Toll-Like Receptor 4 Regulates Platelet Function and Contributes to Coagulation Abnormality and Organ Injury in Hemorrhagic Shock and Resuscitation

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Background—Growing evidence indicates that the presence of toll-like receptor 4 (TLR4) on platelets is a key regulator of platelet number and function. Platelets exposed to TLR4 agonists may serve to activate other cells such as neutrophils and endothelial cells in sepsis and other inflammatory conditions. The functional significance of platelet TLR4 in hemorrhagic shock (HS), however, remains unexplored.

Methods and Results—Using thromboelastography and platelet aggregometry, we demonstrate that platelet function is impaired in a mouse model of HS with resuscitation. Further analysis using cellular-specific TLR4 deletion in mice revealed that platelet TLR4 is essential for platelet activation and function in HS with resuscitation and that platelet TLR4 regulates the development of coagulopathy after hemorrhage and resuscitation. Transfusion of TLR4-negative platelets into mice resulted in protection from coagulopathy and restored platelet function. Additionally, platelet-specific TLR4 knockout mice were protected from lung and liver injury and exhibited a marked reduction in systemic inflammation as measured by circulating interleukin-6 after HS with resuscitation.

Conclusions—We demonstrate for the first time that platelet TLR4 is an essential mediator of the inflammatory response as well as platelet activation and function in HS and resuscitation. (Circ Cardiovasc Genet. 2014;7:615-624.)

Key Words: blood platelets | inflammation | shock, hemorrhagic | toll-like receptor 4

Platelets have been extensively studied as hemostatic regulators and, in the context of hemorrhagic shock (HS), are best recognized for their role in clot formation following vascular endothelial injury. A concept that has taken longer to establish, but is rapidly evolving, is that platelets are also key effector cells in systemic inflammatory processes as both instigators of local and systemic inflammatory reactions and also participants in the inflammation that contributes to tissue injury.1,2 Platelets express several receptors common to other immune cells including members of the toll-like receptor (TLR) family.3–6 These receptors sense not only molecules of microbial origin, but also molecules of host origin released by damage-associated molecular pattern molecules.3–6 Several TLRs, including TLR2, TLR3, TLR4, and TLR9, contribute to the inflammatory response to HS and other forms of sterile injury.6–8

TLR4, the receptor for the bacterial endotoxin lipopolysaccharide, is expressed on platelets, and the effect of lipopolysaccharide signaling on platelet function has been extensively studied.9–14 Through TLR4, platelets act as inflammatory sentinels, surrounding and isolating an infection, while modulating proinflammatory cytokine release. These events endow platelet TLR4 with a pivotal role in sepsis.15,16 However, to date, no work has focused on the function of platelet TLR4 in HS, where activation of TLR4 has also been shown to play a vital role.17–21

In the present work, we report on investigations into the role of TLR4 on the activation of platelets, as well as the role of platelet TLR4 on organ injury and inflammatory cytokine production after HS with resuscitation (HS-R). Using a platelet-specific TLR4 knockout mouse, we demonstrate that TLR4 is necessary for platelet activation and functional changes induced by HS-R as well as for coagulation abnormalities. Furthermore, selective deletion of TLR4 from platelets was sufficient to

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reduce platelet sequestration and organ injury as well as markers of systemic inflammation. Taken together, these data demonstrate a novel and dominant role for TLR4 on platelets and identify a key mechanistic component in the pathogenesis of inflammation and end-organ dysfunction after HS.

Materials and Methods

Animal Care

Animal handling and care complied with published regulations by the National Institutes of Health and were approved by the Institutional Animal Care Committee of the University of Pittsburgh. All mice were 8 to 12 weeks old, weighed 25 to 30 g, and were maintained with a 12:12-hour light–dark cycle and free access to standard laboratory chow and water. Male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Generation of TLR4<sup>loxP/loxP</sup> and Cellular-Specific TLR4<sup>−/−</sup> Mice

TLR4<sup>loxP/loxP</sup> mice were generated as we have previously described. Transgenic mice expressing cre recombinase linked to the platelet factor 4 (Pf4), albumin (Alb), lysozyme (Lyz), and CD11c (cd11c) were obtained from Jackson Laboratory. TLR4<sup>loxP/loxP</sup> mice were interbred with stud transgenic males to generate cellular-specific deletion of TLR4 starting the following 5 cellular-specific groups: (1) wild-type (WT) control (TLR4<sup>Alb-loxP/loxP</sup>), (2) platelet specific (TLR4<sup>Pf4-loxP/loxP</sup>), (3) hepatocyte specific (TLR4<sup>Alb-loxP/loxP</sup>), (4) macrophage specific (TLR4<sup>Lyz-loxP/loxP</sup>), and (5) dendritic cell specific (TLR4<sup>cd11c-loxP/loxP</sup>). Genotyping was performed using standard genetic polymerase chain reaction genotyping techniques. Mice that were globally deficient in TLR4 (TLR4<sup>−/−</sup>) were generated as described.

