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Inhibition of Cyclin-dependent Kinase 2 Signaling Prevents Liver Ischemia and Reperfusion Injury

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Abstract

Background: Liver ischemia and reperfusion injury (IRI) is a major complication of liver transplant, hepatectomy, and hemorrhagic shock. The cyclin-dependent kinase 2 (CDK2) acts as a pivotal regulator of cell cycle and proliferation.

Methods: This study evaluated the modulation and therapeutic potential of CDK2 inhibition in a mouse model of partial liver warm IRI.

Results: Liver IR triggered intrinsic CDK2 expression, peaking by 0.5h of reperfusion, and maintaining a high level throughout 1h-24h. Roscovitine, a specific CDK2 inhibitor, prevented liver IR mediated damage with abolished serum ALT levels and reserved liver pathology. CDK2 inhibition mediated liver protection was accompanied by decreased macrophage/neutrophil infiltration, diminished hepatocyte apoptosis, abolished TLR4 signaling and downstream gene inductions (CXCL-10, TNF-a, IL-1β and IL-6), yet augmented IL-10 expression. In vitro, CDK2 inhibition by Roscovitine suppressed macrophage TLR4 activation, and further depressed downstream inflammatory signaling (MyD88, IRF3, p38, JNK and ERK).

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Conclusions: Our novel findings revealed the critical role of CDK2 in hepatic cytoprotection and homeostasis against liver IRI. As CDK2 inhibition regulated local immune response and prevented hepatocyte death, this study provided the evidence for new treatment approaches to combat IRI in liver transplant.

Introduction

Hepatic ischemia and reperfusion injury (IRI) remains a common problem in liver transplant, hepatic resection, and shock.[1–3] In liver, IR-induced local tissue injury comprises several interrelated yet distinctive phases including ischemia-mediated parenchymal hypoxia, endothelial stimulation, adhesion molecular releasing, activation of Kupffer cells and neutrophils, followed by innate immunity-mediated reperfusion injury, and ultimate hepatic damage. Our team first revealed that macrophage TLR4 activation and its downstream pro-inflammatory profile were essential in the pathophysiology of liver IRI.[2–4]

Cyclin-dependent kinases (CDKs), members of the CMGC (CDK/MAPK/GSK3/CLK) kinase group, are serine/threonine kinases that play an essential role in control of cell cycle, proliferation and transcription by regulating G_1/S transition and S phase progression.[5,6] CDK2 activation requires Cyclin E binding and translocation into the nucleus,[7,8] which relies on the direct physical interaction with mitogen-activated protein kinases (MAPK).[9] Recent studies indicated that CDK2 may regulate adaptive and innate immunity.[10–12] CDK2 activation promoted conventional T cell differentiation and restricted Treg function in mouse cardiac transplantation,[10] while inhibition of CDK2 depressed macrophage function by reducing NF-κB activation.[11] It has been shown that CDK2 inhibition induced apoptosis in human neutrophils and peripheral blood mononuclear cells.[12] In this study, we examined the impact of CDK2 inhibition in liver IRI by using Roscovitine, a CDK2 specific purine inhibitor.[13] By competing the ATP binding site to inhibit CDK2 activity,[14] Roscovitine showed therapeutic potential in leukemia, herpes simplex infection, HIV infection, and breast cancer.[15–18] However, its application in transplant field remains mystery.

In current study, we examined the putative function of Roscovitine-mediated CDK2 inhibition in partial liver IRI. First, we asked whether IR insult triggered CDK2 expression in vivo and then whether CDK2 inhibition was essential to maintain liver homeostasis by controlling pro-inflammation and promoting hepatocytes survival. It may warrant critical evaluation while considering manipulation of CDK2 pathway as a potential treatment target in IRI management.

Materials and Methods

Liver IRI model.

Male C57BL/6 mice (8–12 weeks old, The Jackson Laboratory, Sacramento, CA) were used in an established murine model of partial warm liver IRI (70%). In general, the arterial/portal vessels to the cephalad lobes were clamped for 90min. Mouse body temperature was maintained at 37°C by using a heating pad. No vascular occlusion in sham-controlled mice. Mice were infused with Roscovitine (200μg/kg i.v., Sigma-Aldrich, St. Louis, MO, DMSO (0.6% v/v) as vehicle control) at 1h before ischemia or immediately after ischemia insult.

