Increasing numbers of hepatic dendritic cells promote HMGB1-mediated ischemia-reperfusion injury

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Abstract: Endogenous ligands released from damaged cells, so-called damage-associated molecular pattern molecules (DAMPs), activate innate signaling pathways including the TLRs. We have shown that hepatic, warm ischemia and reperfusion (I/R) injury, generating local, noninfectious DAMPs, promotes inflammation, which is largely TLR4-dependent. Here, we demonstrate that increasing dendritic cell (DC) numbers enhance inflammation and organ injury after hepatic I/R. High-mobility group box 1 (HMGB1), a NF released by necrotic cells or secreted by stimulated cells, is one of a number of ligands promoting TLR4 reactivity. Augmentation of DC numbers in the liver with GM-CSF hydrodynamic transfection significantly increased liver damage after I/R when compared with controls. TLR4 engagement on hepatic DC was required for the I/R-induced injury, as augmentation of DC numbers in TLR4 mutant (C3H/HeJ) mice did not worsen hepatic damage. It is interesting that TLR4 expression was increased in hepatic DC following HMGB1 stimulation in vitro, suggesting a mechanism for the increased liver injury following I/R. It thus appears that functional TLR4 on DC is required for I/R-induced injury. Furthermore, HMGB1 may direct the inflammatory responses mediated by DC, at least in part, by enhancing TLR4 expression and reactivity to it and other DAMPs. J. Leukoc. Biol. 81: 119–128; 2007.

Key Words: inflammation · Toll-like receptor 4 · liver

INTRODUCTION

Ischemia reperfusion (I/R) injury is a pathophysiologic process, whereby hypoxic organ damage is accentuated following return of blood flow and oxygen delivery. Transient episodes of ischemia are encountered during solid organ transplantation, trauma, hypovolemic shock, and elective liver resection, when inflow occlusion or total vascular exclusion is used to minimize blood loss. The pathophysiology of liver I/R injury includes direct cellular damage as the result of the ischemic insult as well as delayed dysfunction and damage resulting from activation of inflammatory pathways [1, 2].

The distal interacting elements in the cascade of inflammatory responses resulting in organ damage following hepatic I/R injury have been studied extensively. Activation of Kupffer cells with production of reactive oxygen species, up-regulation of the inducible NO synthase in hepatocytes, activation of JNK, up-regulation of proinflammatory cytokines, and neutrophil accumulation has been identified as contributing events to the inflammation-associated damage [3–8]. The extent to which the initial cellular injury contributes to propagation of the inflammatory response and leads to further tissue damage is poorly understood. Studies suggest that a key link between the initial damage to cells and the activation of inflammatory signaling involves release of endogenous damage/danger-associated molecular pattern signals (DAMPs) from ischemic cells or disruption of the tissue matrix [9, 10].

Our recent studies suggest that the initial ischemic injury activates inflammatory signaling through the release of high-mobility group box 1 (HMGB1) [9], which is a nuclear protein that is involved in DNA binding and bending, enhancing access to transcriptional factors [11]. In addition to its nuclear role, extracellular HMGB1 has been shown to be a critical mediator of the innate immune response to infection and injury. During the course of experiments to identify late-acting mediators of endotoxemia and sepsis, HMGB1 was found to have cytokine-like properties [12]. HMGB1 is released from activated macrophages and immunostimulated gut epithelial cells in a delayed manner relative to the secretion of the early proinflammatory mediators TNF and IL-1 [13, 14]. HMGB1 is also released from necrotic or damaged cells and promotes inflammation [15, 16]. Our laboratory has shown recently that whereas HMGB1 is a late mediator of systemic inflammation, HMGB1 can also play a role as an early mediator following acute, local organ injury [9]. HMGB1 levels are increased by I/R in the liver, and activation of the innate immune system by HMGB1 in this context requires TLR4-dependent signaling.

