



Role of Toll-like receptor 4 in intravascular hemolysis-mediated injury

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Abstract

Massive intravascular hemolysis is a common characteristic of several pathologies. It is associated with the release of large quantities of heme into the circulation, promoting injury in vulnerable organs, mainly kidney, liver, and spleen. Heme activates Toll-like receptor 4 (TLR4), a key regulator of the inflammatory response; however, the role of TLR4 in hemolysis and whether inhibition of this receptor may protect from heme-mediated injury are unknown. We induced intravascular hemolysis by injection of phenylhydrazine in wildtype and *Tlr4*-knockout mice. In this model, we analyzed physiological parameters, histological damage, inflammation and cell death in kidney, liver, and spleen. We also evaluated whether heme-mediated-inflammatory effects were prevented by TLR4 inhibition with the compound TAK-242, both *in vivo* and *in vitro*. Induction of massive hemolysis elicited acute kidney injury characterized by loss of renal function, morphological alterations of the tubular epithelium, cell death, and inflammation. These pathological effects were significantly ameliorated in the TLR4-deficient mice and in wildtype mice treated with TAK-242. *In vitro* studies showed that TAK-242 pretreatment reduced heme-mediated inflammation by inhibiting the TLR4/NF- κ B (nuclear factor kappa B) axis. However, analysis in liver and spleen indicated that TLR4 deficiency did not protect against the toxic accumulation of heme in these organs. In conclusion, TLR4 is a key molecule involved in the renal inflammatory response triggered by massive intravascular hemolysis. TLR4 inhibition may be a potential therapeutic approach to prevent renal damage in patients suffering from hemolysis.

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Introduction

Intravascular hemolysis is a common characteristic of different pathologies, such as sickle cell disease (SCD), spherocytosis, thrombotic microangiopathies, and malaria [1]. Regardless of the origin, destruction of red blood cells (RBCs) induces the release of large quantities of hemoglobin (Hb) and heme into the circulation. Cell-free Hb and heme are neutralized in plasma by the scavenger proteins haptoglobin and hemopexin [2]. However, massive or chronic intravascular hemolysis

depletes this protective system, resulting in the accumulation of toxic heme-derivatives in kidney, liver, and spleen.

The spleen and liver are involved in the scavenging of senescent RBCs, cell-free Hb, and heme as well as iron recycling [3]. However, massive intravascular hemolysis leads to red pulp fibrosis, inflammation, and functional asplenia [4]. In the liver, Hb and heme affect sinusoidal microcirculation and induces hepatocellular necrosis, causing chronic hepatopathy and cirrhosis [5,6]. The kidney is especially vulnerable to massive

hemolysis [1]. Renal Hb accumulation promotes acute kidney injury (AKI) and further chronic kidney disease (CKD), with the subsequent augmentation in morbidity and mortality [7]. Hemoprotein-induced AKI involves a combination of pathogenic mechanisms, including oxidative stress, cell death, inflammation, and vasoconstriction [8,9]. There is no specific treatment to impair heme-mediated toxicity during massive hemolytic crisis. Therefore, it is necessary to unravel the mechanisms underlying heme-mediated harmful effects to identify new therapeutic targets.

Heme is a damage-associated molecular pattern (DAMP) that activates the innate immune system through a receptor-mediated mechanism [10]. Specifically, Toll-like receptor 4 (TLR4) recognizes and is activated by heme [10]. Activation of TLR4 triggers nuclear factor kappa B (NF- κ B) translocation to the nucleus and the subsequent expression of proinflammatory cytokines and chemokines [11,12]. Several studies have demonstrated that TLR4 targeting improved renal function, ameliorated tubulointerstitial damage, and reduced proinflammatory signaling in experimental models of AKI, including ischemia–reperfusion [13], sepsis [14], cyclosporine nephrotoxicity [15], and rhabdomyolysis [16]. Genetic or pharmacological disruption of TLR4 signaling also reduced liver injury in experimental models of ischemia–reperfusion [17], cholestasis [18], and hepatic sinusoidal obstruction syndrome [19]. However, no previous study had analyzed the role of TLR4 in intravascular hemolysis and whether heme-mediated damage may be prevented by targeting TLR4. To answer these questions, we developed an experimental model of intravascular hemolysis in TLR4 knockout mice and animals treated with the TLR4 inhibitor TAK-242. We analyzed histological and functional changes in the main organs affected by massive intravascular hemolysis (kidney, liver, and spleen) and performed *in vitro* studies to characterize the intracellular pathways involved in heme-mediated harmful effects via TLR4.

Materials and methods

Animal models

Animal experiments were performed in accordance with the Directive 2010/63/EU of the European Parliament and were approved by the Institutional Animal Care and Use Committee (IIS-Fundación Jimenez Diaz). All mice were male, aged 12 weeks, and weighed 25–30 g. In a first set of experiments, intravascular hemolysis was induced in C57BL/6J mice (*Tlr4*^{+/+}; EnVigo, Barcelona, Spain) and TLR4-deficient mice (*Tlr4*^{-/-}; obtained from Dr. Consuelo Guerri, CIPF, Spain) by a single intraperitoneal administration of freshly prepared phenylhydrazine (Phe) solution (150 mg/kg of body weight, Cat#114715 Sigma-Aldrich, St. Louis, MO, USA). In another study, the TLR4 inhibitor TAK-242 (5 mg/kg of body weight, Cat#614316 Sigma-Aldrich) was administered intraperitoneally 1 or 4 h before and 24 and 48 h after

intraperitoneal injection of phenylhydrazine. TAK-242 reaches a high concentration in plasma 3 h after intraperitoneal injection [20]. Mice were euthanized 72 h after phenylhydrazine injection. Blood samples were collected for hematological analysis (Scil Vet ABC hematology analyzer, Scil, Madrid, Spain) and biochemical analysis (ADVIA 2400 Clinical Chemistry System, Siemens Healthcare, Erlangen, Germany). Dissected kidneys, liver, and spleen were fixed in 4% paraformaldehyde and embedded in paraffin wax for histological studies or snap-frozen for RNA and protein isolation.

Cell culture

Murine renal proximal tubular epithelial (MCT) cells were originally obtained from Eric Neilson (Vanderbilt University, Nashville, TN, USA) [21]. MCT were cultured in RPMI 1640 (Cat#R0883, Sigma-Aldrich) supplemented with 10% decomplexed fetal bovine serum (Cat#F7524, Sigma-Aldrich), glutamine (2 mM; Cat#G7513, Sigma-Aldrich), and penicillin/streptomycin (100 U/ml; Cat#P0781, Sigma-Aldrich) in 5% CO₂ at 37 °C. MCT were depleted of fetal bovine serum (FBS) when 70–80% confluent and 24 h after stimulation with hemin/heme (0–1 μ M; Cat#H9039, Sigma-Aldrich). Some cells were pretreated with the TLR4 inhibitor TAK-242 (TAK, 5 μ g/ml; Cat#614316, Sigma-Aldrich) for 4 h or with NF- κ B inhibitor parthenolide (Part, 2.5 μ g/ml; Cat#P0667, Sigma-Aldrich) for 1 h before heme stimulation. TLR4 and TLR2 expression was determined by flow cytometry using a BD FACS Canto II cytometer (BD Biosciences, San Jose, CA, USA).

RNA extraction and RT-qPCR

Total RNA from tissues or cultured cells was isolated using the TRIzol method with TRIzol G (Cat#A4051, Panreac, Barcelona, Spain) and reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Cat#4368813, ThermoFisher, Waltham, MA, USA). Expression of target genes was analyzed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) on an ABI Prism 7500 PCR system (Applied Biosystems, Foster City, CA, USA) using Taqman gene expression assays (see Supplementary materials and methods).

Histology, immunohistochemistry, and immunofluorescence

Histological and immunohistochemistry studies were performed using formalin-fixed paraffin-embedded 3- μ m tissue sections. Signs of histological injury were examined with hematoxylin/eosin (Cat#10034813 and Cat#6766007, ThermoFisher) or periodic acid-Schiff (PAS) staining (Cat#3952016, Sigma-Aldrich). Full methods for F4/80 immunohistochemistry and p65 immunofluorescence are presented in the Supplementary materials and methods. The presence of cell death was determined using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay following

the manufacturer's instruction (Cat#S7110, Millipore, Burlington, MA, USA).

