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REVIEW

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The NLRP3 inflammasome in platelets – form, functions, and future of the complex

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ABSTRACT

Platelets are anucleate cells that primarily facilitate thrombosis and hemostasis but can also act as mediators of vascular inflammation in disease. Platelets are typically understood to do this through the release of pre-formed chemokines coupled with direct heterotypic interactions with a variety of immune cells. However, an alternative mode of action has been described where platelets are able to undertake *de novo* synthesis of the cytokines interleukin-1β (IL-1β) and interleukin-18 (IL-18). The primary mechanism to produce these inflammatory mediators is the activation of the NACHT leucine-rich repeat pyrin domain-containing protein 3 (NLRP3) inflammasome, a multi-protein complex that processes IL-1β and IL-18 through caspase activation. The presence and characteristics of the NLRP3 inflammasome have been widely described in a variety of nucleated cells, although its role in anucleate platelets is less clear. In the last decade, the presence of the inflammasome has been reported in platelets and linked to several diseased states including sickle cell disease, acute coronary syndrome, sepsis, and viral hemorrhagic fever. This emerging new biology of platelets, its role in platelet function, vascular inflammation, and other related areas of exploration are critically reviewed here.

PLAIN LANGUAGE SUMMARY

Platelets are blood cells that are critical for effective hemostasis but also drive pathological thrombosis. While these are considered as the classic roles of platelets in the body, they also play a role in immunity, inflammation, and infection. One recent aspect of the inflammatory activity of platelets has been around the presence and activity of a large protein complex called the NLRP3 inflammasome. When the NLRP3 inflammasome is activated by cellular or pathogen danger signals, several smaller proteins come together to form a large functional unit which allows the cell to produce an inflammatory signal that can activate other cells. It is important to understand what the NLRP3 inflammasome in platelets does in health and disease, as it can drive a hyperactive immune environment that can cause negative effects.

In platelets, the role of the NLRP3 inflammasome has only begun to be explored, and there have been many recent studies on this topic; however, they often have found slightly different or conflicting results. In this review, the current literature on the platelet NLRP3 inflammasome is brought together and critiqued, highlighting common themes, but also differences between these studies. There remains a great deal still to be understood about the presence and activity of the NLRP3 inflammasome in platelets, as well as controversies and new areas which need to be explored.

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A role for platelets beyond haemostasis

It is well established in the field that platelets play a critical role in physiological hemostasis and pathological thrombosis. However, over the last several decades, a range of other biological roles of platelets have emerged including angiogenesis,[1] wound healing,[2] and innate immunity.[3] The role of platelets acting as innate immune cells is perhaps best described in their capacity to directly respond to bacterial components.[4,5] Beyond this, more evidence is now emerging of a role for platelets concurrent to thrombosis and hemostasis as blood-borne sentinel cells which are actively involved in immunity, and platelets are

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hypothesized to be important for the development, progression, and resolution of vascular inflammation.[3] Platelets possess a full repertoire of inflammatory functions and a diverse array of mechanisms for transcellular communication allowing them to coordinate the interactions of, and interact with, endothelial cells and circulating immune cells.[3,6] These inflammatory effects are thought to be mediated by two primary mechanisms, cytokine and chemokine release and the formation of heterotypic cell aggregates. The release of platelet α-granules results in the surface expression of P-selectin and release of pre-formed chemokines such as CCL3, CCL5, CCL5, CXCL4 (PF4), PAF, and CXCL10 among others. These bioactive mediators trigger proinflammatory gene expression in both endothelium and leukocytes.[7] P-selectin facilitates heterotypic interactions with both endothelial cells and leukocytes through P-selectin glycoprotein ligand-1.[8–10] Platelets have been demonstrated to modulate the function of all major immune cell subsets present in the blood: monocytes, neutrophils, and lymphocytes, demonstrating a capacity to regulate both the innate and adaptive immune system. In examples of this, platelets can recruit monocytes via CCN1 and facilitate inflammatory monocyte patrolling in animal models of inflammation,[11] activate neutrophil extracellular trap (NET) formation in sepsis,[12] and mediate lymphocyte activation via α-granule-dependent surface expression of CD40-ligand.[13] Through the release of vasoactive factors and the formation of heterotypic cell complexes, platelets act as a focal point for vascular inflammation enabling the recruitment of leukocytes to the endothelium and their transmigration to the subendothelial space.

