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TLR4-dependent upregulation of the platelet NLRP3 inflammasome promotes platelet aggregation in a murine model of hindlimb ischemia

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Abstract

Platelets play a critical role in the pathophysiology of peripheral arterial disease (PAD). The mechanisms by which muscle ischemia regulates aggregation of platelets are poorly understood. We have recently identified the Nod-like receptor nucleotide-binding domain leucine rich repeat containing protein 3 (NLRP3) expressed by platelets as a critical regulator of platelet activation and aggregation, which may be triggered by activation of toll-like receptor 4 (TLR4). In this study, we performed femoral artery ligation (FAL) in transgenic mice with platelet-specific ablation of TLR4 (TLR4 PF4) and in NLRP3 knockout (NLRP3$^{-/-}$) mice. NLRP3 inflammasome activity of circulating platelets, as monitored by activation of caspase-1 and cleavage of interleukin-1β (IL-1β), was upregulated in mice subjected to FAL. Genetic ablation of TLR4 in platelets led to decreased platelet caspase 1 activation and platelet aggregation, which was reversed by the NLRP3 activator Nigericin. Two weeks after the induction of FAL, ischemic limb perfusion was increased in TLR4 PF4 and NLRP3$^{-/-}$ mice as compared to control mice. Hence, activation of platelet TLR4/NLRP3 signaling plays a critical role in upregulating platelet aggregation and interfering with perfusion recovery in muscle ischemia and may represent a therapeutic target to improve limb salvage.

Keywords

platelets; NLRP3; hindlimb ischemia; peripheral arterial disease

Introduction

Peripheral arterial disease (PAD) is an advanced stage of atherosclerosis that may result in life-threatening conditions such as critical limb ischemia. Millions of Americans are affected

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by PAD, with a worldwide prevalence of over 200 million [1-2]. Failure of revascularization with surgical or endovascular procedures is associated with a significant risk of limb loss [2]; 6 to 12% of patients with revascularization failure die within 30 days of amputation [3].

Platelets play a critical role in the development and progression of PAD [4]. Activation and aggregation levels of circulating platelets are elevated in patients with PAD [5-6] and correlate with disease severity [7]. Current guidelines recommend aspirin as first-line therapy in the prevention of atherothrombotic events in patients with PAD [8]. Various other antiplatelet agents such as ADP receptor antagonists [9] and thromboxane inhibitors [10] have been investigated in patients with symptomatic PAD. Moreover, novel oral anticoagulants, such as low dose rivaroxaban in combination with aspirin, have recently shown promising results in patients with PAD [11]. However, treatment options for PAD are still limited, which is explained in part by an incomplete understanding of the underlying pathophysiological mechanisms.

Recent evidence proposes a critical role of inflammatory processes in the pathophysiology of PAD [12-15]. Platelets employ pattern recognition receptors toll-like receptor 4 (TLR4) and nucleotide-binding domain leucine rich repeat containing protein 3 (NLRP3) [16-19]. NLRP3 may become activated by TLR4 signaling [20-21], resulting in the formation of intracellular inflammasome complexes with the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1-dependent cleavage and secretion of interleukin 1β (IL-1β) [22]. In platelets, activation of TLR4 and NLRP3 promotes platelet activation/aggregation and thrombus formation [18-19, 23-24]. Moreover, activation of platelet TLR4 is involved in regulating limb perfusion and muscle necrosis after hindlimb ischemia [25]. The role of the platelet NLRP3 inflammasome in muscle ischemia, however, remains unknown. In this study, we hypothesized that the platelet NLRP3 inflammasome would be upregulated in a murine model of hindlimb ischemia via TLR4 and regulate platelet aggregation.

Materials and Methods

Animals and femoral artery ligation (FAL) model

The animal protocol complied with the regulation regarding the care and use of experimental animals published by the NIH and was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (protocol number 16037594). C57/BL6 wild-type (WT) mice (male; 8-10 weeks) were purchased from Jackson Laboratories (Bar Harbor, ME). NLRP3$^{-/-}$ mice were obtained from Millennium Pharmaceuticals (Cambridge, MA) and were on a C57BL/6 background [26]. TLR4loxP/loxP (TLR4 Flox) control mice were generated as previously described [27]. Platelet specific TLR4 knockout (TLR4 PF4) mice were generated by crossing TLR4 Flox mice with Pf4-Cre transgenic mice as described [28]. Breeding of both NLRP3$^{-/-}$ and TLR4 PF4 mice were performed in the laboratory of Dr. Timothy Billiar (Department of Surgery, University of Pittsburgh Medical Center).

