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TIFA Upregulation after Hypoxia-Reoxygenation is TLR4 and MyD88-Dependent and Associated with HMGB1 Upregulation and Release

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Abstract

TRAF-interacting protein with a forkhead-associated domain (TIFA) is a tumor necrosis factor receptor-associated factor 6 (TRAF6) binding protein that mediates IL-1 signaling. We recently reported that TIFA mRNA is significantly upregulated early in the liver following trauma and hemorrhagic shock. In the present study, we sought to characterize the upregulation of TIFA by hypoxia-reoxygenation and investigate its role in hypoxia-induced signaling. TIFA expression was detected by qRT-PCR and Western blotting in both mouse hemorrhagic shock with resuscitation (HS-R) and hepatocytes exposed to hypoxia-reoxygenation. Involvement of TLR4 and MyD88 was assessed using cells from TLR4−/− and MyD88−/− mice. The interaction of TIFA with TRAF6 and IRAK-1 was investigated using co-immunoprecipitation *in vitro*. RNAi was performed to knock down the endogenous expression of the TIFA gene in hepatocytes. High mobility group box 1 protein (HMGB1) expression was detected by Western blotting and ELISA, and the activation of NF-κB and MAPK was measured with EMSA and Western blotting. The results showed that TIFA expression was upregulated following HS-R *in vivo* and hypoxia-reoxygenation *in vitro*. Further analysis revealed that hypoxia-reoxygenation-induced upregulation of TIFA was TLR4 and MyD88-dependent. Moreover, TIFA was found to associate with TRAF6 constitutively, whereas its association with IRAK-1 was seen only after hypoxia-reoxygenation. Suppression of TIFA by siRNA reduced NF-κB activation and HMGB1 upregulation and release following hypoxia-reoxygenation. Taken together, these data suggest that TIFA is involved in the regulation of cell signaling in hypoxia-reoxygenation. The increase in TIFA level appears to be a feed-forward mechanism involved in TLR4/MyD88-dependent signaling, leading to NF-κB activation and HMGB1 release.

Keywords

TIFA; TLR4; hypoxia; liver; inflammation

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INTRODUCTION

Hemorrhagic shock and resuscitation (HS-R) is a common feature of traumatic injury and results in tissue and organ hypoxia and reoxygenation [1, 2]. HS-R initiates an inflammatory response characterized by the upregulation of cytokine expression and accumulation of neutrophils in a variety of tissues [3, 4]. The liver, with its crucial involvement in metabolism and homeostasis, is among the most frequently affected organs [5]. Early inflammatory response appears to be crucial for the survival of both patients and experimental animals subjected to HS-R [6]. However, the inflammatory response can also compromise healthy tissue, further exacerbating inflammation [7]. We have shown that high mobility group box1 protein (HMGB1) release contributes to the inflammatory response induced by HS-R [8] and that hypoxia is a potent stimulus for the toll-like receptor (TLR) 4-dependent upregulation and release of HMGB1 from hepatocytes [9].

DNA microarray analysis provides a rapid, efficient method for identifying global changes in mRNA transcription. The data obtained from gene arrays can be utilized to identify candidate genes for further analysis or to identify key pathways that have been altered at the mRNA level [10–12]. In order to characterize the early changes in gene expression that take place following severe injury in a model that incorporates both tissue trauma and HS-R, we previously carried out a DNA microarray analyses on mouse livers using a dual platform (Affymetrix and CodeLink) approach that addressed the reproducibility of detected biomarkers [13]. Significant changes in 6,338 unique genes common to two platforms were identified within six hours of HS-R and trauma after proper gene filtering and normalization. Of the genes identified, TRAF-interacting protein with a forkhead-associated domain (TIFA) was particularly interesting because it was one of the most dramatically upregulated genes induced by HS-R and trauma. TIFA is a recently identified adapter protein that participates in signal transduction of tumor necrosis factor receptor (TNFR) and the interleukin-1 receptor (IL-1R)/TLR super family [14]. Its role in HS-R has never been reported.

In the present study, we investigated the role of upregulation of TIFA in the setting of hypoxia-reoxygenation. The results showed that TIFA expression was upregulated in the liver following HS-R and hypoxia-reoxygenation in cultured hepatocytes. The upregulation of TIFA induced by hypoxia-reoxygenation was TLR4 and myeloid differentiation factor 88 (MyD88)-dependent. Further analysis revealed that TIFA associates with tumor necrosis factor receptor-associated factor 6 (TRAF6) constitutively, whereas it associates with IL-1R-associated kinase 1 (IRAK-1) in a hypoxia-reoxygenation stimulation-dependent manner. Importantly, suppression of TIFA with siRNA transfection decreased HMGB1 expression and release in response to hypoxia-reoxygenation in hepatocytes. Thus, TIFA upregulation induced by hypoxia-reoxygenation appears to be part of a feed-forward signaling mechanism.

