Molecular Cardiology

Nucleotide-Binding Oligomerization Domain 2 Receptor Is Expressed in Platelets and Enhances Platelet Activation and Thrombosis

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Background—Pattern recognition receptor nucleotide-binding oligomerization domain 2 (NOD2) is well investigated in immunity, but its expression and function in platelets has never been explored.

Method and Results—Using reverse transcription polymerase chain reaction and Western blot, we show that both human and mouse platelets express NOD2, and its agonist muramyl dipeptide induced NOD2 activation as evidenced by receptor dimerization. NOD2 activation potentiates platelet aggregation and secretion induced by low concentrations of thrombin or collagen, and clot retraction, as well. These potentiating effects of muramyl dipeptide were not seen in platelets from NOD2-deficient mice. Plasma from septic patients also potentiates platelet aggregation induced by thrombin or collagen NOD2 dependently. Using intravital microscopy, we found that muramyl dipeptide administration accelerated in vivo thrombosis in a FeCl₃-injured mesenteric arteriole thrombosis mouse model. Platelet depletion and transfusion experiments confirmed that NOD2 from platelets contributes to the in vivo thrombosis in mice. NOD2 activation also accelerates platelet-dependent hemorrhosis. We further found that platelets express receptor-interacting protein 2, and provided evidence suggesting that mitogen-activated-protein kinase and nitric oxide/soluble guanylyl cyclase/cGMP/protein kinase G pathways downstream of receptor-interacting protein mediate the role of NOD2 in platelets. Finally, muramyl dipeptide stimulates proinflammatory cytokine interleukin-1β maturation and accumulation in human and mouse platelets NOD2 dependently.

Conclusions—NOD2 is expressed in platelets and functions in platelet activation and arterial thrombosis, possibly during infection. To our knowledge, this is the first study on NOD-like receptors in platelets that link thrombotic events to inflammation. (Circulation. 2015;131:1160–1170. DOI: 10.1161/CIRCULATIONAHA.114.013743.)

Key Words: blood platelets ▪ mitogen-activated protein kinases ▪ nucleotide-binding oligomerization domain 2 ▪ receptor-interacting protein serine-threonine kinase 2 ▪ thrombosis

Humans are constantly challenged by numerous bacteria and viruses. As the primary barrier defending humans against the pathogen invasion, the innate immune system recognizes the conserved molecular structure in microbes and initiates inflammatory and antimicrobial response relying on pattern recognition receptors. Among the major families of pattern recognition receptors, Toll-like receptors and nucleotide-binding oligomerization domain (NOD)—like receptors are the key players in innate immunity. In contrast to Toll-like receptors, which are found on plasma membrane, NOD-like receptors are cytoplasmic receptors. All NOD-like receptors are structurally similar, containing a central NOD, C-terminal leucine-rich repeat domain, and a variable N-terminal protein-protein interaction domain that interacts with downstream effectors. NOD1 and NOD2 are the two important Nod-like receptors fitting the typical structure with NOD1 containing 1 caspase recruitment domain, whereas NOD2 contains 2 caspase recruitment domains.

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NOD1 is broadly distributed, whereas NOD2 is mainly expressed in monocytes, macrophages, dendritic cells, intestinal epithelial cells, and Paneth cells. Although NOD1 and
NOD2 have a high degree of similarity, they recognize different bacterial cell wall peptidoglycan components. NOD1 recognizes d-glutamyl-meso-diaminopimelic acid primarily from gram-negative bacteria, whereas NOD2 detects muramyl dipeptide (MDP), the minimal bioactive motif in peptidoglycan from all bacteria. In addition to their critically important roles in hosts defending pathogen invasion, they are also crucial to the pathogenesis of a myriad of inflammatory diseases. Homozygous mutations of NOD2 are highly correlated with the incidence of Crohn disease.\(^5\,^6\)

Although primarily involved in hemostasis and thrombosis, increasing evidence demonstrates that platelets also play key roles in immune and inflammatory responses and the related diseases. Many pattern recognition receptors, mainly Toll-like receptors, which were originally discovered in classic immune cells, are also expressed in platelets,\(^8\,\,^{10}\) and play important roles in platelet activation, thrombosis, and hemostasis.\(^11\,\,^{13}\)

As the 2 most important members in the NOD-like receptor family, NOD1 and NOD2 have been intensively investigated in classic immune cells and in inflammatory diseases,\(^2\,\,^{14}\,\,^{16}\) but their expression and functions in platelets have never been reported. In this study, we report that NOD2 is also expressed in platelets and functions in platelet activation and thrombosis.

**Materials and Methods**

Detailed Materials and Methods are described in the online-only Data Supplement.

**Platelets and Peripheral Blood Mononuclear Cell Preparation**

All experiments using human subjects were performed in accordance with the Declaration of Helsinki and approved by the Institutional Review Board Fudan University. Platelets and peripheral blood mononuclear cells (PBMCs) were prepared as detailed in the online-only Data Supplement.