For FAL, mice were anesthetized with pentobarbital (0.1 cc/g intraperitoneal). After hair removal and preparation with iodine solution, transverse incisions were made in each groin and the femoral structures were isolated. On the right, the external iliac and femoral veins

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and arteries and all visible branches were ligated with 6-0 silk [29]. Care was taken to preserve the femoral nerve. On the left, the femoral vessels were exposed but not ligated. Animals were kept warm with a heating lamp during the surgical procedure. Mice were euthanized by an overdose of inhaled isoflurane followed by cervical dislocation at designated time points.

Platelet caspase-1 activity assay

Blood from mice was obtained 6 hours after surgery via cardiac puncture [30]. Mice that underwent surgery in the absence of FAL were used as sham controls. Murine platelets were isolated as previously described [18]. For certain experiments, isolated platelets from WT mice were incubated for 30 minutes with an inhibitor against NLRP3 (MCC950; 100 nM, Cayman Chemical, Ann Arbor, MI) [31] or caspase-1 (YVAD; 100 nM, Calbiochem, Darmstadt, Germany) [32]. DMSO was used as a control. In other experiments, isolated platelets derived from TLR4 PF4 or TLR4 Flox control mice were treated for 30 minutes with the NLRP3 inflammasome activator Nigericin (10 μM, Cayman Chemical) [33]. Activation of caspase-1 in isolated platelets was measured using the FAM-FLICA Caspase-1 Assay Kit according to the manufacturer's protocol (Immunochemistry Technologies, Bloomington, MN). Platelets were analyzed in a black 96-well microtiter plate using a plate reader for relative fluorescence units (RFUs).

Detection of IL-1β in platelet supernatants

Platelets isolated from WT mice subjected to FAL or sham surgery were kept untreated or treated with MCC950 (100 nM) for 30 minutes. Platelets were lysed with Pierce IP Lysis Buffer (ThermoFisher Scientific; Pittsburgh, PA) and protein concentrations were determined with the Bradford Concentration Assay (ThermoFisher Scientific). 50 μg of protein was resolved by 8.5% SDS-polyarcylamide gel electrophoresis. Western Blotting onto nitrocellulose membranes (Bio Rad, Hercules, CA) was performed using the Criterion Blotter system (Bio Rad). Membranes were incubated overnight with anti-IL-1β polyclonal antibody (1:2000, rabbit IgG; abcam, Cambridge, MA). Anti-tubulin monoclonal antibody (1:2000, mouse IgM; BD Pharmingen/Biosciences; San Jose, CA) was used as a loading control. Antibody binding was detected with corresponding secondary fluorescence-labeled antibodies, HRP-conjugated Clarity Western Substrate (Bio Rad), and a SRX-101a Film processer (Konica, Cleveland, OH).

Platelet aggregometry

Blood from mice was obtained 6 hours after surgery via cardiac puncture. Platelet aggregation was evaluated using whole blood impedance aggregometry (Model 700, ChronoLog, Havertown, PA) as described previously [18]. Collagen (2 μg/ml; ChronoLog) was used as platelet agonist. Aggregation was measured for 6 minutes at 37°C with a stir speed of 1,200 rpm. Analysis was performed using the Aggrolink-8 software (ChronoLog). Data are reported as area under the curve (AUC), which incorporates the slope and amplitude of the aggregation curves.

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Laser Doppler Perfusion Imaging (LDPI)

LDPI was performed as described previously [25]. In brief, animals were anesthetized with inhaled isoflurane and kept warm on a heating pad for the duration of the procedure. Similar ambient lighting was used for each study for standardization. After preparing each hindlimb with depilatory cream, the blood flow to both hindlimbs was measured using a laser doppler blood flow meter (Perimed, Stockholm, Sweden). Three sequential images of the entire hindlimb were obtained and averaged. Perfusion was expressed as a ratio of the ischemic to nonischemic limb at each time point to confirm adequacy of ligation. Laser doppler perfusion imaging was performed before and 1, 7, and 14 days after FAL.

Statistical analysis

Data show mean ± SD. Results are reported from at least three independent experiments. Two-way factorial ANOVA with post hoc Bonferroni correction was used as appropriate. All statistical analyses were performed using Graph Pad Prism software (GraphPad, San Diego CA).

