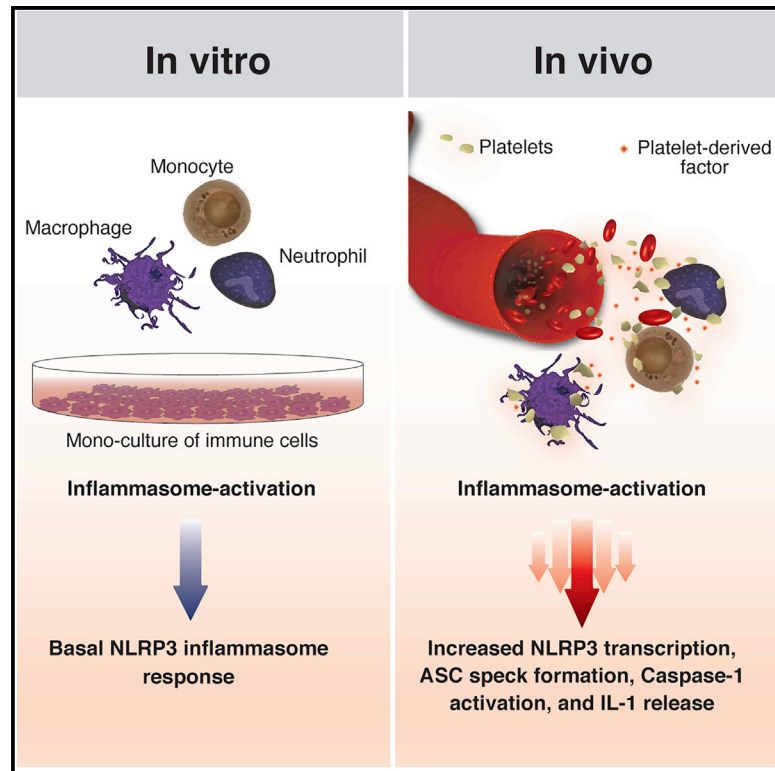


Platelets Fuel the Inflammasome Activation of Innate Immune Cells

Graphical Abstract



Authors

Verena Rolfes, Lucas Secchim Ribeiro, Ibrahim Hawwari, ..., Eicke Latz, Moshe Arditi, Bernardo Simoes Franklin

Correspondence

ribeiro@uni-bonn.de (L.S.R.),
moshe.arditi@cshs.org (M.A.),
franklin@uni-bonn.de (B.S.F.)

In Brief

Rolfes et al. show that platelets license NLRP3 expression in immune cells, boost inflammasome responses *in vitro*, and are critical for full IL-1 responses *in vivo*. A platelet-gene signature is present in NLRP3-related autoinflammatory diseases. Soluble platelet factors enhance NLRP3 transcription and boost IL-1 in macrophages.

Highlights

- Platelets license NLRP3 for inflammasome activation in innate immune cells
- Platelets are required for optimal monocyte inflammasome activation
- Platelets shape IL-1 β *in vivo*, and platelet counts correlate with IL-1 β in plasma
- A constitutive, heat-sensitive soluble platelet-factor boost IL-1 β in macrophages



Article

Platelets Fuel the Inflammasome Activation of Innate Immune Cells

Verena Rolfes,^{1,8} Lucas Secchim Ribeiro,^{1,8,*} Ibrahim Hawwari,¹ Lisa Böttcher,¹ Nathalia Rosero,¹ Salie Maasewerd,¹ Marina Lima Silva Santos,² Tomasz Próchnicki,¹ Camila Meirelles de Souza Silva,⁷ Carlos Wagner de Souza Wanderley,⁷ Maximilian Rothe,¹ Susanne V. Schmidt,¹ H. James Stunden,¹ Damien Bertheloot,¹ Magali Noval Rivas,⁴ Cor Jesus Fontes,³ Luzia Helena Carvalho,² Fernando Queiroz Cunha,⁷ Eicke Latz,^{1,5,6} Moshe Arditi,^{4,*} and Bernardo Simoes Franklin^{1,9,*}

¹Institute of Innate Immunity, Medical Faculty, University of Bonn, 53127 Bonn, NRW, Germany

²Laboratório de Malária, Instituto René Rachou, Fundação Oswaldo Cruz, 30190-002 Belo Horizonte, MG, Brazil

³Departamento de Clínica Médica, Universidade Federal de Mato Grosso, 78060-900 Cuiabá, MT, Brazil

⁴Departments of Pediatrics, Division of Infectious Diseases and Immunology, and Infectious and Immunologic Diseases Research Center (IIDRC), Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA

⁵Department of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, MA 01655, USA

⁶German Center for Neurodegenerative Diseases, 53127 Bonn, NRW, Germany

⁷Center for Research in Inflammatory Diseases, School of Medicine of Ribeirão Preto, University of Sao Paulo, 14049-900 Ribeirão Preto, SP, Brazil

⁸These authors contributed equally

⁹Lead Contact

*Correspondence: ribeiro@uni-bonn.de (L.S.R.), moshe.arditi@cshs.org (M.A.), franklin@uni-bonn.de (B.S.F.)

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SUMMARY

The inflammasomes control the bioactivity of pro-inflammatory cytokines of the interleukin (IL)-1 family. The inflammasome assembled by NLRP3 has been predominantly studied in homogeneous cell populations *in vitro*, neglecting the influence of cellular interactions that occur *in vivo*. Here, we show that platelets boost the inflammasome capacity of human macrophages and neutrophils and are critical for IL-1 production by monocytes. Platelets license *NLRP3* transcription, thereby enhancing ASC oligomerization, caspase-1 activity, and IL-1 β secretion. Platelets influence IL-1 β production *in vivo*, and blood platelet counts correlate with plasmatic IL-1 β levels in malaria. Furthermore, we reveal an enriched platelet gene signature among the highest-expressed transcripts in IL-1 β -driven autoinflammatory diseases. The platelet effect is independent of cell-to-cell contact, platelet-derived lipid mediators, purines, nucleic acids, and a host of platelet cytokines, and it involves the triggering of calcium-sensing receptors on macrophages. Hence, platelets provide an additional layer of regulation of inflammasomes and IL-1-driven inflammation.

INTRODUCTION

An unbalanced production of interleukin-1 (IL-1) cytokines drives the immunopathology of several auto-inflammatory diseases (Sims and Smith, 2010). As nearly all cells express the IL-1 receptor (IL-1R), IL-1 cytokines have the ability to influence innate and adaptive immunity and exert broad effects in the body. IL-1 β is unique in the medical literature: while many human inflammatory diseases are caused by a host of cooperative inflammatory factors, mutations in genes controlling the expression of IL-1 β cause a spectrum of life-threatening auto-inflammatory syndromes (Broderick et al., 2015). Monotherapies blocking IL-1 β activity in patients with auto-inflammatory syndromes result in a rapid and sustained reversal of symptoms and severity (Dinarello et al., 2012) and are currently the first line of intervention against these conditions. A growing bulk of evidence has now characterized other common inflammatory and metabolic condi-

tions as responsive to IL-1 β neutralization (Dinarello, 2011; Dinarello and van der Meer, 2013). This was further validated by the results of the CANTOS Study, which showed that canakinumab, a humanized anti-IL-1 β monoclonal antibody, significantly reduced the risk for recurrent cardiovascular events (Ridker et al., 2017a) and indicated that IL-1 β is associated with increased incidence of fatal lung cancer (Ridker et al., 2017b).

The expression of IL-1 cytokines is tightly regulated. For instance, the production of some members of this family (e.g., IL-1 β and IL-18) is restricted to immune cells. Furthermore, upon induction, these proteins are synthesized as inactive precursors in the cytosol, and a series of intracellular events is required for their maturation and release into the extracellular space. These events include the assembly of inflammasomes—intracellular protein complexes formed by a sensor—such as NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and the cysteine



protease caspase-1 (Latz et al., 2013). Upon activation, inflammasome sensors recruit ASC, which oligomerizes to form a micron-sized structure termed ASC speck, which recruits and activates caspase-1. Active caspase-1 processes pro-IL-1 β and pro-IL-18 into their bioactive forms and drives an inflammatory cell death, termed pyroptosis, through gasdermin-D-induced membrane pore formation and leakage of cytosolic content (Kayagaki et al., 2015; Liu et al., 2016).

The majority of the studies on inflammasomes were performed *in vitro*, predominantly in monocultures of macrophages. Although this approach allowed the discovery of molecular mechanisms controlling inflammasomes, it underestimated the influence of other cell populations on the regulation of IL-1 cytokines *in vivo*. For instance, it has been recently discovered that T cells drive inflammasome-independent IL-1 β production by dendritic cells (DCs) (Jain et al., 2020).

Around one trillion platelets ($150\text{--}450 \times 10^9/\text{L}$) circulate in the blood of a healthy individual, outnumbering all other leukocytes in the vasculature by several folds. Platelets have been increasingly recognized for their roles in immunity (Carestia et al., 2019; Dann et al., 2018; Kral et al., 2016; Passacquale et al., 2011) and were reported to produce IL-1 cytokines (Allam et al., 2017; Denis et al., 2005; Hawrylowicz et al., 1989; Thornton et al., 2010). More recently, they were reported to assemble inflammasomes (Cornelius et al., 2019; Hottz et al., 2013). Platelets could therefore be relevant cellular sources of IL-1 or extracellular ASC specks *in vivo* or could alter the inflammatory responses of other immune cells.

Here, we report that platelets are essential for the full inflammasome activation of innate immune cells. Co-culture with platelets not only boosted the NLRP3 activation and production of IL-1 α , IL-1 β , and IL-18 from human macrophages and neutrophils, but it was also crucial for the optimal production of IL-1 cytokines by human monocytes. Platelets specifically influenced the NLRP3 inflammasome activation of macrophages *in trans* by enhancing the transcription of NLRP3 and IL-1 β . Using a series of complementary techniques that included assays with human platelets and megakaryocytes (MKs), as well as platelets and cells from knockout and transgenic mice, we demonstrated that platelets and MKs do not express the components of the canonical inflammasome (NLRP3, ASC, caspase-1, and IL-1 β). Indeed, the effect of platelets on human monocyte-derived macrophages (hMDMs) was independent of IL-1 signaling and did not require direct cell contact, platelet-derived nucleic acids, lipid mediators, or purines, but it could be neutralized by heat inactivation. We further show that platelet depletion attenuated systemic production of IL-1 β *in vivo*. Furthermore, platelet counts and plasma levels of IL-1 β were positively correlated in naturally infected malaria patients. Moreover, we observed an enriched platelet signature among the highest-expressed genes in a cohort of pediatric patients with gain-of-function mutations in NLRP3 that cause Muckle-Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disease (NOMID).

In summary, platelets fuel NLRP3 activity of immune cells and shape IL-1 inflammation. Thus, platelet-modifying therapies could have widespread implications for autoinflammatory and autoimmune diseases.

RESULTS

Platelets Boost IL-1 Production in Inflammasome-Activated Immune Cells

The NLRP3 inflammasome is primarily assembled in myeloid cells. Although activated platelets are known to form aggregates with and modulate the functions of immune cells (Allen et al., 2019; Carestia et al., 2019; Dann et al., 2018; Kral et al., 2016; Passacquale et al., 2011), their effects on the inflammasome activation of leukocytes remains unexplored. To address this question, we investigated the influence of platelets on the production of IL-1 cytokines elicited by the bona fide NLRP3 inflammasome activators Nigericin and ATP in mouse and human immune cells. Mouse bone marrow-derived macrophages (BMDMs) (Figure 1A), hMDMs (Figure 1B), human neutrophils (Figure 1C), and human CD14⁺ monocytes (Figure 1D) were cultured or not with platelets. Co-cultures were left untreated or primed with lipopolysaccharide (LPS), followed by NLRP3 activation. The levels of IL-1 β and tumor necrosis factor alpha (TNF- α) were measured in cell-free supernatants. A multiplex cytokine assay was used to additionally measure the production of IL-1 α and IL-18, two other members of the IL-1 family, along with several other cytokines and growth factors in hMDMs and human neutrophils (Figures S1A and S1B). Co-culture with platelets boosted the production of IL-1 β from mouse and human inflammasome-activated macrophages and human neutrophils (Figures 1A–1C) without significantly altering the release of lactate dehydrogenase (LDH) from activated cells (Figure S2A). Fitting a sigmoidal curve between the IL-1 β levels produced in inflammasome-activated macrophages and the platelet-macrophage ratio, we found a strong ($R = 0.8886$, $p = 0.0163$) concentration-dependent effect of platelets in these cells (Figure S1C). The addition of platelets also boosted the release of IL-18 and IL-1 α from activated hMDMs (Figure S1A). Extending these results, the addition of platelets to neutrophils also enhanced their production of IL-8 (Figure S1B). Platelets alone produced CCL5 (RANTES) and low levels of IL-18 that were close to the detection limit of the assays. Platelets lacked expression of most of the other investigated cytokines (Figures 1, S1A, and S1B), indicating that they do not directly contribute to the cytokines measured in the co-cultures. Notably, the co-culture of platelets with mouse macrophages, human monocytes, and neutrophils, but not hMDMs, resulted in diminished TNF- α production in response to LPS stimulation. These results suggest that despite NF- κ B being a common transcription factor regulating the production of TNF- α and IL-1 cytokines, platelets differentially regulate the production of these cytokines.