HS Model

To study the influence of HS models on platelet function, WT mice were divided into the following 3 groups (n=8–10): (1) sham (control), (2) HS, and (3) HS and resuscitation (HS-R).

HS was performed as we have previously described. Briefly, bilateral inguinal dissections were performed, a small femoral arteriotomy was made, and the femoral arteries were cannulated with sterile polyethylene catheter. Animals were hemorrhaged to a mean arterial pressure of 25 mm Hg for 120 minutes. The contralateral cannula was used for continuous hemodynamic monitoring. For the resuscitation group, animals were transfused with 3 times the volume of shed blood used for continuous hemodynamic monitoring. For the resuscitation group, animals were transfused with 3 times the volume of shed blood used for continuous hemodynamic monitoring.

Sample Collection

At the conclusion of the experiment, animals were anesthetized with isoflurane and blood was harvested by cardiac puncture. Serum was collected by centrifugation at 13,000g (10 minutes, 4°C). The same liver and lung lobe were harvested, snap-frozen in liquid nitrogen or treated with 2-methylbutane, and then stored at −80°C. The liver and lung were also removed and fixed in 10% neutral formalin for hematoxylin and eosin staining. For immunofluorescence staining, the liver and lung were flushed with PBS and then perfused with 2% paraformaldehyde followed by standard sucrose treatment.

Thromboelastography Analysis

Platelet function and coagulopathy were measured by thromboelastography analysis using Haemoscope 5000 analyzers (Haemonetics, Braintree, MA). An aliquot of 340 μL sodium-citrated whole blood (3.2% sodium citrate added in a ratio of 1:9 [v/v]) was reconstituted with 20 μL of 0.2 mol/L CaCl<sub>2</sub> added to room temperature disposable thromboelastography cups containing 2.0 IU of heparinase I, and the thromboelastography was done simultaneously on 2 channels.

Coagulation profiles (which plot clot amplitude versus time) and clotting velocity curves (first derivative of the amplitude curve) were generated using thromboelastography software (version 4.2.3, Haemonetics). Maximal amplitude (MA), in millimeters was reported as a measurement of clot strength and platelet aggregation/function. Blood samples were obtained at the time of shock initiation and then after resuscitation, thus each mouse served as its own internal control.

Platelet Depletion

Recipient mice were treated with a platelet-depleting antibody (anti-CD41, BD Biosciences, San Diego, CA) at a final concentration of 1 μg/mL. Blood was collected in 200 μL sterile sodium saline (0.9% wt/vol sodium chloride) via penile vein. Injection of this mixture reduced the number of circulating platelets to <0.05×10<sup>6</sup>μL (normal value, 1.0–1.2×10<sup>6</sup>μL; data not shown) consistent with previously published values.

Platelet Isolation and Transfusion

Seventy-two hours after the platelet depletion, the entire circulating blood volume, 1.0 mL/mouse, was collected from donor mice into sterile tubes containing 0.1 mL acid citrate dextrose (Sigma, St. Louis, MO) and 1 μmol/L Prostaglandin E1 (Sigma). Platelet-rich plasma (PRP) was obtained from the mixture by centrifugation at 260g for 8 minutes and 260g for an additional 3 minutes, collecting supernatant after each centrifugation. The PRP was then spun at 740g for 10 minutes, and the pellet was then resuspended in 120 μL of sterile PBS.

Platelet concentration was determined using the Unopette collection system (Becton Dickinson, Franklin Lakes, NJ, 1:100 dilution) and counting the platelets with a hemocytometer. As reported previously, this method of platelet isolation results in <0.01% of leukocytes in the platelet suspensions. Platelets (value=1.0–1.2×10<sup>6</sup>μL) from 2 different strain donor mice were then diluted with PBS to a volume of 200 μL and transfused via penile vein into recipient mice just before the model. HS-R was performed on the transfused mice within 3 hours after transfusion (n=5 recipient mice per group).

Platelet Aggregation

Whole blood samples were used for aggregation studies. Blood was stimulated with 20 μmol/L ADP or 5 μg/mL collagen (Chronolog, Havertown, PA) as indicated, and platelet aggregation was assessed using a Chronolog Lumi-aggregometer (model 490). For HS-R experiments, blood was tested from the time of initial blood draw (pre-shock) or at the termination of resuscitation.

