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Platelet toll-like receptors in thromboinflammation

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1. ABSTRACT

Besides their undiscussed role in hemostasis and thrombosis, platelets are also key effector cells capable of assisting and modulating inflammatory reactions and immune responses. Platelets play a sentinel role in immune surveillance by recognizing danger signals from pathogens and cell damage through the expression of toll-like receptors (TLRs) on its surface and internal compartments. Platelets express all 10 TLRs transcripts and its signalosome, including adaptor proteins and transcription factors. The activation of these receptors in platelets triggers hemostatic and inflammatory responses which participate in the host's response to bacterial and viral infections, linking thrombosis with infection and immunity. Among the responses elicited by the activation of platelet TLRs are platelet adhesion and aggregation, formation of mixed platelet-leukocyte aggregates, expression and secretion of cytokines and quemokines, and thrombin generation. TLRs also are expressed in megakaryocytes, and their activation regulates not only platelet biogenesis, but also pro-inflammatory and antiviral responses. This review will focus on work that has shown the role of platelet TLRs, mainly in thromboinflammatory responses elicited by platelets and megakaryocytes.

2. INTRODUCTION

Historically, platelets have been recognized as cells involved in hemostasis and thrombosis, and their role in both processes was broadly studied. A concept that has taken longer to establish, but is rapidly evolving, is that platelets are also key effector cells in systemic inflammatory processes both as instigators of local and systemic inflammatory reactions and also participants in the inflammation that contributes to tissue injury. Thus, platelets are now recognized as multitasking cells capable of influencing physiologic and pathophysiologic processes like inflammation, immunity and cancer (1–3).

Inflammatory and immune functions played by platelets could be explained, as in lower vertebrates such as fish and birds, platelets and leukocytes share a common ancestral cell, the thrombocyte, which performs hemostatic and immune functions (4).

One of the most relevant characteristics of platelets as immune cells is the expression of toll-like receptors (TLRs). TLRs are critical receptors that transmit danger signals to the innate immune system and mediate inflammatory events that can eventually recruit and activate cells of the adaptive immune

Table 1. Platelet TLRs and potential associated diseases

TLRs	Associated diseases	References
TLR4	Sepsis tissue damage, disseminated intravascular coagulation, thrombocytopenia	9, 17, 34, 38
	Hemorrhagic shock and resuscitation	35
	Trauma and hemorrhagic shock	36
TLR2	Atherosclerosis	15, 60
	Sepsis	57
	Thrombosis	61
	Periodontitis	55, 56
	Platelet turnover	63
TLR3	Thrombocytopenia associated with viral infection	12, 69
TLR7	Thrombocytopenia associated with viral infection	13
TLR9	Acute coronary syndrome	74
	Atherosclerosis	15, 76

system to respond against invading pathogens. TLRs recognize evolutionary conserved structures that are broadly shared among pathogens, termed pathogen-associated molecular patterns (PAMPs) or endogenous molecules associated with cellular damage (DAMPs), such as microbial lipids, carbohydrates, nucleic acids, and proteins. TLRs are widely expressed on both immune cells and non-immune cells, including dendritic cells, macrophages, lymphocytes, and endothelial cells. In humans, 10 TLRs have been identified (5, 6).

Some TLRs are transmembrane receptors expressed on cell surfaces (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) and recognize membrane components of bacterial origin (TLR1, TLR2, TLR4, TLR5, TLR6) as well as structures on fungus (TLR2, TLR4 and TLR6), parasites (TLR2 and TLR4), and in some cases, molecular patterns of viruses (TLR2 and TLR4). The other group of TLRs is located intracellularly in the endosome compartments (TLR3, TLR7, TLR8, and TLR9) and senses pathogens-derived nucleic acids. Endosomal TLRs mostly recognize single-stranded RNA (ssRNA) (TLR7 and TLR8), double-stranded RNA (dsRNA) (TLR3) and double-stranded DNA (TLR9) (5, 6).

In nucleated cells, each TLR triggers its own response depending on the ligand sensed, and throughout the recruitment of different adaptor proteins. All TLRs, except TLR3, recruit the myeloid differentiation primary response gene 88 (MyD88) adaptor protein after ligand recognition and activation. The initiated down-stream signaling cascade involves family members of serine-threonine kinases, interleukin-1 receptor-associated kinase (IRAK) and the tumor necrosis factor (TNF) R-associated factor 6 (TRAF6). This pathway activates the nuclear factor-kappaB (NF-kappaB) and mitogen-activated protein kinases (MAPK), and promotes the expression of various pro-inflammatory cytokines (e.g. interleukin-6 (IL-6), IL-8 and TNF-alpha (5, 6).

TLR3 utilizes the adaptor protein TRIF, instead of MyD88, and after binding to TRAF3 and recruitment of IKK-related kinases, activates interferon regulatory factor-3 (IRF3) stimulating the release of type I interferons (IFNs). Although TLR3 functions independently of MyD88, its activation can also activate NF-kappaB and MAPK through TRIF interaction with TRAF6 (7). The unique TLR capable of utilizing both MyD88 and TRIF-dependent pathways is TLR4 (5).

The expression of TLRs on platelets was described almost simultaneously by three groups. TLR1 and TLR6 were the first TLRs identified on human platelets and in a megakaryocytic cell line (8). Then, the expression of TLR2, TLR4 and TLR9 was reported located not only in the surface but also in the cytoplasm of platelets (9, 10). Different studies dissected the functionality of these receptors on platelets, and also new TLRs such as TLR3, TLR7 and TLR9 were characterized (11–14). Interestingly, all 10 TLRs transcripts are expressed on platelets and its expression is more abundant in women than men. Furthermore, the expression of some platelet TLRs transcripts has a positive correlation with coronary heart disease risk factors, including obesity, gender, and inflammation (15).

With regard to megakaryocytes, few studies have looked into the expression and function of TLRs in megakaryocytes and whether or not these receptors could have a role in platelet production. In recent years, the presence and function of TLR2, TLR3, TLR4 and TLR9 were characterized (1, 11, 12, 14, 16).

This review will focus on work that has shown the role of TLRs in both platelet and megakaryocyte physiology.

