preparations activated with thrombin[180]. However, due to massive amounts of proteins synthesized by leukocytes in comparison to platelets, contamination with as little as one leukocyte per 10^5 platelets led to IL-1β detection in platelet suspensions[180]. In my work, leukocyte contamination was assessed prior to each experiment and platelet suspensions were observed to have a purity of more than 99%, which is equivalent to less than 0.5 x 10^5 of contaminating leukocytes (Fig.4.2a). Thus, the high purity of my platelet preparation could be a reason, why I do not observe any IL-1β secretion or NLRP3 expression in platelets. Most of the studies describing IL-1β and NLRP3 expression in platelets, do not assess or show platelet purity analysis. This makes it difficult to judge the purity of their platelet preparations and the confounding effect it may have on their results. Leukocyte contaminations could lead to deceptive interpretations and constitute one explanation for the discrepant results. Murthy et al. show, for instance, that the NLRP3 inflammasome is upregulated in platelets through assessment of caspase-1 activity and IL-1β secretion after platelet activation[88]. As they do not show platelet purity assessments in their study or platelet marker expression in their immunoblotting, the observed caspase-1 activity and IL-1β expression and secretion could also be due to leukocyte contaminations in their platelet preparations[88].

Another explanation for the different findings could be the chosen analysis methods. Most of the findings reporting NLRP3 expression in platelets were obtained by flow cytometry or confocal analysis in isolated platelets. In contrast to my results, protein expression of inflammasome components was not investigated by quantitative methods, such as immunoblotting of NLRP3 and ASC. As both, flow cytometry and microscopy mostly rely on antibody based visualization of the target, unspecific antibody binding might confound results and constitute another reason for contrasting findings.

Further, different studies describe that newly synthesized IL-1 is mostly retained within the cell[83,181] and Hottz et al. show that IL-1β is mostly secreted in microvesicles from NLRP3 activated platelets[86]. This makes detection of IL-1β in platelet supernatant difficult and might also explain the conflicting reports about IL-1β expression in platelets throughout the literature.

Finally, it is important to note that IL-1β expression in human platelets was reported only after 18 hours of stimulation with thrombin, PAF or ADP *in vitro*, in the range of 0.4 - 1 ng/ml[83]. In my assays, IL-1β secretion from human macrophages was analyzed after a stimulation time of 4.5 hours, in the range of 20-30 ng/ml (Fig. 4.3a). Due to these different IL-1β production

kinetics it is unlikely that platelet-derived IL-1 cytokines cause an effect in my experimental setting. This hypothesis was confirmed by addition of wild-type platelets to NLRP3 activated IL-1R or IL-18R deficient BMDMs, which still led to IL-1β amplification (Fig. 4.7a-b). In line with this, expression of IL-1 cytokines was absent in the proteomic analysis of supernatant from unstimulated or LPS stimulated human platelets (Fig.4.25) and IL-1β secretion was never measured in the 'platelet only' controls in all of my experiments. Consequently, I exclude a role for platelet-derived IL-1 cytokines in my experimental system.

Further studies are necessary to clarify whether platelets are generally sources of IL-1 cytokines and to assess the importance of platelet-derived IL-1 for immune responses in different pathologies.

5.1.4 A soluble, platelet-secreted factor regulates NLRP3 activation in hMDMs

Macrophages are known for their phagocytic functions[139] and different studies have described their ability to phagocytose platelets[104,140]. In contrast to these findings, my results suggest that cell contact is not required for the platelet-mediated regulation of IL-1β secretion from hMDMs (Fig. 4.14 and 4.12).

These findings differ from Scull et al.'s report, showing enhanced cytokine secretion from LPS stimulated macrophages only after phagocytosis of activated platelets[104]. However, it is important to note that Scull et al. measured cytokine secretion only after 24 hours of LPS stimulation and did not cover NLRP3 activation in their study. One possible scenario to explain my findings could be that macrophages rely on platelet-derived soluble factors to boost their cytokine secretion in the presence of both priming and activating inflammasome stimuli, while macrophages start to phagocytose platelets if they sense a single stimulus, like LPS, for a prolonged time. To fully understand how macrophage-platelet interactions develop over time and in response to different stimuli, further time course experiments need to be conducted.

Interestingly, preliminary data suggest that platelet interaction with both neutrophils and monocytes require cell contact (personal communication with Ibrahim Hawwari, Institute of Innate Immunity, University Hospital Bonn), indicating a differential regulation in comparison to platelet - macrophage interactions. Although the mechanism underlying these platelet-immune cell interactions is still under investigation, these preliminary findings highlight the cell-type specific nature of platelet interactions with immune cells.

Further investigation into the nature of the platelet-secreted factor showed that it is most likely not an α-granule (Fig. 4.17 – 4.19) or a COX1/2 or LOX derived lipid mediator (Fig. 4.22).

Platelets from *Nbeal2*-knock out mice, which lack α-granules, and a combination of techniques including recombinant human proteins, blocking antibodies and specific inhibitors, were employed to exclude a role for α-granule-derived factors. However, when interpreting these results some experimental limitations should be considered.

Although platelets from *Nbeal2*-knock out mice lack α-granules, they were still shown to contain low levels of α-granule derived factors[18], making this mouse model less reliable. Platelets could still up-regulate P-selectin expression and secrete vWF after stimulation, although at much lower levels than wild-type platelets[18]. Thus, while my results show that the absence of α-granules and a significantly decreased α-granule content do not inhibit the platelet-mediated NLPR3 regulation in hMDMs, they do not completely exclude a role for α-granule derived factors in my experimental setting.

When considering my experiments with recombinant proteins, it should be noted, that while they constitute a powerful means to test the effect of single factors on hMDMs *in vitro*, this reductionist approach might lack decisive factors that are necessary for full signaling activity. Platelets might mediate NLPR3 activation in hMDMs through the secretion of multiple different factors, which could either act on hMDMs directly or form heteromers and act on hMDMs subsequently. Recently, Alard et al. showed for instance that heteromers of platelet-derived RANTES and neutrophil-borne HNP1 are formed and play a role in monocyte recruitment[97]. Therefore, this reductionist approach should be considered with caution.

Despite their limitations, the results from these different experimental approaches complement each other and together, they most likely exclude a role for α-granule derived factors in the platelet-mediated boost of IL-1β secretion from NLRP3 activated hMDMs.