Western blotting

Proteins from tissues or cultured cells were isolated in lysis buffer and analyzed by western blotting. Primary antibodies (see Supplementary materials and methods) were detected using an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody and developed using Luminata Crescendo Western HRP substrate (Cat#WBLUR0500, Millipore). Densitometry analysis was performed using Quantity One 1-D Analysis Software (RRID:SCR_014280) and quantification was expressed as arbitrary densitometric units (AU).

Statistical analysis

All collected data were first tested for normality using the Shapiro–Wilk test. Data comparisons between experimental groups were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test for normally distributed data or the Kruskal–Wallis test with Dunn's correction for nonnormally distributed data. Group measurements were expressed as mean \pm SEM. Values of $p < 0.05$ were considered statistically significant. Statistical analysis was performed using GraphPad Prism 8.0 statistical software (San Diego, CA, USA) (RRID:SCR_002798).

Results

TLR4 deficiency protects against intravascular hemolysis-mediated systemic inflammatory response

To study whether TLR4 deficiency protected against heme-induced injury, we developed an experimental model of massive intravascular hemolysis in wildtype (*Tlr4*^{+/+}) and TLR4 knockout (*Tlr4*^{-/-}) mice (Figure 1A). Mice were injected with phenylhydrazine (Phe) to promote the lysis of erythrocytes and the massive release of heme into the intravascular space, as previously reported [22]. Hemolytic effects were visible in the serum of Phe-treated mice, which showed a reduction in hematocrit and erythrocyte number. However, no apparent effect of TLR4 genotype on hemolysis degree was reported (Figure 1B–D). Intravascular hemolysis has been associated to systemic inflammatory response [23]. In our study, Phe-injection increased circulating levels of leukocytes, including lymphocytes, monocytes, and granulocytes (Figure 1E–H). This inflammatory systemic response was significantly attenuated in *Tlr4*^{-/-} mice. Therefore, TLR4 deficiency seems to be related to a lower immune activation in response to intravascular hemolysis.

Lack of TLR4 did not affect hepatic and splenic injury caused by massive intravascular hemolysis

The spleen is one of the main organs responsible for the elimination of damaged erythrocytes [4]. Since massive intravascular hemolysis induces splenic injury [24–26], we investigated whether the TLR4 absence could have a protective effect on this organ. Hemolysis caused splenomegaly (Figure 2A,B) and important structural alterations in the spleen, including reduction of white pulp area, as well as disorganization and loss of cellular density of the red pulp (Figure 2C). In this line, quantitative analysis of total spleen cell number depicted an impairment of splenic cells in hemolytic mice (Figure 2D), although no increased TUNEL staining was observed 72 h after hemolysis induction (supplementary material, Figure S1A). All these histological findings were similar in wildtype and TLR4 knockout mice with hemolysis. However, in basal conditions we observed higher mortality and increased expression of the apoptotic markers caspase-3 and PARP-1 in *Tlr4*^{-/-} (supplementary material, Figure S1A,B). We next studied the inflammatory response in the spleen. Massive intravascular hemolysis upregulated gene expression of the proinflammatory cytokines and chemokines *Il6* and *Ccl2*, whereas no elevation was found in *Tnf* (Figure 2E). Changes in proinflammatory cytokines were TLR4-independent, whereas western blot analysis showed a reduction of ERK1/2 phosphorylation levels in *Tlr4*^{-/-} mice after induction of hemolysis in comparison with hemolytic *Tlr4*^{+/+} animals (supplementary material, Figure S1C). Immunohistochemical studies showed a reduced presence of F4/80+ infiltrating macrophages in the spleen of Phe-injected mice, with no differences according to the TLR4 genotype (Figure 2F). As expected, hemolysis increased the splenic expression of antioxidant proteins involved in reactive oxygen species (ROS) elimination, such as catalase (*Cat*) and superoxide dismutase (*Sod1*), heme oxygenase 1 (*Hmox1*) (intracellular heme degradation), and ferritin (*Fth1*) (iron storage), although these differences were independent of TLR4 genotype (Figure 2G).

The liver is also susceptible to heme toxicity [6,27]. In our study, induction of hemolysis increased serum levels of the liver injury marker aspartate aminotransferase levels (AST) and lactate dehydrogenase (LDH), but no changes were observed in alanine aminotransferase (ALT) (Figure 2H–J). TLR4 deficiency reduced the hemolysis-mediated elevation of AST and LDH. Histologic analysis did not reveal significant alterations in hepatic tissue (Figure 2K). In contrast to the spleen, we observed an increased number of death cells in the liver (Figure 2L), and elevation of proteins related to the apoptotic process, such as caspase-3 and PARP1 (supplementary material, Figure S1E). Nevertheless, lack of TLR4 did not prevent hepatic cells death in TLR4 knockout mice. In the liver, increased expression of *Tnf* and *Ccl2* were detected in Phe-injected mice, whereas *Il6* levels remained unchanged (Figure 2M). However, changes in proinflammatory cytokines were