3. TLR4

For more than 30 years, it has been well-described that patients with sepsis often have low platelet counts, and that the intravenous injection of lipopolysaccharide (LPS) into mice induces thrombocytopenia and platelets localize to lung and liver microvasculature (17). However, the mechanism for thrombocytopenia development was not fully understood. The concept that platelets might recognize antigens directly via TLRs was first reported by Shiraki *et al.*, who found that human platelets and a megakaryocytic cell line, Meg-01, expressed TLR1 and TLR6 mRNA and the transcribed proteins on the membrane surface (8). Additionally, besides TLR1 and TLR6, the authors were not able to detect any other member of the TLR family in platelets (8). In contrast, one year later, the expression of TLR4 and the binding of its specific ligand, LPS, was described in humans and mice, both in platelets and megakaryocytes (9, 10).

3.1. TLR4-mediated platelet activation *in vitro*

Some of the most important TLR4 ligands include LPS, viral motifs and DAMPs such as soluble hyaluronan, beta-defensin 2, high-mobility group box 1 (HMGB1) protein and histones (18). LPS is the main structural component of gram-negative bacterial wall and an important player in the ability of host cell detection of these foreign pathogens (19). Since sepsis is a major clinical problem with few therapeutic options, much focus has been devoted to the implication and mechanism of LPS-mediated thrombocytopenia *via* platelet TLR4.

LPS recognition by TLR4 involves a multiprotein interaction. First, a plasma protein, LPS-binding protein binds LPS and transfers LPS monomers to CD14. CD14 is a high-affinity receptor for LPS, present both as a soluble form in blood or as a glycophosphoinositol-anchored protein on the surface of myeloid lineage cells. CD14 deficient cells do not respond to LPS, but might respond to this stimulus in the presence of soluble CD14 (sCD14) in the circulation (19).

Effects of LPS on platelet function are controversial. It has been reported that LPS can directly promote platelet activation responses including aggregation, dense and alpha granule release and platelet adhesion to fibrinogen (9). However, other studies show that although LPS *per se* does not trigger platelet activation, it enhances classical agonist-induced platelet activation (20–22). Moreover, this effect also appears to be dependent on the LPS-stimulation time since LPS does not initiate rapid platelet responses, but over time it primes platelet aggregation to soluble agonists (23). Despite these studies showing a direct or indirect LPS-mediated activation of platelets, it has also been reported that on the contrary, LPS inhibits platelet function, or that activation of platelets with LPS did not induce any platelet response nor potentiate the effects of classical agonists (24–26).

Besides inducing hemostatic platelet responses, activation mediated by LPS also includes triggering of platelet pro-inflammatory responses, as the splicing of unprocessed platelet IL-1beta with translation and accumulation of IL-1beta protein, a potent pro-inflammatory cytokine that initiates and amplifies a wide variety of effects associated with innate immunity and host responses to microbial invasion and tissue injury (23). Furthermore, LPS signaling promotes the production of platelet microparticles that induce endothelial cell activation by virtue of the caspase-1-dependent IL-1beta they express (27).

The explanation for these broad arrays of *in vitro* responses is still not entirely clear but seems to rely on several considerations. Among them, platelet preparation is a critical point. Although platelets express TLR4 in sufficient quantities to physically bind LPS, they do not express CD14 (28). Thus, the presence of low amounts of plasma or serum or sCD14 is required in order to observe LPS-mediated platelet responses (21, 23, 28). The requirement of plasmatic CD14 might also explain the more reproducible platelet-mediated effects of LPS observed *in vivo*. The strain and concentration of LPS employed are also important variables to be considered when analyzing platelet activation by LPS. Human platelets can discern various isoforms of bacterial LPS *via* TLR4 engagement, resulting in distinct cytokine secretion profiles and different strains trigger platelet responses, with different potency (22, 29). In addition, from the molecular point of view, another plausible explanation for the LPS-mediated platelet stimulatory or inhibitory effect could be related to a biphasic response of cGMP signaling. In previous studies it was shown that in contrast to the widely accepted platelet inhibitory role of nitric oxide (NO)/soluble guanylate cyclase (sGC)/cGMP/cGMP-dependent protein kinase (PKG) pathway, low concentrations of cGMP generated during platelet activation induced by von Willebrand factor (VWF) or subthreshold thrombin concentrations are stimulatory (30, 31). In agreement with this data, Zhang *et al.* reported that LPS added together or after low thrombin or collagen concentrations, triggers platelet activation associated with a rapid and moderate increase of cGMP levels and activation of PKG (22). Moreover, the authors argued that the previous reported LPS-mediated platelet-inhibitory effects (24–26) were due to the prolonged exposure and high LPS concentrations used to activate platelets which could have induced high inhibitory levels of cGMP (22). Although the concept of a dual action of cGMP in platelets is a very challenging issue and could explain

the different effects of LPS on platelets, this concept still remains to be definitively proof considering that the general accepted idea is that elevation of cGMP always results in platelet inhibition (32). Collectively, these data suggest that LPS-mediated platelet activation responses require CD14 as cofactor and are selectively triggered by LPS strains, concentration and stimulation time.

3.2. TLR4-mediated platelet activation *in vivo*

The functional significance of TLR4 in platelets was first demonstrated by Andonegui *et al.*, who showed that LPS-induced thrombocytopenia was platelet-TLR4-dependent (9). Using adoptive transfer of wild-type or TLR4-deficient platelets into wild-type or LPS-treated mice, platelet TLR4 was identified as an essential receptor for the LPS-induced thrombocytopenia and platelet accumulation into the lungs. Platelet TLR4 has a role in not only the modulation of LPS-induced thrombocytopenia, but also in the TNF-alpha production. The increased TNF-alpha levels that occurs after LPS administration are significantly lower in mice depleted of platelets and restored by the infusion of platelets (33). Nevertheless, how platelets contribute to augment TNF-alpha levels is still not clear.

Disseminated intravascular coagulation (DIC) is a frequent consequence of sepsis and is associated with organ failure. Although the pathogenic mechanisms of DIC are still not completely elucidated, a hyper-coagulation state and circulating activated platelets are considered to contribute to vascular occlusion. Interestingly, platelet TLR4 have been shown to be sufficient to accelerate microvascular thrombosis in the cremaster venules during endotoxemia (34), suggesting that the presence of TLR4 on platelets could be a link between DIC and sepsis.

The role of platelet TLR4 in thrombomodulation is not restricted to sepsis. Activation of platelet TLR4 is necessary for platelet activation and functional changes as well as for coagulation abnormalities, systemic inflammation and organ injury induced by hemorrhagic shock and resuscitation (35). In addition, the HMGB1, a nuclear protein released by dying cells or activated platelets, and a TLR4 recognized DAMP, has been recently identified as a potent mediator of platelet activation and thrombus formation both *in vitro* and *in vivo* (36, 37). Intriguingly, the mechanistic *in vitro* study of these effects in human platelets revealed that they are mediated via TLR4-NF-kappaB and cGMP decreased levels (37). However in the *in vivo* studies, HMGB1 mediated platelet responses were dependent on TLR4/MyD88 dependent recruitment of GC toward the platelet plasma membrane, followed by MyD88/GC complex formation, GC activation, and cGMP-dependent activation of PKG (36).