Results

The platelet NLRP3 inflammasome is upregulated following FAL via TLR4

We studied activation of the platelet NLRP3 inflammasome in a hindlimb ischemia mouse model (FAL). Caspase-1 activity was significantly elevated in circulating platelets from WT mice subjected to FAL as compared to sham controls (Fig. 1A). Incubation of platelets with the NLRP3 inhibitor MCC950 significantly suppressed caspase-1 activation in platelets derived from FAL and sham mice. The caspase-1 inhibitor YVAD served as a positive control and significantly downregulated caspase-1 activation. We also studied cleavage of IL-1β in platelets, which allows monitoring of caspase-1 activity [18]. Platelet expression of cleaved IL-1β was increased in mice subjected to FAL and decreased in the presence of the NLRP3 inhibitor (Fig. 1B).

TLR4 signaling is involved in activating the NLRP3 inflammasome in immune cells and platelets [20-21, 23]. Hence, we next investigated whether FAL-induced activation of the platelet NLRP3 inflammasome depends on platelet TLR4. FAL-induced upregulation of platelet caspase-1 activity was significantly suppressed in mice whose platelets were lacking TLR4 (TLR4 PF4) as compared to platelets from TLR4 Flox control mice (Fig. 1C). Platelet caspase-1 activity at baseline (sham) also was significantly decreased in TLR4 PF4 mice as compared to control mice. Downregulated platelet caspase-1 activity in platelet-specific TLR4 knockout mice was significantly reversed by the NLRP3 activator Nigericin, which did not occur in platelets derived from TLR4 Flox controls (Fig. 1D), indicating that activation of platelet NLRP3 is mediated via TLR4.

Platelet TLR4/NLRP3 signaling promotes platelet aggregation in FAL

Next, we investigated the effect of FAL-induced TLR4-dependent platelet NLRP3 inflammasome activation on aggregation of platelets. Platelet aggregation was significantly increased in WT (Fig. 2A) and TLR4 Flox control mice (Fig. 2B) after FAL. Aggregation of platelets in the presence or absence of FAL was significantly suppressed in NLRP3$^{-/-}$ mice

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(Fig. 2A) and TLR4 PF4 mice (Fig. 2B). Downregulated platelet aggregation in mice with platelet-specific TLR4 knockout was significantly restored by the NLRP3 activator Nigericin, which did not occur in platelets derived from TLR4 Flox control mice, indicating a functionally active link between platelet TLR4 and NLRP3 regulating platelet aggregation (Fig. 2C).

Absence of platelet TLR4 and NLRP3 improves perfusion recovery two weeks after FAL

We tested the effect of platelet TLR4 and NLRP3 ablation on perfusion recovery following FAL. Perfusion of the femoral artery subjected to ligation and that of the contralateral nonligated femoral artery was evaluated by LDPI at baseline (day 0), shortly after FAL induction (day 1), and on days 7 and 14 (Fig. 3A). Two weeks after the induction of FAL, the ischemia/nonischemia perfusion ratio was significantly increased in mice whose platelets were lacking TLR4 (TLR4 PF4) as compared with that in TLR4 Flox control mice (Fig. 3B). Similar results were obtained in NLRP3$^{-/-}$ mice (Fig. 3C).

Discussion

In this study, we show that hindlimb ischemia following FAL induces activation of the platelet NLRP3 inflammasome via TLR4. We further identify a critical role of platelet TLR4/NLRP3 signaling in upregulating platelet aggregation and interfering with perfusion recovery after FAL.

While revascularization procedures may restore large vessel patency in PAD, upregulation of platelet aggregation and accumulation of platelet aggregates in the microvasculature are critical determinants of perfusion recovery and ongoing muscle injury [4, 34]. Hyperreactive platelets may form aggregates with immune cells, cause platelet microembolism, and significantly impair reperfusion therapy outcome, exacerbating vascular and tissue damage [7, 34-35]. Upon activation, platelets upregulate an arsenal of potent thromboinflammatory mechanisms, resulting in surface expression or release of adhesion proteins, growth factors, cytokines, chemokines, and coagulation factors into the local microenvironment [35-37].

