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Ron receptor-dependent gene regulation in a murine model of endotoxin-induced acute liver failure

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Abstract

Background—Prior experimentation has shown that loss of the tyrosine kinase (TK) signaling domain of the Ron receptor leads to marked hepatocyte protection in a model of lipopolysaccharide (LPS)-induced acute liver failure (ALF) in D-galactosamine (GalN)-sensitized mice.

Methods—To elucidate the role of Ron in the regulation of hepatic gene expression, microarray analyses were performed on liver RNA isolated temporally from wild type (WT) and TK^{−/−} mice during the progression of ALF. Gene array data was validated using Western analyses and immunohistochemistry analyses as well as by *ex vivo* culture systems.

Results—At baseline, 101 genes were differentially expressed between WT and TK^{−/−} livers, which regulated processes involved in hypoxia, proliferation, apoptosis and metabolism. One hour after ALF induction, WT livers exhibited increased cytokine expression compared to TK^{−/−} livers and after 4 hours, an induction of suppressor of cytokine signaling (SOCS) genes as well as JAK-STAT pathway activation were prominent in TK^{−/−} livers compared to controls.

Conclusions—In conclusion, our studies suggest a novel hepato-protective mechanism in the Ron TK^{−/−} mice wherein increased and sustained SOCS production and JAK-STAT activation in the hepatocyte may inhibit the destructive proinflammatory milieu and promote survival factors which blunt hepatic death and ensuing development of ALF.

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Keywords

Mst1R; suppressors of cytokine signaling; Met Receptor; Hepatocyte growth factor-like protein

INTRODUCTION

Acute liver failure (ALF) affects approximately 2500 people in the United States annually with mortality rates for these individuals being as high as 80% without a successful liver transplant⁽¹⁾. The most common causes of ALF include drug toxicity (e.g. acetaminophen and idiosyncratic reactions) indeterminate liver disease, and viral hepatitis (e.g. Hepatitis A and B)⁽¹⁾. Liver failure is heavily dependent on cytokine signaling and inflammation⁽²⁾. The major cell type mediating the production and release of pro- and anti-inflammatory cytokines and chemokines are the resident liver macrophages (Kupffer cells)^(3–6). Although ALF has been studied for many years, little is known about the regulation of genes involved in the initial steps of promoting liver dysfunction and hepatocyte death.

The Ron receptor tyrosine kinase is a single-spanning membrane bound heterodimeric glycoprotein that is expressed in most epithelial cells and on tissue resident macrophages^(7, 8). Ron is a member of the Met family and has been implicated in a variety of biological processes including cancer progression and inflammation^(7–14). The ligand for Ron is the hepatocyte growth factor-like (HGFL) protein, also known as macrophage stimulating protein^(15–17). HGFL binding induces Ron activation and the stimulation of downstream secondary messengers, which culminate to regulate changes in gene transcription^(7, 8).

Mice lacking the tyrosine kinase (TK) signaling domain of Ron, referred to as Ron TK^{–/–} mice⁽¹⁸⁾, are viable and have been utilized to study Ron function *in vivo*. In a model of ALF induced by LPS and D-Galactosamine (GalN), Ron TK^{–/–} mice displayed a hepatoprotective phenotype compared to controls⁽¹⁰⁾. In this model, the TK^{–/–} mice displayed mild aminotransferase elevation, minimal hepatocyte apoptosis and relatively normal liver histology in contrast to WT mice. Recent studies have further documented important cell-type specific roles for Ron in regulating both Kupffer cell-dependent cytokine production and in inhibiting hepatocyte survival after injury⁽¹⁹⁾.

The goal of the present study was to identify Ron-dependent genes involved in the progression of ALF. To accomplish this, RNA was temporally isolated from the livers of WT and Ron TK^{–/–} mice after LPS/GalN treatment. Microarray assays were then utilized to define the genes that were altered between the genotypes during the progression of ALF. Our studies show that the expression of suppressors of cytokine signaling (SOCS) genes that regulate cytokine secretion, as well as the JAK-STAT signaling pathway were increased in the TK^{–/–} livers compared to controls. In total, our analyses point to a potentially important mechanism by which Ron signaling influences the production of and response to cytokines during the progression of ALF.