Next, we asked whether platelets also influence the activity of other inflammasomes. For this, we activated AIM2 or NLRC4, respectively, by intracellular delivery of double-stranded DNA (dsDNA) (Poly[dA:dT]) (Figure S1D) or the rod protein of the T3SS apparatus from *Salmonella typhimurium* (PrgI) (STAR Methods; Figure S1E) in co-cultures of human platelets and macrophages. Stimulation of the AIM2 or NLRC4 inflammasomes in hMDMs resulted in IL-1 β secretion, which was not significantly altered by the addition of platelets (Figures S1D and S1E), indicating that the platelet effect was NLRP3-specific. Co-culture with platelets also boosted IL-1 β production from

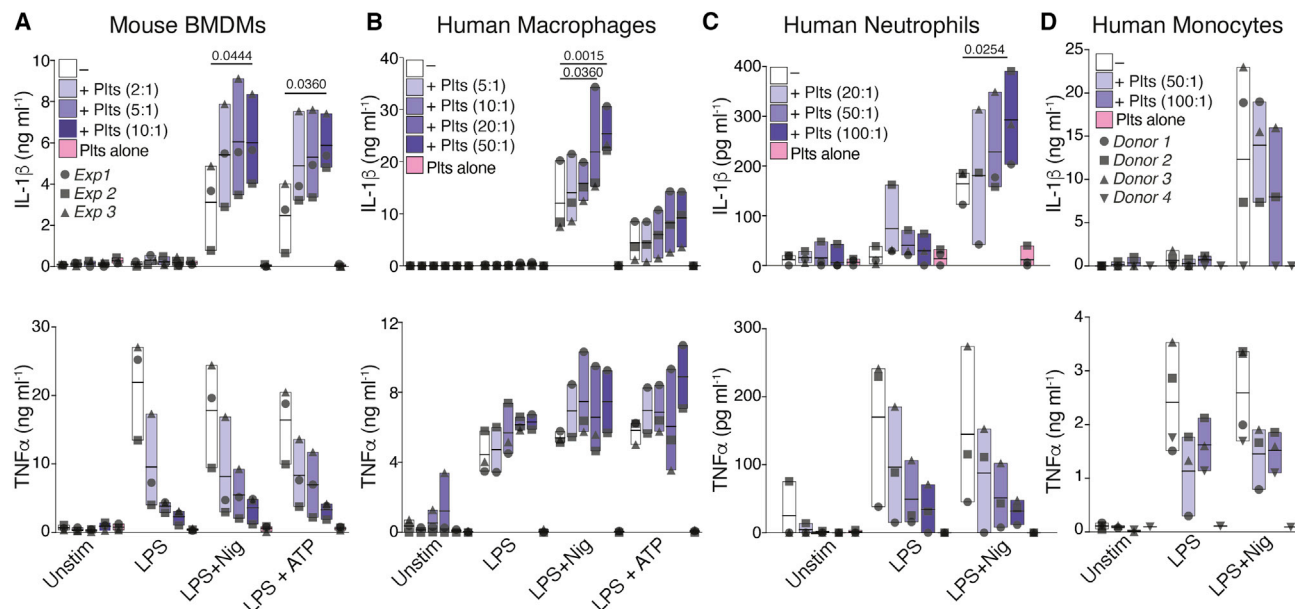


Figure 1. Platelets Boost Inflammasome Activation of Innate Immune Cells

(A–D) IL-1 β and TNF- α levels released by unstimulated (Unstim), LPS-primed, and Nigericin, or ATP-activated (A) WT BMDMs, (B) human macrophages (hMDMs), (C) neutrophils, or (D) monocytes. Cells were cultivated alone (–) or with platelets (+Plts) in the indicated ratios. Data is presented as floating bars (with mean and minimum to maximum values) and pooled from at least three independent experiments with cells and platelets from different donors. Each symbol represents the average from technical triplicates per blood donor or mouse.

See also [STAR Methods](#) and [Figure S1](#).

hMDMs primed with Toll-like receptor (TLR)2 or TLR7/8 agonists (Pam3Csk4 and R848, respectively) ([Figure S1F](#)), indicating that the platelet effect is not exclusively mediated through TLR4. However, blockade of TLR4 signaling on hMDMs with resatorvid (TAK-242) ([Matsunaga et al., 2011](#)) partially prevented the effect of platelets ([Figure S1G](#)), indicating that the platelet effect is in part orchestrated by TLR4. Together, these data show that platelets boost the production of IL-1 cytokines in NLRP3-activated innate immune cells.

Platelets Are Critical for the Inflammasome Activation of Human Monocytes

Notably, co-culture with platelets did not influence the production of IL-1 β by inflammasome-activated monocytes ([Figure 1D](#)), likely due to the steady-state presence of contaminating platelets on the preparations of freshly isolated monocytes. To address that, we isolated CD14⁺ monocytes from peripheral blood using magnetic separation kits, added or not with a platelet removal cocktail ([STAR Methods](#)). Cell purity was assessed by fluorescence-activated cell sorting (FACS) ([Figure 2A](#)). Platelet depletion efficiently reduced the numbers of free platelets (CD41⁺CD14[–]) or platelets associated with monocytes (CD41⁺CD14⁺), while enriching the frequency of platelet-free monocytes (CD14⁺CD41[–]) ([Figures 2A and 2B](#)). Importantly, platelet depletion was not detrimental to monocytes, assessed by LDH release ([Figure S2B](#)). However, platelet removal impaired the cytokine response of NLRP3-activated monocytes. Notably, monocyte responses could be restored by the re-addition of autologous platelets ([Figures 2C and S2C](#)). These data indicate

that platelets are crucial for monocytes to trigger an optimal inflammatory response.

Human monocytes can also engage an alternative inflammasome activation, in which LPS alone is sufficient to trigger IL-1 β maturation and secretion ([Gaidt et al., 2016](#)). To test the effect of platelet removal in alternatively activated monocytes, we stimulated standard or platelet-depleted CD14⁺ monocytes with LPS (1 μ g mL^{–1}) for 16 h. Similar to the effect on the canonical inflammasome ([Figure 2C](#)), platelet depletion impaired the IL-1 β response from alternatively activated cells ([Figure 2D](#)), and the re-addition of platelets rescued their IL-1 β production ([Figure 2D](#)).

Next, we asked whether co-culture with platelets could also enhance NLRP3 activation in the monocytic cell line THP-1, which has been used extensively to characterize the biology of the inflammasomes. As expected ([Gaidt et al., 2016, 2017](#)), NLRP3-activated THP-1s were able to produce IL-1 β on their own. Nevertheless, co-culture with platelets boosted the production of IL-1 β by THP-1s to levels comparable to those produced by primary monocytes ([Figure 2E](#)). No IL-1 β was detected on platelets cultivated alone. Together, these findings reveal that platelets are critical for optimal inflammasome activation by human monocytes.

Platelets Influence IL-1 Responses *In Vivo*

As monocytes and macrophages are relevant sources of IL-1, we reasoned that platelets might influence IL-1 responses *in vivo*. Therefore, we investigated the effects of platelet depletion using a mouse model of systemic IL-1-dependent inflammation

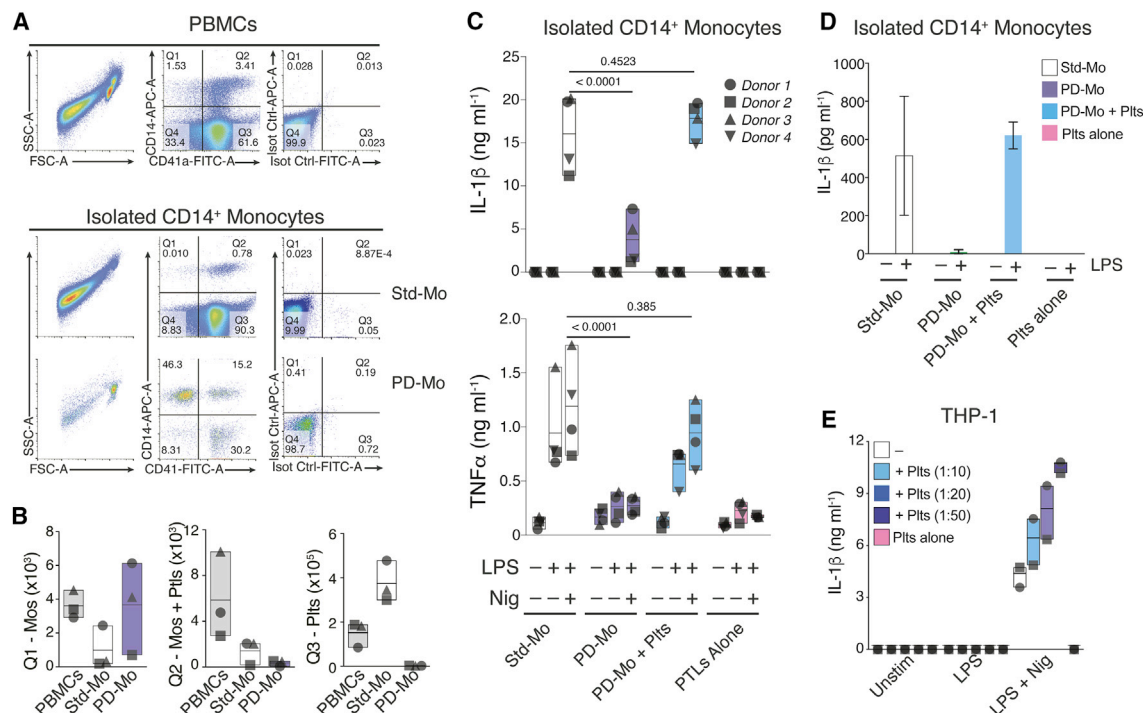


Figure 2. Platelets Are Critical for the Production of IL-1 Cytokines by Human Primary Monocytes

(A) Representative FACS dot plots of CD41 and CD14 expression in human PBMCs, standard (Std-Mo), or platelet-depleted (PD-Mo) CD14⁺ monocytes (see STAR Methods).

(B) Quantification of platelets (CD41⁺CD14⁻), platelet-monocyte aggregates (CD41⁺CD14⁺), and platelet-free monocytes (CD41⁻CD14⁺) in PBMCs and isolated monocytes.

(C) IL-1 β and TNF- α levels in cell-free supernatants of Std-Mo versus PD-Mo, or in platelet-depleted monocytes replenished with autologous platelets (PD-Mo + Plts, 50:1 Plt:Cell), and stimulated as indicated.

(D and E) IL-1 β levels released by non-canonically activated Std-Mo, PD-Mo, or PD-Mo + Plts, stimulated with LPS (1 μ g mL⁻¹) for 16 h (D) or by inflammasome-activated THP-1 $\pm$ platelets (E). Data is presented as floating bars (with mean and minimum to maximum values) and pooled from independent experiments. Each symbol represents the average from technical triplicates per blood donor. Data in (D) shows bar graph with Mean + SD, from a representative of two independent experiments.

See also Figure S2.

triggered by imidazoquinolinone compounds (Kanneganti et al., 2006). We induced thrombocytopenia in mice by intravenous (i.v.) injection of a GPIIb monoclonal antibody (α CD42b). Treatment with α CD42b has been widely used in literature (Barrett et al., 2019; Boilard et al., 2010; Sreeramkumar et al., 2014; Xiang et al., 2013) and results in a drop of the blood platelet counts (Figures 3A, S3A, and S3B), with no reported effects on the blood counts of other leukocytes (Barrett et al., 2019; Packham et al., 2014). As previously reported (Kanneganti et al., 2006), intraperitoneal (i.p.) injection of R848 in combination with LPS induced robust IL-1 β systemically, measured in sera (Figure 3B), and locally, in peritoneal lavage (PEL) (Figure 3C). Notably, this response was attenuated by platelet depletion. Although levels of IL-1 β were significantly lower in the sera of platelet-depleted mice (Figure 3B), we could not detect IL-1 β in the PEL of all mice (Figure 3C). Importantly, platelet depletion did not affect the migration of neutrophils and monocytes to the peritoneum (Figures 3D and S3B). These data suggest that the decrease in IL-1 β levels was not due to reduced peritoneal leukocyte counts.

Platelets were reported to amplify IL-1 inflammation in rheumatoid arthritis (RA) (Boilard et al., 2010) and more recently in atherosclerosis (Barrett et al., 2019), two inflammatory diseases characterized by strong IL-1 inflammation (Di Giovine et al., 1987; Joosten et al., 2009; Mantovani et al., 2019; Ridker et al., 2017a), supporting their role in modulating IL-1 levels *in vivo*. In line with this hypothesis, a positive correlation between blood platelet counts and plasma IL-1 β concentrations was recently reported in a cohort of 500 healthy, Caucasian volunteers (Netea et al., 2016; Tunjungputri et al., 2018). However, blood leukocyte counts were equally correlated with plasma levels of IL-1 β in that study, hindering the precise contribution of platelets for the IL-1 β concentrations (Tunjungputri et al., 2018). To investigate the relation between platelet counts and IL-1 β concentrations in the context of disease, we studied a cohort of human subjects naturally infected with *Plasmodium vivax*, the predominant cause of malaria in the Brazilian Amazon (Howes et al., 2016). Infections with *P. vivax* commonly cause pro-inflammatory cytokine imbalance (Andrade et al., 2010), with thrombocytopenia and anemia being the two most commonly associated complications. We

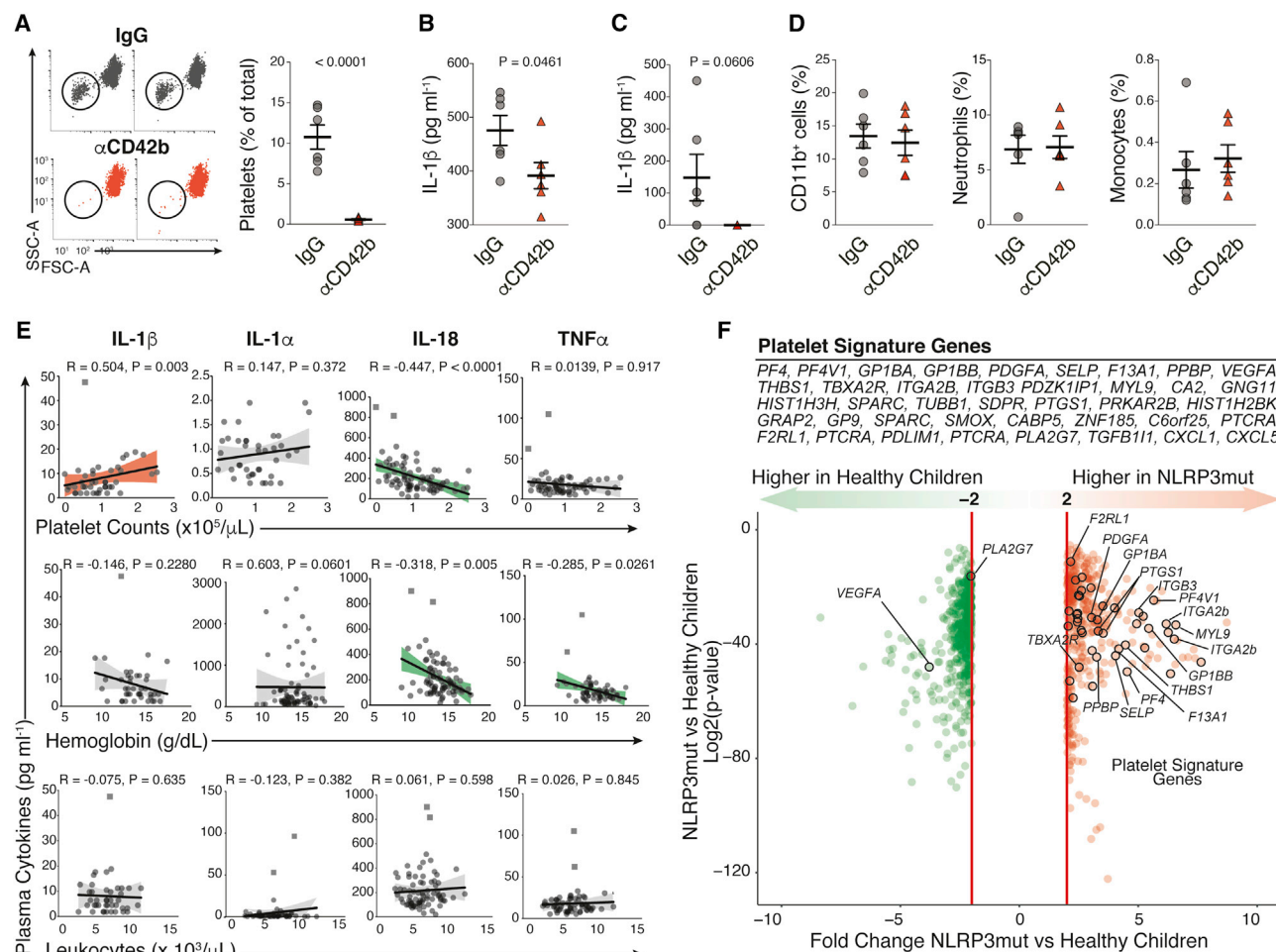


Figure 3. Platelets Influence IL-1 Levels In Vivo

(A) Representative FACS plots and quantification of platelets in the blood of mice treated with a platelet-depleting Ab (α CD42b) or IgG control. (B and C) IL-1 β levels in sera (B) or peritoneal lavage (C) from platelet-depleted mice after i.p. challenge with LPS + R848 (see STAR Methods). (D) FACS quantification of neutrophils and monocytes in peritoneal lavage of mice treated as in (C). (E) Correlation between plasma cytokines and blood cell counts or hemoglobin levels in naturally infected malaria patients ($n = 78$). Red lines, positive correlations; shaded areas, 95% confidence; squared symbols, outliers (see STAR Methods). (F) Volcano plot of log $_2$ fold change versus significance (p value) highlighting the platelet-signature genes within transcripts of healthy ($n = 14$) versus pediatric patients ($n = 22$) with NLRP3 mutations (NLRP3mut) causing MWS/NOMID (Balow et al., 2013). See also Figure S3.