**Preparation of Platelet-Poor Plasma From Patients With Bacterial Infection**

Blood from patients with bacteremia and clinical sepsis, but without disseminated intravascular coagulation and clinical septic shock, was obtained from Fudan University Zhongshan Hospital. Ethical permission for all donations was obtained from the Zhongshan Hospital ethics board. Of 14 patients, 7 were infected with *Staphylococcus aureus*, 4 with *Escherichia coli*, and 3 with enterococci. All patients were clinically cured after appropriate treatment. The control blood was obtained from the same donors at least 2 weeks after the cure of infection. Platelet-poor plasma was prepared and stored at \(-80^\circ\)C before use.

**Animal Studies**

NOD2-deficient mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animal procedures were performed according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). Mouse platelets were prepared as described previously.\(^17\)

**Platelet Functional Studies**

Platelet aggregation, secretion, spreading, and clot retraction were measured as previously described\(^16\,\,^{19}\) and detailed in the online-only Data Supplement.

**Bleeding Assay**

The bleeding assay was measured as described previously\(^20\) with more information in the online-only Data Supplement.

**cGMP Assay**

cGMP in platelets was assayed by using commercially available cGMP \(^125\)I radioimmunoassay kit as reported previously.\(^25\)

**Nitric Oxide Detection**

Platelet nitric oxide (NO) level was measured by using fluorescent NO sensor DAF-FM DA on a collagen matrix as previously reported. Briefer, platelets were first preincubated with NO fluorescent indicator DAF-FM DA (1 \(\mu\)mol/L) in Tyrode buffer at 37°C for 30 minutes. After stimulation with MDP for 15 minutes, platelets were immobilized on a collagen matrix slide, and fluorescence was observed by confocal microscopy with 495 nm excitation and 515 nm emission filters.

**Reverse Transcription Polymerase Chain Reaction**

Total RNA was isolated and 1 \(\mu\)g of total RNA was reversely transcribed to cDNA by using RNA isolation kit and reverse transcription polymerase chain reaction (RT-PCR) kit (TaKaRa, Japan), respectively. PCR reactions were performed by using specific primers (Table I in the online-only Data Supplement).

**Intravital Microscopy of FeCl₃-Injured Thrombus Formation in Mouse Mesenteric Arteriole**

Intravital microscopy of FeCl₃ thrombus formation in mouse mesenteric arteriole was performed as described previously.\(^18\,\,^{23}\) More information is provided in the online-only Data Supplement.

**Lethal Pulmonary Thromboembolism Mouse Model Induced by Epinephrine and Collagen**

C57BL/6 mice were administrated rabbit anti-mouse platelet serum via the tail vein to produce an \(\approx 90\%\) decrease of the count of circulating platelets. On the second day, the wild type (WT) and NOD2\(^\sim\) mice were anesthetized with 10% chloral hydrate and blood was collected from the abdominal aorta. Blood was centrifuged at 200g for 3 minutes to obtain platelet-rich plasma, followed by centrifugation at 700g for 3 minutes to get platelet pellets, and then platelets were resuspended in 1 mL of Tyrode solution. Then, each 0.5-mL platelet suspension was incubated with MDP (5 \(\mu\)g/mL) or normal saline at 37°C for 30 minutes and centrifuged, and finally, platelet pellets were resuspended in Tyrode solution. The MDP- or saline-treated platelets were transfused into the platelet-depleted mice (0.125 mL per mouse). After 30 minutes, the mice were injected with a mixture of recombinant collagen (4 mg/kg) and epinephrine (0.3 mg/kg) dissolved in 0.1 mL of saline via the tail vein. The death rate was recorded within 15 minutes.

**Interleukin-1β Assay**

Platelet interleukin-1β (IL-1β) was assayed by flow cytometry, Western blot, and enzyme-linked immunosorbent assay. More information is available in the online-only Data Supplement.

**Statistical Analysis**

All data are expressed as mean±standard deviation. Differences between the groups were analyzed by 1-way analysis of variance, followed by a Tukey test for multiple comparisons unless otherwise stated. The Mann-Whitney test was used for comparison between 2 groups, and the Fisher exact test was used for comparison of death rate. Statistical analysis was performed by using Prism 5 (GraphPad Inc, San Diego). \(P<0.05\) was considered to be statistically significant.
Results

Human and Mouse Platelets Express NOD2
Human platelets express NOD2 receptor both at the RNA and protein levels as detected by RT-PCR (Figure 1A1) and Western blot (Figure 1B), similarly to PBMC (Figure 1). In contrast to PBMC, which expresses both NOD1 and NOD2, only NOD2 was detected in human platelets. The detected NOD2 on platelets is not from the contaminating PBMC, because the PBMC-specific marker CD14, which is robustly expressed in PBMC, is not detectable from platelet sample (Figure 1A2). Similarly, we also detected robust expression of NOD2, but not NOD1, in mouse platelets by RT-PCR (Figure 1C) and Western blot (Figure 1D).