METHODS

Mice and Endotoxin-Induced Acute Liver Failure

Mice containing a germline deletion of the Ron tyrosine kinase signaling domain (TK^{-/-} mice) have been previously described and were backcrossed onto a C57BL6 genetic background^(18, 19). Wild type (WT) and TK^{-/-} mice were generated from heterozygous TK^{+/-} crosses and then intercrossed to produce the experimental (TK^{-/-}) and control (WT) mice. Male mice from 8 to 11 weeks were utilized. Acute liver failure was induced by intraperitoneal injection of lipopolysaccharide (LPS, 27ng/gm body wt, Sigma Chemical, St. Louis MO) and D-galactosamine (GalN, 1mg/gm body wt, Sigma Chemical, St. Louis MO) as previously described and animals were sacrificed at the time points indicated⁽¹⁰⁾.

RNA Extraction and Microarray Analysis

Total RNA was isolated from liver tissue using TRIzol reagent (Invitrogen, Carlsbad, CA) as previously described⁽¹⁹⁾. RNA samples from two livers per genotype per time point were submitted to the Cincinnati Children's Hospital Medical Center Affymetrix Core (Cincinnati, OH). RNA quality was assessed and quantified using an Agilent Bioanalyzer 2100 (Hewlett Packard, Palo Alto, CA). 400–500ng of total RNA per sample was used in the TargetAmp 1-Round Aminoallyl-aRNA amplification kit (Epicentre Biotechnologies, Madison, WI) to generate cRNA following the manufacturer's instructions. Biotin-X-X-NHS (Epicentre Biotechnologies, Madison, WI) was used to label the aminoallyl-aRNA with biotin. The biotin-labeled cRNA target was then chemically fragmented and hybridized to the Affymetrix MOE-430 GeneChip array containing approximately 39000 targets. The probe arrays were scanned using the Affymetrix GeneChip Scanner 3000 and GeneChip Operating Software 1v4 (Affymetrix, Santa Clara, CA).

Data Analysis

Changes in gene expression levels from the microarray signals were determined using algorithms in the Microarray Analysis Suite (Affymetrix, Santa Clara, CA) and GeneSpring GX 7.3.1 software (Silicon Genetics, Redwood City, CA). Gene lists from the microarray data were obtained based on 1.5 fold expression difference using the Welch T Test and 2-tailed Student's t test (with or without Benjamini and Hochberg false discovery rate with FDR (P < 0.01)). Correlation of gene expression with numeric parameters was assessed using the Pearson correlation test with P value. Lists were filtered based on fold change and P value. Statistical significance among groups were determined using an unpaired 2-tail Welch T test or Mann-Whitney U test with Bonferroni correction. Gene clustering analyses were performed using GeneSpring GX 7.3 and K-means algorithms. The gene lists generated from the Gene Spring analyses were uploaded onto the DAVID Bioinformatics Resources 2008 National Institute of Allergy and Infectious Diseases, NIH website (<http://david.abcc.ncifcrf.gov/>) and pathway and functional annotation analyses were performed. The gene lists for each Figure are contained in the supplemental information.

Kupffer Cell and Hepatocyte Isolation and Culture

Primary Kupffer cells and hepatocytes were isolated as previously described⁽¹⁹⁾. Kupffer cells were treated with bacterial LPS (500 ng/ml) and hepatocytes were pretreated with 200ng/ml of Actinomycin D (ActD) followed by TNF- α (5ng/ml) (R&D Systems, Minneapolis) for the times indicated⁽¹⁹⁾.

Quantitative Real Time PCR

RNA isolation and cDNA preparation was performed as described previously⁽¹⁹⁾. Quantitative Real time (qRT-)PCR was performed using FastStart SYBR Green with an ABI 7900HT machine (Applied Biosystems, Foster City, CA). Expression levels were normalized to beta-glucuronidase (GusB) as internal control. The experiments were repeated twice with Kupffer cells pooled from 2 mice per isolation. Relative gene expression results are reported. The primers used were: GusB (5'-TTGAGAACTGGTATAAGACGCATCAG-3'; 5'-TCTGGTACTCCTCACTGAACATGC-3'), and SOCS3 (5'-TGGGCAGTGGGAGTGGTTATTTCT-3'; 5'-ACTTTCCCGCTGGAACTTGTTTGC-3').

