Published in final edited form as:

Hepatology. 2018 December ; 68(6): 2380–2404. doi:10.1002/hep.30093.

HIGH MOBILITY GROUP BOX-1 DRIVES FIBROSIS PROGRESSION SIGNALING VIA THE RECEPTOR FOR ADVANCED GLYCATION END-PRODUCTS IN MICE

Xiaodong Ge^{1,2}, Elena Arriazu^{2,*}, Fernando Magdaleno^{1,**}, Daniel J. Antoine³, Rouchelle dela Cruz^{4,***}, Neil Theise^{4,5}, and Natalia Nieto^{1,2,6}

¹Department of Pathology, University of Illinois at Chicago, 840 S. Wood St., Suite 130 CSN, MC 847, Chicago, IL 60612, USA

²Division of Liver Diseases, Department of Medicine, Icahn School of Medicine at Mount Sinai, Box 1123, 1425 Madison Ave., Room 11-70, New York, NY 10029, USA

³MRC Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh, EH16 4TJ, GB

⁴Division of Digestive Diseases, Mount Sinai Beth Israel Medical Center, First Avenue at 16th Street, New York, NY 10003

⁵Department of Pathology, New York University Langone Medical Center, 550 First Ave., New York, NY 10016

⁶Department of Medicine, Division of Gastroenterology and Hepatology, University of Illinois at Chicago, 840 S. Wood St., Suite 1020N, MC 787, Chicago, IL 60612, USA

Abstract

Background & Rationale—High-mobility group box-1 (HMGB1) is a damage-associated molecular pattern (DAMP) increased in response to liver injury. Since HMGB1 is a ligand for the receptor for advanced glycation end-products (RAGE), we hypothesized that induction of HMGB1 could participate in the pathogenesis of liver fibrosis via RAGE cell-specific signaling mechanisms.

Results—liver HMGB1 protein expression correlated with fibrosis stage in patients with chronic Hepatitis C virus (HCV) infection, primary biliary cirrhosis (PBC) and alcoholic steatohepatitis (ASH). Hepatic HMGB1 protein expression and secretion increased in five mouse models of liver fibrosis due to drug-induced liver injury, cholestasis, alcoholic steatohepatitis or non-alcoholic steatohepatitis. HMGB1 was up-regulated and secreted mostly by hepatocytes and Kupffer cells (KC) following chronic carbon tetrachloride (CCl₄) treatment. Neutralization of HMGB1 protected whereas injection of recombinant HMGB1 promoted liver fibrosis. *Hmgb1* ablation in hepatocytes (*Hmgb1*^{Hep}) or myeloid cells (*Hmgb1*^{Mye}) partially protected while ablation in both

Contact Information: Natalia Nieto, Department of Pathology, University of Illinois at Chicago, 840 S. Wood St., Suite 130 CSN, MC 847, Chicago, IL 60612, USA. Fax: +1 (312) 996-1217. nnieto@uic.edu.

*Center for Applied Medical Research, Avda. Pío XII 55, 31008 Pamplona, Navarre, Spain.

**University Hospital Bonn, Sigmund Freud Str. 25, 53105 Bonn, Germany.

***Department of Laboratories, The Medical City, Ortigas Avenue, Pasig City, 1605 Metro Manila, Philippines.

(*Hmgb1*^{Hep Mye}) prevented liver fibrosis *in vivo*. Co-culture with hepatocytes or KC from CCl₄-injected wild-type (WT) mice up-regulated Collagen type I production by hepatic stellate cells (HSC); yet, co-culture with hepatocytes from CCl₄-injected *Hmgb1*^{Hep} or with KC from CCl₄-injected *Hmgb1*^{Mye} mice partially blunted this effect. *Rage* ablation in HSC (*Rage*^{HSC}) and RAGE neutralization prevented liver fibrosis. Lastly, we identified that HMGB1 stimulated HSC migration and signaled via RAGE to upregulate Collagen type I expression by activating the pMEK1/2/pERK1/2/pJNK signaling pathway.

Conclusion—hepatocyte and KC-derived HMGB1 participates in the pathogenesis of liver fibrosis by signaling via RAGE in HSC to activate the pMEK1/2/pERK1/2/pJNK pathway and increase Collagen type I deposition.

Keywords

Collagen type I; hepatic stellate cells; hepatocytes; macrophages

A large majority of patients with chronic liver disease of diverse etiologies progresses to fibrosis, cirrhosis and hepatocellular carcinoma. Identification of the key mediators, receptors and signaling pathways they activate, particularly at the very early stages, along with better understanding of how they promote fibrosis is critical to prevent disease progression, design new therapies, improve the quality of life of these patients and make headway in this field of research.

HMGB1 is a DAMP that rises in response to sterile and non-sterile tissue damage (1–4). Increased nuclear export due to injury drives HMGB1 cytoplasmic localization and ultimately secretion (2, 5, 6). Our recent studies reveal that HMGB1 is up-regulated and secreted largely by hepatocytes and KC, both anatomically located in close proximity to HSC, the key pro-fibrogenic cells in the liver (1, 2). Hence, since HMGB1 could be detrimental for neighboring cells, increased production by these two cellular sources could target HSC to eventually elicit scarring (1, 2, 7–10).

RAGE, a multi-ligand receptor long implicated in homeostasis, development and inflammation, is one of the main receptors for HMGB1 (11). RAGE engagement results in activation of numerous downstream signals impinging on canonical pathways that regulate transcription and translation as well as cell proliferation, survival, differentiation and migration. Yet, the functional consequences of RAGE engagement and the signaling cascades turned on are strongly dependent on the cell type, disease context, ligand identity and local concentration that may also impact the composition and even organization of the liver matrix.

So far, the role of HMGB1 and how it signals in liver fibrosis has not been fully elucidated. In this study, our central hypothesis was that cell-specific induction of HMGB1 could participate in the pathogenesis of liver fibrosis via RAGE cell-dependent mechanisms to up-regulate Collagen type I production in HSC; thus, contributing to scarring. We aimed to demonstrate this using human samples, several mouse models of liver fibrosis along with genetic *in vitro* and *in vivo* complementary approaches.

EXPERIMENTAL PROCEDURES

Human samples

Dr. Theise provided paraffin-embedded archival human liver biopsies from de-identified controls (healthy liver explants) and patients with clinically proven HCV, PBC or ASH-induced fibrosis, which were treatment *naïve*. The modified Ishak staging for fibrosis, which also parallels the Metavir staging system for chronic hepatitis, was used for HCV (12), the Scheuer system for PBC (13) and the Brunt classification for ASH (40).

Study approval

The procurement of archived human samples was IRB approved and no patient information was disclosed. All animals received humane care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health. Housing and husbandry conditions were approved by our IACUC office prior to initiation of the studies. All *in vivo* experiments were carried out according to the ARRIVE guidelines.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical comparisons among groups and treatments *in vitro*, were performed using paired Student's *t*-test. All *in vivo* experiments were performed in triplicate and data were analyzed by a two-factor analysis of variance and the number of mice are indicated in the legend to each figure.

Please, see Supplementary Information.

RESULTS

Hepatic HMGB1 protein expression correlates with fibrosis stage in chronic HCV, PBC and ASH patients

Since we hypothesized that HMGB1 could drive liver fibrosis, to determine whether there is correlation between HMGB1 induction, localization and disease stage, we analyzed the expression of HMGB1 in archived human liver biopsies from de-identified healthy controls and patients with clinically proven HCV-induced fibrosis that advanced in disease stage (Scheuer/Batts-Ludwig classification) (12). Liver biopsies from HCV-infected patients showed progressive increase in HMGB1 protein expression with disease stage (Figure 1A, first row). Similar results were observed in patients with PBC or ASH (2) (Figure 1A, second and third rows, respectively). Computer-assisted morphometry analysis demonstrated association between HMGB1 nucleocytoplasmic shuttling and fibrosis stage in all three conditions (2) (Figure 1B). Serum HMGB1 increased in patients with HCV (15.1 ± 3.1 ng/ml) and ASH (16.7 ± 6.6 ng/ml) compared to healthy individuals (1.1 ± 0.2 ng/ml). Therefore, the increase in HMGB1 expression, translocation into the cytoplasm and secretion correlates with fibrosis progression in chronic HCV, PBC and ASH (2) patients.