Platelet TLR4 not only has a critical role in thrombomodulation, but also is a critical mediator for pathogen trapping and killing during sepsis (38). Platelets, *via* TLR4, function as a barometer for systemic infection. Once platelets recognize LPS, they bind avidly to neutrophils that are sequestered in the liver sinusoids, and trigger the release from neutrophils of DNA decorated with bactericidal proteins (neutrophil extracellular traps, NETs) that ensnare bacteria from the circulation. Major components of NETs are histones, which are DAMPs. These basic proteins are potent inducers of platelet hemostatic and inflammatory effector responses (39, 40). Furthermore, histone-activated platelets possess a procoagulant phenotype that drives plasma thrombin generation (41). Histone H3 and H4-mediated platelet responses are decreased by the blockade of TLR4 and TLR2 (40). Collectively, these findings support the notion that platelet TLR4 is not a vestigial receptor, but rather, a relevant sensor of TLR4 ligands that allows platelets to quickly interact with invading microorganisms and/or damage signals, that are increased during septic or sterile inflammatory conditions, and trigger hemostatic and/or innate immune responses. Interestingly, in humans, Asp299Gly polymorphism in TLR4 is associated with a lower risk of carotid atherosclerosis, less intimal media thickness, and with a decreased risk of acute coronary events independent of standard coronary risk factors (42, 43).

In contrast to these studies, a recent challenging work demonstrated that mice deficient in platelet MyD88, the TLR common adaptor protein, does not show hallmarks sepsis responses such as thrombocytopenia, coagulation, endothelial activation or distant organ injury in response to *Klebsiella pneumoniae* (a gram-negative bacteria) suggesting that platelet MyD88-dependent TLR signaling does not contribute to the host response during gram-negative sepsis, except for a moderate decrease in TNF-alpha and keratinocyte chemoattractant production (44). More studies are required to determine whether the reported effects are specific for *Klebsiella pneumoniae* or shared by other gram-negative bacteria as well as by LPS in a sepsis model with platelets deficient in MyD88. Furthermore, since TLR4 is unique for its ability to signal through both MyD88 and TRIF (5), the role of this adaptor molecule should also be investigated in the context of TLR-mediated platelet´s physiopathology.

3.3. TLR4 involvement in megakaryocytopoiesis

In addition to playing a relevant role in the regulation of platelet function, TLR4 is also involved in the regulation of platelet production. Early work has shown TLR4 surface expression on Meg-01 cells; however, in this study the functional role of this receptor was not addressed (26). Further confirmation of TLR4 on megakaryocyte cell surface and increased

expression with maturation of the cells was shown in murine megakaryocytes isolated from fetal livers (9). Additionally, TLR4 and TLR2 were identified in human megakaryocytes isolated from patients with myelodysplasia (45). Interestingly, mice with deletion of a 74,723-bp DNA fragment in the third exon of the TLR4 gene, exhibit decreased platelet number, turnover, and thrombin-stimulated expression of P-selectin (46). These findings highlighted several relevant issues, including the concept of TLR4 requirement for genomic regulation of platelet production (turnover) from megakaryocytes, and also that TLR4 regulates an intracellular mechanism(s) leading to alpha-granule secretion. The decrease in platelet numbers of mice deficient in TLR4 was also observed by Andonegui *et al.* (9), but not in the study of Aslam *et al.* (33). The first study employed the C57BL/6J mice strain, while in the latter BALB/c mice were used. Therefore, genetic background might be a determinant for the role of TLR4 in platelet biogenesis.

3.4. Signaling Pathways involved in platelet TLR4 activation

The TLRs signalosome in nucleated cells activates transcription factors as NF-kappaB and IRF3. As platelets are enucleated cells, it was not clear how TLRs in platelets might trigger activation. Several groups, including ours, described the expression of cytosolic proteins belonging to TLRs signalosome such as the transcription factors NF-kappaB, IRF3, the adaptor proteins between TLRs MyD88, TRIF, TRAF3, TRAF6, TBK-1, IRAK1 and IKK-I (47–49). The observation that platelet effector responses triggered by LPS, histones or HMGB1 are inhibited pharmacologically, or by genetic ablation of TLR4, MyD88 or NF-kappaB, supports the notion that TLR4 and its downstream molecules mediate platelet activation (9, 21, 22, 36, 37). Due to the lack of nucleus, how do these transcription factors transduce the LPS signal and trigger selected platelet responses? Some studies that addressed this query found that platelet TLR4 ligation by LPS or HMGB1 results in cGMP increases (22, 36). Specifically, TLR4 stimulation results in the recruitment of sGC towards the platelet plasma membrane followed by MyD88/GC complex formation, sGC activation, and cGMP dependent activation of PKG (36). This pathway was not turned on in TLR4- or MyD88-deficient animals, suggesting that TLR4 ligands activate platelets through a non-canonical TLR4/MyD88 and cGMP/PKG dependent pathway (36). The role of cGMP in TLR4-mediated platelets responses was further expanded by Yang *et al.* who found that NF-kappaB was also involved in the TLR4/MyD88 and cGMP/PKG pathway. Remarkably, and in contrast with the previous studies, they had observed cGMP levels decreased upon HMGB1-mediated TLR4 stimulation (37). Undeniable, cGMP/PKG appears to be a potential final downstream signaling pathway involved in platelet TLR4 transduction signaling triggered either by PAMPs or DAMPs. However, due to the current controversy, more data from independent groups are necessary to elucidate the biphasic role of sGC/cGMP/PKG in platelet activation.

So far, the molecular mechanisms described in histone-mediated platelet activation include PI3K/AKT, ERK1/2 and p38 MAPK signaling pathways as well as NF-kappaB activation, however is still not clear whether they are activated *via* TLR4 and TLR2 (40). On the other hand, studies addressing the molecular mechanism involved in IL-1beta production found that the splicing of the IL-1beta gene requires MyD88 and TIRAP, and IRAK1/4, AKT and JNK phosphorylation and activation. Moreover, TRAF6, an intermediate between IRAK1/4 and the AKT and MAPK cascades couples MyD88 to the AKT pathway (27).

In conclusion platelet TLR4 activation appears to affect multiple intracellular targets and protein kinases which are known to “cross-talk” and may have in-built-mechanisms to limit their effects (Figure 1).