found that platelet counts positively correlated with plasma IL-1 β concentrations (Spearman's $R = 0.504$, $p = 0.0028$). Despite a positive trend, no correlations were found between platelet counts and IL-1 α ($R = 0.15$, $p = 0.37$) or TNF- α ($R = 0.014$, $p = 0.917$) (Figure 3E). Unexpectedly, IL-18 levels were negatively correlated with platelet counts ($R = -0.45$, $p < 0.0001$), which suggests that different mechanisms regulate the production of this cytokine *in vivo*. Supporting this hypothesis, heme (Li et al., 2014) and oxidized hemoglobin were shown to activate the NLRP3 inflammasome (Nyakundi et al., 2019). Indeed, IL-18 ($R = -0.32$, $p = 0.005$) and TNF- α ($R = -0.29$, $p = 0.003$) correlated with hemoglobin levels (Figure 3E), suggesting that anemia may be a contributing factor for the regulation of these cytokines, but not for the associations between platelet counts and IL-1 β . Importantly, none of the cytokines investigated correlated with

leukocyte counts (Figure 3E) in the malaria cohort. As leukocytes are a relevant source in IL-1 cytokines in the blood, these data support that platelets are major contributors in the regulation of IL-1 levels in this context. IL-1 β levels also had no correlation with parasitemia (data not shown), suggesting that IL-1 β production may not be caused by *Plasmodium*-associated danger molecules (Dostert et al., 2009).

Next, we asked whether there is a relationship between platelets and inflammation in exclusively IL-1 β -driven human autoinflammatory syndromes (Broderick et al., 2015). We generated a platelet gene signature comprising 45 transcripts known to be either platelet-specific or strongly associated with platelet activity from direct comparisons of publicly available gene expression analyses of purified human platelets (Eicher et al., 2016; Rowley et al., 2011). Remarkably, 42 out of 45 platelet-signature

genes were upregulated in the whole blood of pediatric patients harboring *NLRP3* mutations (*NLRP3mut*) associated with the IL-1-driven autoinflammatory disorders MWS and NOMID (Balow et al., 2013; Goldbach-Mansky et al., 2006), compared to healthy children (Figure 3F). Importantly, as reported in the original cohort (Goldbach-Mansky et al., 2006), the baseline clinical manifestations and blood cell counts of MWS/NOMID patients were within the pediatric reference ranges, supporting that differences in blood cell counts are likely not responsible for the changes we observed. Collectively, these findings support that platelets influence IL-1 β *in vivo*.

Platelet Effect on Immune Cells Is Independent of Platelet-Derived IL-1 or Platelet Inflammasomes

Previous reports have indicated that platelets express IL-1 cytokines, including IL-1 α (Thornton et al., 2010), IL-1 β (Denis et al., 2005), and IL-18 (Allam et al., 2017). However, as shown in Figures 1, S1A, and S1B, IL-1 α and IL-1 β were not detected in monocultures of platelets in our experimental settings. To exclude the possibility that platelets directly contribute to the IL-1 cytokines measured in our assays, we performed co-culture experiments using platelets or BMDMs from mice with targeted genetic deficiency in IL-1 proteins or IL-1 receptors. First, we activated the NLRP3 inflammasome in wild-type (WT) BMDMs co-cultured with platelets from either WT or IL-1 β -deficient (*Il1b*^{-/-}) mice. We found that *Il1b*^{-/-} platelets were equally able to boost IL-1 β production in inflammasome-activated BMDMs as WT platelets (Figure 4A). Similarly, the addition of WT platelets to BMDMs from IL-1R-deficient mice (*Il1r*^{-/-}) also boosted their IL-1 β production upon inflammasome activation (Figure 4B), thus excluding a role for platelet-derived IL-1 α in mediating the response. Finally, the addition of platelets to *Il-18*^{-/-} BMDMs equally boosted their IL-1 β production toward LPS + Nigericin (Figure 4C), indicating that neither the platelet-derived IL-1 α / β nor the IL-18 cytokines could be responsible for the platelet effect.

Recent studies reported the assembly of the NLRP3 inflammasome in platelets and their involvement in the pathogenesis of dengue (Hottz et al., 2013) and sickle cell disease (Vogel et al., 2018). To determine whether the co-activation of platelet NLRP3 could contribute to the IL-1 production in co-cultures, we tested if the addition of WT platelets could compensate for the impaired IL-1 response of *Nlrp3*^{-/-} or *Pycard*^{-/-} (*Asc*^{-/-}) BMDMs. We stimulated LPS-primed *Nlrp3*^{-/-} or *Pycard*^{-/-} BMDMs with Nigericin or ATP, alone or in co-culture with WT platelets. As expected, macrophages from *Nlrp3*^{-/-} and *Pycard*^{-/-} mice failed to produce IL-1 β in response to inflammasome activation (Figure S2D). The addition of WT platelets to *Nlrp3*^{-/-} or *Pycard*^{-/-} macrophages had no effect on the IL-1 β production by these BMDMs (Figure S2D), indicating that the activation of inflammasomes in platelets is not involved in the amplification of macrophage IL-1 β responses. To determine whether the NLRP3/ASC inflammasome is expressed and assembled in platelets, we imaged inflammasome assembly in bone marrow (BM) cells from transgenic (Tg) ASC-mCitrine mice (Tzeng et al., 2016). ASC-mCitrine⁺ BM cells were stained for leukocytes (CD45), neutrophils (Ly6G), and platelets (CD41) and assessed by flow cytometry and confocal microscopy.

While ASC was clearly visualized and assembled into fluorescent specks in inflammasome-activated leukocytes (CD45⁺), macrophages (CD45⁺F4/80⁺), and neutrophils (CD45⁺Ly6G⁺), it was not observed in platelets and MKs (CD41⁺ cells) (Figures S3C and S3D). These results were confirmed using flow cytometry and confocal imaging of BM cells from ASC-mCherry knock-in reporter mice (Figure S3E).

Most studies reporting the presence (Brown et al., 2013; Denis et al., 2005) or activity (Hawrylowicz et al., 1989; Kaplanski et al., 1993) of IL-1 cytokines or inflammasome molecules used preparations of washed platelets without assessment of purity, which could not rule out the presence of contaminating leukocytes in their platelet preparations (Pillitteri et al., 2007). We therefore re-evaluated the expression of the canonical inflammasome components in platelets and peripheral blood mononuclear cells (PBMCs) from healthy volunteers. The purity of platelet preparations was assessed by flow cytometry, microscopy, and qPCR using platelet (PF4) and leukocyte markers (*CD45*, *CD14*) (Figures S4A–S4E and Table S1). Importantly, both freshly isolated human and mouse platelets remained viable after purification and upregulated P-selectin (CD62P) upon thrombin stimulation (Figures S3F and S4E). Nevertheless, human platelets did not express the inflammasome components NLRP3, ASC, and caspase-1, nor IL-1 β at the mRNA (Figure S4D) and protein level (Figures S4F–S4H), although these molecules were promptly detected in PBMCs from the same donors and increased upon LPS stimulation. Furthermore, unlike the human monocytic line THP-1, no expression of IL-1 β and inflammasome molecules was detected in the human megakaryocytic cell MEG-01 (Figure S3G), which also failed to secrete mature IL-1 β upon inflammasome activation (Figure S3H).

Finally, we re-analyzed publicly available transcription profiles of purified platelets from five independent studies (Devignot et al., 2010; Gnatenko et al., 2005; Londin et al., 2014; Raghavachari et al., 2007; Spivak et al., 2014), including the gene expression profiles of platelets from patients with dengue (Devignot et al., 2010) and sickle cell disease (Raghavachari et al., 2007; Figures S5A–S5E). In all these studies, we re-assessed the expression of IL-1 β and the inflammasome components NLRP3, ASC, and caspase-1 using the expression of platelet markers PF4 (CXCL4) or platelet-derived growth factor A (PDGFA) as comparisons (Figures S5A–S5E). None of the NLRP3, ASC, caspase-1, or IL-1 β transcripts were detected in human platelets in these studies (Figures S5A–S5E). Together with our previous findings, these observations support the conclusion that human and mouse platelets do not express the components of the canonical NLRP3 inflammasome and are therefore unable to assemble inflammasomes.

The Platelet Effect Is Mediated by a Soluble and Constitutive Factor

Phagocytosis of activated platelets has been shown to regulate platelet and neutrophil function, survival, and differentiation (Badrnya et al., 2014; Chatterjee et al., 2015; Lang et al., 2002; Senzel and Chang, 2013). To determine whether phagocytosis is responsible for the effect of platelets on inflammasome activation, we performed confocal imaging of hMDMs (Figure 4D) and purified blood neutrophils (Figure S2E) incubated with freshly

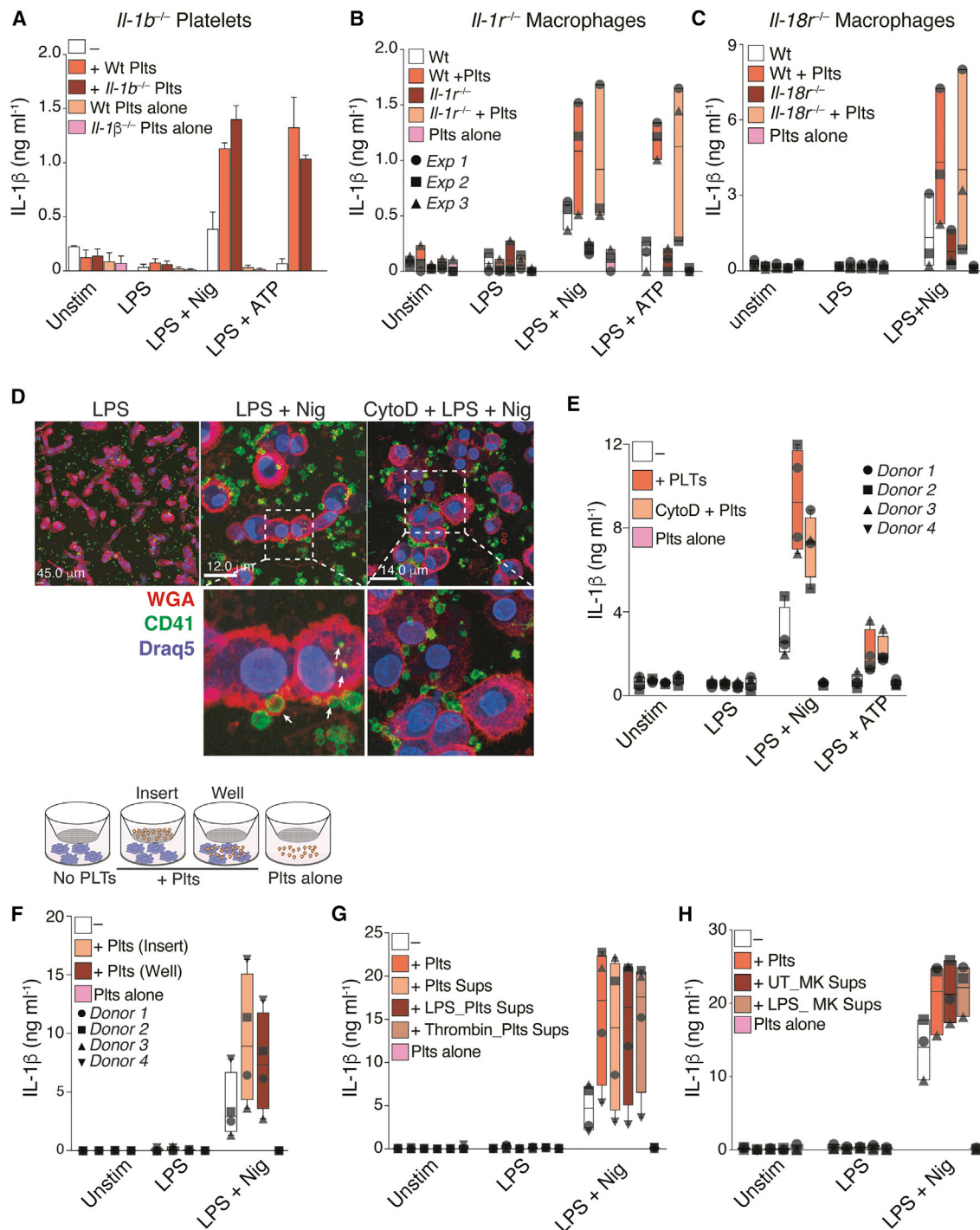


Figure 4. The Effect Is Independent of Platelet-Derived IL-1 Cytokines or Inflammasomes

(A) IL-1β released by WT BMDMs ± platelets (5:1 ratio) from WT or *Il1b^{-/-}* mice. Bar graph with Mean and SD from one experiment with three mice. (B and C) IL-1β levels in cell-free supernatants of WT or *Il1r^{-/-}* (B) or *Il18r^{-/-}* (C) BMDMs ± platelets (5:1 ratio) from WT mice. (D) IL-1β levels in cell-free supernatants of WT, *Nlrp3^{-/-}*, or *Pycard^{-/-}* BMDMs ± WT platelets (5:1 ratio). Confocal imaging of hMDMs co-cultured with platelets (50:1 ratio). Cells were pretreated or not with Cytochalasin D before the addition of platelets. Blue (Draq5, nuclei); red (wheat germ agglutinin [WGA], plasma membrane); green (anti-CD41, platelets). (E) IL-1β released by hMDMs treated as in (D). (F) Schematics of *trans*-well and *well* and IL-1β released by hMDMs cultured alone, in direct co-cultures with platelets (Well), or in *trans*-well (insert). (G and H) IL-1β released by hMDMs ± platelets or platelet (G) and MK (H) supernatants. Graphs show floating bars (with mean and minimum to maximum values) from pooled data from two to four independent experiments. Each symbol represents the average of technical triplicates from different donors.

isolated platelets. Co-cultures were stimulated with LPS, followed by inflammasome activation. Both hMDMs (Figure 4D) and neutrophils (Figure S2E) phagocytosed platelets; however, pretreatment of hMDMs with the phagocytosis inhibitor Cytochalasin D did not prevent the platelet-mediated boost of IL-1 β (Figure 4E). Furthermore, platelets were able to boost the IL-1 β responses of hMDMs in both direct and in *trans*-well co-cultures (Figure 4F), and cell-free supernatant from resting, LPS-stimulated, or thrombin-activated platelets also enhanced IL-1 β production from hMDMs (Figure 4G). Importantly, thrombin had no effect on macrophages stimulated in the absence of platelets (Figure S2F). Thus, the platelet influence on IL-1 β production by inflammasome-activated macrophages is independent of cell contact. In contrast, the transfer of platelet supernatants to cultures of human neutrophils or monocytes did not promote a significant increase in the production of IL-1 β (Figure S2G; data not shown), suggesting that cell-to-cell contact may be required for the platelet-boosting effects in these cells.