Hepatic HMGB1 protein expression and secretion correlates with liver fibrosis in mice

To determine if mouse models of liver fibrosis would mirror the above-mentioned findings in humans, we injected WT mice with mineral oil (MO) or CCl₄ for 1 mo. hematoxylin and eosin (H&E) staining, HMGB1 and Collagen type I immunohistochemistry (IHC), HMGB1 western blot and Collagen type I morphometry assessment revealed that CCl₄ increased HMGB1 and Collagen type I proteins compared to MO-injected mice (Figure 1C).

Furthermore, the IHC findings were validated in four additional models of liver fibrosis induced by another drug (thioacetamide, TAA) (Figure 1D, left), cholestasis (bile duct ligation, BDL) (Figure 1D, right), ASH (*ad libitum* and intra-gastric infusion “hybrid” feeding regime of high cholesterol fat diet and ethanol liquid diet with a weekly binge of alcohol (14)) (Supplementary Figure 1A, left) and NASH (methionine and choline deficient diet, MCD) (Supplementary Figure 1A, right).

Since HMGB1 translocation into the cytoplasm and ultimately secretion are critical for driving downstream events (1, 2, 15–20), its localization was quantified by morphometry analysis in all five models. As in humans, there was a significant increase in HMGB1 nucleocytoplasmic shuttling in all the fibrotic compared to their respective control mice (Figure 1E and Supplementary Figure 1B). Importantly, liver and serum HMGB1 significantly increased in all fibrotic mice compared to their controls (Figure 1E). Hence, the rise in HMGB1 expression, translocation into the cytoplasm and secretion correlates well with the extent of liver fibrosis in mice too. Thus, HMGB1 could play a major role in the pathogenesis of liver fibrosis; yet, the cellular source of the protein and how it acts as a pro-fibrogenic factor remains unknown.

Identification of the cellular source of HMGB1 in liver fibrosis

To identify the precise hepatic cellular source of HMGB1 in liver fibrosis, immunofluorescence (IF) and co-localization studies were carried out using specific markers of HSC (desmin, cytoplasmic), KC plus infiltrating macrophages (F4/80, cytoplasmic), hepatocytes (HNF4 α , nuclear), endothelial cells (CD31, cytoplasmic) and biliary epithelial cells (CK7, cytoplasmic). As a second approach, HMGB1 IF and fluorescent *in situ* hybridization (FISH) with cell markers (*Des*, *Emr1*, *Hnf4a*, *Pecam1* and *Krt19*) was performed. In MO-injected mice, HMGB1 co-localized with markers of HSC, KC plus infiltrating macrophages and hepatocytes. Following chronic CCl₄ injection, HMGB1 expression increased in HSC, KC plus infiltrating macrophages and hepatocytes (Figures 2A, 2C and 2E; 2B, 2D and 2F left). These findings were further confirmed by ESI-LS-MS in primary HSC, KC and hepatocytes isolated from these mice (Figures 2B, 2D and 2F right). There was minimal co-localization of HMGB1 with markers of endothelial cells (Supplementary Figure 2A–2B) or biliary epithelial cells (Supplementary Figure 2C–2D) under CCl₄ treatment. In summary, these *in vivo* results suggest that HMGB1 is highly induced in liver fibrosis, particularly in HSC, KC plus infiltrating macrophages and hepatocytes; yet, if it plays a role in the pathogenesis of the disease and the specific mechanism whereby it could act as a pro-fibrogenic DAMP still remains to be defined.

Neutralization of HMGB1 prevents whereas injection of HMGB1 promotes liver fibrosis in mice

To dissect if blocking HMGB1 prevents the fibrogenic response to liver injury, WT mice were injected non-immune IgG or an HMGB1 neutralizing Ab before each MO or CCl₄ dose for 1 mo. Liver injury was assessed by H&E staining (Figure 3A, first row), the pathology scores (Figure 3B, left) and alanine aminotransferase (ALT) activity (Figure 3C). HMGB1 expression was detected by IHC (Figure 3A, second row) and quantified in liver and serum (Figure 3B, middle). The extent of fibrosis was determined by Collagen type I IHC and Sirius red/Fast green staining (Figure 3A, third and fourth rows), morphometry, Collagen type I and α -smooth muscle actin (α SMA) western blot, hepatic 4-hydroxyproline levels (Figure 3B, right and bottom), and by quantitative real-time polymerase chain reaction (qRT-PCR) analysis for key fibrosis-related genes (i.e. *Colla1*, *Colla2* and *Des*) (Supplementary Figure 3A, top rows). All these parameters were significantly reduced in CCl₄-injected mice pre-treated with the HMGB1 neutralizing Ab compared to mice pre-treated with non-immune IgG. Given this, we asked if injection of HMGB1, as a reverse approach, will enhance liver fibrosis. WT mice were injected HMGB1 before each CCl₄ dose for 1 mo (21). H&E staining, HMGB1 expression, Collagen type I IHC, Sirius red/Fast green staining, the number of HSC (Desmin⁺) (Figure 3D), the pathology scores, total liver and serum HMGB1, morphometry, Collagen type I and α SMA western blot, hepatic 4-hydroxyproline levels (Figure 3E), fibrosis-related genes (Supplementary Figure 3A, bottom rows) and ALT activity (Figure 3F) were greater in mice injected with HMGB1 and CCl₄ compared to mice injected with CCl₄ alone. Thus, HMGB1 increases fibrosis progression but the mechanism whereby it occurs is still unknown.

Hmgb1 ablation in hepatocytes or myeloid cells partially protects while ablation in both prevents CCl₄-induced liver fibrosis

We have previously described the role of intracellular HSC-derived HMGB1 on Collagen type I expression *in vitro* (1). Here, we demonstrate that ablation of *Hmgb1* in HSC *in vivo* using two different targeting strategies (i.e. *Hmgb1*^{fl/fl}*Gfap-Cre* and *Hmgb1*^{fl/fl}*Lrat-Cre* mice) confers some (~25%) yet not full protection from hepatic fibrosis (Supplementary Figure 4); therefore, pointing at a more prominent contribution of HMGB1 from other liver cell types.

Since we identified that the major sources of HMGB1 were hepatocytes and KC plus infiltrating macrophages, to further establish if hepatocyte- and/or myeloid cell-derived HMGB1 contributes to liver fibrosis, WT, *Hmgb1*^{Hep}, *Hmgb1*^{Mye} and *Hmgb1*^{Hep Mye} were injected MO or CCl₄ for 1 mo. Liver injury was assessed by H&E staining (Figure 4A, first row), the pathology scores (Figure 4B, left) and ALT activity (Figure 4C). HMGB1 expression was detected by IHC (Figure 4A, second row), the subcellular localization analyzed by morphometry and quantified in liver and serum (Figure 4D, left). The extent of fibrosis was determined by Collagen type I IHC and Sirius red/Fast green staining (Figure 4A, third and fourth rows), morphometry (Figure 4B, middle), Collagen type I and α SMA western blot, hepatic 4-hydroxyproline levels (Figure 4E) and fibrosis-related genes (Supplementary Figure 3B). All these parameters revealed partial protection from liver fibrosis in CCl₄-injected *Hmgb1*^{Hep} or *Hmgb1*^{Mye} and almost full prevention in

Hmgb1^{Hep Mye} compared to WT mice. Vascularization (CD31, Figure 4A, fifth row), CD31 index (Figure 4B, right) and *Vegf* mRNA (not shown) were alike in all groups of CCl₄-injected mice, suggesting that changes in angiogenesis did not account for the fibrosis stage in these mice. Importantly, since HMGB1 binds several receptors including RAGE, toll-like receptors (TLRs2/4/9), Mac-1, syndecan-1, phosphacan protein-tyrosine phosphatase- ζ/β and CD24 (22), their expression was evaluated by qRT-PCR; however, no significant changes were found in any of the HMGB1 receptors' mRNA levels in the livers from all the MO- or CCl₄-injected mice (not shown). Equal RAGE expression was confirmed by western blot (Figure 4E, bottom). Overall, these findings suggest that *Hmgb1* ablation in hepatocytes or myeloid cells partially protects while ablation in both prevents CCl₄-induced liver fibrosis.