Animals were sacrificed at reperfusion for sample collection. In another parallel study, mice were received Roscovitine (200μg/kg i.v., DMSO as ontrol) at 1h before ischemia, subjected to 100min liver ischemia and followed 6h of reperfusion. All animal experiments were approved by UCLA Animal Research Committee.

Liver damage measurement.

Serum alanine aminotransferase (sALT) levels were assessed for hepatocellular damge by ALT kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instruction.

Histopathology.

Hematoxylin/eosin stained liver sections were assessed by Suzuki's score. Liver specimens, stained with Alexa Fluor 488 anti-CD68 (FA-11; 5μg/ml, BIO-RAD, Hercules, CA), FITC anti-Ly-6G (1A8; 5μg/ml, BD Biosciences, San Jose, CA), and PE anti-CDK2 (78B2; 5μg/ml, Cell Signaling Technology, Danvers, MA) were analyzed blindly.

Myeloperoxidase (MPO) assay.

Liver tissue MPO activity was measured by MPO Activity Assay Kit (Abcam, Cambridge, MA) according to the manufacturer's instruction.

Real-time RT-PCR.

We designed primers by Roche Universal ProbeLibrary, tested primers' annealing temperature by melt curve, and confirmed by DNA gel electrophoresis. Sequences were published.[1,19] Platinum SYBR qPCR kit (Invitrogen, Waltham, MA) was used. Gene expression was calculated by 2^(-Delta Ct). The ratio of target gene induction to housekeeping gene HPRT was calculated.

Western blots.

Liver proteins (30μg/sample) were separated by gel electrophoresis and transferred to nitrocellulose membrane. Anti-mouse NF-κB, Bcl-2, and β-actin mAbs (Cell Signaling Technology) were used for probe.

Caspase-3/7 analysis.

Caspase-3/7 green detection kit (Thermo Fisher Scientific) was used to stain liver section according to the manufacturer's instruction.

Bone marrow-derived macrophages (BMM).

We isolated bone marrow cells from C57BL/6 mice, then cultured (5×10^6/well) them with 10% L929 cell medium (GM-CSF) for 6 days. High purity (95–99%) of $CD11b^+F4/80^+$ cells can be observed. Lipopolysaccharides (LPS, 10ng/ml) were used to activate BMM, supply with Roscovitine (10μg/ml) or DMSO for 24h.

TNF-α concentration in supernatants was measured by ELISA kit (Thermo Fisher Scientific). Cells RNA was assayed for gene inductions (Real-time RT-PCR). Cell protein lysis was screened for MAPK family proteins (ELISA kit, Thermo Fisher Scientific).

Statistical analysis.

Results were indicated as mean ± standard deviation, analyzed by Student's-t test and ANOVA test. $P<0.05$ is considered significant.

Results

Hepatic CDK2 activation pattern in IRI.

We first documented the CDK2 induction profile in livers subjected to 90min of partial warm ischemia insult and followed 0–24h of reperfusion. Interestingly, CDK2 levels increased sharply after the ischemia, peaking by 0.5h of reperfusion, and maintaining relatively high level during 1h-24h of reperfusion, as compared with sham group (Fig 1a). Colocalization of CDK2 and CD68 indicated CDK2 expression in $CD68^+$ macrophages in the liver with 90min of ischemia and 6h of reperfusion (Fig 1b: green fluorescence indicated CD68, red represented CDK2 expression; white arrow pointed dual positive cells).

CDK2 inhibition ameliorated liver IR damage.

We then suppressed CDK2 function by using Roscovitine, a specific CDK2 inhibitor in our liver IRI model. Unlike DMSO control group, mice livers administrated with Roscovitine were resistant against IR-mediated tissue damage with decreased sALT values (Fig 1c, $p<0.05$), and well-preserved liver pathology (Fig 1d: no edema, congestion or necrosis; Fig 1e: reduced Suzuki's score).

CDK2 inhibition abolished macrophage/neutrophil infiltration in IRI.