Platelet-derived lipid mediators should also be considered more carefully. Although the most prominent platelet-derived lipid mediators were excluded in this work by using COX1/2 and LOX inhibitors (Fig. 4.22), platelets contain more lipid families that could produce lipid mediators. These families include aminophospholipids, phosphatidylinositides, oxidized phospholipids, lysophospholipids, sphingolipids and neutral lipids including glycerides, free cholesterol and cholesteryl ester[118]. Some of these lipid families have already been linked to inflammasome activation and immune regulation. For instance, Yeon et al. showed that oxidized phosphatidylcholine activate the NLRP3 inflammasome in macrophages[182]. Platelets have been shown to store the lysophospholipid sphingosine 1-phosphate (S1P), which they can

secrete in huge amounts upon activation[183]. Stimulation of the S1P receptor on macrophages has been linked to NLRP3 activation in tumor-associated macrophages and microglia[184,185], suggesting a role for S1P in macrophage inflammasome activation. Thus, further analysis of platelet-derived lipid mediators is needed to completely exclude their involvement in my experimental setting.

My results also demonstrated that the dense granule resident molecules ATP and ADP are most likely not involved in the platelet-mediated NLRP3 regulation of hMDMs (Fig.4.21). However, similar to the addition of recombinant proteins, it is important to consider that the addition of ADP to hMDMs on its own might not lead to IL-1β amplification in hMDMs because a decisive factor from platelets is missing, that would interact with ADP (Fig. 4.21b). Moreover, I was not able to assess the correct function of apyrase or Benzonase treatment due to experimental restrictions. Thus, only further complementary experiments will show the functional impact of ADP and ATP in my experimental setting.

In contrast to these findings, I showed that extracellular but not intracellular calcium plays a role in the platelet-mediated NLRP3 regulation of hMDMs (Fig.4.20).

Calcium could either function as co-factor for hMDM or platelet activation, or act on calcium sensitive proteins secreted by platelets. Supporting the latter hypothesis, direct addition of calcium chloride to NLRP3 activated hMDMs did not yield IL-1β amplification (Fig. 4.20c). Also, I detected calcium binding S100 proteins among the highly abundant proteins in my proteomic analysis of platelet supernatants (Fig.4.25a). These cytosolic, calcium binding proteins have a broad range of intracellular functions, including the regulation of cell migration, proliferation, differentiation, migration and inflammation[153]. Recently, Sunahori et al. showed that the heterodimeric complex of S100A8/A9 (Calprotectin) proteins amplifies proinflammatory cytokine secretion from macrophages in rheumatoid arthritis[186]. These findings are similar to the observations in this work, making S100 proteins, and specifically Calprotectin, highly promising candidates for the platelet-secreted factor regulating NLRP3 activation in hMDMs. Thus, calcium could play an indirect role in my experimental setting, facilitating the function of other platelet-derived proteins. Further experiments investigating the role of S100 proteins on NLRP3 activated hMDMs will be necessary to address this hypothesis.

Alternatively, extracellular calcium could also affect platelets. Platelets rely heavily on calcium signalling for platelet activation and can actively import calcium from the extracellular compartment[187]. Therefore, the lack of extracellular calcium through the use of extracellular

calcium chelators in my experimental setting (Fig. 4.20) could have led to diminished platelet activation and less secreted platelet factors, thereby partly inhibiting IL-1β secretion from NLPR3 activated hMDMs. Treating platelets with calcium chelators before co-culturing them with hMDMs could offer new insights into this effect.

Interestingly, heat inactivation of platelet supernatant abolished the boost in IL-1β secretion from NLRP3 activated hMDMs (Fig. 4.22c), indicating the involvement of a heat sensitive factor, most likely of protein nature. Proteins are well-known for their heat-sensitivity. High temperatures denature the highly organized structure of proteins, rendering them incapable of performing their functions. Complementary experiments using proteinases could be employed to verify the involvement of proteins in my experimental setting.

Altogether, my data show that platelets mediate NLRP3 activation in hMDMs through a soluble, secreted factor, which is partly calcium sensitive and most likely of protein nature. Testing different, well-known platelet-secreted factors for regulating NLRP3 activity was not successful and further experiments are necessary to fully address this issue.

5.1.5 Platelets induce genome wide transcriptional changes in hMDMs

RNA sequencing analysis demonstrated that the presence of platelets or platelet supernatant drastically changes the gene expression profile of hMDMs, with the majority of DEGs changed after platelet addition. Three major pathway groups were associated with these changes: metabolism, cancer and TGF-β signaling pathways (Fig. 4.23c). With the exception of TGF-β signaling, the same pathway groups were regulated by platelet supernatant (Fig. 4.24b).

While TGF-β signaling was still detected in the pathway analysis of DEGs after addition of platelet supernatant to hMDMs, it was not among the most regulated pathways anymore. In general, significantly more DEGs were detected in hMDMs in the presence of platelets (Fig. 4.23) than in the presence of platelet supernatant (Fig. 4.24). These results suggest, that either platelets per se regulate more genes in hMDMs than their secretome or, althernatively, that platelets themselves are the source of these additional DEGs.

Given that platelets are the major source of TGF-β in the body, which can be released rapidly upon activation[91,188], it is possible that the source of DEGs associated with TGF-β signaling are mainly platelets and not hMDM. Yet, I still detected DEGs associated to TGF-β signaling in hMDMs after addition of platelet supernatant which supports a possible role for platelet-secreted TGF-β in my experimental setting. This is reinforced by data from my proteomic analysis of platelet supernatant, where I found different proteins associated to TGF-β signaling

being among the highest abundant proteins secreted from platelets (Fig. 4.25a). Recently, Guido et al. also showed that TGF-β signaling can drive tumor growth by promoting metabolic reprogramming in cancer-associated fibroblasts[189]. These findings link all three groups of highly regulated pathways found in my RNA sequencing analysis with each other in a physiologically relevant manner. Additionally, proteomic analysis revealed thrombospondin 1 as the highest abundant protein in platelet supernatant (Fig. 4.25a). Besides its importance for hemostasis, thrombospondin 1 is a key mediator of TGF-β in systemic sclerosis[190], connecting the highest abundant protein found in my proteomics analysis to TGF-β signaling pathways in an autoimmune disease. Thrombospondin 1 has also been described to regulate IL-1β production in macrophages[191]. Depending on the respective receptor, it can have context-specific pro- or anti-inflammatory effects on the IL-1β pathway in macrophages. While the CD36, CD47 and integrin-binding domains of thrombospondin 1 can independently enhance the inflammasome dependent IL-1β secretion from THP-1 monocyte derived macrophages, the CD47-binding domain can also inhibit LPS-induced IL-1β expression in human macrophages[191].

These recent findings together with my proteomics and RNA sequencing data mark both TGF-β and Thrombospondin 1 as interesting candidates for the platelet-derived factor and further research should address their involvement in the platelet-mediated NLRP3 regulation of hMDMs.

Besides TGF-β signaling, metabolism and cancer associated genes were regulated in hMDMs by both platelet presence or platelet supernatant addition (Fig. 4.23 and 4.24).

