

TLR4 Regulates Platelet Function and Contributes to Coagulation Abnormality and Organ Injury in Hemorrhagic Shock and Resuscitation

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Abstract

Background - Growing evidence indicates that the presence of 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, however, remains unexplored.

Methods and Results - Using thromboelastography and platelet aggregometry, we demonstrate that platelet function is impaired in a mouse model of hemorrhagic shock with resuscitation (HS-R). Further analysis using cellular specific TLR4 deletion in mice revealed that platelet TLR4 is essential for platelet activation and function in HS-R 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 knock-out mice were protected from lung and liver injury and exhibited a marked reduction in systemic inflammation as measured by circulating IL-6 following HS-R.

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 hemorrhagic shock and resuscitation.

Key words: platelet, coagulopathy, inflammation, hemorrhagic shock, TLR4

Introduction

Platelets have been extensively studied as hemostatic regulators, and in the context of hemorrhagic shock 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 both as instigators of local and systemic inflammatory reactions and also participants in the inflammation that contributes to tissue injury.^{1,2} Platelets express a number of receptors common to other immune cells including members of the toll-like receptor family (TLRs).³⁻⁶ These receptors not only sense molecules of microbial origin but also molecules of host origin released by damage associated molecular pattern molecules (DAMPs).³⁻⁶ Several TLRs, including TLR2, TLR3, TLR4, and TLR9 contribute to the inflammatory response to hemorrhagic shock and other forms of sterile injury.⁶⁻⁸

TLR4, the receptor for the bacterial endotoxin lipopolysaccharide (LPS), is expressed on platelets and the effect of LPS 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 hemorrhagic shock, 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 following 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 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 following hemorrhagic shock.



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 Use and Care Committee of the University of Pittsburgh. All mice were 8–12 weeks old, weighed 25–30g, and were maintained with a 12:12-h 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^{loxP/loxP} and cellular specific TLR4^{-/-} mice

TLR4^{loxP/loxP} mice were generated as we have previously described.²² 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^{loxP/loxP} mice were interbred with stud transgenic males to generate cellular-specific deletion of TLR4 creating the following five cellular-specific groups: 1) Wild-type control (TLR4^{loxP/loxP}); 2) platelet specific (TLR4^{loxP/Pf4-cre});

3) hepatocyte specific (TLR4^{loxP/Alb-cre}); 4) macrophage specific (TLR4^{loxP/Lyz-cre}); and 5) dendritic cell specific (TLR4^{loxP/CD11c-cre}).

Genotyping was performed using standard genomic PCR genotyping techniques. Global TLR4^{-/-} mice were generated as described.²²

Hemorrhagic shock model

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

HS was performed as we have previously described.²³ Briefly, bilateral inguinal dissections were performed, a small femoral arteriotomy was made, and the femoral arteries were cannulated with sterile PE-10 catheter. Animals were hemorrhaged to a mean arterial pressure (MAP) of 25 mmHg for 120 min. The contralateral cannula was used for continuous hemodynamic monitoring. For the resuscitation group, animals were transfused with three times the volume of shed blood with Ringer lactate after HS and body temperature was maintained at 37°C. Animals were sacrificed 6 hours after HS-R and blood and tissues were collected for analysis. Control animals received cannulation only without hemorrhage.

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 13,000×g (10min, 4°C). The same liver and lung lobe was 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 (H&E) staining. For immunofluorescence staining, liver and lung were flushed with PBS and then perfused with 2% paraformaldehyde followed by standard sucrose treatment.

Thromboelastography (TEG) analysis

Platelet function and coagulopathy were measured by TEG analysis using Haemoscope 5000 analyzers (Haemonetics, Braintree, MA). An aliquot of 340 μ l sodium-citrated whole blood [(3.2% sodium citrate added in a ratio of 1:9 (v/v)] recalcified with 20 μ l of 0.2 mol/l CaCl_2 was added to room temperature disposable TEG cups containing 2.0 IU of Heparinase I, and the TEG were done simultaneously on two channels. Coagulation profiles (which plot clot amplitude vs. time) and clotting velocity curves (first derivative of the amplitude curve) were generated using TEG software (version 4.2.3, Haemonetics). Maximal amplitude (MA, in mm) was reported as a measurement of clot strength and platelet aggregation/function. Blood samples were obtained at the time of shock initiation and then following 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 μ g/g body weight, diluted in 200 μ l sterile normal saline (0.9% [wt/vol] sodium chloride) via penile vein. Injection of this mixture reduced the number of circulating platelets to less than $0.05 \times 10^6 \mu\text{L}$ (normal value, 1.0 to $1.2 \times 10^6 \mu\text{L}$) (data not shown) consistent with previously published values.²⁴

Platelet isolation and transfusion

Seventy-two hours after the platelet depletion, the entire circulating blood volume, approximately 1.0ml/mouse, was collected from donor mice into sterile tubes containing 0.1ml acid citrate dextrose(ACD, Sigma, St. Louis, MO) and 1 μ M Prostaglandin E1(PGE1,Sigma). Platelet-rich plasma (PRP) was obtained from the mixture by centrifugation at 260 \times g for 8 minutes and 260 \times g for an additional 3 minutes, collecting supernatant after each centrifugation. The PRP was then spun at 740 \times g for 10 minutes and the pellet was then re-suspended in 120 μ l 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.²⁵ Platelets (value=1.0 to 1.2 $\times 10^6$ μ L) from 2 same strain donor mice were then diluted with PBS to a volume of 200 μ l, and transfused via penile vein into recipient mice just prior to the model.²⁶ HS-R was performed on the transfused mice within 3h following transfusion (n=5 recipient mice per group).

Platelet aggregation:

Whole blood samples were utilized for aggregation studies. Blood was stimulated with 20 μ M adenosine diphosphate (ADP) or 5ug/ml collagen (Chronolog, Haverton, Pennsylvania) 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% FBS) and centrifuged for 5 minutes at 2,000×g. The pellet was resuspended with 500µl platelet washing solution (PBS containing 2 mM 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 (20-100µM), thrombin (0.1-0.5U/ml), or collagen (5µg/ml).

Platelet counting

Before and after the induction of HS-R, 300µl 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^{loxP/loxP} mice or TLR4^{loxP/Pf4-cre} mice was centrifuged at 800×g 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×RIPA cell lysis buffer (Cell Signaling, Danvers, MA). Protein content of cell lysates was determined by BCA protein assay (Pierce, Rockford, IL). Equal protein amounts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunostaining with optimized dilutions of a rabbit monoclonal anti-mouse 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-F-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 six 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 (MPO) activity in lung tissue lysates was determined with a mouse MPO ELISA kit (Hycult Biotech, Plymouth Meeting, PA) according to the manufacturer's protocol. Briefly, 10mg of frozen lungs were homogenized in lysis buffer with a Tissue Tearor (Biospec Products, Bartlesville, OK) and then centrifuged at 1500×g for 15 min. MPO 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 1ml of 3% thioglycollate by washing the peritoneum with sterile PBS (5ml) and culturing cells in DMEM (GIBCO, 11995). Adherent macrophages were cultured overnight prior to exposure to LPS (50ng/ml, 3hrs). Supernatant was isolated for analysis of cytokine production.

Endotoxemia

Endotoxemia was induced by intraperitoneal injection of lipopolysaccharide (LPS) (*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 following sacrifice.

Assessment of IL-6 protein levels

Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) were used to determine IL6 concentrations in cell supernatant or serum according to the manufacturer's instructions. All samples were assayed in duplicate.

Measurement of serum ALT and AST levels

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

Statistical analysis

Results are expressed as means±standard error (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 two groups under the same treatment were performed by Student's *t*-test, and one-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 TEG. Maximum amplitude was chosen as the primary parameter for assessing platelet function by TEG.²⁷ HS-R led to a significantly lower maximum amplitude (MA=38.6±9.7mm, $p < 0.05$), indicating smaller and weaker clot formation compared to control mice (60.4±6.2mm). This was not seen with HS without resuscitation where the MA value did not show significant changes compared to normal control (56.4±8.5mm, $p > 0.05$) (Figure 1A). Representative TEG tracings of each group are displayed (Figure 1B-D). 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±9.7mm, $p < 0.05$), no significant changes of MA values were observed in global TLR4^{-/-} mice subjected to HS-R compared to control (Figure 2A, tracings in 2B).