NOD2 Receptor Agonist MDP Potentiates Platelet Aggregation and Dense Granule Release
NOD2 function has been extensively studied in white blood cells, and NOD2 activation elicits proinflammatory responses in white blood cells, playing a critical role in innate immunity. NOD2 functions in platelets have not been studied. NOD2 receptor agonist MDP alone did not induce platelet aggregation, in washed human platelets, even at 100 μg/mL (data not shown), but in the range of 1 to 10 μg/mL, MDP concentration-dependently potentiated human platelet aggregation and ATP release induced by low concentrations of thrombin or collagen (Figure 2A). NOD2 activation induced by MDP was confirmed by the dimerization of NOD2 on the treatment of platelets with 10 μg/mL MDP (Figure 2B). Similarly, 10 μg/mL MDP also significantly potentiated mouse platelet aggregation and ATP release induced by low concentrations of thrombin or collagen. The potentiating effect of MDP on platelet activation is NOD2 dependent, because it did not occur in NOD2–/– mice (Figure 2C and 2D). In an ex vivo study, MDP intraperitoneally given to mice also potentiated platelet aggregation and ATP release induced by thrombin and collagen in a NOD2-dependent manner (data not shown).

NOD2 Receptor Agonist MDP Potentiates Platelet Clot Retraction
Platelet-dependent clot retraction is a late-phase outside-in signaling event associated with a second wave of interaction between talin and integrin-β3 intracellular domain during platelet activation. When clot retraction was examined, we found that the NOD2 agonist MDP accelerated clot retraction in human platelet suspension (Figure 3). Similarly, MDP also accelerated clot retraction in mouse platelet suspension (Figure 3). Consistent with the effects of NOD2 activation on platelet aggregation and ATP release, MDP-induced clot retraction acceleration is NOD2 dependent, because it did not occur in NOD2–/– mice (Figure 3). Taken together, these data clearly indicate that NOD2 activation induced by MDP potentiates platelet activation.

NOD2 Agonist MDP Does Not Affect Platelet Spreading
Platelet spreading is an early-phase outside-in signaling event downstream of platelet αIIbβ3 integrin activation. In contrast to enhancing clot retraction, NOD2 agonist MDP had no effect on human platelet spreading on fibrinogen at 10 μg/mL (Figure I in the online-only Data Supplement), the concentration that activates NOD2 receptor as evidenced by receptor dimerization (Figure 2B). Similarly, mouse platelet spreading was also unaffected by MDP stimulation (Figure II in the online-only Data Supplement). Moreover, NOD2 deficiency also did not affect mouse platelet spreading (Figure II in the online-only Data Supplement), contrasting to the impaired clot retraction in NOD2–/– mice (Figure 3).

Figure 1. Both human and mouse platelets express NOD2. A, RT-PCR detection of NOD1, NOD2 (A1) and monocyte-specific CD14 (A2) in human platelets and peripheral blood mononuclear cells (PBMCs). B, Western blot detection of NOD1 and NOD2 in human platelets and PBMC. CHO cells were used as a negative control. C, RT-PCR detection of NOD1, NOD2 (C1), and monocyte-specific CD14 (C2) in mouse platelets; 299 bp NOD1 and 273 bp NOD2 are expected. D, Western blot detection of NOD1 and NOD2 in mouse platelets. CHO indicates Chinese hamster ovary cell line; NOD1, nucleotide-binding oligomerization domain 1; NOD2, nucleotide-binding oligomerization domain 2; and RT-PCR, reverse transcription polymerase chain reaction.
NOD2 Deficiency Impairs Thrombus Formation and Hemostasis In Vivo

To explore the role of NOD2 in thrombus formation in vivo, we examined the FeCl₃-injured thrombus formation in mesenteric arteriole in WT and NOD2⁻/- mice by using intravital microscopy. As shown in Figure 4A and 4B, WT mice receiving MDP exhibit increased thrombus formation in mesenteric arterioles, whereas NOD2⁻/- mice do not, indicating the enhancing role of NOD2 activation in thrombosis. Decreased blood loss after tail snip was observed consistently in WT mice receiving MDP, but not in NOD2-deficient mice receiving the same dose of MDP (Figure 4C), suggesting that NOD2 contributes to hemostasis, and thrombosis, as well, under MDP stimulation. These results are consistent with the potentiating effects of MDP on platelet aggregation and secretion.

NOD2 is also expressed in endothelial cells and macrophages, which may also contribute to thrombosis and hemostasis on MDP stimulation in septic settings. To corroborate that the NOD2 expressed in platelets prompts thrombosis and hemostasis in vivo after MDP dosing, we preincubated platelets with normal saline or MDP in vitro and transfused into platelet-depleted mice. We then challenged the mice with epinephrine and collagen, and examined the mortality with the use of the lethal lung thromboembolism mouse model. The death rate in mice receiving MDP-treated platelets is 69.2% (n=13), significantly higher than 28.6% in the mice receiving saline-treated platelets (n=14; P<0.05, 1-tailed Fisher exact test). In contrast, MDP-treated platelets from NOD2-deficient mice did not significantly increase the death rate in platelet-depleted WT mice challenged with epinephrine and collagen in comparison with the mice receiving saline-treated NOD2⁻/- platelets (30.8% versus 25%, n=13 and 12, respectively; P>0.05, 1-tailed Fisher exact test). We also examined the FeCl₃-injured thrombus in mesenteric arteriole in platelet-depleted mice. Mice receiving MDP-treated platelets exhibited increased thrombus formation in comparison with the mice receiving saline-treated platelets. In contrast, MDP-treated platelets from NOD2-deficient mice did not.
significantly increase thrombus formation in platelet-depleted WT mice in comparison with the mice receiving saline-treated NOD2–/– platelets (Figure III in the online-only Data Supplement). These data clearly indicate that platelet-expressed NOD2 plays a significant role in thrombosis and hemostasis, correlating well with the NOD2-potentiating effect on platelet activation (Figure 2).