A third, and currently more poorly defined, mechanism of platelet-driven inflammation occurs through their synthetic capacity. Platelets possess pre-mRNA that can be spliced and translated to mature proteins.[14] Experiments performed *in vitro* demonstrate that platelets produce several proteins that potentially influence the thrombo-inflammatory process including tissue inhibitors of metalloproteinases,[15] tissue factor,[16] interleukin-1β (IL-1β),[17,18] and IL-18.[19] Interestingly, protein translation by platelets occurs for many hours and potentially days after they are activated.[20] Therefore, it is conceivable that once activated, they could contribute to the low-grade inflammation associated with several diseases. This ability is driven by their capability to respond directly to dangerous and pathogen-associated molecular patterns (D/PAMPs).[5,21] In other cells, the synthesis of IL-1β and IL-18 is a result of the activation of the NLRP3 inflammasome.[22] Evidence is now emerging to suggest the presence of a competent NLRP3 inflammasome in platelets, although its mechanism of activation, role in platelet activation, and wider inflammatory effects are still in their infancy. In the present review, we will examine the evidence for NLRP3 inflammasome expression and activity in platelets, and its potential functional roles.

The NLRP3 inflammasome

The inflammasomes are a family of large multimeric protein complexes with enzymatic activity related to their ability to synthesize cytokines or interferons.[22] Inflammasomes respond to both DAMPs and PAMPs, but distinct inflammasome complexes are often specific to individual ligands. Here, we concentrate primarily on the NLRP3 inflammasome and the cells of the blood which have been shown to express this including monocytes, neutrophils, and more recently, platelets.[23,24]

The NLRP3 inflammasome has been comprehensively described in leukocytic models since the first report of the complex in 2004,[25] and this has provided a robust understanding of the structure of the complex and each subunit of the activated multimer. When activated, the associated complex includes the NACHT-leucine-rich repeat pyrin domain containing protein 3 (NLRP3 or cryopyrin) subunits, the apoptosis-associated speck-like protein containing a CARD (ASC) and the thiol protease caspase-1 (or interleukin converting enzyme-1). Activation of the complex leads to the interaction of the NLRP3 pyrin domain with ASC and subsequent recruitment of caspase-1 via the ASC caspase recruitment domain (CARD). This results in caspase-1 activation via auto-cleavage. Activated caspase-1 cleaves both gasdermin D and pro-IL-1β to facilitate secretory pore formation and synthesis of IL-1β, respectively.

The NLRP3 inflammasome responds to a wide range of ligands including the bacterial toxin nigericin, bacterial membrane component lipopolysaccharide (LPS), cellular stress signal adenosine triphosphate (ATP), gout monosodium urate crystals, atherosclerotic plaque cholesterol crystals, oxidized LDL (oxLDL), and amyloid-β protein.[25,26] The wide range of the stimuli has been attributed to the targeting of a common signaling node, which involves reactive oxygen species (ROS),[27] paired with mitochondrial dysfunction.[28–30] This plethora of ligands likely accounts for its known role in many diseases, notably in

multiple inflammatory diseases including gout,[31] systemic lupus erythematosus (SLE),[32] cardiovascular disease,[33] rheumatoid arthritis,[34] systemic viral infections,[24] and bacterial sepsis,[35] all of which can be linked to one or more of the above ligands. This broad range of stimuli is atypical of other inflammasomes, where the majority respond to a single specific agonist. For example, the AIM2 inflammasome is only stimulated by viral double-stranded DNA (dsDNA) present when cells are infected by dsDNA viruses,[36] and the IPAF inflammasome only by type III or IV secretion systems employed by gram-negative bacteria to deliver toxins.[22] Ligand specificity typically permits regulation of inflammasome activity and limits the potential for severe inflammatory reactions associated with dysregulated control.[37]