Two additional mouse models confirm the role of HMGB1 in liver fibrosis

To further prove the role of hepatocyte and myeloid cell-derived HMGB1 in the CCl₄ model, we used two other well-established models of liver fibrosis. To this end, WT and *Hmgb1*^{Hep Mye} mice were injected TAA for 1 mo or underwent BDL for 2 wk. Liver injury was assessed by H&E staining (Supplementary Figure 5A, first row) and the pathology scores (Supplementary Figure 5B, left). HMGB1 expression was detected by IHC (Supplementary Figure 5A, second row), the subcellular localization analyzed by morphometry and quantified in liver and serum (Supplementary Figure 5C). The extent of fibrosis was determined by Collagen type I IHC (Supplementary Figure 5A, third row) and morphometry (Supplementary Figure 5B, right). Together, the data demonstrated more liver injury, fibrosis and ductular reaction in TAA-treated or BDL WT than in *Hmgb1*^{Hep Mye} mice. Thus, these data confirm the results obtained in the CCl₄ model in two additional models of liver fibrosis.

Hepatocytes and KC are a major source of extracellular HMGB1 signaling to HSC to increase Collagen type I production

Since the IHC from human and mouse liver suggested that hepatocytes and KC plus infiltrating macrophages are a major source of HMGB1 (2), to further define their paracrine involvement in the upregulation of Collagen type I production by HSC, co-cultures of primary hepatocytes or KC from MO- or CCl₄-treated WT, *Hmgb1*^{Hep} and *Hmgb1*^{Mye} mice and WT HSC were established. Western blot analysis demonstrated that co-culture with hepatocytes or KC from CCl₄-injected WT mice significantly enhanced total Collagen type I expression in HSC over that of MO-injected mice (Figure 5A, lanes 2 and 6, total Collagen type I written in red); however, co-culture with hepatocytes or KC from CCl₄-injected *Hmgb1*^{Hep} or *Hmgb1*^{Mye}, respectively, partially prevented this effect (Figure 5A, lanes 4 and 8, total Collagen type I written in red). Total HMGB1 was quantified in cell lysate and culture medium from all cells seeded alone or in co-culture. The culture medium from HSC co-cultured with hepatocytes from CCl₄-injected mice showed a 5-fold increase in the concentration of HMGB1 compared to that from HSC co-cultured with KC from CCl₄-injected mice, suggesting that hepatocytes secrete more HMGB1 than KC. Notably, HSC also appeared to signal to hepatocytes and KC in co-culture to increase HMGB1 production and secretion; hence creating a pro-fibrogenic loop (Figure 5B). Therefore, HSC are responsive to hepatocyte- and KC-derived HMGB1 increasing total Collagen type I

protein; yet, the receptor(s) involved and the signals conveyed to HSC to up-regulate Collagen type I protein remain unknown.

HMGB1 stimulates HSC migration without altering their viability or cell cycle

To dissect if HMGB1 could also affect the chemotaxis of HSC, cells were treated with 0–1 nM HMGB1 for 24 h in the presence or absence of mitomycin to inhibit mitosis. HMGB1 caused a significant increase in cell migration, an important function gained by HSC during their activation that contributes to their pro-fibrogenic phenotype. This result was specific as mitomycin blocked the effects of HMGB1 on HSC migration (Figure 5C). However, HMGB1 did not alter HSC viability or cell cycle (Figure 5D).

Rage ablation in HSC and RAGE neutralization prevent liver fibrosis *in vivo*

Among the receptors HMGB1 binds to, RAGE and TLR4 are expressed in HSC and participate in liver fibrosis (22–34). To establish the role of HSC RAGE in the effects mediated by HMGB1 in liver fibrosis, WT control, WT injected a RAGE neutralizing Ab before CCl₄, *Tlr4*^{HSC} and *Rage*^{HSC} mice were treated with CCl₄ for 1 mo. Liver injury was assessed by H&E staining (Figure 6A, first row), the pathology scores (Figure 6B, left) and ALT activity (Figure 6C). HMGB1 expression was detected by IHC (Figure 6A, second row), the subcellular localization analyzed by morphometry and quantified in liver and serum (Figure 6D). Validation of the targeting strategy was demonstrated by co-localization of RAGE and Desmin in *Rage*^{HSC} mice (Figure 6A, bottom left) and by co-localization of TLR4 and Desmin in *Tlr4*^{HSC} mice (Figure 6A, bottom right). The extent of fibrosis was determined by Collagen type I IHC and Sirius red/Fast green staining (Figure 6A, third and fourth rows), morphometry (Figure 6B, right), Collagen type I and α SMA western blot, hepatic 4-hydroxyproline levels (Figure 6E) and fibrosis-related genes (Supplementary Figure 3C). The results revealed major reduction of fibrosis in WT with RAGE neutralization and in *Rage*^{HSC} but minimal protection in *Tlr4*^{HSC} mice; therefore, validating the role of HMGB1 signaling via RAGE in HSC for its pro-fibrogenic effects. In addition, RAGE neutralization or ablation in HSC reduced HMGB1 levels further protecting the liver. To make sure that the protection observed in *Rage*^{HSC} mice was recapitulating protection due to lack of HMGB1 signaling via this receptor, we administered HMGB1 into WT mice treated with a RAGE neutralizing Ab throughout the chronic CCl₄ treatment. Overall, our results suggest that blockade of RAGE signaling prevents the pro-fibrogenic effect of HMGB1 (Supplementary Figure 6).

Since RAGE neutralization will block RAGE signaling in all liver cells expressing it, we then looked into whether *Rage* ablation in hepatocytes or myeloid cells could also protect from the pro-fibrogenic effects of HMGB1 in the liver. WT, *Rage*^{Hep} and *Rage*^{Mye} mice were injected CCl₄ for 1 mo. Liver injury was assessed by H&E staining (Figure 7A, first row), the pathology scores (Figure 7B, left) and ALT activity (Figure 7C). HMGB1 expression was detected by IHC (Figure 7A, second row), the subcellular localization analyzed by morphometry and quantified in liver and serum (Figure 7D). Validation of the targeting strategy was demonstrated by co-localization of RAGE and HNF4 α in *Rage*^{Hep} mice (Figure 7A, bottom left) and by co-localization of RAGE and F4/80 in *Rage*^{Mye} mice (Figure 7A, bottom right). The extent of fibrosis was determined by Collagen type I IHC and

Sirius red/Fast green staining (Figure 7A, third and fourth rows), morphometry (Figure 7B, right), Collagen type I and α SMA western blot, hepatic 4-hydroxyproline levels (Figure 7E) and fibrosis-related genes (Supplementary Figure 3D). Overall, the data revealed a slight protection in *Rage*^{Hep} but no protection in *Rage*^{Mye} compared to WT mice. Nonetheless, the *Rage*^{Mye} mice showed reduced necrosis and ALT but more inflammation (Figure 7B) perhaps pointing to a specific subset of immune cells infiltrating the liver to clear necrotic cells.