The TLRs are major sensors of the innate immune system, which recognizes the invasion of pathogenic microorganisms or tissue damage. Specific molecules that are present in microbial products [pathogen-associated molecular pattern molecules (PAMPs)] or endogenous DAMPs are the proximal ligands for these and other DAMP receptors [17]. Perhaps more than any of the other TLR family members, TLR4 sits at the interface of
microbial and sterile inflammation by responding to bacterial endotoxin and multiple other endogenous ligands, including hyaluronic acid [18], heparan sulfate [19], fibrinogen [20], HMGB1 [9, 21], and heat shock proteins (hsp) [22]. Whereas the role of TLR4 in the recognition of LPS is well established [23], only recently has it become apparent that TLR4 also participates in the recognition of several DAMPs. In vivo evidence for TLR4-mediated danger signaling comes from studies of acute tissue injury in hemorrhagic shock [24] as well as cardiac [25], renal [25, 26], and hepatic [9, 10] I/R models. In each case, TLR4 mutant animals exhibited reduced injury or inflammation when compared with wild-type controls. We and others reported recently that liver damage following warm I/R was decreased markedly in TLR4 mutant animals [9, 10] and restored by repletion with TLR4-competent bone marrow.

Much of the work about activation of the immune system by endogenous molecules has been with immune cells, specifically dendritic cells (DC), which are the nominal, professional APCs, detecting and amplifying innate immune responses, thereby promoting the development of the adaptive immune responses [27, 28]. Activation of TLRs on DC by danger signals may be partly responsible for the induction of cytokine and chemokine production by this cell population. Necrotic cell death appears to be primarily responsible for recruiting and activating DC in various tissues [29–31]. The aim of this study was to determine whether the TLR4-dependent injury in hepatic I/R is enhanced by increasing DC numbers. We show, using a model of sterile inflammation, that DC are responsible for recognizing the initial damage from ischemic cells and for activating TLR4-dependent signaling required for I/R-induced injury. In addition, HMGB1 may direct DC responses by enhancing TLR4 reactivity.

MATERIALS AND METHODS

Materials

Recombinant HMGB1 (rHMGB1) was provided by Kevin J. Tracey (North Shore University Hospital, Manhasset, NY) and prepared as described previously [32].

Animals

Male wild-type (C57BL/6; C3H/HeJ) and TLR4 mutant (C3H/HeJ) mice (8–12 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). All animals were maintained in a laminar flow-specific, pathogen-free atmosphere at the University of Pittsburgh (PA). The Institutional Animal Care and Use Committee of the University of Pittsburgh approved all animal protocols, and the experiments were performed in adherence to the National Institutes of Health (NIH) Guidelines for the use of Laboratory Animals.

Isolation and purification of liver DC

Rapid tail-vein injection of naked plasmids carrying the GM-CSF gene was used to expand DC within the mouse liver [33]. Plasmid containing GM-CSF cDNA, driven by the CMV promoter, was constructed and purified using a plasmid mega kit (Qiagen, Inc., Valencia, CA). The purity of the plasmid preparations was checked by absorbance at 260 and 280 nm and 1% agarose gel electrophoresis. Plasmids (5 μg in 2.0 ml normal saline), carrying genes encoding GM-CSF, empty plasmid, or PBS control were injected into mice i.v., 7 and 3 days prior to ischemia. Mice also received rHMGB1 or vehicle PBS i.v., 1 h prior to ischemia. The HMGB1 used for these studies contained undetectable amounts of LPS, as measured by the chromogenic Limulus amoeboocyte lysate assay (Associates of Cape Cod, East Falmouth, MA, or BioWhittaker, Walkersville, MD). Sham animals underwent anesthesia, laparotomy, and exposure of the portal triad without hepatic ischemia. Animals were killed at predetermined time-points (1–6 h) after reperfusion for serum and liver samples.