4. TLR2-MEDIATED PLATELET ACTIVATION *IN VITRO* AND *IN VIVO*

TLR2 is a cell surface receptor that binds a wide range of microbial components, such as gram-positive-derived lipoteichoic acid, bacterial lipoproteins, and zymosan (50). TLR2 forms functional heterodimers with either TLR1 or TLR6, which is the prerequisite to recognizing a wide spectrum of microbial pathogen-associated molecules. While triacylated lipoproteins are considered ligands for TLR2/1, diacyl lipoproteins are recognized by TLR2/6 (51). DAMPs have also been described as ligands for TLR2 which include human beta-defensin-3, hyaluronan fragments, heat shock proteins, histones and HMGB1 (52).

Cognasse *et al.* first described the presence of TLR2 receptors in human platelets. Later, several studies revealed the platelet responses elicited by activation of TLR2, and delineated the molecular pathways involved in these events. Pam3CSK4, a synthetic triacylated lipopeptide that mimics the amino terminus of bacterial lipopeptides and a TLR2/1 agonist, was employed in most studies. The stimulation of human or murine platelets with Pam3CSK4 triggers adhesion, aggregation, release and expression of pro-inflammatory substances such as CXCL4, CD40L, VWF and P-selectin and formation of mixed aggregates between platelets and polymorphonuclear leukocytes (21, 53). These responses are specifically mediated by TLR2 as they are abolished in the presence of TLR2-blocking antibodies and absent in platelets from $TLR2^{-/-}$ mice (54). Under non-aggregating conditions, Pam3CSK4 also induces

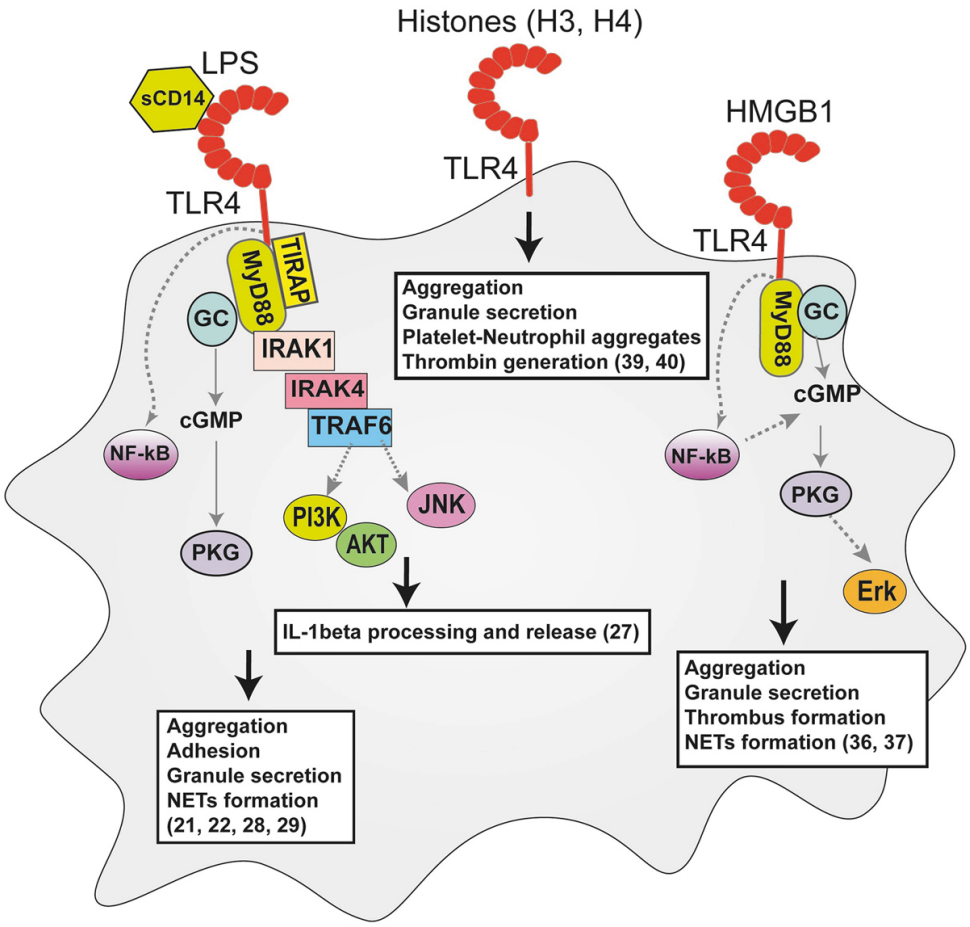

Figure 1. Platelet TLR4 signaling pathways and effector responses. The Figure shows the signaling pathways in platelets stimulated by the different TLR4 ligands so far identified. After engagement to LPS, TLR4 interacts with myeloid differentiation primary response protein (MyD88) and TIR domain containing adaptor protein (TIRAP); stimulating downstream signal pathways that involve interactions between IL-1R-associated kinases 1 and 4 (IRAK1/IRAK4) and the adapter molecule TNF receptor-associated factor 6 (TRAF6) and activation of JUN N-terminal kinases (JNK) and PI3K/AKT pathways. Besides, LPS activates nuclear factor-κappaB (NF-kB). MyD88 also forms a complex, with the enzyme guanylate cyclase (GC) leading to cGMP-protein kinase (PKG) activation. Histones H3 and H4 binding to TLR4 activate several platelet activation responses. It is not yet known the signal molecules involved. HMGB1 binding promotes a MyD88/GC complex formation and PKG activation. cGMP modulation is controversial in the HMGB1 signaling. Main platelet activation responses by TLR4 stimulation with each ligand are indicated in white boxes.

neutrophil-platelet heterotypic aggregates in a P-selectin-dependent manner (54). The same effect is observed when wild-type mice are challenged with live *Porphyromonas gingivalis,* a gram-negative bacterium that uses TLR2, but not in TLR2$^{-/-}$ mice (54, 55). The interaction of platelets with neutrophils in these mixed aggregates increases bacteria binding and the phagocytic activity of leukocytes (55). Moreover, the activation of platelets with periodontopathogens directly induces platelet surface expression of CD40L associated with activation of the TLR2 and TLR4. Importantly, CD40L is a critical mediator of thrombotic and inflammatory processes that are associated with a high risk for cardiovascular disease and patients with periodontitis and *Porphyromonas gingivalis,* which also presents a higher risk of cardiovascular disease, has a statistically significant increase of soluble CD40L (56). Interestingly, platelet–neutrophil interactions also promote NETs formation not only by platelet TLR4 stimulation, but also when platelet TLR2 recognizes Pam3CSK4 or *Staphylococcus aureus* (57).