Flow Cytometry

PRP was isolated as described above from either sham or HS-R mice and was incubated with FITC-anti-mouse-TLR4, PE-anti-mouse-CD41, and APC-anti-mouse-CD62P (BD Bioscience, San Diego, CA) antibodies following the manufacturer’s protocol. After incubation, the platelets were washed in washing solution (PBS containing 0.1% sodium azide and 1% fetal bovine serum) and centrifuged for 5 minutes at 2000g. The pellet was resuspended with 500 μL platelet washing solution (PBS containing 2 mmol/L EDTA) and read on the Guava easyCyte 8HT flow cytometry system (Millipore, Billerica, MA) using Guava soft 2.2.2. For ex vivo activation studies, PRP was activated with ADP (20–100 μmol/L), thrombin (0.1–0.5 U/mL), or collagen (5 μg/mL).

Platelet Counting

Before and after the induction of HS-R, 300 μL whole blood was collected and circulating platelets counting was performed using an Advia 120 hematology analyzer (Bayer Diagnostics, Tarrytown, NY).

Western Blotting

PRP from TLR4<sup>loxP/loxP</sup> mice or TLR4<sup>Pf4-loxP/loxP-Cre</sup> mice was centrifuged at 800g for 20 minutes. Pellets were resuspended in platelet lysis buffer (20 mmol/L Tris [pH, 7.4], 150 mmol/L NaCl, 1% Triton X-100, and 1 mmol/L EDTA). Liver and lung samples were homogenized in 1×radioimmunoprecipitation assay buffer cell lysis buffer (Cell
Protein content of cell lysates was determined by bicinchoninic assay protein assay (Pierce, Rockford, IL). Equal protein amounts were separated by SDS-PAGE followed by immunostaining with optimized dilutions of a rabbit monoclonal anti-rabbit CD41 (Abcam, Cambridge, MA) or TLR4 (Abcam). Horseradish peroxidase-conjugated secondary antibodies were used in a standard enhanced chemiluminescence reaction according to manufacturer’s instructions (Pierce, Rockford, IL).

**Immunofluorescence**

Livers and lung were fixed in 2% paraformaldehyde solution and stained with rat anti-CD41 antibody and rabbit anti–p–actin antibody (Abcam, Cambridge, MA). Samples were washed using PBS+0.5% BSA followed by incubation in the appropriate Cy3 (1:1000, Invitrogen, Carlsbad, CA) and Cy5 (1:1000, Jackson Immunoresearch Laboratories, West Grove, PA) secondary antibodies. Samples were then thoroughly washed before incubation with Hoechst nuclear stain. Positively stained cells in 6 random fields were imaged with Olympus Fluoview 1000 microscopy (Olympus, Melville, NY).

**Histopathology**

Ethanol-fixed samples were processed in a Shandon Excelsior ES tissue processor (Thermo Scientific, Waltham, MA) with alcohol dehydration and xylene infiltration. Standard hematoxylin-eosin staining was performed to assess for necrosis and inflammatory infiltrate.

**Lung Myeloperoxidase Activity**

As a marker for neutrophil infiltration, myeloperoxidase activity in lung tissue lysates was determined with a mouse myeloperoxidase ELISA kit (Hycult Biotech, Plymouth Meeting, PA) according to the manufacturer’s protocol. Briefly, 10 mg of frozen lungs were homogenized in lysis buffer with a Tissue Tearor (Biospec Products, Bartlesville, OK) and then centrifuged at 1500g for 15 minutes. Myeloperoxidase activity was measured in the supernatant.

**Peritoneal Macrophage Isolation and Treatment**

Peritoneal macrophages were isolated from mice 4 days after the sterile intraperitoneal injection of 1 mL of 3% thioglycollate by washing the peritoneum with sterile PBS (5 mL) and culturing cells in DMEM (GIBCO, 11995). Adherent macrophages were cultured overnight before exposure to lipopolysaccharide (50 ng/mL) for 3 hours. Supernatant was isolated for analysis of cytokine production.

**Endotoxemia**

Endotoxemia was induced by intraperitoneal injection of lipopolysaccharide (Escherichia coli 0111:B4 purified by gel filtration chromatography, >99% pure, Sigma-Aldrich) at a dose of 3 mg/kg for 6 hours into 8-week-old male mice. Control animals received saline injections as vehicle alone. Serum was obtained for cytokine analysis after euthanasia.

**Assessment of Interleukin-6 Protein Levels**

Quantikine ELISA kits (R&D Systems, Minneapolis, MN) were used to determine interleukin-6 (IL-6) concentrations in cell supernatant or serum according to the manufacturer’s instructions. All samples were assayed in duplicate.