Experimental design

In hepatic DC expansion experiments, plasmids (5 μg in 2.0 ml normal saline) carrying genes encoding GM-CSF, empty plasmid, or PBS control were injected into mice i.v., 7 and 3 days prior to ischemia. Mice also received rHMGB1 or vehicle PBS i.v., 1 h prior to ischemia. The HMGB1 used for these studies contained undetectable amounts of LPS, as measured by the chromogenic Limulus amoeboocyte lysate assay (Associates of Cape Cod, East Falmouth, MA, or BioWhittaker, Walkersville, MD). Sham animals underwent anesthesia, laparotomy, and exposure of the portal triad without hepatic ischemia. Animals were killed at predetermined time-points after reperfusion for serum and liver samples.

Flow cytometry

Expression of DC surface molecules was determined by flow cytometric analysis, using an Epics Elite flow cytometer (Coulter Corp., Hialeah, FL). Cells were stained with mAb against CD40, CD80, and CD86 (all rat IgG2a). MHC Class II antigens were detected with mAb against IA<sup>α</sup> (mouse IgG2a, all from BD PharMingen, San Diego, CA). Expression of B7-H1 was identified by anti-B7-H1-specific mAb (rat IgG2a, eBioscience, San Diego, CA). The appropriate isotype control antibodies were used in the experiments.

MLR

Nylon, wool-eluted spleen T cells (2×10⁵/well in 100 μl) were cultured in triplicate in 96-well, round-bottom microculture plates with graded doses of β-irradiated (20 Gy; X-ray source) DC in RPMI-1640 complete medium for 5–4 days in 5% CO₂ in air. [³H]TdT (1 μCi/well) was added for the final 18 h, and incorporation of [³H]TdT into DNA was assessed by liquid scintillation counting. Results were expressed as mean cpm ± SD.

Cell culture and treatment

DC were isolated from the liver of mice and cultured at 2 × 10⁶ cells/ml in 24-well plates for 18–48 h. In some experiments, DC were stimulated with...
HMGB1 (1 μg/ml) or LPS (1 μg/ml). The supernatants of DC culture were subjected to cytokine detection by ELISA, and the cells were collected for detection of cytokine mRNA with real-time RT-PCR.

Liver damage assessment

To assess hepatic function and cellular injury following liver ischemia, serum alanine aminotransferase (ALT) levels were measured using the Opera Clinical Chemistry System (Bayer Co., Tarrytown, NY).

Cytokine measurement

Culture supernatants and serum were harvested and analyzed for the presence of cytokines using commercial ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocols. A standard curve using recombinant cytokine was generated for each assay.

Immunohistochemical staining

The cryostat sections (4 μm) were blocked with 0.3% H2O2, followed by goat serum, and then stained with biotin-conjugated anti-CD11c or -CD11b (both from BD PharMingen). The isotype- and species-matched, irrelevant mAb were used as controls. The color was developed by an enzyme reaction using avidin-biotin-alkaline phosphatase complex as the substrate. The slides were counterstained with Harris’ hematoxylin and mounted with a Crystal mount (Biomeda Corp., Foster City, CA).

SYBR Green real-time RT-PCR

Total RNA was extracted from cultured DC or whole liver using the TRIzol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer’s instruction. mRNA for TNF-α, IL-6, TLR4, TLR9, and GAPDH was quantified in duplicate by SYBR Green two-step, real-time RT-PCR. After removal of potentially contaminating DNA with DNase I (Life Technologies), 1 μg total RNA from each sample was used for RT with an oligo dT (Life Technologies) and a Superscript II (Life Technologies) to generate first-strand cDNA. PCR reaction mixture was prepared using SYBR Green PCR master mix (PE Applied Biosystems, Foster City, CA), using the primers as described previously [9]. Thermal cycling conditions were 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min on an ABI PRISM 7000 sequence detection system (PE Applied Biosystems). Each gene expression was normalized with GAPDH mRNA content.