MATERIAL AND METHODS

Animals

Male C57BL/6 wild-type (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TLR4 knockout (TLR4−/−) mice and MyD88−/− mice were bred at the University of Pittsburgh. All mice used were on a C57BL/6 background. Mice were 8 to 12 weeks of age at the time of the experiments. Animal handling and care complied with the regulations for the care and use of experimental animals published by the National Institutes of Health and experimental protocols were approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh.

Mice hemorrhagic shock protocol

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and inhaled isoflurane (Abbott Labs, Chicago, IL). Both femoral arteries were cannulated, one for continuous blood pressure monitoring and the other for blood withdrawal or fluid administration. Blood was withdrawn over 10 minutes to achieve a mean arterial pressure (MAP) of 25 mmHg. MAP was maintained at 25 to 30 mmHg. After two hours of hemorrhagic shock, animals were restored to a MAP of ≥ 80 mmHg by administration of three times the volume of shed blood with lactated Ringer's solution over designed time intervals.

Hepatocyte isolation and cell culture

Hepatocytes were isolated from mice by an *in situ* collagenase (type VI; Sigma) perfusion technique, as described previously with modifications [15]. Hepatocyte purity exceeded 98% and viability exceeded 95% as determined by standard testing. Highly purified hepatocytes were suspended in Williams' E medium supplemented with calf serum, HEPES, insulin, L-glutamine, penicillin, and streptomycin. The cells were plated on collagen-coated cell culture dishes and cultured overnight at 37°C under normoxic conditions (21% O_2).

Hepatocyte hypoxia-reoxygenation model

Hypoxic conditions were obtained by placing the cells into a modular incubator chamber (Billups-Rothenburg, Del Mar, CA) flushed with a hypoxic gas mixture containing 1% O_2, 5% CO_2, and 94% N_2. Duplicate hypoxic cultures were returned for reoxygenation in the normoxic incubator. Hepatocytes incubated under normoxic conditions served as controls.

Reverse transcription PCR and quantitative real-time PCR

Total RNA was extracted from homogenization of snap frozen livers using RNeasy Mini Kit (Qiagen, Valencia, CA). iScript™ reverse transcription supermix for RT-qPCR kit (Bio-Rad, Hercules, CA) was used for reverse transcription (RT). PCR amplification mixtures were prepared using iTaq™ Fast SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA). Sequences for TIFA primers were as follows: Forward: 5′-AGAAGGCTCCGCAAGACTC-3′, Reverse 5′-ACTGGCACATGTTGGAATTG-3′. Real-time PCR was performed using Mx3000p system (Stratagene, La Jolla, CA). All samples were run in triplicate. Quantification of gene expression was calculated relative to β-actin.

Preparation of cell lysates and Western blotting

Hepatocytes that subjected to hypoxia for two hours and reoxygenation for six hour were washed twice in PBS and lysed with 1× cell lysis buffer (Cell Signaling, Danvers, MA) on ice for 10 minutes. Liver samples were homogenized with a glass dounce homogenizer in 1×RIPA cell lysis buffer. Protein content of cell lysates was determined by BCA protein assay (Pierce, Rockford, IL). For Western blotting, equal protein amounts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane followed by immunostaining with primary antibody specific for TIFA, IRAK1, TRAF6, p-IκBα, IκBα, p-p38, p38, p-c-JUN N-terminal kinase (JNK), JNK, p-extracellular signal-regulated kinase (ERK) 1/2, ERK1/2 (Cell Signaling), HMGB1, p-IκB kinase (IKK) β and IKKβ (Abcam, Cambridge, MA). Horseradish peroxidase-conjugated secondary antibodies were then used in a standard enhanced chemiluminescence reaction according to the manufacturer's instructions (Pierce).

Co-immunoprecipitation analysis

Whole cell lysates were incubated overnight with anti-TIFA antibody and immune complexes were then precipitated with protein A/G agarose beads for six hours and then

washed three times with immunoprecipitation buffer. Immunoprecipitated proteins were eluted with 2×SDS loading buffer and analyzed by Western blotting as described above.

Immunocytofluorescence

Hepatocytes plated on coverslips were treated as described and then fixed with 2% (W/v) paraformaldehyde. Cells were then permeabilized with 0.1% Triton X, and blocked with 2% BSA in PBS with further blocking with whole mouse IgG. The samples were then incubated with primary antibody specific for TIFA (Abcam) and F-actin rhodamine phalloidin (Invitrogen, Eugene, OR). Samples were washed followed by incubation in the appropriate Cy3 (Invitrogen) and Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies. Samples were then incubated with Hoechst nuclear stain. The nuclear stain was removed and samples were washed before visualizing with Olympus Fluoview 1000 microscope.