To avoid aberrant activation, full activation of the NLRP3 inflammasome requires two distinct signals, a common feature of immune cell activation that protects from abnormal activation. In the context of the NLRP3 inflammasome, this requirement ensures careful regulation of secretion of the inflammatory cytokines IL-1β or IL-18. The two-step activation model involves a priming signal, which increases the expression of constituent proteins, followed by a second signal that induces complex formation and activation.[22] It is now understood that priming is a combination of two concurrent events and can be subdivided into transcriptional and non-transcriptional priming. Transcriptional priming is transcription of inflammasome genes to elevate inflammasome component proteins, which of the five components, pro-IL-1β is required in particular abundance.[23] Non-transcriptional priming can include post-translational modifications such as ubiquitination,[38–40] and phosphorylation of multiple inflammasome components including ASC[41] and NLRP3.[42] This two-step model of NLRP3 inflammasome activation can be reproduced in the laboratory by stimulation with *e.g.*, LPS (PAMP) followed by *e.g.*, ATP (cell stress), as priming and then an activating signal respectively.[43]

Dysregulation of NLRP3 can be driven by mutations which remove or weaken the critical activatory checkpoint of two-step activation, or excess bioavailability of exogenous PAMPs or endogenous DAMPs such as LPS or oxLDL, respectively. Generally, conditions with a mutation of NLRP3 fall within the cryopyrin-associated periodic syndrome (CAPS) group. CAPS is characterized as an inflammatory state marked clinically by increases in traditional markers of inflammation such as C-reactive protein with a range of other systemic inflammatory symptoms which can be lethal.[44] Mutations in NLRP3 which cause CAPS have since been translated into murine models to better understand the disease and the cellular and systemic phenotypes that are driving the morbidities.[45] In the first instance, a mutation in parallel to the human R260W was reproduced in a murine model, and this drove unregulated production of IL-1β but without the typical necessity of an activating signal.[46] In a separate study, mutations corresponding to human A352V and L353P were mutated in a cre-dependent murine model, and the role of myeloid cells versus all tissues was compared – myeloid cell lineages alone were able to induce a severe and lethal phenotype.[47] In a large population-wide study examining genetic variants of NLRP3, several variants were shown to be associated with adverse cardiovascular mortality underpinned by increased low-grade systemic inflammation, however this was low-grade without the typically severe CAPS phenotype.[48] These studies demonstrate the significant potential that the NLRP3 inflammasome has to promote systemic inflammation and adversely affect clinical outcomes; this underpins the requirement to fully understand the potential role of the NLRP3 inflammasome in platelets.

Function of the platelet NLRP3 inflammasome

While several publications have now demonstrated a role for the NLRP3 inflammasome in platelets, it remains an emerging field with many aspects of expression, regulation, and function of the complex in platelets not yet understood. The field of the platelet NLRP3 inflammasome was initiated by several early studies which identified a role for platelet-produced cytokines. These include reports that platelets stimulated by LPS produce IL-1β which plays a role in autocrine activation,[17] platelets in the context of human immunodeficiency virus infection produce IL-18,[19] and that platelets stimulated with SLE serum produce IL-1β which drives endothelial cell activation.[18] While these three publications all described the production of cytokines which are known to be downstream of the NLRP3 inflammasome, the direct role of the complex in these studies was not examined, however they provide substantial evidence of the capacity of platelets to produce cytokines that are typically downstream of the NLRP3 inflammasome. The publications to date which explore the role of the NLRP3 inflammasome in platelets can be broadly divided into two

groups – those which describe that the NLRP3 inflammasome primarily alters platelet function itself and those which explain a systemic inflammatory role for the complex in platelets. These are examined in the following sub-sections.