Statistical analysis

Results are expressed as the mean ± SEM. Group comparisons were performed using Student’s t-test or ANOVA. Differences were considered significant at P < .05.

RESULTS

GM-CSF hydrodynamic transfection induces massive expansion of DC within the liver

DC are professional APCs, which induce and regulate immune responses. The role of hepatic DC in immunity is poorly understood as a result of the relative paucity of DC found within the liver and the difficulty of isolating them from normal mice. Most previous studies of hepatic DC were obtained via in vitro propagation with various cytokines and stimuli. In this study, we adopted an established method of retrograde gene transfection in the liver by rapid tail-vein injection of naked plasmid-carrying genes, so-called hydrodynamic injection. Transfection with plasmid GM-CSF greatly expanded in vivo B220–CD205–CD11b+CD11c+ LMDC [35]. The increase in CD11c+ cells in the liver was confirmed by immunohistochemistry (Fig. 1A). Whereas treatment of mice with vehicle PBS or control plasmid GFP did not increase the number of CD11c+ cells, overexpression of GM-CSF produced massive expansion of DC within the liver, and the livers from GM-CSF-treated and control PBS were also harvested, and hepatic NPC were isolated. Following hydrodynamic transfection of plasmid GM-CSF, the average number of NPC per liver increased more than 20- to 30-fold to more than 200 million cells (data not shown). The majority of these cells morphologically resembled MDC, forming large aggregates and localizing in periportal areas (Fig. 1A). Flow cytometry analysis revealed that the percentage of CD11c+/CD11b+ cells of NPC from GM-CSF-treated mice increased to 17.7% compared with 5% in PBS-treated mice (Fig. 1B). Thus, this method of DC expansion using GM-CSF overexpression resulted in a 60- to 90-fold increase of hepatic

Fig. 1. GM-CSF overexpression produces massive expansion of DC in the liver. (A) Plasmids carrying genes encoding GM-CSF or GFP were injected into mice using hydrodynamic technique. PBS injection was also used as a control. The increase in CD11c+ cells was confirmed by liver section staining. Images are representative liver sections from six mice per group. (B) Flow cytometry analysis of NPC isolated from mice treated with GM-CSF or PBS. The NPC were double-stained with FITC-CD11b and PE-CD11c. The sorted, double-positive cells, which showed typical DC morphology, were used for further characterization. Experiment shown is representative of three separate experiments with similar results.

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DC to 20–30 million cells compared with 0.5–2 million cells in normal animals.

DC expansion with GM-CSF does not affect apparent phenotype or function

We determined if GM-CSF overexpression affected the phenotype or function of DC in the liver prior to performing other studies. The liver NPC were isolated from plasmid GM-CSF- and vehicle PBS-treated mice. Hepatic DC were purified further with anti-CD11c mAb-coated magnetic beads. The phenotype of liver DC was determined by flow cytometric analysis. We found no significant difference in expression of MHC Class II or costimulatory molecules on hepatic DC when comparing GM-CSF- and PBS-treated animals (Fig. 2A). In addition to phenotype, we examined allostimulatory activity in a MLR (Fig. 2B). Hepatic DC from GM-CSF- and PBS-treated mice induced proliferative responses in allogeneic T cells to a similar degree. These results confirm that DC expansion with GM-CSF hydrodynamic transfection not only increased the number of hepatic DC but that this procedure did not grossly affect the phenotype or function of these cells.

Augmentation of liver DC numbers worsens hepatic I/R injury

To determine if the inflammation and organ injury following hepatic I/R were mediated by DC, mice with livers expanded with GM-CSF and control livers were subjected to warm I/R. There was minimal hepatic damage as assessed by serum ALT levels in sham-treated animals receiving GM-CSF or control PBS, indicating that our approach to expand DC did not result in hepatic injury by itself (Fig. 3A). In control mice treated with PBS, sixty minutes of ischemia and six hours of reperfusion resulted in significant hepatocellular injury when compared with sham treated animals. Further worsening of hepatic injury was observed in mice treated with GM-CSF undergoing I/R when compared with control animals subjected to ischemia alone.