HMGB1 signals via RAGE to upregulate Collagen type I protein expression in HSC

To dissect the mechanism involved in the increase in Collagen type I by HMGB1 in HSC; first, we evaluated if the effect was receptor-mediated. Upon successful *Rage* (Figure 8A, left) or *Tlr4* silencing (not shown), HSC were challenged with 0–1 nM HMGB1 for 24 h. Western blot analysis revealed that RAGE (Figure 8, right) but not TLR4 (not shown) was critical for the effects of HMGB1 on Collagen type I increase in HSC. Therefore, HMGB1 upregulated Collagen type I in HSC via RAGE; yet, the specific signal(s) transduced remained elusive. Since RAGE signaling leads to activation of protein kinases, to further investigate their contribution to the effects of HMGB1 on Collagen type I, we evaluated their expression in the presence of HMGB1. Western blot analysis proved that HMGB1 phosphorylated MEK1/2 and its downstream targets ERK1/2 and cJun besides activating pAkt signaling as previously identified by us (1). Both pathways are known to increase Collagen type I (1, 35). Moreover, cJun, a component of the heterodimer AP1, is a potent transactivating factor for the *Col1a1* (36) and *Col1a2* (37) genes. Importantly, these events were blocked in *Rage*^{-/-} HSC (Figure 8B). Last, incubation of cells with the pMEK1/2 inhibitor PD98095, blocked pERK1/2, pcJun and pAkt at 30 min and lowered intra- and extracellular Collagen type I at 24 h (Figure 8C), suggesting that these two signaling pathways, with pMEK1/2 likely upstream of pAkt, were involved in the pro-fibrogenic response to HMGB1 via RAGE in HSC.

DISCUSSION

Numerous mediators and signaling pathways that induce, propagate and sustain liver fibrosis have been identified in the past; still, there is no definitive treatment for well-established fibrosis. This study addresses such unmet need and challenges our current view of the mechanisms driving liver disease by reinforcing the role of HMGB1, an intrahepatic sterile DAMP, in the pathogenesis and progression of liver fibrosis. Here, we tested the novel hypothesis that during injury, hepatocytes and KC plus infiltrating macrophages increase and secrete HMGB1, which acts as a paracrine alarmin via RAGE signaling in HSC to up-regulate Collagen type I production to trigger and/or sustain scarring.

First, we highlighted that hepatic HMGB1 protein induction, translocation to the cytoplasm and secretion correlate with fibrosis progression in patients with chronic HCV, PBC and ASH and in five mouse models of liver fibrosis (CCl₄, TAA, BDL, ASH and diet-induced NASH). The overall contribution of HMGB1 to the pathogenesis of liver fibrosis was then demonstrated by injecting mice with an HMGB1 neutralizing Ab or recombinant HMGB1, which blunted or increased liver injury and fibrosis, respectively; thus, stressing the

significance of these findings. Overall, HMGB1 levels appear clearly associated with clinical outcome.

Second, we asked which cellular source of HMGB1 could drive the fibrogenic response. We had previously described that while HSC secretion of HMGB1 is minimal, in response to an osteopontin (OPN) challenge or to increased endogenous production of OPN, as it occurs in liver fibrosis, HSC are able to acetylate HMGB1 *in vitro*, an event that contributes to Collagen type I deposition. In the present study, we show that HMGB1 also stimulates HSC migration *in vitro* and *in vivo*, an event largely contributing to progression of liver fibrosis. Importantly, we demonstrate that ablation of *Hmgb1* in HSC *in vivo* using two conditional targeting strategies partially (~25%) prevents scar formation. This finding unlocked the possibility that additional cellular sources of HMGB1 may have a more meaningful impact in liver fibrosis.

Next, we identified that hepatocytes and KC plus infiltrating macrophages, are the main hepatic cellular source of HMGB1 in the setting of liver fibrosis and that HSC stimulate these cells to increase HMGB1 production, as shown in the co-culture studies, therefore creating a pro-fibrogenic loop. Since HMGB1 expression, nucleocytoplasmic shuttling and secretion was maximal in hepatocytes followed by KC plus infiltrating macrophages in mice with liver fibrosis, we endeavored to determine their specific and combined contribution to fibrogenesis *in vivo*. We show that ablation of *Hmgb1* in hepatocytes or myeloid cells partially protects while ablation in both prevents CCl₄-induced liver injury and fibrosis, which was validated in two additional mouse models of liver fibrosis (i.e. TAA and BDL). Likewise, we demonstrate that both cell types are a source of HMGB1 signaling to HSC to increase Collagen type I production.

Mechanistically, we previously described that recombinant HMGB1 can bind RAGE in HSC *in vitro* and activate the PI3K/pAkt signaling pathway to up-regulate Collagen type I production (1). We have now identified that HMGB1 signals via RAGE in HSC *in vivo* to increase Collagen type I protein expression and that *Rage* but not *Tlr4* ablation in HSC prevents liver fibrosis. RAGE engagement by HMGB1 leads to phosphorylation and thus activation of the MEK1/2, ERK1/2 and cJun signaling pathway that up-regulates Collagen type I protein expression (Figure 8D). This pathway is relevant for liver fibrosis as it also drives TGFβ production and signaling via Smad2/3 in HSC feeding back into the MEK1/2/ERK1/2/cJun pathway (38, 39) in addition to TGFβ also being known for stimulating HSC migration (40). Notably, phosphorylation of MEK1/2 occurred within 30 min with a subsequent increase in Collagen type I within 24 h. Since PD98095 inhibited pMEK1/2 and this caused blockade of pAkt, pMEK1/2 is likely upstream of pAkt in regulating Collagen type I production.

The specificity of HMGB1 signaling via RAGE in HSC *in vivo* was further confirmed by co-injecting a RAGE neutralizing Ab along with HMGB1 to WT mice or by using *Rage*^{HSC} mice with chronic CCl₄ treatment. Notably, RAGE neutralization or ablation also decreased HMGB1 production itself, perhaps further protecting the liver as a parallel mechanism; yet, the mechanism behind this effect remains an open question. Interestingly, since this effect was observed only in *Rage*^{HSC} mice and not in *Rage*^{Hep} or *Rage*^{Mye}

mice, it could suggest that RAGE signaling in HSC may affect HMGB1 production by hepatocytes and myeloid cells, although the potential mediator and/or mechanisms involved still remain to be determined. Of note, *Rage* ablation in myeloid cells triggered an unexpected surge in the number of inflammatory cells, which perhaps resulted from either HMGB1 *per se* or from other RAGE ligands increasing their extravasation into the liver maybe to phagocytose necrotic cells. Post-translational modifications of HMGB1 and their association with pro-inflammatory events that drive fibrosis are currently under investigation in our laboratory and may also be critical in this setting.

Last, we are aware that some discrepancy may exist between the potential role of TLR4 in liver fibrosis previously suggested by others (41) and our findings. An explanation may be that our studies used conditional ablation of *Tlr4* in HSC *in vivo* for the first time as this strategy was not previously available. Moreover, a de-lipidated LPS-binding region in position 3–15 and a lipid A-binding region in position 80–96 containing four and one acetylation sites, respectively, have been predicted in HMGB1; thus, it is also possible that HMGB1 sequesters LPS precluding it from binding to TLR4, which could be further regulated by protein acetylation; yet, this has not been proven. It is also conceivable that lack of TLR4 in HSC causes a paracrine effect on neighboring cells that sustains fibrosis. Finally, it is possible, though more speculative, that in some settings HMGB1 may enhance TLR4 expression, which could sustain TLR4 signaling in key cells such as KC and infiltrating macrophages. While these studies suggest a more prominent role for RAGE, and specifically HSC RAGE, in fibrosis onset and progression, we do not rule out that in the later stages of the disease, when HMGB1 undergoes post-translational modifications, or even during fibrosis resolution, TLR4 may be part of the picture. Nonetheless, while the RAGE binding site has been localized to residues 150–183 in HMGB1, the TLR4 binding site has not been identified so far.

