LIVER TRANSPLANTATION 21:969–981, 2015

ORIGINAL ARTICLE

The Protective Function of Galectin-9 in Liver Ischemia and Reperfusion Injury in Mice

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Galectin-9 (Gal-9) has gained attention as a multifaceted player in adaptive and innate immunity. To elucidate the role of Gal-9, we used a mouse model of partial liver ischemia/reperfusion injury (IRI) with wild type (WT) and Gal-9 knockout (KO) mice as well as a recombinant galectin-9 (reGal-9) protein. We found that the expression of Gal-9 was enhanced endogenously in the liver especially by hepatocytes and Kupffer cells during warm IRI for a mouse liver, which causes massive destruction of liver tissue. Gal-9 was released into the extracellular space in the liver and the highest levels in the plasma at 1 hour after reperfusion. The present study elucidates a novel role of Gal-9 signaling in mouse liver IRI, by using Gal-9–deficient mice and a stable form of reGal-9 protein. In the circumstance of Gal-9 absence, liver damage due to ischemia/reperfusion (IR) exacerbated the severity as compared with WT. On the other hand, exogenously administered reGal-9 significantly ameliorated hepatocellular damage. It decreased the local infiltration of the inflammatory cells such as T cells, neutrophils, and macrophages, and it reduced the expression of proinflammatory cytokines/chemokines; then, it strongly suppressed the apoptosis of the liver cells. Interestingly, severe liver damage due to IR in Gal-9 KO mice was improved by the administration of reGal-9. In conclusion, Gal-9 engagement ameliorated local inflammation and liver damage induced by IR, and the present study suggests a significant role of Gal-9 in the maintenance of hepatic homeostasis. In conclusion, targeting Gal-9 represents a novel approach to protect from inflammation such as liver IRI. Exogenous Gal-9 treatment will be a new therapeutic strategy against innate immunity-dominated liver tissue damage. ***Liver Transpl 21:969-981, 2015***. **© 2015 AASLD.**

Received February 10, 2015; accepted April 7, 2015.

Galectins are involved in various processes including embryonic development, tumor biology, and regulation of the immune system,[1] and they are evolutionarily conserved glycan-binding proteins with diverse roles in innate and adaptive immune responses.[2] At least 15 galectins have been identified in mammals.[3] Notably,

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Abbreviations: Ab, antibody; CXCL, chemokine ligand; CXCL1, keratinocyte chemoattractant; CXCL2, macrophage inflammatory protein-2; Gal-9, galectin-9; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H & E, hematoxylin-eosin; HPF, high-power field; IFN-γ, interferon-γ; IgG, immunoglobulin G; IL, interleukin; IR, ischemia/reperfusion; IRI, ischemia/reperfusion injury; KD, kilodalton; KO, knockout; Ly6-G, lymphocyte antigen 6 complex locus G; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; reGal-9, recombinant galectin-9; sALT, serum alanine aminotransferase; SEM, standard error of mean; T_h, T helper; TIM, T cell immunoglobulin mucin; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor α; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; WT, wild type.

This work was funded by Grants-in-Aid for Scientific Research, Kitano Research Scholarships, and Research Scholarships of Uehara Memorial Foundation.

Potential conflict of interest: Nothing to report.

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DOI 10.1002/lt.24159
View this article online at wileyonlinelibrary.com.
LIVER TRANSPLANTATION.DOI 10.1002/lt. *Published on behalf of the American Association for the Study of Liver Diseases*

galectin-9 (Gal-9), a member of the galectin family, is ubiquitously expressed in a variety of tissues and is particularly abundant in the liver.[4] Gal-9 was first identified as an apoptosis-inducing factor for thymocytes[5] and an eosinophil-activating factor.[6] Recently, several articles have reported that Gal-9 treatment ameliorated in mouse experimental models of autoimmune encephalomyelitis,[7] arthritis,[8] myocarditis,[9] polymicrobial sepsis,[10] diabetes,[11,12] and hepatitis.[13,14]

Ischemia/reperfusion injury (IRI) still remains an important problem in clinical transplantation. IRI in the liver causes approximately 10% of early graft failure, can lead to a higher incidence of acute and chronic rejection, and contributes to the acute shortage of donor organs available for transplantation.[15] The mechanisms underlying liver IRI are complex but are known to involve the activation of T cells and macrophages including Kupffer cells and neutrophils, leading to the formation of reactive oxygen species, secretion of proinflammatory cytokines/chemokines, complement activation, and vascular cell adhesion molecule activation.[16] The acute inflammation response during liver reperfusion consists of 2 phases: acute and subacute response. In the acute phase at 3 to 6 hours after reperfusion, hepatocellular injury associates with T lymphocyte and Kupffer cell activation.[17] In the subacute phase at 18 to 24 hours, massive neutrophil accumulation takes place.[18] Especially, at 1 hour after reperfusion, CD4 positive T lymphocytes are the key regulator in initiating ischemia/reperfusion (IR)–induced liver inflammation.[19,20] T cell–Kupffer cell interaction constitutes a key event in liver IRI[21]; however, there is not enough data to prove this mechanism.

Molecules of the T cell immunoglobulin mucin (TIM) family represent relatively newly described immune regulators. Recent experiments have revealed that Gal-9 has been identified as a ligand for TIM-3. Binding of Gal-9 to TIM-3 causes an inhibitory signal that results in the induction of apoptosis of T helper (T_h) 1 cells and downregulated T_h1-type immunity.[22] The activation of the TIM-3/Gal-9 pathway negatively regulates activated CD4+ and CD8+ alloreactive T cells and results in the prolonged survival of allogeneic skin grafts.[23] It has been reported that the TIM-1/TIM-4 or TIM-3/Gal-9 pathway represents one of the key mechanisms underlying T cell–Kupffer cell crosstalk in the pathophysiology of liver IRI.[24,25]

This study was focused on examining the putative role of Gal-9 in the pathophysiology of a mouse liver model for warm IRI. Our results demonstrate that Gal-9 engagement ameliorated local inflammation and liver damage due to IR, suggesting the importance of Gal-9 signaling in the maintenance of hepatic homeostasis, expansion of the organ donor pool, and improvement of the overall success of liver transplantation.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (9 to 12 weeks, 25 to 30 g weight) were purchased from CLEA Japan, Inc. (Osaka, Japan). Gal-9 knockout (KO; Gal-$9^{-/-}$) mice[8] were kindly provided by GalPharma (Takamatsu, Japan). All animals were maintained under specific pathogen-free conditions and received humane care according to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication, 8th edition, revised, 2011). All experimental protocols were approved by the Animal Research Committee of Kyoto University.

Liver IRI Model

We used an established mouse model of partial warm hepatic IRI.[24-26] An atraumatic clip was used to interrupt the artery/portal venous blood supply to the left/middle liver lobes. After 90 minutes of ischemia, the clamp was removed, initiating reperfusion (Supporting Fig. 1A). To investigate the function of Gal-9, mice were given an intravenous injection of a stable form of recombinant galectin-9 (reGal-9) protein (60 μg/body), provided by GalPharma (Takamatsu, Japan)[8,27] at 30 minutes before the ischemia insult, and then were killed 6 and 24 hours after reperfusion. Control mice were pretreated with phosphate-buffered saline (PBS). Sham-operated mice underwent the same procedure but without vascular occlusion.