Generation of TLR4^{loxP/Pf4-cre} mice

To study the role of TLR4 specifically on platelets, TLR4^{loxP/Pf4-cre} mice were generated as

described above³. Deficiency in platelet TLR4 was confirmed by Western blotting and flow cytometry. TLR4 protein was detectable in lysates of TLR4^{loxP/loxP} but not TLR4^{loxP/Pf4-cre} platelets (Supplemental Figure 1A). Staining for TLR4 in TLR4^{loxP/loxP} and TLR4^{loxP/Pf4-cre} platelets demonstrated specific deletion in TLR4^{loxP/Pf4-cre} platelets (Supplemental Figure 1B). Importantly, TLR4^{loxP/Pf4-cre} mice showed intact TLR4 signaling capacity on other cells types confirming similar characterization performed by others³. Intraperitoneal injection of LPS, resulted in a significant increase in serum IL-6 levels in both TLR4^{loxP/loxP} and TLR4^{loxP/Pf4-cre} mice (TLR4^{loxP/loxP} 224±26 pg/ml vs TLR4^{loxP/Pf4-cre} 217±19 pg/ml, p=NS) confirming the finding of others³. Furthermore, to show that TLR4 signaling is intact on other cell types, peritoneal macrophages harvested from both TLR4^{loxP/loxP} and TLR4^{loxP/Pf4-cre} mice were stimulated with LPS and demonstrated non-significant differences in IL-6 production (Supplemental Figure 1C).

Platelet TLR4 contributes to platelet function impairment in HS-R

To define cell type specificity of TLR4 involved in platelet dysfunction in HS-R, four kinds of cell specific TLR4^{-/-} mice, including TLR4^{loxP/Pf4-cre} mice (platelet), TLR4^{loxP/Lyz-cre} mice (myeloid), TLR4^{loxP/Alb-cre} mice (hepatocyte), TLR4^{loxP/CD11c-cre} (dendritic cell) mice and their control (TLR4^{loxP/loxP}) mice were subjected to HS-R and the TEG 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 TLR4^{loxP/Lyz-cre} mice, TLR4^{loxP/Alb-cre} mice and TLR4^{loxP/CD11c-cre} mice compared with control mice, but HS-R failed to induce significant changes of MA values in TLR4^{loxP/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 due to dilutional thrombocytopenia, circulating platelet counts were performed in WT, global TLR4^{-/-}, TLR4^{loxP/loxP} and TLR4^{loxP/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 TLR4^{loxP/Pf4-cre} mice and control TLR4^{loxP/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 B demonstrate that platelets from TLR4^{loxP/Pf4-cre} mice did not show significantly increased CD62P expression in response to HS-R, as compared with TLR4^{loxP/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 TLR4^{loxP/loxP}: 41±5.8 vs TLR4^{loxP/Pf4-cre}: 48±9.7, p=NS) and 50uM ADP (%CD62p expression TLR4^{loxP/loxP}: 72±4.5 vs TLR4^{loxP/Pf4-cre}: 68±8.2, p=NS).

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



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deficient platelets response appropriately to traditional aggregation agonists. Similar results were obtained using 20uM ADP and 0.1U/ml thrombin (data not shown). Following 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 following HS-R in mice lacking TLR4 on platelets. Taken together, these findings indicate impaired platelet function following 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 liver and lung during HS-R, the expression of CD41 was assessed in TLR4^{loxP/Pf4-cre} mice and TLR4^{loxP/loxP} mice by western blotting. In models of sepsis, platelets accumulate in lung and liver where they may contribute to organ damage.^{28,29} CD41 significantly increased in HS-R group compared to unmanipulated control group in TLR4^{loxP/loxP} mice. However, the expression of CD41 did not show significant changes in TLR4^{loxP/Pf4-cre} mice between HS-R group and control group (Figure 6A,B). Platelets were also labeled with anti-CD41 antibody and examined by immunofluorescence in these tissues. In untreated control TLR4^{loxP/loxP} mice, there was no accumulation of platelets into any tissues. However, a significant accumulation of platelets was noted in liver and lung of TLR4^{loxP/loxP} mice subjected to HS-R. In contrast, there was minimal platelet accumulation into the liver and lung of TLR4^{loxP/Pf4-cre} mice both subjected to HS-R and untreated control (Figure 6C,D).

Platelet TLR4 contributes to organ injury and cytokine release in HS-R

To assess the involvement of platelet TLR4 in liver and lung injury, TLR4^{loxP/Pf4-cre} mice and TLR4^{loxP/loxP} mice were subjected to HS-R and liver and lung injury was assessed by histology,

circulating ALT and AST concentrations, and lung MPO activity. As shown in Figure 7, HS-R induced significantly hepatic necrosis (arrows) and lung inflammatory injury in TLR4^{loxP/loxP} mice as compared with control. Strikingly, HS-R caused minimal microscopic changes in liver and lung of TLR4^{loxP/Pf4-cre} mice. Serum AST and ALT levels increased significantly after HS/R in TLR4^{loxP/loxP} mice but not in TLR4^{loxP/Pf4-cre} mice (Figure 7C,D). Additionally, HS-R significantly increased the lung MPO activity in TLR4^{loxP/loxP} but not TLR4^{loxP/Pf4-cre} mice (Figure 7E). In accordance with the reduced liver and lung injury, TLR4^{loxP/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 TLR4^{loxP/loxP} mice (Figure 7F).

Pre-shock 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 following HS-R using adoptive transfer. We reduced native platelets from wild type (TLR4^{loxP/loxP}) mice using an anti-platelet antibody to platelet counts of less than $0.05 \times 10^6/\mu\text{L}$ (normal value, 1.0 to $1.2 \times 10^6/\mu\text{L}$). Platelets were then harvested from either wild type or mice lacking TLR4 on platelets (TLR4^{loxP/PF4cre}) and transfused into thrombocytopenic recipients. Post transfusion platelet values were consistently 0.5 - $0.7 \times 10^6/\mu\text{L}$, indicating that native platelets were less than 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, TLR4^{loxP/loxP} will be reported as LOXP and TLR4^{loxP/PF4cre} will be reported as PF4-cre alone. Mice with TLR4(+) platelets after transfusion

developed significant coagulopathy (LOXP^{donor}LOXP^{recipient}, pre-HS-R MA: 57.3±4.2mm vs post-HS-R MA: 36.1±6.8mm, p<0.01) which was not seen in mice lacking TLR4 on platelets (PF4-cre^{donor}PF4-cre^{recipient}, pre-HS-R MA: 61.2±6.9mm vs post-HR-R MA: 59.4±4.9mm, 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 wild type animals after HS-R (PF4-cre^{donor}LOXP^{recipient}, pre-HS-R MA: 58.7±5.9mm vs post-HR-R MA: 60.1±8.1mm, p<0.05) (Figure 8A,B). The effect of platelet transfusion was also reflected in the degree of liver injury, as mice transfused with TLR4(+) platelets had significantly elevated AST levels, while transfusion of TLR4(-) platelets reduced these effects (Figure 8C).

Discussion

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Toll-like receptors 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 LPS- induced thrombocytopenia, cytokine production and platelet accumulation in the liver and lung.^{11,14,31} Both platelet activation and TLR4 signaling on other cell types have been shown to contribute to the pathogenesis of organ injury following hemorrhagic shock and resuscitation,^{2,18-20} however, 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 TEG and platelet aggregometry assays. TEG provides comprehensive clotting profile analysis but is also a reliable indicator of clot strength and platelet function using the maximum amplitude.²⁷ The finding of reduced MA in HS-R is directly supported by a substantial impairment in platelet aggregation following 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 TLR4^{loxP/Pf4-cre} mice while wild-type and transgenic, cell specific deletion controls demonstrated severe functional impairment in HS-R, indicating the specificity of our findings to platelet TLR4 as opposed to other cell types. HS-R also resulted in an up-regulation of CD62P, a marker of platelet activation, in wild type mice while activation was significantly mitigated in TLR4^{loxP/Pf4-cre} mice. This suggests that at least a subset of platelets may be activated through TLR4 signaling following HS-R, although the functional significance of this remains unexplored. Collectively, these data indicate a critical role for platelet TLR4 in platelet function following HS-R. In support of this observation, we have shown further that transfusion of platelets lacking TLR4 into thrombocytopenic wild type mice resulted in reversal of coagulopathy following HS-R as measured by TEG. Interestingly, the deposition of platelets in liver and lung, the increase of circulation IL-6 concentration, and liver and lung injury were also attenuated in TLR4^{loxP/Pf4-cre} mice compared with control in response to HS-R. The reduced pro-inflammatory cytokine production likely represents an overall reduction in injury severity following HS-R as opposed to a direct effect of platelet TLR4 on IL-6 production, as the TLR4^{loxP/Pf4-cre} mice demonstrated intact TLR4 signaling and IL-6 production following exposure to LPS³. These data for the first time suggest that platelet TLR4 is necessary and

sufficient for the development of platelet dysfunction and coagulopathy following HS-R and may regulate the accompanying systematic inflammation and organ injury.