**Measurement of Serum Alanine Aminotransferase and Aspartate Aminotransferase Levels**

To assess hepatic function and cellular injury after HS-R, plasma levels of alanine aminotransferase and aspartate aminotransferase were measured using the Dri-Chem 7000 Chemistry Analyzer (Heska Co, Loveland, CO; slides from Fujifilm Japan).

**Statistical Analysis**

Results are expressed as means±SEM. Normality of sampled data was assessed using the Shapiro–Wilk test, and analysis was performed using SigmaPlot 11.0 (Systat Software, Point Richmond, CA). Comparisons of 2 groups under the same treatment were performed by Student t test, and 1-way ANOVA analysis was used for multiple group comparisons. Significance was established at $P<0.05$.

**Results**

**Platelet Function Is Impaired in HS-R Mouse Model**

To explore platelet function changes in HS and HS-R, coagulation was measured by whole-blood thromboelastography. MA was chosen as the primary parameter for assessing platelet function by thromboelastography. HS-R led to a significantly lower MA (MA=38.6±9.7 mm; $P<0.05$), indicating smaller and weaker clot formation compared with control mice (60.4±6.2 mm). This was not seen with HS without resuscitation where the MA value did not show significant changes compared with normal control (56.4±8.5 mm; $P>0.05$; Figure 1A). Representative thromboelastography tracings of each group are displayed (Figure 1B–1D). Thus, HS-R leads to a marked change in the coagulation profile primarily consistent with altered platelet function.

**TLR4 Contributes to Platelet Function Impairment in HS-R**

We next investigated whether TLR4 was involved in the impairment of platelet function in the HS-R mouse model. Whereas MA values dropped significantly in WT mice (38.6±9.7 mm; $P<0.05$), no significant changes of MA values were observed in global TLR4+/- mice subjected to HS-R compared with control (Figure 2A, tracings in 2B).

**Generation of TLR4+/-Pf4-cre Mice**

To study the role of TLR4 specifically on platelets, TLR4+/-Pf4-cre mice were generated as described above. Deficiency in platelet TLR4 was confirmed by Western blotting and flow cytometry.

**Figure 1.** Changes of platelet function in hemorrhagic shock mouse models. Thromboelastography was used to investigate platelet function via maximal amplitude (MA) after hemorrhagic shock (HS) or HS with resuscitation (HS-R). A, MA value significantly decreased in HS-R mice compared with control and HS alone. Data shown are means±SEM; n=8 to 10 mice/group. *P<0.05 vs control. B, Representative tracing of control mice. C, Representative tracing of HS-R mice. D, Representative tracing of HS group. x axis indicates time (minutes); and y axis, MA (millimeters).
TLR4 protein was detectable in lysates of TLR4loxP/loxP but not TLR4loxP/Pf4-cre platelets (Figure IA in the Data Supplement). Staining for TLR4 in TLR4loxP/loxP and TLR4loxP/Pf4-cre platelets demonstrated specific deletion in TLR4loxP/Pf4-cre platelets (Figure IB in the Data Supplement). Importantly, TLR4loxP/Pf4-cre mice showed intact TLR4 signaling capacity on other cell types confirming similar characterization performed by others.3 Intraperitoneal injection of lipopolysaccharide resulted in a significant increase in serum IL-6 levels in both TLR4loxP/loxP and TLR4loxP/Pf4-cre mice (TLR4loxP/loxP, 224±26 pg/mL versus TLR4loxP/Pf4-cre, 217±19 pg/mL; *P<0.05 vs control) confirming the finding of others.3 Furthermore, to show that TLR4 signaling is intact on other cell types, peritoneal macrophages harvested from both TLR4loxP/loxP and TLR4loxP/Pf4-cre mice were stimulated with lipopolysaccharide and demonstrated nonsignificant differences in IL-6 production (Figure IC in the Data Supplement).

Platelet TLR4 Contributed to Platelet Function Impairment in HS-R

To define cell-type specificity of TLR4 involved in platelet dysfunction in HS-R, 4 kinds of cell-specific TLR4−/− mice, including TLR4loxP/Pf4-cre mice (platelet), TLR4loxP/Pf4-cre mice (myeloid), TLR4loxP/AI4-cre mice (hepatocyte), TLR4loxP/CD11c-cre (dendritic cell) mice, and their control (TLR4loxP/loxP) mice, were subjected to HS-R and the thromboelastography was measured. The additional cell-specific deletion mice were included as controls to demonstrate that the effects of removal of TLR4 from platelets were specific to platelet function rather than an epiphenomenon of transgenic manipulation. As expected, significantly lower MA values were observed in the HS-R in TLR4loxP/Pf4-cre mice, TLR4loxP/Pf4-cre mice, and TLR4loxP/CD11c-cre mice compared with control mice, but HS-R failed to induce significant changes of MA values in TLR4loxP/Pf4-cre mice (Figure 3A), indicating a preservation of platelet function when TLR4 is removed specifically from platelets rather than other cell types (Figure 3B). To exclude that platelet function impairment was simply attributable to dilutional thrombocytopenia, circulating platelet counts were performed in WT, global TLR4−/−, TLR4loxP/loxP, and TLR4loxP/Pf4-cre mice subjected to HS-R or untreated control. Importantly, there were no significant differences of platelet counts (Figure 3C).