Hepatocellular Function

Serum alanine aminotransferase (sALT) levels in peripheral blood, an indicator of hepatocellular injury, were measured by a standard spectrophotometric method with an automated clinical analyzer (JCA-BM9030; JEOL, Ltd., Tokyo, Japan).

Histology

Liver paraffin sections (4-μm thick) were stained with hematoxylin-eosin (H & E). The severity of liver IRI (necrosis, sinusoidal congestion, and centrilobular ballooning) was blindly graded with a modified Suzuki's criteria on a scale from 0 to 4.[28]

Immunohistochemistry

Rat monoclonal antibodies (mAbs) against mouse Gal-9 (108A2; Biolegend, San Diego, CA), CD3 (17A2; TONBO biosciences, Irvine, CA), CD68 (FA-11; AbD Serotec, Kidlington, UK), or lymphocyte antigen 6 complex locus G (Ly6-G; RB6-8C5; Tonbo Biosciences, Irvine, CA) were applied on liver paraffin-embedded sections. Then, biotinylated rabbit anti-rat immunoglobulin G (IgG) and goat anti-rabbit IgG were applied. After incubation, immunoperoxidase (VECTASTAIN Elite ABC Kit, Vector Labs, Burlingame, CA) was applied to the sections. Positive cells were counted blindly at 10 high-power field (HPF)/section (×400). Negative controls were prepared by incubation with normal rat IgG or rabbit IgG (sc-2026, 2027; Santa Cruz Biotechnology, Santa Cruz, CA) instead of the first antibody (Ab).

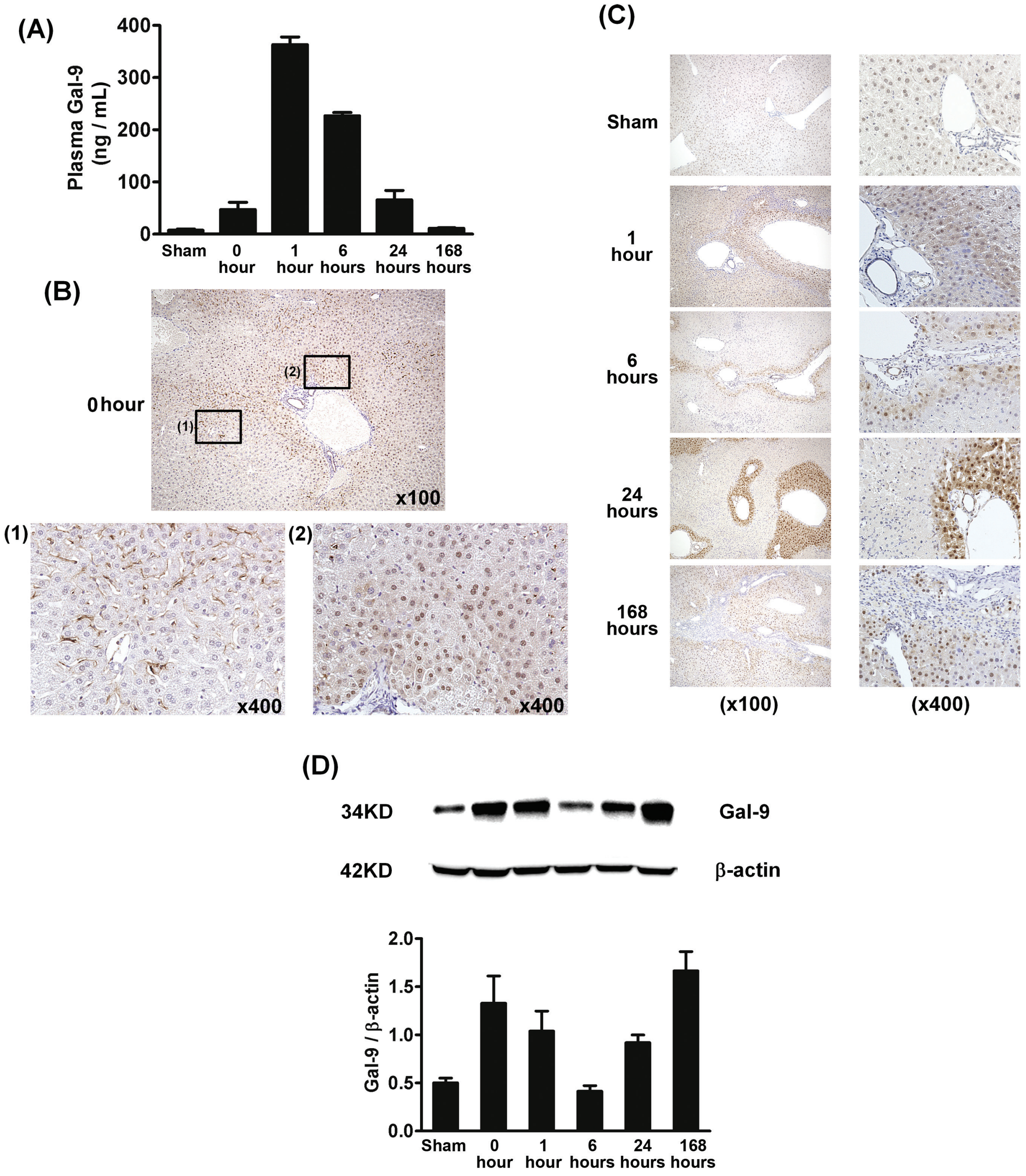

Figure 1. Expression of Gal-9 in IRI livers. (A) The concentration of Gal-9 in mouse plasma at 0 to 168 hours of liver reperfusion followed by 90 minutes of local warm ischemia; (B) The data show 0 hours, that is, 90 minutes after ischemia and just before reperfusion; (C) 1 to 168 hours after reperfusion. (D) Top: the changes in expression of Gal-9 were measured by western blot analysis. Bottom: quantitation of the Gal-9/β-actin ratio. Means and SEM are shown (n = 3 to 5/group).

Enzyme-Linked Immunosorbent Assay for Gal-9

Plasma Gal-9 level was quantified by enzyme-linked immunosorbent assay as reported previously.[29] Anti-mouse Gal-9 mAb (GalPharma), following the addition of anti-rabbit IgG-biotin (Southern Biotech, Birmingham, AL), was used for detection. Samples were quantified by using streptavidin (SA)-HRP (Thermo Scientific, Rockford, IL) and tetramethylbenzidine (Biolegend) and were measured by a spectrophotometer.

Quantitative Reverse-Transcription Polymerase Chain Reaction (PCR)

Total RNA was extracted from the liver tissue using the RNeasy Kit (Qiagen, Venlo, the Netherlands) and complementary DNA was prepared by Omniscript RT kit (Qiagen). Quantitative PCR was performed using the StepOnePlus Real-Time PCR System (Life Technologies, Tokyo, Japan). Primers used to amplify specific gene fragments have been listed (Supporting Table 1).