HS-R causes a global ischemia-reperfusion injury which can result in systemic and end-organ inflammation and eventually organ failure.^{32,33} Strong evidence for a proinflammatory role for platelets exists,³⁴ and platelet depletion has a beneficial effect on the outcome of ischemia/reperfusion injury.³⁵⁻³⁸ 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.^{39,40} Early coagulopathy is also a key component of HS-R.²⁷ In the present study, mice deficient in TLR4 showed intact platelet function compared to 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 LPS induced platelet aggregation was abolished by an anti-TLR4-blocking antibody or TLR4 knockout in mice.^{3,10,41}

Interestingly, platelets were shown to accumulate in the liver and lungs during HS-R in a TLR4 dependent manner, which was consistent with an inductive effect of HS-R on liver and lung injury and IL-6 production. Previously, the infusion of LPS into animals and humans has been shown to induce severe thrombocytopenia and platelet aggregation formation in the lung

and liver microvascular circulation,⁴¹ followed by degradation of the platelets and acute inflammation accompanied by tissue destruction.^{42,43} Furthermore, infused platelets from wild-type but not TLR4 knockout mice accumulate in the lung of LPS-treated wild-type mice.¹⁶ Despite the finding of increased CD62p expression and increased platelet sequestration following HS-R, a significant aggregation defect and coagulopathy was present, which may represent similar platelet response to severe sepsis, which presents with both microvascular thrombosis and systemic coagulopathy.^{16,39} Although we did not establish the ligand or mechanism of TLR4 activation in platelets in HS-R, we suggest in the present study that platelet TLR4 signaling is likely related to the cascade of endogenous mediators that has been extensively characterized.⁴⁴⁻⁴⁷ Candidates include any number of DAMP TLR4 agonists such as HMGB1 which is known to be involved in organ injury and inflammation in HS-R⁴⁸ or even LPS which can escape from the gut in injury.⁴⁹

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.^{1,2,7,8-12,14,28} In addition, platelets can adhere to neutrophils or monocytes to induce transcellular biosynthesis between the two cell types to produce mediators that each cell is unable to synthesize alone.⁵⁰ These mediators could cause ample tissue dysfunction and systematic inflammation. In support of this, TLR4 knock-out animals had reduced neutrophil activity within the lung following HS-R compared to WT animals as measured by MPO levels.

In summary, we have shown for the first time that platelet TLR4 contributes to platelet function impairment, and the deposition of platelets in liver and lung following hemorrhage. Furthermore, deletion of TLR4 specifically from platelets prevents the increase of circulating IL-6, and liver and lung injury following 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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Conflict of Interest Disclosures: None.

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Figure Legends:



Figure 1. Changes of platelet function in hemorrhagic shock mouse models.

Thromboelastography (TEG) was used to investigate platelet function via maximal amplitude (MA) after HS or HS-R. A: MA value significantly decreased in HS-R mice compared with control and HS alone. Data shown are means \pm SEM, n = 8-10 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, time (min); y axis, maximum amplitude (mm).

Figure 2. TLR4 contributes to platelet function impairment in HS-R. A: TEG from wide type (WT) mice and TLR4^{-/-} subjected to HS-R or unmanipulated control. A: MA values showing reduction WT but not TLR4^{-/-} mice after HS-R. Data shown are means \pm SEM, n= 8-10 mice/group. *p<0.05 vs. control. B: Representative TEG tracings. x axis, time (min); y axis, maximum amplitude (mm).

Figure 3. Platelet TLR4 contributes to platelet function impairment in HS-R. A: Whole blood of cell specific TLR4^{-/-} mice were subjected to HS-R or unmanipulated control and analyzed by

TEG. Data shown are means \pm SEM, n= 4-6 mice/group. *p<0.01, **p<0.02 vs. control. B: Representative TEG tracings. x axis, time(min); y axis, maximum amplitude(mm). C: Platelet counts from WT, global TLR4^{-/-}, TLR4^{loxP/loxP} and TLR4^{loxP/Pf4-cre} mice following HS-R or control. Data are means \pm SEM, n= 4-6 mice/group. *p<0.05 vs. control.

Figure 4. Assessment of platelet activation by flow cytometry. Platelet rich plasma from TLR4^{loxP/Pf4-cre} and TLR4^{loxP/loxP} mice was isolated and stained for CD41 and CD62p (100uM ADP). A: Percentage of CD62p positive. Data are means \pm SEM, n = 8-10 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 TLR4^{loxP/Pf4-cre} and TLR4^{loxP/loxP} mice with 0.1U/ml thrombin confirms equal CD62p upregulation. Data are means \pm SEM, n = 5 mice/group. p=NS between thrombin groups.

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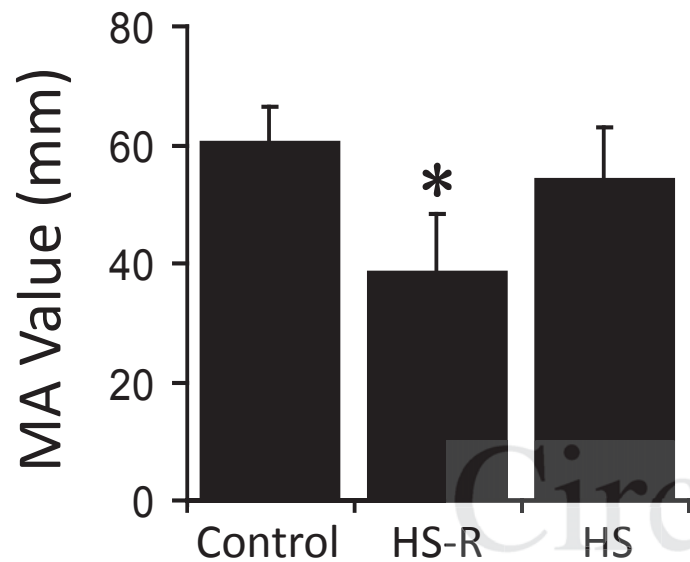
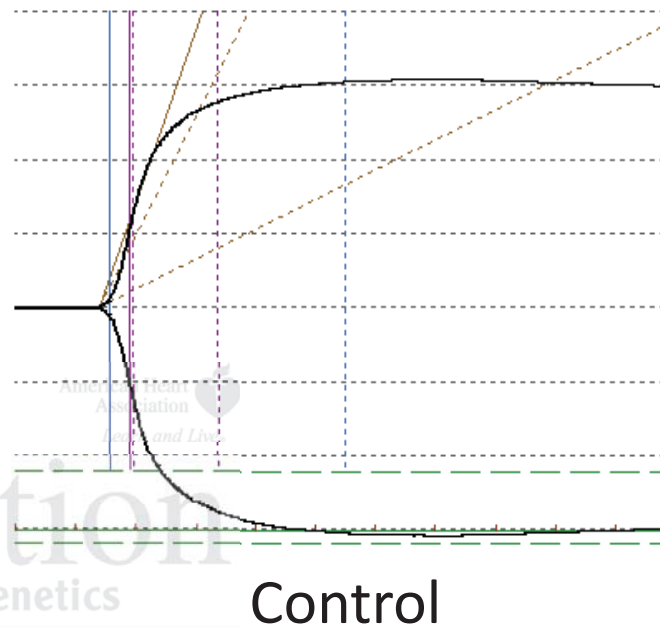
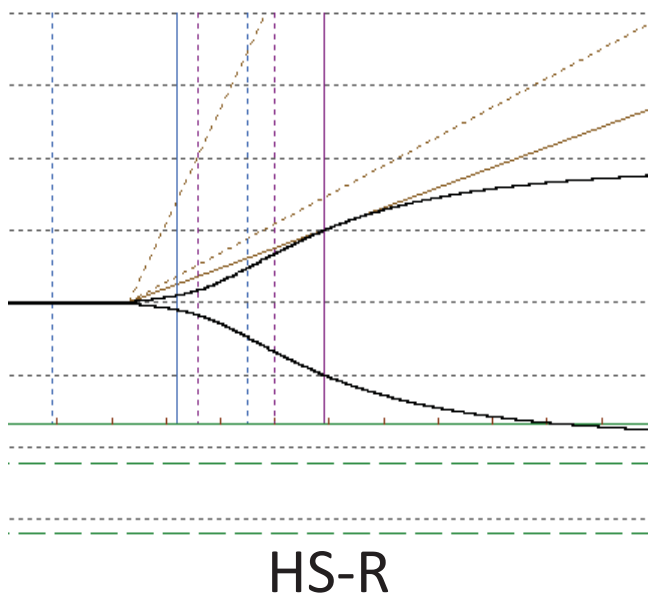
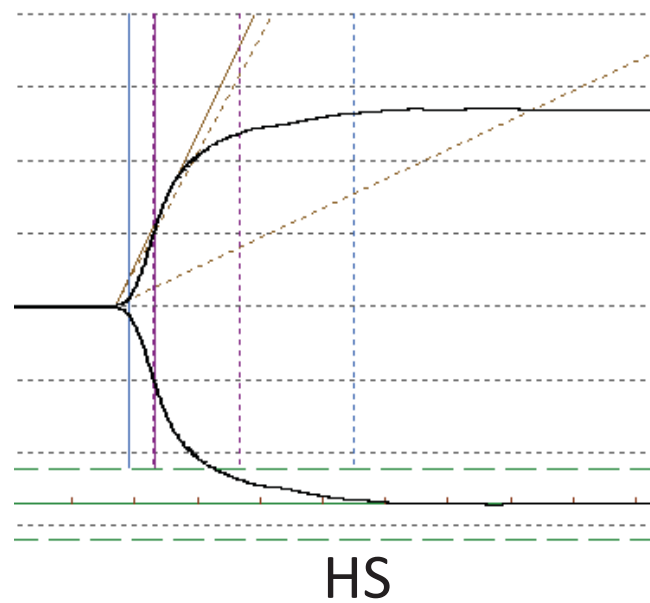
Figure 5. TLR4 regulates platelet aggregation following HS-R. Aggregometry was performed on wild-type (TLR4 +) or mice lacking TLR4 on their platelets (TLR4 -). Results are expressed as the area under the aggregometry curve, measured as arbitrary units/minute (AU*min). A: Ex vivo collagen (5ug/ml) treatment of platelets demonstrates no significant difference in aggregation between groups. N=8 mice/group. p=NS. B: Aggregometry from either control (pre-shock blood draw) vs HS-R (post-shock). Collagen (5ug/ml) was present as the agonist in all samples. N=5 mice/group. C: Representative aggregometry tracings from control and HS-R. Each y axis block represents 10% change and each x axis block is 1 minute.