These reports show that in sharp contrast with LPS, *in vitro* platelet activation of TLR2 by components of the gram-positive bacterial wall induces all the activation effector responses in a direct manner. These differences could be associated with the lack of TLR2 for a cofactor requirement such as CD14 for LPS. Noteworthy, it was reported that *Streptococcus pneumoniae*, recognized by several TLRs including TLR2, triggers platelet activation responses in a TLR2-independent manner (58). Furthermore, platelets from TLR2$^{-/-}$, TLR4$^{-/-}$, TLR9$^{-/-}$, TLR2/4$^{-/-}$, and also MyD88$^{-/-}$ mice respond to *Streptococcus pneumoniae* in a similar manner to wild-type platelets, suggesting that *Streptococcus pneumoniae* activates platelets by a

mechanism that is independent of TLRs signaling in platelets (58). Whether the relevance of platelet TLRs in the host response is dependent on the bacteria strain remains to be elucidated.

Besides bacteria lipoproteins, TLR2 also recognizes viral envelope glycoproteins from human cytomegalovirus (HCMV) and DAMPs (59). HCMV interacts with platelets through TLR2 and induces platelet degranulation, the release of pro-inflammatory and pro-angiogenic molecules. HCMV-activated platelets bind to neutrophils and enhance neutrophil extravasation in a TLR2-dependent manner. These responses triggered by platelet TLR2-HCMV interactions exacerbate tissue damage and might contribute to accelerating atherosclerosis (60).

Among DAMPs, some are generated during pathophysiological conditions associated with oxidative stress, such as dyslipidemia, diabetes, and acute or chronic infections. These highly reactive products, generated by lipid peroxidation, modified autologous proteins and lipids generating biologically active derivatives. Modifications of phosphatidylethanolamine generate carboxyalkylpyrrole-phosphatidylethanolamine derivatives (CAP-PEs) which are elevated in plasma of hyperlipidemic mice. The activation of TLR2 by CAP-PEs promotes platelet hyperactivity *in vitro* and accelerates thrombosis *in vivo* in the settings of hyperlipidemia (61).

Thus, like TLR4, platelet TLR2 activation can be triggered by several ligands, and therefore activation of this platelet receptor may be relevant in the generation of pro-thrombotic and pro-inflammatory platelet-mediated responses under different acute or chronic inflammatory clinical conditions.

4.1. TLR2 involvement in megakaryocytopoiesis

The effect of TLR2 on megakaryocytes was studied in the megakaryocytic cell lines Meg-01 and Dami (62, 63). Stimulation of Meg-01 with Pam3CSK4 trigger the activation of NF-kappaB, AKT and ERK1/2 pathways and, up-regulation of transcription factors involved in megakaryocyte maturation, accompanied by increases in megakaryocyte ploidy (index of maturation), thrombotic markers and adhesion to extracellular matrices (63). In addition, stimulation of Dami cells with heat killed lacto bacillus (HKL), another TLR2 ligand, results in up-regulation of TLR2 together with cytokine secretion, mainly IL-6, which is a positive regulator of megakaryocyte generation and CD41 expression. These effects were mediated by activation of beta-catenin components suggesting a cross talk between Wnt and TLR pathway leading to maturation of megakaryocytes (62). Studies in mice showed that although platelets initially drop after Pam3CSK4 infusion, platelet numbers return to normal levels after five days, accompanied by an increase in megakaryocyte maturation (63). Although more studies are warranted in human primary megakaryocytes, these findings suggest that TLR2 platelet activation regulates platelet turnover through a direct effect on megakaryocytes.

4.2. Signaling Pathways involved in platelet TLR2 activation

Downstream signaling of TLR2/1 involves the activation of the Src family kinases, Syk, and the linker for activation of T cells (LAT) phosphorylation, leading to the activation of PLCgamma2, PI3K/AKT, thromboxane A_2 (TXA_2) formation and Ca^{2+} mobilization (54, 64). Activation of platelets by stimulation of TLR2/1 also includes P2X1-mediated Ca^{2+} mobilization, and ADP receptor activation but not the phosphorylation of IRAK1, the MyD88 downstream signal that is operative in nucleated cells upon TLR2 activation. Interestingly, the diacylated macrophage activating lipoprotein-2 (MALP-2), which selectively induces TLR2/6 complex signaling, does not induce any activation of human platelets but rather exerts inhibitory effects on platelets subsequently challenged with Pam3CSK4 (65).

Comparison between classical platelets' agonists, such as collagen or thrombin, revealed that while collagen-mediated platelet activation responses were almost similar to those elicited by Pam3CSK4, thrombin-mediated platelet activation showed specific differences between the two stimuli. Although both agonists trigger the activation of PLC and PI3K/AKT pathways, the kinetic of protein phosphorylation by thrombin is faster. The binding of focal adhesion kinase (FAK) to factor XIIIa is decreased in response to TLR2 activation vs. thrombin stimulation, and while platelet activation by thrombin results in a small and stable clot, TLR2 activation does not induce any significant clot formation (66). Thus, different effector platelet responses might be triggered in the context of inflammation and thrombosis, according to the triggering stimuli. This concept is very important, especially when platelet inhibitors are considered as therapeutic agents.

Platelet Ca^{2+} responses coupled to either GPVI or TLR2/1 activation persists in the presence of the major endothelium-derived inhibitors of platelet function, prostacyclin and NO. This effect is due to the secondary activation of P2X1 receptors and influx of Ca^{2+} mediated by activation of TLR2/1 or GPVI receptor. Platelet responses induced by Pam3CSK4 are more resistant to inhibition than those induced by collagen (67). Whether the ability of TLR2/1 to evoke platelet aggregation and secretion in the presence of prostacyclin and NO offers the potential for enhanced platelet responses during infection and inflammation is not clear.