Hemostasis- and thrombosis-linked platelet NLRP3 inflammasome

The more classical role for the platelet NLRP3 inflammasome from the point of view of the platelet biologist concentrates on the complex modulation of the normal hemostatic activity of platelets, primarily driving hyperactivity. This was initially identified through exploring the importance of Bruton's tyrosine kinase (BTK) in the activation of the NLRP3 inflammasome downstream of the classical platelet agonists collagen and thrombin. This study went on to suggest an important role in platelet hemostatic activation for the NLRP3 inflammasome, with specific pharmacological inhibitors of the inflammasome components and with transgenic NLRP3 or BTK murine models confirming these findings.[49] In 2018, NLRP3 inflammasome-mediated regulation of platelet function was further explored and was established to be key to the regulation of integrin αIIbβ3 outside-in signaling, and when tested in an NLRP3 knockout model, platelet thrombus formation was significantly reduced and bleeding times increased,[50] suggesting an additional role for NLRP3 inflammasome activity in classical platelet function. Although these two studies agree on the importance of the NLRP3 inflammasome in hemostatic platelet activity, they disagree on key points such as the phenotype associated with α-granule secretion. A study utilizing a patient cohort and transgenic IL-1R murine models identified a key role for IL-1β in platelet hyperactivity.[51] Another study was published which reinforced the previously suggested aggregatory role of platelet NLRP3 inflammasome in a murine model of hind limb ischemia downstream of platelet Toll-like receptor (TLR) 4 ligation and caspase-1 cleavage.[52] The same group then followed this up with a study which identified the platelet NLRP3 inflammasome in the development of pancreatic cancer in a murine model, again hypothesized to be through increased platelet aggregation.[53] The role of NLRP3 and BTK was further developed in a sickle cell disease (SCD) model and supports earlier findings suggesting that inhibition of either BTK with ibrutinib or NLRP3 with MCC950 both led to a protective reduction in platelet aggregation in SCD platelets.[54] The same group explored SCD murine models and demonstrated that Syk is a component upstream of NLRP3 inflammasome activity, where pharmacological blockade led to reduced platelet activity.[55] Utilizing further murine models targeting NLRP3, ASC, and caspase-1 in a PF4-cre model neither ASC nor caspase-1 were identified to modulate platelet function, but NLRP3 independent of classical inflammasome activity modulated GP1b function, this is a critical study as inflammasome components were directly assessed through PF4-cre-dependent expression.[56]

Beyond directly modulating platelet function but still having an effect on hemostasis, the platelet NLRP3 inflammasome via increased caspase-1 activity has been implicated in driving NETosis and the linked increase in deep vein thrombosis.[57] In support of this, a more recent murine study on deep vein thrombosis critically found that platelet-specific NLRP3 inflammasome activity regulated IL-1β levels within the thrombi which in turn supported NETosis and venous thrombosis.[58]

Despite the findings commented on above, which all demonstrate a role for platelet NLRP3 inflammasome activity in platelet activation, a recent study using ASC knockout murine models demonstrated an opposite effect, where ASC expression actively appeared to control platelet activation via modulation of GPVI signaling.[59] Whether this is somehow linked to the previously mentioned work of Qiao et al., who suggested a knockout of NLRP3 reduced platelet activity,[50] potentially increasing the pool of ASC to further inhibit GPVI-mediated function has not been considered but may be worth exploring. Intriguingly, this group was not able to confirm expression of either NLRP3 or caspase-1 in their murine platelets, however ASC was demonstrated by immunoblot.[59]

In a more translational study, the role of platelet NLRP3 inflammasome activity was explored in an SCD clinical cohort, and this was paired with a murine model of the disease. A series of experiments were performed concentrating on fluorescent measurements of caspase-1 activity with the fluorochrome-labeled inhibitors of caspase (FLICA).[60] They were able to describe that the platelet-derived TLR4 ligand high-mobility group box 1 (HMGB1)[61] through BTK drives NLRP3 inflammasome activation.[62] Furthermore, HMGB1 was shown to be present in the plasma of SCD patients, and this correlated with basal platelet caspase activity and plasma swap experiments could stimulate naive platelets from healthy donors.[62] This

was linked back to a murine model of SCD, which demonstrates increased NLRP3 inflammasome-driven platelet aggregation, and thereby supports their previous findings of the role of BTK/NLRP3 in classical platelet activity.[49]