Compared with DMSO control group, CDK2 inhibition reduced the frequency of macrophages (Fig 2b, d, $p<0.05$) and neutrophils (Fig 2c, e, $p<0.05$) in the ischemic liver lobes. In parallel, Roscovitine treatment significantly diminished neutrophil function, indicated by MPO activity (Fig 2a, $p<0.05$).

CDK2 inhibition regulated IR-triggered hepatic pro-inflammatory programs.

To analyze the immunomodulatory mechanism of CDK2 inhibition, we next screened liver chemokine/cytokine expression patterns in liver IRI. Roscovitine infusion markedly suppressed neutrophil/macrophage-derived pro-inflammatory chemokines and cytokines programs (Fig 3a, b, $p<0.05$). Interestingly, IL-10 levels were promoted selectively after Roscovitine treatment, as compared with DMSO treatment group (Fig 3c).

CDK2 inhibition depressed IR-induced liver apoptosis.

As CDK2 modulation may affect cell cycle, we then assessed the IR-mediated hepatic apoptosis. Indeed, Roscovitine treatment abolished hepatocellular apoptosis, indicated by diminished frequency of Caspase $3/7^+$ cells (Fig 4a,b) and reduced NF-κB activation (Fig

4c). On the other hand, CDK2 inhibition enhanced anti-apoptotic Bcl-2 expression in IR-livers (Fig 4c).

CDK2 inhibition prevented macrophage TLR4 response.

Activation of macrophage TLR4 signaling is the pivotal phase in local immune response in liver IRI (2). We then determined how CDK2 inhibition may regulate TLR4 response in BMM culture. TLR4 ligand LPS were used to activate BMM in the presence of Roscovitine or DMSO. The administration of Roscovitine sharply reduced TLR4 expression (Fig 5a, $p<0.05$), and downstream pro-inflammatory cytokine profile (Fig 5c: TNF-α, IL-6, and IFN-β, $p<0.05$), but augmented IL-10 level (Fig 5b, $p<0.05$). These results document the negative regulation of CDK2 inhibition in macrophage TLR4 response *in vitro*.

CDK2 inhibition regulated macrophage MAPK pathway.

We next analyzed the mechanism by which CDK2 inhibition regulated macrophage TLR4 activation. Cultured BMM were activated by LPS, supplied with Roscovitine or DMSO. Interestingly, addition of Roscovitine reduced myeloid differentiation factor 88 (MyD88) and interferon regulatory transcription factor 3 (IRF3) expressions (Fig 6a, $p<0.05$), accompanied by diminished levels of phosphorylated p38 (p-p38), phosphorylated c-Jun N-terminal kinase (p-JNK), and phosphorylated extracellular-regulated kinases (p-ERK1/2) (Fig 6c, $p<0.05$). Consistently, Roscovitine depressed macrophage-induced TNF-α levels in the culture supernatant (Fig 6b, $p<0.05$).

Discussion

CDK2, a Ser/Thr kinase, characterized for its role in cell cycle progression, controls peripheral immune tolerance, promotes T cell differentiation and restricts Treg function in adaptive immune responses.[10,20–22] However, the role of CDK2 signaling in innate immunity *in vivo* remains elusive. In the current study, we documented that CDK2 inhibition with Roscovitine ameliorated the hepatocellular damage by preventing macrophage activation and depressing TLR4-driven inflammation in a well-established murine liver IRI model. Hence, CDK2 inhibition is essential to maintain liver homeostasis in IRI. In our series, CDK2 inhibition reduced sALT levels and reserved the tissue architecture in liver IRI. The therapeutic effect of Roscovitine was also demonstrated by reduced local macrophage and neutrophil recruitment/activation; decreased parenchyma cell apoptosis; diminished inflammatory cytokines production with promoted IL-10 expression. The therapeutic effect of Roscovitine has been shown in leukemia, herpes simplex infection, HIV infection, and breast cancer.[15–18] As Roscovitine is currently being developed into phase II clinical trials for Cushing's Disease and Cystic Fibrosis,[23,24] it should be considered to prevent liver IRI in clinical.