Small interference RNA treatment and knockdown of TIFA

For transient knockdown experiments, hepatocytes were transfected with specific small interference RNA to TIFA (siTIFA) or control siRNA-A (Santa Cruz Biotechnology, Santa Cruz, CA). Described briefly, primary mouse hepatocytes were plated onto six-well plates. The TIFA and control siRNA duplexes were diluted to a final concentration of 10 μM in Opti-Mem (Invitrogen, San Diego, CA) and incubated with Lipofectamine 2000 transfection reagent (Invitrogen) at room temperature for 15 minutes. The mixture was then incubated with hepatocytes in serum and antibiotic-free conditions for 18 hours at 37°C. After the cells were washed twice with sterile PBS, they were incubated in William's E medium supplemented with 5% calf serum for up to 24 hours before exposure to hypoxia.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Following treatment of cells as described above, the cells were scraped into 1 mL PBS using a cell scraper. Cells were centrifuged, the cell pellet was resuspended in buffer A, and incubated on ice for 15 minutes before centrifuged for five minutes. Nuclei were washed once in buffer A. Nuclear proteins were extracted by gentle resuspension of the nuclei in buffer C together with buffer D added in a dropwise fashion. Samples were kept on ice for a further hour, followed by centrifugation and collection of the supernatants. Protein concentration was determined by BCA protein assay. Double-stranded nuclear Factor-κB (NF-κB) specific oligonucleotide was end-labeled by incubation with [γ-^{32}P]ATP using T4 polynucleotide kinase (U.S. Biochemicals, Cleveland, OH), and purified on a G-50 Sephadex column. Nuclear proteins were incubated with ^{32}P-labeled oligonucleotide for 45 minutes at room temperature. The DNA-protein complexes were resolved on a 4% nondenaturing polyarylamide gel in 0.5× Tris-borate-EDTA (TBE) buffer. The gels were dried using a vacuum-assisted gel-dryer and then subjected to autoradiography.

ELISA assay

HMGB1 concentrations in cell culture supernatants were detected by ELISA according to the manufacturers' instructions (IBL, Hamburg, Germany). All samples were assayed in duplicate.

Statistical Analyses

All statistical analyses were performed using SigmaStat or InStat software (GraphPad, San Diego, CA). Data are expressed as mean ± standard deviation (SD). Differences between groups were analyzed for statistical significance by Student's t-test or factorial ANOVA followed by a Fisher's LSD post hoc test. Significance was determined at the 95% confidence level ($p < 0.05$).

RESULTS

TIFA is up-regulated in mouse liver tissues by HS-R

We have previously characterized the changes in hepatic gene expression over six hours in the setting of hemorrhagic shock with bilateral femur fracture (HS/T). Among the 6,338 mRNAs showing significant change on Affymetrix and CodeLink gene arrays from baseline, the upregulation of TIFA was among the most dramatic. TIFA mRNA levels were found to increase over 10 fold by six hours (Figure 1A).

We first verified the gene array results by assessing TIFA mRNA and protein levels in the livers of mice subjected to HS-R. TIFA mRNA levels were found to increase up to 7.8 fold by the six hour time point (Figure 1B). Likewise, increases in TIFA protein levels could be detected by six hours in liver tissue following HS-R(Figure 1C). Thus, TIFA expression is markedly increased in the liver in the setting of HS-R.

TIFA expression increased in hypoxic hepatocytes in a TLR4- and MyD88- dependent manner

To determine if a similar response occurred in cultured hepatocytes, primary hepatocytes isolated from WT mice were exposed to hypoxia for two hours and reoxygenated for up to 24 hours. Figures 2A and 2B show that TIFA mRNA and protein levels were significantly increased in cell lysates at the six hour reoxygenation time point after hypoxia.

TIFA has been reported to be involved in cell signal transduction downstream of the Toll/IL-1R family and MyD88 complex upon IL-1 stimulation [14]. To examine whether TLR4 and MyD88-mediated hypoxia-reoxygenation induced TIFA expression, primary hepatocytes isolated from WT, TLR4−/− and MyD88−/− mice were treated with hypoxia for two hours followed by six hours of reoxygenation. Changes in TIFA protein levels were assayed with Western blotting and immunofluorescence. As expected, the expression of TIFA in response to hypoxia-reoxygenation was significantly upregulated in WT cells; however, TIFA was not increased in cell lysates from TLR4 −/− and MyD88−/− hepatocytes subjected to hypoxia-reoxygenation (Figures 2C and 2D).

Interactions between TIFA and TRAF6/IRAK-1

It has been shown that TIFA acts as an adapter protein that links TRAF6 to IRAK-1 upon IL-1 stimulation [14]. We next addressed whether TIFA interacts with TRAF6 and/or IRAK-1 during hypoxia-reoxygenation. The potential association between TIFA and TRAF6/IRAK-1 in mouse hepatocytes was evaluated by co-immunoprecipitation. Following the immunoprecipitation of TIFA, Western blotting of the precipitated fraction showed TIFA and TRAF6 were present in both control and hypoxia-reoxygenated samples, but interaction with IRAK-1 occurred only in hypoxia-reoxygenated samples. These results indicate that TIFA interacts with TRAF6 constitutively in cultured hepatocytes, whereas its association with IRAK-1 under hypoxia-reoxygenation occurs in a stimulation-dependent manner (Figure 3).