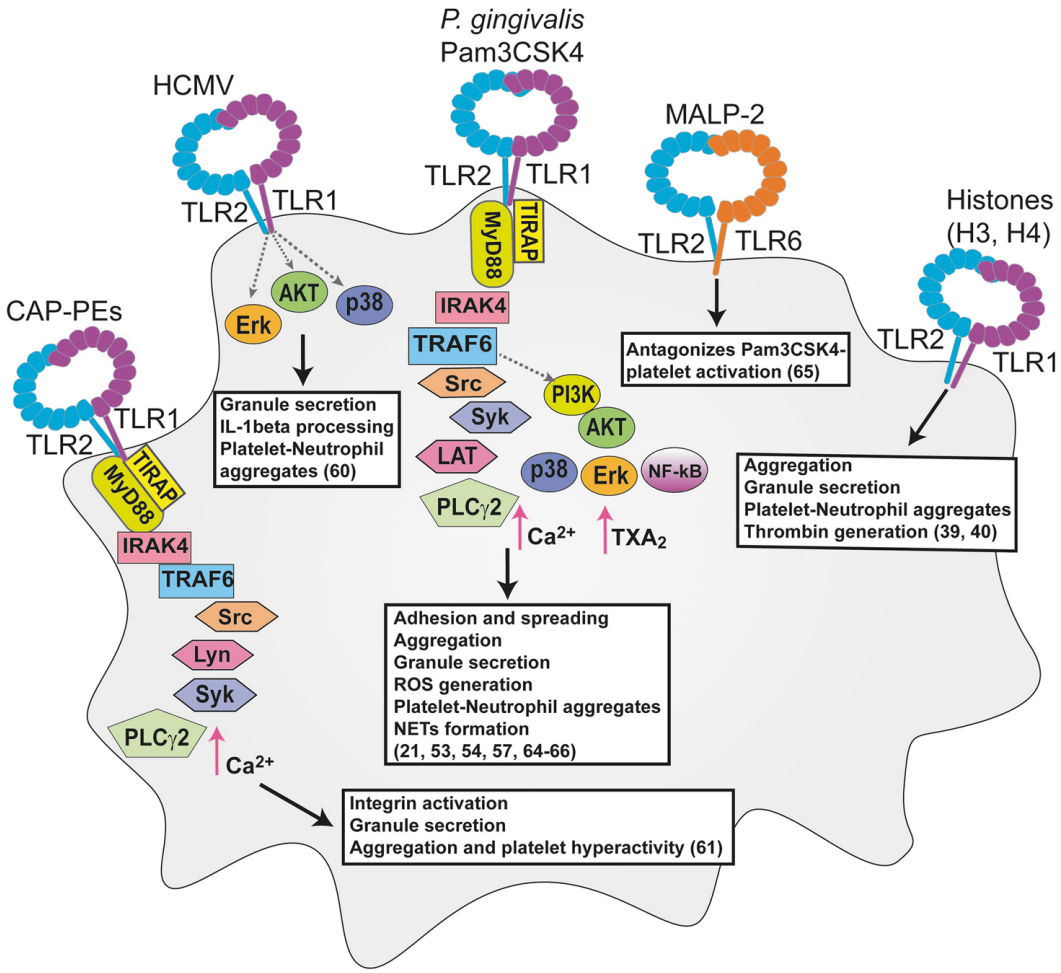

Figure 2. Platelet TLR2 signaling pathways and effector responses. The Figure shows the signaling pathways in platelets stimulated by different TLR2 ligands so far identified. TLR2 builds a complex, with TLR1 or TLR6 in response to different agonists. TLR2 forms a heterodimer, with TLR1 in response to triacylated lipopeptides like Pam3CSK4 or lipoproteins from gram-positive bacteria. In platelets, this complex recruits myeloid differentiation primary response protein (MyD88) and TIR domain containing adaptor protein (TIRAP) leading to activation of interleukin-1 receptor-associated kinase 4 (IRAK4) followed by TNF receptor-associated factor 6 (TRAF6). This pathway conduces to the activation of the Src family kinases, Syk and the linker for activation of T-cells (LAT), Phospholipase C gamma 2 (PLCgama2) triggering Ca^{2+}-mobilization, PI3K/AKT, ERK, p38, NF-kappaB activation, and thromboxane A_2 (TXA_2) formation. Src signaling pathway is also triggered by carboxyalkylpyrrole-phosphatidylethanolamine derivatives (CAP-PEs) generated under oxidative conditions. Human cytomegalovirus (HCMV) recognition by TLR2 activates PI3K/AKT, ERK and P38 MAPK pathways. Histones H3 and H4 binding to TLR2 activate the indicated platelet responses. The molecular pathways involved are still not identified. In contrast to TLR2/1 responses, when TLR2 forms a complex with TLR6 in response to a synthetic derivative of the macrophage-activating lipopeptide (MALP-2), platelet responses induced by Pam3CSK4 are inhibited. Main platelet activation responses identified by TLR2 stimulation with each ligand are indicated in white boxes

TLR2/1 activation by CAP-PEs, involves MyD88 and TIRAP activation, phosphorylation of IRAK4 and subsequent activation of TRAF6. This, in turn, activates the Src kinases, Syk and PLCgamma2 pathway, leading to integrin alphaIIbbeta3 activation (61). In addition, it was recently showed that activation of TLR2/1 by Pam3CSK4 triggers IkappaBalpha degradation and p65 phosphorylation as well as ERK1/2, p38 and AKT phosphorylation (21, 53).

Collectively these findings suggest that TLR2/1 and TLR2/6 on platelets are functional receptors that link oxidative stress, innate immunity and thrombosis (Figure 2).

5. VIRUS-RECOGNIZING TLRS IN PLATELETS (TLR3, TLR7 AND TLR9)

The presence of viruses in platelets and their capacity of entry within those cells has been acknowledged for more than half a century. Several types of viral infections led to sometimes severe and even life-threatening bleeding. In some of these infections, the platelet count drops dramatically; however, the precise mechanisms involved either in platelet destruction or impairment are still largely unclear (60, 68).

The response of TLRs in platelets to viral PAMPs has been poorly studied. Four members of

TLRs family have shown pivotal roles in recognizing viral nucleic acids. TLR3 recognizes dsRNA resulting from the genetic material or produced during the life cycle of some viruses. TLR7 and TLR8 recognize ssRNA, and TLR9 recognizes viral DNA containing unmethylated CpG sequence. Among the four members, all except for TLR8 are involved in platelet activation (5).

5.1. TLR3

The expression and functional role of platelet TLR3 have been recently examined. Junin virus impairs *in vitro* thrombopoiesis by decreasing proplatelet formation and platelet release, as well as the functionality of *in vitro* generated platelets (69). This decrease in platelet release could be mimicked by Poly(I:C), a synthetic analog of dsRNA replication products and a ligand of TLR3, and by type I IFNs, IFN-alpha and beta (69). These data strongly suggested that megakaryocytes might express TLR3. In fact, the presence of TLR3 transcripts and protein was identified through the megakaryocyte lineage (12, 70). TLR3 is slightly expressed in the surface of $CD34^+$ cells, but not in megakaryocytes or on the platelet surface. However, intracellular TLR3 is detected in $CD34^+$ cells and the receptor expression decreases through the megakaryocyte lineage being the levels of TLR3 in platelets very low (12). Although the reason for this phenomenon is not clear, a similar observation was reported for TLR1 and TLR6 in Meg-01 cells and platelets (8).