In line with this, I detected many metabolism related proteins, such as PKM, among the highest abundant proteins in my proteomic analysis of platelet supernatant (Fig. 4.25a). PKM is a glycolytic enzyme, that regulates HIF-1α activity and IL-1β induction in LPS activated macrophages[192] and, more recently, was shown to induce NLRP3 and AIM2 inflammasome activation[193]. However, since PKM is a glycolytic enzyme, all of these findings describe the role of intracellular PKM. My proteomic results suggest that PKM is secreted by platelets, which has not been described before. HMDMs might be able to sense extracellular PKM as a danger signal and endocytose it. However, it remains to be tested whether extracellular PKM could induce metabolic changes in hMDMs.

Further experiments assessing the role of platelets on the metabolism of hMDMs showed that platelets but not platelet supernatant increase the SRC of unstimulated hMDMs, most likely in a MPC dependent manner (Fig. 4.26 and 4.27). Interestingly, glycolytic capacity of hMDMs

was not enhanced by addition of platelets or platelet supernatant (Fig. 4.26d). The fact that only the presence of platelets and not platelet supernatant induces an increased SRC is mirrored my RNA sequencing results, where more metabolic pathways are regulated in hMDMs if platelets are present in comparison to platelet supernatant, specifically in unstimulated hMDMs (Fig. 4.23c, 4.24b). This might indicate a differential regulation of hMDM metabolism pathways by platelets, with most metabolic pathway changes in hMDMs relying on direct contact with platelets. Although I showed that the platelet-mediated regulation of NLRP3 in hMDMs is regulated by a secreted, soluble, platelet-derived factor, I still tested the effect of MPC inhibition by UK5099 treatment on the platelet-mediated IL-1β amplification by NLRP3 activated hMDMs. Interestingly, MPC inhibition reduced the platelet-mediated boosting of IL-1β from hMDMs by approximately 20% (Fig. 4.28), suggesting a partial involvement of MPC in the platelet-mediated NLPR3 regulation in hMDMs. To understand how much of this effect is contact dependent, these findings have to be validated through additional experiments with platelet supernatant instead of platelets. If future experiments show, that MPC involvement in the platelet-mediated NLPR3 regulation of hMDMs was contact dependent, this would indicate a sequential regulation of NLRP3 in hMDMs by platelets. In this scenario, platelets could regulate hMDM through a secreted, soluble factor(s) first, followed by mediating hMDM metabolism through direct interaction, possibly via MPC. However, this needs to be validated further.

Cancer associated genes were also regulated in hMDMs by both platelet presence and especially by platelet supernatant addition (Fig. 4.23 and 4.24). Specifically, DEGs associated with the pathways “Proteoglycans in cancer” and “Wnt signaling in cancer” were detected.

Syndecans, members of the conserved family of transmembrane heparan sulfate proteoglycans, have recently been shown to be involved in a novel LPS uptake pathway leading to caspase-11 activation and the formation of NLPR3 inflammasome foci[194]. Interestingly, Syndecans can also bind to different growth factors, including VEGFs, PDGFs and TGF-β[195], all of which were found in my proteomic analysis of platelet supernatant (Fig.4.25a). Thus, one possibility by which platelets (or platelet derived factors) might impact NLRP3 signaling could include binding syndecans expressed on the cell membrane of hMDMs. Again, this needs to be tested. Besides their role as transmembrane receptors, syndecans can also mediate extracellular signaling through shedding of their extracellular domain after proteolytic cleavage, which is accelerated under inflammatory conditions[196], leading to secreted syndecans.

Since I detected syndecan 4 in my proteomic analysis in platelet supernatant, platelets seem to shed syndecans into their environment (Fig.4.25a). While the exact role of soluble syndecans remains unclear, it has been shown that they retain their ability to bind growth factors[197]. Hence, platelet-derived, soluble syndecan 4 could bind to platelet-secreted growth factors, forming complexes that might be sensed by hMDMs and could potentiate their NLRP3 response. So far, however, there is no experimental evidence for this hypothesis and further studies are necessary to understand the role of soluble syndecans in inflammation.

Wnt signaling is a highly conserved signaling mechanism and has been mostly associated with cellular differentiation, proliferation, apoptosis and motility[198]. Although no Wnt proteins were found in my proteomics analysis of platelet supernatant, Wnt proteins might affect macrophage signaling in an autocrine matter. BMDMs as well as hMDMs were shown to express basal levels of Wnt5a and of its receptor Fzd5[199]. Detection of a bacterial stimulus, such as LPS, or of the proinflammatory cytokines IL-1β, IL-6 and CCL2 induces macrophages to upregulate Wnt5a[199]. Thus, although Wnt signaling proteins were not detected in platelet supernatant in my experimental setting, macrophages could upregulate Wnt proteins upon detection of proinflammatory cytokines and induce other macrophages to do the same in an autocrine manner. This would explain the upregulation of DEGs associated with the Wnt signaling pathway in my RNA sequencing experiment.

Together, the results of my RNA sequencing analysis of hMDMs (co-) cultured with or without platelets or platelet supernatant and my proteomics analysis of platelet supernatant complement each other highlighting many differentially regulated pathways and promising proteinous candidates, including TGF-β, thrombospondin, PKM and proteoglycans. To validate these findings both *in vitro* and also *in vivo* experiments will be necessary to understand the molecular basis for platelet dependent NLRP3 activation in hMDMs.

5.1.6 Assessment of platelet activation status

Platelets are well known for their fragile nature and are easily activated by mechanical stress. Therefore, I assessed platelet activation and viability after each platelet isolation in this work by P-selectin measurement to assure that platelets were not activated prior to experiments. I demonstrated that platelets express low levels of P-selectin after isolation, but upregulate P-selectin expression drastically after thrombin stimulation (Fig. 4.1b). Since P-selectin is stored in α-granules in resting platelets and is only transported to the plasma membrane upon platelet activation[135], these results indicate the absence of α-granule secretion in my platelet

preparation and suggest that platelets are not activated prior to the experiments conducted in this work.