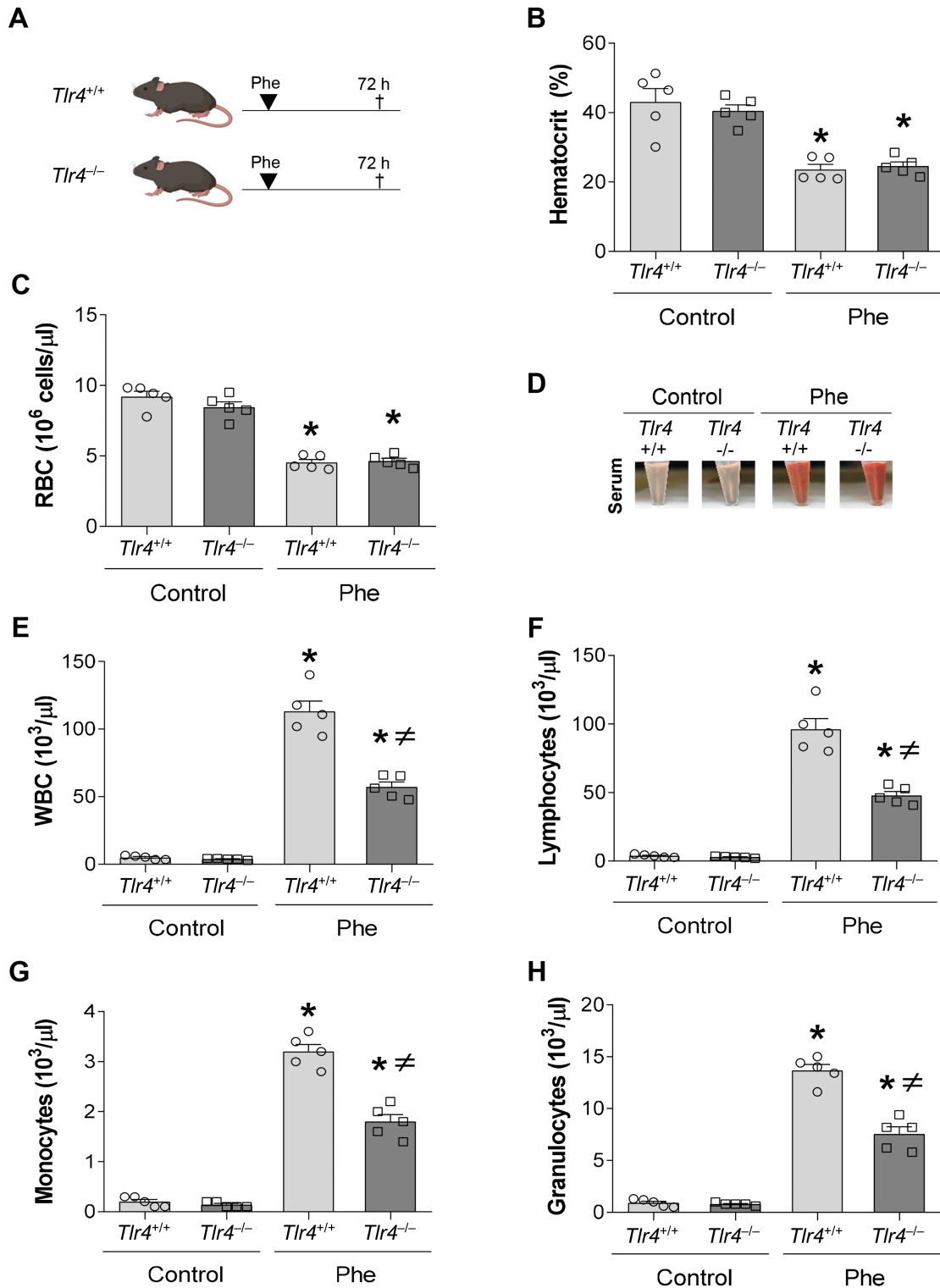


Figure 1. TLR4 deficiency protects against intravascular hemolysis-mediated systemic inflammatory response. (A) Schematic representation of intravascular hemolysis mouse model. C57BL/6 *Tlr4*^{+/+} or *Tlr4*^{-/-} mice were injected i.p. with saline (vehicle) or phenylhydrazine (Phe, 150 mg/kg of body weight) to induce intravascular hemolysis and sacrificed 72 h later. (B) Hematocrit and (C) total red blood cells (RBCs) were measured in fresh blood. (D) Representative images of serum after intravascular hemolysis in *Tlr4*^{+/+} and *Tlr4*^{-/-} mice. (E) Circulating white blood cells (WBC), (F) lymphocyte, (G) monocyte, and (H) granulocyte populations were detected in fresh blood, measured in a hematological cell counter analyzer. Results are expressed as mean ± SEM ($n = 5$). * $p < 0.05$ versus *Tlr4*^{+/+} control mice, ≠ $p < 0.05$ versus *Tlr4*^{+/+} Phe-injected mice.

TLR4-independent. Western blot analysis showed hepatic activation of NF- κ B and ERK1/2 after Phe administration, with significant reduction of NF- κ B activation in *Tlr4*^{-/-} mice as compared with *Tlr4*^{+/+}

(supplementary material, Figure S1F). Immunohistochemical detection of F4/80+ cells in the liver revealed a remarkable morphological alteration in macrophages from Phe-injected mice, with greater cellular surface

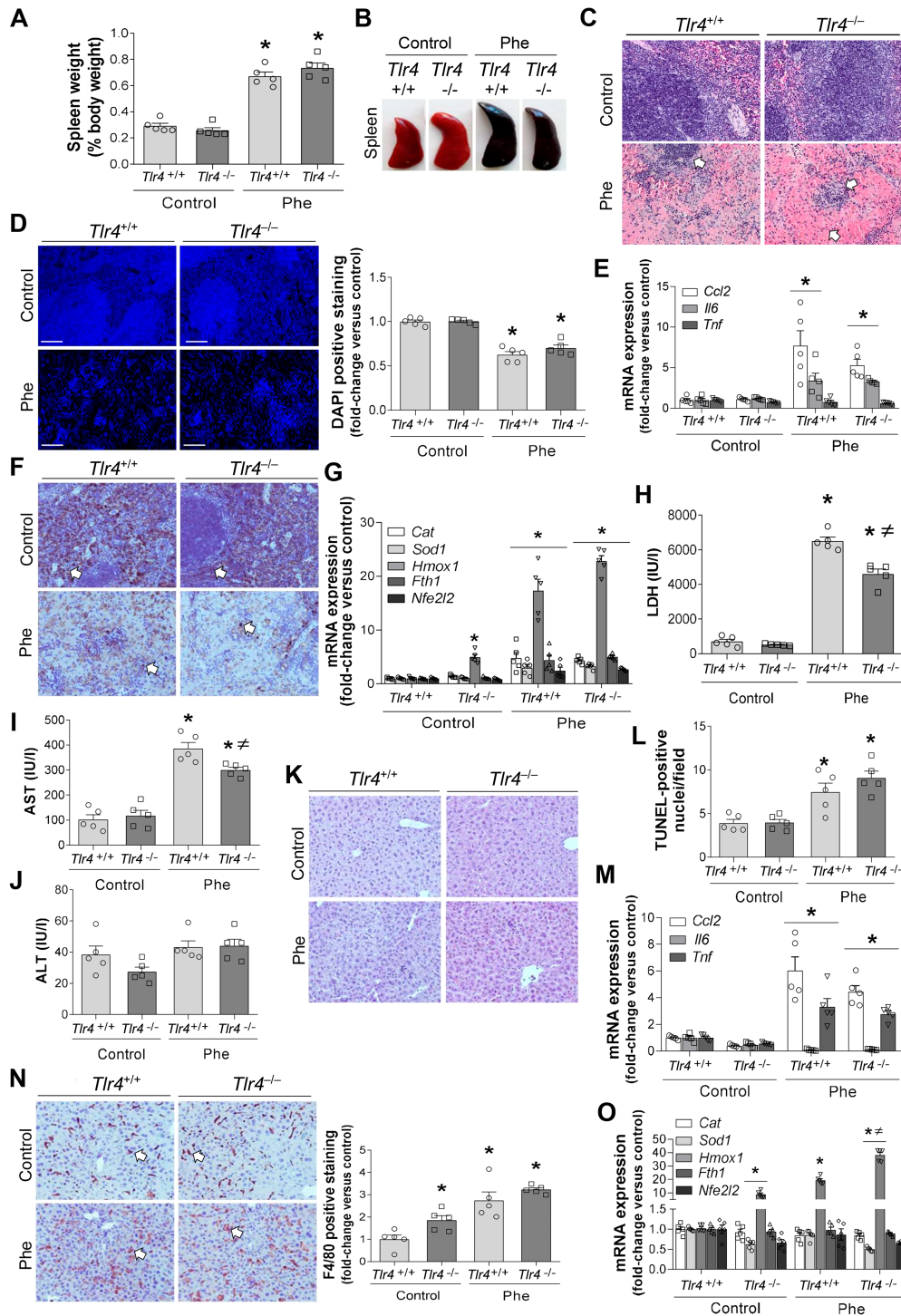


Figure 2. TLR4 deficiency has a limited impact in histological and functional preservation of the liver and spleen after hemolysis. (A) Relative spleen weight and (B) representative images of the spleen after intravascular hemolysis in *Tlr4*^{+/+} and *Tlr4*^{-/-} mice. (C) Representative images showing hematoxylin/eosin staining of spleen at 20× objective magnification. Arrows indicate signs of splenic injury: reduction of white pulp area, tissular disorganization, and loss of cellular density of the red pulp. (D) Representative confocal microscopy images and quantification of splenic DAPI-positive cells (blue). Scale bar, 100 μm. (E) Splenic gene expression of *Ccl2*, *Il6*, and *Tnf* as determined by RT-qPCR. (F) Representative immunohistochemistry images of F4/80+ cells in paraffin-embedded spleen sections at 20× objective magnification. Arrows indicate F4/80+ representative cells. (G) Splenic gene expression of catalase (*Cat*), superoxide dismutase (*Sod1*), heme oxygenase 1 (*Hmox1*), ferritin (*Fth1*), and Nrf2 (*Nfe2l2*) as determined by RT-qPCR. Serum levels of (H) lactate dehydrogenase (LDH), (I) aspartate transaminase (AST), and (J) alanine transaminase (ALT). (K) Representative images showing hematoxylin/eosin staining of paraffin-embedded liver section at 20× objective magnification. (L) Quantification of liver TUNEL-positive cells. (M) Hepatic gene expression of *Ccl2*, *Il6*, and *Tnf* as determined by RT-qPCR. (N) Immunohistochemistry quantification and representative images of F4/80+ cells in paraffin-embedded liver sections at 20× objective magnification. Arrows indicate F4/80+ representative cells. (O) Gene expression of *Cat*, *Sod1*, *Hmox1*, *Fth1*, and *Nfe2l2* in liver as determined by RT-qPCR. Results are expressed as mean ± SEM (*n* = 5). **p* < 0.05 versus *Tlr4*^{+/+} control mice, ≠ *p* < 0.05 versus *Tlr4*^{+/+} Phe-injected mice.

area and a reduction in labeling intensity, independently of TLR4 genotype (Figure 2N). In the liver, *Tlr4*^{-/-} mice showed increased expression of *Hmox1* in basal conditions, an effect that was magnified after hemolysis induction (Figure 2O). Altogether, our data suggest that TLR4 deficiency does not protect against heme-associated toxicity in liver and spleen during intravascular hemolysis.