Western Analyses and Immunohistochemistry (IHC)

Western analyses were performed on the liver tissues and isolated hepatocytes as described previously⁽¹⁹⁾. The Western membranes from three independent experiments were quantified using ImageQuant software (GE Healthcare, Piscataway, NJ). IHC analyses were performed on liver tissue sections as described previously⁽¹⁹⁾. The primary antibodies were phospho(p)- and total JAK2 (Millipore, Billerica, MA), p- and total STAT-3 (Cell Signaling Technology, Danvers MA) and Actin⁽¹⁹⁾.

RESULTS

We have previously shown that the absence of the Ron receptor tyrosine kinase diminished progression of acute liver failure (ALF) in mice; this finding was associated with dysregulation of cytokine production and decreasing hepatocyte death through Ron regulation in both Kupffer cells and hepatocytes respectively^(10, 19). Therefore, we hypothesized that Ron signaling would have a significant impact on the expression pattern of inflammatory and survival genes in the livers of injured mice. In this study, we proceeded to define the dysregulated gene expression profiles, which potentially lead to the alteration of liver pathobiology between Ron deficient (TK-/-) and wild type (WT) animals.

Six Ron TK-/- and six WT control mice were injected with lipopolysaccharide (LPS) and D-galactosamine (GalN), a proven liver-toxic regimen that initiates ALF in rodents^(3, 10, 20, 21). Two animals per genotype were sacrificed at time points of 0 hours (hr), 1 hr and 4 hrs post-injection. Liver RNA was isolated and subjected to microarray analyses to identify differentially expressed genes between WT and TK-/- livers basally and during the progression of ALF. We identified 101 genes that were differentially expressed between genotypes basally (Figure 1A). Web-based DAVID gene function organizing tool⁽²²⁾ allowed grouping of most of these 101 genes into 45 overlapping biological process categories. Five categories from this list include response to hypoxia, cell proliferation,

transcription, apoptosis and metabolism that account for two thirds of the identified genes (Figure 1B). Representative genes within these categories and their relative fold changes are indicated (Figure 1C).

Two important components of the EGF signaling pathway were altered basally in the TK^{-/-} livers compared to WT livers. The epidermal growth factor receptor (EGFR) and the janus kinase 1 (JAK1) genes were upregulated 2.5 fold and 1.8 fold respectively in TK^{-/-} livers. These pathways have been shown to influence cytokine and chemokine levels, thereby promoting as well as diminishing inflammatory responses^(23, 24). One of the most highly dysregulated genes basally was B-cell leukemia/lymphoma 6 (Bcl6), which was expressed about 5-fold more in Ron TK^{-/-} livers than in WT livers (Figure 1C). The Bcl6 gene encodes for a POZ/zinc finger transcriptional repressor that is ubiquitously expressed in adult murine tissue and negatively regulates the expression of select cytokines⁽²⁵⁾. Recent studies have shown that Bcl6 expression in pancreatic B-cells reduces cytokine induced-inflammation, Fas and iNOS expression as well as NO production, which protects against cytokine-induced apoptosis⁽²⁶⁾. Similarly, higher Bcl6 levels in TK^{-/-} liver may protect hepatocytes from cytokine-induced apoptosis in this model of LPS/GalN-induced ALF.

Phosphatidylinositol 3-kinase (PI3K) is a key downstream mediator of many receptor tyrosine kinase signaling pathways. The alpha subunit of hypoxia inducible factor-1 (HIF-1alpha), one of the downstream targets of the PI3K pathway⁽²⁷⁾, was basally high in Ron TK^{-/-} deficient livers (Figure 1C). HIF-1 transcription factor consists of alpha and beta subunits but only the alpha subunit's expression is regulated whereas the beta subunit is constitutively expressed^(27, 28). HIF-1 encodes genes that respond to hypoxia. Three important functional categories of HIF-1α targets include cell proliferation, cell survival and apoptosis^(27, 28). It is possible that high basal HIF-1alpha in the TK^{-/-} livers offers protection from ALF.