Target gene expression was calculated by the ratio to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western Blot Assay

Ab recognizing Gal-9 (Biolegend), Ab to toll-like receptor 4 (TLR4; Santa Cruz Biotechnology), Ab to cleaved caspase-3 (Asp175; CST, Danvers, MA), and β-actin (PM053; MBL, Nagoya, Japan) were used for detection of each molecule, respectively. The intensity of the bands was quantified with imaging analysis software (CS Analyzer, Atto Corporation, Tokyo, Japan).

Apoptosis Assay

Apoptosis in 4-μm liver paraffin sections was detected by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method using the In Situ Apoptosis Detection Kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. Negative control was prepared by the omission of terminal deoxynucleotidyl transferase. Positive controls were generated by treatment with deoxyribonuclease. TUNEL-positive cells were counted in 10 HPF/section under light microscopy.

Statistical Analysis

All data are expressed as means ± standard error of mean (SEM). Differences between experimental groups were analyzed using 1-way analysis of variance or Student *t* test for unpaired data. All differences were considered statistically significant at the P value of <0.05.

RESULTS

Gal-9 Protein Expression Was Up-Regulated in the Liver and Blood Upon IR Insult

The concentration of Gal-9 in blood and the expression of Gal-9 in liver tissues of mice that underwent 90 minutes of warm ischemia followed by 168 hours of reperfusion were examined. The concentration of Gal-9 in plasma showed a high level 1 hour after reperfusion, despite its low level just before reperfusion. Then, it gradually decreased with the duration up to 168 hours after reperfusion (Fig. 1A). On the contrary, as shown in immunohistochemistry (Fig. 1B) and western blot analysis (Fig. 1D), the expression of Gal-9 in the liver had already started during ischemia insult before reperfusion, which was clearly detected in nonparenchymal cells as well as in hepatocytes. Because the feature of Gal-9–positive cells in the nonparenchymal region was consistent with F4/80-positive cells (unpublished data), it is plausible that the Gal-9–positive cells in the nonparenchymal area are thought to be the activated resident Kupffer cells in the liver. One hour after the start of reperfusion, the Gal-9 expression in the liver increased more clearly in the area of the nucleus and cell cytoplasm of hepatocytes (Fig. 1C). Notably, the Gal-9 expression in the liver and the number of Gal-9–positive cells bottomed at 6 hours after reperfusion, which indicated the maximum liver injury due to IR. However, the Gal-9–positive cells and the amounts of Gal-9 in the liver then increased continuously up to 168 hours after reperfusion (Fig. 1C, D). These results indicated a significant involvement of Gal-9 in mouse liver IRI.

Absence of Gal-9 Exacerbated Liver IRI

Hepatocellular damages in warm IRI were analyzed by using wild type (WT) mice and Gal-9–deficient mice. The damage due to IR was profoundly exacerbated in Gal-9$^{-/-}$ mice, compared with WT. As shown in Fig. 2A, the sALT levels in Gal-9$^{-/-}$ mice were significantly increased both 6 and 24 hours after reperfusion. These data correlated well with Suzuki's histological criteria of the hepatocellular damages, composed of necrosis, sinusoidal congestion, and centrilobular ballooning (Fig. 2B). The livers in Gal-9$^{-/-}$ mice showed severe liver tissue damages, which were comparable with or higher than those in wild mice. The results indicated that the absence of Gal-9 exacerbated liver damages caused by IR injury.

ReGal-9 Pretreatment Ameliorated Liver IRI

To elucidate the function of Gal-9 during liver IR, we applied reGal-9 in our mouse IRI model.[26] ReGal-9 was intravenously administered into wild or Gal-9$^{-/-}$ mice 30 minutes before the ischemia insult (Supporting Fig. 1A). As shown in Fig. 2A, the sALT levels in IR-treated WT mice which had been pretreated with reGal-9 were remarkably decreased both 6 and 24 hours after reperfusion, as compared to IR-injured wild mice without Gal-9 administration. Moreover, the sALT levels in IR-injured Gal-9$^{-/-}$ mice pretreated with reGal-9 were also significantly decreased. Notably, although the alanine aminotransferase level in the Gal-9$^{-/-}$ mice pretreated with Gal-9 was still high at 6 hours after reperfusion, it was dramatically reduced 24 hours after reperfusion and became at an almost equal level to the reGal-9 pretreated wild mice. Thus, the pretreatment with reGal-9 ameliorated IR-induced hepatocellular damages in wild mice as well as Gal-9–deficient mice. Moreover, even when reGal-9 was intravenously administered after a 90-minute ischemia insult (Supporting Fig. 1A), the sALT levels were significantly improved (Supporting Fig. 1B). In histological findings as shown in Fig. 2B, the livers in PBS-pretreated WT or Gal-9$^{-/-}$ mice revealed severe lobular edema, congestion, ballooning, and hepatocellular necrosis. In contrast, the exacerbated hepatocellular damages in wild or Gal-9$^{-/-}$ mice were improved both 6 and 24 hours after reperfusion by the pretreatment with reGal-9. Livers in reGal-9-pretreated WT or Gal-9$^{-/-}$ mice showed good preservation of architecture