Inflammation-linked platelet NLRP3 inflammasome

Papers which support the role of platelets as a driver of systemic inflammation include the initial report of the NLRP3 inflammasome in platelets, which was in 2013, where the authors described a role for platelets in the pathogenicity of viral hemorrhagic fever caused by Dengue virus infection.[24] With the application of flow cytometry, the group was able to demonstrate Dengue-virus-driven shedding of IL-1β-rich platelet microparticles which correlated with caspase-1 activation. Using pharmacological inhibitors against caspase-1, mitochondrial ROS, and RIP1 kinase, these mediators were shown to be vital in the activation of NLRP3.[24] This paper suggests a systemic role for platelet IL-1β driving the pathogenesis of the viral hemorrhagic fever by increasing vascular permeability. Later, studies continued to explore this axis and confirmed that platelet–Dengue virus exposure led to increased platelet pyroptosis.[63] Further studies in the role of the platelet NLRP3 inflammasome included understanding the role it may play in sepsis, using a rodent model of cecal ligation and puncture (CLP).[64] The research group showed that inflammasome components co-localized in rat platelets on induction of sepsis, that more platelets were basally active, and that levels of NLRP3 produced cytokines IL-1β and IL-18 were also raised in plasma.[64] In a subsequent study by this same group, they further developed their previous findings using the same rodent model of CLP. Here, they were able to show that inhibition of the NLRP3 inflammasome in combination with CLP prevented excess platelet activation and reduced it to sham treatment levels.[65] The inhibition of the inflammasome also reduced systemic expression of NLRP3 inflammasome-derived cytokines, IL-1β and IL-18, which protected the CLP models from sepsis-induced organ damage and failure. Further support for a primary role of platelet NLRP3 inflammasome as contributing to vascular inflammation elegantly demonstrated that platelets *ex vivo* from SCD patients release extracellular vesicles which contribute to the pathogenesis of SCD through a platelet cytokine-mediated inflammatory cascade, ultimately culminating in neutrophil activation.[66] A study, which focused on an *in vitro* model of prion disease, also noted that platelets undergo NLRP3 inflammasome activation, caspase-1 activity, which generated increased platelet-leukocyte aggregates.[67] In counterpoint to these studies, Rolfe *et al*., described that platelets are critical in the activation of innate immune cell NLRP3 but themselves do not contribute to IL-1β production directly; indeed, genetic ablation of IL-1β in platelets did not reduce IL-1β production by innate immune cells which were dependent on platelet-calcium signaling for NLRP3 activation.[68]

Our own clinical studies on cohorts of patients with chronic low-grade inflammation demonstrated that platelets support the ongoing release of IL-1β, measured by intracellular platelet cytokine levels which correlate with C-reactive peptide.[69] In a similar study to this in a cohort of patients with Crohn's disease, platelets demonstrated increased expression of NLRP3, ASC, and caspase-1 linked to increased serum and platelet IL-1β.[70] Together, the two studies suggest platelets may play a role in the propagation of low-grade vascular inflammation. In a separate report, acute-coronary syndrome (ACS) patients were examined to understand how expression of the platelet NLRP3 inflammasome changes in disease and if this may provide a prognostic platform to evaluate inflammatory progression. In this study, using a combination of western blot and quantitative polymerase chain reaction (PCR), the authors were able to link increased platelet NLRP3 inflammasome expression not only to the disease group, as ACS versus stable angina, but also to adverse outcomes within the ACS group.[71] This presents a convincing clinical study where for the first time platelet NLRP3 inflammasome expression can be shown to have prognostic value in cardiovascular disease and likely indicates a role in disease progression. Focusing instead on hematological disease, a recent study identified how platelets, via the well-described ROS-NLRP3 pathway,[27] contributed to immune thrombocytopenia (ITP). The study was based on two key observations: first, NLRP3 inflammasome expression was increased, and second, antioxidant capacity decreased in ITP platelets. They were then able to show through experiments either inducing ROS, and therefore NLRP3 inflammasome activity, or scavenging ROS, thus inhibiting NLRP3 inflammasome activity, that this effect was ROS dependent. Finally, through specific inhibition of the NLRP3 inflammasome with MCC950 or caspase-1 with Z-YVAD-FMK, pyroptosis and cell death were decreased in ITP platelets.[72] These clinical studies all suggest that the NLRP3 inflammasome

likely plays an important role in the inflammatory homeostasis of the blood and vasculature, and at least some of this role is dependent on platelets and their inflammatory dysregulation.