TLR4 is involved in intravascular hemolysis-associated AKI

In a further step, we determined the role of TLR4 in the kidney after induction of hemolysis. Heme overload was visible in both kidneys and urine samples from mice with hemolysis (Figure 3A). Phe-injected mice showed a decline of renal function, with increased serum creatinine and blood urea nitrogen (BUN) levels (Figure 3B,C). Histological studies confirmed the presence of severe renal alterations in mice with intravascular hemolysis, including loss of brush border and flattening of the tubular epithelium, accumulation of debris and dilatation of the tubular lumen, and the presence of cell death (Figure 3D,E). Interestingly, decline of renal function and histological injury was less severe in *Tlr4*^{-/-} mice, indicating a key role of TLR4 in renal injury associated with intravascular hemolysis. In the same way, hemolytic *Tlr4*^{-/-} mice showed a reduced mRNA expression of the tubular injury markers *Kim1* (*Havcr1*) and *Ngal* (*Lcn2*) as compared to *Tlr4*^{+/+} mice with hemolysis (Figure 3F).

Hb- and heme-overload may trigger proximal tubular cell death [9]. We observed increased TUNEL staining in Phe-injected mice, an effect that was significantly reduced in *Tlr4*^{-/-} mice (Figure 3G). To identify the molecular pathways involved in Hb- and heme-induced cell death, we evaluated the presence of apoptotic markers. No changes in cleaved caspase 3 levels were detected after Phe administration (supplementary material, Figure S2A). Furthermore, although levels of cleaved PARP1 were increased in mice with intravascular hemolysis, this elevation was similar in both genotypes, suggesting that other types of cell death may be involved in this pathological condition (supplementary material, Figure S2A). To further investigate the mechanism involved in protection against intravascular hemolysis in TLR4-deficient mice, we analyzed the renal expression of antioxidant proteins. Interestingly, hemolysis impaired the expression of *Cat* and *Sod1* in *Tlr4*^{+/+} mice, but not in *Tlr4*^{-/-} (Figure 3H). This difference between genotypes could imply a greater capacity to eliminate ROS in *Tlr4*^{-/-} mice. In this connection, the *Tlr4*^{-/-} control group showed a higher basal level of HO-1 gene and protein expression (Figure 3H and Supplementary material, Figure S2B).

Intravascular hemolysis elicited the renal expression of inflammatory cytokines and chemokines (*Il6*, *Ccl2*, *Tnf*), and cell adhesion molecules (*Icam1* and *Vcam1*), an effect that was reduced in TLR4-deficient mice (except for *Tnf*) (Figure 3I,J). In line with these results,

we observed augmented phosphorylation of p65 NF- κ B subunit and ERK1/2 in the kidney of Phe-injected *Tlr4*^{+/+} mice, but not in *Tlr4*^{-/-} mice (Figure 3K). Finally, we evaluated the presence of infiltrating immune cells in the renal tissue. Surprisingly, the number of F4/80+ infiltrating macrophages was reduced in kidneys from mice with intravascular hemolysis, an effect that may be associated with the cytotoxic effects of heme in these cells [28] (Figure 3L). Therefore, these results suggest that TLR4 deficiency reduces production of proinflammatory cytokines and chemokines in the kidney by decreasing the activation of NF- κ B signaling pathways in intravascular hemolysis.

We next performed experiments in cultured tubular epithelial cells (MCTs) to further analyze the mechanisms involved in renal damage associated with the heme-TLR4 interaction. First, we analyzed the effect of heme on TLR4 expression by flow cytometry. We observed a dose-dependent increase of TLR4 expression after exposure to heme for 24 h (Figure 4A). We next evaluated the consequences of heme-mediated TLR4 activation on acute inflammatory response in MCTs. Heme increased the expression of *Ccl2* and *Il6* in a dose-dependent manner (Figure 4B) and promoted time-dependent activation of NF- κ B and ERK1/2 intracellular pathways (Figure 4C,D). Addition of the NF- κ B inhibitor parthenolide and the TLR4 inhibitor TAK-242 significantly decreased heme-mediated NF- κ B and ERK activation as well as proinflammatory cytokine expression (Figure 4E-G). None of these inhibitors affected heme-mediated effects on TLR4 protein and gene expression (supplementary material, Figure 3B,C). Taken together, these results suggest that the presence of heme in renal cells activates the TLR4 pathway, triggering an inflammatory response.

TLR4 inhibition protects against intravascular hemolysis-associated AKI

Having demonstrated the key role of TLR4 in hemolysis-mediated renal injury, we next evaluated whether TLR4 pharmacological targeting may be protective *in vivo*. To that end, we administered TAK-242 to mice 1 h prior to induction of hemolysis (Figure 5A). Neither serum (Figure 5B), hematological parameters (supplementary material, Figure S4A,B), nor systemic inflammatory response associated with hemolysis (supplementary material, Figure S4E-H) changed after TAK-242 administration. However, TLR4 inhibition protected against heme-mediated decline of renal function (Figure 5C,D). Histological studies confirmed the presence of lower structural damage in the kidneys after TLR4 inhibition (Figure 5E). In the same way, treatment with TAK-242 effectively reduced hemolysis-mediated expression of the tubular injury markers *Havcr1* and *Lcn2* (Figure 5F) and tubular cell death (Figure 5G). As shown in Figure 3H, massive intravascular hemolysis impaired the gene expression of the antioxidants *Sod1*, and *Cat*, while increased HO-1 gene and protein expression, this last effect partially