Upon LPS stimulation, Kupffer cells release a variety of inflammatory mediators including IL-6, IL-10, IFN-γ and TNF-α^(5, 6, 29). Correspondingly, the transcriptional levels of various cytokine and chemokine genes changed in the livers of WT and TK^{-/-} livers 1 hour after LPS/GalN injection (Table 1). The most highly upregulated cytokine gene at 1 hr was IL-6, showing nearly a 40-fold increase in mRNA levels in WT and TK^{-/-} livers. IL-6 is one of the most prevalent inflammatory cytokines and mediates a multitude of effects in the cell including cell proliferation, cell survival and apoptosis^(30, 31). However, the upregulation of IL-6 was very short-lived in WT and TK^{-/-} livers. From 1 hr to 4 hrs, the expression of IL-6 returned to near baseline levels (Table 1). Another cytokine gene that was upregulated 1 hour after LPS/GalN treatment was TNF-α. TNF-α is required for the proper function of T cells, B cells, NK cells as well as macrophages including Kupffer cells⁽³²⁾. TNF-α was upregulated 2.5 fold in WT livers compared to 1.9 fold in TK^{-/-} livers (Table 1), but returned to baseline levels after 4 hrs in both genotypes. CXCL-2 (MIP-2) and CCL-3 (MIP-1alpha), chemokines involved in immune cell trafficking and function, were strongly expressed at 1 hour after ALF induction and the levels of each were approximately 2-fold higher in WT livers compared to TK^{-/-} livers (Table 1). From 1 to 4 hours after ALF induction, CCL-3 level was not changed in WT livers, but was increased 1.8-fold in TK^{-/-} livers.

In addition to cytokines, dramatic alterations in gene expression profiles of transcriptional regulators were observed between WT and TK^{-/-} livers (Table 1). The expression of Jun oncogene was upregulated 2.8-fold in the TK^{-/-} livers compared to a 2.8-fold down regulation in WT livers 1 hour after ALF induction. The expression of Jun oncogene is controlled by cytokines and it also acts as downstream mediator of cytokine signaling and controls the induction of suppressor of cytokine signaling (SOCS) genes^(33, 34). Overall, 312 genes were differentially expressed (Figure 2A, 2B) while 83 genes were altered to the same extent (Figure 2C, 2D) in WT and TK^{-/-} livers. Utilizing K-means algorithms, the 312 differentially expressed genes were clustered into 6 groups (Figure 2B, 2D). Similar clustering was performed on the 83 genes regulated similarly in both genotypes. Amongst the differentially regulated gene clusters, some exhibited increased expression in the WT livers, but decreased expression in the TK^{-/-} livers, some showed decreased expression in the WT livers, but showed increased expression in the TK^{-/-} livers, while others either showed increased or decreased expression in the genotypes, albeit to different extents, 1 hr after ALF induction (Figure 2B, 2D). A completed listing of all gene sets in this report is available as supplementary material.

In our model, many cytokines, including interleukins such as IL-6, were highly upregulated within the first 60 minutes. These cytokines signal through the common janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, which can be negatively regulated by SOCS proteins⁽³⁵⁾. SOCS gene expression is induced by various cytokines in a classical negative feedback loop. This inhibition occurs at the base of the signaling receptor by direct binding to proteins such as JAK2⁽³⁶⁾. Further, the SOCS gene family members also inhibit the production of LPS-induced chemokines and cytokines⁽³⁷⁾. As depicted in Figure 3, upregulation of SOCS genes was observed in the livers of WT and TK^{-/-} mice within 1 hour after LPS/GalN exposure. In this time frame, SOCS1 was unchanged in WT animals, whereas SOCS2 and SOCS3 expression were upregulated 2.7 and 2.2 fold, respectively (Figure 3A). In livers of TK^{-/-} mice, both SOCS1 and SOCS2 were unchanged while SOCS3 expression was upregulated by 2.9 fold. However, from 1hr to 4 hrs of ALF induction, there was a significant difference in the expression levels of the SOCS 1, 2 and 3 genes between different genotypes (Figure 3B). All three SOCS genes were upregulated in TK^{-/-} livers, while only SOCS1 was upregulated in WT livers. In TK^{-/-} livers, SOCS1, SOCS2 and SOCS3 expressions were increased by 3.5 fold, 2.7 fold and 1.9 fold respectively. The study of SOCS genes may provide a mechanistic rationale for the protected phenotype observed in the TK^{-/-} livers. Increased levels of all 3 SOCS genes may downregulate cytokine signaling pathways in TK^{-/-} hepatocytes leading to a hepatoprotective phenotype in the Ron TK^{-/-} mice.