Involvement of the NF-κB pathway in TIFA-mediated HMGB1 release

To determine the functional relevance of TIFA in hypoxia-reoxygenation responses and explore the potential signaling pathway, we treated mouse hepatocytes with a TIFA specific siRNA (siTIFA) and then exposed samples to hypoxia-reoxygenation. Suppression of the expression of TIFA mRNA and protein was confirmed by qRT-PCR and Western blotting. Figure 4A and 4B shows siTIFA transfection led to a reduction in TIFA of up to 50 % after hypoxia-reoxygenation treatment.

It has been previously shown in transient transfection assays that TIFA activates NF-κB and JNK [14]. Furthermore, we have shown that hypoxia induced HMGB1 release from cultured hepatocytes in a TLR4 dependent manner [16]. Therefore, we determined whether TIFA was involved in hypoxia-reoxygenation-induced mitogen-activated protein kinase (MAPK) and NF-κB activation or HMGB1 release.

As expected, hypoxia-reoxygenation induced an increase in HMGB1 release into the culture supernatant (measured by ELISA, Figure 4C) and cellular HMGB1 levels (measured by Western blotting, Figure 4D). Suppression of TIFA levels by siRNA treatment prevented hypoxia-reoxygenation-induced increases in HMGB1 release and cellular content (Figures 4C, 4D).

We next examined the activation of IKK, IκBα, p38, ERK1/2, and JNK by Western blotting with phospho-specific antibodies to determine the role of the increases in TIFA levels on these signaling pathways. NF-κB activation, assessed by EMSA and IKK and IκBα phosphorylation showed a marked increase in NF-κB in control siRNA-treated hepatocytes exposed to hypoxia-reoxygenation (Figures 4E, 4F). Likewise, levels of phospho- p38, ERK1/2, and JNK were increased in control-transfected cells after hypoxia-reoxygenation (Figure 4F). However, the suppression of TIFA levels partially suppressed only hypoxia-reoxygenation-induced NF-κB activation and not MAPK phosphorylation. These data suggest that TIFA lies upstream of NF-κB activation but not MAPK activation in the setting of hypoxia-reoxygenation.

DISCUSSION

This study describes an important function for TIFA in hypoxia-reoxygenation and confirms the upregulation of TIFA in HS-R. Among 6,338 genes identified using the dual platform DNA microarray, TIFA was one of the mRNAs most dramatically increased in the early stages following HS and trauma in the livers of WT mice. The upregulation in HS-R of TIFA mRNA and protein expression was verified by qRT-PCR and Western blotting in independent *in vivo* experiments. To further explore the mechanisms of TIFA upregulation and function, we moved to an *in vitro* system of primary mouse hepatocytes. Again, TIFA mRNA and protein levels were significantly increased after hypoxia-reoxygenation. Additional experiments confirmed that hypoxia-reoxygenation-induced upregulation of TIFA is TLR4 and MyD88 dependent. Additionally, immunoprecipitation assay indicated that TIFA associated with TRAF6 constitutively, whereas it interacted with IRAK-1 only under hypoxia-reoxygenation. TIFA knockdown with siRNA shows that increases in TIFA contribute to the upregulation and release of HMGB1 in response to hypoxia-reoxygenation. TIFA also participates in NF-κB activation, but not MAPK activation. Our results indicate that TIFA is rapidly upregulated in a TLR4/MyD88-dependent manner by hypoxia-reoxygenation and that TIFA lies upstream of NF-κB activation and HMGB1 upregulation and release.

Our previously published DNA microarray study utilizing a murine model that incorporated peripheral trauma and HS-R was undertaken to define the early changes in gene expression in the liver, a central organ that uniquely integrates metabolic and immunologic responses to systemic insults [13]. To define only significant and reproducible changes, the study measured expression patterns using two separate microarray platforms (Affymetrix and CodeLin), followed by meta-analysis to identify highly concordant genes common to both platforms, which led to the identification of the potential HS/T associated gene TIFA [13]. Here, we confirm the upregulation of TIFA mRNA and protein by six hours in a model of HS-R alone without peripheral tissue injury, as well as in isolated hepatocytes under

hypoxia. These observations show that hypoxia is a potent stimulus for TIFA upregulation in liver cells.

TIFA was identified as a TRAF6-interacting protein in a yeast two-hybrid screen [14]. The same molecule also was isolated from an independent mammalian two-hybrid screen as a TRAF2-binding protein [17]. TRAFs are a family of proteins that participate in signal transduction of TNFR and IL-1R/TLR superfamilies [18–22]. TIFA activates NF-κB and JNK by linking TRAF6 to IRAK-1 in the IL-1 pathway [14]. TIFA had also recently been shown to be involved in lipopolysaccharide (LPS)-mediated signaling [23], suggesting that TIFA is part of a general response to proinflammatory stress. We found that mouse hepatocytes express little or no TIFA under normoxic conditions, but were significantly upregulated after hypoxia-reoxygenation treatment. The rapid upregulation of TIFA suggests that TIFA levels may normally be limiting for the signaling pathways involving TIFA and that its upregulation could be an important feed-forward mechanism.