Similar to other nucleated cells, the stimulation of TLR3 in megakaryocytes by Poly (I:C), triggers the activation of NF-kappaB, ERK1/2, p38 MAPK, and PI3K/AKT signaling pathways (12). Poly(I:C) is recognized by both endosomal TLR3 and cytosolic receptors, including the helicases RIG-1 and MDA5. However, similar results are obtained when megakaryocytes were stimulated with Poly(A:U), which only signals through TLR3, indicating the specificity of TLR3 in these responses. Poly(I:C) and Poly(A:U) decrease platelet biogenesis in culture through the release of IFN-beta, indicating that activation of TLR3 in megakaryocytes might be another mechanism involved in thrombocytopenia-mediated by virus (12). Interestingly, mice treated with Poly(I:C) have thrombocytopenia, an increase in mean platelet volume associated with an increase in size and an abnormal distribution of bone marrow megakaryocytes within the vascular niche. These effects were directly correlated with the plasmatic and bone marrow IFN-beta levels (71). However, the contribution of megakaryocyte TLR3 to these effects remains to be investigated.

Anabel *et al.* were the first group to show that human platelets express TLR3 mRNA and protein under basal conditions, and that thrombin activation of platelets increased TLR3 surface expression, suggesting an interaction between thrombotic stimuli and inflammatory function of platelets. Similar to TLR4 activation, a direct stimulation of TLR3 by Poly(I:C) results in the secretion of mature IL-1beta and CXCL4 cytokine release through increases in Ca^{2+} levels. In addition, Poly(I:C) increases P-selectin and TLR4 expression (11). It has also been shown that although Poly(I:C) and Poly(A:U) do not directly trigger platelet aggregation, they can potentiate the binding of fibrinogen, aggregation and the release of ATP mediated by classical agonists such as thrombin, ADP, collagen and arachidonic acid. Similar to megakaryocytes, the molecular pathways involved in this synergistic response involve the activation of AKT, ERK1/2 and NF-kappaB (12), (Figure 3).

These findings suggest that both megakaryocytes and platelets could act as linkers between immune and hemostatic responses triggered by viral infections. Megakaryocytes may play a role in antiviral defenses as IFN-beta producers in response to TLR3 activation, but this mechanism could be counterproductive since it results in impaired thrombopoiesis. Therefore, the different signal transduction pathways and effector molecules triggered by the activation of different TLRs appear to determine the positive or negative fate of megakaryocyte development and platelet production, as well as the thrombocytosis or thrombocytopenia frequently observed in bacterial or viral infections, respectively.

5.2. TLR7

Human and mouse platelets express endosomal functional TLR7. The analysis of platelet RNA derived from a large cohort of the Framingham Heart Study, including 839 men and 1050 women, showed that 60 % of individuals had the TLR7-mRNA transcript. Furthermore, the presence of TLR7 protein, although in a variable manner, was also observed in healthy donors (15). In contrast to this variable pattern in humans, mice persistently show TLR7 receptors in their platelets. Injection of encephalomyocarditis virus (EMCV), a ssRNA picornavirus, in wild-type mice but not in $TLR7^{-/-}$ mice, results in a rapid significant drop in platelets count associated with the formation of large platelet-neutrophil aggregates (13). A series of mixing experiments using platelets and granulocytes from wild-type and $TLR7^{-/-}$ mice demonstrated that only platelet TLR7 was able to initiate the formation of heterotypic aggregates with granulocytes, suggesting that when TLR7 is activated, platelets are the blood cells that initiate communication with the neutrophil population. Similar results were observed in human platelets. The mechanism of mixed aggregate formation includes TLR7-mediated platelet granule release, translocation of P-selectin to the cell surface, and a consequent