We observed that supernatants from quiescent and activated platelets had similar effects on the inflammasome activation of hMDMs (Figure 4G). To determine whether this capacity of quiescent platelets was due to undetectable activation during isolation, we transferred supernatants from resting MEG-01 to cultures of hMDMs. Similar to platelets, supernatants from resting MEG-01 boosted the inflammasome activity in hMDMs (Figure 4H). This finding points toward the presence of a soluble and constitutively expressed factor secreted by both platelets and MKs, which modulates the inflammasome activity of macrophages.

Platelets License NLRP3 Transcription to Boost Inflammasome Activation on Human Macrophages

In most immune cells, the activation of NLRP3 requires a priming stimulus, such as the engagement of pattern recognition receptors, that initiates the transcription of *NLRP3*. The amount of NLRP3 available in the cytosol is a key limiting factor regulating its activation. Since we observed that the platelet effect was specific to the activation of the NLRP3 inflammasome (Figures S1D and S1E), we investigated if the platelet effect on macrophages occurs at the transcriptional level during priming or afterward during activation of the NLRP3 inflammasome. To this end, we co-cultured NLRP3-overexpressing immortalized mouse macrophages (NLRP3FiM ϕ s) with platelets and directly stimulated these cells with Nigericin. As the LPS priming signal is also required for the upregulation of pro-IL-1 β , we assessed caspase-1 activity using a specific caspase-1 luminescence assay as a readout. The addition of platelets to NLRP3FiM ϕ s did not enhance their caspase-1 activity (Figure 5A), supporting that platelets influence the inflammasome activation of hMDMs by enhancing the transcription of NLRP3. To determine whether platelets modulate the expression of other inflammasome molecules, we evaluated hMDMs cultivated or not with platelet supernatants, which were used to minimize the detection of transcripts arising from platelets. The addition of supernatants from resting platelets to macrophages boosted their expression of IL-1 β (Figure 5B and Table S1). Surprisingly, the platelet supernatant was as effective as LPS to induce the expression of NLRP3 and pro-IL-1 β protein (Figure S2H, lanes 1 and 3). However, the platelet

supernatant did not alter the expression of pro-caspase-1 or ASC (Figure 5B). In conformity with increased NLRP3 availability in the cytosol, exposure to platelets boosted the formation of ASC specks in hMDMs (Figure 5C). Importantly, cells stained with isotype-matched immunoglobulin G (IgG), as well as platelets cultured alone, did not show ASC specks (Figures 5C and S6A). Co-culture with platelets or exposure to their supernatants increased caspase-1 activation and maturation of IL-1 β in supernatants of hMDMs (Figures 5D–5F). Again, these proteins were not present in platelets (Figures 5D and 5E; Plts alone). We also detected pro-IL-1 β in cell-free supernatants of LPS-primed hMDMs, likely due to cell death during LPS priming. Furthermore, despite lacking pro-IL-1 β mRNA (Figure S4D) and protein (Figures 5D, lane 11, and S4G), platelets and their supernatants increased the concentrations of pro-IL-1 β even on unstimulated hMDMs (Figure 5D, lanes 3 and 4). Contrary to NLRP3, which requires priming for its transcription, NLRC4 and AIM2 are constitutively expressed in the cytosol of hMDMs, which might explain why platelets had minor effects on the AIM2- or NLRC4-dependent IL-1 β secretion (Figures S1D and S1E). Altogether, these findings indicate that platelets boost inflammasome activation by enhancing the transcription of pro-IL-1 β and NLRP3 in hMDMs.

The Platelet-Mediated Amplification of Inflammasome Activity Requires Calcium and Can Be Prevented by Heat Inactivation

Platelets are sources of lipid mediators (Hinz et al., 2016) such as prostaglandins (COX-1/2-derived mediators), which regulate LPS-induced pro-IL-1 β transcription (Zaslon et al., 2017) and inhibit TNF- α in macrophages (Chandra et al., 1995; Xiang et al., 2013; Zaslon et al., 2017). We therefore inhibited COX-1/2 by pre-treating platelets with aspirin (Xiang et al., 2013). Aspirin had no effect on platelet ability to boost IL-1 β production in hMDMs (Figure 6A). Similarly, pre-treatment of platelets with Zileuton (Zt), an inhibitor of leukotriene synthesis, did not block the platelet effect. Of note, aspirin and Zt had no effect on IL-1 β production when used directly on hMDMs (Figure 6A).

Activated platelets can also release nucleic acids and nucleosides that display inflammatory properties (Qin et al., 2016). However, degradation of free nucleic acids by Benzonase (Bz) did not prevent the effect of platelet co-culture on IL-1 β production (Figure 6B). As platelets store other purines including ADP and ATP, we added apyrase (Apy) to the co-culture of hMDMs with platelets. Apyrase did not alter the effect of platelets on the inflammasome activity of hMDMs (Figure 6C). Likewise, the direct addition of extracellular ADP to inflammasome-activated hMDMs also failed to recapitulate the platelet effect (Figure 6D). This is in line with our findings that platelets boost macrophage IL-1 β production through transcriptional regulation during the priming phase of inflammasome activation (Figures 5A and 5B). Altogether, our findings suggest that the platelet-derived factor responsible for the inflammasome boosting does not belong to the classes of nucleic acids, purines, or derivatives of arachidonic acid.

Therefore, we speculated whether the platelet-derived factor could be a protein. Ratifying this hypothesis, both heat inactivation and fixation of platelets (Figure 6E) or platelet supernatants

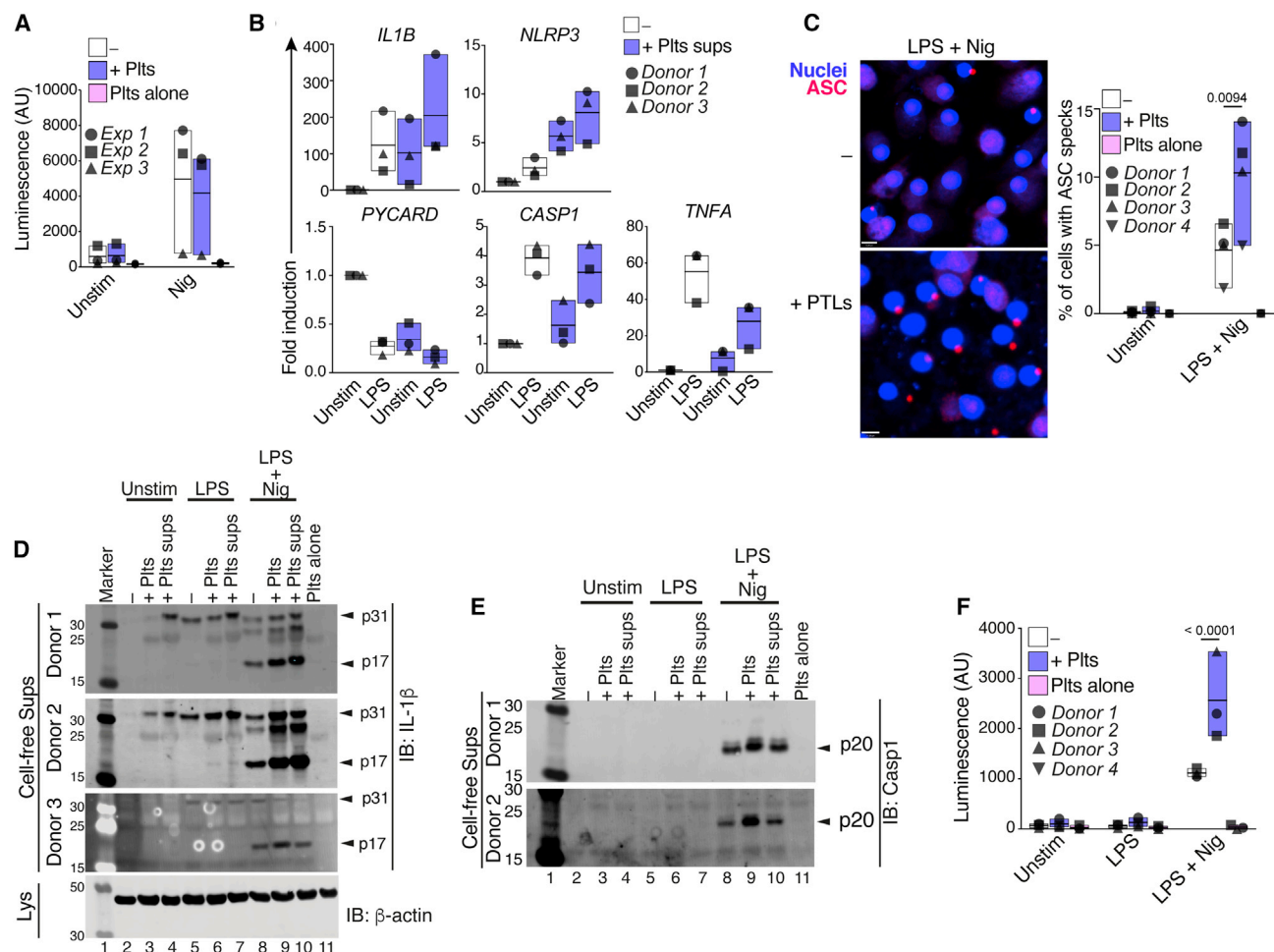


Figure 5. Platelets Enhance NLRP3 and Pro-IL-1 Transcription in Human Macrophages

(A) Caspase-1 activity in cell-free supernatants of NLRP3-overexpressing mouse macrophages $\pm$ platelets (50:1 ratio). Cells were left resting (Unstim) or activated with Nigericin (10 μ M, 90 min) without LPS priming.

(B) qPCR analysis of the indicated genes in hMDMs $\pm$ platelet supernatants.

(C) Confocal imaging and quantification of ASC specks in LPS-primed and Nigericin-activated hMDMs $\pm$ platelets (50:1 ratio). Scale bar, 11 μ m.

(D and E) Immunoblotting for IL-1 β (D) or caspase-1 (E) in cell-free supernatants, as well as β -actin in cell lysates of resting, LPS-primed hMDMs $\pm$ platelets (50:1 ratio), or platelet supernatants. Different donors are indicated.

(F) Caspase-1 activity in cell-free supernatants of hMDMs treated as in (C). Graphs show floating bars (with mean and minimum to maximum values) from pooled data from three or four independent experiments. Each symbol represents the average of technical triplicates from different donors.

(Figure 6F) extinguished their effects on inflammasome activation in hMDMs. Multiple cytokines were detectable in supernatants of resting and activated platelets in our assays (Figure S1A), including CCL5 (RANTES), CXCL12 (SDF1 α), IL-18, and PDGF-BB. Additional literature reported that platelets are cellular sources of CD40L, PF4, brain-derived neurotrophic factor (BDNF), P-selectin, and CXCL7 (Kral et al., 2016; Semple et al., 2011). After the direct addition of human recombinant proteins to hMDMs (Figures 6G, S6B, and S6C), inhibitors or blocking antibodies against platelet-derived cytokines (Figures S6D and S6E), or co-cultures involving cells from animals deficient for chemokine receptors (Figure S6F), we excluded a role for CXCL1, CCL5, CXCL12, CXCL7, PDGF-BB, epidermal growth factor (EGF), vascular endothelial growth factor (VEGFA), CD40L, PF4, BDNF, and P-selectin as contributors to the platelet effect.

The failure of the above-mentioned α -granule-derived proteins to boost IL-1 β production in hMDMs suggested that molecules contained in the dense granules of platelets could mediate the IL-1 β boosting in activated hMDMs. Platelet-dense granules are rich stores of serotonin (5-HT) and Ca²⁺. However, we found that neither the addition of 5-HT (Figure S6G) nor the blockade of its uptake by hMDMs with fluoxetine (Du et al., 2016; Marcinkiewicz et al., 2016) altered IL-1 β production by inflammasome-activated hMDMs (Figure S6H).

We next examined the effect of chelating extracellular or intracellular Ca²⁺ with 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) and BAPTA-AM, respectively. Notably, treatment of co-cultures with BAPTA, but not with the membrane-permeable BAPTA-AM, prevented the platelet effect without interfering with the basal response of hMDMs

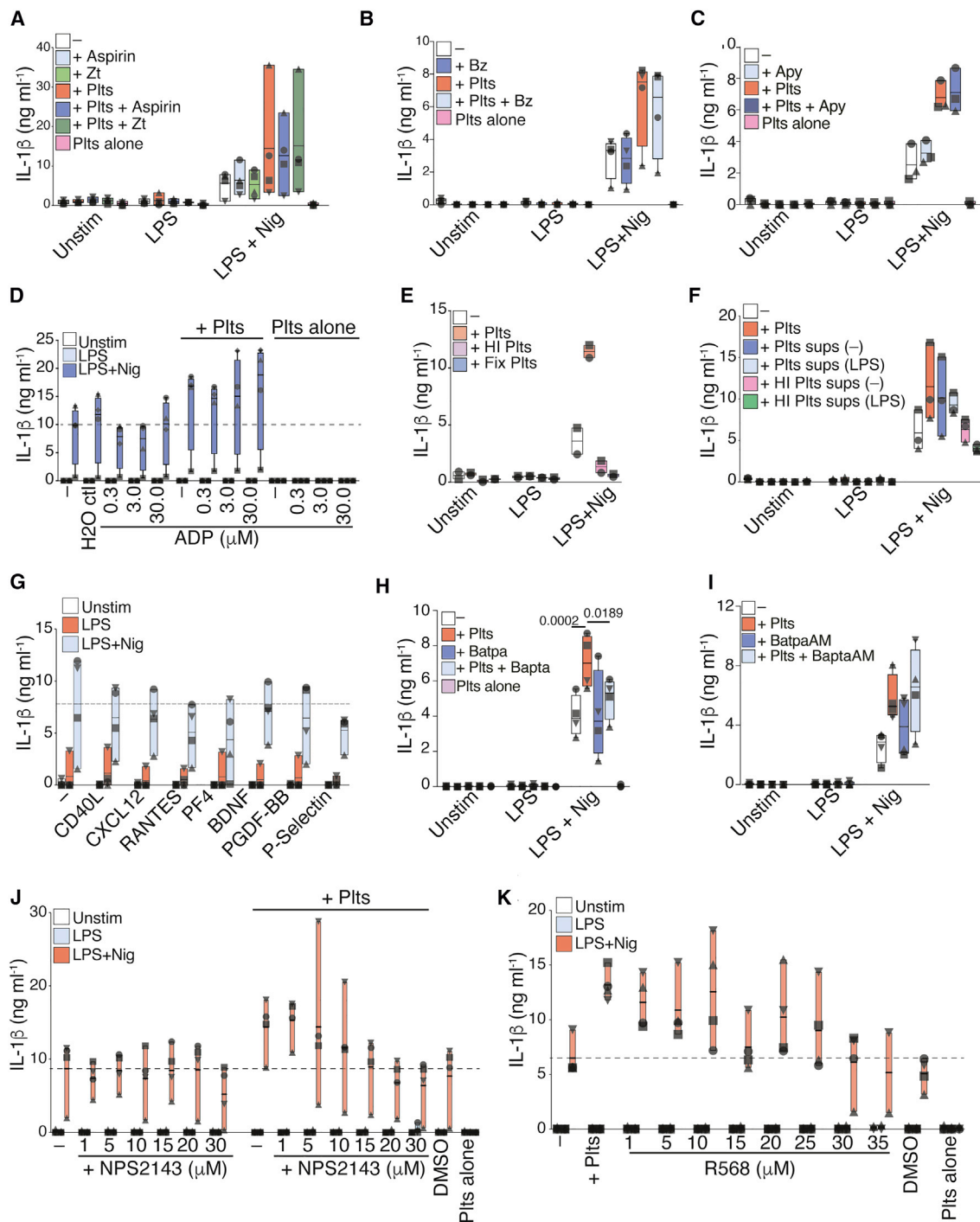


Figure 6. A Platelet-Derived Ca²⁺-Dependent Protein Boosts NLRP3 Activity in Human Macrophages