It is well known that stimulation by TLR4 results in the recruitment of MyD88, MyD88-adaptor-like (Mal), and TIR domain-containing adaptor inducing IFN-β (TRIF) to the cytoplasmic domain of TLR4. MyD88/Mal recruits IRAK and the activated IRAK, then dissociates from the receptor complex, after which it associates with TRAF6, triggering the activation of several different pathways involving NF-κB and MAPKs [24, 25]. TIFA is thought to link IRAK-1 to TRAF6 upon stimulation [14]. Thus, TIFA may play a role in signaling from IL-1R and some members of the TLR family. To determine whether TLR4 and MyD88 mediated hypoxia-reoxygenation induced TIFA expression, we treated WT, TLR4−/−, and MyD88−/− primary hepatocytes with hypoxia-reoxygenation. As expected, hypoxia-reoxygenation induced significant increase of TIFA expression in WT cells; however, no marked changes of TIFA were observed in either TLR4 −/− or MyD88−/− hepatocytes. It has been shown that TIFA acts as an adapter protein that links TRAF6 to IRAK-1 upon IL-1 stimulation, but whether TIFA interacts with TRAF6 and/or IRAK-1 in the presence of hypoxia-reoxygenation has never been investigated. In the present study, the potential association between either TRAF6 or IRAK-1 and TIFA in hepatocytes was evaluated by co-immunoprecipitation. We found that the expression of TRAF6 corresponded with TIFA, indicating that TRAF6 interacts with TIFA constitutively. However, IRAK-1 only immunoprecipitated with TIFA after hypoxia-reoxygenation. These results indicate that TIFA interacts with IRAK-1 in a hypoxia-reoxygenation stimulation-dependent manner, and suggest that TIFA links IRAK-1 to TRAF6 following hypoxia-reoxygenation stimulation. TLR4 signaling assumes an important role in systemic inflammation after HS-R and subsequent hepatic injury, but the mechanism of TLR4 in HS-R-induced liver inflammation remains unclear [26–27]. Thus, our results suggest the upregulation and interaction of TIFA with TRAF6/IRAK-1 promotes TLR4/MyD88-TRAF6/IRAK-1/TIFA signaling as one of the mechanisms leading to signaling after HS-R.

To further study the functional relevance of TIFA in HS-R, we used siRNA to suppress the increase of TIFA and were able to demonstrate that siTIFA-transfected hepatocytes did not increase HMGB1 levels or release in response to hypoxia-reoxygenation. This suppression of HMGB1 release was associated with an inhibition of NF-κB activation. Yoshimura *et al.* [23] showed that an inhibitory TIFA-binding protein, ZCCHC11, interacted with TIFA after LPS treatment and suppressed TLR4-mediated TRAF6-dependent activation of NF-κB and cytokine production. Inoue *et al.* [21, 28] performed a homology search of human and mouse genome databases and identified a gene that encodes a protein with structural similarity to TIFA. This TIFA-related protein associated with TIFA and blocked TIFA-mediated activation of NF-κB and cell proliferation and maturation. Ea *et al.* [29] further confirmed that TIFA activates IKK by promoting oligomerization and ubiquitination of

TRAF6. Therefore, our results are consistent with a specific role for TIFA in NF-κB activation.

Data presented in this manuscript provide novel insights into the signaling pathways activated in hepatocytes during hypoxia-reoxygenation. Our findings indicate that TIFA is involved not only in IL-1 and LPS-induced signaling, as previously shown, but also in hypoxia-reoxygenation-activated pathways. Furthermore, this signaling includes a feed-forward mechanism resulting from an increase in TIFA gene expression. Thus, it is likely that TIFA upregulation is part of a general response to proinflammatory stress and warrants further study.

Acknowledgments

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Highlights

- The expression of TIFA upregulated in response to ischemia/hypoxia-reperfusion.
- Hypoxia-reoxygenation(H/R) induced TIFA upregulation was TLR4 and MyD88-dependent.
- TIFA associates with TRAF6 constitutively, but with IRAK1 only after H/R challenge.
- Suppression of TIFA reduced H/R-induced NF-κB activation and HMGB1 release.