Function of the platelet NLRP3 inflammasome synopsis

While these papers summarized in the two sub-sections above agree on the core hypothesis, that the NLRP3 inflammasome plays an important role in platelet biology, they do not agree on the role that the NLRP3 inflammasome may play, ranging from a systemic effect of secreted IL-1β driving vascular inflammation in Dengue infection,[24] SLE,[18] sepsis,[64,65] prion disease,[67] or SCD,[66] a thrombotic mediator role in deep vein thrombosis,[57,58] to an autocrine effect which is vital for platelet activation,[17,50] or control of platelet activation.[49,52–54,59,62] There is also some evidence that constitutive NLRP3 activity may be important in thrombopoiesis, as thrombocytopenia has been shown to be significantly associated with anti-IL-1β therapy,[73] and it is well described that IL-1β plays a role in megakaryocyte maturation.[51]

Many of these manuscripts report conflicting findings on the roles of the NLRP3 inflammasome, its components' relative expression, and downstream products. Which of these effects are the primary role of the NLRP3 inflammasome in platelets is yet to be determined, what is clear is that this complexity is important in homeostasis of the systemic vasculature, hemostasis, and thrombosis, as well as in several aspects of platelet biology.

NLRP3 inflammasome expression in platelets

Platelet transcriptomic and proteomic studies are a rich resource to explore the expression of RNA transcripts and proteins in platelets. These studies have been performed on both human and murine platelets within the last decades. The primary human proteomic study reported that of the five NLRP3 inflammasome components, NLRP3, ASC, caspase-1, IL-1β, and gasdermin D, only ASC and gasdermin D were present. Copy numbers of these proteins were estimated to not exceed 1500 per cell (Table 1).[74] The human transcriptome reported expression of all of the above components, however they were all ranked below the 3000 most common transcripts (Table 1).[75] In replication of the findings in human platelets, very similar observations were made in murine platelets. The murine platelet proteome showed that again only ASC and gasdermin D are present (Table 1),[76] identical to the human report. The murine transcriptome is again a replication of the human platelet transcriptome, where all components were considered expressed, although all were ranked below the 4000 most common transcripts (Table 1).[75]

The proteomic reports alone suggest that the presence of the complete NLRP3 inflammasome in platelets may be unlikely. However, a lack of detection could be attributed to either low copy numbers of these proteins or transcripts that were below technological detection thresholds. In support of this theory, the human proteome does not detect the presence of TLRs, but their expression in platelets has been evidenced by traditional biochemical approaches.[77–80] One could also consider that the complexes are upregulated

Table 1. Relative expression or copy number of NLRP3 inflammasome components in human and murine platelets, respectively.[72–74] Rowley et al. rank transcripts on the assumed count from highest to lowest. Burkhart et al. and Zeiler et al. use an estimated copy number of each protein.

	Protein or transcript	Rowley, human rank	Burkhart, human copy number	Rowley, murine rank	Zeiler, murine copy number
Components	NLRP3	5913	–	10955	–
	ASC	3005	1000	4178	3743
	Caspase-1	3365	–	6354	–
	Interleukin 1-β	4972	–	5773	–
	Gasdermin D	4114	1500	9645	308
Upstream	TLR2	5465	–	10865	–
	TLR4	7930	–	11414	–
	TLR6	11362	–	12714	–
	MyD88	3858	940	1906	1044
Controls	GPIX	48	32400	16	63503
	CD36	615	16700	6093	–
	GPVI	367	9600	155	7822
	BTK	599	11100	1590	12146
	Fyn	548	6800	490	4145

after an inflammatory insult, making detection a significant challenge in healthy donor samples. An alternative hypothesis is that the NLRP3 inflammasome components are differentially expressed across platelet subpopulations, while in healthy conditions there may only be a proportionally small positive NLRP3 inflammasome population which falls below detection thresholds, however in response to inflammatory environments the proportions of these subpopulations may dynamically change.