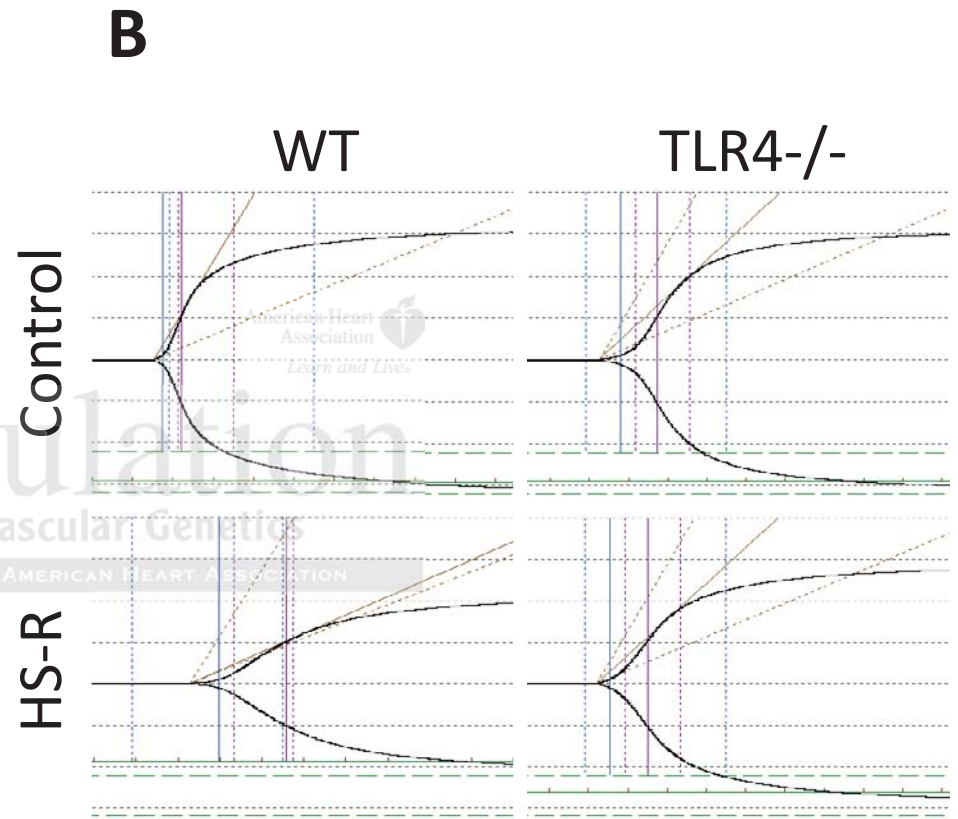
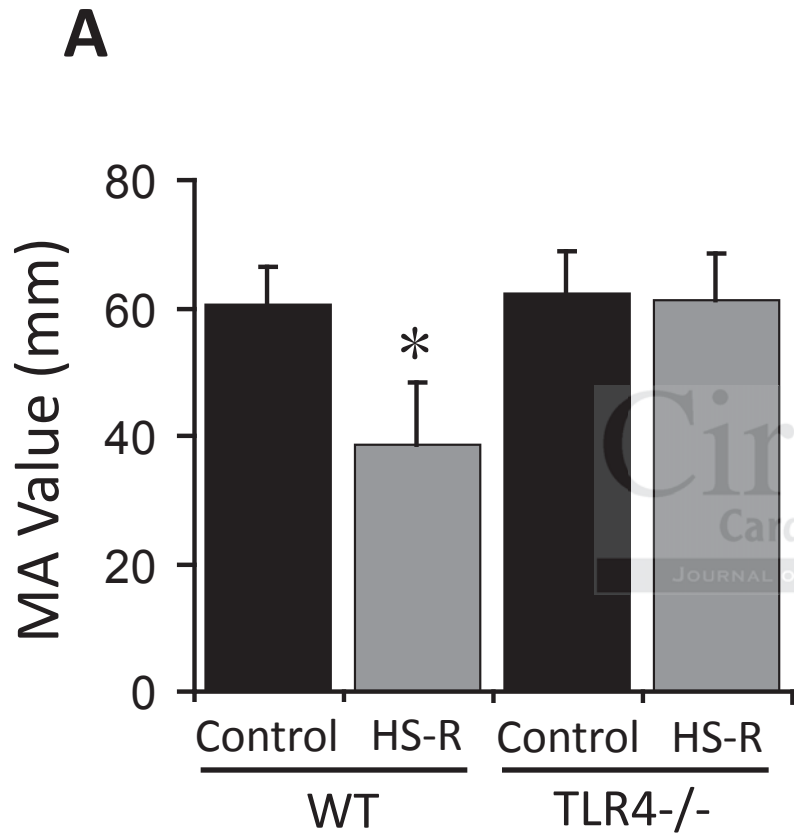
Figure 6. Effect of HS-R on platelet sequestration into lung and liver. TLR4^{loxP/Pf4-cre} mice and TLR4^{loxP/loxP} mice were subjected to HS-R or unmanipulated control. Platelet sequestration into lung and liver of the mice were assessed by measuring the expression of CD41 using western blotting and immunofluorescence. A: The expression of CD41 in liver (A) and lung (B), analyzed by Western blotting. Data are means \pm SEM, n = 8-10 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 x200). Representative images of five individual experiments.