NOD2 Deficiency Attenuated Platelet Hyperreactivity Induced by Bacterial Infection
During bacterial infection, MDP is shed from bacteria and released into the blood, resulting in a strong increase in MDP concentration and activation of NOD2 signal cascade in vivo. To check whether NOD2 activation affects platelet activity during bacterial infection, we treated plateau-rich plasma from WT or NOD2–/– mice with plasma from patients with bacteremia, and then detected platelet aggregation. NOD2 deficiency attenuated the potentiating effects of septic plasma on platelet aggregation induced by thrombin and collagen (Figure 5). Our results demonstrated a critical role of NOD2 in platelet hyperreactivity caused by bacterial infection.

Platelets Express Receptor-Interacting Protein 2, Receptor-Interacting Protein 2/Mitogen-Activated Protein Kinase Pathway Is Activated Downstream of NOD2
Our results thus far indicate that NOD2 activation enhances platelet activation and thus prompts thrombosis and hemostasis. Next, we sought to elucidate the mechanism of NOD2-mediated platelet activation. Mitogen-activated protein kinase (MAPK) is well documented in platelet activation and thrombosis. Receptor-interacting protein 2 (RIP2)/MAPK pathway activation has been reported to mediate NOD2-induced inflammation and anti-infection response in white blood cells.1,2 We therefore examined whether the RIP2/MAPK pathway is involved in NOD2-mediated platelet activation.

Although a variety of cells express RIP2, it is not clear whether platelets express RIP2. Using RT-PCR and Western blot, we found that both human and mouse platelets express robust RIP2, similarly to PBMC (Figure 6A). To our knowledge, this is the first report that platelets express RIP2. Furthermore, on MDP (10 μg/mL) stimulation, platelet RIP2 was phosphorylated at 5 minutes lasting for >10 minutes (Figure 6B). Erk, p38, and JNK were also phosphorylated with the peak phosphorylation apparently later than RIP2 (Figure 6B). These results agree with a previous study with white blood cells and indicate that the MAPK pathway is activated downstream of RIP2 in platelets on NOD2 activation triggered by MDP.

MAPK Inhibition Abolishes the Potentiating Effects of NOD2 on Platelet Activation
After showing the activation of RIP2/MAPK pathway in platelets downstream of MDP-induced NOD2 activation, we further examined whether MAPK signaling mediates the potentiating effects of NOD2 on platelet activation. As shown in Figure 6C, the inhibition of Erk, p38, and JNK with PD98059, SB203580, and SP600125, respectively, abolished the potentiating effect of MDP on platelet aggregation induced by thrombin or collagen. Similarly, the accelerated platelet-dependent clot retraction elicited by MDP was also prevented by inhibitors for Erk, p38, and JNK (Figure 6D). These data confirmed that MAPK phosphorylation downstream of NOD2/RIP2 mediates the potentiating effects of MDP on platelet activation.

MDP Elevates cGMP and NO in Platelets in a NOD2-Dependent Manner
cGMP elevation has been observed in platelets stimulated with collagen, thrombin, and von Willebrand factor.26,27 cGMP-protein kinase G (PKG)-MAPK pathway has been reported to mediate platelet activation downstream of glycoprotein Ib-IX.28–30 After showing MAPK phosphorylation downstream of NOD2 activation on MDP stimulation, we measured cGMP levels in platelets stimulated with MDP. As shown in Figure 7A, NOD2 agonist MDP concentration-dependently increased cGMP in human platelets up to 3.4 times in the range of 1 to 10 μg/mL, the concentrations that activated NOD2 and potentiated platelet activation concentration-dependently (Figure 2). MDP also increased cGMP in mouse platelets. At 10 μg/mL, MDP increased intracellular
cGMP 3.7 times in mouse platelets, similarly to human platelets (Figure 7B). The cGMP-enhancing effect of MDP is mediated by NOD2, because it was abolished by NOD2 deficiency (Figure 7B). These results show that cGMP is downstream of NOD2, and may contribute to platelet activation as in the case of glycoprotein Ib-IX–mediated platelet activation, which was confirmed by PKG inhibitor KT5823 abolishing the potentiating effect of MDP on platelet aggregation (Figure 7C). However, we did not observe the inhibition of KT5823 on MAPK phosphorylation elicited by MDP (data not shown), suggesting a MAPK-independent mechanism for the stimulatory role for cGMP/PKG in platelet activation.29 Platelets express functional inducible nitric oxide synthase (iNOS) and NO/soluble guanylyl cyclase (sGC) which mediate cGMP elevation during platelet activation.31 As expected, MDP-treated human platelets showed elevated intracellular NO levels, as detected by fluorescent NO sensor DAF-FM DA (Figure 7D). NOD2 deficiency abolished MDP-induced elevation of intracellular NO levels, showing NOD2 dependency (Figure 7E). Thus, we propose that cGMP elevation in platelets elicited by NOD2 activation is mediated by RIP2/iNOS/NO/sGC signaling32 (Figure 7F).
NOD2 Receptor Agonist MDP Stimulates Platelet Processing of IL-1β