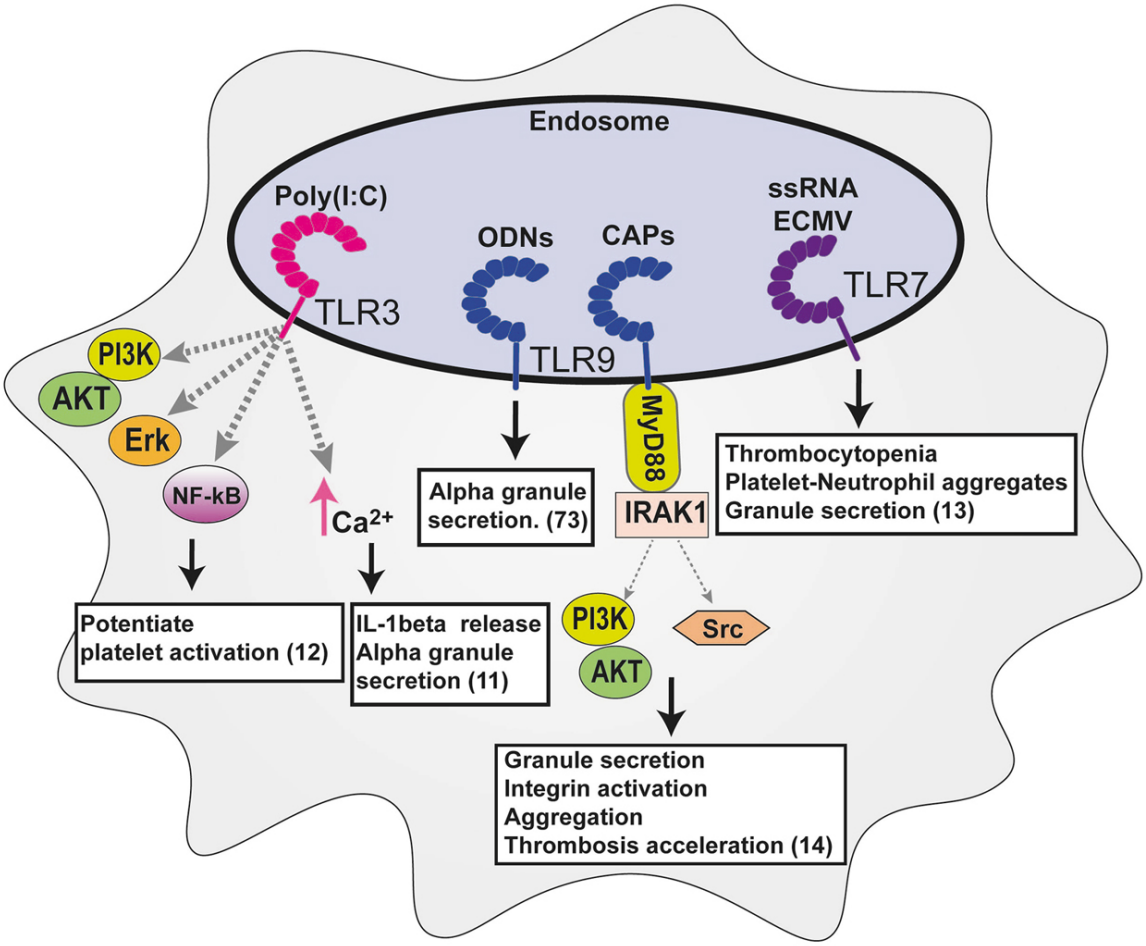

Figure 3. Platelet-endosome TLRs signaling pathways and effector responses. The Figure shows the signaling pathways in platelets stimulated by different endosome TLRs ligands so far identified. Virus recognition by platelet TLRs involves TLR3 and TLR7 in the endosome compartment. Double stranded (dsRNA) from replicating virus binds to TLR3 and activates PI3K/AKT, ERK and NF-kappaB pathways and induces Ca^{2+} mobilization. Encephalomyocarditis virus (EMCV), a single stranded (ssRNA) virus, activates platelet TRL7 by undisclosed signaling pathways. Intracellular pathways triggered by unmethylated CpG dinucleotides or synthetic oligodeoxynucleotides (ODN) recognition by platelet TLR9 remain to be elucidated. However, carboxyalkylpyrrole protein adducts (CAPs) engagement to platelet TLR9 involve myeloid differentiation primary response protein (MyD88) recruitment and activation of interleukin-1 receptor-associated kinase 1 (IRAK1) leading to PI3K/AKT and Src kinase activation. Main platelet activation responses identified by TLR-3, TLR7 and TLR9 stimulation with each ligand are indicated in white boxes.

increase in platelet-neutrophil adhesion (Figure 3). Interestingly, platelet-TLR7 stimulation (from human or mouse origin) does not induce platelet aggregation, nor potentiate the activation responses triggered by thrombin or collagen, suggesting that direct pro-thrombotic events do not appear to be platelet TLR7-dependent (13). This effect is in clear distinction to stimulation of the other platelet TLRs, TLR2, TLR4 and TLR9, which are predominantly activated by bacteria and results in platelet hemostatic responses. Notably, viral infection of platelet-depleted mice also led to increased mortality, indicating that platelets are necessary for survival during viral infection. In agreement with these observations, and although the role of TLR7 was not addressed, a recent study showed that mice infected with coxsackievirus B3, another ssRNA picornavirus, displayed a rapid thrombocytopenia that correlated with an increase in platelet phosphatidylserine exposure and platelet-leukocyte aggregates (72).

5.3. TLR9

TLR9 recognizes unmethylated 2´-deoxyribo (cytidine-phosphate guanosine) DNA motifs, commonly referred to as CpG motifs, found in bacterial and viral DNA. Human and murine platelets express TLR9. Resting platelets express significant amounts of TLR9, and in contrast to TLR4 or TLR2, it is markedly up-regulated by platelet stimulation with thrombin but not LPS, suggesting that TLR9 also exists in intracellular compartments. Interestingly, the increased levels of TLR9 after thrombin stimulation were not observed in mice platelets, indicating species differences in the ability of platelets to regulate TLRs expression (33). TLR9 is expressed through all stages

of platelets production (megakaryocytes, proplatelets and platelets) and TLR9 partly localizes with VAMP8, a SNARE protein that directs TLR9 to the membrane. The activation of platelets with ODN (class B CpG), results in the surface increase of TLR9 and P-selectin and ODN sequestration (73).

Remarkably, a clinical study shown that TLR9 basal expression on platelet surface is greater in acute coronary syndrome patients compared to healthy subjects. However, when platelets of healthy subjects or patients treated with dual anti-platelet therapy (aspirin and $P2Y_{12}$ receptor antagonist) were stimulated with ODN, P-Selectin and CD63 expression were increased in a similar manner indicating that the TLR9 pathway is not susceptible to the current anti-platelet therapy (74). To understand the relevance of TLR9 in coronary syndrome patients, these interesting findings should be confirmed in a larger cohort of patients.

Besides recognizing viruses, TLR9 senses endogenous ligands generated under pathophysiological conditions associated with oxidative stress (14). Proteins modified by lipid peroxidation are known as carboxyalkylpyrrole protein adducts (CAPs), which are detected in atherosclerotic plaques, tumors, and in healing wounds. Furthermore, plasma levels of CAPs are elevated in diabetes and atherosclerosis, diseases frequently associated with a pro-thrombotic state and an increased platelet reactivity (75, 76). CAPs represent a novel and unconventional ligand for platelet TLR9. In fact, CAPs promote platelet activation and aggregation *in vitro* and accelerate thrombosis *in vivo* in a TLR9/MyD88-dependent manner, and require IRAK1 signaling and PI3K/AKT and Src kinase phosphorylation (17) (Figure 3). This work highlights platelet TLR9 and TLR2 as functional platelet receptors that sense danger signals associated with oxidative stress, and link that with innate immunity and thrombosis.

6. CONCLUDING REMARKS

Thrombus formation is inseparably linked with inflammation, and the recent emphasis on the role of platelets as sentinel innate immune cells demonstrates that platelets provide a unique link between coagulation and immune responses.

Human and murine platelets and megakaryocytes variably express functional TLRs. The interaction of bacteria, virus and sterile danger molecules with human platelet TLRs seems to play an important role in the pathogenesis of several diseases, including cardiovascular disease, hemorrhagic shock, infective endocarditis, DIC, immune and viral-mediated thrombocytopenia. Although increasing pre-clinical and clinical studies present evidence of a relevant role of platelet TLRs, linking thrombosis with the innate immune responses during infectious or sterile inflammatory diseases, there are several questions still that require exploration and answering. Furthermore, the recent discoveries of expression and functionality of NOD-like receptors in platelets (77–79) strengthens the relevance of pattern recognition receptors in platelets. A deeper understanding of the role that these molecules play in platelet physiology is necessary to better determine the importance of these receptors in the crossroads of hemostasis and innate immunity.

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