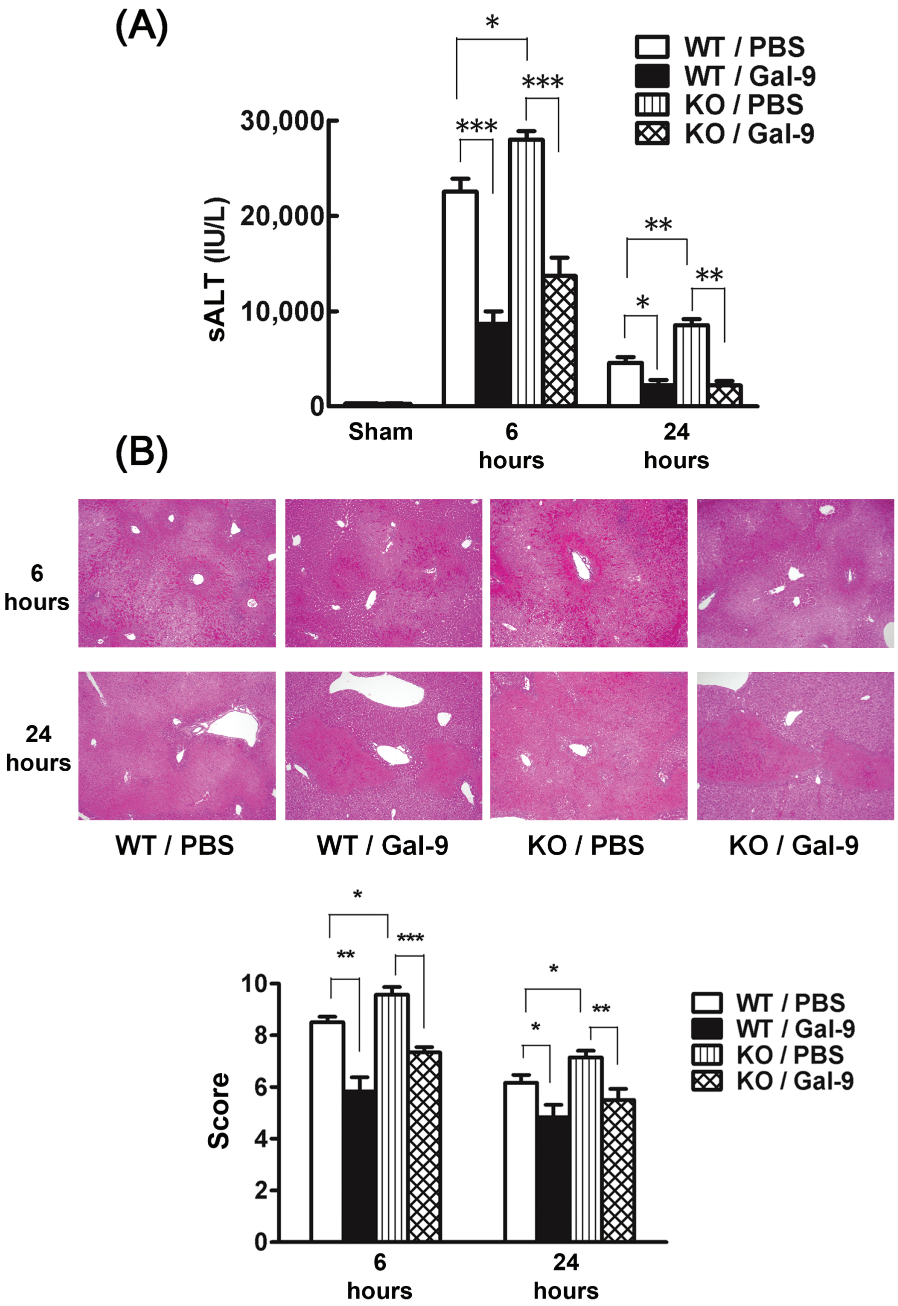

Figure 2. Liver damage induced by IRI in wild mice or Gal-9 deficient mice with or without reGal-9 pretreatment. (A) Hepatocellular damage measured by sALT levels at 6 and 24 hours. (B) Top: representative liver histology (H & E staining; magnification ×100) of liver lobes harvested 6 and 24 hours after reperfusion. Bottom: quantitation of Suzuki's histological criteria of the hepatocellular damage. Means and SEM are shown (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n = 6 to 8/group).

and histological detail. Indeed, the Suzuki's score was significantly improved both 6 and 24 hours after reperfusion in the reGal-9–pretreated WT and Gal-9$^{-/-}$ mice (Suzuki score; Fig. 2B). Thus, the protective effect of reGal-9 was confirmed in the mouse IR injury. The Gal-9 expression in the liver tissues clearly increased at 6 hours after reperfusion by the pretreatment with reGal-9 (Fig. 3). Although the Gal-9 expression was markedly reduced at 6 hours in the IR-induced wild mice, pretreatment with reGal-9 profoundly enabled enhanced Gal-9 expression in the IR-induced wild mice 6 hours after reperfusion. Surprisingly, such enhanced production of Gal-9 was remarkable even 24 hours after the start of reperfusion during IR insult (Fig. 3B). These data indicated that the changes in Gal-9 protein level in the livers of IR-injured mice are strictly related to the damages of the liver and that exogenous administration of reGal-9 could efficiently protect liver from the damages caused by IR.

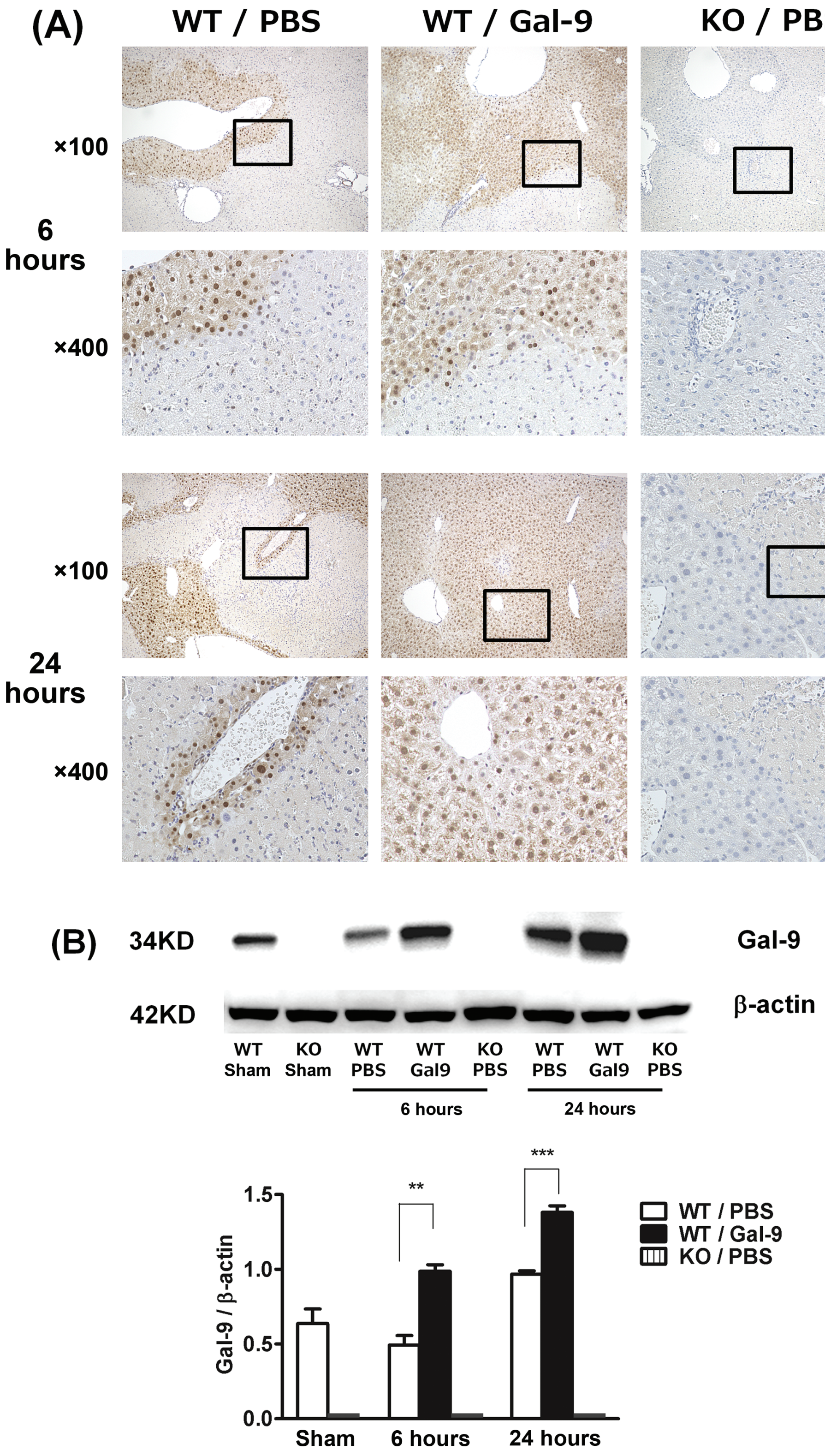

Figure 3. Exogenous reGal-9 administration enhanced Gal-9 expression on liver tissue. Actual Gal-9 expression was accessed (top) by immunohistochemistry (magnification ×100 and ×400) and (bottom) by western blot analysis. Means and SEM are shown ($^{**}P < 0.01$; $^{***}P < 0.001$; $n = 3$/group).