By controlling the G1/S transition, CDK2 directly regulates the proliferation and apoptosis of immune cells, thus serving as a potent target to a diverse range of immune responses. Indeed, CDK2 inhibitor depressed inflammatory markers and increased survival in carrageenan-induced pleurisy and bleomycin-induced lung injury.[25] Here, we first found that warm ischemia itself triggered steep elevation of CDK2 expression in IR-stressed liver as

early as 0.5h of reperfusion. It may indicated that intrinsic CDK2 was required in liver damage. As IR-exacerbated hepatocellular damage peaking at 6h,[19] we then examined CDK2 inhibition by Roscovitine at this time point. Interestingly, a single dose of Roscovitine (at −1h) diminished the hepatocellular damage and preserved liver architecture. Consistently, Roscovitine treatment post ischemia insult also prevented liver IRI (Fig S1a). CDK2 inhibition may protected liver in severe liver IRI subjected by prolonged warm ischemia (Fig S1b).

As IR stress-mediated endothelial cells activation produced adhesion molecular, peripheral neutrophils and macrophages turned active and emigrated into liver during reperfusion. Circulatory immune cells further enhanced local tissue destruction by releasing inflammatory cytokines in liver IRI.[2–4,26,27] In this study, CDK2 inhibition blocked neutrophil infiltration and abolished MPO activity in IR-stressed liver. Roscovitine further diminished CXCL-1 expression, which in turn suppressed neutrophil chemoattraction cascade in liver IRI. Consistently, inhibition of CDK2 depressed IR-mediated macrophage emigration, and along with deprivation of downstream inflammation profiles.

In liver IRI, innate pro-inflammatory cytokine TNF-α activated downstream procaspase 3, and leaded to hepatocyte apoptosis, featured as cellular shrinkage, chromatin condensation and apoptotic body formation.[19] Interestingly, Roscovitine treatment not only decreased hepatocellular apoptosis and NF-κB activation, but also increased anti-apoptotic Bcl-2 level. As Bcl-2 can block mitochondrial membrane permeabilization and release of cytochrome C, it implies that Roscovitine may trigger cellular survival pathway in liver IRI. We also observed somewhat high frequency of caspase3/7^{+} apoptotic cells in CDK2 inhibition group compared with B7-H1Ig protected liver,[19] which indicated the minimal effect of Roscovitine on IR-damaged hepatocytes.

Activation of sentinel TLR family was identified as the initial stimulus in the infectious/inflammatory disease development.[28,29] In previous studies, we have shown that endogenous TLR4-mediated inflammation response was essential in the pathogenesis of liver IRI.[2–4,30] Inhibition of CDK2 not only depressed IR-triggered TLR4 expression, but also diminished the release of TNF-α, IL-6, IL-1β, yet increased the IL-10 level.

TLR4 promotes innate immune response through adaptor MyD88, and Toll/IL-1 receptor domain containing adaptor inducing IFN-β (TRIF) dependent pathway.[31] In this study, reduced MyD88/IRF3 expressions were observed in LPS-stimulated BMM cultures supplemented with Roscovitine, indicating CDK2 inhibition blunted pro-inflammatory program by MyD88 and TRIF signaling. Indeed, MyD88 and IRF3 did contribute to TLR4-induced NF-κB activation and pro-inflammatory profiles in mice models of intestinal IRI and heart transplant.[32,33] To further investigate the mechanism of CDK2 inhibition, we monitored the downstream MAPK signaling in vitro. Consistently, inhibition of CDK2 not only depressed the expression levels of p-p38, p-ERK1/2 and p-JNK, but also reduced the TNF-α level. Hence, our findings document the pivotal role of CDK2 inhibition in regulating macrophage inflammation by suppressing MyD88/TRIF-IRF3 and downstream MAPK (p38 /JNK/ERK) pathway (Fig 7).