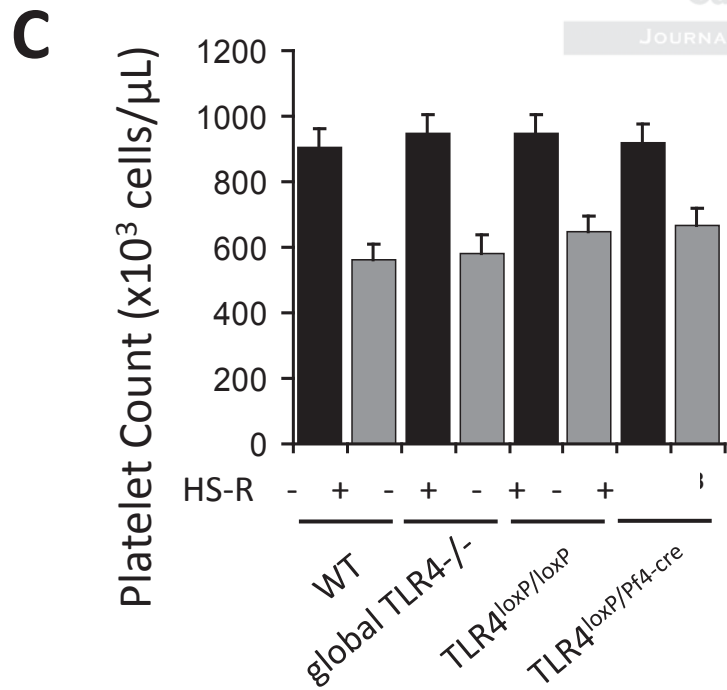
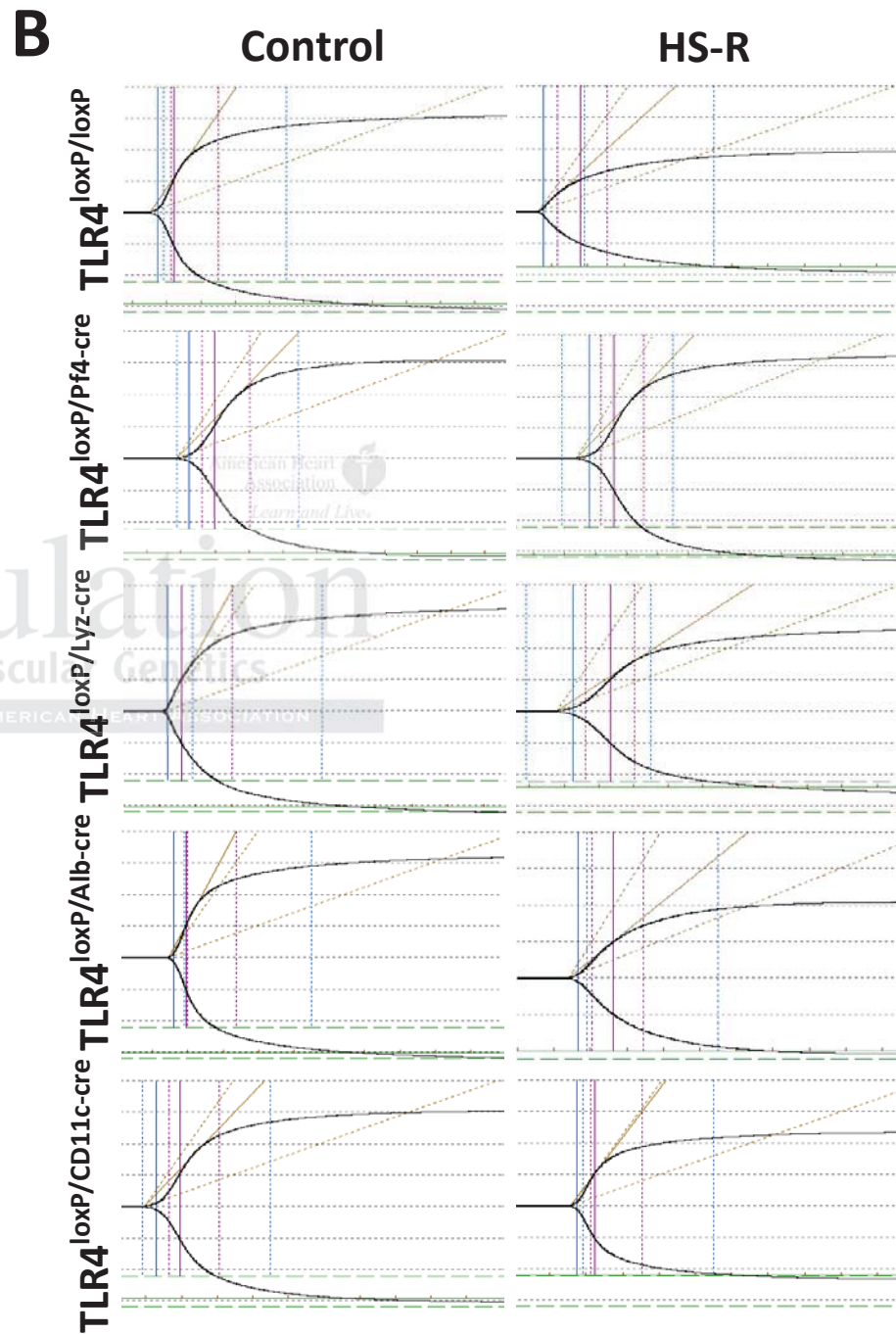
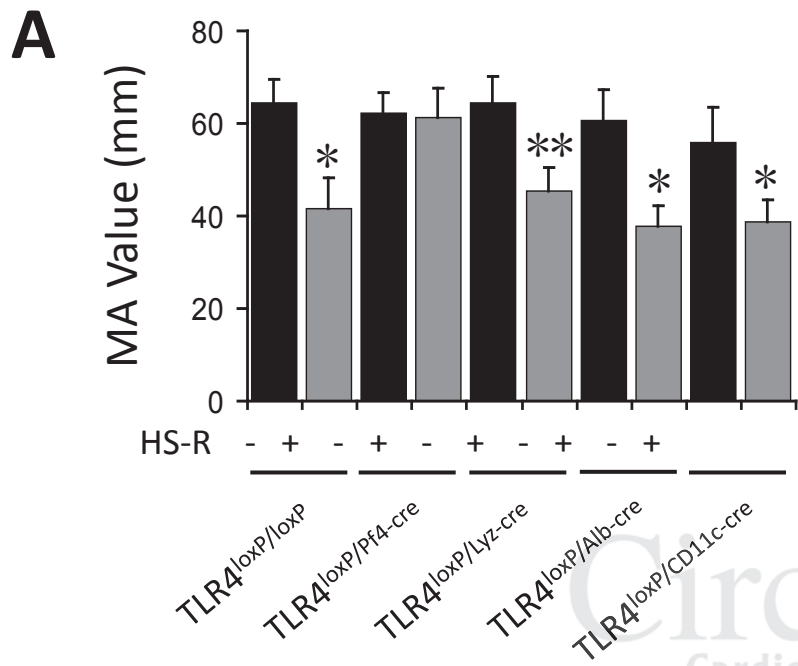
Figure 7. Effect of HS-R on lung and liver injury and cytokine release. TLR4^{loxP/Pf4-cre} mice and TLR4^{loxP/loxP} mice were subjected to HS-R or unmanipulated control and liver and lung injury was assessed by histology, circulating AST and ALT concentrations, lung MPO activity and circulating IL-6. Liver (A) and lung (B) were sectioned and stained with H&E and tissue damage induced by HS-R was assessed by light microscopy (Nikon FX series). Black arrows show the regions of necrosis. (Original magnification \times 100). Serum AST (C) and ALT (D) levels were assayed and are presented as means \pm SEM (n = 8-10 mice/group). Aliquots of the mouse lung tissues were homogenized to prepare total protein samples and MPO levels were assayed by ELISA (E). The levels are presented as means \pm 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 \pm SEM (n = 8-10 mice/group). *p<0.05 vs. control. #p<0.05 vs. HS-R group of TLR4^{loxP/loxP} mice.

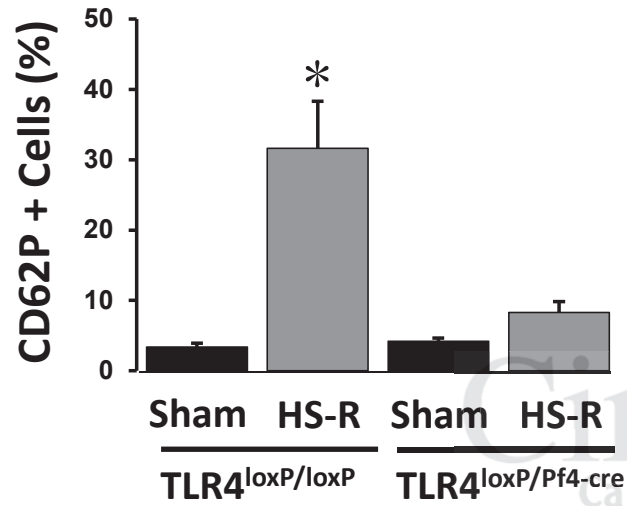
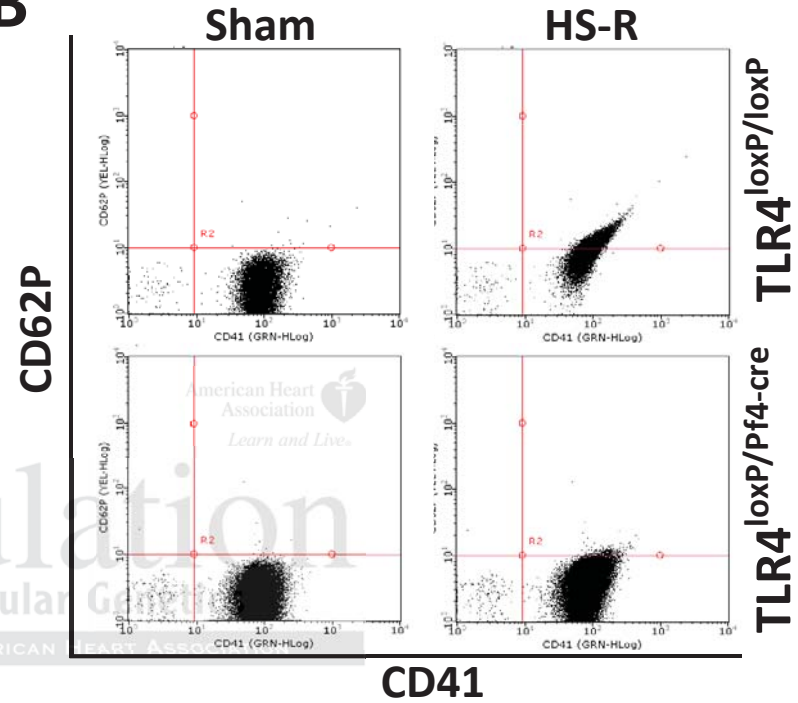
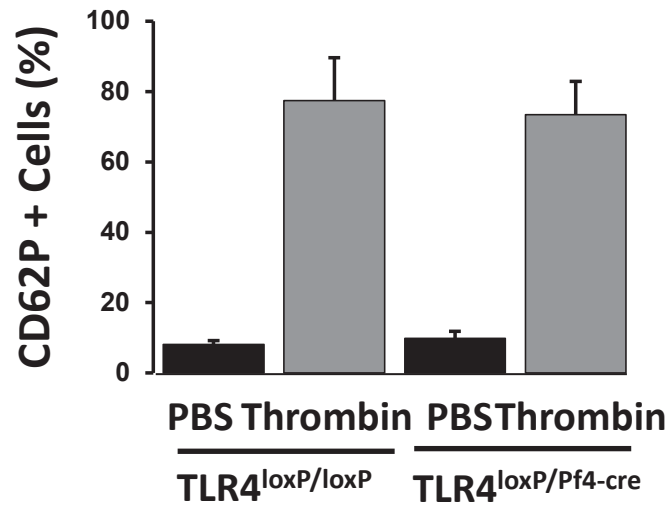
Figure 8. Platelet TLR4 is necessary and sufficient for the induction of platelet dysfunction and organ injury in HS-R. Wild-type (LOXP) and mice lacking TLR4 on platelets (PF4-cre) were depleted of their platelets using anti-CD41 antibody and subsequently transfused donor platelets isolated from either their littermates (LOXP^{trans}LOXP, PF4-cre^{trans}PF4-cre) as controls or from donors with opposite TLR4 status with respect to their platelets (LOXP^{trans}PF4-cre, PF4-cre^{trans}LOXP) to test the specific role of TLR4 on platelets in HS-R. (A) TEGs pre and post HS-R are shown and the MA are quantified in (B) as means \pm SEM (n = 5 mice/group). (C) rSerum AST levels including both sham and HS-R (means \pm SEM (n = 5 mice/group)). *p<0.01 vs. control. #p<0.05 vs. HS-R group of LOXP^{trans}LOXP mice.