The current papers reviewed here all provide convincing functional evidence of the NLRP3 inflammasome, and there is prior evidence in platelets of the cytokines downstream of NLRP3, IL-1β[17,18] and IL-18.[19] However, among the above studies, few have shown traditional detection of NLRP3 component by SDS-PAGE and immunoblot,[66] which is performed as best practice in leukocytes.[25] However, there are also reports that state they cannot detect the components in platelets. The recent publication of Huang *et al.*, showed no expression of NLRP3 inflammasome components in murine platelets,[81] and Watanabe *et al.*, who were able to show that only ASC was expressed in murine platelets and other components were absent.[59] Whether this is due to the relatively low abundance of the components and difficulty in detection, a lack of expression in platelets requiring the necessity of inflammatory environments, or the presence of platelet subpopulations requires further careful examination with particular care over contaminating innate immune cells in the platelet lysates. Indeed, others have indicated that IL-1β secretion from platelets occurs from a pre-matured pool of the cytokine, which is entirely NLRP3 independent.[82] Gasdermin D remains a relatively unexplored candidate in platelet biology compared with other components of the NLRP3 inflammasome, however, considering its capacity to produce small pores on the cells' surface[83] and the fact that it is considered expressed by proteomic studies, it may well have a physiological function in platelets. Recent studies on the gasdermin D platelet axis have suggested it is a key player in platelet pyroptosis,[84] which ties with the canonical role of gasdermin D. Due to limitations on the detection of caspase-1 cleavage by immunoblot, activation of the NLRP3 inflammasome has been demonstrated primarily using FLICA, which has been extensively criticized in regard to its sensitivity and has been shown to bind to apoptotic cells with diminished sensitivity for the active site of caspase.[85,86] Further methods have since been published for using the apoptotic specificity of FLICA reagents and repurposing them into an assay for cell death.[86] Where proteins such as HMGB1 are thought to be released by necrotic cells,[87] there is a risk that an observation of increased platelet HMGB1 release paired with increased FLICA signal,[62] may be a detection of necrotic or apoptotic platelets. This open question over the sensitivity of the FLICA assay to detect *bona fide* caspase-1 activity reinforces the need for either alternative techniques or the inclusion of orthogonal validation by traditional protein biochemistry methods and carefully designed control experiments to confirm caspase-1 cleavage products in future studies,[66] alongside presence of other NLRP3 inflammasome components.

The paradox of priming

The two-step, signal 1 followed by signal 2, mechanism of priming then activation is understood to be vital for NLRP3 inflammasome-mediated synthesis of IL-1β. However, this has not been established in platelet NLRP3 inflammasome studies. Currently, the majority of studies suggest that platelets are seemingly able to demonstrate activation with only a single signal. However, this diverges on whether this single signal is inflammatory,[62] pathogenic,[24] or thrombotic[50] in source. This is inconsistent with the current literature in nucleated cells and could suggest that within platelets, there is a unique mechanism of action.[14] It is possible to assume that the megakaryocyte may receive the transcriptional priming signal, producing platelets with additional inflammatory transcripts, and the platelet may receive the non-transcriptional priming and secondary activatory signal. This would indicate a complex cross-cellular process regulating and controlling a platelet (sub)population which is pre-programmed by megakaryocytes that have adapted to the systemic environment and produced tailored platelets containing the requisite components for NLRP3 inflammasome activation. A component of this hypothesis is a study which described that IL-1β is upregulated in cardiovascular disease, obesity, and infection, and these conditions led to megakaryocyte activation and platelet hyperactivity.[51] This suggests that there may be a cyclical relationship where an increasingly inflammatory environment drives megakaryocytes to produce platelets pre-programmed toward inflammation. In additional support of platelet pre-programming by megakaryocytes, the concept of disease altering the platelet proteome has already been demonstrated in severe obesity[88] and Dengue infection.[89]

This would further suggest that the lack of inflammasome components in the Burkhart,[74] Rowley,[75] and Zeiler[76] studies may be due to the use of naïve and healthy donors for analysis. It is tempting to suggest that the pulmonary megakaryocyte subset,[90] which must be continually exposed to airborne DAMPs and PAMPs, may be a unique reservoir that produces "inflammatory" platelets. Early work on this megakaryocyte subset suggested that blood concentration of platelets was changing in response to respiratory insults,[91] and it could be hypothesized that this increase in cell count was paired with a change in platelet proteome; this avenue of platelet subpopulation research should be pursued, and recent progress toward high-resolution subpopulation discovery has already been made by cytometry approaches.[92–94] This theory may be supported by murine studies utilizing platelet-specific NLRP3 A350V mutants, where platelets expressed increased levels of caspase-1 in the mutant, indicating that megakaryocytic changes in the bone marrow led to hyperinflammatory platelets.[95] Post-myocardial infarction also led to increased megakaryocyte IL-1β expression and thrombopoiesis, which led to poorer survival outcomes,[96] linking megakaryocyte NLRP3 activity with platelet hyperactivity and thrombosis.