Platelets represent an important linkage between thrombosis and inflammation. To assess the role of platelet NOD2 in inflammation, we measured proinflammatory cytokine IL-1β production in human platelets stimulated with MDP. In the range of 10 to 100 ng/mL, MDP concentration-dependently stimulates IL-1β accumulation at protein level in human platelets (Figure 8A). The effect of MDP is time dependently from 1 to 4 hours (Figure 8A). This effect appeared to be mediated by caspase-1 activation, because caspase-1 inhibitor FMK002 abolished MDP-stimulated IL-1β maturation (Figure 8B) and accumulation (Figure 8C) in human platelets. MDP also stimulated IL-1β accumulation in mouse platelets, which is NOD2 dependent (Figure 8D). MDP NOD2-dependently stimulates platelets IL-1β maturation provided direct evidence that NOD2 links the prothrombotic and proinflammatory roles of platelets.

Discussion

Beyond the well-recognized pivotal role in thrombosis and hemostasis, platelets also play a critical role in inflammatory and infectious diseases, and increasing evidence indicates that bacterial infection predisposes atherosclerosis and thrombotic events. Among the mechanisms underlying the infection-associated prothrombotic state, Toll-like receptors have been reported to mediate platelet activation in response to bacterial challenge. As a pattern recognition receptor, although NOD2 is well investigated in immunity, its expression and function in platelets have never been reported. The present study demonstrates that (1) both human and mouse platelets express robust NOD2; (2) NOD2 receptor activation potentiates platelet activation and enhances in vivo thrombosis; (3) plasma from septic patients potentiates platelet aggregation NOD2 dependently; (4) MAPK and iNOS/NO/sGC/cGMP/PKG pathways downstream of RIP2 mediate the role of NOD2 in platelet activation; and (5) NOD2 receptor activation triggers platelet processing of proinflammatory cytokine IL-1β. To our knowledge, this is the first report on NOD-like receptor in platelets; our findings provided novel insight into platelet activation mechanism, NOD2 function, and further highlighted platelet NOD2 as a critical receptor bridging thrombosis/hemostasis and immunity.
MAPK is activated during platelet stimulation and plays critical roles in platelet activation. Previously MAPK activation had been attributed to NOD2 signaling downstream of RIP2 in immune cells.\textsuperscript{1,2} Therefore, we checked MAPK pathway activation downstream of NOD2 in platelets. We found that Erk, p38, and JNK were phosphorylated in MDP-stimulated platelets. Importantly, we found that platelets expressed robust RIP2, and RIP2 was phosphorylated earlier than Erk, p38, and JNK on MDP stimulation, indicating that MAPK is activated downstream of RIP2 activation. In addition, we found that MAPK inhibition abolished the potentiating effects of MDP on platelet aggregation, further confirming the critical roles of MAPK in NOD2-mediated platelet activation. These results also suggest that p38, Erk, or JNK may be sequentially activated or coactivation of them is needed for platelet aggregation induced by low concentration of agonists. This hypothesis is supported by the previous studies showing that p38 sequentially activates Erk, JNK1 deficiency impairs Erk2 phosphorylation,\textsuperscript{37,38} and p38 or JNK inhibition abolished platelet aggregation under a low concentration of agonist stimulation.\textsuperscript{39,40}

The biphasic role of cGMP has been reported in agonist-induced platelet activation; a low concentration of cGMP stimulates platelet activation during the early process\textsuperscript{41} via the cGMP/PKG/MAPK pathway.\textsuperscript{29} After showing that NOD2 mediates platelet activation via the MAPK pathway downstream of RIP2, we next studied the role of cGMP in NOD2-mediated platelet activation. We found that NOD2 mediates platelet activation via the MAPK pathway downstream of RIP2 and NOD2 expression.