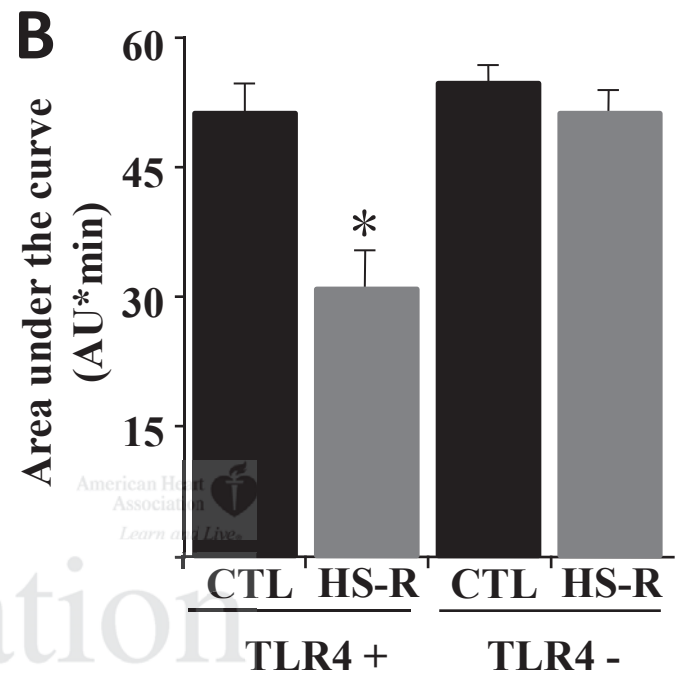
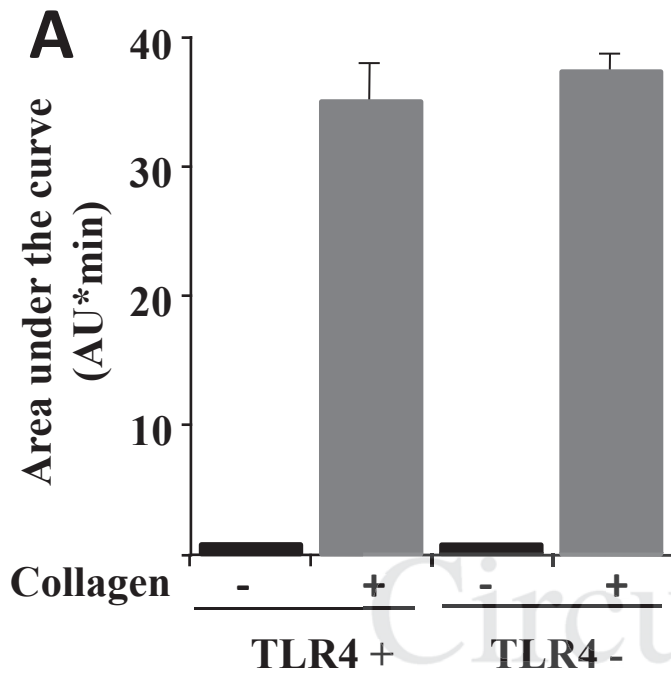