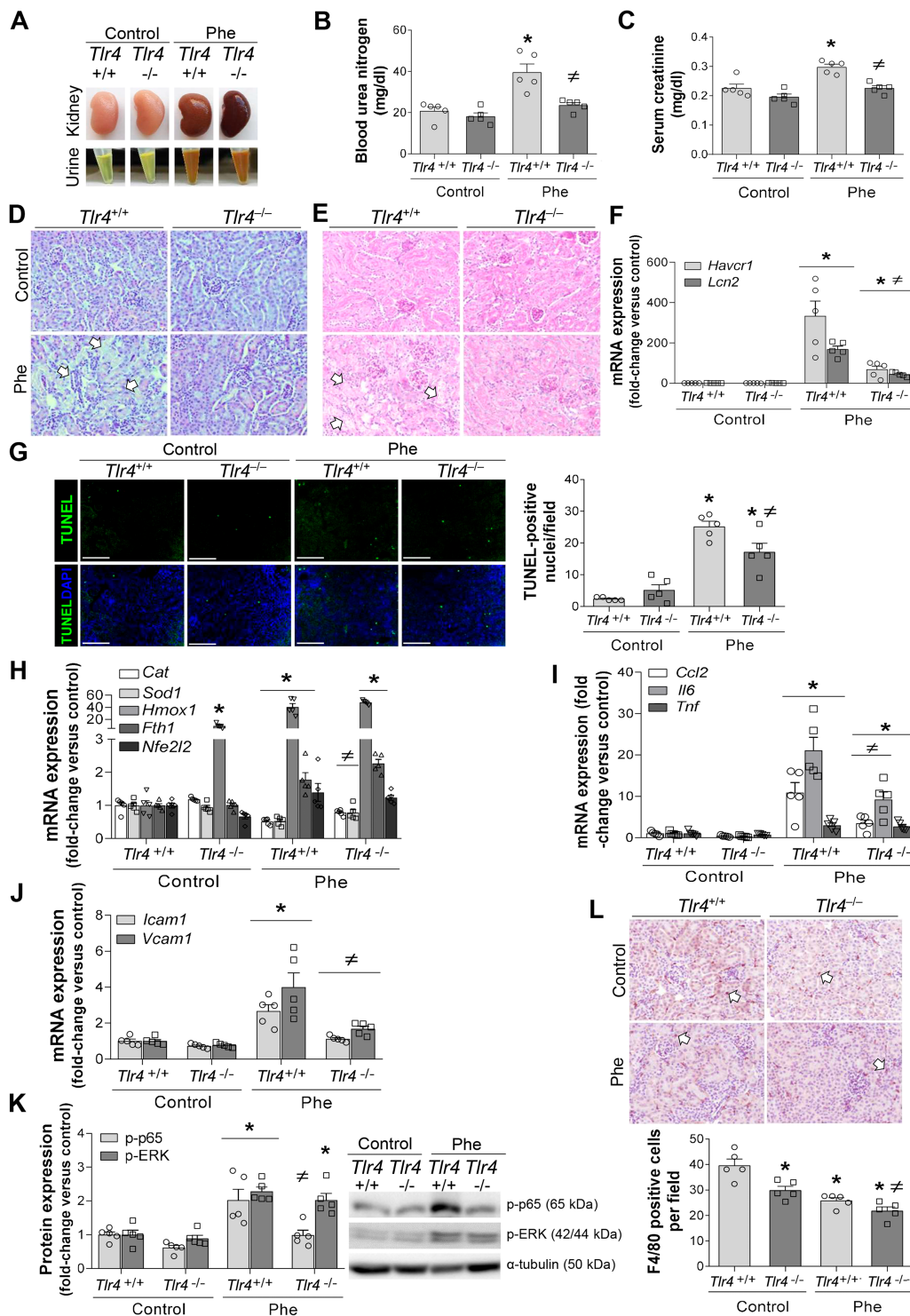


Figure 3. TLR4 deficiency protects against intravascular hemolysis-mediated AKI. (A) Representative images of kidney and urine after intravascular hemolysis in *Tlr4*^{+/+} and *Tlr4*^{-/-} mice. (B) Serum levels of blood urea nitrogen (BUN) and (C) creatinine. Representative images showing (D) hematoxylin/eosin and (E) periodic-acid Schiff (PAS) staining of paraffin-embedded renal sections at 20× objective magnification. Arrows indicate signs of acute tubular injury: loss of brush border, flattening of the tubular epithelium, accumulation of debris, and dilatation of the tubular lumen. (F) Gene expression of tubular injury markers KIM1 (*Havcr1*) and Ngal (*Lcn2*) as determined by RT-qPCR. (G) Representative confocal microscopy images and quantification of kidney TUNEL-positive cells (green). Nuclei were stained with DAPI (blue). Scale bar, 200 μm. Renal gene expression of catalase (*Cat*), superoxide dismutase (*Sod1*), heme oxygenase 1 (*Hmox1*), ferritin (*Fth1*), and Nrf2 (*Nfe2l2*) (H), *Ccl2*, *Il6*, and *Tnf* (I), *Icam1* and *Vcam1* (J) as determined by RT-qPCR. (K) Renal phosphorylated p65 NFκB and ERK1/2 protein levels quantification and representative western blot images. (L) Quantification and representative immunohistochemistry images of F4/80+ cells in paraffin-embedded renal sections at 20× magnification. Arrows indicate representative F4/80+ cells. Results are expressed as mean ± SEM (n = 5). *p < 0.05 versus *Tlr4*^{+/+} control mice, ≠ p < 0.05 versus *Tlr4*^{+/+} Phe-injected mice.

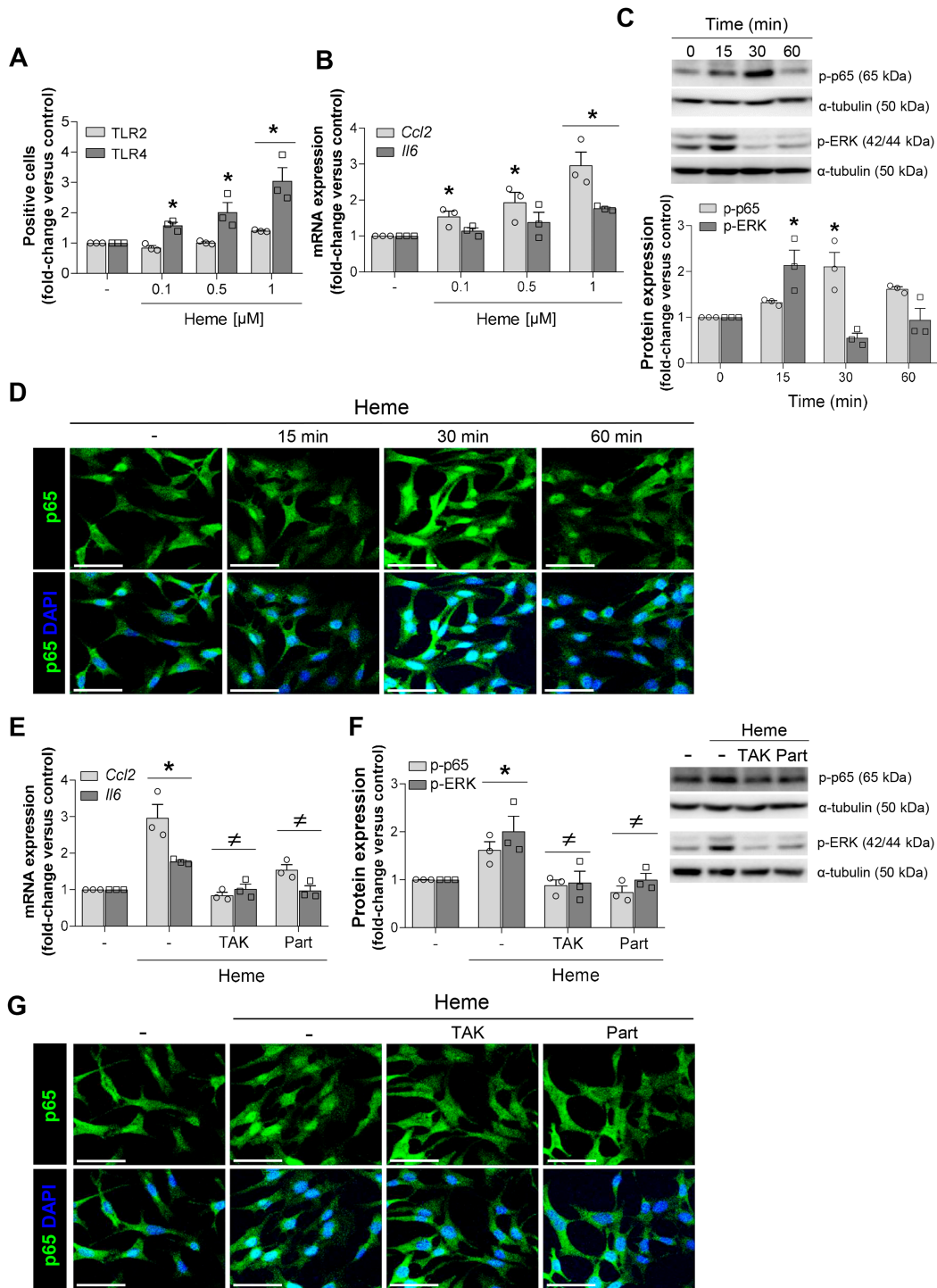


Figure 4. *In vitro* TLR4 pharmacological inhibition reduces heme-promoted inflammation. (A) TLR4 and TLR2 levels on cell surface were measured by flow cytometry in MCTs cells treated with heme (1 μ M) for 24 h. (B) *Ccl2* and *Il6* mRNA expression measured by RT-qPCR in MCTs cells exposed to different heme doses (0–1 μ M). (C) Quantification and representative images of phospho-p65 and phospho-ERK1/2 protein levels assessed by western blotting after exposure to heme (1 μ M) for different times (0–60 min). (D) Representative confocal microscopy images showing p65 NF κ B (green) nuclear translocation in MCTs cells treated with heme (1 μ M) for different times (0–60 min). Nuclei were stained with DAPI (blue). Scale bar, 50 μ m. MCTs were pretreated with TAK-242 (TAK) for 4 h or parthenolide (Part) for 1 h and then stimulated with heme (1 μ M). (E) *Ccl2* and *Il6* mRNA expression as determined by RT-qPCR after heme stimulation for 6 h. (F) Quantification and representative blot of p65 NF κ B and ERK1/2 phosphorylation levels 15 and 30 min after heme stimulation as assessed by western blotting. (G) Representative confocal microscopy images showing p65 NF κ B (green) nuclear translocation in MCTs cells 30 min after heme exposure with and without TAK-242 and parthenolide pretreatment. Nuclei were stained with DAPI (blue). Scale bar, 50 μ m. Results are expressed as mean \pm SEM from $n = 3$ independent experiments. * $p < 0.05$ versus nontreated cells, $\neq p < 0.05$ versus heme-treated cells.