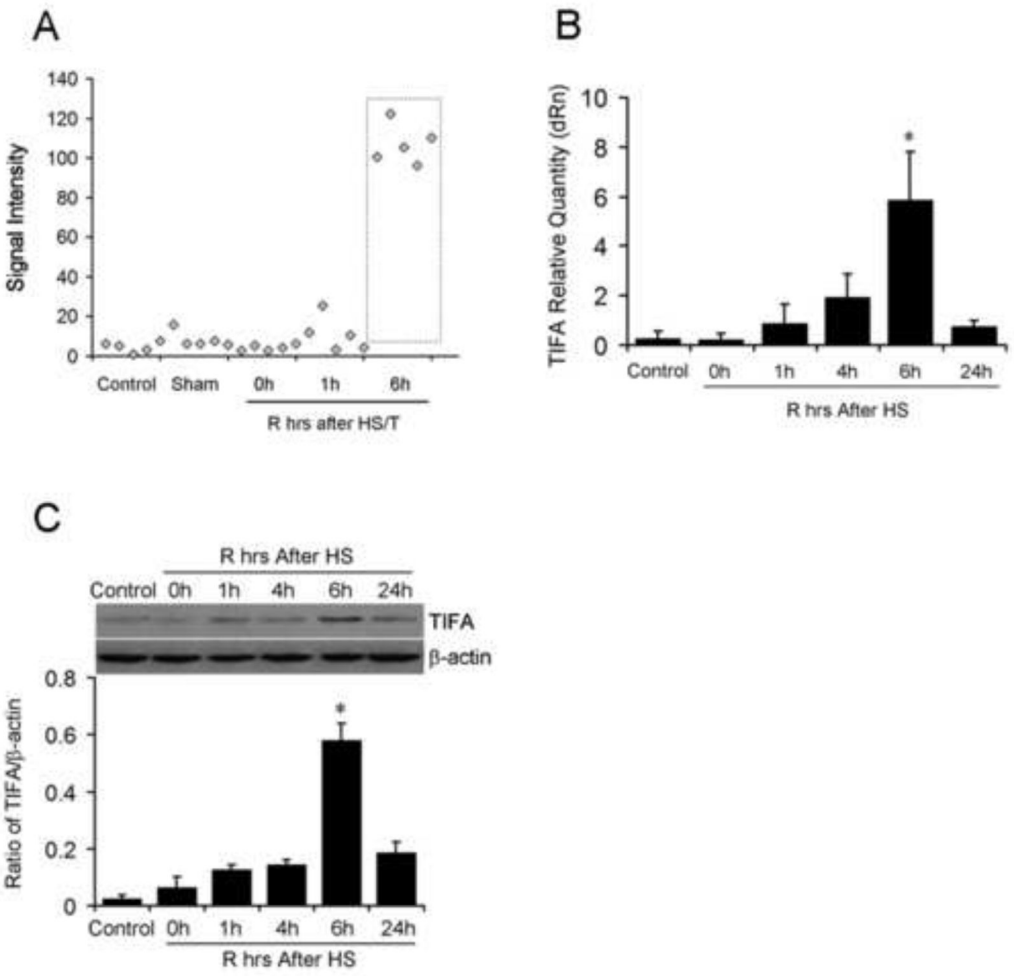

Fig. 1. TIFA is upregulated in mouse liver by HS-R

(A): Effect of HS/T and resuscitation on TIFA gene level in mouse liver, analyzed with Affymetrix and CodeLink microarray. (B): Effect of HS-R on TIFA mRNA expression in mouse liver, analyzed by qRT-PCR using primers for TIFA and β-actin (internal control). (C): Effect of HS-R on TIFA protein expression in mouse liver, analyzed by Western blotting and shown as the intensity ratio of TIFA to β-actin. Data expressed as mean ± SD, n= 5–6 samples/group. * $p < 0.05$ vs. control.

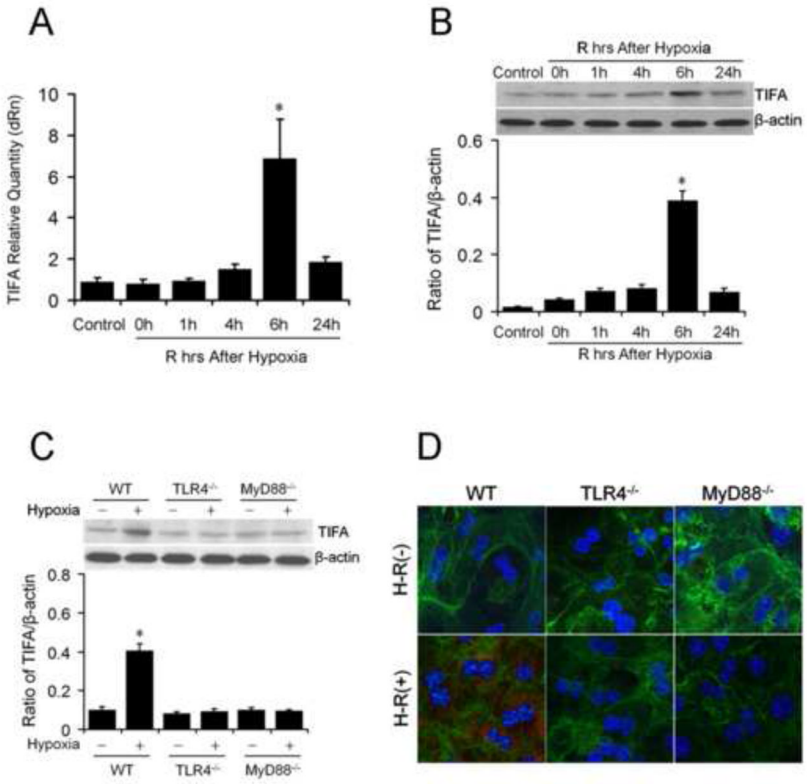

Fig. 2. Hypoxia-reoxygenation induced TIFA upregulation is TLR4 and MyD88-mediated