In summary, this study provides evidence that CDK2 inhibition prevented liver IRI by suppressing macrophage activation and depressing TLR4-driven inflammation. In the context of ongoing phase II clinical trials in Cushing disease and cystic fibrosis,[23,24] our current preclinical results identified Roscovitine as a novel principle for prevention of sterile inflammation in liver.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BMM	Bone marrow-derived macrophages
CDK2	cyclin-dependent kinase 2
IRF3	interferon regulatory transcription factor 3
IRI	ischemia/reperfusion injury
MAPK	mitogen-activated protein kinases
MPO	myeloperoxidase
MyD88	myeloid differentiation factor 88
p-p38	phosphorylated p38
p-JNK	phosphorylated c-Jun N-terminal kinase
p-ERK1/2	phosphorylated extracellular-regulated kinases
sALT	serum alanine aminotransferase
TRIF	Toll/IL-1 receptor domain containing adaptor inducing IFN-β

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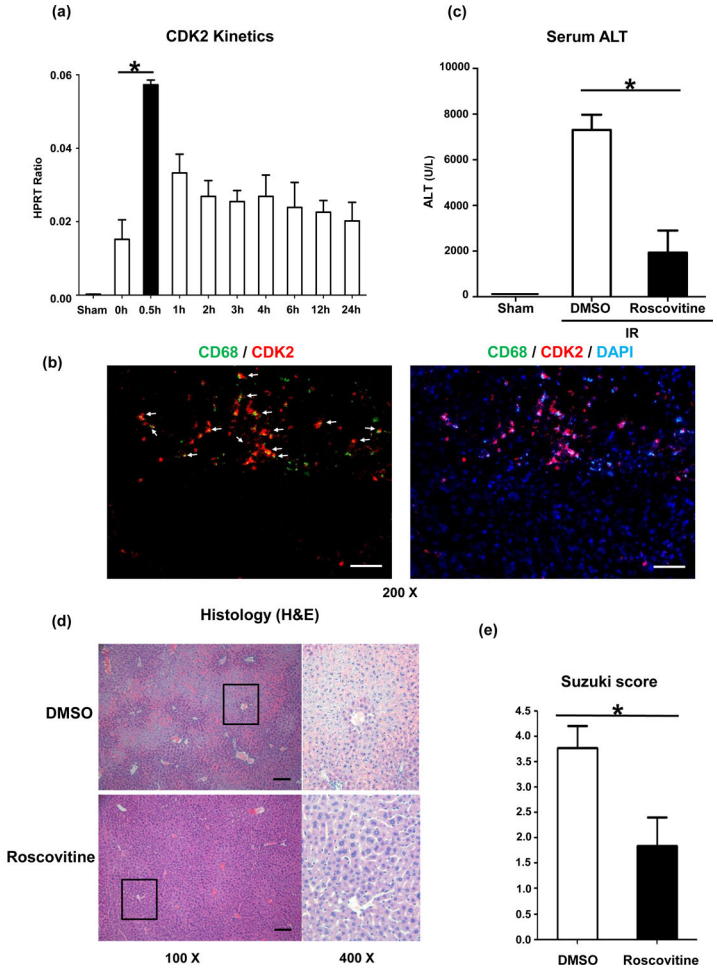

Figure 1.

(a) Liver IRI–triggered CDK2 gene expression. Liver samples were retrieved from C57/B6 mice that were subjected to 90 min of warm ischemia, followed by different lengths of reperfusion. Sham-operated group as control (n = 6–8/group, *p < 0.05). (b) Dual staining of CD68 and CDK2 in liver subjected to 90 min of ischemia and 6 h of reperfusion (magnification x200, scale bar represents 100 μm).

Livers in WT mice pretreated with Roscovitine or DMSO were subjected to 90 min of ischemia, followed by 6 h of reperfusion. The hepatocellular damage was assessed by (c) sALT levels and (d, e) liver histology (H&E staining: magnification x100 and x400, scale bar represents 100 μm) (n = 6–8/group, *p < 0.05).