Platelet TLR4 Contributed to Platelet Activation in HS-R

We next assessed the activation of platelets isolated from TLR4loxP/Pf4-cre mice and control TLR4loxP/loxP mice subjected to HS-R. The activation state of the platelets was measured by examining surface CD62P expression using standard flow cytometry analysis. Figure 4A and 4B demonstrate that platelets from TLR4loxP/Pf4-cre mice did not show significantly increased CD62P expression in response to HS-R, as compared with TLR4loxP/loxP mice, suggesting that HS-R modulates platelet activation through platelet TLR4. Importantly, platelets lacking TLR4 expression were still capable of upregulating CD62P in response to thrombin (Figure 4C). Similar results were obtained using both collagen (%CD62p expression TLR4loxP/loxP: 41±5.8 versus TLR4loxP/Pf4-cre: 48±9.7; *P=NS).

Figure 2. Toll-like receptor 4 (TLR4) contributes to platelet function impairment in hemorrhagic shock with resuscitation (HS-R). A, Thromboelastography from wild-type (WT) mice and TLR4−/− subjected to HS-R or unmanipulated control. Maximal amplitude (MA) values showing reduction in WT but not TLR4−/− mice after HS-R. Data shown are means±SEM; n=8 to 10 mice/group. *P<0.05 vs control. B, Representative thromboelastography tracings. x axis indicates time (minutes); and y axis, MA (millimeters).

Figure 3. Platelet toll-like receptor 4 (TLR4) contributes to platelet function impairment in hemorrhagic shock with resuscitation (HS-R). A, Whole blood of cell-specific TLR4−/− mice were subjected to HS-R or unmanipulated control and analyzed by thromboelastography. Data shown are means±SEM; n=4 to 6 mice/group. *P<0.01, **P<0.02 vs control. B, Representative thromboelastography tracings. x axis indicates time (minutes); and y axis, maximal amplitude (MA; millimeters). C, Platelet counts from wild-type (WT), global TLR4−/−, TLR4loxP/loxP, and TLR4loxP/Pf4-cre mice after HS-R or control. Data shown are means±SEM; n=4 to 6 mice/group. *P<0.05 vs control.
and 50 μmol/L ADP (%CD62p expression TLR4loxP/loxP: 72±4.5 versus TLR4loxP/Pf4-cre: 68±8.2; *P<0.05 vs control).

Platelet Aggregation Is Impaired in HS-R and Regulated by TLR4

Platelet function following HS-R was assessed using platelet aggregometry. As shown in Figure 5A, ex vivo collagen treatment of blood isolated from unmanipulated mice shows equivalent aggregation response between TLR4-positive and -negative mice, indicating that TLR4-deficient platelets respond appropriately to traditional aggregation agonists. Similar results were obtained using 20 μmol/L ADP and 0.1 U/mL thrombin (data not shown). After HS-R, there was a substantial reduction in the area under the curve for WT mice, indicating impaired platelet aggregation (Figure 5B). Strikingly, however, there was no significant difference in aggregation after HS-R in mice lacking TLR4 on platelets. Taken together, these findings indicate impaired platelet function after HS-R that is not seen in TLR4-deficient mice.

Platelet TLR4 Contributed to Platelet Sequestration Into Liver and Lung in HS-R

To determine whether platelets localized in the liver and lung during HS-R, the expression of CD41 was assessed in TLR4loxP/loxP mice by Western blotting. In models of sepsis, platelets accumulate in the lung and liver where they may contribute to organ damage.28,29 CD41 significantly increased in HS-R group compared with unmanipulated control group in TLR4loxP/loxP mice. However, the expression of CD41 did not show significant changes in TLR4loxP/Pf4-cre mice between HS-R group and control group (Figure 6A and 6B). Platelets were also labeled with anti-CD41 antibody and examined by immunofluorescence in these tissues. In untreated control TLR4loxP/loxP mice, there was no accumulation of platelets into any tissues. However,