Figure 7. MDP elevates platelet cGMP and NO levels NOD2 dependently. A, MDP concentration-dependently increases cGMP in human platelets. Human platelets were treated with MDP at 37°C for 15 minutes, the cGMP in platelets was detected by \textsuperscript{125}I radioimmunoassay. B, MDP elevates cGMP in platelets from WT, but not in NOD2\textsuperscript{−/−} mice. Data are expressed as mean±SD by using platelets from different donors or mice (n=4). C, MDP potentiation of platelet aggregation induced by low concentration of thrombin was abolished by PKG inhibitor KT5823. Human washed platelets were used. Tracings shown are representative of 3 experiments using platelets from different donors. D, MDP elevates NO in human platelets. Platelets preincubated with fluorescent NO sensor DAF-FM DA were stimulated with MDP. Top shows platelet fluorescence under confocal microscope (scale bar=5 μm); bottom is the quantification of NO expressed as fluorescence per platelet in arbitrary unit (mean±SD of 4 experiments). E, MDP elevates platelet NO in WT but not in NOD2\textsuperscript{−/−} mice. NO was assayed as in D. Top is representative of 4 experiments using platelets from different mice; bottom is the quantification of NO expressed as mean±SD, n=4. The concentrations for MDP, thrombin, and KT5823 were 10 μg/mL, 0.02 U/mL, and 8 μmol/L, respectively. F, MAPK and NO/cGMP/PKG pathways downstream of RIP2 mediate the role of NOD2 in platelets. iNOS indicates inducible nitric oxide synthase; MAPK, mitogen activated-protein kinase; MDP, muramyl dipeptide; NO, nitric oxide; NOD2, nucleotide-binding oligomerization domain 2; PKG, protein kinase G; RIP2, receptor-interacting protein 2; sGC, soluble guanylyl cyclase; and WT, wild type.
activation dramatically enhanced platelet cGMP. In line with our findings, the activation of Toll-like receptor, another pattern recognition receptor that potentiates platelet activation, also increases platelet cGMP levels and activates platelets via cGMP-dependent protein kinase (PKG) pathway.\(^{11}\) We also consistently found that NOD2 agonist MDP potentiates platelet aggregation PKG dependently.

Previously, Shimada et al\(^{16}\) even reported that macrophages from NOD2-deficient mice exhibited impaired NO production iNOS-dependently in response to Chlamydophila pneumoniae. NO production from RIP2-deficient mice was also decreased in response to C pneumoniae, suggesting the RIP2-dependent NO production.\(^{16}\) Platelets express functional iNOS and NO/sGC mediates cGMP elevation during platelet activation;\(^{11}\) thus, we propose that cGMP elevation in platelets elicited by NOD2 activation is mediated by RIP2/iNOS/NO/sGC signaling,\(^{32}\) which is supported by our findings that MDP increases platelet NO level NOD2 dependently. In general, RIP2 activation is regarded as the result of its phosphorylation via direct interaction with dimerization form of NOD2 after NOD2 activation.\(^{42}\) Therefore, we propose that NOD2 promotes platelet activation via MAPK and iNOS/NO/sGC/cGMP pathways downstream of RIP2. However, how RIP2 mediates iNOS activation in platelets is still not clear and deserves further investigation.

As a key proinflammatory cytokine, IL-1\(\beta\) is linked to the pathogenesis of a variety of thromboinflammatory diseases including sepsis, Crohn disease, ulcerative colitis, type 2 diabetes mellitus, and atherosclerosis. To exploit the proinflammatory role of platelet NOD2 and its connecting role in thrombosis and inflammation/immunity during bacterial infection, we provided data showing that MDP stimulates IL-1\(\beta\) accumulation in human and mouse platelets NOD2 dependently; we also found that plasma from patients with bacteremia enhances platelet activation NOD2 dependently. In line with our findings, Porphyromonas gingivalis challenge has been reported to promote atherogenesis via IL-1 signaling,\(^{43}\) Streptococcus pneumoniae infection worsens atherosclerosis and exacerbates ischemic brain injury platelet and IL-1 dependently,\(^{35}\) whereas IL-1\(\beta\) deletion decreases the severity of atherosclerosis.\(^{44}\)

It is well known that platelets participate in the pathogenesis of atherosclerosis.\(^{45}\) The role of NOD2 in atherosclerosis is limited and inconsistent. Although 2 groups found that NOD2 prompts atherosclerosis,\(^{15,46}\) Yuan et al\(^{47}\) recently reported that MDP/NOD2 axis activation reduces atherosclerosis. The role of platelet NOD2 in the growth and destabilization of atherosclerosis is not clear. The prothrombotic and proinflammatory roles of platelet NOD2 demonstrated by our present study suggest that platelet NOD2 may facilitate the initiation and development of atherosclerosis.

The different MDP concentrations needed for NOD2-mediated prothrombotic and proinflammatory response attracted our attention. At low MDP concentrations (10–100 ng/mL), platelet NOD2 triggers IL-1\(\beta\) maturation; whereas at high MDP concentrations (1–10 \(\mu\)g/mL), platelet NOD2 potentiates...
Platelets activation, but does not stimulate IL-1β maturation (data not shown). Although the underlying mechanism accounting for this different concentration dependence remains to be elucidated, the present study implies that platelet NOD2 is likely to switch its roles at different stages of infection. At the initial stage of infection, in response to low concentrations of MDP from pathogens, platelet NOD2 appears to mainly exert proinflammatory effects. With the pathogen accumulation as the infection progresses, a high concentration of MDP from bacteria triggers the prothrombotic response of platelet NOD2. In conclusion, NOD2, which is mainly expressed in white blood cells and plays critical roles in innate immune against infection, is also expressed in platelets and potentiates platelet activation and thrombosis, and prompts proinflammatory cytokines. NOD2 in platelets is crucial for pathogenesis and treatment of inflammatory and thrombotic diseases.

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Disclosures
None.