To validate the microarray findings, Western analyses were performed on whole livers from WT and TK^{-/-} mice 4 hours after LPS/GalN treatment. As shown in Figure 4A and 4B, there is a significant increase of JAK2 level in the livers of TK^{-/-} mice compared to WT mice. Immunohistochemical (IHC) staining was performed on liver sections from LPS/GalN treated mice 4 hours after ALF induction. The TK^{-/-} livers showed stronger hepatocyte JAK2 staining compared to WT livers (Figure 4C). IHC analyses also demonstrated increased nuclear STAT3 staining in TK^{-/-} hepatocytes compared to the controls (Figure 4C). To examine the impact of selective Ron loss in hepatocytes, primary hepatocytes were

isolated from WT and TK^{-/-} livers. Primary hepatocytes were subsequently treated *ex vivo* with actinomycin D (ActD) and TNF- α , to induce an ALF response⁽¹⁹⁾, and cell lysates were isolated at the time points noted in Figure 4D. The increasing phosphorylation and total expression levels of JAK2 were analyzed by Western blot in the TK^{-/-} hepatocytes compared to controls (Figure 4D).

To directly test if Ron activation regulates SOCS expression, Kupffer cells from WT and TK^{-/-} mice were isolated and stimulated with LPS *ex vivo*. As depicted in Figure 5, TK^{-/-} Kupffer cells exhibited exaggerated SOCS3 expression with no differences observed between genotypes in the absence of LPS. In total, our studies suggest a novel hepatoprotective mechanism in TK^{-/-} mice after LPS/GalN induced liver failure. We propose that increased and/or temporally altered activation of the JAK-STAT-SOCS pathway in TK^{-/-} hepatocytes promotes their survival and increased SOCS in TK^{-/-} Kupffer cells blunts the production of an inflammatory cytokine milieu.

DISCUSSION

In the present study, we investigated the transcriptional regulation of genes induced in the liver of wild type and Ron TK^{-/-} mice during progression of ALF modeled by endotoxin exposure. Microarray analyses were performed to define gene expression changes in any of the genes within the mouse genome. Our studies identified genes that were differentially expressed basally between WT and TK^{-/-} livers as well as genes which were differentially regulated by Ron receptor signaling during ALF. Under basal conditions, 101 genes were different in Ron replete and Ron deficient livers. Genes that are basally regulated by Ron in the liver fall into 5 biological processes relating to the response to hypoxia, secretion, cell proliferation, cell death, and metabolism. The differential gene expression in these categories may be important for determining the response of the liver during injury.

Within 1 hour of exposure to LPS/GalN treatment, significant differential expression of 312 genes were noted in the livers of WT and TK^{-/-} mice, while a number of other genes were also identified which are modulated during ALF but did not change between genotypes. Of the genes induced during early injury, numerous cytokine and chemokine gene expressions were dysregulated to varying degrees. Dramatic upregulation of IL-6 was observed in both genotypes while the levels of CXCL2 (MIP-2) and CCL-3 (MIP-1 α) were more highly (2- fold) expressed in WT livers versus TK^{-/-} livers. Increasing levels of CXCL-2 and CCL-3 may help attract leukocytes contributing to the hepatic inflammatory environment^(38, 39). This data is consistent with our previous observation showing that WT livers display increased neutrophil recruitment compared to TK^{-/-} livers⁽¹⁰⁾. Although recruitment of leukocytes is a key component of immune response and host-defense mechanism, in various disease conditions including endotoxemia, the activation and infiltration of leukocytes may also be a factor leading to increased tissue damage in WT livers⁽⁴⁰⁾.