In conclusion, the increase in hepatic HMGB1 during fibrogenesis contributes to the pathogenesis of the disease by driving scar formation. HMGB1, unlike other pro-fibrogenic mediators, provides a much wider time-frame for clinical intervention due to its remarkable cellular mobility, prolonged half-life and accordingly, longer receptor activation (3). Hence, the increase in HMGB1 is not a mere result of liver damage but also a contributing event and thus a possible target to prevent the onset and progression of liver fibrosis. It is hoped that the innovative nature of this concept could have great impact for the detection and potentially the treatment of liver fibrosis in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support: American Association for the Study of Liver Diseases, Pinnacle Research Award G3156 (X. G.). Bancaja Short-Term Fellowship and Asociación Española para el Estudio del Hígado Postdoctoral Fellowship (E. A.). Chicago Biomedical Consortium Postdoctoral Fellowship (F. M. and N. N.). Wellcome Trust Research Fellowship (D. J. A.) and Medical Research Council Centre for Drug Safety Science Grant (D. J. A. & N. N.). US Public Health Service Grants R01 DK069286, R56 DK069286, R56 DK069286-06S1 and R01 DK111677 from the National Institute of Diabetes and Digestive and Kidney Diseases (N. N.). US Public Health Service Grants P20

AA017067, P20 AA017067-01S1, P20 AA017067-03S1 and U01 AA021887 from the National Institute on Alcohol Abuse and Alcoholism (N. N.).

The authors are very grateful to Dr. Timothy R. Billiar (University of Pittsburgh, Pittsburgh, PA) for donating the *Hmgb1^{fl/fl}* mice, Dr. Bernd Arnold (German Cancer Research Center, Heidelberg, Germany) for providing the *Rage^{fl/fl}* mice, Dr. David J. Hackam (University of Pittsburgh, Pittsburgh, PA) for donating the *Tlr4^{fl/fl}* mice, Dr. Robert Schwabe (Columbia University, New York, NY) for donating the *Lrat-Cre* mice and Dr. Hidekazu Tsukamoto (University of Southern California, Los Angeles, CA) for donating the samples from the *ad libitum* and intragastric infusion “hybrid” feeding regime of high cholesterol fat diet and ethanol liquid diet with a weekly binge of alcohol. We are also very thankful to all past and current members from the Nieto Laboratory for their helpful comments, support and suggestions throughout the course of this project.

Abbreviations

αSMA	α-smooth muscle actin
Alb	albumin
ALT	alanine aminotransferase
ASH	alcoholic steatohepatitis
AUC	area under the curve
BDL	bile duct ligation
CCl₄	carbon tetrachloride
DAMP	damage-associated molecular pattern(s)
ELISA	enzyme-linked immunosorbent assay
ESI-LC-MS	electrospray ionization-liquid chromatography-mass spectrometry
FISH	fluorescent <i>in situ</i> hybridization
Gfap	glial fibrillary acidic protein
H&E	hematoxylin and eosin
HCV	Hepatitis C virus
HMGB1	high-mobility group box-1
<i>Hmgb1^{fl/fl}</i>	<i>Hmgb1</i> floxed mice
<i>Hmgb1^{Hep}</i>	conditional <i>Hmgb1</i> knockout mice in hepatocytes
<i>Hmgb1^{HSC}</i>	conditional <i>Hmgb1</i> knockout mice in hepatic stellate cells
<i>Hmgb1^{Hep Mye}</i>	conditional <i>Hmgb1</i> knockout mice in hepatocytes and myeloid cells
<i>Hmgb1^{Mye}</i>	conditional <i>Hmgb1</i> knockout mice in myeloid cells
HSC	hepatic stellate cells

IF	immunofluorescence
IHC	immunohistochemistry
KC	Kupffer cell(s)
<i>Lrat</i>	lecithin retinol acyltransferase
<i>Lyz2</i>	lysozyme 2
MCD	methionine and choline deficient diet
MCS	methionine and choline sufficient diet
MMP	matrix metalloproteinase
MO	mineral oil
OPN	osteopontin
PBC	primary biliary cirrhosis
qRT-PCR	quantitative real-time polymerase chain reaction
RAGE	receptor for advanced glycation end-products
<i>Rage</i>^{fl/fl}	<i>Rage</i> floxed mice
<i>Rage</i>^{Hep}	conditional <i>Rage</i> knockout mice in hepatocytes
<i>Rage</i>^{HSC}	conditional <i>Rage</i> knockout mice in hepatic stellate cells
<i>Rage</i>^{Mye}	conditional <i>Rage</i> knockout mice in myeloid cells
SEM	standard error of the mean
shRNA	small hairpin RNA
TAA	thioacetamide
TLR(s)	toll-like receptor(s)
<i>Tlr4</i>^{fl/fl}	<i>Toll-like receptor 4</i> floxed mice
<i>Tlr4</i>^{HSC}	conditional <i>Tlr4</i> knockout mice in hepatic stellate cells
WT	wild-type

References

1. Arriazu E, Ge X, Leung TM, Magdaleno F, Lopategi A, Lu Y, Kitamura N, et al. Signalling via the osteopontin and high mobility group box-1 axis drives the fibrogenic response to liver injury. *Gut*. 2017; 66:1123–1137. [PubMed: 26818617]
2. Ge X, Antoine DJ, Lu Y, Arriazu E, Leung TM, Klepper AL, Branch AD, et al. High mobility group box-1 (HMGB1) participates in the pathogenesis of alcoholic liver disease (ALD). *J Biol Chem*. 2014; 289:22672–22691. [PubMed: 24928512]

3. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, et al. HMGB-1 as a late mediator of endotoxin lethality in mice. *Science*. 1999; 285:248–251. [PubMed: 10398600]
4. Huebener P, Pradere JP, Hernandez C, Gwak GY, Caviglia JM, Mu X, Loike JD, et al. The HMGB1/RAGE axis triggers neutrophil-mediated injury amplification following necrosis. *J Clin Invest*. 2015; 125:539–550. [PubMed: 25562324]
5. Wu CX, Sun H, Liu Q, Guo H, Gong JP. LPS induces HMGB1 relocation and release by activating the NF-kappaB-CBP signal transduction pathway in the murine macrophage-like cell line RAW264.7. *J Surg Res*. 2012; 175:88–100. [PubMed: 21571302]
6. Tang D, Shi Y, Kang R, Li T, Xiao W, Wang H, Xiao X. Hydrogen peroxide stimulates macrophages and monocytes to actively release HMGB1. *J Leukoc Biol*. 2007; 81:741–747. [PubMed: 17135572]
7. Chen R, Hou W, Zhang Q, Kang R, Fan XG, Tang D. Emerging role of high-mobility group box 1 (HMGB1) in liver diseases. *Mol Med*. 2013; 19:357–366.
8. Maugeri N, Franchini S, Campana L, Baldini M, Ramirez GA, Sabbadini MG, Rovere-Querini P, et al. Circulating platelets as a source of the damage-associated molecular pattern HMGB1 in patients with systemic sclerosis. *Autoimmunity*. 2012; 45:584–587. [PubMed: 22928592]
9. Jube S, Rivera ZS, Bianchi ME, Powers A, Wang E, Pagano I, Pass HI, et al. Cancer cell secretion of the DAMP protein HMGB1 supports progression in malignant mesothelioma. *Cancer Res*. 2012; 72:3290–3301. [PubMed: 22552293]
10. Pisetsky D. Cell death in the pathogenesis of immune-mediated diseases: the role of HMGB1 and DAMP-PAMP complexes. *Swiss Med Wkly*. 2011; 141:w13256. [PubMed: 21877298]
11. Hori O, Brett J, Slattery T, Cao R, Zhang J, Chen JX, Nagashima M, et al. The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *J Biol Chem*. 1995; 270:25752–25761. [PubMed: 7592757]
12. Theise ND. Liver biopsy assessment in chronic viral hepatitis: a personal, practical approach. *Mod Pathol*. 2007; 20(Suppl 1):S3–14. [PubMed: 17486049]
13. Scheuer PJ. Ludwig Symposium on biliary disorders--part II. Pathologic features and evolution of primary biliary cirrhosis and primary sclerosing cholangitis. *Mayo Clin Proc*. 1998; 73:179–183. [PubMed: 9473003]
14. Lazaro R, Wu R, Lee S, Zhu NL, Chen CL, French SW, Xu J, et al. Osteopontin deficiency does not prevent but promotes alcoholic neutrophilic hepatitis in mice. *Hepatology*. 2015; 61:129–140. [PubMed: 25132354]
15. Carneiro VC, de Moraes Maciel R, de Abreu da Silva IC, da Costa RF, Paiva CN, Bozza MT, Fantappie MR. The extracellular release of *Schistosoma mansoni* HMGB1 nuclear protein is mediated by acetylation. *Biochem Biophys Res Commun*. 2009; 390:1245–1249. [PubMed: 19879244]
16. Youn JH, Shin JS. Nucleocytoplasmic shuttling of HMGB1 is regulated by phosphorylation that redirects it toward secretion. *J Immunol*. 2006; 177:7889–7897. [PubMed: 17114460]
17. Evankovich J, Cho SW, Zhang R, Cardinal J, Dhupar R, Zhang L, Klune JR, et al. High mobility group box 1 release from hepatocytes during ischemia and reperfusion injury is mediated by decreased histone deacetylase activity. *J Biol Chem*. 2010; 285:39888–39897. [PubMed: 20937823]
18. Dhupar R, Klune JR, Evankovich J, Cardinal J, Zhang M, Ross M, Murase N, et al. Interferon regulatory factor 1 mediates acetylation and release of high mobility group box 1 from hepatocytes during murine liver ischemia-reperfusion injury. *Shock*. 2011; 35:293–301. [PubMed: 20856174]
19. Oh YJ, Youn JH, Ji Y, Lee SE, Lim KJ, Choi JE, Shin JS. HMGB1 is phosphorylated by classical protein kinase C and is secreted by a calcium-dependent mechanism. *J Immunol*. 2009; 182:5800–5809. [PubMed: 19380828]
20. Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, Rubartelli A, et al. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *EMBO J*. 2003; 22:5551–5560. [PubMed: 14532127]
21. Agalave NM, Larsson M, Abdelmoaty S, Su J, Baharpoor A, Lundback P, Palmblad K, et al. Spinal HMGB1 induces TLR4-mediated long-lasting hypersensitivity and glial activation and regulates pain-like behavior in experimental arthritis. *Pain*. 2014; 155:1802–1813. [PubMed: 24954167]