However, my proteomic analysis of platelet supernatant generated from both unstimulated and LPS stimulated platelets showed that the most abundant, secreted proteins were associated with platelet degranulation and activation pathways (Fig.4.25b). Interestingly, further analysis of platelets after supernatant generation demonstrated that unstimulated or LPS stimulated platelets were mainly P-selectin negative after 3 hours of incubation, similar to freshly isolated platelets (Fig. 4.13c). These results suggest that unstimulated, P-selectin negative platelets might be activated in a differential way, lacking α-granule content release and P-selectin transfer to the plasma membrane. In this context it is important to consider that α-granules do not represent a homogeneous population. Several groups have demonstrated the existence of different classes of α-granules, with distinct morphological appearances and protein compositions[200-202]. Additionally, platelet can release their thematically different granule contents in sequential steps according to the stimuli they detect in their surroundings[201,203]. Thus, although platelets in my experimental setting only show low P-selectin expression, they could still be partially degranulated and activated. In line with my results, Mirlashari et al. showed that platelet stimulation with LPS does not influence platelet degranulation but increases platelet aggregation in platelet-rich plasma[204]. Contrasting these results, Shashkin et al. demonstrated that LPS stimulation of platelets can up-regulate P-selectin over time and lead to RNA splicing in platelets[181]. To fully understand the activation status of my platelet preparations, different additional platelet activation parameters could be assessed including platelet adhesion to leukocytes or to natural or artificial surfaces, platelet aggregation or conformational changes[205]. Further, the release of dense and α-granules or lysosomes and the shedding of microvesicles could be measured[205]. Recently, Södergren et al. established a comprehensive flow cytometry protocol, which simultaneously measures the expression of six different platelet activation markers[206]. They assess the expression of the platelet activation markers LAMP-1, indicating lysosomal exocytosis; PAC-1, a platelet aggregation marker; Annexin V, nessecary for the pro-thrombinase complex assembly; $DiIC_1$, which indicates preserved mitochondrial membrane potential; the relative platelet size, as a change of phenotype is associated with platelet activation; and P-selectin expression, as a sign of α-granule release[206]. This six-color flow cytometry could be used to characterize the platelets in my experimental setting and help to understand how and to what extend platelets are activated.

These results would also contribute valuable information about the platelet secreted factor(s), which leads to IL-1β amplification in NLRP3 activated hMDMs.

Here, I demonstrate that although unstimulated or LPS stimulated platelets do only express low levels of P-selectin, they secrete proteins associated with platelet degranulation and activation pathways. Thus, the activation status of platelets in my experimental setting remains unclear and further experiments are necessary to elucidate platelet activation and understand functional consequences.

5.1.7 Relevance of the observed effect *in vivo*

While my work establishes that platelets mediate NLRP3 activation of hMDMs *in vitro*, the *in vivo* relevance of these findings remains unclear. Different studies and preliminary *in vivo* data suggest, however, that platelets also influence IL-1β secretion from leukocytes *in vivo*.

Recently, Tunjungputri et al. found a direct correlation between blood platelet numbers and plasma IL-1β concentrations in a cohort of Caucasian, healthy individuals from the 500-Human Functional Genomics Project[89]. They also linked the capacity of leukocytes to produce IL-1β to the platelet degranulation capacity[89]. These findings indicate a modulatory role for platelets *in vivo*, linking platelet activation to leukocyte IL-1β production, which is in line with my *in vitro* findings. While the involvement of healthy donors without co-morbidities is a strength of this study, it does not address the interaction of platelets and IL-1 inflammation in disease states.

This was addressed by Boilard et al., who reported that platelets can amplify IL-1β inflammation in a rheumatoid arthritis mouse model[207]. This study reported platelets to amplify inflammation via microparticle production and proposed IL-1β as the amplifying factor[207]. However, it was impossible to distinguish whether the enhanced IL-1β response in rheumatoid arthritis was elicited by platelet-derived IL-1β or by platelet interaction with other immune cells, as suggested by my *in vitro* results (Fig. 4.3). Thus, although platelets are involved in the development of inflammation during rheumatoid arthritis, the exact mechanism by which platelets influence this disease remains elusive.

Besides these recent findings, preliminary data from our institute shows that platelet depletion in mice attenuated LPS-induced serum concentrations of IL-1β *in vivo*, while increasing TNFα and IL-6 concentrations (data not shown, personal communication with Dr. Lucas Ribeiro). These results complement my *in vitro* findings, where platelet addition to BMDMs led to

increased IL-1β and decreased TNFα secretion and suggest that platelets influence IL-1β responses *in vivo*.

Consistent with this hypothesis, preliminary data from a collaborating group in Brazil shows that high blood platelet counts are associated with increased plasma concentrations of IL-1β but not IL-1α and TNFα in samples from human subjects naturally infected with *Plasmodium vivax*, the predominant cause of malaria in the Brazilian amazon basin (data not shown, personal communication with Dr. Marina Lima S. Santos, Instituto René Rachou, Fundação Oswaldo Cruz). These results highlight platelets as a contributing factor in the regulation of IL-1β in human disease, adding functional relevance to my *in vitro* data and confirming our preliminary *in vivo* data. Additionally, the results suggest that IL-1α and TNFα might be regulated by different mechanisms in malaria patients.

Together, these recent studies and the preliminary data from our institute and collaborators in Brazil complement my *in vitro* data and suggest a role for platelets in the regulation of IL-1β responses *in vivo*. To understand the extend of this platelet-mediated regulation of IL-1β secretion from leukocytes, further *in vivo* experiments are necessary. Especially once the platelet-derived factor(s) that induces NLRP3 regulation in hMDMs is identified *in vitro*, more targeted *in vivo* studies will help to explore the relevance of this effect in humans.

5.1.8 Perspectives

In this study, I show that platelets regulate the NLRP3 activation in BMDMS, hMDMs and human neutrophils. Additionally, I identify platelets as crucial factors for cytokine production from NLRP3 activated human monocytes.

Comparing my RNA sequencing results with the proteomic analysis of platelet supernatant yielded different promising candidates that would be worth testing in my experimental setting, such as thrombospondin 1, different s100 proteins, TGF-β and PKM. Possible ways to examine their involvement would be the use of recombinant human proteins, blocking antibodies or mice, that are genetically deficient of these factors. Thrombospondin 1 and different S100 proteins, including Calprotectin were found in platelet supernatant, too. Since these were both shown to bind to the CD36 receptor[191,208], it would be interesting to test its involvement in my experimental setting as well.

Further transcriptome analysis indicated a possible role of proteoglycans in the platelet-mediated NLRP3 regulation in hMDMs. As syndecan 4 was detected in my proteomic analysis of platelet supernatant, it should also be tested as a promising platelet-secreted factor in my

experimental setting. Importantly, it can bind other platelet-secreted factors forming complexes that could be detected by hMDMs[197]. In general, it will be important to consider complex formation of different factors, which might not elicit higher IL-1β secretion by NLPR3 activated hMDMs on their own but could act synergistically. Naturally, testing all possible platelet factors in different combinations would not be feasible experimentally but adding the most promising platelet-derived factors and hMDMs together and probing for synergistic effects would be doable. Since Syndecan 4 is known to interact with VEGF and PDGF, I would test a combination of these factors in my experimental setting first.

Further, I observed that platelets increase the maximal oxygen consumption of hMDMs, most likely in a pyruvate carrier dependent manner. Inhibition of the pyruvate carrier also partly affected the platelet-mediated IL-1β amplification by NLRP3 activated hMDMs. Since pyruvate, a product of the glycolytic pathway, seems to contribute to the platelet-mediated effect in hMDMs, it would be interesting to understand if the effect is also dependent on glucose, an important fuel upstream of pyruvate.