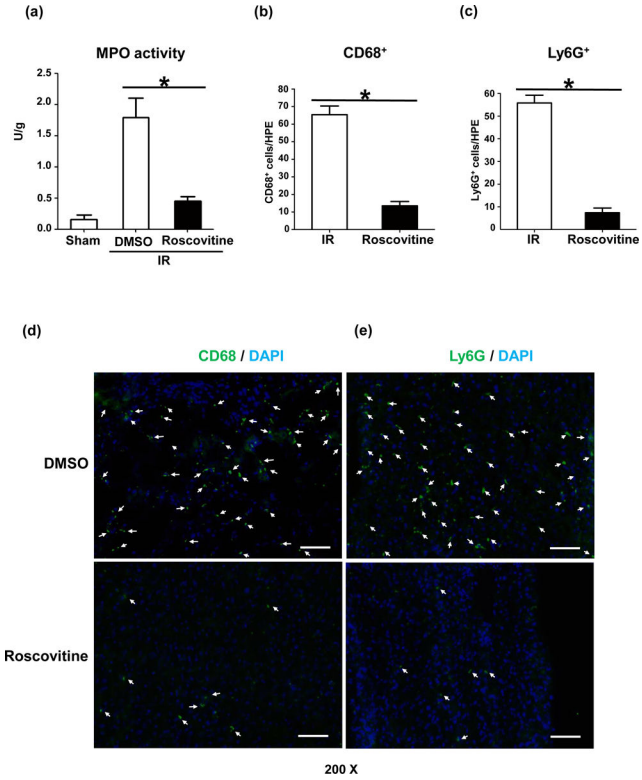

Figure 2.

Recruitment of neutrophils/macrophages in livers insulted by 90 min of ischemia and 6 h of reperfusion. (a) MPO levels (b) and (d) CD68+ macrophages (c) and (e) Ly-6G+ neutrophils in IR liver lobes (magnification x200, scale bar represents 100 μm) (n = 6–8/group, *p < 0.05).

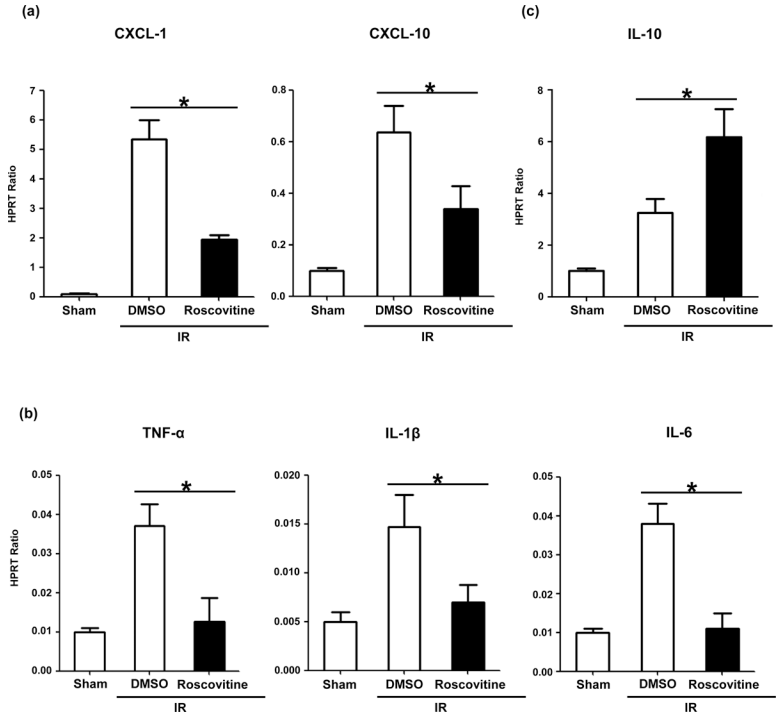

Figure 3.

Quantitative RT-PCR–measured cytokines/chemokines gene programs in livers subjected by 90 min of ischemia and 6 h of reperfusion. (a) CXCL-1 and CXCL-10; (b) TNF-α, IL-1β, and IL-6; and (c) IL-10. Values were normalized to HPRT gene induction (n = 6–8/group, *p < 0.05).