The NLRP3 inflammasome is a critical thromboinflammatory mechanism employed by immune cells, which was described in platelets for the first time in the context of dengue fever [17]. In this study, the dengue virus triggered activation of the platelet NLRP3 inflammasome, which resulted in platelet shedding of IL-1β-rich microparticles and endothelial dysfunction. We have demonstrated recently that activation of platelets by collagen or thrombin also upregulates platelet NLRP3 inflammasome activity [18]. In another recent study, we show that the NLRP3 inflammasome in platelets is upregulated in sickle cell disease, which was mediated via the damage-associated molecular pattern molecule high-mobility group box 1 (HMGB1) and TLR4, promoting aggregation of circulating platelets [23]. We and others have shown that activation of platelet TLR4 and NLRP3 promotes platelet activation/aggregation and thrombus formation, proposing that upregulation of these pattern recognition receptor signals in platelets plays a critical role in disease states associated with abnormal coagulation and inflammation [18-19, 23-24].

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In this study, the platelet NLRP3 inflammasome was upregulated in mice subjected to hindlimb ischemia, which was mediated via platelet TLR4 and resulted in elevated platelet aggregation levels. These findings were associated with improved perfusion in the muscle tissue of TLR4 PF4 and NLRP3$^{-/-}$ mice two weeks after FAL, indicating possible involvement of platelet TLR4/NLRP3 signaling in regulating perfusion recovery. The potential role of TLR4/NLRP3-mediated platelet aggregation in interfering with perfusion recovery from muscle ischemia, however, is currently unknown. Recent evidence supports a critical role of the NLRP3 inflammasome in immune cells in regulating progression of atherosclerosis [38]. It is possible that elevated platelet aggregation levels mediated by platelet TLR4/NLRP3 signaling increase microvascular thrombus formation and further exacerbate tissue damage as part of a vicious cycle occurring in chronic muscle ischemia, which is currently under investigation. Further studies are needed to investigate the role of platelet NLRP3 inflammasome activation in PAD and study this mechanism as a potential therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations:

ASC	apoptosis-associated speck-like protein containing a CARD
FAL	femoral artery ligation
IL-1β	interleukin-1β
NLRP3	nucleotide-binding domain leucine rich repeat containing protein 3
PAD	peripheral arterial disease
TLR4	toll-like receptor 4
WT	wild-type

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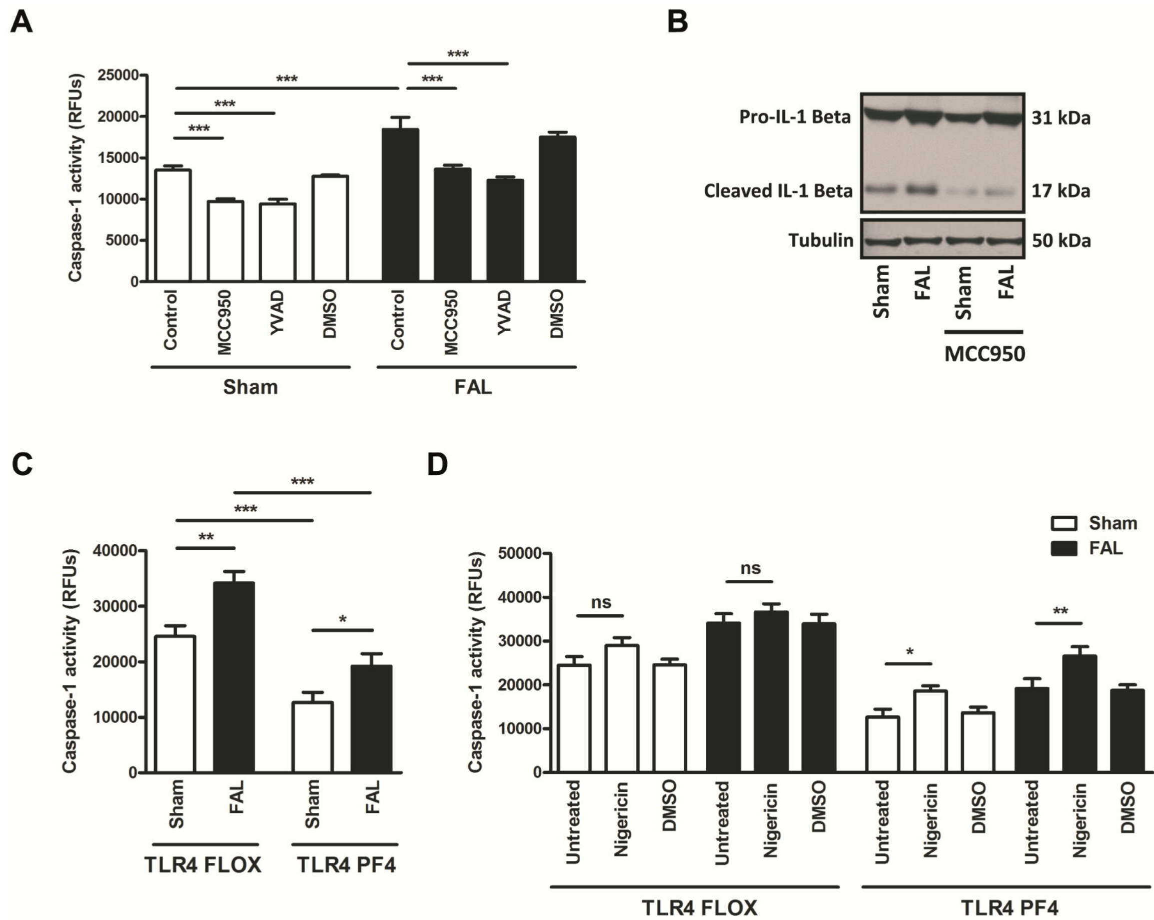