Figure 4. Assessment of platelet activation by flow cytometry. Platelet-rich plasma (PRP) from TLR4loxP/Pf4-cre and TLR4loxP/loxP mice was isolated and stained for CD41 and CD62p (100 μmol/L ADP). A, Percentage of CD62p-positive cells. Data shown are means±SEM; n=8 to 10 mice/group. *P<0.05 vs control. B, Representative dual parameter dot-plot figures obtained by flow cytometry for CD41 and CD62p. C, Treatment of PRP from both TLR4loxP/Pf4-cre and TLR4loxP/loxP mice with 0.1 U/mL thrombin confirms equal CD62p upregulation. Data shown are means±SEM; n=5 mice/group. P=NS between thrombin groups. HS-R indicates hemorrhagic shock with resuscitation.

Figure 5. Toll-like receptor 4 (TLR4) regulates platelet aggregation following hemorrhagic shock with resuscitation (HS-R). Aggregometry was performed on wild-type (TLR4+) or mice lacking TLR4 on their platelets (TLR4−). Results are expressed as the area under the curve (TLR4+/ CTL HS-R). A, Aggregometry from either control (CTL; preshock blood draw) vs HS-R (postshock). Collagen (5 μg/mL) was present as the agonist in all samples. n=5 mice/group. B, Representative aggregometry tracings from CTL and HS-R. Each y axis block represents 10% change, and each x axis block is 1 minute.

Figure 6. Platelet TLR4 regulates injury after hemorrhage. A, Percentage of CD62p-positive cells. Data shown are means±SEM; n=8 to 10 mice/group. *P<0.05 vs control. B, Representative dual parameter dot-plot figures obtained by flow cytometry for CD41 and CD62p. C, Treatment of PRP from both TLR4loxP/Pf4-cre and TLR4loxP/loxP mice with 0.1 U/mL thrombin confirms equal CD62p upregulation. Data shown are means±SEM; n=5 mice/group. P=NS between thrombin groups. HS-R indicates hemorrhagic shock with resuscitation.
a significant accumulation of platelets was noted in the liver and lung of TLR4loxP/loxP mice subjected to HS-R. In contrast, there was minimal platelet accumulation into the liver and lung of TLR4loxP/Pf4-cre mice both subjected to HS-R and untreated control (Figure 6C and 6D).

Platelet TLR4 Contributes to Organ Injury and Cytokine Release in HS-R

To assess the involvement of platelet TLR4 in the liver and lung injury, TLR4loxP/Pf4-cre and TLR4loxP/loxP mice were subjected to HS-R, and liver and lung injury was assessed by histology, circulating alanine aminotransferase and aspartate aminotransferase concentrations, and lung myeloperoxidase activity. As shown in Figure 7, HS-R induced significantly hepatic necrosis (arrows) and lung inflammatory injury in TLR4loxP/loxP mice as compared with control. Strikingly, HS-R caused minimal microscopic changes in liver and lung of TLR4loxP/Pf4-cre mice. Serum aspartate aminotransferase and alanine aminotransferase levels increased significantly after HS/R in TLR4loxP/loxP mice but not in TLR4loxP/Pf4-cre mice (Figure 7C and 7D). In addition, HS-R significantly increased the lung myeloperoxidase activity in TLR4loxP/loxP but not TLR4loxP/Pf4-cre mice (Figure 7E). In accordance with the reduced liver and lung injury, TLR4loxP/Pf4-cre mice had a marked reduction in systemic inflammation as measured by levels of IL-6 in the circulation after HS-R, as compared with TLR4loxP/loxP mice (Figure 7F).

Preshock Transfusion of TLR4-Negative Platelets Eliminates Coagulopathy and Organ Injury in HS-R