(A): Effect of hypoxia-reoxygenation on TIFA mRNA expression in hepatocytes, analyzed by qRT-PCR. (B): Effect of hypoxia-reoxygenation on TIFA protein expression in hepatocytes, analyzed by Western blotting. Primary hepatocytes isolated from WT, TLR4−/−, and MyD88−/− mice were treated with hypoxia-reoxygenation and the expression of TIFA protein was analyzed by Western blotting (C) or immunofluorescence (200× magnification) (D). Red, TIFA; green, actin; blue, Hoechst. H, hypoxia; R, reoxygenation. Data are expressed as mean ± SD, n= 5–6 samples/group. * $p < 0.05$ vs. control or hypoxia (−).

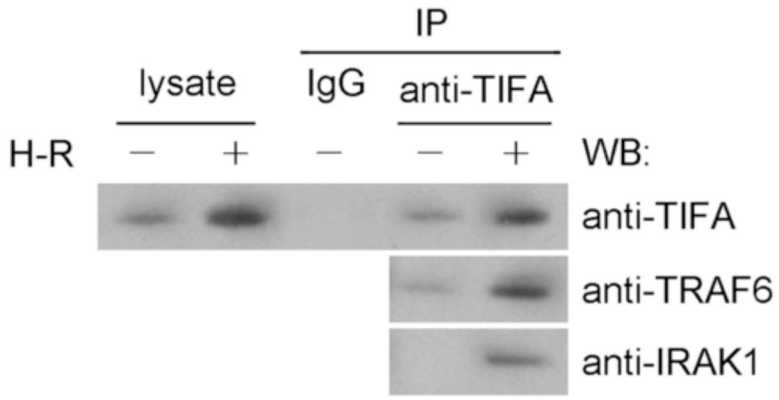

Fig. 3. TIFA associates with TRAF6 and IRAK-1 in mouse hepatocytes

Lysates isolated from mouse hepatocyte exposed to hypoxia-reoxygenation (+) or without hypoxia-reoxygenation (−) were immunoprecipitated (IP) in the presence of an anti-TIFA or control IgG antibody, followed by Western blotting (WB) with anti-TIFA, anti-TRAF6, or anti-IRAK-1 antibodies.