22. Bianchi ME, Manfredi AA. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. *Immunol Rev.* 2007; 220:35–46. [PubMed: 17979838]
23. Yang H, Tracey KJ. Targeting HMGB1 in inflammation. *Biochim Biophys Acta.* 2010; 1799:149–156. [PubMed: 19948257]
24. Yu M, Wang H, Ding A, Golenbock DT, Latz E, Czura CJ, Fenton MJ, et al. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock.* 2006; 26:174–179. [PubMed: 16878026]
25. Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, Abraham E. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem.* 2004; 279:7370–7377. [PubMed: 14660645]
26. Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, Sohn JW, et al. High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am J Physiol Cell Physiol.* 2006; 290:C917–924. [PubMed: 16267105]
27. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med.* 2007; 13:1050–1059. [PubMed: 17704786]
28. Tian J, Avalos AM, Mao SY, Chen B, Senthil K, Wu H, Parroche P, et al. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat Immunol.* 2007; 8:487–496. [PubMed: 17417641]
29. Chen GY, Tang J, Zheng P, Liu Y. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. *Science.* 2009; 323:1722–1725. [PubMed: 19264983]
30. Li J, Kokkola R, Tabibzadeh S, Yang R, Ochani M, Qiang X, Harris HE, et al. Structural basis for the proinflammatory cytokine activity of high mobility group box 1. *Mol Med.* 2003; 9:37–45. [PubMed: 12765338]
31. Goodwin M, Herath C, Jia Z, Leung C, Coughlan MT, Forbes J, Angus P. Advanced glycation end products augment experimental hepatic fibrosis. *J Gastroenterol Hepatol.* 2013; 28:369–376. [PubMed: 23173780]
32. Miura K, Yang L, van Rooijen N, Brenner DA, Ohnishi H, Seki E. Toll-like receptor 2 and palmitic acid cooperatively contribute to the development of nonalcoholic steatohepatitis through inflammasome activation in mice. *Hepatology.* 2013; 57:577–589. [PubMed: 22987396]
33. Vespasiani-Gentilucci U, Carotti S, Perrone G, Mazzarelli C, Galati G, Onetti-Muda A, Picardi A, et al. Hepatic toll-like receptor 4 expression is associated with portal inflammation and fibrosis in patients with NAFLD. *Liver Int.* 2015; 35:569–581. [PubMed: 24649857]
34. Miura K, Kodama Y, Inokuchi S, Schnabl B, Aoyama T, Ohnishi H, Olefsky JM, et al. Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1 β in mice. *Gastroenterology.* 2010; 139:323–334. e327. [PubMed: 20347818]
35. Urtasun R, Lopategi A, George J, Leung TM, Lu Y, Wang X, Ge X, et al. Osteopontin, an oxidant stress sensitive cytokine, up-regulates collagen-I via integrin α (V) β (3) engagement and PI3K/pAkt/NF κ B signaling. *Hepatology.* 2012; 55:594–608. [PubMed: 21953216]
36. Vergeer WP, Sogo JM, Pretorius PJ, de Vries WN. Interaction of Ap1, Ap2, and Sp1 with the regulatory regions of the human pro- α 1(I) collagen gene. *Arch Biochem Biophys.* 2000; 377:69–79. [PubMed: 10775443]
37. Sherwood AL, Bottenus RE, Martzen MR, Bornstein P. Structural and functional analysis of the first intron of the human α 2(I) collagen-encoding gene. *Gene.* 1990; 89:239–244. [PubMed: 2129528]
38. Chang HH, Chang MC, Wu IH, Huang GF, Huang WL, Wang YL, Lee SY, et al. Role of ALK5/Smad2/3 and MEK1/ERK Signaling in Transforming Growth Factor β 1-modulated Growth, Collagen Turnover, and Differentiation of Stem Cells from Apical Papilla of Human Tooth. *J Endod.* 2015; 41:1272–1280. [PubMed: 26001858]
39. Hayashida T, Decaestecker M, Schnaper HW. Cross-talk between ERK MAP kinase and Smad signaling pathways enhances TGF- β -dependent responses in human mesangial cells. *FASEB J.* 2003; 17:1576–1578. [PubMed: 12824291]
40. Li L, Wang JY, Yang CQ, Jiang W. Effect of RhoA on transforming growth factor β 1-induced rat hepatic stellate cell migration. *Liver Int.* 2012; 32:1093–1102. [PubMed: 22498718]

41. Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, Schwabe RF. TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med.* 2007; 13:1324–1332. [PubMed: 17952090]

B

		HMGB1 Ratios				
		Nuclear	Cytoplasmic	Nuclear/Total	Cytoplasmic /Total	
Healthy		1.0±0.09	1.0±0.04	1.0±0.00	ND	
HCV	Stage 1	1	1.2±0.08	33.3±3.35***	0.8±0.02*	0.2±0.02*
		2	1.1±0.14	55.1±8.65***	0.7±0.04*	0.3±0.04*
		3	1.1±0.09	84.8±8.43***	0.6±0.02**	0.4±0.03*
		4	1.1±0.11	132.8±12.13***	0.5±0.04**	0.5±0.04**
PBC	Stage 2	1	1.2±0.03	69.4±0.71***	0.7±0.00*	0.3±0.00*
		2	1.6±0.05*	133.6±17.62***	0.6±0.05**	0.4±0.05*
		3	1.4±0.13*	170.3±10.76***	0.5±0.04**	0.5±0.04**
		4	1.2±0.08	205.0±11.99***	0.4±0.03**	0.6±0.03**
ASH	Stage 3	1	1.0±0.10	85.4±16.20***	0.7±0.06*	0.3±0.07*
		2	1.7±0.20*	96.0±11.76***	0.6±0.08*	0.4±0.07*
		3	1.4±0.13*	137.6±17.64***	0.6±0.05*	0.4±0.05*
		4	1.6±0.10*	296.0±29.47***	0.4±0.02**	0.6±0.05**

Figure 1. Hepatic HMGB1 protein expression correlates with fibrosis stage in chronic HCV, PBC and ASH patients

IHC on formalin-fixed paraffin-embedded human liver biopsies from HCV, PBC and ASH patients shows increased HMGB1 (400x oil, green arrow) expression with disease stage (A). Quantification of nuclear, cytoplasmic and the ratios of nuclear-to-total and cytoplasmic-to-total HMGB1 protein expression in these patients according to disease stage. Results are given as fold-change of the healthy liver explants assigned a value of 1 and are expressed as mean ± SEM; $n=10$ /disease and stage; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs. healthy (B).