Finally, there are numerous platelet-derived factors that have not yet been tested in the scope of this work. One could for example analyze the platelet secretome in more detail to determine the involvement of microvesicles or dense granule derived factors. Since PMVs are the most abundant cell-derived microvesicles in the body and have already been shown to reprogram macrophage gene expression and function before[92,105], one would expect them to be involved in the regulation of NLPR3 activation in hMDMs as well. Additionally, it would be interesting to test the general involvement of platelet derived dense granules. For this, one could employ *Unc13d* knockout mice, which lack dense granule secretion[209]. Together with further analysis of dense granule derived factors, such as serotonin, one could clarify the role of platelet-derived dense granule content in context of my results.

The sheer abundance of possible pathways which are highlighted in my transcriptomic and proteomic analysis, together with the possibility of individual factors acting synergistically complicate the search for a mechanism by which platelets regulate NLRP3 activation in hMDMs. Testing the role of key factors identified in my RNA sequencing results, which are also supported by my proteomic analysis, promises to be the most efficient and rewarding approach.

5.1.9 Conclusion

Although inflammasomes have been studied extensively over the last years, the majority of *in vitro* studies was conducted in monocultures of monocytes or macrophages, neglecting networking, regulatory and synergistic effects of other immune cells. Since platelets are the second-most abundant cell type in the human blood and have been recognized for their immune functions over the past decade, this study signaling during interaction with immune cells in the context of inflammasome activation.

Here, I show for the first time that platelets modulate the inflammasome response of innate immune cells. Platelets enhanced the NLRP3 inflammasome response in BMDMs, hMDMs, and human neutrophils, and proved to be crucial for IL-1β cytokine production by NLPR3 activated human monocytes. In contrast to recent findings, I show that platelets alone do not express NLPR3 inflammasome components or secrete IL-1β, excluding a role of platelet NLRP3 activation or platelet-derived IL-1 cytokines in the observed effect.

Furthermore, I demonstrate that platelets regulate NLRP3 activation in hMDMs by enhancing NLRP3 and IL-1β transcription, increasing caspase-1 activity and promoting the assembly of ASC specks. Thereby, platelets boost proinflammatory cytokine secretion by NLRP3 activated hMDMs. This effect is independent of cell-contact and mediated by a platelet-derived, soluble and heat sensitive factor. Further analysis excluded a role of platelet α-granule derived factors and demonstrated that other classical platelet-related factors, such as ATP, ADP, nucleic acids and COX1/2 and LOX derived lipid mediators, were also dispensable in the regulation of NLRP3 in hMDMs by platelets. The platelet-mediated effect is partly dependent on extracellular calcium and on the activity of the mitochondrial pyruvate carrier. Further investigations will be necessary to show how these factors mediate NLPR3 activation in hMDMs.

Finally, RNA sequencing analysis identified genome wide transcriptional changes in hMDMs in the presence of platelets or platelet supernatant. Together with a proteomic analysis of platelet supernatant, this study offers an array of new possible candidates that could be involved in the platelet-mediated NLPR3 regulation of hMDMs. Identifying the underlying mechanism of this effect will be instrumental to develop new therapeutic approaches for IL-1 blockage in patients with NLRP3 dependent diseases, such as CAPS.

5.2 Platelets express PD-L1 in cancer patients

Next to their function in inflammatory processes, platelets are also well-known for their role in cancer development and metastasis. They promote tumor growth, angiogenesis and the survival of CTCs and can also be employed as liquid cancer markers[114]. Through interaction with tumor cells, platelets get 'educated' and display an altered transcriptomic profiles which can be used to predict the tumor occurrence in patients[116,130].

In the cancer field, checkpoint inhibition therapies antibodies have proved to be a promising treatment for different cancer types, including HNSCC. Although the use of monoclonal antibodies targeting the PD-L1/PD-1 immune checkpoints has shown unprecedented and durable effects in some HNSCC patients, in general, only 10-20% of HNSCC patients responded favorably and the mortality rates in this cancer still remain very high[119,120]. Therefore, new and easily accessible biomarkers are needed to identify eligible patients for therapy and predict their response. Since platelets are important for cancer development and they were shown to be tumor educated in some cancer types, this study investigated the potential of platelets as new therapeutic target in HNSCC patients in collaboration with Prof. Barbara Wollenberg's group in Lübeck.

5.2.1 Platelets from HNSCC patients express PD-L1

In a study from Kim et al. it was shown that PD-L1 is a favorable marker in HNSCC and expressed on immune cells but not on tumor cells[160]. Confirming these results, PD-L1 expression was observed in PBMCs from HNSCC cancer patients, which was independent of the presence of locoregional or distant metastases or the incidence of the tumor (Fig. 4.29). Also, PBMCs from smokers showed PD-L1 expression at an intermediate level in comparison to cancer patients and healthy donors (Fig.4.29).

These results suggest, that PBMCs from HNSCC patients constitutively express PD-L1, independent of the cancer location or metastatic state of the patients. The fact that smokers already express an intermediate level of PD-L1 could indicate their elevated risk to develop HNSCC. Cigarette smoking has been linked to increased IFN-g production and subsequent induction of PD-L1 expression in immune cells[161,162]. Since smoking is also one of the biggest risk factors for HNSCC development, my results suggest that PD-L1 expression on PBMCs could act as predictive factor for HNSCC.

Imaging of PBMCs from HNSCC patients showed that the PD-L1 expression was located on platelets (Fig. 4.29c). Further analysis of freshly isolated platelets from HNSCC patients

demonstrated that PD-L1 protein expression was significantly higher in platelets from HNSCC patients than in platelets from healthy donors (Fig. 4.31).

Although platelets have been described to interact with tumors and become tumor educated through various changes in their RNA and protein content[116,210,211], this is the first description of PD-L1 expression on human platelets.

Further stratification of my results showed that PD-L1 expression on platelets from HNSCC patients was also independent of the metastatic stage or the incidence of the cancer (Fig. 4.31b-c). As in PBMCs, platelets from smokers also displayed enhanced PD-L1 expression similar to the level on platelets from HNSCC patients. PD-L1 expression on platelets could therefore function as a predictive factor for the development of HNSCC. In this study, smokers were not stratified further according to their smoking habits. Analyzing the PD-L1 expression on platelets from people with different smoking habits, either smoking several cigarettes per day or only occasionally, would improve the understanding of the PD-L1 expression dynamics on platelets. Further experiments will hopefully show what triggers are necessary to induce PD-L1 expression in platelets from healthy donors.