reverted after TLR4 inhibition (Figure 5H). Finally, we observed that the renal inflammatory response was significantly reduced in TAK-242-treated mice, as

demonstrated by lower levels of *Il6*, *Ccl2*, and decreased NF- κ B activation (Figure 5I–K). However, no differences were observed in endothelial activation markers

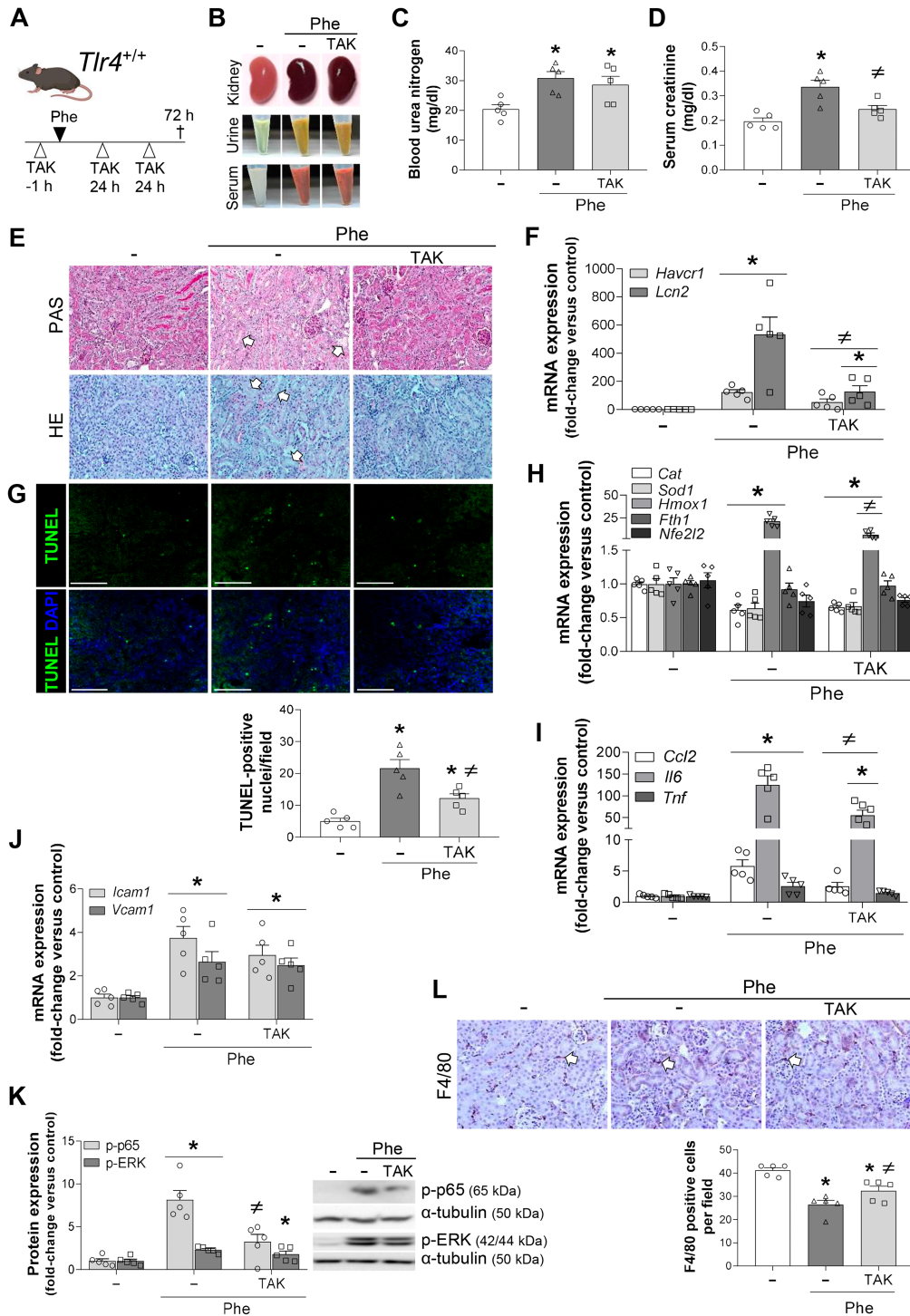


Figure 5. TLR4 pharmacological inhibition attenuates acute kidney injury induced by intravascular hemolysis. (A) Schematic representation of intravascular hemolysis mouse model. C57BL/6 mice were injected i.p. with saline (vehicle) or phenylhydrazine (Phe, 150 mg/kg of body weight) to induce intravascular hemolysis and sacrificed 72 h later. The TLR4 inhibitor TAK-242 was administered i.p. 1 h before and 24 and 48 h after Phe injection. (B) Representative images of kidney, urine, and serum. (C) Serum measurement of blood urea nitrogen (BUN) and (D) creatinine. (E) Representative images showing hematoxylin/eosin and periodic-acid Schiff (PAS) staining of paraffin-embedded renal section at 20× objective magnification. Arrows indicate signs of acute tubular injury: loss of brush border, flattening of the tubular epithelium, accumulation of debris, and dilatation of the tubular lumen. (F) Expression of tubular injury biomarkers *Kim1* (*Havcr1*) and *Ngal* (*Lcn2*) determined by RT-qPCR. (G) Representative images and quantification of TUNEL-positive cells (green) in renal sections. Nuclei were stained with DAPI (blue). Scale bar, 200 μm. Renal gene expression of catalase (*Cat*), superoxide dismutase (*Sod1*), heme oxygenase 1 (*Hmox1*), ferritin (*Fth1*), and Nrf2 (*Nf2l2*) (H), *Ccl2*, *Il6* and *Tnf* (I), *Icam1*, and *Vcam1* (J) as determined by RT-qPCR. (K) Western blotting quantification and representative images showing phosphorylation levels of p65 NFκB and ERK1/2. (L) Quantification and representative images at 20× objective magnification of F4/80 immunostaining in paraffin-embedded renal tissue sections. Arrows indicate representative F4/80+ cells. Results are expressed as mean ± SEM (*n* = 5). **p* < 0.05 versus control mice, ≠ *p* < 0.05 versus Phe-injected mice.