Inflammatory cytokines, including TNF and IL-6, play key roles in the pathophysiology of hepatic I/R injury [36, 37]. We measured serum levels of these cytokines following I/R (Fig. 3B). Compared with sham-treated animals, liver I/R in control animals resulted in increased levels of TNF and IL-6, 6 h following reperfusion. Animals treated with GM-CSF displayed even higher levels of serum TNF and IL-6 when compared with control animals subjected to I/R. The worsened injury seen in animals following DC expansion suggests that DC in the liver play a key role in the inflammation and organ damage after I/R.

I/R activates hepatic DC

As increasing DC numbers within the liver appear to enhance injury following I/R, we sought to determine the effect of I/R on DC found within the liver. The DC from livers of hydrodynamically GM-CSF-transfected animals undergoing sham or I/R procedures were harvested. After I/R, hepatic DC were characterized by a mature phenotype with up-regulation of MHC Class II and costimulatory molecules (Fig. 4A). Similarly, I/R also enhanced the function of DC, as they were able to induce a greater proliferative response from allogeneic T cells (Fig. 4B). In addition to DC phenotype and function, we examined the effect of I/R on cytokine expression derived from hepatic DC (Fig. 5). Using real-time RT-PCR, we quantitatively measured mRNA levels for TNF and IL-6 in hepatic DC harvested from livers subjected to the sham or I/R procedure. Production of TNF and IL-6 by DC was higher after I/R when compared with sham procedures. As we have shown that TLR4 plays a critical role in the pathogenesis of liver I/R injury [9, 10], we also examined the change in expression of TLR4 in DC following I/R. It is interesting that TLR4 expression in hepatic DC, in addition to IL-6 and TNF mRNA, increased significantly after I/R when compared with mice undergoing the sham procedure.

DC-mediated hepatic I/R injury is dependent on functional TLR4

Although TLR4-mediated inflammation plays a key role in I/R injury [9, 10], the initiating events by which damaged liver cells activate TLR4 remain to be elucidated. It is important that the cell type responsible for recognizing the endogenous ligands released from damaged liver cells is unknown. Our recent work demonstrates that TLR4 engagement on nonparen-
chymal cells of the liver was required for the I/R-induced injury [38]. To determine if DC in the liver were important in mediating TLR4-mediated inflammation and injury following I/R, TLR4 wild-type and mutant mice were treated with GM-CSF or control PBS and subjected to 60 min of liver ischemia and 6 h of reperfusion. In agreement with our previous reports [9, 10], control TLR4 mutant mice were protected from liver I/R compared with wild-type mice, as measured by serum ALT levels (Fig. 6A). In addition, mice transfected with GM-CSF expanded their liver DC and displayed worsened hepatic injury when compared with PBS-treated mice. It is interesting that this worsening of organ damage was not seen in TLR4 mutant mice treated with GM-CSF and undergoing hepatic I/R. Of note, we confirmed that the TLR4 mutant mice responded similarly to GM-CSF transfection as wild-type mice. TLR4 wild-type and mutant mice exhibited similar increases in the
number of NPC and DC from harvested livers after GM-CSF treatment (data not shown).

We also examined the production of inflammatory cytokines from hepatic DC after I/R in these animals. Whereas hepatic DC, harvested from TLR4 wild-type mice and transfected with GM-CSF, exhibited increased production of TNF and IL-6 compared with control I/R animals, there were no significant differences in production of these cytokines after I/R from hepatic DC in GM-CSF-transfected TLR4 mutant mice when compared with control animals (Fig. 6B). Thus, intact TLR4 expression on hepatic DC is necessary to mediate the enhanced inflammatory response observed after liver I/R injury.