The data also indicate that pPD-L1 expression is not limited to HNSCC patients but also occurs in other cancer types, such as lung cancer. Future work will show if pPD-L1 expression is a general cancer related platelet phenotype or if it is restricted to those two cancer types. Then it will also be possible to assess the predictive capacity of PD-L1 expression on platelets in a better way.

5.2.2 PD-L1 expression on platelets is affected by immunotherapy

Since thrombocytopenia has been shown to be a common side effect of checkpoint inhibition therapies[163], pPD-L1 in cancer patients might be a susceptible target for antibody-based anti-PD-L1 therapies leading to platelet clearance. Additionally, two cancer drug trials, including anti-PD-L1 therapy, were halted due to bleeding incidences (Kestrell Study – https://uk.reuters.com/article/us-astrazeneca-cancer/astrazeneca-pauses-two-cancer-drug-trials-enrolment-due-to-bleeding-idUKKCN12R2D4), indicating a possible connection between anti-PD-L1 therapy and platelet in cancer patients.

Platelets showed significantly diminished PD-L1 expression for seven days after atezolizumab treatment (Fig.4.32), indicating that pPD-L1 was indeed targeted by anti-PD-L1 antibodies. Consistently, PD-L1 expression was also reduced in PBMCs. Since PBMCs still contain large numbers of platelets, it was not possible to distinguish the origin of their PD-L1 expression.

Other cells next to platelets might express PD-L1 in PBMCs from cancer patients. Further analyses will be necessary to clarify this point in the future.

At the end of one atezolizumab treatment cycle (21 days), PD-L1 expression was fully reconstituted in platelets and PBMCs to levels before treatment (Fig.4.32a-b). These results highlight the need for close PD-L1 expression monitoring in the blood during therapy. Since PD-L1 levels were reconstituted by day 21, a more frequent dose of anti-PD-L1 antibodies might yield better results, keeping PD-L1 levels on platelets and PBMCs low, thereby possibly increasing therapy success. Experiments with a larger patient cohort will show whether this hypothesis is true.

The results of this work also show that whole blood cell and platelets counts were not affected by atezolizumab treatment (Fig.4.32c). Since the clinical automatic whole blood counters only assess the number of free platelets in the blood, these results suggest that free platelets are not affected by atezolizumab therapy. The anti-PD-L1 antibody might only bind to platelets complexed with immune or cancer cells, which are not measured by conventional clinical cell counters. Different publications have shown that activated platelets readily interact with immune cells during inflammation[108] or with tumor cells and CTCs in cancer[114]. Therefore, it would not be surprising if PD-L1 expressing platelets would from complexes with other cells and do not get counted in the automatic whole blood counters[132]. Further studies about the nature of PD-L1 expressing platelets and their interaction with other cells will clarify this point.

These results also show that neither whole blood nor platelet counts are reliable biomarkers to assess the success of immune checkpoint therapies in cancer patients. However, the measurement of pPD-L1 could function as blood-based biomarker for cancer and might also be utilized to select suitable patients for anti-PD-L1 therapies. So far, insurance companies in Germany only use the percentage of PD-L1 expression in the tumor as a criterion to determine which patients are eligible for anti-PD-L1 or anti-PD-1 treatment[132]. However, many HNSCC patients successfully treated with checkpoint inhibition therapies were only partly PD-L1 positive or did not express PD-L1 in the tumor at all[212]. Thus, blood-based liquid biopsies measuring pPD-L1 expression might proof to be more reliable. It is important to consider, though, that this work is only based on a small sample size of only 4 patients, which might result in misleading interpretations. Further studies with bigger patient cohorts with different cancer types will be necessary to validate these hypotheses and correlate clinical response rates of cancer patients to immunotherapy with pPD-L1 expression.

5.2.3 Perspectives

In the second part of my thesis, I report that platelets from HNSCC patients show PD-L1 expression, which is affected by immunotherapy with the anti-PD-L1 antibody atezolizumab[132].

This work only describes the presence of PD-L1 on platelets but does not elucidate the mechanisms underlying this PD-L1 expression. Therefore, it would be interesting to examine how PD-L1 is regulated in platelets from cancer patients. Since platelets from healthy donors did not express PD-L1, platelets must either upregulate PD-L1 themselves or acquire it from their surroundings. Different studies have shown that platelets interact with tumors and can become tumor educated through changes in their RNA and protein content[116,210,211]. Thus, platelets might acquire PD-L1 from interactions with tumors and display it on their membrane independent of their activation status. Alternatively, platelets might also be produced from megakaryocytes containing PD-L1, which they can upregulate during cancer development. This hypothesis suggests that megakaryocytes produce disease specific, different platelet populations. The concept of platelet heterogeneity has been discussed in the platelet field for a while[213]. Recent studies provide evidence that the platelet transcriptome changes when the environment of megakaryocytes changes, such as during inflammation or diabetes[213-216]. Thus, megakaryocytes in cancer patients might produce cancer-specific, PD-L1 expressing platelets. To test this hypothesis, megakaryocytes cell lines could be incubated with tumor conditioned medium and their PD-L1 expression could be examined afterwards. Additionally, immunoprotein profiling of megakaryocytes from HNSCC patients will aid these investigations[132].

Further investigations will also be needed to understand the function of PD-L1 expressing platelets in the tumor environment. So far, high platelet counts and platelet-lymphocyte ratios have been associated with poor prognosis for cancer patients[112]. Activated platelets are mainly known for their contribution to angiogenesis, tumor growth, survival of CTCs and metastasis, thus, generally aiding cancer development[114]. Since PD-L1 is normally expressed by tumor cells to interact with PD-1 on T cells to inhibit their immune response, pPD-L1 might act in a similar way. Further investigations will show whether this hypothesis holds true and whether pPD-L1 promotes or inhibits cancer development.

Finally, the potential of pPD-L1 as a general tumor biomarker remains to be answered. Best et al. showed that RNA profiles from tumor educated platelets provide accurate information about the cancer signature of patients and can be used as blood based cancer diagnostics [116,130]. Thus,

platelets in general seem to be suitable blood based cancer biomarker for the cancer cell types tested in their study. To determine if pPD-L1 expression on platelets could be used as a general or more specific cancer biomarker, larger cohort studies with different cancer types are necessary.

5.2.4 Conclusion

Despite the recent successes of checkpoint inhibition therapy in many cancer, including HNSCC, a substantial amount of patients was not susceptible to these therapies[119,120]. So far no easily accessible biomarker has been identified yet, which could predict the patients response to therapy. This has made a more personalized treatment with checkpoint inhibitors difficult.