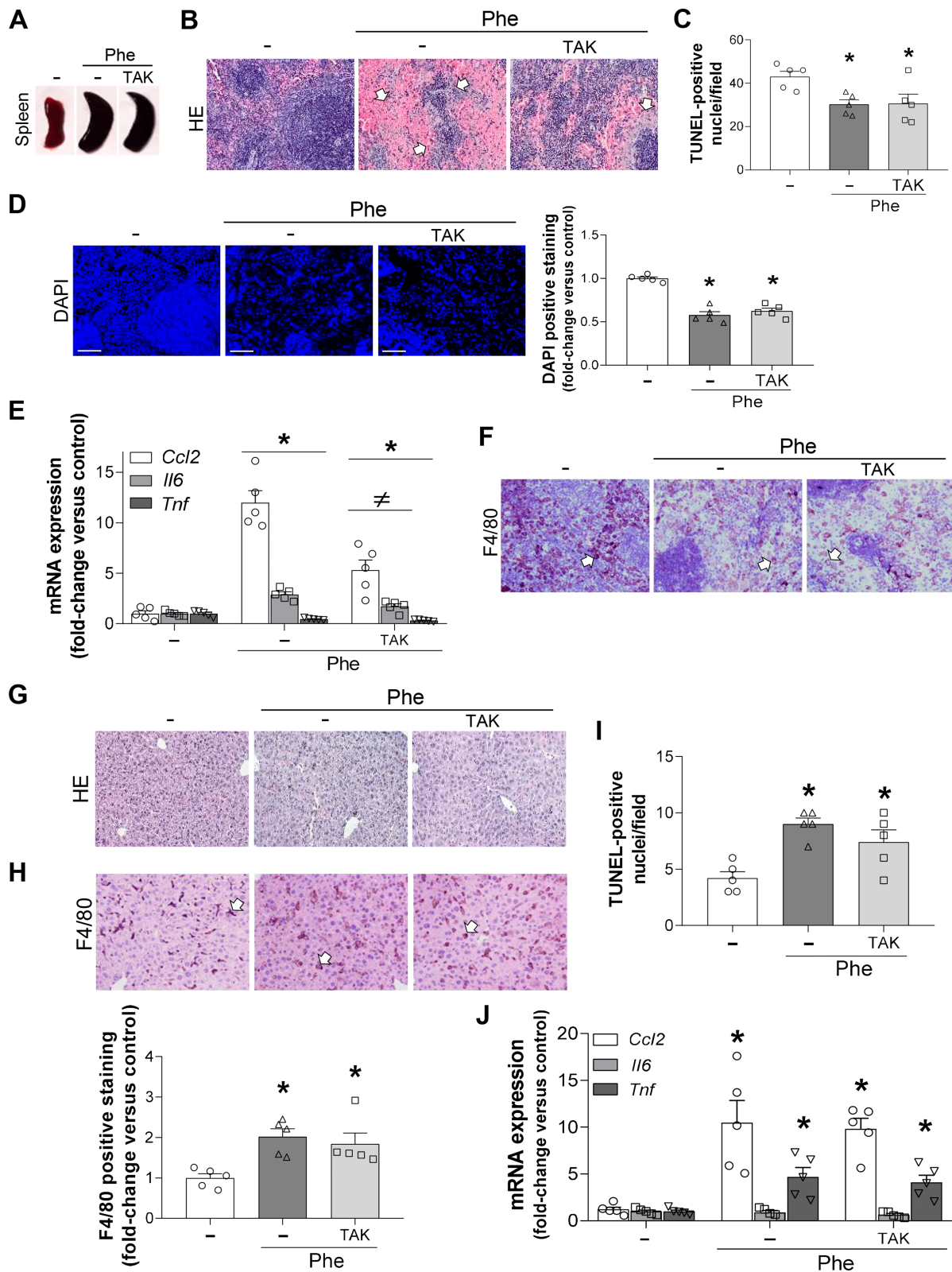


Figure 6. Effect of TLR4 inhibition in spleen and liver after induction of hemolysis. (A) Representative images of the spleen in control and Phe-injected mice. (B) Representative images showing hematoxylin/eosin staining of spleen at 20× objective magnification. Arrows indicate signs of splenic injury: reduction of white pulp area, tissular disorganization, and loss of cellular density of the red pulp. (C) Quantification of splenic TUNEL-positive cells after intravascular hemolysis. (D) Representative confocal microscopy images and quantification of splenic DAPI-positive cells (blue). Scale bar, 100 μm. (E) Splenic gene expression of *Ccl2*, *Il6*, and *Tnf* as determined by RT-qPCR. (F) Representative immunohistochemistry images of F4/80+ cells staining in paraffin-embedded spleen sections at 20× objective magnification. Arrows indicate representative F4/80+ cells. (G) Representative images showing hematoxylin/eosin staining of paraffin-embedded liver section at 20× objective magnification. (H) Immunohistochemistry quantification and representative images of stained F4/80+ cells in paraffin-embedded liver sections at 20× objective magnification. Arrows indicate representative F4/80+ cells. (I) Quantification of liver TUNEL-positive cells. (J) Hepatic gene expression of *Ccl2*, *Il6*, and *Tnf* as determined by RT-qPCR. Results are expressed as mean ± SEM (n = 5). *p < 0.05 versus control mice, ≠p < 0.05 versus Phe-injected mice.

(Figure 5J). TLR4 pharmacological inhibition significantly attenuates F4/80+ reduction (Figure 5L).

TAK-242 treatment did not revert hemolysis-associated splenomegaly (Figure 6A), histological splenic alterations (Figure 6B), or cell death (Figure 6C,D; supplementary material, Figure S5A), but significantly reduced splenic gene expression of proinflammatory cytokines (Figure 6E) and activation of proinflammatory the ERK1/2 signaling pathway (supplementary material, Figure S5B). Additionally, hemolysis induced a marked decrease of F4/80+ cells in the spleen regardless the pharmacological treatment (Figure 6F). On the other hand, the integrity of liver structure was not compromised in hemolyzed mice, independently of TLR4 inhibitor treatment (Figure 6G). Mice given TAK-242 showed similar numbers of TUNEL-positive cells, expression of proinflammatory cytokines and F4/80+ cells content in the liver compared with nontreated mice (Figure 6H,I). However, this inhibitor reduced the activation of the proinflammatory signaling pathways ERK1/2 and NF- κ B in the liver (supplementary material, Figure S5C). We next analyzed whether the failure of the preventive effect of TAK-242 in the spleen/liver was due to the short time of its administration (1 h before Phe-injection). However, similar results were observed when we administered TAK-242 4 h before induction of hemolysis (supplementary material, Figures S6–S8). Taken together, these data suggest that the beneficial effects of TLR4 inhibition during intravascular hemolysis are tissue-specific, with a significant reduction of the inflammatory response in the kidney and spleen, and a partial effect in the liver.

Discussion

To the best of our knowledge, this is the first study focusing on the role of TLR4 in intravascular hemolysis-mediated injury. We demonstrated that TLR4 is involved in heme-mediated renal damage, whereas it contributed in a lesser way in the spleen or liver injury.

Massive intravascular hemolysis has a deleterious impact on different tissues, including the kidney, liver, and spleen [6,24,29–32]. In our experimental model, phenylhydrazine administration lysed RBCs, with the ensuing release of large quantities of heme into the circulation, thus promoting AKI [33]. In our study, TLR4 targeting reduced renal inflammation and ameliorated histological damage and renal dysfunction associated with hemolysis. In agreement with our data, previous studies demonstrated a protective effect of TLR4 inhibition in different types of AKI, with reduced inflammatory response in the kidney [13,15,16,34–36]. In the same way, we observed reduced expression of proinflammatory cytokines and chemokines, as well as decreased activation of NF- κ B in kidneys of hemolytic TLR4 knockout mice or mice treated with the TLR4 inhibitor, TAK-242. This compound, also known as resatorvid, binds selectively to TLR4 and interferes with protein–protein interactions between the receptor and its

adaptor molecules [37]. It has been proposed that TLR4 activation promotes and exacerbates proinflammatory response upon renal injury and may contribute to kidney dysfunction [13,38]. A recent study published by Belcher *et al* reported that CD14 and MD-2 were required for heme-mediated TLR4 recognition and activation, although these interactions were different in LPS-mediated TLR4 activation [39]. Heme has proinflammatory properties, increasing the production of cytokines and chemokines by parenchymal renal cells throughout TLR4 activation [11,12,40]. Our *in vitro* results support these observations, since heme triggered TLR4 expression in tubular epithelial cells and promoted Ccl2 and Il6 expression as well as NF- κ B and ERK1/2 activation via TLR4. Based on these data and on the leukocytosis associated with phenylhydrazine [23], an increase in infiltrating inflammatory cells into the kidney would be expected. However, hemolysis decreased the number of F4/80+ macrophages in renal tissue. Hb and heme accumulation in the kidney could elicit monocyte recruitment and further macrophage differentiation to decrease extracellular heme concentration [22]. Macrophages are able to take up Hb and degrade intracellular heme via HO-1 [41]. However, heme overload may be toxic for these cells, as reported in HO-1-deficient macrophages [28]. This fact may explain our results, since massive accumulation of heme in the kidney may saturate the capacity of macrophages to degrade heme, triggering cell death. These results are apparently contradictory with the increased macrophage content observed in other models of renal heme accumulation, such as rhabdomyolysis [42]. However, it is important to note that heme renal content is higher in hemolysis than in rhabdomyolysis [22,43].