Figure 1. The platelet NLRP3 inflammasome is upregulated following FAL via TLR4.
(A) Platelet caspase-1 activity (measured by FLICA assay) is upregulated in mice subjected to FAL. Platelet caspase-1 activity is suppressed in the presence of the NLRP3 inhibitor MCC950 and the caspase-1 inhibitor YVAD. (B) Expression of cleaved IL-1β in platelets is increased in mice subjected to FAL and decreased in the presence of the NLRP3 inhibitor MCC950. (C) Platelet caspase-1 activity in the presence or absence of FAL is downregulated in TLR4 PF4 mice as compared to TLR4 Flox controls. (D) Downregulated platelet caspase-1 activity in TLR4 PF4 mice is restored by the NLRP3 activator Nigericin,which does not occur in platelets derived from TLR4 Flox controls. Data are presented as mean ±SD for N 3 and at least three separate experiments in all studies. *p< 0.05, **p < 0.01,***p < 0.001 (2-way ANOVA with Bonferroni post-hoc test in A, C, D).

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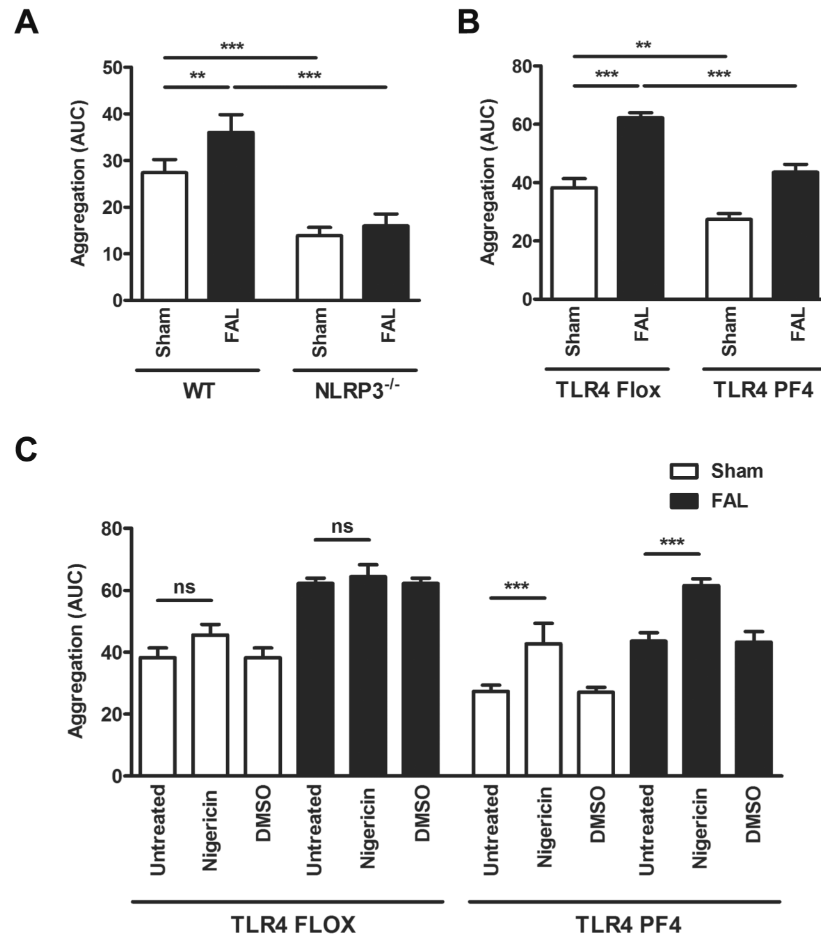

Figure 2. Platelet TLR4/NLRP3 signaling promotes platelet aggregation in FAL.