Hepatic HMGB1 protein expression and secretion correlates with liver fibrosis in mice.

WT mice were injected MO or CCl₄ for 1 mo. H&E staining (200x) shows greater necrosis (blue arrow) and inflammation (yellow arrow) in CCl₄- than in MO-injected WT mice. IHC shows increased and translocated HMGB1 (400x, green arrows) and up-regulated Collagen type I expression (200x; perivenular fibrosis, closed pink arrow; bridging fibrosis, open pink arrow) in CCl₄- compared to MO-injected WT mice (C, left). Western blot of HMGB1 (C, top right) and total Collagen type I morphometry (C, bottom right) in MO- and CCl₄- injected mice. Results are given as fold-change of the MO-injected mice assigned a value of 1 and are expressed as mean ± SEM; $n=8$ /group; ** $p<0.01$ and *** $p<0.001$ vs. MO. H&E (200x), HMGB1 (400x) and Collagen type I (200x) IHC in WT mice given water or TAA

and with sham operation or BDL. Both treatments caused liver injury along with increased plus translocated HMGB1 and up-regulated Collagen type I (**D**). Quantification of nuclear, cytoplasmic and the ratios of nuclear-to-total and cytoplasmic-to-total HMGB1 protein given as fold change of the MO-injected mice assigned a value of 1. Total liver (area under the curve (AUC)) and serum (ng/ml) HMGB1 quantified by electrospray ionization-liquid chromatography-mass spectrometry (ESI-LC-MS). Results are expressed as mean $\pm$ SEM; $n=8$ /group; * $p<0.05$, ** $p<0.01$ and *** $p<0.01$ vs. its own control (**E**).

Figure 2. Identification of the cellular source of HMGB1 in liver fibrosis

IF, FISH and morphometry analysis in mouse liver following CCl₄-injections demonstrates co-localization and induction of HMGB1 with markers of HSC (desmin protein – cytoplasmic- and *Des* mRNA) (A, B), KC and infiltrating macrophages (F4/80 protein – cytoplasmic- and *Emr1* mRNA) (C, D) and hepatocytes (HNF4α protein – nuclear- and *Hnf4a* mRNA) (E, F). All panels are at 630x oil (H: hepatocyte). Primary cells were isolated from these mice and HMGB1 was quantified by ESI-LC-MS (AUC) (B, D, F, right). Results are expressed as mean ± SEM; *n*=8/group; **p*<0.01 and ***p*<0.001 vs. MO.

Figure 3. Neutralization of HMGB1 prevents while injection of HMGB1 promotes liver fibrosis in mice

WT mice were injected CCl₄ for 1 mo along with non-immune IgG or a HMGB1 neutralizing Ab (both at 0.6 µg/g b. wt.) administered 1 h before each CCl₄ dose. Liver injury and fibrosis assessed by H&E staining (200x; necrosis, blue arrow; inflammation, yellow arrows), HMGB1 (400x oil, green arrows), Collagen type I IHC and Sirius Red/Fast Green staining (200x; perivenular fibrosis, closed pink arrows; bridging fibrosis, open pink arrows) (A), the pathology scores, total liver (AUC) and serum (ng/ml) HMGB1, Collagen type I and Sirius red/Fast green morphometry, Collagen type I and αSMA western blot and 4-hydroxyproline content (B) and ALT activity (C) were significantly reduced in CCl₄-injected mice pre-treated with the HMGB1 neutralizing Ab compared to mice pre-treated with non-immune IgG. Results are expressed as mean ± SEM; *n*=6/group; **p*<0.05, ***p*<0.01 and ****p*<0.001 vs. CCl₄ + IgG. WT mice were injected CCl₄ or 0–0.2 µg/g HMGB1 (1 h before each dose of CCl₄) for 1 mo. H&E staining (200x), HMGB1 IHC (400x), Collagen type I IHC (200x), Sirius red/Fast green staining (200x) and desmin IHC (200x) show more necrosis (blue arrows), inflammation (yellow arrows), perivenular fibrosis (closed pink arrows), bridging fibrosis (open pink arrows) and desmin (white arrows) in mice injected HMGB1 compared to controls (D). Pathology scores, total liver (AUC) and

serum (ng/ml) HMGB1, Collagen type I and Sirius red/Fast green morphometry, Collagen type I and α SMA western blot and 4-hydroxyproline content (E). ALT activity (F). Results are mean $\pm$ SEM; $n=6$ /group; * $p<0.05$ and ** $p<0.01$ vs CCl₄ + Albumin.

B

Treatment	Scores				Morphometry		Index
	Necrosis	Ballooning	Inflammation	Fibrosis	Collagen type I	Sirius red/Fast green	
WT + CCl ₄	2.18±0.14	0.96±0.13	2.33±0.16	2.72±0.14	1.00±0.07	1.0±0.13	1,213±52
Hmgb1 ^{ΔHep} + CCl ₄	1.42±0.15**	0.63±0.11*	1.41±0.11**	1.72±0.16**	0.46±0.80**	0.57±0.07*	1,217±49
Hmgb1 ^{ΔMye} + CCl ₄	1.08±0.13**	0.41±0.10**	1.83±0.11*	1.82±0.13**	0.36±0.50**	0.33±0.06**	1,130±44
Hmgb1 ^{ΔHepΔMye} + CCl ₄	0.34±0.12***	0.28±0.12**	1.27±0.13**	1.24±0.19**	0.15±0.04***	0.18±0.04***	1,161±57

Figure 4. *Hmgb1* ablation in hepatocytes or myeloid cells partially protects while ablation in both prevents CCl₄-induced liver fibrosis

H&E staining (200x), HMGB1 IHC (400x), Collagen type I IHC (200x), Sirius red/Fast green staining (200x) and CD31 IHC (630x oil) in 1 mo CCl₄-injected WT show more necrosis (blue arrows), inflammation (yellow arrows), HMGB1 expression and translocation (green arrows), perivenular fibrosis (closed pink arrows) and bridging fibrosis (open pink arrows) than in *Hmgb1*^{Hep}, *Hmgb1*^{Mye} or *Hmgb1*^{Hep Mye} mice. Vascularization (CD31, red staining, white arrows) was similar in all mice (A). Scores for necrosis, hepatocyte ballooning degeneration, inflammation, fibrosis, Collagen type I and Sirius red/Fast green morphometry and CD31 index (B). Serum ALT (C). Quantification of nuclear, cytoplasmic and the ratios of nuclear-to-total and cytoplasmic-to-total HMGB1 protein given as fold change of the MO-injected mice assigned a value of 1. Total liver (AUC) and serum (ng/ml) HMGB1 quantified by ESI-LC-MS (ND: not detected) (D). Western blot analysis for Collagen type I, αSMA and RAGE and quantification of 4-hydroxyproline (E). Results are expressed as mean ± SEM; *n*=8/group; **p*<0.05, ***p*<0.01 and ****p*<0.001 vs. WT + CCl₄.