This work provides the first description of PD-L1 expression in platelets from HNSCC and lung cancer patients. PD-L1 expression in platelets from HNSCC patients was not associated with disease stage, the occurrence of metastasis or with the incidence of the cancer (primary or recurrent). The level of PD-L1 expression in platelets was similar in all cancer patients in comparison to healthy donors, which did not show any or very low PD-L1 expression. Interestingly, this study showed that platelets from healthy donors with smoking habits also expressed PD-L1 at a significantly higher level than healthy donors. Since smoking is one of the prominent risk factors for the development of HNSCC, PD-L1 expression on platelets could therefore function as a biomarker for early HNSCC development.

Further analysis showed that the PD-L1 expression on platelets from lung cancer patients was affected by immunotherapy with the anti-PD-L1 antibody atezolizumab, while whole blood and platelets counts in the same patients remained unchanged. Further investigations with larger cohorts and in different cancer types will be necessary to validate and correlate PD-L1 expression on platelets with clinical response rates.

Taken together, these results suggest that PD-L1 expression on platelets could be a valuable and easily accessible biomarker for the detection of early cancer development.

6 Abbreviations

BMDMs	Bone-marrow derived macrophages
hMDMs	Human monocyte derived macrophages
CTC	Circulating tumor cells
NLRP	NOD, LRR and pyrin containing
NLRC	NOD, LRR and Card containing
NOD	Nucleotide-binding and oligomerization domain
COX	cyclooxygenase
LOX	lipoxygenase
IL	Interleukin
HNSCC	Head and Neck Squamous Cell Carcinoma
PD-L1	Programmed cell death ligand 1
DC	Dendritic cell
NK cell	Natural Killer cell
PRR	Pattern recognition receptor
PAMPs	Pathogen-associated molecular patterns
ROS	Reactive oxygen species
NLR	NOD-like receptor
TLR	Toll-like receptor
ALR	AIM2 like recptor
CLR	C-type lectin receptor
RIG-1	Retinoic acid inducible gene 1
RLR	RIG-1Like receptor
CD	Cluster of Differentiation
GM-CSF	Granulocyte-macrophage colony-stimulating factor
NET	Neutrophil extracellular traps
ATP	Adenosine triphosphate
NAD	Nicotinamide adenine dinucleotide
TCA	Tricarboxylic acid
FAD	Flavin adenine dinucleotide
HIF-1α	Hypoxia-inducible factor 1 a
LRR	Leucine-rich repeat
TIR	Toll-IL-1 receptor
LPS	Lipopolysaccharide
MyD88	Myeloid differentiation primary response protein 88

TRIF	TIR domain-containing adaptor protein-inducing IFN-β
TIRAP	TIR-associated protein
TRAM	TRIF-related adaptor molecule
MAP	Mitogen activated protein
NF-κB	Nuclear factor kappa B
NAIP	NLRB or neuronal apoptosis inhibitor proteins
NLRX	NLR family and CARD-related X effector domain containing
CAPS	Cryopyrin-associated periodic fever syndromes
MWS	Muckle-Wells syndrome
FCAS	Familial cold autoinflammatory syndrome
NOMID	Neonatal Onset Multisystem Inflammatory Disease
IFN	Interferons
TNF	Tumor necrosis factor
GPCRs	G protein-coupled receptors
PG	Prostaglandin
TXA_2	Thromboxane A2
vWF	Van Willebrand factor
ADP	Adenosine diphosphate
GPIbα	platelet glycoprotein (GP)Ibα
PARs	Protease-activated receptors
PMVs	Platelet-derived microvesicles
PAF	Platelet activating factor
mtDNA	Mitochondrial DNA
mRNA	Messenger RNA
MIP	Macrophage inflammatory protein
PF4	Platelet factor 4
PDGF	Platelet derived growth factor
TGF-β	Transforming growth factor-β
PLA	Platelet-leukocyte aggregates
PSGL-1	P-selectin glycoprotein ligand-1
MCP-1	Monocyte chemotactic protein 1
MPO	myeloperoxidase
PMAs	Platelet-monocyte aggregates
ICAM-1	Intercellular adhesion molecule 1
HNP1	Human neutrophil peptide 1
MIF	Migration inhibiting factor

SDF-1	Stromal cell-derived growth factor 1
MET	Macrophage extracellular trap
PNC	Platelet-neutrophil complexes
LFA-1	Lymphocyte-function-associated-antigen 1
HMGB1	High-mobility group box 1
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
NSAID	Non-steroidal anti-inflammatory drug
OCS	Open canalicular system
DTS	Dense tubular system
EGFR	Epidermal growth factor receptor
CTLA4	Cytotoxic T lymphocyte-associated protein 4
PD-1	Programmed cell death protein 1
NSCLC	Non-small cell lung cancer
BDNF	Brain-derived neurotrophic factor
PA	Anthrax protective antigen
ACD	Acid citrate dextrose
WGA	Wheat germ agglutinin
FCS	Fetal calf serum
DEG	Differentially expressed genes
HTRF®	Homogeneous time resolved fluorescence
LDH	Lactate dehydrogenase
BCA	Bicinchoninic acid assay
LFQ	Label-free quantification
EFA	Extracellular Flux analyzer
PLGF / PIGF	Placental growth factor
SRC	Spare respiratory capacity
OCR	Oxygen consumption rate
ECAR	Extracellular acidification rate
RT-PCR	Reverse transcription PCR
PCR	Polymerase chain reaction
qPCR	Quantitative real time PCR
CRID3	cytokine release inhibitory drug 3
MPC	Mitochondrial pyruvate carrier
S1P	Lysophospholipid sphingosine 1-phosphate
MHC	Major Histocompatibility Complex