A**B****C****D**

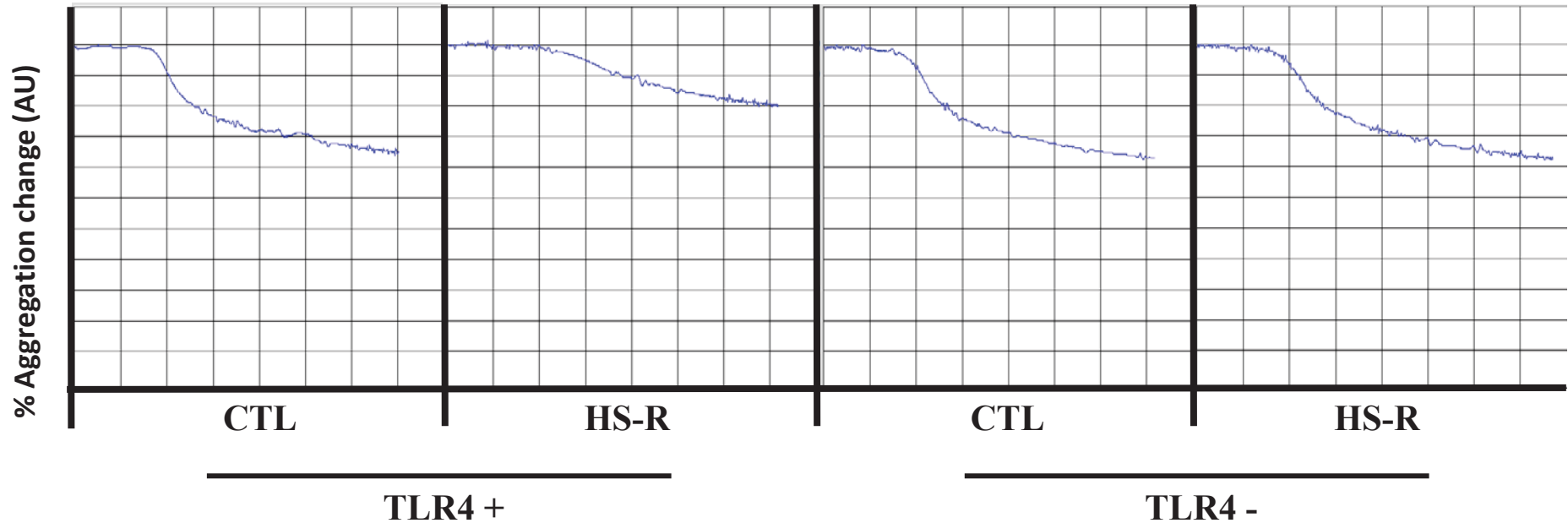


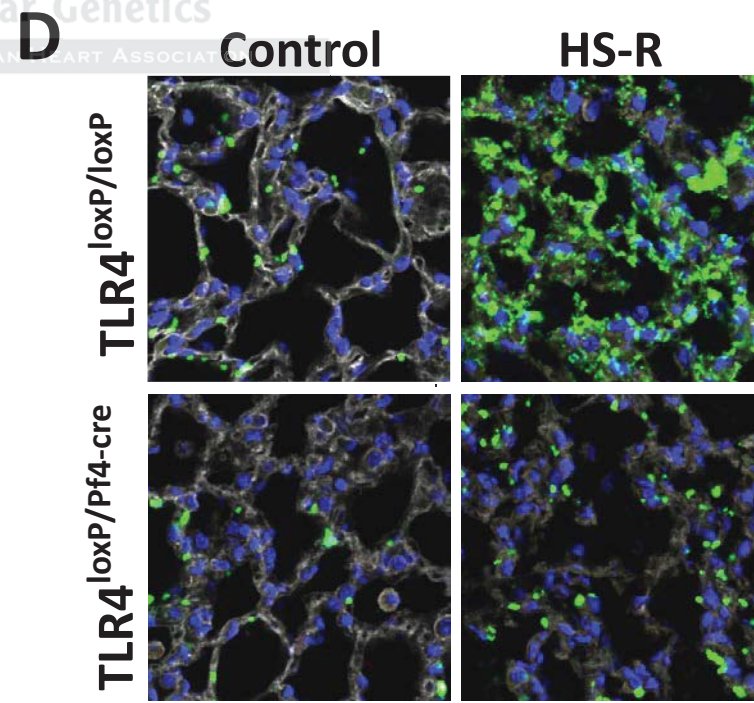
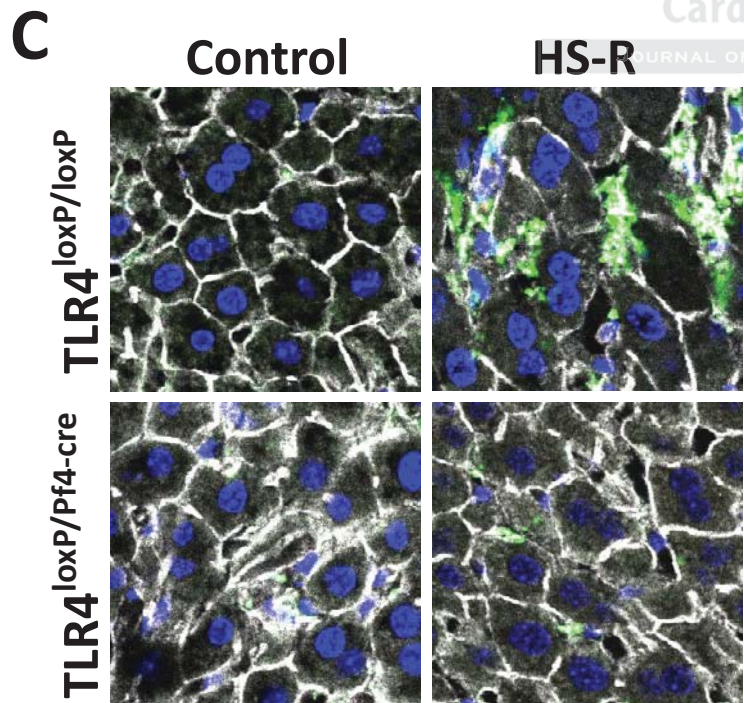
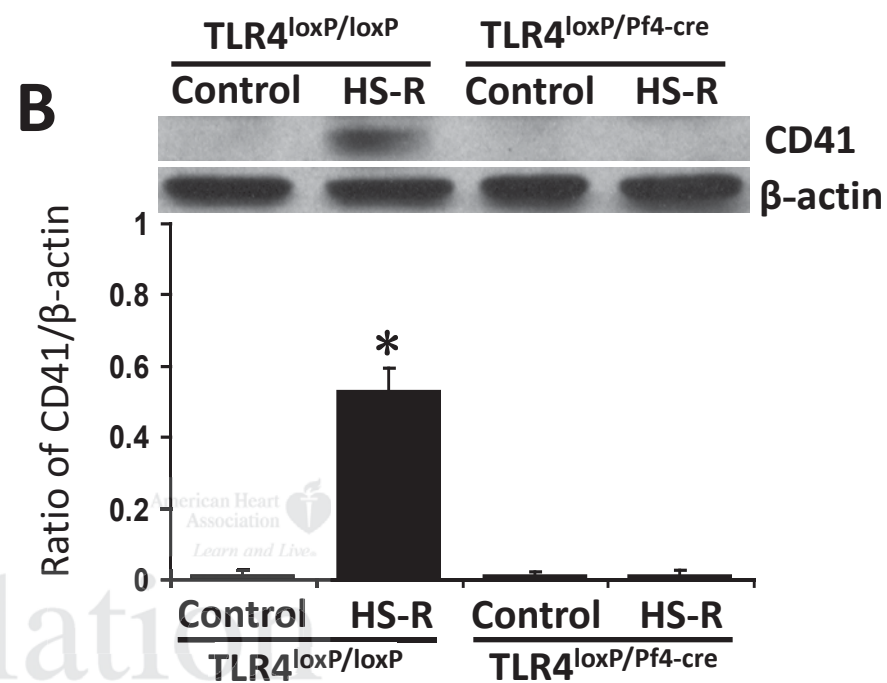
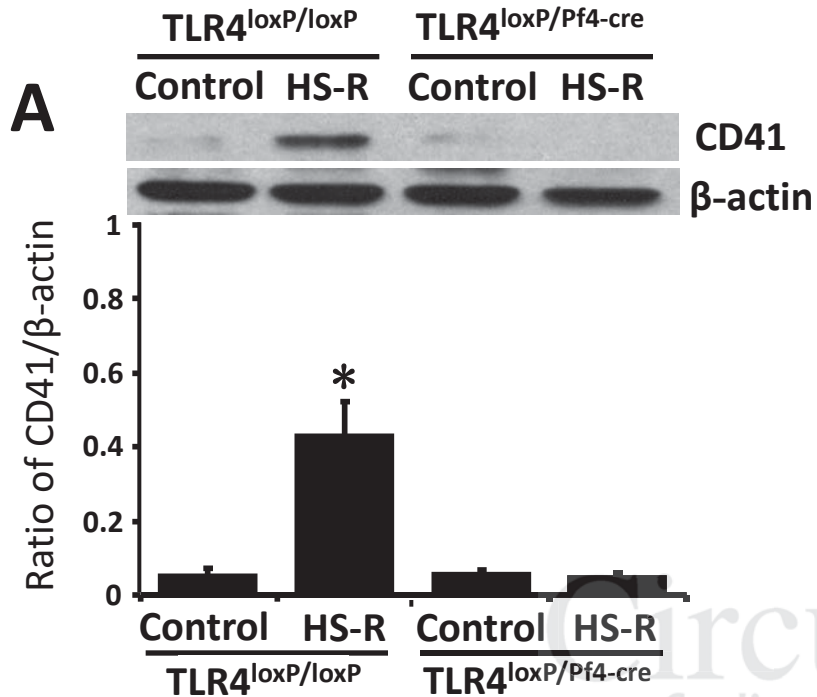


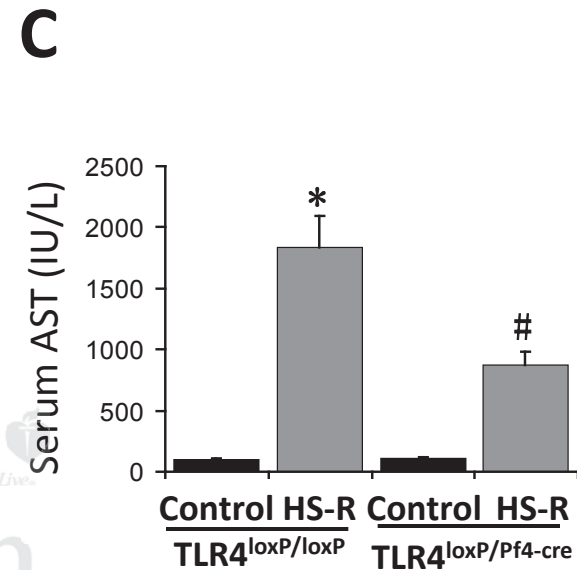
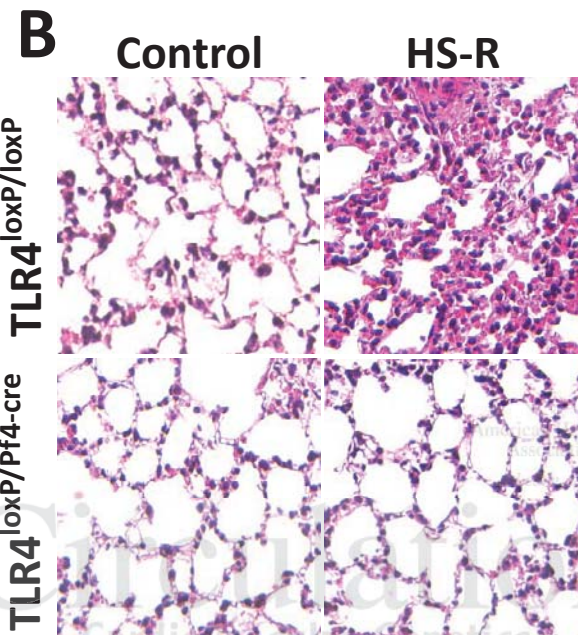
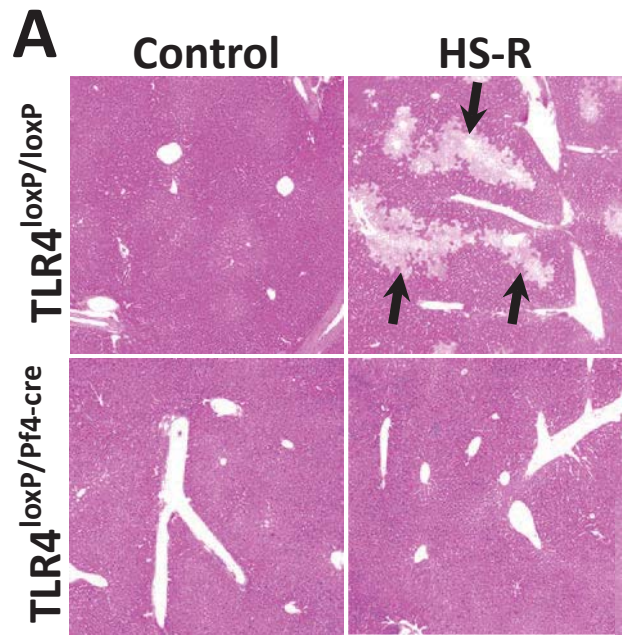
A**B****C**