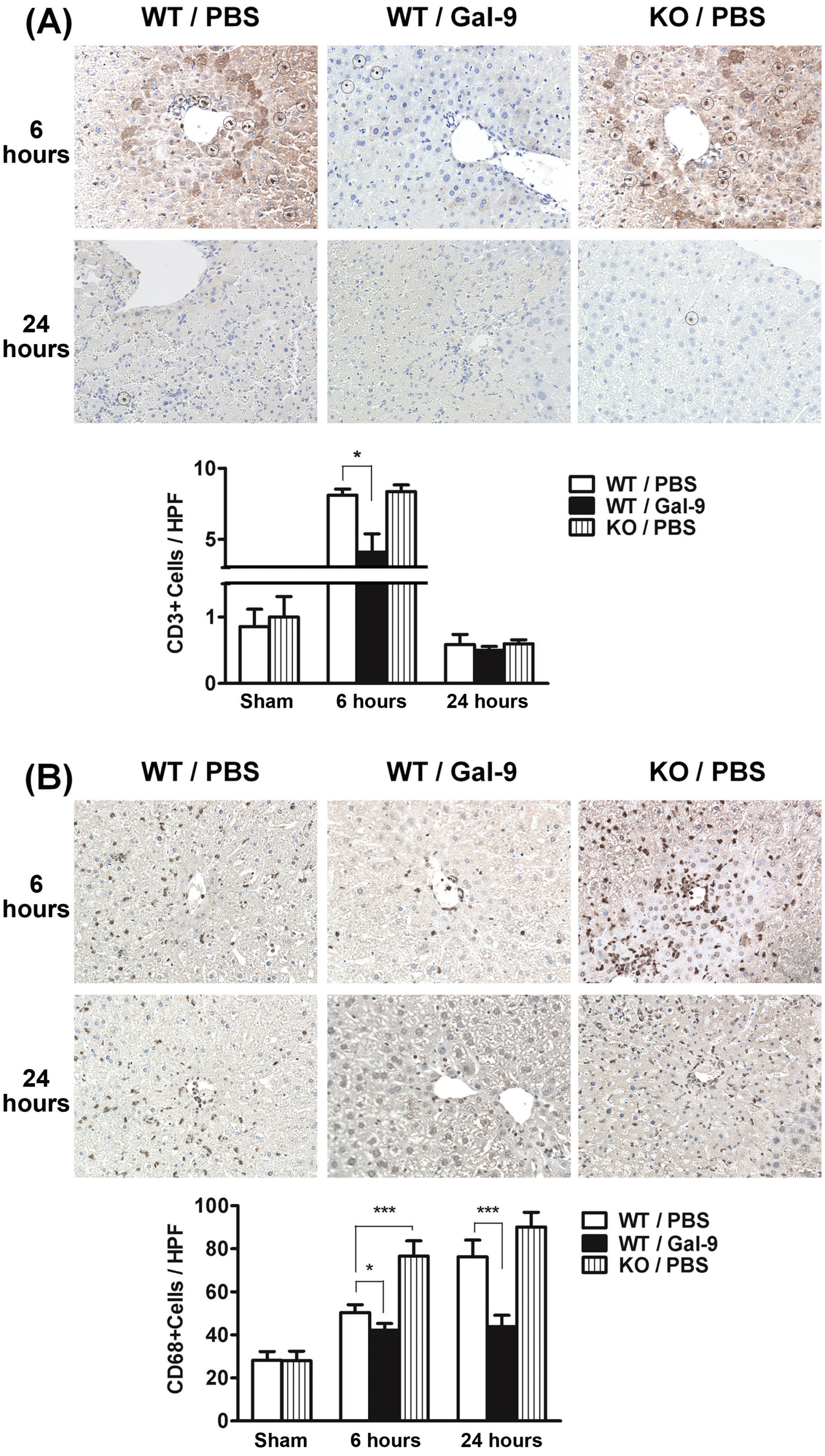

Figure 4. Immunohistochemical staining for CD3-, CD68-, and Ly6-G–expressing cells in IRI livers. (A) Top: representative liver sections stained by CD3 (dark brown spots, as shown by circle). Bottom: quantitation of hepatic CD3 accumulation. (B) Top: representative liver sections stained by CD68 (dark brown spots). Bottom: quantitation of hepatic CD68 accumulation. (C) Top: representative liver sections stained by Ly6-G (dark brown spots; magnification ×400). Bottom: quantitation of hepatic Ly6-G accumulation. Means and SEM are shown (*$P < 0.05$; ***$P < 0.001$; n = 3/group).

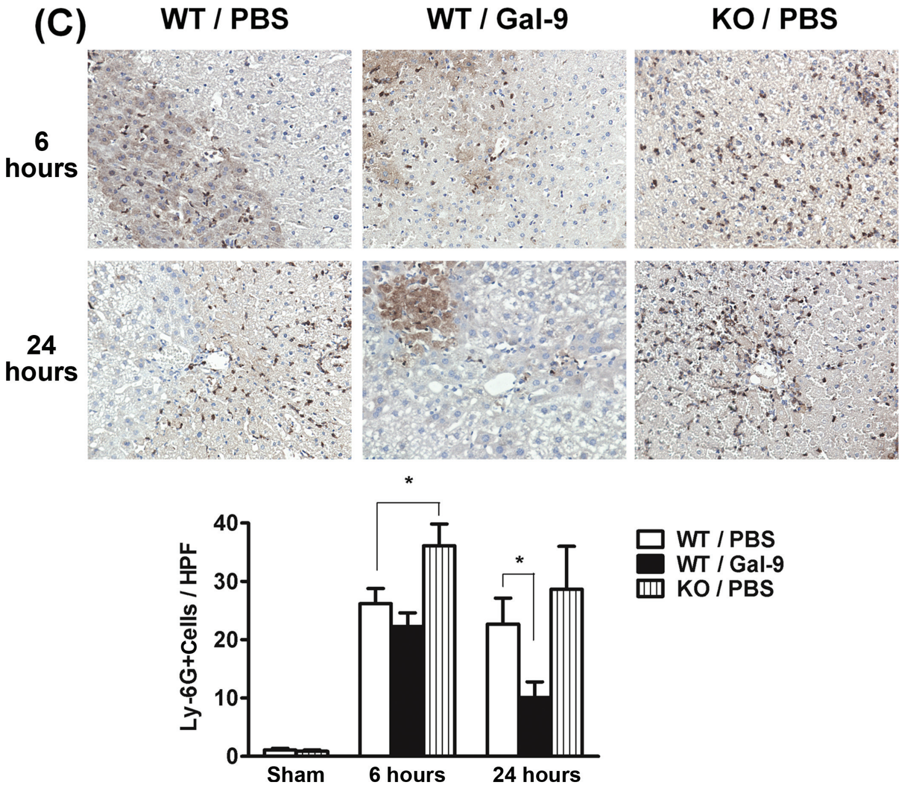

Figure 4. Continued.