References
poorly to current antiplatelet drugs. 

Arterial thrombosis, especially in infectious and inflammatory conditions where hyperactive platelets are known to respond

NOD2 also triggers platelet interleukin-1β production via caspase-1 activation and activating mitogen activated-protein kinase and nitric oxide/soluble guanylyl cyclase/cGMP/protein kinase G signal pathway. Under infectious and inflammatory conditions, platelets usually become hyperactive to a variety of agonists including thrombin, collagen, and bacterial products. This increased platelet activation contributes to the increased atherosclerosis and thrombosis risk, but its underlying mechanisms remain poorly deciphered. In this study, we show that nucleotide-binding oligomerization domain 2 (NOD2), an intracellular pattern recognition receptor critical to innate immunity, is also expressed in platelets. With the use of NOD2-specific agonist and NOD2-deficient mice, we find that NOD2 promotes platelet activation and thrombosis by activating mitogen activated-protein kinase and nitric oxide/soluble guanylyl cyclase/cGMP/protein kinase G signal pathway downstream of receptor-interacting protein 2. NOD2 also triggers platelet interleukin-1β maturation via caspase-1 activation. These data demonstrate a nonimmune activity of NOD2 that bridges immunity and hemostasis/thrombosis in platelets. This crosstalk sheds novel insights into the mechanism accounting for the increased platelet reactivity and thrombotic risk in infectious and inflammatory diseases. Blocking NOD2 and its downstream pathway could potentially reduce and prevent arterial thrombosis, especially in infectious and inflammatory conditions where hyperactive platelets are known to respond poorly to current antiplatelet drugs.

**CLINICAL PERSPECTIVE**

Under infectious and inflammatory conditions, platelets usually become hyperactive to a variety of agonists including thrombin and collagen. This increased platelet activation contributes to the increased atherosclerosis and thrombosis risk, but its underlying mechanisms remain poorly deciphered. In this study, we show that nucleotide-binding oligomerization domain 2 (NOD2), an intracellular pattern recognition receptor critical to innate immunity, is also expressed in platelets. With the use of NOD2-specific agonist and NOD2-deficient mice, we find that NOD2 promotes platelet activation and thrombosis by activating mitogen activated-protein kinase and nitric oxide/soluble guanylyl cyclase/cGMP/protein kinase G signal pathway downstream of receptor-interacting protein 2. NOD2 also triggers platelet interleukin-1β maturation via caspase-1 activation. These data demonstrate a nonimmune activity of NOD2 that bridges immunity and hemostasis/thrombosis in platelets. This crosstalk sheds novel insights into the mechanism accounting for the increased platelet reactivity and thrombotic risk in infectious and inflammatory diseases. Blocking NOD2 and its downstream pathway could potentially reduce and prevent arterial thrombosis, especially in infectious and inflammatory conditions where hyperactive platelets are known to respond poorly to current antiplatelet drugs.
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SUPPLEMENTAL MATERIAL

Expanded Materials and Methods

Materials
Muramyl dipeptide (MDP) was from Invivogen (San Diego, CA). Fibrinogen, thrombin, and equine collagen (collagen) were purchased from Chrono-Log (Havertown, PA). FITC-labeled phalloidin, apyrase, PGE1, PD98059, SB203580, SP600125, Bornstein and Traub Type I recombinant human collagen (recombinant collagen) and NO sensor DAF-FM DA were from Sigma-Aldrich (St Louis, MO). The anti-NOD1, anti-NOD2, anti-RIP2, and anti-GAPDH antibodies were from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). The anti-p38, anti-phospho-p38 (Thr180/Tyr182), anti-Erk1/2, anti-phospho-Erk1/2 (Thr202/Tyr204), anti-JNK, and anti-phospho-JNK (Thr183/Tyr185) were from Cell Signal Inc (Beverly, MA). The anti-IL-1β-FITC and isotype antibodies, caspase-1 inhibitor Z-WEHD-FMK (FMK002) were from R & D Systems (Minneapolis, MN). Epinephrine was from Shanghai Harvest Pharmaceutical (Shanghai, China).

Platelets and peripheral blood mononuclear cells (PBMC) preparation
Healthy volunteers who had not taken aspirin or other non-steroidal anti-inflammatory drugs for at least 14 days were recruited and informed consent was obtained. Blood was drawn from the antecubital vein and mixed with acid-citrate dextrose buffer (9:1 vol/vol). Platelet-rich plasma prepared as previously described was filtered through a Sepharose 2B column (Sigma-Aldrich) equilibrated in Tyrode's solution (pH 7.35) to isolate platelets. PBMC were isolated from whole blood by density centrifugation (Ficoll-Hypaque; Sigma-Aldrich) according to the manufacturer’s instructions.
Platelet functional studies

Platelet aggregation and secretion in response to agonists were measured as previously described\(^3\). Analysis of platelet spreading on immobilized fibrinogen was done as described previously\(^4\).