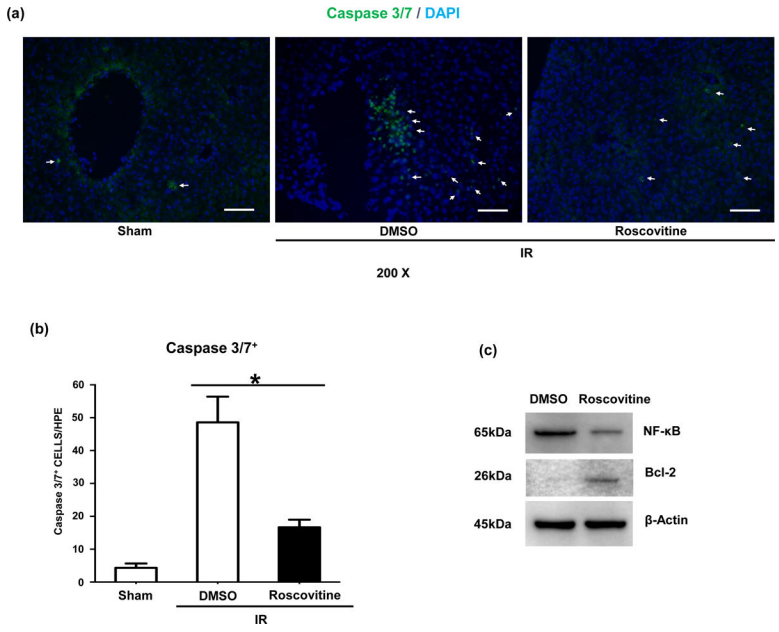

Figure 4.

Apoptosis in livers insulted by 90 min of ischemia and 6h of reperfusion. (a) and (b) Caspase 3/7–assisted detection of hepatic apoptosis (white arrow) in ischemia liver lobes (magnification x200, scale bar represents 100 μm). (c) Western blots measured NF-κB, Bcl-2, and β-actin in liver samples (n = 4–6/group, *$p < 0.05$).

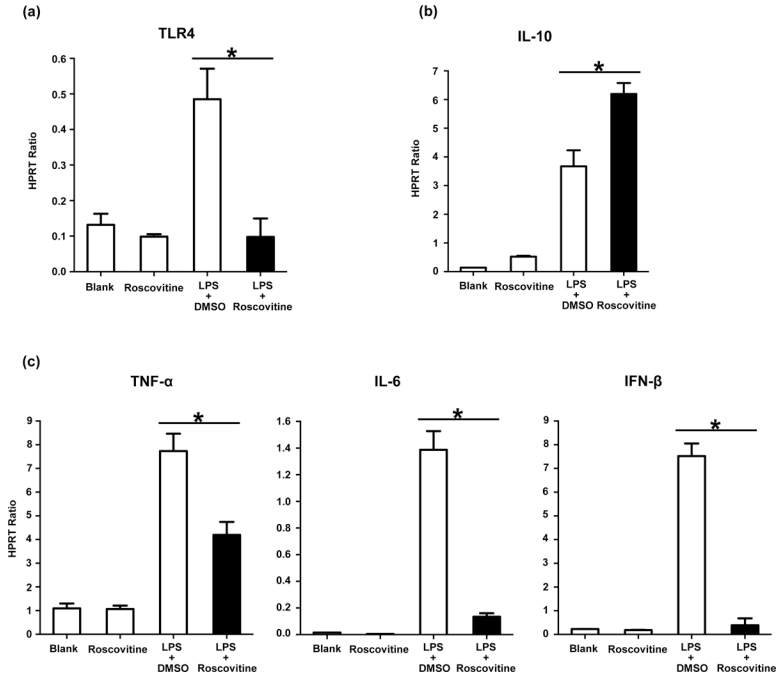

Figure 5.

The effect of CDK2 inhibitor upon macrophage TLR4 activation in vitro. Bone marrow–derived macrophages were stimulated with LPS in the absence or presence of Roscovitine or DMSO (control). Gene inductions of (a) TLR4, (b) IL-10, and (c) TNF-α, IL-6, IFN-β in culture cells ($n = 4–6$/group, $*p < 0.05$).