Depletion of Gal-9 Increased T Cell, Macrophage, and Neutrophil Infiltration, Whereas ReGal-9 Pretreatment Suppressed the Infiltration

Although relatively small but significant numbers of CD3-positive T cells could be found in IR-injured wild mice, the numbers of T cells further decreased in the mice pretreated with reGal-9 at 6 hours after reperfusion (Fig. 4A). On the other hand, the numbers of T cells in Gal-9$^{-/-}$ mice increased as compared to the wild mice. As shown in Fig. 4B, reGal-9 pretreatment to the IR-injured wild mice lowered CD68 positive macrophage infiltration into the liver as compared with wild mice without reGal-9 pretreatment. In the IR-injured Gal-9$^{-/-}$ mice, the numbers of the CD68 positive macrophages were significantly increased at 6 hours after reperfusion. Numbers of Ly6-G positive cells, which are activated neutrophils, were significantly decreased in the livers of wild mice pretreated with reGal-9, especially at 24 hours after the reperfusion, as compared to mice without the reGal-9 pretreatment. On the contrary, the infiltration of neutrophils into the livers of Gal-9$^{-/-}$ mice increased 6 hours after reperfusion (Fig. 4C). Hence, Gal-9 suppressed the liver infiltration of inflammatory cells such as T cells, macrophages, and neutrophils and played a role in the inhibition of liver damage during IR.

Gal-9 Pretreatment Suppressed Proinflammatory Cytokines and Chemokines

We then examined the effects of a Gal-9 deficiency and the reGal-9 pretreatment on the expression of proinflammatory cytokines and chemokines, which have been reported to be important in the mechanism of liver IRI. Cytokines (tumor necrosis factor α [TNF-α], interleukin [IL]-6, IL-1β, and interferon [IFN]-γ) and chemokine ligand (CXCL; CXCL1 and CXCL2, which are chemotactic for macrophages) were measured during liver IRI by quantitative reverse-transcription PCR (Fig. 5).[30] Six hours after reperfusion, reGal-9 pretreatment of the wild mice profoundly decreased the expression of TNF-α, IL-6, IL-1β, and IFN-γ. In addition, in Gal-9$^{-/-}$ mice, the expression of these cytokines was clearly increased compared to those of wild mice. The expression of CXCL1 and CXCL2 at 6 hours was significantly reduced in wild mice pretreated with reGal-9. The expression of CXCL1 in Gal-9$^{-/-}$ mice was significantly increased at 24 hours, and the expression of CXCL2 in Gal-9$^{-/-}$ mice was also increased significantly at 6 and 24 hours.

Gal-9 Pretreatment Suppressed IR-Induced Apoptosis of Hepatocytes

We examined how Gal-9 deficiency and treatment with reGal-9 affect the apoptosis of liver cells induced

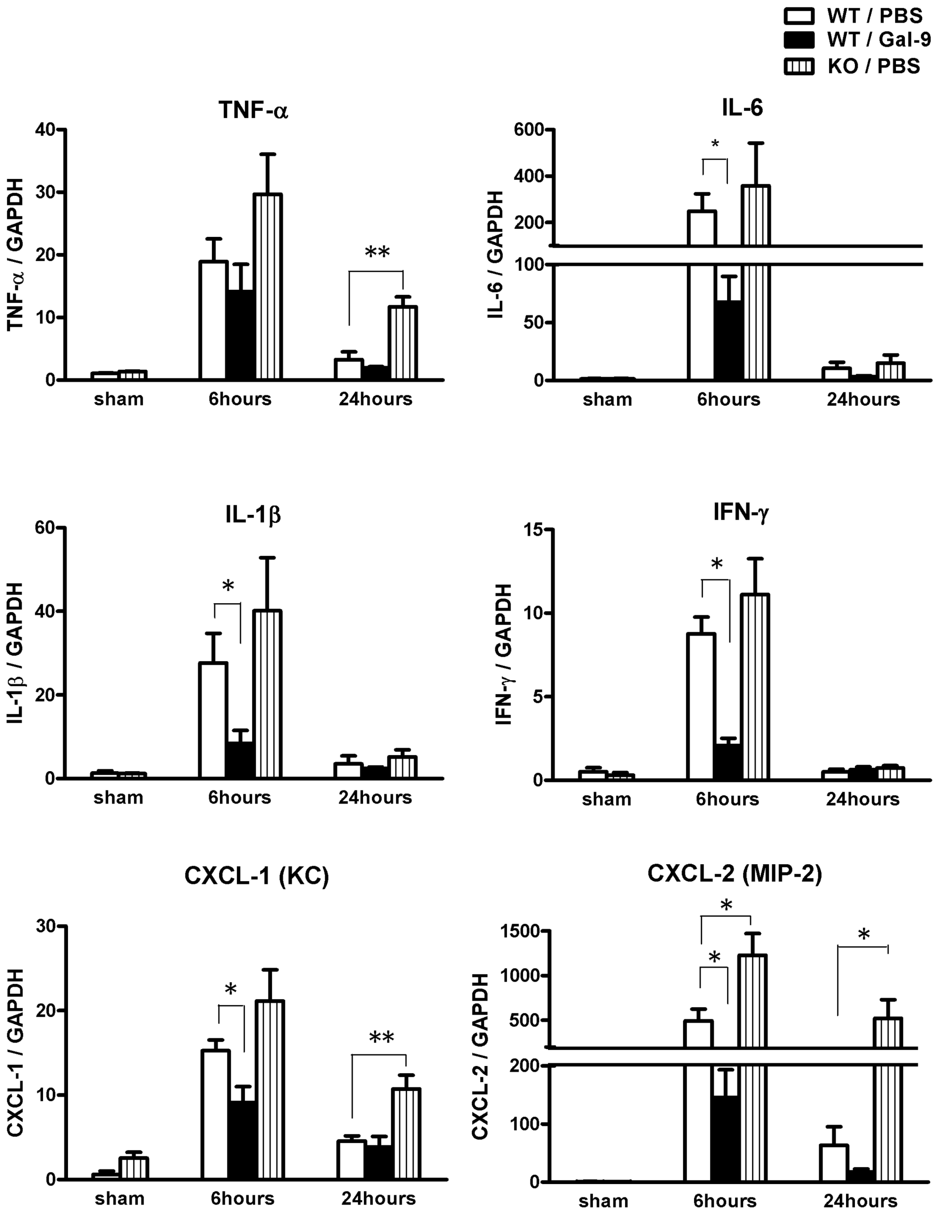

Figure 5. Quantitative real-time PCR–assisted detection of gene expression of proinflammatory cytokines and chemokines in IRI livers. The induction ratios of proinflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ) and chemokines (CXCL1 and CXCL2) 6 and 24 hours after IR in mouse liver tissues. Data were normalized to GAPDH gene expression. Means and SEM are shown (*$P < 0.05$; **$P < 0.01$; n = 4 to 5/group).

by IRI (Fig. 6A). The number of cells in apoptosis (TUNEL-positive cells/field) induced by IR in wild mice significantly decreased in cases that were pretreated with reGal-9. In contrast, TUNEL-positive cells increased in Gal-9$^{-/-}$ mice compared with WT. In addition, pretreatment with reGal-9 inhibited the expression of cleaved caspase-3 at 24 hours after reperfusion (Fig. 6B). On the contrary, the expression of a cleaved caspase-3 level in the liver significantly increased in Gal-9$^{-/-}$ mice at both 6 and 24 hours after reperfusion. These data clearly demonstrate that Gal-9 plays a crucial role in the protection of liver damage induced by IR and in the maintenance of liver homeostasis.