We next sought to establish whether TLR4 specifically on the platelet was necessary and sufficient to the development of coagulopathy and organ injury after HS-R using adoptive transfer. We reduced native platelets from WT (TLR4loxP/loxP) mice using an antiplatelet antibody to platelet counts of <0.05×10^6/μL (normal value, 1.0–1.2×10^6/μL). Platelets were then harvested from either WT or mice lacking TLR4 on platelets (TLR4loxP/PF4-cre) and transfused into thrombocytopenic recipients. After the transfusion, platelet values were consistently 0.5 to 0.7×10^6/μL, indicating that native platelets were <10% of circulating in the transfused recipients (data not shown). The tested groups of donor and recipient platelets are noted in Figure 8. For the purpose of clarity in reporting these data, TLR4loxP/loxP will be reported as LOXP and TLR4loxP/PF4-cre will be reported as PF4-cre alone. Mice with TLR4(+) platelets after transfusion developed significant coagulopathy (LOXPdonorLOXPrecipient, pre–HS-R MA: 57.3±4.2 mm versus post–HS-R MA: 36.1±6.8 mm; *P<0.01) which was not seen in mice lacking TLR4 on platelets (PF4-credonorPF4-crerecipient, pre–HS-R MA: 61.2±6.9 mm versus post–HR-R MA: 59.4±4.9 mm; P=NS). Transfusion of TLR4(+) platelets into thrombocytopenic TLR4(−) platelet mice resulted in a significant reduction in clot strength (Figure 8B). Strikingly, however, transfusion of TLR4(−) platelets completely reversed the coagulation abnormality seen in WT animals after HS-R (PF4-credonorLOXPrecipient, pre–HS-R MA: 58.7±5.9 mm versus post–HR-R MA: 60.1±8.1 mm; *P<0.05; Figure 8A and 8B). The effect of platelet transfusion was also reflected in the degree of liver injury, as mice transfused with TLR4(+) platelets had significantly elevated aspartate aminotransferase levels, whereas transfusion of TLR4(−) platelets reduced these effects (Figure 8C).

Figure 6. Effect of hemorrhagic shock with resuscitation (HS-R) on platelet sequestration into lung and liver. TLR4loxP/Pf4-cre and TLR4loxP/loxP mice were subjected to HS-R or unmanipulated control. Platelet sequestration into lung and untreated control of the mice were assessed by measuring the expression of CD41 using Western blotting and immunofluorescence. A, The expression of CD41 in the liver (A) and lung (B), analyzed by Western blotting. Data shown are means±SEM; n=8 to 10 mice/group. *P<0.05 vs control. Livers (C) and lungs (D) were stained with CD41 (green), F-actin (white) antibody, and counterstained with Hoechst to detect nuclei (blue) and imaged by confocal microscopy (Olympus FV1000 confocal; magnification, ×200). Representative images of 5 individual experiments.

Discussion

TLRs transmit danger signals to the innate immune system and mediate inflammatory events that can recruit and activate cells of the adaptive immune system to respond against pathogens.30 Many studies have identified TLR4 on platelets, and the presence of TLR4 on platelets is essential for the
lipopolysaccharide-induced thrombocytopenia, cytokine production, and platelet accumulation in the liver and lung. Both platelet activation and TLR4 signaling on other cell types have been shown to contribute to the pathogenesis of organ injury following HS-R, but the specific role of TLR4 expressed on platelets had not previously been addressed.

Our results demonstrate that platelet function is impaired in the mouse model of HS-R, as measured by thromboelastography and platelet aggregometry assays. Thromboelastography not only provides comprehensive clotting profile analysis, but is also a reliable indicator of clot strength and platelet function using the MA. The finding of reduced MA in HS-R is directly supported by a substantial impairment in platelet aggregation after HS-R. Further analysis using global TLR4−/− mice revealed that TLR4 is essential for platelet function impairment in HS-R. Interestingly, platelet function was intact in TLR4loxP/Pf4-cre mice, whereas WT and transgenic, cell-specific deletion controls demonstrated severe functional impairment in HS-R. This suggests that at least a subset of platelets may be activated through TLR4 signaling after HS-R, although the functional significance of this remains unexplored. Collectively, these data indicate a critical role for platelet TLR4 in platelet function after HS-R. In support of this observation, we have shown further that transfusion of platelets lacking TLR4 into thrombocytopenic WT mice resulted in reversal of coagulopathy after HS-R as measured by thromboelastography. Interestingly, the deposition of platelets in the liver and lung, the increase of circulation IL-6 concentration, and liver and lung injury were also attenuated in TLR4loxP/Pf4-cre mice compared with control in response to HS-R. The reduced proinflammatory cytokine production likely represents an overall reduction in injury severity after HS-R as opposed to a direct effect of platelet TLR4 on IL-6 production, because the TLR4loxP/Pf4-cre mice demonstrated intact TLR4 signaling and IL-6 production after exposure to lipopolysaccharide.

Figure 7. Effect of hemorrhagic shock with resuscitation (HS-R) on lung and liver injury and cytokine release. TLR4loxP/Pf4-cre and TLR4loxP/loxP mice were subjected to HS-R or unmanipulated control, and liver and lung injury was assessed by histology, circulating aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations, lung myeloperoxidase (MPO) activity, and circulating interleukin-6 (IL-6). Liver (A) and lung (B) were sectioned and stained with hematoxylin and eosin, and tissue damage induced by HS-R was assessed by light microscopy (Nikon FX series). Black arrows show the regions of necrosis (magnification, ×100). Serum AST (C) and ALT (D) levels were assayed and are presented as means±SEM (n=8–10 mice/group). Aliquots of the mouse lung tissues were homogenized to prepare total protein samples, and myeloperoxidase levels were assayed by ELISA (E). The levels are presented as means±SEM (n=8–10 mice/group). Serum samples from mice treated as described above were used to assay IL-6 concentration by ELISA (F), and the levels are presented as means±SEM (n=8–10 mice/group). *P<0.05 vs control, #P<0.05 vs HS-R group of TLR4loxP/loxP mice.