(A) Collagen-induced platelet aggregation is increased when WT mice are subjected to FAL and decreased in NLRP3$^{-/-}$ mice. (B) Collagen-induced platelet aggregation is increased when TLR4 Flox control mice mice are subjected to FAL and decreased in TLR4 PF4 mice. (C) Downregulated platelet aggregation in TLR4 PF4 mice is restored by the NLRP3 activator Nigericin, which does not occur in platelets derived from TLR4 Flox controls. Data are presented as mean ± SD for N 3 and at least three separate experiments in all studies. **p < 0.01, ***p < 0.001 (2-way ANOVA with Bonferroni post-hoc test in A-C).

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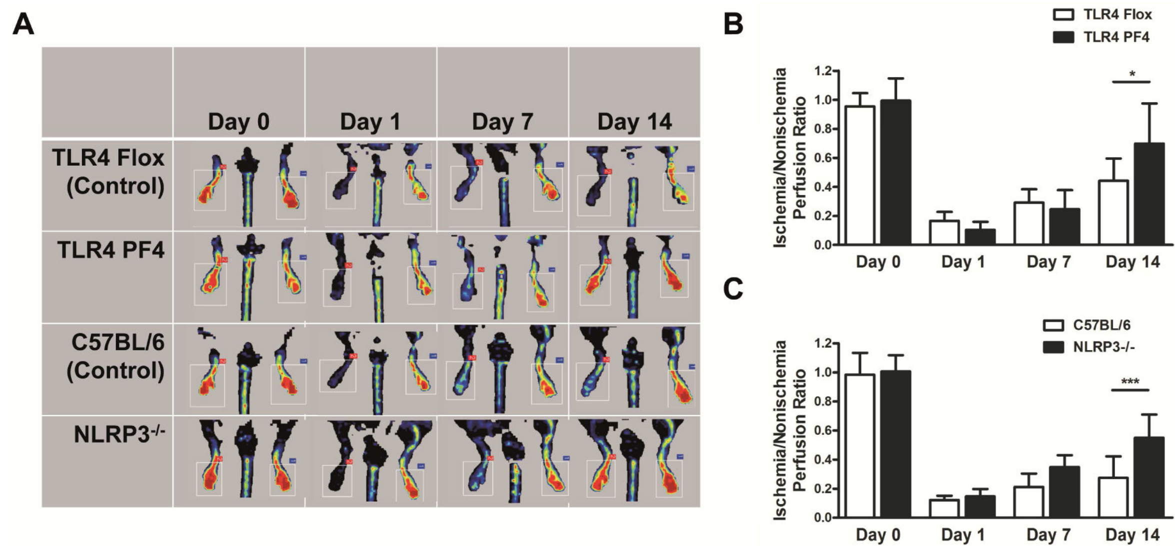

Figure 3. Absence of platelet TLR4 and NLRP3 improves perfusion recovery two weeks after FAL.

(A) Perfusion of the femoral artery subjected to ligation and that of the contralateral nonligated femoral artery is evaluated by LDPI in TLR4 Flox, TLR4 PF4, WT (C57BL/6), and NLRP3$^{-/-}$ mice at baseline (day 0), shortly after FAL induction (day 1), and on days 7 and 14. (B) Two weeks after the induction of FAL, the ischemia/nonischemia perfusion ratio is increased in TLR4 PF4 mice as compared with that in TLR4 Flox control mice. (C) Two weeks after the induction of FAL, the ischemia/nonischemia perfusion ratio is increased in NLRP3$^{-/-}$ mice as compared with that in WT (C57BL/6) control mice. Data are presented as mean ± SD for N 3 and at least three separate experiments in all studies. *$p < 0.05$, ***$p < 0.001$ (2-way ANOVA with Bonferroni post-hoc test in B, C).