Briefly, washed platelets in Tyrode's solution were preincubated with MDP for 15 min, and transferred onto Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) precoated with 20 μg/mL of fibrinogen, and then allowed to adhere for 30 - 120 min at 37°C. After washing with PBS, attached platelets were fixed, permeabilized, and then stained with FITC-labeled phalloidin. Adherent platelets were viewed using a Leica SPE confocal microscope.

Clot retraction in platelet suspension was assayed as described by Su et al\(^4\). Briefly, 2 mg/mL fibrinogen was added to the platelets suspended in Tyrode's solution, and platelet suspension was dispensed in 0.3 mL aliquots into cuvettes. Clot retraction was initiated by the addition of 1 U/mL thrombin and allowed to proceed at 37°C. Clot retraction was monitored by taking photographs at indicated time points using a digital camera. Sizes of retracted clots on photographs were quantified using NIH Image J software.

Western blotting

To detect NOD1, NOD2 and RIP2 expression, PBMC and platelets prepared as described above were lysed using RIPA buffer and subjected to immunoblotting using specific antibodies. Phosphorylation of RIP2, Erk1/2, p38 and JNK in MDP-stimulated platelets was analyzed using phosphorylation-specific antibodies; samples were similarly processed as described before\(^1\). To detect NOD2 dimerization, electrophoresis of platelet lysates was run under non-reducing conditions.

Intravital microscopy of FeCl\(_3\)-injured thrombus formation in mouse mesenteric arteriole
Intravital microscopy of FeCl$_3$ thrombus formation in mouse mesenteric arteriole was performed as described previously$^3,5$. Briefly, calcein-labeled platelets were injected into C57BL/6 wild type (WT) or NOD2-deficient (NOD2-/-) mice via tail vein; thrombosis was induced by FeCl$_3$, and recorded with intravital microscopy. MDP (100 μg/0.1 mL per mouse) or saline was injected intraperitoneally 12 hours before FeCl$_3$ injury.

**Bleeding assay**

Bleeding assay was measured as described previously$^6$. Briefly, the distal 1 mm of the mouse tail was amputated and the tail was immersed in 1 mL of 0.9% NaCl warmed to 37$^\circ$C for 10 min. Blood loss was determined by measuring the absorbance of the saline at 560 nm and comparing the result to a standard curve constructed using known volumes of mouse blood.

**IL-1β assay**

Human platelets were treated with 100 ng/mL MDP for indicated times, or treated with MDP for 4 hours at indicated concentrations, permeabilized, and stained with either anti-IL-1β-FITC or an isotype antibody for 30 min. Platelets were washed and analyzed by flow cytometry. To detect IL-1β in mouse platelets, platelets were treated with 100 ng/mL MDP for 4 hours, and then lysed and measured for mature IL-1β using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.
**Supplementary Table 1.**

**Primers used for RT-PCR**

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<th>Accession No.</th>
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Supplemental Figure legends

Figure S1. MDP stimulation does not affect human platelet spreading on fibrinogen. Human washed platelets were seeded on fibrinogen-coated cover slips for 30 - 120 min at 37°C, then permeabilized and stained with FITC-phalloidin. The results shown are representative of three experiments using platelets from different donors. Scale bar = 10 μm.

Figure S2. MDP stimulation and NOD2 deficiency do not affect mouse platelet spreading on fibrinogen. Washed platelets from WT or NOD2-/- mice pretreated with normal saline as control or MDP were plated on fibrinogen-coated cover slips for 45 min at 37°C, then permeabilized and stained with FITC-phalloidin and observed under a 40 x water-immersion objective with magnification of 5 (upper panel) using a Leica microscope. The results shown are representative of at least three experiments using platelets from different mice. Scale bar = 10 μm.

Figure S3. NOD2 from platelets mediates the prothrombotic role of MDP: platelet depletion/reconstitution study on mouse mesenteric arteriole thrombosis model. A, Platelet-depleted mice receiving MDP-treated platelets exhibit increased thrombus formation than those receiving saline-treated platelets. In contrast, MDP-treated platelets from NOD2-deficient mice do not significantly increase thrombus formation in platelet-depleted WT mice compared with those receiving saline-treated NOD2-/- platelets. Platelets from WT or NOD2-/- mice pretreated with normal saline or MDP (10 μg/mL) were transfused into platelet-depleted mice. Thrombosis was induced by FeCl₃ injury, and recorded with intravital microscopy. Calcein was used to label platelets. B, Statistical analysis of FeCl₃-induced thrombus formation. Occlusion time and time to first thrombus (larger than 20 μm and stable for more than 2 min) were analyzed. Data are
expressed as mean ± SD, n = 10 - 13. Determination of significant differences was performed by one-way ANOVA, followed by Bonferroni's Multiple Comparison Test for multiple comparisons.
Zhang et al, Fig. S1.
<table>
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Zhang et al, Fig. S2.
A

Platelet-depleted WT mice

<table>
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<th>Platelets transfused</th>
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</table>

B

Zhang et al, Fig. S3

Occlusion time (min)

WT       | NOD2-/-
---|---
P < 0.01

Time to form 1st thrombus more than 20 μm (min)

WT       | NOD2-/-
---|---
P < 0.05

control  | MDP  | control  | MDP
---|---|---|---

---|---|---|---
References