HS-R causes a global ischemia/reperfusion injury which can result in systemic and end-organ inflammation and eventually organ failure. Strong evidence for a proinflammatory role for platelets exists, and platelet depletion has a beneficial effect on the outcome of ischemia/reperfusion injury. In the present study, impaired platelet function was only observed in HS-R mice, but not in mice subjected to HS alone. The result suggests that either adequate time had not elapsed in the HS alone group to alter platelet activation, or,
more likely, that events associated with reperfusion contribute to platelet activation.

Coagulation abnormalities are common in severe sepsis, and it has been suggested that the presence of TLR4 on platelets could be a link between disseminated intravascular coagulation and sepsis. Early coagulopathy is also a key component of HS-R. In the present study, mice deficient in TLR4 showed intact platelet function compared with the platelet impairment seen in WT mice when subjected to HS-R, suggesting that HS-R may also modulate platelet function in a TLR4-dependent manner. Similar observations have been reported in sepsis models where lipopolysaccharide-induced platelet aggregation was abolished by an anti–TLR4-blocking antibody or TLR4 knockout in mice. The beneficial effects of TLR4 knockout could be related to reduced recruitment of activated platelets into lungs and liver, where they could cause tissue damage. Indeed, platelets have the capacity to release many different inflammatory mediators to affect local tissue and other inflammatory cells. In addition, platelets can adhere to neutrophils or monocytes to induce transcellular biosynthesis between the 2 cell types to produce mediators that each cell is unable to synthesize alone. These mediators could cause ample tissue dysfunction and systemic inflammation. In support of this, TLR4 knockout animals had reduced neutrophil activity within the...
lung after HS-R compared with WT animals as measured by myeloperoxidase levels.

In summary, we have shown for the first time that platelet TLR4 contributes to platelet function impairment and the deposition of platelets in the liver and lung after hemorrhage. Furthermore, deletion of TLR4 specifically from platelets prevents the increase of circulating IL-6 and liver and lung injury after by HS-R. These results suggest that TLR4 expression on platelets may play a previously unrecognized role in inflammatory signaling and that platelet TLR4 signaling may contribute a link between hemostasis and inflammation in HS-R.

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Disclosures
None.

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**CLINICAL PERSPECTIVE**

The multifactorial role of platelets in response to inflammation is an evolving area of clinical interest. In addition to the known function in hemostasis, the platelet is now recognized as an innate immune cell that is subject to a complex signaling and response mechanism. In the present work, we describe that toll-like receptor 4 (TLR4) mediates the sterile injury response by platelets seen after hemorrhagic shock and resuscitation in mice. Using both thromboelastography and whole-blood aggregometry, we characterize the extent of platelet dysfunction after hemorrhage. Excessive platelet sequestration within lung and liver seem to be linked to organ injury in mice. Through a series of adoptive transfer studies and the use of transgenic deletion of TLR4 specifically from the platelet, we show here for the first time that TLR4 signaling on platelets regulates platelet function after hemorrhage. Deletion of TLR4 from the platelet reduced end-organ injury and prevented platelet deposition in lung and liver. Although confirmation in humans is needed, TLR4 signaling on platelets may represent an important link between platelet dysfunction and the sterile inflammation seen after severe hemorrhage.
Toll-Like Receptor 4 Regulates Platelet Function and Contributes to Coagulation Abnormality and Organ Injury in Hemorrhagic Shock and Resuscitation
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Supplemental Figure 1. Specific deletion of TLR4 in platelets. A: Platelets from TLR4^loxP/loxP^ and TLR4^loxP/Pf4-cre^ mice were isolated and lysates analyzed by western blotting for TLR4. B: Platelet rich plasma from TLR4^loxP/loxP^ and TLR4^loxP/Pf4-cre^ mice was isolated and stained for TLR4 and CD41. Representative dual parameter dot-plot figures obtained by flow cytometry are shown. C: LPS treatment of peritoneal macrophages isolated from TLR4^loxP/loxP^ and TLR4^loxP/Pf4-cre^ reveals intact TLR4 signaling with no significant differences in HS-R values between groups as measured by IL-6 concentration by ELISA. N=7 mice/group.