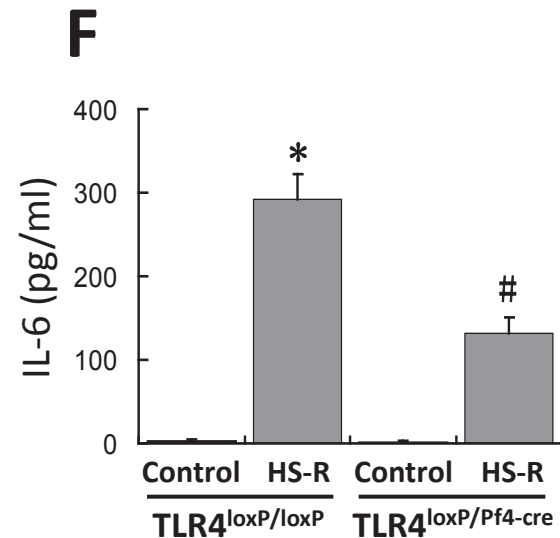
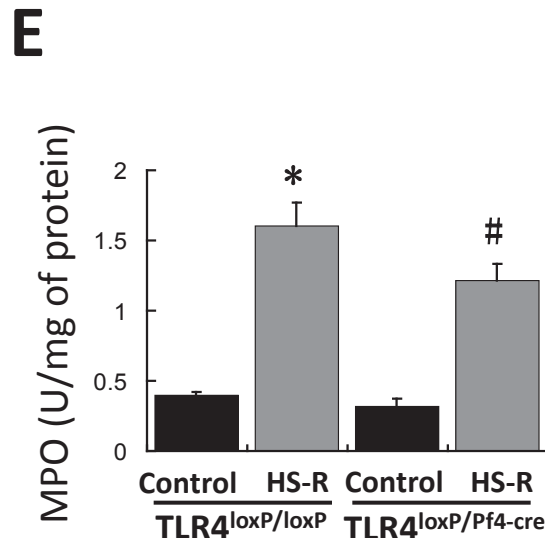
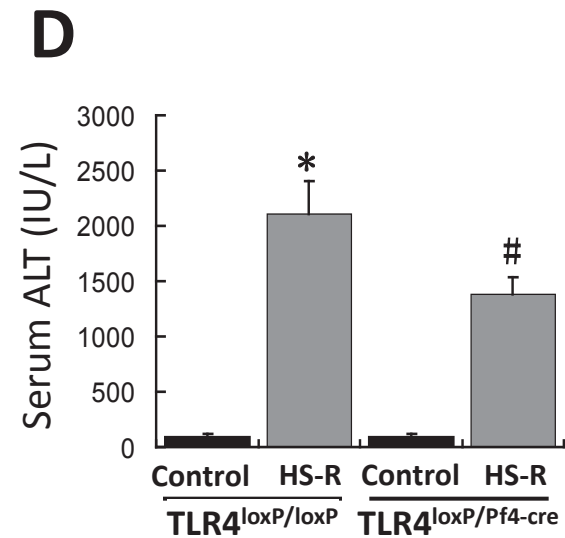
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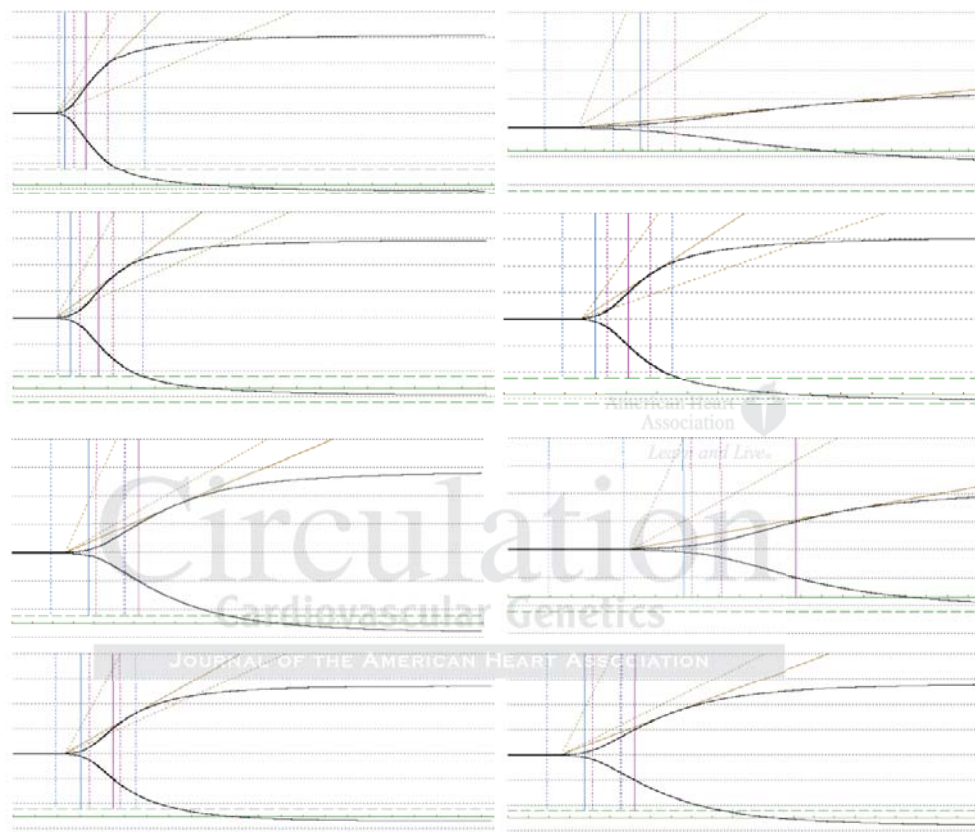
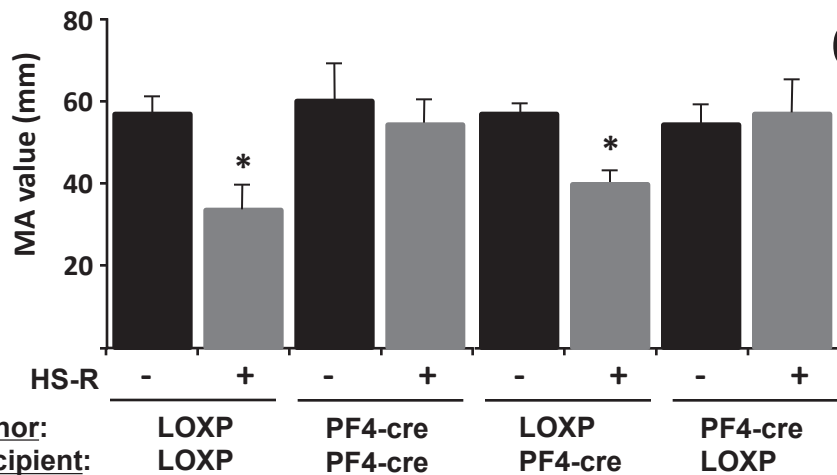
A**Control****HS-R**

Donor: LOXP
Recipient: LOXP

Donor: PF4-cre
Recipient: PF4-cre

Donor: LOXP
Recipient: PF4-cre

Donor: PF4-cre
Recipient: LOXP

**B****C**