**HMGB1 increases TLR4 reactivity of liver DC**

Inflammation and injury responses in warm hepatic I/R are partially TLR4-dependent. Although our recent studies [9] suggest a central role for HMGB1 in TLR4 dependent hepatocyte damage, the precise mechanism by which HMGB1 mediates the resultant inflammation has not been elucidated. HMGB1 expression was up-regulated markedly in hepatocytes in the warm I/R model and in hepatocytes made ischemic in vitro, suggesting that stressed or damaged hepatocytes provide DAMPs in hepatic I/R to neighboring immune cells in the liver. We thus asked whether HMGB1 may contribute to the activation of DC following I/R. Whereas LPS stimulation of liver DC resulted in the production of TNF and IL-6, it is surprising that there was minimal production of these inflammatory cytokines from HMGB1-stimulated DC (Fig. 7A). Addition of HMGB1 to LPS-stimulated DC markedly augmented cytokine production when compared with LPS alone. To elucidate the mechanism by which HMGB1 might increase TLR4 reactivity in liver DC, we examined the expression of TLR4 in these cells. In the presence of HMGB1, TLR4 expression on hepatic DC was enhanced significantly compared with that in the absence of HMGB1 (Fig. 7B). We also examined the effect HMGB1 may have on other TLRs. In contrast to TLR4, HMGB1 did not increase TLR9 expression in liver DC.

**HMGB1 treatment worsens hepatic I/R and increases TLR4 expression in the liver**

We have shown that HMGB1 is an early mediator of injury and inflammation following hepatic ischemia and that exogenous HMGB1 administration alters hepatic injury in I/R [9]. A nonlethal dose (20 μg) of HMGB1 was given to mice immediately following reperfusion. There was no difference in apparent liver injury as assessed by serum ALT levels when comparing sham animals given vehicle PBS (Fig. 8A). As expected, I/R increased serum ALT levels in PBS-treated mice; however, animals also given HMGB1 had significantly greater increases in liver enzyme levels. We next sought to determine if HMGB1 treatment enhanced TLR4 expression. Although there was no difference in hepatic injury, as assessed by serum ALT levels when comparing HMGB1 and vehicle-treated sham mice, administration of HMGB1 resulted in enhanced hepatic TLR4 expression when compared with controls in the sham-treated mice (Fig. 8B). Similarly, HMGB1 administration in mice undergoing I/R also increased TLR4 expression in the liver. These results suggest that HMGB1 may indeed mediate hepatic injury after I/R by enhancing TLR4 expression on cells involved in TLR4-mediated inflammation, such as liver DC. Furthermore, we hypothesize that this allows for increased TLR4 reactivity and activation of the innate immune system in non-infectious settings by recognizing DAMPs. As has been suggested recently, there may be a memory element to such damage, mediated by hepatic NK cells, which we are investigating currently [39].
DISCUSSION

TLR signaling is one of the major mechanisms by which the innate immune system senses damaged, pathogenic microorganisms, recognizing PAMPs, which are released microbial products [17]. Increasing evidence suggests that TLRs may also be involved in the activation of the innate immune system in noninfectious settings by recognizing DAMPs [9, 10, 25, 40]. TLR4 plays a critical role in the pathogenesis of liver I/R injury [9, 10]. Furthermore, our recent work using TLR4 bone marrow chimeras demonstrates that the liver NPC, not the parenchymal hepatocytes, are responsible for recognizing the damage from ischemic cells and for activating TLR4-dependent signaling required for I/R-induced injury [38]. The purpose of the studies performed here was to identify the initial events responsible for TLR4-dependent inflammation following I/R in the liver. We found that hepatic DC expansion could be achieved using hydrodynamic plasmid GM-CSF transfection without affecting apparent DC phenotype or function; increasing DC numbers promote TLR4-dependent damage in hepatic I/R; I/R is associated with increased DC TLR4 expression; and provision of exogenous HMGB1 enhances DC TLR4 expression in vivo, further promoting inflammation.