Three members of the SOCS gene family (SOCS1, SOCS2 and SOCS3) were differentially regulated in the WT and TK^{-/-} livers. SOCS proteins are induced by cytokines and act in a negative feedback loop to inhibit cytokine gene expression. SOCS1, 2 and 3 exhibited

increased and prolonged expression in the TK^{-/-} livers compared to controls. Given the high expression of IL-6R in hepatocytes⁽⁴¹⁾, it is interesting to speculate that IL-6 signaling varies between the WT and Ron TK^{-/-} livers. Elevated IL-6 during ALF in Ron TK^{-/-} livers leads to increases in SOCS expression blocking the deleterious effects of cytokine signaling in the TK^{-/-} hepatocyte.

Analyses of liver tissues showed that the JAK2-STAT3 pathway was hyperactivated in the TK^{-/-} livers compared to control livers. Further, recent studies in mice have shown that the JAK2/STAT3 pathway initiates survival signals and that the activation of this pathway in hepatocytes is essential to protect these cells from LPS/GalN-induced apoptosis⁽⁴²⁾. SOCS proteins, by themselves, also activate survival/anti-apoptotic pathways in various cell types⁽³⁵⁾.

In the liver, Kupffer cells are the major cell types responding to LPS resulting in a robust increase in cytokine chemokine secretion^(5, 6, 29). LPS binds to and activates toll like receptor (TLR-4) initiating a signaling cascade that results in NF- κ B phosphorylation and activation of cytokine chemokine secretion^(5, 6, 29). SOCS3 has been shown to inhibit LPS-induced cytokine chemokine secretion by interacting with the TLR-4 pathway^(43, 44). SOCS3 is upregulated by LPS in macrophages and acts in a negative feedback loop to attenuate inflammatory responses. Prior studies have shown that intracellular delivery of a recombinant cell-penetrating form of SOCS3 protein in different murine models of ALF, including the LPS/GalN model, significantly increased survival and decreased inflammatory responses with reduction in hepatic hemorrhage and necrosis⁽⁴⁵⁾. Similarly, we speculate that over expressing SOCS3 specifically in the WT hepatocytes may make them resistant to LPS/GalN induced death, similar to the TK^{-/-} hepatocytes. Increasing SOCS3 in the WT Kupffer cells may similarly decrease the high levels of cytokines observed after LPS/GalN treatment. Increased levels of SOCS3 seen in TK^{-/-} Kupffer cells over time may alter the nature of the inflammatory cytokine/chemokine milieu, making it less toxic. Based on this combined data, Figure 6 outlines a proposed model for the role of Ron signaling in regulating ALF. In this model, the Kupffer cell activation (through LPS stimulation) leads to the production of cytokines and chemokines. In Kupffer cells, loss of Ron signaling may lead to increasing SOCS expression, which may alter cytokine gene expression. Upregulation of the JAK2/STAT3 signaling pathway in TK^{-/-} hepatocytes alters the response of these cells to the cytokine and chemokine milieu. Hyperactivation of the JAK2/STAT3 pathway, along with increases in pro-survival genes such as Bcl6 in TK^{-/-} livers may confer hepato-protective effects after LPS/GalN-induced ALF. This implies that Ron receptor signaling in Kupffer cells, as well as hepatocytes, regulates the expression of SOCS genes. Further, SOCS induction in Kupffer cells and hepatocytes in TK^{-/-} livers, plays a role in resistance to ALF. In summary, this report has identified a novel signaling mechanism by which the Ron receptor regulates ALF progression that may be utilized to design targeted therapeutic interventions for decreasing the morbidity and mortality associated with this disease.