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8 Appendix

8.1 Supplementary figure

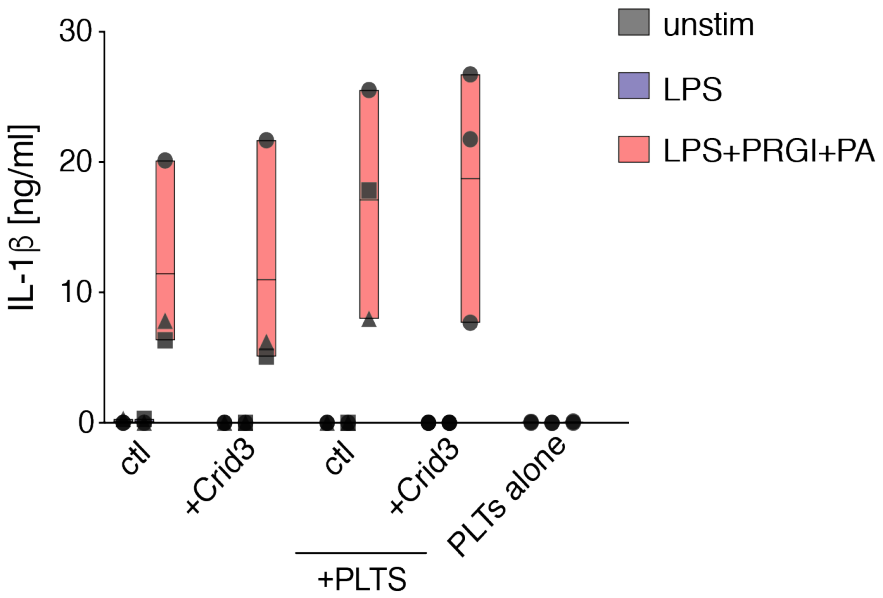

Figure S1 CRID3 does not affect the platelet-mediated cytokine amplification from NRLC4 activated hMDMs. HTRF® measurement of IL-1β in cell-free supernatants of unstimulated (Unstim), LPS stimulated hMDMs (200 ng/ml, 3 hours), or LPS primed and PrgI and PA (2 µg/ml and 0.5 µg/ml respectively, 2 hours) treated hMDMs. Cells were cultivated alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs). The NLRP3 inhibitor CRID3 (2.5 µM) was added to the macrophage-platelet co-culture 30 minutes prior PrgI and PA treatment. Floating bars (with mean and minimum to maximum values) are shown from pooled data from three independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

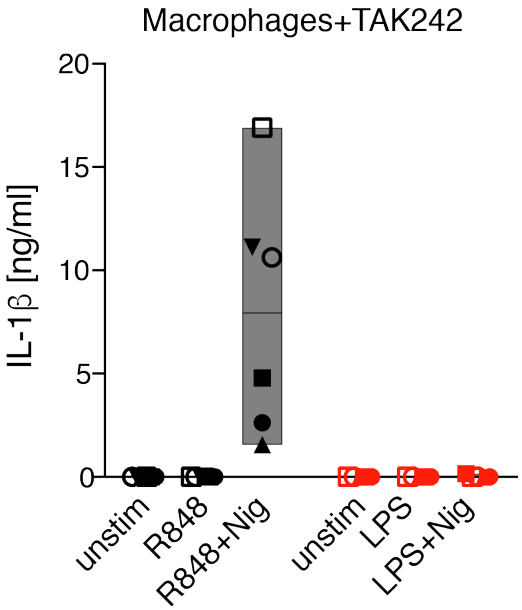

Figure S2 TAK242 treatment blocks TLR4 signalling in hMDMs. HTRF® measurement of IL-1β in cell-free supernatants of unstimulated (Unstim), LPS stimulated hMDMs (200 ng/ml, 3 hours), or R848 (10 µM, 3 hours) primed and nigericin activated (Nig, 10 µM, 90 minutes) hMDMs. TAK242 (0.5 µg/ml) was added to the hMDMs 5 minutes prior to the start of the assay. Floating bars (with mean and minimum to maximum values) are shown from pooled data from four to six independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

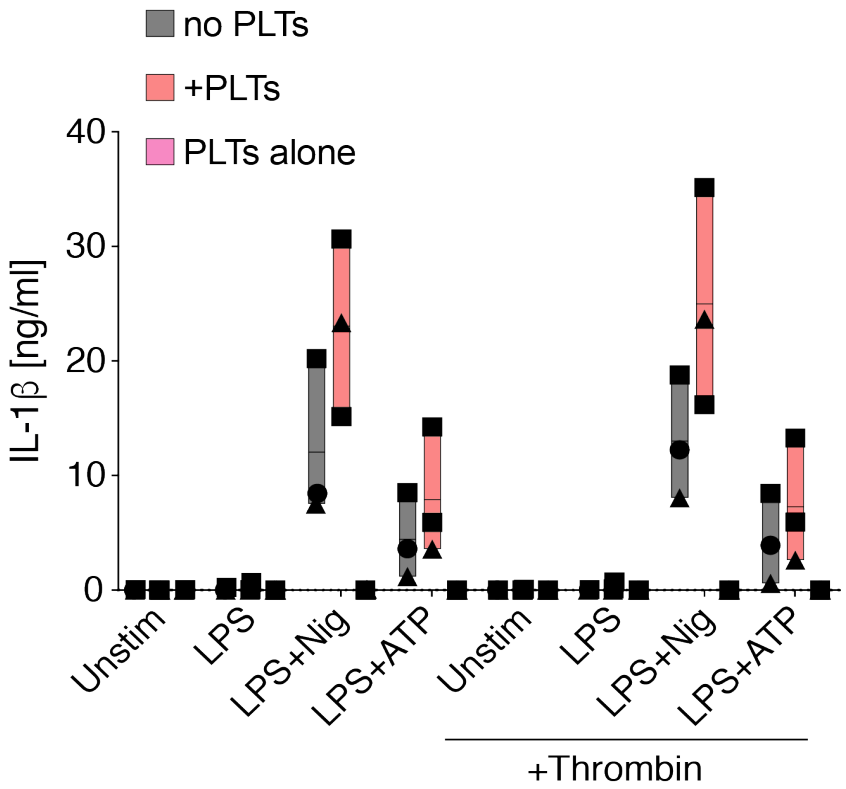

Figure S3 IL-1β secretion of hMDMs is not affected by Thrombin. HTRF® measurement of IL-1β in cell-free supernatants of unstimulated (Unstim), LPS stimulated hMDMs (200 ng/ml, 3 hours), or LPS primed and nigericin (10 µM, 90 minutes) treated hMDMs. Cells were cultivated alone (No PLTs) or in the presence of platelets (+PLTs, 50:1 ratio PLTs to hMDMs). Some of cells were treated with thrombin (0.1 U/ml) prior to the start of the inflammasome assay. Floating bars (with mean and minimum to maximum values) are shown from pooled data from three independent experiments. Each symbol in this figure represents the average of technical triplicates from different donors.

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ABSTRACT

Cancer immunotherapy has been revolutionised by drugs that enhance the ability of the immune system to detect and fight tumors. Immune checkpoint therapies that target the programmed death-1 receptor (PD-1), or its ligand (PD-L1) have shown unprecedented rates of durable clinical responses in patients with various cancer types. However, there is still a large fraction of patients that do not respond to checkpoint inhibitors, and the challenge remains to find cellular and molecular cues that could predict which patients would benefit from these therapies. Using a series of qualitative and quantitative methods we show here that PBMCs and platelets from smokers and patients with head and neck squamous cell carcinoma (HNSCC) or lung cancer express and up-regulate PD-L1 independently of tumor stage. Furthermore, treatment with Atezolizumab, a fully humanised monoclonal antibody against PD-L1, in 4 patients with lung cancer caused a decrease in PD-L1 expression in platelets, which was restored over 20 days. Altogether, our findings reveal the expression of the main therapeutic target in current checkpoint therapies in human platelets and highlight their potential as biomarkers to predict successful therapeutic outcomes.

PII: *25446*

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