(A) IL-1 β released by resting (Unstim), LPS-primed, and Nigericin-activated hMDMs $\pm$ aspirin or Zileuton (Zt) pre-treated platelets (50:1 ratio)
(B and C) IL-1 β levels released by unstimulated or LPS-primed hMDMs $\pm$ platelets. Cells were incubated with Benzonase (B) or Apyrase (C) before LPS priming and inflammasome activation.
(D) IL-1 β levels in co-cultures of platelets and hMDMs added with the indicated concentrations of ADP or equivalent dilutions in water.
(E and F) IL-1 β released by hMDMs stimulated as in (A) and co-cultured with heat-inactivated (HI), fixed platelets, or fresh or HI platelet supernatants.
(G) IL-1 β released by hMDMs activated as in (A) and added with the indicated recombinant proteins.
(H and I) IL-1 β levels in co-cultures of platelets and hMDMs added with BAPTA in Ca²⁺-free medium (H) or BAPTA AM (I) in normal RPMI medium before activation.

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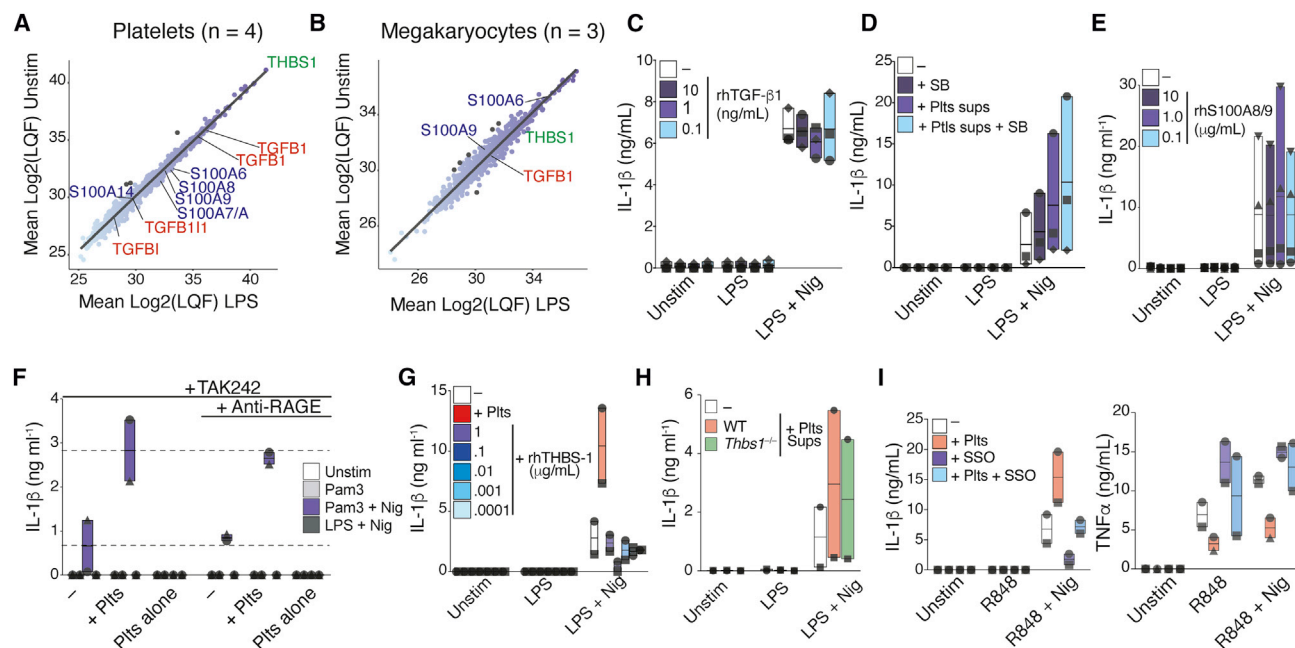


Figure 7. Platelets Have Broad Effects on Macrophages That May Contribute to Boosting of Inflammasome Activation

(A and B) Proteomics of cell-free supernatants from resting (Unstim) or LPS-treated (A) human platelets ($n = 4$) or MK (B) ($n = 3$). Darker points show proteins with $\log_2$ fold ≥ 2 between LPS and Unstim.

(C–G) IL-1 β released by resting or inflammasome-activated hMDMs added with (C) rhTGF- β 1 or (D) pre-treated with SB-431542, (E) added with rhS100A8/9, (F) pre-treated with TAK242 ($0.5 \mu\text{g mL}^{-1}$) and an α RAGE mAb ($10 \mu\text{g mL}^{-1}$), or (G) added with rhTHBS1.

(H) IL-1 β released by inflammasome-activated BMDMs cultured with WT or *Thbs1* $^{-/-}$ platelet supernatants.

(I) IL-1 β and TNF- α levels released by R848-primed and Nigericin-activated hMDMs $\pm$ platelets. hMDMs were pre-treated with the CD36 inhibitor SSO. Graphs show floating bars (with mean and minimum to maximum values) from pooled data from two to four independent experiments

See also Figure S7.

(Figures 6H and 6I). However, the addition of calcium chloride (CaCl_2) to inflammasome-activated hMDMs did not evoke additional IL-1 β secretion (Figure S6I), suggesting that Ca^{2+} is required but not sufficient for the platelet effect.

Cells sense extracellular Ca^{2+} primarily through calcium sensing receptors (CaSR), which are G-protein-coupled receptors (GPCRs). To test the role of CaSR, we pre-treated hMDMs with the CaSR-selective inhibitor NPS2143. We found that NPS2143 blocked the platelet effect on hMDMs (Figure 6J). Of note, recapitulating the platelet effect, allosteric activation of CaSR with the calcimimetic compound R568 boosted the IL-1 β response of inflammasome-activated hMDMs (Figure 6K). These findings indicate that a heat-sensitive protein that induces calcium signaling via CaSR is involved in the platelet-mediated increase of IL-1 β from inflammasome-activated hMDMs.

A Combination of Platelet-Derived Proteins Might Boost Inflammasome Activation of Macrophages

Next, we assessed the protein secretome of platelets and MKs and identified additional candidates similarly secreted by these cells

that could mediate the IL-1 boosting effect (Figures 7A, 7B, S7A, and S7B). Supernatants from quiescent or LPS-treated human platelets (Figure 7A; $n = 4$), as well as from MEG-01 cells (Figure 7B, $n = 3$) were evaluated by liquid chromatography-tandem mass spectrometry (LC-MS). As our findings in Figures 4G and 4H indicated the presence of a factor ubiquitously present in the supernatants of resting and activated platelets, we directed further analysis to proteins that remained unchanged (fold change > -1.5 and < 1.5) following LPS stimulation in both cell types. Among the proteins similarly secreted by platelet and MKs, we identified members of the TGF- β and S100 family and thrombospondin-1 (THBS1), which figured as the most abundant protein in platelet supernatants (Figures 7A and 7B). Notably, TGF- β , S100, and THBS1 require Ca^{2+} for their activity (Bertheloot and Latz, 2017; Cailotto et al., 2011; Misenheimer and Mosher, 1995). Additionally, in line with our findings that TLR4 signaling is partially involved in the platelet effect (Figure S1G), THBS1 can activate TLR4 (Li et al., 2013), can regulate IL-1 β in macrophages (Stein et al., 2016), and is necessary for the full activity of TGF- β (Crawford et al., 1998). We therefore tested the effect of these proteins on the

(J) IL-1 β levels released by inflammasome-activated hMDMs incubated with the indicated concentrations of calcium chloride (CaCl_2) in Ca^{2+} -free medium. Graphs show floating bars (with mean and minimum to maximum values) from pooled data from two to four independent experiments. Each symbol represents the average of technical triplicates from different donors.

See also Figure S6.

platelet-mediated boosting of inflammasomes. We tested the addition of recombinant human TGF- β (rhTGF- β 1) to inflammasome-activated hMDMs. Consistent with previous reports of an antagonistic effect of TGF- β on TNF- α signaling (Vaday et al., 2001; Verrecchia and Mauviel, 2004; Yamane et al., 2003), the addition of rhTGF- β 1 to hMDMs inhibited their production of TNF- α triggered by LPS (Figure S7C). However, rhTGF- β 1 did not influence the IL-1 β response (Figure 7C). Likewise, selective inhibition of the TGF- β receptor by pre-treating hMDMs with SB431542 (Matsuyama et al., 2003) did not prevent the platelet effect (Figure 7D), despite preventing the downregulation of TNF- α (Figure S7C). These data indicate that TGF- β is likely not the platelet-derived factor enhancing the NLRP3 activity of hMDMs, but it might underlie the regulation of TNF- α .

S100 proteins are important regulators of Ca²⁺ homeostasis, and platelet-derived S100A8/9 has been shown to induce inflammation (Lood et al., 2016; Wang et al., 2014). S100A8/9 is enriched in platelets and binds to membrane receptors such as TLR4, receptor for advanced glycation endproducts (RAGE), and CD36 (Bertheloot and Latz, 2017). TLR4 and CD36 are also known receptors for THBS1 (Li et al., 2013; Stein et al., 2016). However, we found that addition of neither rhTHBS1 (Figures 7G and S7E) nor rhS100A8/9 (Figure 7E) to hMDMs influenced their basal response to LPS + Nigericin. Of note, rhS100A8/9 induced TNF- α secretion from hMDMs (Figure S7D). Furthermore, supernatants from *Thbs1*^{-/-} platelets were as efficient as WT platelets in boosting IL-1 β release and caspase-1 activity from inflammasome-activated BMDMs (Figure 7H), and RAGE inhibition antibodies had no effect on the platelet boosting of IL-1 β (Figures 7F and S7G). These findings led us to conclude that platelet-derived THBS1 and S100A8/9 are not, individually, responsible for the platelet effect on macrophages.

As THBS1 and S100 proteins bind to CD36, we tested the effect of CD36 inhibition with Sulfosuccinimidyl oleate (SSO) (Kuda et al., 2013). SSO interfered with IL-1 β and TNF- α release (Figure 7I) and caspase-1 activity (Figure S7F) in both the presence and absence of platelets. Nevertheless, even in the presence of SSO, platelets increased both the release of IL-1 β and the activity of caspase-1 from macrophages compared to SSO-pre-treated macrophages in monoculture.

As single inhibition of TLR4, RAGE, and CD36 did not completely abrogate the platelet effect, we speculated that a combined activity of platelet-derived S100 and THBS1 could mediate the boosting of IL-1 β on hMDMs. An experimental strategy to block TLR4/RAGE and CD36 resulted in complete inhibition of IL-1 β , TNF- α , and caspase-1 activity of hMDMs (Figure S7G), thus precluding us to conclude whether these proteins are synergistically involved in the platelet-mediated enhancement of IL-1 β response of hMDMs. Hence, additional work is required to delineate the mechanisms by which platelets affect the inflammasome activation of hMDMs.

DISCUSSION

Here, we report that the interaction with platelets licenses NLRP3, potentiates inflammasome activation and production of IL-1 cytokines by innate immune cells, and is critical for NLRP3-activation in human monocytes. The platelet effect is

mediated by a yet unidentified soluble factor (or factors), which enhances NLRP3 transcription and boosts inflammasome activation. Hence, our work identifies platelets as additional players in governing the production of IL-1 cytokines and emphasizes that full inflammasome activity *in vivo* is too complex to be modeled by *in vitro* monocultures of single-cell populations.

Platelets are key players in inflammation (Carestia et al., 2019; Dann et al., 2018; Kral et al., 2016; Passacquale et al., 2011) and express TLRs that sense infection and initiate inflammatory responses (Andonegui et al., 2005). While platelets are known to react to LPS (Cognasse et al., 2008; Andonegui et al., 2005), they do not constitutively express membrane CD14 (Damien et al., 2015) and require soluble CD14 from plasma for TLR4 signaling (Brown and McIntyre, 2011; Damien et al., 2015).

Our work adds to a growing bulk of evidence that platelet parameters, such as their blood counts, can be additional causes of variation in the concentrations of circulating cytokines (Hu et al., 2018; Schirmer et al., 2018; Tunjungputri et al., 2018). Platelets secrete a large array of proteins (Coppinger et al., 2004; Maynard et al., 2007), which affect leukocytes in different ways (Kral et al., 2016; Sreeramkumar et al., 2014). They have also been proposed as sources of IL-1 cytokines and, more recently, to assemble inflammasomes (Cornelius et al., 2019; Hottz et al., 2013; Vogel et al., 2018).

Our work challenges these findings with a series of complementary techniques that demonstrate that platelets lack expression of NLRP3, ASC, and caspase-1 and are therefore incapable of assembling inflammasomes. Those included experiments using platelets and BMDMs from mice lacking IL-1 proteins or their receptors (Figures 4A–4C); mice lacking inflammasome molecules (Figure S2D), megakaryocytes, and platelets from humans (Figures S3F, S3G, and S4); and transgenic or knockin inflammasome reporter mice (Figures S3C–S3E), as well as a MS-based proteomics analysis of supernatants from human platelets and MK cell line (Figures 7 and S7). Our conclusions were further supported by a meta-analysis of transcriptomics from five independent studies with human platelets (Figure S5). We believe that the discrepancies are likely because the studies reporting inflammasomes on platelets relied on fluorescence assessment of inflammasome components, using antibodies of weak specificity (Beilharz et al., 2016), and without quantitative measurement of proteins in platelet lysates by immunoblotting. Likewise, IL-1 expression on platelets is reported with conflicting results in literature. Some studies describe expression of IL-1 in activated platelets (Denis et al., 2005; Lindemann et al., 2001; Thornton et al., 2010), while others find IL-1 activity in bioassays (Hawrylowicz et al., 1989; Kaplanski et al., 1993), with no evidence of the cytokine itself. Notably, throughout all our experiments, we assessed the purity (leukocyte contamination CD14 and/or CD45; Figures S3C–S3E, S4D, and S4F) as well as activation capacity (ability to upregulate CD62p upon thrombin stimulation; Figures S3F and S4E) of our platelet preparations. These important controls were not performed in the aforementioned studies. Hence, it cannot be ruled out that their platelet preparations are free of contaminating leukocytes (Pillitteri et al., 2007). For example, a preparation of 5×10^8 platelets, commonly used in previous studies for RNA isolation, with a 98% purity (assessed by FACS) could easily contain $\sim 2 \times 10^6$ contaminating leukocytes,

which could account for a significant portion of RNA within a sample. Supporting this, IL-1 β expression on platelet preparation is closely correlated to the presence of contaminating leukocytes (Pillitteri et al., 2007).