The liver is a complex organ with important roles in immune surveillance and clearance of toxins as well as bacteria and their products. Liver cells express pattern recognition molecules, including members of the TLR system, which recognize molecular patterns of invading pathogens and endogenous DAMPs. TLR4, present on hepatocytes and NPC (i.e., Kupffer cells, sinusoidal endothelial cells, stellate cells, hepatic DC), is involved in the response to LPS and several endogenous ligands including hyaluronic acid, heparan sulfate, fibrinogen, HMGB1, and hsp [9, 18, 19, 22, 41].

We have recently reported that TLR4 expression on hepatic NPC is critical for the full manifestation of hepatic I/R injury. However, the NPC cell type and the mechanism of its activation by potential DAMPs, such as HMGB1, were not identified. We focused here on the DC, as they play a critical role in responding to endogenous signals of cellular damage [29, 30, 42, 43]. Liver DC play an important role in responses to pathogens [44, 45] and modulate the immune response in the setting of tissue injury and release of DAMPs [46, 47]. Deep understanding of the role these cells play remains limited as a result of their scarcity for isolation. To date, much of the work about hepatic DC consists of studying DC propagated in vitro from liver NPC. However, there are concerns regarding possible alterations in DC bioactivity with in vitro culture [48]. Attempts at direct isolation of liver DC have resulted in poor yields and the necessity of pooling DC from 20 to 40 mice [48]. Recently, multiple approaches have been used to expand liver DC in vivo. These include injection of fms-like tyrosine kinase 3 ligand [49, 50] or systemic administration of adenoviral-mediated GM-CSF [51]. In this study, we used an approach consisting of hydrodynamic injection of plasmid DNA. This method of rapid injection of a large volume of DNA solution into animals via the tail vein achieves high levels of gene

Fig. 7. HMGB1 increases TLR4 reactivity of liver DC, which were isolated from the liver of mice and cultured at 2 × 10⁶ cells/ml in 24-well plates for 24 h. DC were stimulated with HMGB1 (1 µg/ml), LPS (1 µg/ml), or a combination of both. The supernatants of DC culture were subjected to TNF and IL-6 cytokine detection by ELISA (A), and the cells were collected for detection of TLR4 and TLR9 mRNA with real-time RT-PCR (B). Experiments shown are representative of two separate experiments with similar results. CM, control media.
expression in the liver [52, 53]. The advantages of using plasmid DNA compared with adenoviral vectors include the ease of obtaining large amounts of plasmid DNA, safety of administration, and lack of foreign protein delivery from the adenoviral particle. With plasmid DNA use, there is less immune response, as with adenoviral use, causing additional destruction of target cells. DC generated from plasmid GM-CSF injection were phenotypically and functionally similar to DC generated from plasmid GM-CSF transfection. The findings that DC expansion worsens liver injury after I/R, only when there is functional TLR4 present, suggest that DC are indeed one of the key cells responding to potential DAMPs released after acute injury. Our results demonstrate that after I/R, hepatic DC are activated, as evidenced by a change in their phenotype and an increase in their antigen-presenting function. In addition, activated DC from livers after I/R produce increased levels of inflammatory cytokines and have increased expression of TLR4. We then asked whether the NF-κB may play a role in activating DC during TLR4-mediated inflammation after I/R. This question was based on our previous studies showing that HMGB1 is up-regulated in the liver after I/R and that the activity of HMGB1 as an early mediator of inflammation and cell injury after hepatic I/R requires TLR4 [9]. It is surprising that unlike LPS, stimulation of cultured DC with HMGB1 did not increase inflammatory cytokine production. However, TLR4 expression was increased threefold after HMGB1 stimulation when compared with LPS alone. Increased TLR4 expression in DC following HMGB1 stimulation was associated with enhanced TLR4 reactivity, as combined HMGB1 and LPS stimulation resulted in significantly greater cytokine production when compared with LPS alone. Similarly, HMGB1 administration in vivo also resulted in up-regulation of hepatic TLR4 and resulted in increased liver injury after I/R. We thus propose that HMGB1 is a superadaptor molecule, enhancing the responsiveness to environmental signals.