Figure 5. Hepatocytes and KC are a major source of HMGB1 signaling to HSC to increase Collagen type I production

Western blot of intra- and extracellular Collagen type I in HSC co-cultured with hepatocytes or KC from MO- or CCl₄-injected WT, *Hmgb1*^{Hep} and *Hmgb1*^{Mye} mice. Results are given as fold-change of the MO groups assigned a value of 1 and are expressed as mean ± SEM; *n*=3/group; **p*<0.05, ***p*<0.01 and ****p*<0.001 vs. MO or control and •*p*<0.05, ••*p*<0.01 and •••*p*<0.001 vs. WT (A). Total cell lysate (AUC) and cell culture (ng/ml) HMGB1 in HSC, hepatocytes and KC seeded alone or in co-culture. In the HSC/HEP and HSC/KC co-cultures, the cell lysate is from hepatocytes and from KC, respectively (ND: not detected). Results are expressed as mean ± SEM; *n*=3/group; ***p*<0.01 and ****p*<0.001 vs. WT + MO (B). *HMGB1 stimulates HSC migration without altering their viability or cell cycle*. HSC were treated with 0–1 nM HMGB1 in the presence or absence of mitomycin for 24 h. HSC migration is induced by HMGB1 but it is not fully prevented by co-treatment with mitomycin (C). HSC viability and cell cycle (D). *n*=3/condition.

B

Treatment	Scores				Morphometry	
	Necrosis	Ballooning	Inflammation	Fibrosis	Collagen type I	Sirius red/ Fast green
WT + CCl ₄	2.72±0.11	1.8±0.2	2.4±0.4	2.99±0.2	1.0±0.05	1.0±0.14
RAGE Ab + CCl ₄	1.2±0.1***	1.5±0.2	2.6±0.3	1.9±0.2**	0.61±0.07**	0.56±0.08**
Tlr4 ^{ΔHSC} + CCl ₄	2.86±0.21	2.3±0.2	2.17±0.1	2.7±0.2	0.93±0.09	0.79±0.12
Rage ^{ΔHSC} + CCl ₄	1.5±0.2**	1.7±0.1	2.18±0.2	1.8±0.1**	0.31±0.03***	0.38±0.07***

Figure 6. RAGE ablation in HSC and RAGE neutralization prevent liver fibrosis *in vivo* WT control or i.p. injected 0.4 μg/g b. wt. of RAGE neutralizing Ab (2 h before CCl₄), *Tlr4*^{HSC} and *RAGE*^{HSC} mice were injected CCl₄ for 1 mo. H&E staining (200x; necrosis, blue arrows; inflammation, yellow arrows), HMGB1 IHC (400x oil, green arrows), Collagen type I IHC and Sirius red/Fast green staining (200x; perivenular fibrosis, closed pink arrows; bridging fibrosis, open pink arrows) and co-localization of RAGE and TLR4 with Desmin (white arrows) (A). Scores for necrosis, hepatocyte ballooning degeneration, inflammation, fibrosis, Collagen type I and Sirius red/Fast green morphometry (B). Serum ALT (C). Quantification of nuclear, cytoplasmic and the ratios of nuclear-to-total and cytoplasmic-to-total HMGB1 protein given as fold change of the MO-injected mice assigned a value of 1. Total liver (AUC) and serum HMGB1 (ng/ml) quantified by ESI-LC-MS (ND: not detected) (D). Western blot analysis for Collagen type I and αSMA and quantification of 4-hydroxyproline (E). Results are expressed as mean ± SEM; *n*=8/group. **p*<0.05, ***p*<0.01 and ****p*<0.001 vs. WT + CCl₄.

B

Treatment	Scores				Morphometry	
	Necrosis	Ballooning	Inflammation	Fibrosis	Collagen type I	Sirius red/ Fast green
WT + CCl ₄	2.8±0.2	1.9±0.1	2.5±0.1	2.75±0.2	1.0±0.09	1.0±0.13
<i>Rage</i> ^{ΔHep} + CCl ₄	3.0±0.26	2.28±0.2	2.7±0.2	2.5±0.3	0.81±0.17	0.83±0.1*
<i>Rage</i> ^{ΔMye} + CCl ₄	0.5±0.1	1.7±0.1	3±0.1*	2.8±0.2	0.97±0.12	1.2±0.19

Figure 7. Rage ablation in hepatocytes slightly reduces whereas ablation in myeloid cells does not protect from liver fibrosis

WT, *Rage*^{Hep} and *Rage*^{Mye} mice were injected CCl₄ for 1 mo. H&E staining (200x; necrosis, blue arrows; inflammation, yellow arrows), HMGB1 IHC (400x oil, green arrows), Collagen type I IHC and Sirius red/Fast green staining (200x; perivenular fibrosis, closed pink arrows; bridging fibrosis, open pink arrows) and co-localization of RAGE with HNF4α or F4/80 (white arrows) (**A**). Scores for necrosis, hepatocyte ballooning degeneration, inflammation, fibrosis, Collagen type I and Sirius red/Fast green morphometry (**B**). Serum ALT (**C**). Quantification of nuclear, cytoplasmic and the ratios of nuclear-to-total and cytoplasmic-to-total HMGB1 protein given as fold change of the MO-injected mice assigned a value of 1. Total liver (AUC) and serum (ng/ml) HMGB1 quantified by ESI-LC-MS (**D**). Western blot for Collagen type I and αSMA and quantification of 4-hydroxyproline (**E**). Results are expressed as mean ± SEM; *n*=8/group. **p*<0.05 and ***p*<0.01 vs. WT + CCl₄.

Figure 8. HMGB1 signals via RAGE to upregulate Collagen type I protein expression in HSC

Western blot for RAGE expression in HSC infected with control or *Rage* small hairpin RNA (shRNA) lentiviral particles (**A**, left). Western blot for intra- and extracellular Collagen type I and intracellular MMP1 expression in HSC with and without *Rage* ablation and treated with 0–1 nM HMGB1 for 24 h (**A**, right). WT and *Rage*^{−/−} HSC were treated with HMGB1 for 30 min. Western blot for pMEK1/2, pERK1/2, ERK1/2, pcJun, pAkt1/2/3, Akt, RAGE and Actin (**B**). HSC were treated with 0–2 μM PD98059 for 1 h prior to addition of 0–1 nM HMGB1. Samples were analyzed by western blot for pMEK1/2, pERK1/2, total ERK1/2, pcJun, pAkt1/2/3⁴⁷³Ser, total Akt1/2/3, RAGE and actin at 30 min (**C**, top) and for Collagen type I and Calnexin at 24 h (**C**, bottom). Results are given as fold-change of the control or WT HSC assigned a value of 1 and are expressed as mean ± SEM (omitted under the blots due to lack of space); numbers under the blots are ratios of any given protein expression against the specific internal loading control or the native protein. *n*=3/group; **p*<0.05, ***p*<0.01 and ****p*<0.001 vs. control and •*p*<0.05, ••*p*<0.01 and •••*p*<0.001 vs. WT HSC or HMGB1-treated. **Working model:** we previously showed that intracellular acetylation of HMGB1 due to OPN stimulation in HSC also contributes to Collagen type I deposition (1). We now demonstrate that during liver fibrosis, hepatocytes and KC plus infiltrating macrophages increase HMGB1 expression and secretion. Extracellular HMGB1 binds

RAGE on HSC, activates both the pMEK1/2/pERK1/2/pJNK and the PI3K/pAkt signaling pathways to up-regulate Collagen type I production. pMEK1/2 appears to be upstream of pAKT in these events. *Rage* ablation in HSC or RAGE neutralization prevents the HMGB1-mediated effects on HSC to up-regulate Collagen type I production; hence, preventing fibrosis. Notably, *Rage* ablation in HSC or neutralization reduces HMGB1 production by hepatocytes and myeloid cells via unknown mechanisms further protecting the liver (Note that the strategies we used to prove each step have been integrated in the working model and are written using black font).