In line with our findings, platelets have been reported to amplify IL-1 β -mediated inflammation in RA (Boilard et al., 2010), an IL-1-driven disease (Dinarello, 2011), and a recent study demonstrated pro-atherogenic effects of platelets by and skewing monocytes into a pro-inflammatory macrophage phenotype (Barrett et al., 2019). Platelet depletion in mice with RA (Boilard et al., 2010) or in *Ldlr*^{-/-} mice fed a western diet (Barrett et al., 2019) attenuated inflammation and improved clinical outcomes. However, it was not possible in these studies to distinguish whether these effects were caused by decreased platelet-derived cytokines or by the influence of platelets on other immune cells, as we report here.

Our data confirm their relevance to human physiology and disease, as high blood platelet counts are found in patients (Cicarelli et al., 2014) and mouse models of IL-1-driven auto-inflammatory disorders (Bonar et al., 2012). In Kawasaki disease (KD), an acute systemic vasculitis in children for which thrombocytosis is a common feature, human and experimental mouse models have determined a critical role of IL-1 β in the cardiovascular pathogenesis. Furthermore, thrombocytosis and platelet activation are predictive of the development of coronary aneurysm in KD (Burns et al., 2017; Lee et al., 2012b).

As both IL-1 α and IL-1 β induce thrombocytosis in mice (Kimura et al., 1990; Nishimura et al., 2015; Trinh et al., 2015), platelets could help feed an inflammatory loop by potentiating IL-1 signaling, which in turn triggers platelet biogenesis. However, whether thrombocytosis is a marker of disease or a contributor to pathogenesis remains to be investigated. Platelet counts have been correlated with the concentrations of other cytokines that affect platelet production in immune thrombocytopenia (ITP) and other diseases. Those include CD40L, CXCL5, CCL5, EGF, VEGF, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN) γ , monocyte chemoattractant protein 1 (MCP-1), IL-8, and PDGF-BB (Feng et al., 2012; Pourcelet et al., 2014). Thus, the contribution of platelets to cytokine production in human biology warrants further investigation.

Notably, co-culture with platelets impaired the LPS-induced TNF- α production in several immune cells. This observation is in line with previous studies (Aslam et al., 2006; Gudbrandsdottir et al., 2013; Xiang et al., 2013) and may be explained by the reported ability of platelets to sequester TNF- α and IL-6 released from LPS-stimulated monocytes (Carestia et al., 2019). Platelets have a protective role in sepsis through the regulation of TNF- α and IL-6 via the secretion of a COX1/PGE2/EP4-dependent lipid mediator (Xiang et al., 2013). In line with these findings, we observed that TNF- α is regulated via a platelet-derived COX1-dependent lipid mediator and is related to TGF β signaling.

Despite our efforts, we were unable to identify a unique platelet factor that boosts the inflammasome activation of innate immune cells. However, we show that the regulation of IL-1 β production is likely mediated through a constitutively expressed protein factor that engages a CaSR on primary human macrophages. This is consistent with previous reports of roles for CaSR on inflammasome activation (Lee et al., 2012a; Rossol

et al., 2012). These findings will need further validation using genetic deletion of these receptors. It is possible that a combination of factors acting in synergy and affecting different pathways underlie the platelet effect.

Our findings also strengthen recent discoveries of a direct link between the mammalian immune and coagulation systems (Burzynski et al., 2019; Wu et al., 2019; Yang et al., 2019) by showing a direct participation of platelets in the inflammasome output of innate immune cells.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2020.107615>.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

E.L. is co-founder and consultant of IFM Therapeutics.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human Caspase-1 (p20), mAb (Bally-1)	Adipogen International	AG-20B-0048-C100; RRID:AB_2490257
Anti-human CD14-APC (61D3)	Thermo Fisher Scientific	17-0149-42; RRID:AB_10669167
Anti-human CD41 FITC (HIP8)	Thermo Fisher Scientific	11-0419-42; RRID:AB_10718234
Anti-human CD45 PE (2D1)	Thermo Fisher Scientific	12-9459-42; RRID:AB_10718238
Anti-human CD66b	Thermo Fisher Scientific	11-0666-42; RRID:AB_2572461
Anti-human NAP2 (CXCL7)	PeproTech	300-14-10; RRID:AB_147531
Anti-human RAGE	R&D Systems	MAB11451; RRID:AB_2224344
Anti-mouse CD41 eFluor450 (eBioMWRReg30)	Thermo Fisher Scientific	48-0411-82; RRID:AB_1582238
Anti-mouse CD45 PE (30-F11)	Thermo Fisher Scientific	12-0451-83; RRID:AB_465669
Anti-human CXCL12 / SDF-1a	R&D Systems	AF-310-SP; RRID:AB_354461
Anti-human IL-1 beta / IL-1F2 Antibody, goat	R&D Systems	AF-201-NA; RRID:AB_354387
Anti-mouse IRDye 680	Li-Cor Biosciences	925-68080; RRID:AB_2814917
Anti-mouse Ly6G-AF488 (RB6-8C5)	Thermo Fisher Scientific	53-5931-82; RRID:AB_469918
Anti-mouse Ly6G-APC (1A8-ly6g)	Thermo Fisher Scientific	17-9668-82; RRID:AB_2573307
Anti-mouse NLRP3/NALP3, mAb (Cryo-2)	Adipogen International	AG-20B-0014; RRID:AB_2490202
Anti-human/mouse CD62p (P-selectin) APC (Psel.KO.2.3)	Thermo Fisher Scientific	12-0628-42; RRID:AB_10804401
Anti-human/mouse Beta-Actin (Host: mouse)	Li-Cor Biosciences	926-42212; RRID:AB_2756372
Anti-human/mouse Beta-Actin (Host: rabbit)	Li-Cor Biosciences	926-42210; RRID:AB_1850027
Anti-human/mouse Beta-Tubulin (Host: rabbit)	Li-Cor Biosciences	926-42211; RRID:AB_1850029
Anti-rabbit IRDye 800	Li-Cor Biosciences	926-32213; RRID:AB_621848
FcR Blocking Reagent, human	Miltenyi Biotec	130-059-901
FcR Blocking Reagent, mouse	Miltenyi Biotec	130-092-575
Mouse IgG1 K Iso Control APC (P3.6.2.8.1)	Thermo Fisher Scientific	17-4714-42; RRID:AB_1603315
Mouse IgG1 K Iso Control FITC (P3.6.2.8.1)	Thermo Fisher Scientific	11-4714-42; RRID:AB_10596964
Mouse IgG1 K Iso Control PE (P3.6.2.8.1)	Thermo Fisher Scientific	12-4714-42 RRID:AB_1944423
Normal Goat IgG control	R&D systems	AB-108-c; RRID:AB_354267
OneComp eBeads	Thermo Fisher Scientific	01-1111-42
Polyclonal anti-GPIb alpha	Emfret Analytics	R300; RRID:AB_2721041
Polyclonal non-immune rat immunoglobulins	Emfret Analytics	C301; RRID:AB_2734715
Purified anti-ASC (TMS-1) Antibody	BioLegend	653902; RRID:AB_2561778
Purified anti-human CD42b Antibody (HIP1)	BioLegend	303902; RRID:AB_314382
Purified Mouse IgG, k Isotype	BioLegend	401402; RRID:AB_2801451
Rat Anti-mouse CD14-FITC (Sa14-2)	BioLegend	123308; RRID:AB_940580
Rat IgG1 K Iso Control eFluor450 (eBRG1)	Thermo Fisher Scientific	48-4301-82; RRID:AB_1271984
Rat IgG2a K Iso Control APC (eBR2a)	Thermo Fisher Scientific	17-4321-81; RRID:AB_470181
Rat IgG2b, K Iso Control PE (eB149/10H5)	Thermo Fisher Scientific	12-4031-82; RRID:AB_470042
Chemicals, Peptides, and Recombinant Proteins		
2-mercaptoethanol	Sigma-Aldrich	M6250-250ML
Acetylsalicylic acid (Aspirin)	Sigma-Aldrich	A5376-100G
Acid-Citrate-dextrose solution (ACD)	Sigma-Aldrich	C3821-50ML
Adenosine 5-diphosphate sodium salt (ADP)	Sigma-Aldrich	A2754-500MG
Adenosine triphosphate (ATP)	Sigma-Aldrich	A2382

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Apyrase from potatoes, High Activity, ATPase > 600 units/mg protein	Sigma-Aldrich	A2230-100UN
BAPTA AM	Enzo Life Sciences Inc	BML-CA411-0025
BAPTA, Tetrasodium salt	Santa Cruz Biotechnology	sc-278716
Benzonase® Nuclease	Sigma-Aldrich	E1014-5KU
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A9647
Calcium chloride	Sigma-Aldrich	21115-100ML
Chloroform	Merck	1.02445.1000
cOmplete EDTA-free protease inhibitor Cocktail Tablets	Sigma-Aldrich	4693132001
CRID3 (CP-456773-02, MCCP90)	Sigma-Aldrich	PZ0280
Cutasept® F Haut-Desinfiziens	Hartmann	9803640
Cytochalasin D	Sigma-Aldrich	C8273-1MG
D-(+)-Glucose solution	Sigma-Aldrich	G7021-100 g
Di-Sodium hydrogen phosphate (Na ₂ HPO ₄)	Sigma-Aldrich	255793
Dimethyl sulfoxide (DMSO), cell culture grade	PAN-Biotech	P60-36720100
Dithiothreitol (DTT)	Thermo Fisher Scientific	Y00147
dNTP Mix (10 nM each)	Thermo Fisher Scientific	R0192
DRAQ5	Thermo Fisher Scientific	65-0880-96
Ethanol (EtOH) absolute	AppliChem	147194.1214
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific	26140079
Ficoll-Paque PLUS	GE Healthcare	17-1440-03
GIBCO Dulbecco's Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific	41965039
GIBCO Dulbecco's Phosphate Buffered Saline (PBS)	Thermo Fisher Scientific	14190144
GIBCO GlutaMAX 100X	Thermo Fisher Scientific	35050061
GIBCO Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	15140122
GIBCO RPMI 1640 Medium	Thermo Fisher Scientific	21875091
GIBCO Sodium Pyruvate 100mM	Thermo Fisher Scientific	11360070
GIBCO UltraPure 0.5M EDTA, pH 8.0	Thermo Fisher Scientific	15575020
Hoechst 34580	Thermo Fisher Scientific	H21486
L929 cell line supernatant	Gift from Prof. Latz and Ms. Engels (University of Bonn)	N/A
Lipopolysaccharide (LPS), ultrapure, <i>E. coli</i> 0111:B4	Invivogen	ttrl-3pelps
Magnesium chloride (MgCl ₂) solution	Sigma- Aldrich	M1028-100ML
Methanol	Merck	1.06007.2500
Met-RANTES trifluoroacetate salt, human	Bachem	H-6544.0050
Nigericin, free acid	Invitrogen	N1495
Oligo(dT) ₁₈ primer	Thermo Fisher Scientific	SO132
PageRuler™ Plus Prestained Protein Ladder, 10 to 250kDa	Thermo Fisher Scientific	26619
Pam3Cysk4	InvivoGen	P2200.0005
PhosSTOP Easypack Phosphatase Inhibitor Cocktail Tablets	Sigma-Aldrich	04906837001
Piperazine-1,4-bis(2ethanesulfonic acid) (PIPES)	Sigma-Aldrich	P6757-25G
Poly-L-Lysine solution	Sigma-Aldrich	P4707
Potassium chloride (KCl)	Sigma-Aldrich	P9327-100ML
(LFn-)Prgl fusion protein	Gift from Prof. Geyer and Dr. Fußhöller (University of Bonn)	N/A
Prostaglandin E1 (PGE-1)	Sigma-Aldrich	P5515-1MG

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Protective antigen (PA), recombinant from <i>Bacillus anthracis</i>	List Biological Laboratories	171C
R848	Invivogen	tlrl-r848-5
Recombinant human BDNF	Tocris Bioscience	2837
Recombinant human CD40L / CD154, soluble	Enzo Life Sciences	ALX-522-015-C010
Recombinant human EGF	Sigma-Aldrich	E9644
Recombinant human GM-CSF	Immunotools	11343125
Recombinant human GRO-alpha / MGSA (CXCL1)	PeptoTech	300-11-5UG
Recombinant human NAP-1 (CXCL7)	PeptoTech	300-14-10
Recombinant human P-selectin	R&D systems	ADP3
Recombinant human PF4 (CXCL4)	PeptoTech	300-16-5UG
Recombinant human PGDF-BB	BioLegend	577304
Recombinant human PLGF-1 (PIGF-1)	Thermo Fisher Scientific	PHG0296
Recombinant human RANTES (CCL5)	Thermo Fisher Scientific	PHC1054
Recombinant human S100A8/S100A9 heterodimer	R&D Systems	8226-S8-050
Recombinant human SDF-alpha (CXCL12)	PeptoTech	300-28a-10UG
Recombinant human TGF-b1	PeptoTech	100-21C-2UG
Recombinant human THBS-1	Sigma-Aldrich	ECM002-50UG
Recombinant human VEGF	Thermo Fisher Scientific	PHC9394
SB431542	Invivogen	inh-sb43
Sodium chloride (NaCl)	Sigma- Aldrich	S5150-1L
Sodium deoxycholate	Sigma- Aldrich	D-6750-10G
Sodium dodecyl sulfate (SDS)	Sigma- Aldrich	75746-1KG
Sodium hydroxide (NaOH)	Sigma- Aldrich	S2770-100ML
Sulfo-N-succinimidyl Oleate sodium (SSO)	BioMol	Cay11211-5
TAK242 (Resatorvid)	Invivogen	tlrl-cli95
Thrombin from human plasma	Sigma-Aldrich	T6884-100UN
Triton X-100	Carl Roth	3051.3
Tween® 20	Sigma- Aldrich	P2287
UK5099	BioMol	Cay16980-5
Wheat Germ Agglutinin (WGA) Alexa Fluor 555	Thermo Fisher Scientific	W32464
Zileuton	Tocris	3308
GIBCO Opti-MEM	Thermo Fisher Scientific	31985070
Lipofectamine 2000 Transfection Reagent	Thermo Fisher Scientific	11668019
Poly [dA:dT]	Sigma	P0883
Critical Commercial Assays		
Alexa Fluor 647 Antibody labeling kit	Thermo Fisher Scientific	A20186
Caspase-Glo® 1 Inflammsome Assay	Promega	G9952
CD14 MicroBeads, human	Miltenyi Biotech	130-050-201
EasySep Direct Human Neutrophil Isolation Kit	STEMCELL Technologies	19666
EasySep Human Monocyte Isolation Kit	Stemcell	19359
Human IL1 beta HTRF kit	Cisbio	62IL1PEB
Human TNF alpha HTRF kit	Cisbio	62TNFPEB
Maxima SYBR Green/ROX qPCR Master Mix	Thermo Fisher Scientific	K0221
Mouse IL1 beta HTRF kit	Cisbio	63ADK010PEB
Mouse IL6 HTRF kit	Cisbio	63ADK043PEB
Mouse TNF alpha HTRF kit	Cisbio	6FMTNPEB
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	23225