Gal-9 Suppressed Expression of TLR4 in Liver IRI

Finally, the expression of TLR4, which has been reported to be critical in the pathogenesis of liver IRI,[31] was measured by a western blot analysis (Supporting Fig. 2). Administration of reGal-9 suppressed the expression of TLR4 in the liver of the IR-injured wild mice both 6 and 24 hours after reperfusion. On the contrary, the expression level of TLR4 in the liver in the IR-injured Gal-9$^{-/-}$ mice significantly increased both 6 and 24 hours after reperfusion as compared with wild mice. Thus, reGal-9 suppressed TLR4 upregulation induced by IRI. The data suggest that

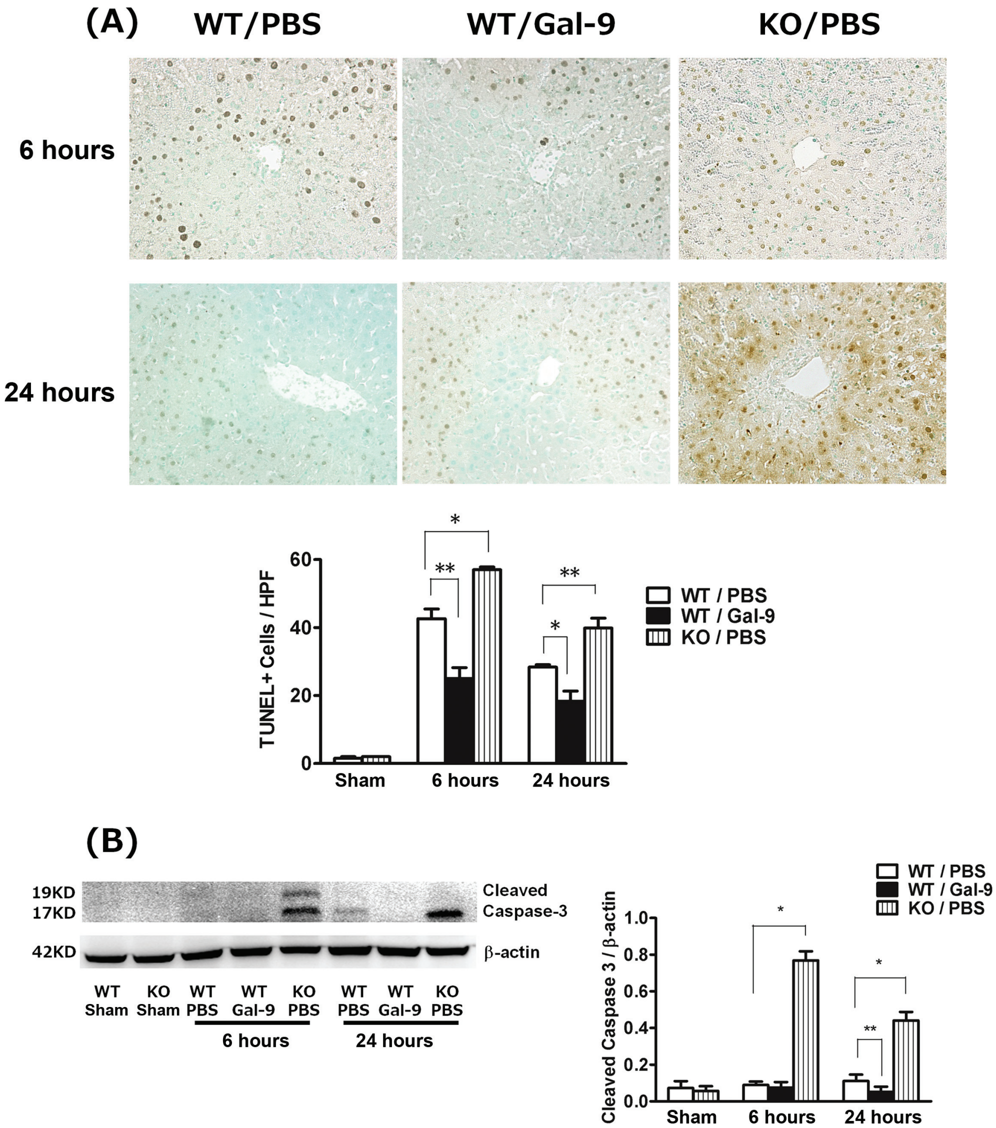

Figure 6. TUNEL-assisted detection of intrahepatic apoptosis in IRI livers. (A) Upper panel: Representative liver sections were stained by TUNEL method in liver tissue (TUNEL positive cells as shown by dark brown spots: ×400 magnification). Lower panel: Quantitation of intrahepatic apoptosis by TUNEL method. Means and SEM are shown ($^*P < 0.05$, $^{**}P < 0.01$; n = 5 to 6/group). (B) Left side: The expression of cleaved caspase-3 in liver tissue was measured by western blot analysis. Right side: Quantitation of cleaved caspase-3/β-actin ratio. Means and SEM are shown ($^*P < 0.05$, $^{**}P < 0.01$; n = 3 to 5/group).

Gal-9 plays a crucial role in reducing the inflammation caused by IR through the down-regulation of TLR4 expression.

DISCUSSION

The present study provides clear evidence that Gal-9 has a crucial role as an endogenous protective factor in the pathogenesis of warm IRI in a mouse liver. First, Gal-9 expression in the liver rapidly increased at the early stage of liver IRI and continued during the course. Second, absence of Gal-9 exacerbated profoundly the IR-induced hepatocellular damage. On the contrary, administration of reGal-9 significantly improved liver damage because of IR in the wild as well as in the Gal-$9^{-/-}$ mice.

The most interesting data in this study are that the expression of endogenous Gal-9 was rapidly enhanced in the liver right after the onset of the liver IR insult, and this fast and furious expression started from an early time during IR, especially at the ischemic stage that is just before reperfusion. The expression reached to the peak at a very early time of the reperfusion stage, and it bottomed at 6 hours after reperfusion when the liver suffered severe damage as shown in Fig. 1D. Moreover, even 168 hours after the onset of reperfusion, hepatocytes still keep producing endogenous Gal-9 protein (Fig. 1C). Gal-9 concentration in plasma was the highest 1 hour after the start of reperfusion, and the concentration gradually reduced over time (Fig. 1A). These data imply that Gal-9 may recover homeostasis of the liver tissue