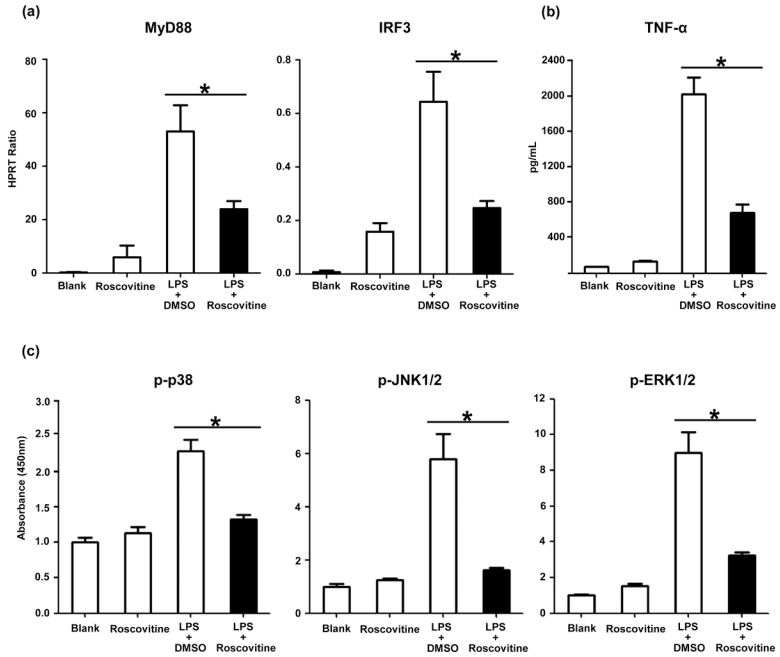

Figure 6.

The anti-inflammatory function of CDK2 inhibition upon macrophage in vitro. Bone marrow–derived macrophages were stimulated with LPS in the absence or presence of Roscovitine or DMSO (control). Roscovitine treatment depressed MyD88/IRF3 and MAPK signalings in macrophage. (a) MyD88 and IRF3; (b) TNF-α; (c) phosphorylated p38, phosphorylated JNK1/2, and phosphorylated ERK1/2 (*$p < 0.05$, $n = 4–6$/group).

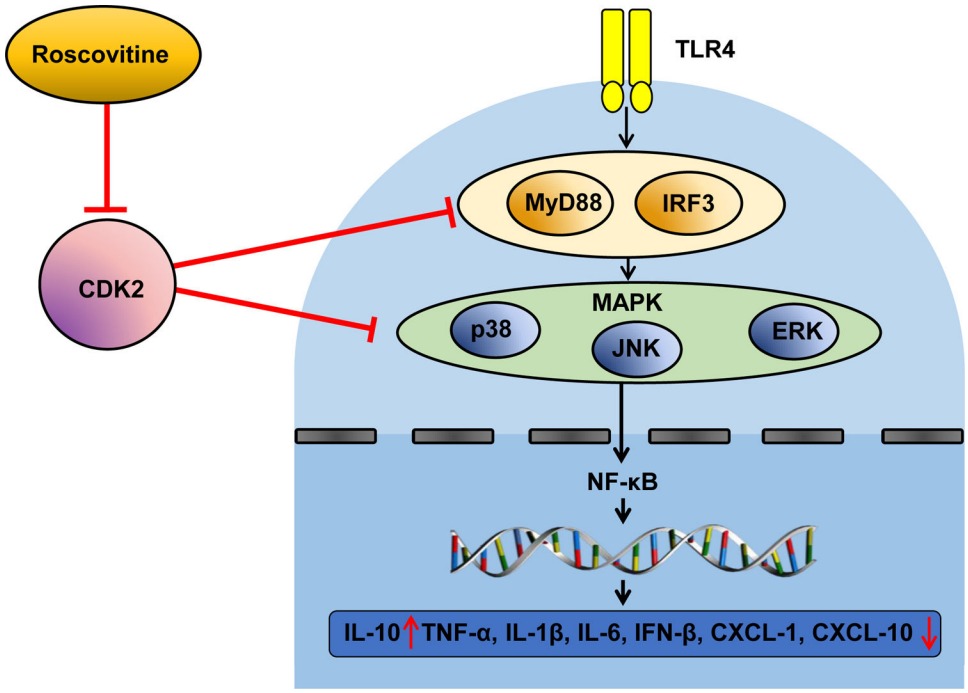

Figure 7.

A scheme of putative molecular mechanism by which CDK2 inhibition may depress TLR4 signaling and proinflammatory macrophage programs in liver IRI.