Previous studies have shown that injury can result in an enhanced, TLR-mediated, proinflammatory response mediated by DC [54]. However, whether this injury-induced, enhanced TLR reactivity is dependent on HMGB1 is unknown. TLR4 plays a critical role in endotoxin signaling and participates in the recognition of several DAMPs such as hyaluronic acid, heparan sulfate, and fibrinogen and perhaps hsp [18–20, 22]. The release of these ligands stimulates inflammatory activity through TLR4 signaling. We hypothesize that HMGB1 may direct the inflammatory responses mediated by DC by enhancing TLR4 sensitivity to potential DAMPs and other environmental signals. The mechanism by which HMGB1 mediates TLR4 expression remains to be elucidated. HMGB1, in vitro, interacts with TLR2 and -4 as well as the receptor for advanced glycation end-products (RAGE), all seemingly important for HMGB1-induced inflammatory signaling [21, 55–57]. We hypothesize that HMGB1 interacting with RAGE signals to up-regulate TLR4 expression. Indeed, using neutralizing antibodies and cells from RAGE–/– animals, Dumitriu et al. [58] demonstrated that RAGE is required for the effect of HMGB1 downstream activation of MAPKs and NF-κB in DC. RAGE also modulates hepatic I/R injury, as animals treated with soluble RAGE, the extracellular ligand-binding domain of RAGE, display increased survival after I/R [59]. Whether RAGE-induced inflammation is in part a result of up-regulation of TLR4 in these as well as our own models is as-yet unknown but a rich area for further study. Alternatively, HMGB1 may interact directly with TLR4, resulting in a positive feedback enhancing TLR4 expression. Further studies are necessary to determine the mechanism by which HMGB1 mediates TLR4 up-regulation as well as to determine the role of other hepatic NPC including NK and NKT cells. Manipulating DC in vivo, as we have shown previously in murine tumor models, may also be important in preventing or ameliorating hepatic I/R responses [58]. In addition, targeting HMGB1 itself [59], using recently available mAb to neutralize its effects, as has been done in the setting of sepsis or arthritis, could be useful strategies in several clinical settings.

**Fig. 8.** HMGB1 treatment worsens hepatic I/R and increases TLR4 expression in the liver. (A) Serum ALT levels were measured in sham mice and mice undergoing ischemia and 6 h of reperfusion. Animals were treated with a nonlethal dose of rHMGB1 (20 ug) or vehicle PBS i.v., 1 h prior to ischemia. Data represent means ± se; n = 6 mice per group. *, P < .05, versus mice subjected to I/R given vehicle PBS. (B) Hepatic TLR4 mRNA level expression was measured in mice following ischemia and 6 h of reperfusion or sham operation. Animals were treated with HMGB1 (20 ug) or vehicle PBS i.v., 1 h prior to ischemia. Results were obtained using real-time RT-PCR and expressed as relative increase of mRNA expression compared with normal, untreated animals. Data represent means ± se; n = 6 mice per group. *, P < .05, versus mice subjected to sham given vehicle PBS; †, P < .05, versus mice subjected to I/R given vehicle PBS.
In summary, this study documents that increasing DC numbers expressing functional TLR4 are critical to the full manifestation of hepatic I/R injury. DC are likely to be one of the initial responders to the release of DAMPs from damaged or necrotic cells with subsequent signaling and activation of TLR4. The release of HMGB1 may contribute to the inflammation and injury after I/R by enhancing TLR4 reactivity of DC to itself as well as other DAMPs. Interventions that inhibit TLR4 or HMGB1 activity on DC may be effective in settings of ischemic liver injury to minimize organ damage and may be useful in other clinical settings associated with inflammation and cellular necrosis.

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