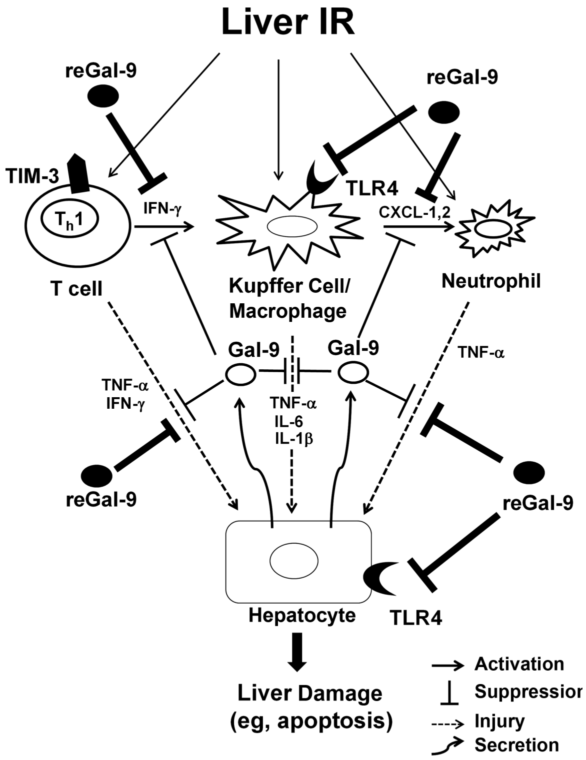

Figure 7. Cross-talk interactions underlying the protective effects of Gal-9 in liver IRI. This schema depicts mechanisms underlying the protective effects of Gal-9 in liver IRI. Liver IR triggers at first activation of T_h1 cells, macrophages, and neutrophils, sequentially. The production of IFN-γ by activated T_h1 cells also activates macrophages. The activated macrophages elaborate inflammatory cytokines (TNF-α and IL-6) and chemokines (CXCL1 and CXCL2), and up-regulate TLR4 expression. The expression of Gal-9 is induced endogenously in especially hepatocytes and Kupffer cells by IR insult at early stage, and works as protective factor. Exogenous Gal-9 such as reGal-9 administration suppresses the signal from activated T_h1 cells, macrophages and neutrophils including TLR4 expression to hepatocytes, reduces apoptosis in the liver, and finally facilitates liver damage.

through a rapid protective reaction against the damage caused by reperfusion. Previous reports revealed that Gal-9 production was induced and elevated from monocytes and macrophages by IFN-γ in the case of hepatitis C infection.[13] Unlike the case of chronic liver damage by a virus, in the acute injury instance such as liver IRI, hepatocytes, and nonparenchymal cells such as Kupffer cells appeared to directly produce endogenous Gal-9 independently of the presence of IFN-γ.

Thus, the question arises as to how Gal-9 affects IR-caused liver damage. The function of endogenous Gal-9 was examined by using Gal-9 KO mice. As shown in Fig. 2, liver cell damage was exacerbated in both sALT level and in the histological finding as compared with those of WT mice. The absence of Gal-9 significantly increased the production of proinflammatory cytokines (TNF-α, IL-6, IFN-γ, and IL-1β) and chemokines (CXCL1 and CXCL2) in the liver upon IR. These cytokine/chemokine mediators are known to influence T cell/macrophage/neutrophil trafficking patterns in liver tissue. TNF-α triggers neutrophil-attracting CXC chemokines to express adhesion molecules on vascular endothelial cells.[32] Neutrophil adhesion to endothelial cells leads to their transmigration into the liver parenchyma. Especially, neutrophil-derived neutrophil elastase induces inflammatory chemokines (CXCL1, CXCL2) and accelerates IR-mediated damage via the feedback mechanism with recruited neutrophils,[33] which results in the direct injury to the membrane components.[34] The data in the present study strongly support the critical "protective" role of Gal-9 against liver IRI by suppressing these vicious circles. Notably, the expression of Gal-9 has increased at an early stage of reperfusion before an increase in sALT as shown in Figs. 1 and 2. The rise of Gal-9 may provide an early warning signal; in other words, Gal-9 may play a crucial role as an alarmin in the case of liver IRI.

One of the key events after reperfusion is apoptosis of hepatocytes.[35] In the setting of liver IRI related to Gal-9, activated caspase-3 also plays a key role in the final stages of the apoptotic cascade.[36] As shown in Fig. 6, the liver tissues in the absence of Gal-9 showed an increased frequency of TUNEL cells, accompanied by an increased cleaved caspase-3 expression. This phenomenon was strongly suppressed by the administration of reGal-9. Gal-9 has been well known for induction of apoptosis in IFN-γ and IL-17 producing T cells and amelioration of autoimmunity in murine models. Gal-9 is a strong modulator of T cell immunity through its apoptotic effects on T_h1 and T_h17 cells in case of autoimmunity.[37] Therefore, Gal-9 down-regulates the production of IFN-γ and other proinflammatory cytokines through induction of apoptosis in T_h1 cells, which is consistent with our present finding of decreased IFN-γ induction and T cell infiltration in the liver after reGal-9 administration.

It has been reported that Gal-9 is a ligand of TIM-3 that is expressed on T_h1 and T_h17 cells[7] and that Gal-9 signaling induced the death of these cells, resulting in the suppression of T_h1- and T_h17-related cytokine production in vivo and in vitro.[8] In addition, TIM-3/Gal-9 signaling may exert a "protective" function by depressing IFN-γ production in liver IRI settings, and anti–Gal-9 Ab treatment in vivo slightly exacerbated liver damage due to IR.[25] The exogenous administration of reGal-9 administration may further facilitate the apoptosis of T_h1 cells through the activation of the Gal-9/TIM-3 pathway in T_h1 cells.[12] A recent study shows that harnessing TIM-3/Gal-9 signaling at the T cell–hepatocyte interface facilitates homeostasis in IR-stressed orthotopic liver transplants.[36] Our data strongly suggest that the interaction of Gal-9 with TIM-3 in the underlying mechanism will be essential in the regulation of liver IRI.

TLR4 has been reported to be involved in the initiation of IRI, as evidenced by the full protection of TLR4-deficient livers.[38] The parenchymal hepatocyte is an active participant in the sterile inflammatory response after IR through TLR4-mediated activation of proinflammatory signaling.[39] Administration of reGal-9 strongly down-regulated TLR4 expression and vice versa the Gal-9 deficiency induced up-regulation of TLR4 expression during IR injury (Supporting Fig. 2).

Figure 7 depicts the mechanisms underlying the liver IR injury on the basis of the present study and other previous reports. Thus, it will be plausible that IR insult induces the production of Gal-9 in the liver, which works as a protective function against tissue damage. However, the function and amounts of endogenous Gal-9 are not enough to recover the damages or maintain homeostasis of the liver environment caused by an IR insult; besides, endogenous Gal-9 is highly susceptible to proteolysis in the biological process. In order to overcome the problem, we used reGal-9, which is composed of tandem-repeat–type and is a highly stable galectin.[27] The present study indicates that the administration of stable reGal-9 should be useful for inadequate amounts of endogenous Gal-9 and that it possess a therapeutic potential for the prevention or treatment of IRI in the liver.

In conclusion, our data indicate an essential role of Gal-9 in the pathophysiology of liver IRI. The endogenous Gal-9 in the liver works as a protective factor and plays a crucial role in the maintenance of liver homeostasis. Administration of exogenous Gal-9, such as stable reGal-9, decreases liver damage due to IR. This study suggests a previously unrecognized function of Gal-9 and a new therapeutic strategy for liver IRI.

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