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References

1. Stravitz RT, Kramer DJ. Management of acute liver failure. *Nat Rev Gastroenterol Hepatol*. 2009; 6:542–553. [PubMed: 19652652]
2. Igonin AA, Armstrong VW, Shipkova M, Lazareva NB, Kukes VG, Oellerich M. Circulating cytokines as markers of systemic inflammatory response in severe community-acquired pneumonia. *Clin Biochem*. 2004; 37:204–209. [PubMed: 14972642]
3. Galanos C, Freudenberg MA, Reutter W. Galactosamine-induced sensitization to the lethal effects of endotoxin. *Proc Natl Acad Sci U S A*. 1979; 76:5939–5943. [PubMed: 293694]
4. Hishinuma I, Nagakawa J, Hirota K, Miyamoto K, Tsukidate K, Yamanaka T, et al. Involvement of tumor necrosis factor-alpha in development of hepatic injury in galactosamine-sensitized mice. *Hepatology*. 1990; 12:1187–1191. [PubMed: 2227817]
5. Jirillo E, Caccavo D, Magrone T, Piccigallo E, Amati L, Lembo A, et al. The role of the liver in the response to LPS: experimental and clinical findings. *J Endotoxin Res*. 2002; 8:319–327. [PubMed: 12537690]
6. Seki E, Brenner DA. Toll-like receptors and adaptor molecules in liver disease: update. *Hepatology*. 2008; 48:322–335. [PubMed: 18506843]
7. Leonis MA, Thobe MN, Waltz SE. Ron-receptor tyrosine kinase in tumorigenesis and metastasis. *Future Oncol*. 2007; 3:441–448. [PubMed: 17661719]
8. Wagh PK, Peace BE, Waltz SE. Met-related receptor tyrosine kinase Ron in tumor growth and metastasis. *Adv Cancer Res*. 2008; 100:1–33. [PubMed: 18620091]
9. Caldwell CC, Martignoni A, Leonis MA, Ondiveeran HK, Fox-Robichaud AE, Waltz SE. Ron receptor tyrosine kinase-dependent hepatic neutrophil recruitment and survival benefit in a murine model of bacterial peritonitis. *Crit Care Med*. 2008; 36:1585–1593. [PubMed: 18434891]
10. Leonis MA, Toney-Earley K, Degen SJ, Waltz SE. Deletion of the Ron receptor tyrosine kinase domain in mice provides protection from endotoxin-induced acute liver failure. *Hepatology*. 2002; 36:1053–1060. [PubMed: 12395314]
11. Mallakin A, Kutcher LW, McDowell SA, Kong S, Schuster R, Lentsch AB, et al. Gene expression profiles of Mst1r-deficient mice during nickel-induced acute lung injury. *Am J Respir Cell Mol Biol*. 2006; 34:15–27. [PubMed: 16166746]
12. McDowell SA, Mallakin A, Bachurski CJ, Toney-Earley K, Prows DR, Bruno T, et al. The role of the receptor tyrosine kinase Ron in nickel-induced acute lung injury. *Am J Respir Cell Mol Biol*. 2002; 26:99–104. [PubMed: 11751209]
13. Tsutsui S, Noorbakhsh F, Sullivan A, Henderson AJ, Warren K, Toney-Earley K, et al. RON-regulated innate immunity is protective in an animal model of multiple sclerosis. *Ann Neurol*. 2005; 57:883–895. [PubMed: 15929040]
14. Wetzel CC, Leonis MA, Dent A, Olson MA, Longmeier AM, Ney PA, et al. Short-form Ron receptor is required for normal IFN-gamma production in concanavalin A-induced acute liver injury. *Am J Physiol Gastrointest Liver Physiol*. 2007; 292:G253–261. [PubMed: 17008558]
15. Bezerra JA, Witte DP, Aronow BJ, Degen SJ. Hepatocyte-specific expression of the mouse hepatocyte growth factor-like protein. *Hepatology*. 1993; 18:394–399. [PubMed: 8340069]
16. Gaudino G, Follenzi A, Naldini L, Collesi C, Santoro M, Gallo KA, et al. RON is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP. *Embo J*. 1994; 13:3524–3532. [PubMed: 8062829]
17. Wang MH, Ronsin C, Gesnel MC, Coupey L, Skeel A, Leonard EJ, et al. Identification of the ron gene product as the receptor for the human macrophage stimulating protein. *Science*. 1994; 266:117–119. [PubMed: 7939629]