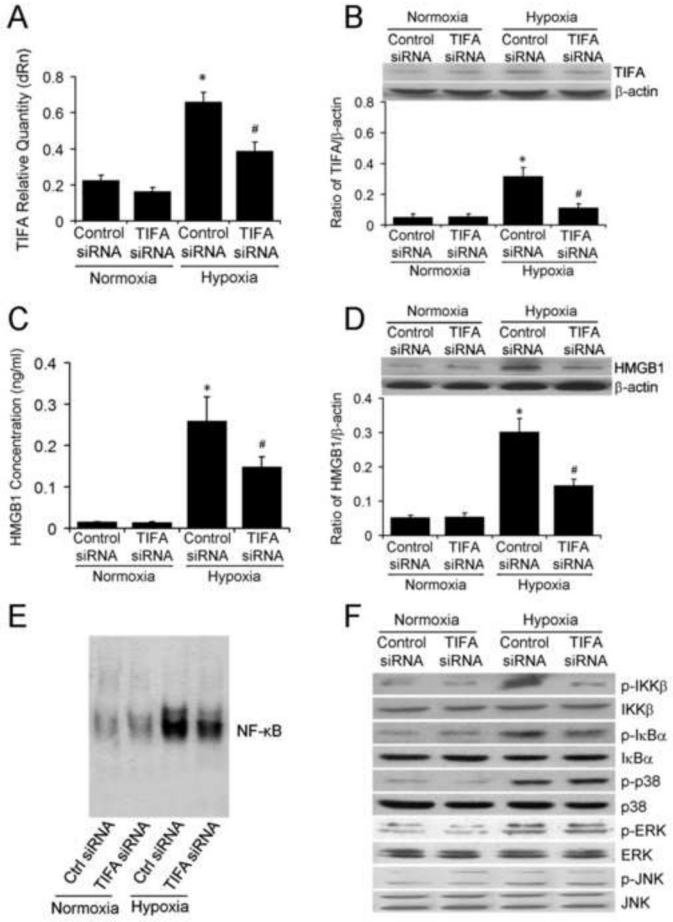

Fig. 4. NF-κB involved in TIFA-mediated HMGB1 expression and release

Hepatocytes were transient transfected with TIFA specific siRNA (siTIFA) and the expression of TIFA mRNA and protein were detected by qRT-PCR (A) and Western blotting (B). The effect of siTIFA transfection on HMGB1 expression and release in hepatocytes in response to hypoxia-reoxygenation were analyzed by ELISA in cell culture supernatant (C) and by Western blotting in cell lysates (D). The effect of siTIFA transfection on NF-κB activation in hepatocytes in response to hypoxia-reoxygenation was detected by EMSA (E). The effect of siTIFA transfection on IKK, IκBα and MAPKs activation in hepatocytes in response to hypoxia-reoxygenation was detected by Western blotting (F). Data are expressed as mean ± SD, n= 5–6 samples/group. * $p < 0.05$ vs. normoxia, # $p < 0.05$ vs. control siRNA.

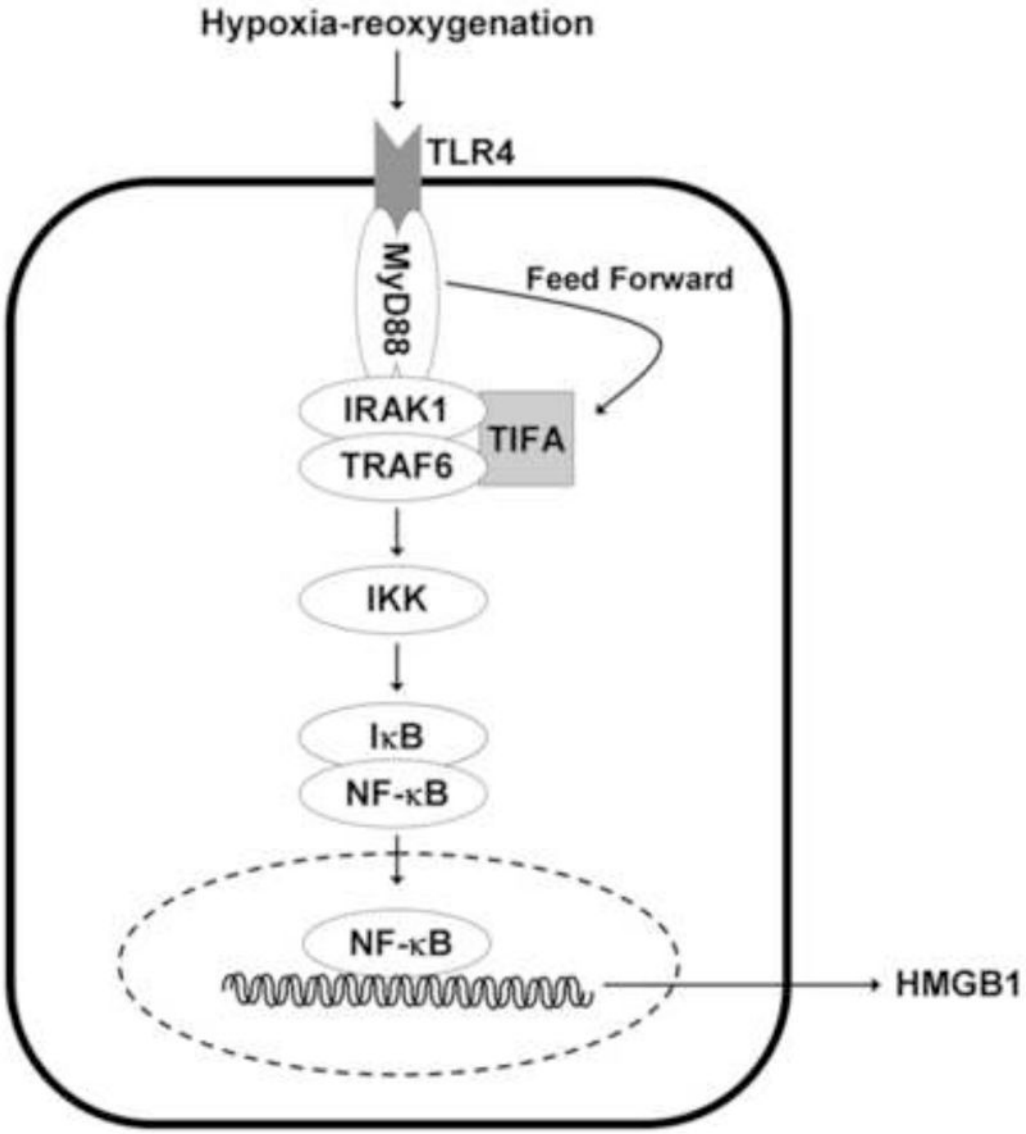

Fig. 5.
Proposed schematic model for TIFA signaling in response to hypoxia-reoxygenation