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pierce LDH Cytotoxicity Assay Kit	Thermo Fisher Scientific	B3026-88954
ProcartaPlex Human Cytokine/Chemokine/ Growth Factor Panel 1 45plex	Thermo Fisher Scientific	EPX450-12171-901
RNase-Free DNase Set	QIAGEN	79254
RNeasy Mini Kit	QIAGEN	74106
S-Monovette 9NC tubes	Sarstedt	01.1606.001
S-Monovette K3EDTA tubes	Sarstedt	02.1066.001
SuperScript III First-Strand Synthesis SuperMix	Thermo Fisher Scientific	18080-400
Deposited Data		
Transcription profiling of whole blood from pediatric patients Muckle-Wells disease and healthy children	Balow et al., 2013	Accession Nr.: GSE43553
Transcription profiling of human platelets from patients with essential thrombocythemia	Gnatenko et al., 2005	Accession Nr.: E-GEOD-2006
Transcription profiling of human platelets from patients with dengue shock syndrome	Devignot et al., 2010	Accession Nr.: GEOD17924
Transcription profiling of human platelets from patients with sickle cell disease	Raghavachari et al., 2007	Accession Nr.: E-GEOD-11524
Transcription profiling of human platelets from patients with sepsis-induced acute lung injury (ALI)	Freishtat et al., 2009	Accession Nr.: E-GEOD-10361
Transcription profiling of human platelets from patients with Polycythemia Vera (PV)	Spivak et al., 2014	Accession Nr.: E-GEOD-47018
Raw Mass Spectrometry Data Files	This paper	Data are available via ProteomeXchange: PXD017976
Experimental Models: Cell Lines		
NLRP3-FLAG overexpressing immortalized NLRP3 KO		(Franklin et al., 2014; Stutz et al., 2013)
THP-1	ATCC	ATCC TIB-202
MEG01	ATCC	CRL2021
Experimental Models: Organisms/Strains		
<i>Mus musculus</i> , wild-type (C57BL/6j)	The Jackson Laboratory	Stock 000664
<i>Mus musculus</i> , ASC-mCitrine Tg (B6.Cg-Gt(ROSA)26Sortm1.1(CAG-Pycard/mCitrine*,-CD2*)Dtg/J)	The Jackson Laboratory	Stock 030744
<i>Mus musculus</i> , <i>Nlrp3</i> ^{-/-} mice (B6.B6N-Nlrp3tm1Vmd)	Mariathasan et al., 2006	N/A
<i>Mus musculus</i> , <i>Asc</i> ^{-/-} mice (B6.129-Pycardtm1Vmd)	Mariathasan et al., 2004	N/A
<i>Mus musculus</i> , <i>Il1b</i> ^{-/-} mice (B6.129S2-Il1b1tm1Dch)	Shornick et al., 1996	N/A
<i>Mus musculus</i> , <i>Il1r</i> ^{-/-} mice (B6.129S7-Il1r1tm1Imx/J)	The Jackson Laboratory	Stock 003245
<i>Mus musculus</i> , <i>Il18r</i> ^{-/-} mice (B6.129P2-Il18r1tm1Aki/J)	The Jackson Laboratory	Stock 004131
<i>Mus musculus</i> , <i>Cd36</i> ^{-/-} mice (B6.129S1-Cd36tm1Mfe/J)	The Jackson Laboratory	Stock 019006
<i>Mus musculus</i> , <i>Tsp1</i> ^{-/-} mice (B6.129S2-Thbs1(tm1Hyn)/J)	The Jackson Laboratory	Stock 006141
<i>Mus musculus</i> , <i>Cxcr3</i> ^{-/-} mice (B6.129P2-Cxcr3tm1Dgen/J)	The Jackson Laboratory	Stock: 005796
<i>Mus musculus</i> , <i>Ccr5</i> ^{-/-} mice (B6.129P2-Ccr5tm1Kuz/J)	The Jackson Laboratory	Stock: 005427
The knock in ASC-mCherry reporter mouse is available upon request	Eicke Latz, Institute of Innate Immunity	N/A
Oligonucleotides		
hACTB-Fwd: 5'-ccaccatgtaccctggcatt-3'	Metabion	N/A
hACTB-Rev: 5'-cggagtacttgcgctcagga-3'	Metabion	N/A
hNLRP3-Fwd: 5'-tcggagacaaggggatcaaa-3'	Metabion	N/A
hNLRP3-Rev: 5'-agcagcagtgtgacgtgagg-3'	Metabion	N/A
hCD14-Fwd: 5'-gagctcagaggttcgaaga-3'	Metabion	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
hCD14-Rev: 5'-cttcacgtccagctcaca-3'	Metabion	N/A
hPYCARD-Fwd: 5'-gagctcaccgtaacgtgct-3'	Metabion	N/A
hPYCARD-Rev: 5'-actgaggagggcctggat-3'	Metabion	N/A
hPF4- Fwd: 5'-ctgaagaagatggggacctg-3'	Metabion	N/A
hPF4- Rev: 5'-gtggctatcagttggcag-3'	Metabion	N/A
hCASP1-Fwd: 5'-acaaccagctatgccaca-3'	Metabion	N/A
hCASP1-Rev: 5'-gtgcggcttgactgtccat-3'	Metabion	N/A
hBP1BA-Fwd: 5'-ctgctcttgcctctgtgt-3'	Metabion	N/A
hBP1BA-Rev: 5'-ctccaggtgtgtgtgtg-3'	Metabion	N/A
hL1B-Fwd: 5'-tgggcagactcaaattccagct-3'	Metabion	N/A
Software and Algorithms		
GraphPad Prism 8.1.2 (227)	https://www.graphpad.com	RRID:SCR_002798
FlowJo 10.4	https://www.flowjo.com	RRID:SCR_008520
Velocity 3D Image Analysis Software v6.0.1	https://www.perkinelmer.com	RRID:SCR_002668

RESOURCES AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Bernardo S Franklin (franklin@uni-bonn.de).

Materials Availability

This study did not generate new unique reagents. The knockin ASC-mCherry reporter mouse is available upon request.

Data and Code Availability

The mass spectrometry proteomics data are available via ProteomeXchange: PXD017976.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study Subjects

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.

Malaria patients naturally infected with *Plasmodium vivax* in the Amazon area of Cuiaba (Mato Grosso, Brazil) were invited to participate in the study. Seventy-eight individuals (aged 18 - 78 years old) who sought care at the Julio Muller Hospital, whose thick blood smear was positive for *P. vivax* were included. Another 9 healthy volunteers from the same endemic area who tested negative for Plasmodium infection were recruited and served as healthy donor controls. Exclusion criteria included: (i) refuse or inability to sign the informed consent; (ii) age < 18 years; (iii) pregnant women; (iv) mixed infection with *P. falciparum* or *P. malariae*, tested by both microscopic examination and a nested-PCR; (v) any other comorbidity that could be traced. Clinical and demographical data were acquired through a standardized questionnaire, and the hematological profiles were assessed by automated complete blood count carried out at the site hematology facility. Plasma samples were isolated immediately after blood sampling and stored at -80°C until use. The study was approved by the Ethical Review Board of the René Rachou Research Center, FIOCRUZ, Brazilian Ministry of Health (Reporter CEPESH/CPqRR N. 05/2008 and N. 01/2018).

All participants were instructed about the objectives of the study and signed an informed consent in accordance with guidelines for human research, as specified by the Brazilian National Council of Health (Resolution 196/96). Patients diagnosed with *P. vivax* malaria were treated according to the standard protocols recommended by the National Malaria Control Program (chloroquine + primaquine).

Mice

Mice were housed under standard conditions at 22°C and a 12 h light-dark cycle with free access to food and water. Animal care, handling and experimentation was approved by the ethical committee in Bonn: LANUV-NRW #84-02.04.2016.A487, and in Ribeirão Preto, São Paulo (149/2019). Male 8 - 12 week old C57BL/6j mice were used for experimentation. Male and female 8 week old C57BL/6j, *Nlrp3*^{-/-}, *Asc*^{-/-}, *Casp-1*^{-/-}, *Il1b*^{-/-}, *Il1r*^{-/-}, *Il18r*^{-/-}, *Tsp1*^{-/-}, *Cd36*^{-/-}, *Cxcr3*^{-/-}, *Ccr5*^{-/-}, and ASC-mCitrine Tg mice were used as source of primary cells.

Cell lines

The monocytic cell line THP-1 (ATCC TIB-202), and the megakaryocytic cell line MEG-01 (ATCC CRL-2021) were cultured in RPMI-1640 medium supplemented with 10% FBS, 1% Penicillin/Streptomycin and GlutaMAX (1x) at 37°C with 5% CO₂.

METHODS DETAILS

Data Presentation

Unless indicated otherwise (in Figure legends), all graphs are represented as Floating Bars (with mean, and minimum to maximum values) and are built from pooled data from a minimum of two independent experiments (biological replicates), performed in triplicates (technical replicates) with immune cells or platelets from different donors. For experiments performed less than 3 times, bar graphs with mean and standard deviation (SD) are shown. Each symbol represents the average from 3 technical replicates per each donor, experiment, or mouse. Symbols are coded (●, □, ▲, ▼, etc.) to indicate donors so readers can track the internal variability between different donors, mice, or experiments. Dots are semi-transparent, with darker symbols indicating overlapping points.

Generation of human primary macrophages

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 obtained through differentiation of CD14⁺ monocytes in a medium complemented with 500 U ml⁻¹ rhGM-CSF (Immunotools) for 3 days. In brief, human peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy donors by density gradient centrifugation in Ficoll-Paque PLUS (Healthcare). PBMCs were incubated at 4°C with magnetic microbeads conjugated to monoclonal anti-human CD14 antibodies according to the manufacturer's instructions (Miltenyi Biotec). CD14⁺ monocytes were thereby magnetically labeled and isolated using a MACS column placed in a magnetic field. CD14⁺ monocytes were cultivated in complete medium (RPMI1640 medium with 10% FBS, 1% Penicillin-Streptomycin, 1% GlutaMAX and 1% Sodium Pyruvate) complemented with 500 U ml⁻¹ rhGM-CSF at a concentration of 2x10⁶ ml⁻¹ in 6-well plates to generate monocyte-derived macrophages. Cells were harvested at day 3, counted using a hemocytometer and seeded at a concentration of 1x10⁵/well in complete medium complemented with 125 U ml⁻¹ rhGM-CSF in 96-well flat-bottom plates and incubated overnight for experiments on the next day.

Generation of mouse bone marrow macrophages

Mice were anaesthetized 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 at 400 x g for 5 minutes. Cells were resuspended in DMEM supplemented with 20% L929 supernatant and cultured for 6 days to differentiate into macrophages (BMDMs). On day six, cells were harvested using cold PBS containing 5 mM EDTA and 2% FBS and scraping. After centrifugation at 350 x g for 5 minutes, the BMDM were seeded at 1x10⁵/well in DMEM with 20% L929 supernatants in flat-bottom 96-well plates and incubated overnight for experiments on the next day.

Isolation of CD14⁺ human Monocytes

Venous blood was collected in S-Monovette® K3EDTA tubes and PBMCs were obtained by density gradient centrifugation in Ficoll-Paque PLUS. Monocytes were isolated from PBMCs using the EasySep™ Human Monocyte Isolation Kit according to the manufacturer instructions (STEMCELL Technologies™). PBMCs were washed twice with PBS complemented with 2% FBS and 1mM EDTA before incubated with the supplied monocyte isolation cocktail and the platelet removal cocktail for 5 minutes. Magnetic beads were added to this suspension for another 5 minutes before magnetic separation in an EasySep™ Magnet. After 2.5 minutes of incubation, the enriched suspension was poured into a new tube. The isolated monocytes were counted using a hemocytometer and resuspended in RPMI medium at a concentration of 1x10⁶ ml⁻¹. The purity of the purified monocytes was assessed by flow cytometry using CD14 (monocyte) and CD41 (platelet) markers.

Isolation of Human Neutrophils

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™). Whole blood was incubated with the Neutrophil Isolation Cocktail and RapidSpheres™ for 5 minutes and diluted with neutrophil isolation buffer (1mM EDTA in PBS). After 5 minutes of incubation in the EasySep™ Magnet, the enriched cell suspension was poured into a new tube and incubated again with RapidSpheres™ for another 5 minutes, followed by a second, and third round of magnetic separation. The obtained neutrophils were counted using a hemocytometer and pelleted by centrifugation at 350 x g for 5 minutes. Cells were resuspended in RPMI-1640 medium supplemented with 10% FBS, 1% GlutaMAX and 1% Penicillin-Streptomycin. Neutrophil suspension was adjusted to 1x10⁶ ml⁻¹ and 100 μL (1x10⁵ cells/well) were seeded in a 96-well round-bottom plate. The purity of the purified neutrophils was assessed by flow cytometry using CD66b (neutrophil marker) and CD41 (platelet marker).

Isolation of Human Platelets

Human platelets were isolated as previously described (Alard et al., 2015) with slight modifications. In brief, venous blood was drawn into S-Monovette® 9NC collection tubes. The blood was centrifuged for 5 minutes at 330 x g without brake to obtain platelet-rich plasma (PRP). All following centrifugation steps were performed without brake and in the presence of 200 nM PGE₁ to inhibit platelet activation. PRP was transferred to a new tube and diluted 1:1 with phosphate-buffered saline (PBS) to reduce leukocyte contamination and centrifuged for 10 minutes at 240 x g. Platelets were pelleted by centrifugation at 430 x g for 15 minutes and washed once with PBS. Total platelets were counted using a hemocytometer and resuspended in RPMI medium to a concentration of $1 \times 10^8 \text{ ml}^{-1}$ unless otherwise indicated. The purity of the purified platelets was assessed by flow cytometry using CD45 (leukocyte) and CD41 (platelet) markers.