Alongside the theory of platelet pre-programming, there is some evidence that platelets have a synthetic capacity,[14] and they are packaged with mRNA for pro-IL-1β,[17] and pro-IL-18,[19] among other mRNAs,[14] which have been shown to be spliced upon activation. This suggests that there is flexibility within platelets to splice-selected mRNAs and upregulate proteins;[97] therefore, platelets may also undergo post-megakaryocyte proteomic changes in response to circulating DAMPs or PAMPs priming them for inflammatory activation.

What remains clear is that because there are 150–400 000 platelets per microliter of human blood, any inflammatory event that this significant number of platelets can initiate must be tightly controlled and aberrant activation avoided. It is likely that the relationship between parent megakaryocytes and platelets in an inflammatory environment is vital to the pro-inflammatory platelet phenotype in which the NLRP3 inflammasome evidences. This strongly suggests that understanding the changes which megakaryocytes undergo in inflammation will be just as vital as those which the platelets, acting as the effector cells, demonstrate.

The future of the platelet inflammasome

The major question posed to the proponents of the immune-competent platelets is: are platelets, subsets or all, able to produce IL-1β at levels that can drive systemic inflammation. If not true, an alternative but also important answer is that platelet IL-1β plays a local and autocrine role for platelet activation. Both are backed by many of the recent publications summarized above, however which of these may be the primary purpose of the platelet NLRP3 inflammasome remains unclear and substantial future work is required to confirm which phenotype may be predominant, or indeed most relevant clinically.

Typically, the current emphasis on platelet NLRP3 inflammasome studies has concentrated on IL-1β as the effector molecule; however, there is also evidence now that IL-18 or pyroptosis can drive phenotypes associated with CAPS,[98] a fact that must not be ignored in future platelet NLRP3 inflammasome studies. While the clinical role of the platelet NLRP3 inflammasome has been shown to be broad, there remain many conditions which have not been explored. Any condition where systemic inflammation is present, whether acute or chronic, is a viable model for the study of platelet NLRP3 inflammasome activity. In terms of translation, a role for systemic NLRP3 inflammasome-driven inflammation linking with cardiovascular disease has been shown in large population studies of NLRP3 variants,[48] however, in an anti-IL-1β therapy study only a minor effect on recurrent cardiovascular events was shown,[73] perhaps hinting at the supportive but not the defining role that IL-1β may play in atherothrombosis. Intriguingly, the same study did suggest that the blockade of IL-1β reduced the incidence of lung cancer.[99] If a role for platelets in sterile systemic inflammation can be identified, this could pave the way for novel therapeutics targeting circulating platelets and early systemic inflammation prior to pathogenesis, atherogenesis, or indeed metastasis.

What remains to be confirmed and validated is the inflammatory potential of platelets via the NLRP3 inflammasome, and what mechanisms or further ligands control the expression, priming, and activation of the complex in both platelets and megakaryocytes. The field currently describes many NLRP3 inflammasome-related findings in platelets, this is of particular import to be addressed as the field describes roles for the NLRP3 inflammasome that are either pro-aggregatory or pro-inflammatory, and the true answer may be

that both are equally relevant under different conditions, however this must be fully elucidated. Ultimately, these outstanding questions on the platelet NLRP3 inflammasome must be carefully assessed and validated by the platelet research community to fully appreciate the impact that platelet NLRP3 inflammasome activity may have on health and disease.

Author contributions

MSH: conceptualization, writing original draft, review, editing draft and addressing reviewer comments. MB: review and editing draft. KMN: review and editing draft.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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