Hemolysis induces endothelial activation, vasoconstriction, and reduces renal blood flow, representing an additional hit that could increase renal injury [1,12]. In our experimental model, hemolysis increased *Icam1* and *Vcam1* mRNA levels, an effect that was attenuated in TLR4-deficient mice, but not after TAK-242 treatment. TLR4 deficiency protected against the elevation of renal vascular permeability in experimental sepsis-induced AKI [38]. In agreement with our results, TAK-242 administration failed to prevent renal microvascular alterations, even though TLR4 inhibition significantly improved creatinine clearance and reduced neutrophil recruitment in sepsis-induced AKI [34]. These observations suggest a limited vascular impact of TLR4 pharmacological inhibition.

Heme induces oxidative stress [44]. To restrain these pathological effects, there are antioxidant enzymes such as superoxide dismutase (SOD), catalase, and HO-1 [45,46]. HO-1 levels in the kidney were found elevated in experimental models with massive heme release and in renal biopsies of patients with hemolysis, as we have observed [47,48]. Induction of HO-1 protected against rhabdomyolysis-induced AKI [49], whereas HO-1-deficient mice showed greater impairment of renal function after Hb exposure [46]. In our study, TLR4 knockout control mice had higher basal levels of HO-1 than the wildtype controls, which may imply a greater capacity

for heme degradation in the case of hemolysis. In contrast to HO-1, hemolysis decreased *Sod1* and *Cat* mRNA levels in wildtype kidneys, but not in TLR4-deficient mice. Prolonged exposure to heme-induced oxidative stress diminished the expression of these enzymes in SCD patients [50]. However, the relationship between TLR4 and these antioxidant proteins is not clear.

In agreement with previous studies, heme accumulation in the kidney resulted in massive renal cell death [1,51]. Importantly, TLR4 targeting reduced heme-mediated cell death in mice with hemolysis. Apoptosis markers analyzed were not increased in the kidneys of hemolytic mice. It should be noted that TUNEL staining detects DNA breakage and, therefore it is not a marker of apoptosis [52]. This observation could explain the apparent discrepancy between results obtained for TUNEL and apoptosis markers. Similarly, renal hemo-protein overload has been associated with other types of cell death, such as ferroptosis and necroptosis [43,53], suggesting a potential role of caspase-independent cell death in AKI induced by intravascular hemolysis.

Massive intravascular hemolysis is deleterious for the spleen and the liver, as reported in patients with SCD [24,29], hereditary spherocytosis [25], and other congenital hemolytic anemias [26]. These patients show a wide range of splenic manifestations, from congestive enlargement to fibrotic atrophy [24]. As consequence of hemolysis, we observed splenomegaly, reduced cellular density in the red pulp, tissular disorganization, and decreased white pulp area. Recent studies have reported similar splenic alterations after hemolysis [24,54]. However, these pathological effects were independent of TLR4. Similarly, no significant differences in expression of proinflammatory cytokines or antioxidant enzymes were found according to the *Tlr4* genotype. However, TAK-242 administration successfully decreased proinflammatory cytokines in the spleen. TLR4 has an essential role in regulation of the innate immune system and inflammatory response, thus playing a key role in splenic functionality [4,55]. Systemic and permanent TLR4 deficiency may have a deleterious effect on the spleen, whereas transient TLR4 pharmacological inhibition could decrease these potentially damaging effects and effectively reduce inflammation. Nevertheless, the TAK-242 compound failed to preserve splenic tissue integrity, so the potential therapeutic strategy of TLR4 inhibition in spleen seems to be limited. In the spleen, red pulp macrophages (RPM) remove damaged erythrocytes from the circulation [55]. In SCD, these macrophages may be overwhelmed by excessive erythrophagocytosis and are unable to further engulf defective RBCs [24]. In our study, hemolysis decreased the abundance of RPMs, a phenomenon that may be related to excessive erythrophagocytosis and the consequent saturation of the heme degrading mechanisms, thus promoting cell death and consequent release of their intracellular content, aggravating splenic injury. This proposed mechanism is supported by other publications reporting increased macrophage death after massive erythrophagocytosis [56].

Massive intravascular hemolysis also affects liver injury and is associated with mortality risk in SCD patients [31].

Hemolytic crises trigger liver dysfunction and fibrosis [5]. Intravascular hemolysis causes hepatic inflammation and impairs the hepatic microvasculature [6,39]. Our results demonstrated a reduction of the liver injury markers AST and ALT after TAK-242 administration in Phe-injected mice, although this pharmacological approach did not prevent heme-induced hepatocyte death. The liver has an important role in iron recycling and RBC clearance [4]. Recent research emphasizes that the liver, but not the spleen, is the primary site of RBC clearance under certain pathological conditions [3]. That study identified a population of macrophages specialized in erythrophagocytosis that are rapidly recruited to the liver in the presence of damaged RBCs [3]. We detected increased expression of cytokines and elevated F4/80+ staining in the liver of Phe-injected mice, supporting data obtained in other models of intravascular hemolysis [3]. However, all these effects were TLR4-independent. Contrasting with the promising findings on renal protection, the results obtained in liver and spleen highlight the complexity of the systemic impact of intravascular hemolysis and the different susceptibility to inflammatory response of each organ separately. In this sense, it is important to note that TLR4 expression was higher in spleen than liver than kidney in healthy conditions (supplementary material, Figure S9). However, induction of hemolysis significantly increased TLR4 expression in the kidney, whereas focal or no elevation was observed in the liver and spleen, respectively (supplementary material, Figure S9). Unlike the kidney, the spleen and liver are exposed to elevated heme concentrations after induction of hemolysis (supplementary material, Figure S10). Although inhibition of TLR4 may protect against heme-mediated harmful effects, massive heme-overload may induce huge tissue injury, thus limiting the possible beneficial effects of TLR4 inhibition in the spleen and liver.

In summary, our results identify TLR4 as a key molecule in intravascular hemolysis-induced AKI. TLR4 deficiency and pharmacological inhibition decreased the severity of kidney injury, reducing functional and structural injury, inflammation, and cell death. Our *in vitro* and *in vivo* studies with TAK-242 suggest that this protective effect could be associated with inhibition of the TLR4/NF κ B signaling pathway. However, TLR4 inhibition failed to prevent hemolysis-derived injury in spleen and exhibited a limited effect on preserving hepatic functionality. Thus, TLR4 inhibition may be a potential therapeutic approach to prevent renal damage in patients with severe hemolytic crisis, but its combination with other pharmacological targets should be considered to prevent the harmful effects of massive intravascular hemolysis in other organs.

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Author contributions statement

CVC and JAM designed the study. CVC, CH, MGH, CGC, SRM, JLMP, LOR, CGG, MVM, IC and BA performed research. CVC, MGH, MLG, IC, BA, JE and JAM performed data analysis and data interpretation. CVC, JE and JAM wrote the article. All authors critically revised the article for important intellectual content.

Data availability statement

No multi-omics data were obtained in this article. Results used in this publication will be shared upon reasonable request addressed to the corresponding author.

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. TLR4 deficiency has a limited effect in cell-death and inflammation in spleen and liver after hemolysis

Figure S2. TLR4 deficiency did not protect against hemolysis-mediated apoptosis in kidney

Figure S3. Inhibition of NF- κ B and ERK1/2 pathways did not modify heme-mediated TLR4 expression in renal cells

Figure S4. TLR4 pharmacological inhibition did not modify hematological parameters, circulating immune cells or hemolysis-mediated apoptosis of renal cells

Figure S5. Apoptosis and inflammatory pathways in spleen and liver after TLR4 inhibition in mice with hemolysis

Figure S6. TLR4 pharmacological inhibition reduces renal injury associated to hemolysis

Figure S7. Effects of TLR4 pharmacological inhibition in spleen after induction of hemolysis

Figure S8. Effects of TLR4 pharmacological inhibition in liver after induction of hemolysis

Figure S9. *Tlr4* gene expression in tissues after induction of hemolysis

Figure S10. Hematological parameters and heme content in tissues after induction of hemolysis