Isolation of Mouse Platelets

Blood was drawn by puncturing the vena facialis of anaesthetized 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 citrate-dextrose solution (ACD). PRP was prepared by centrifugation at 330 x g for 5 minutes without break. All following centrifugation steps were performed without brake. PRP was transferred to a new tube and diluted in twice as much volume of PIPES/saline/glucose (PSG) buffer with the final concentration of 1.5 μM PGE₁. The suspension was centrifuged at 240 x g for 10 minutes to reduce leukocyte and erythrocyte contamination. The supernatant was transferred into a tube with PGE₁ in a final concentration of 0.7 μM in PSG buffer. The platelets were pelleted by centrifugation at 1000 x g for 5 min, washed with 1.5 μM PGE₁ in PSG buffer. The washed platelets were resuspended in DMEM, counted in a hemocytometer, and the platelet suspension was adjusted to $5 \times 10^6 \text{ ml}^{-1}$ unless otherwise indicated. Purity and viability of the prepared platelets were assessed by flow cytometry.

Purity Assessment of Cells Populations

Samples of isolated neutrophils and platelets were analyzed for purity, platelet pre-activation and platelet viability after each experiment. Isolated murine and human platelets were activated with 0.5 or 1 U ml^{-1} thrombin respectively for 30 min. Cells were blocked with 1:10 mouse or human Fc blocking reagent for 10 minutes at room temperature (RT). The samples were stained with fluorochrome-conjugated monoclonal anti-mouse or anti-human Ig antibodies against CD41/CD41, CD62p, CD14, CD45 or CD66b as indicated for 30 minutes in the dark. Cells were washed and resuspended in flow cytometry buffer (1% FBS in PBS) for analysis. Compensation beads (OneComp Beads) and isotype controls were prepared in the same way. Flow cytometry was performed with a Macs Quant® Analyzer10 (Miltenyi Biotec) and analyzed using the Flowtop software (Tree Star). The applied gating strategy was based on doublet discrimination and isotype-matched control antibodies.

Platelet depletion and LPS + R848 model

Platelet depletion was performed in male, 6-10 week-old C57BL/6j mice by intravenous injection of an anti-CD42b (GPIIb-alpha) monoclonal antibody or its isotype IgG control (Emfret Analytics, 2 $\mu\text{g/g}$ animal, 100 μL). 6 hours after injection, a drop of blood was taken from the tail vein to confirm the efficiency of platelet depletion by flow cytometry. Next, the animals were intraperitoneally injected with 200 μg of R848 (Resiquimod, Invivogen) or a combination of 200 μg of R848 and 200 μg of LPS (E.coli O111:B4, Invivogen) in equal volumes (100 μL) as described (Kanneganti et al., 2006). The collection of samples was conducted 6 hours after the inflammatory stimuli. Blood was centrifuged for 10 minutes at 5000 rpm, 4°C for collection of serum. Peritoneal lavage was centrifuged for 5 minutes at 500 g, 4°C and the cells from the peritoneal lavage were fixed and stained for phenotyping. Blood and cell-free peritoneal exudate were used for the cytokine evaluation (R&D Systems).

Immuno-staining of Whole Bone Marrow Cells

Wild-type, ASC-mCitine Tg or ASC-mCherry *knock in* reporter mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Femurs and tibias 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 staining buffer and blocked with 1:10 mouse FcR blocking reagent for 10 minutes at RT before staining with fluorochrome-conjugated anti-mouse CD41 (platelet marker), Ly6G (neutrophil marker), CD14 (monocyte marker) or CD45 (leukocyte marker) (1:5 dilution each). Staining was performed for 30 min at room temperature. Cells were washed twice in PBS and resuspended in staining buffer before flow cytometry analysis or confocal microscopy imaging. Compensation beads (OneComp eBeads) and isotype controls were prepared in the same way. Flow cytometry was performed on MacsQuant® VYB and analyzed using FlowJo. The gating strategy was based on doublet discrimination and isotype-matched control antibodies or unstained controls.

Generation of Platelet and MK supernatants

After platelet isolation, the cell suspension was adjusted to 5×10^7 platelets ml^{-1} (for human experiments) or 5×10^6 platelets ml^{-1} (for mouse BMDM experiments), in order to fit the macrophage:platelets proportion of 1:50 (human) or 1:5 (mouse). For the generation of supernatants from the megakaryocytic cell line MEG-01, 1×10^6 cells ml^{-1} were used. Next, platelets or MEG-01 were left untreated (Unstim) or incubated at 37°C with LPS (200 ng ml^{-1}) or thrombin (1.0 U ml^{-1}) for 3 hours. After stimulation, the platelet cell suspension

was centrifuged for 10 minutes at 3000 x g for the generation of the cell-free supernatants. MEG-01 cells were first centrifuged for 7 minutes at 170 x g and the obtained supernatants were centrifuged again for 10 minutes at 3000 x g. The absence of cells was confirmed by microscopic visualization in a hemocytometer. The supernatants were immediately used to stimulate hMDMs in RPMI, or immediately frozen at -80°C until further use.

Stimulation of THP-1 and MEG-01 cells

For stimulation assays, THP-1 cells were treated overnight with 100 nM phorbol 12-myristate 13-acetate, then were primed for 2 h with $1\text{ }\mu\text{g ml}^{-1}$ of LPS and were subsequently activated with $10\text{ }\mu\text{M}$ of Nigericin.

Stimulation assays

Seeded BMDMs, human macrophages or neutrophils were centrifuged at 350 x g for 5 minutes prior to replacing the supernatant by fresh, serum-free DMEM (for BMDMs) or RPMI (for human neutrophils and macrophages) as control or platelet suspension. For NLRP3 stimulation, cells were primed with 200 ng ml^{-1} LPS for 3 hours and activated with $10\text{ }\mu\text{M}$ Nigericin or 5 mM ATP for 90 minutes unless otherwise indicated. Human monocytes were primed with 2 ng ml^{-1} of LPS before being activated with Nigericin ($10\text{ }\mu\text{M}$, 90 min).

For the stimulation of the NLRC4 inflammasome, human macrophages were primed with 200 ng ml^{-1} LPS for 3 hours before $2\text{ }\mu\text{g ml}^{-1}$ LFn-Prgl and $0.5\text{ }\mu\text{g ml}^{-1}$ PA (from *Bacillus anthracis*) were added to the culture medium for 2 hours (Zhao et al., 2011).

For the stimulation of the AIM2 inflammasome, human GM-CSF-derived macrophages were primed with LPS (200 ng ml^{-1} , 3 hours), followed by stimulation with transfected Poly(dA:dT). Briefly, vesicles were formed using a 1:50 dilution of the transfection agent Lipofectamine 2000 in OptiMEM. After 15 minutes of incubation, the empty vesicles were combined in 1:1 proportion of $4\text{ }\mu\text{g ml}^{-1}$ Poly(dA:dT), and further incubated for 15 minutes. The dsDNA-containing vesicles were added directly on the cell culture for a final volume of $100\text{ }\mu\text{L}$ of medium (final stimulatory concentration of $1\text{ }\mu\text{g ml}^{-1}$). Cells were stimulated for 3 hours before cell-free supernatants were harvested for cytokine measurements.

R848 and Pam3Cysk4 were also used for priming in some experiments at $10\text{ }\mu\text{M}$ and $1\text{ }\mu\text{g ml}^{-1}$ respectively. After stimulation, cells were centrifuged and cell-free supernatants were collected to measure cytokine levels by HTRF. In experiments where the activity of COX1/2 and LOX was inhibited, platelets were incubated with Aspirin, or Zileuton (both at $100\text{ }\mu\text{M}$) for 60 min at 37°C , before their addition to hMDMs. SB-431542 ($10\text{ }\mu\text{M}$) was added to the macrophage culture 60 minutes before stimulation. The drugs were diluted in RPMI 1640, without supplements.

Trans-well assays

hMDMs were seeded at a concentration of $1 \times 10^5/100\text{ }\mu\text{L}$ in HTS $0.4\text{ }\mu\text{m}$ Trans-well 96-well plates on the day prior to the experiment. Platelets were added to the upper wells at a concentration of 6.25×10^7 in $80\text{ }\mu\text{L}$ of DMEM. After addition of platelets, inflammasome activation was carried out as described above and the levels of IL-1 β and TNF α were assessed in cell-free supernatants by HTRF.

Calcium chelation experiments

The calcium chelator BAPTA or the cell-permeable calcium chelator BAPTA-AM (see STAR Methods) 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. Cells were primed with 200 ng ml^{-1} LPS for 3 hours before the addition of either 0.5 mM BAPTA or $5\text{ }\mu\text{M}$ BAPTA-AM. Then, cells were stimulated with $10\text{ }\mu\text{M}$ Nigericin for NLRP3 inflammasome activation. Cell-free supernatants were analyzed afterward by HTRF for cytokine secretion.

Quantitative PCR

Total RNA containing small RNAs from purified human platelets, or PBMCs was purified using the miRNeasy kit (QIAGEN), DNA was digested with DNase I (QIAGEN), and cDNA was generated by using 500 ng RNA with SuperScript III Reverse Transcriptase kit (Thermo Fisher) following the manufacturer's instructions. cDNA was diluted (1:10 for PBMCs, and 1:3 for platelets) and qPCR was performed with Maxima SYBR Green on a QuantStudio 6 Flex RT PCR machine (Thermo Fisher) for 40 cycles and followed by a melt curve analysis for off-target products. The primers used were ACTB-fwd-ccaccatgtaccctggcatt, ACTB-rev-cggagtacttgcgctcagga, NLRP3-fwd-tcggagacaaggggatcaaa, NLRP3-rev-agcagcagtgtagctgagg, CD14-fwd-gagctcagaggttcggaaga, CD14-rev-cttcacgtccagctcaca, PYCARD-fwd-gagctcaccgctaactgtgct, PYCARD-rev-actgaggaggggcttgat, PF4-fwd-ctgaagaa gatggggacctg, PF4-rev-gtggctatcagttgggcagt, CASP1-fwd-acaaccagctatgccaca, CASP1-rev-gtgcggcttgactgtccat, GP1BA-fwd-ctgctcttgcctctgtgtgt, GP1BA-rev-ctccaggtgtgtgtgtgtgt, IL1B-fwd-tgggcagactcaaattccagct, IL1B-rev-ctgtacctgtcgtcgtgttga.

Imaging and quantification of ASC specks

To examine ASC specking, hMDMs were seeded at a concentration of $2 \times 10^5/\text{well}$ 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 $5 \times 10^7\text{ ml}^{-1}$. Cells were stimulated with 200 ng ml^{-1} LPS for 3 hours and the NLRP3 inflammasome was activated with $10\text{ }\mu\text{M}$ Nigericin for 45 minutes. Afterward, 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, cells were blocked with 1:10 human FcR blocking reagent for 10 minutes at room temperature and

stained with directly labeled anti-ASC-647 or the same amount of directly labeled IgG1 control 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 re-suspended in PBS and imaged using an 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 a Leica TCS SP5 SMD confocal system. The quantification of ASC specks per nuclei was carried out using the image analyzer software CellProfiler 3.0 (Open source software: <https://cellprofiler.org>). A pipeline was constructed to convert the fluorescent images into binary code. Through this conversion, the nuclei and ASC specks were identified and quantified. ASC specks were identified using the EnhanceFeature tool.

Cytokine measurements

Levels of human, or murine IL-1 β , IL-6, and TNF α in cell-free supernatants were quantified using commercially available HTRF® (homogeneous time-resolved fluorescence) kits. The HTRF was performed according to manufacturer instructions (Cisbio). Multiplex Cytokine array was used for the detection of several human cytokines and growth factors according to the manufacturer's instructions (Thermo Fisher).

Caspase-1 activity

Caspase-Glo® 1 Inflammasome Assay (Promega) provides a luminogenic caspase-1 substrate, Z-WEHD-amino luciferin, in a lytic reagent optimized for caspase-1 activity and luciferase activity. The reaction buffer was mixed with the cell-free supernatant and the luminescent signal was evaluated 30 minutes later.

Lactate dehydrogenase (LDH) assay

The extracellular lactate dehydrogenase (LDH) concentrations in cell-free supernatants were assessed using the Pierce LDH Cytotoxicity Assay, according to the manufacturer's instructions. Samples were measured at an absorbance of 490 nm using a Spectra-max i3 plate reader (ThermoFisher). 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.

Confocal laser scanning microscopy

Platelets and immune cells were imaged in a Leica TCS SP5 SMD confocal system (Leica Microsystems, Wetzlar, Germany). Images were acquired using a 63X objective, with a numerical aperture of 1.2, and analyzed using the Volocity 6.01 software (PerkinElmer, Waltham, Massachusetts, U.S.A.).

Meta-analysis of microarray data

Pre-processed microarray data of platelets and whole peripheral blood cells under steady state and certain disease states (GSE2006, GSE11524, GSE10361, GSE50858, GSE47018, GSE17924) were down-loaded from the Gene Expression Omnibus (GEO) data base (<https://www.ncbi.nlm.nih.gov/geo/>). Expression values for the house-keeping gene GAPDH and platelet- as well as inflammasome-associated genes (*CXCL4/PFA4*, *PDGFA*, *IL1B*, *PYCARD*, *NLRP3*, *TIMP1*, *CASP1*, *ARG2*, *TP2A*, *SELENB1*) were extracted for each dataset. In case multiple probe sets for GAPDH were present on the microarray chip, a mean expression value for GAPDH was calculated. The expression values for probe sets of platelet and inflammasome-associated genes were normalized for each dataset according to the respective GAPDH mean expression value. Log2 transformed normalized expression data were plotted as bar charts by the Tidyverse package in R (v3.4.2).

Proteomics (LC-MS)

Suspensions of freshly isolated human platelets were adjusted to a concentration of 5×10^7 ml⁻¹ in RPMI. Platelets were left untreated, or stimulated with 200 ng ml⁻¹ LPS, 1 U ml⁻¹ Thrombin for 3 hours. Platelets were pelleted by centrifugation (3000 x g, 10 minutes) and the cell-free supernatants were harvested. Cell-free supernatants were added with 1x complete protease inhibitor cocktail prior to freezing at -80°C. Proteomics analysis was carried out at the CECAD/CMC Proteomics Core Facility (University Cologne, Germany) on a Q Exactive Plus Orbitrap (Thermo Scientific) mass spectrometer that was coupled to an EASY-nLC (Thermo Scientific). Briefly, peptides were loaded in 0.1% formic acid in water onto an in-house packed analytical column (50 cm ϕ 75 μ m I.D., filled with 2.7 μ m Poroshell EC120 C18, Agilent) and 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 columns. The mass spectrometer was operated in data-dependent acquisition mode. All mass spectrometric raw data were processed by the CECAD/CMC Proteomics Core Facility using Maxquant (version 1.5.3.8) with default parameters. Briefly, 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. Figures were assembled using the R Tidyverse package. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD017976.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed with GraphPad Prism Version 7.0f. All statistical analysis were preceded by Normality tests, followed by the recommended parametric or nonparametric tests. For most *in vitro* experiments with several groups (hMDMs alone versus with Platelets, stimulated or not), P values were determined by two-way ANOVA with Tukey's or Sidak's or multiple comparison tests. For *in vivo* experiments, in which two groups of mice are compared ($n = 6/\text{group}$), (Figure 3) we used two-tailed Unpaired t test to calculate differences. Correlations between cytokines and cell counts were computed with Two-tail Spearman. Outliers were detected using the ROUT method, and were excluded from the statistical analysis, but not removed from the figures. Additional statistical details are given in the respective figure legends, when appropriate.