18. Waltz SE, Eaton L, Toney-Earley K, Hess KA, Peace BE, Ihlendorf JR, et al. Ron-mediated cytoplasmic signaling is dispensable for viability but is required to limit inflammatory responses. *J Clin Invest.* 2001; 108:567–576. [PubMed: 11518730]
19. Stuart WD, Kulkarni RM, Gray JK, Vasilias J, Leonis MA, Waltz SE. Ron receptor regulates Kupffer cell-dependent cytokine production and hepatocyte survival following endotoxin exposure in mice. *Hepatology.* 2011; 53:1618–1628. [PubMed: 21520175]
20. Decker K, Keppler D. Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. *Rev Physiol Biochem Pharmacol.* 1974;77–106. [PubMed: 4375846]
21. Konishi Y, Shinozuka H, Farber JL. The inhibition of rat liver nuclear ribonucleic acid synthesis by galactosamine and its reversal by uridine. *Lab Invest.* 1974; 30:751–756. [PubMed: 4835601]
22. Delker DA, Geter DR, Roop BC, Ward WO, Ahlborn GJ, Allen JW, et al. Oncogene expression profiles in K6/ODC mouse skin and papillomas following a chronic exposure to monomethylarsonous acid. *J Biochem Mol Toxicol.* 2009; 23:406–418. [PubMed: 20024957]
23. Hamilton LM, Torres-Lozano C, Puddicombe SM, Richter A, Kimber I, Dearman RJ, et al. The role of the epidermal growth factor receptor in sustaining neutrophil inflammation in severe asthma. *Clin Exp Allergy.* 2003; 33:233–240. [PubMed: 12580917]
24. Rocourt DV, Mehta VB, Besner GE. Heparin-binding EGF-like growth factor decreases inflammatory cytokine expression after intestinal ischemia/reperfusion injury. *J Surg Res.* 2007; 139:269–273. [PubMed: 17291530]
25. Jardin F, Ruminy P, Bastard C, Tilly H. The BCL6 proto-oncogene: a leading role during germinal center development and lymphomagenesis. *Pathol Biol (Paris).* 2007; 55:73–83. [PubMed: 16815642]
26. Igoillo-Esteve M, Gurzov EN, Eizirik DL, Cnop M. The transcription factor B-cell lymphoma (BCL)-6 modulates pancreatic {beta}-cell inflammatory responses. *Endocrinology.* 2011; 152:447–456. [PubMed: 21190961]
27. Hellwig-Burgel T, Stiehl DP, Wagner AE, Metzen E, Jelkmann W. Review: hypoxia-inducible factor-1 (HIF-1): a novel transcription factor in immune reactions. *J Interferon Cytokine Res.* 2005; 25:297–310. [PubMed: 15957953]
28. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer.* 2003; 3:721–732. [PubMed: 13130303]
29. Su GL. Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation. *Am J Physiol Gastrointest Liver Physiol.* 2002; 283:G256–265. [PubMed: 12121871]
30. Hodge DR, Hurt EM, Farrar WL. The role of IL-6 and STAT3 in inflammation and cancer. *Eur J Cancer.* 2005; 41:2502–2512. [PubMed: 16199153]
31. Hodge DR, Peng B, Cherry JC, Hurt EM, Fox SD, Kelley JA, et al. Interleukin 6 supports the maintenance of p53 tumor suppressor gene promoter methylation. *Cancer Res.* 2005; 65:4673–4682. [PubMed: 15930285]
32. Aggarwal BB, Shishodia S, Takada Y, Jackson-Bernitsas D, Ahn KS, Sethi G, et al. TNF blockade: an inflammatory issue. *Ernst Schering Res Found Workshop.* 2006:161–186. [PubMed: 16331857]
33. Lo YY, Wong JM, Cruz TF. Reactive oxygen species mediate cytokine activation of c-Jun NH2-terminal kinases. *J Biol Chem.* 1996; 271:15703–15707. [PubMed: 8663189]
34. Qin H, Roberts KL, Niyongere SA, Cong Y, Elson CO, Benveniste EN. Molecular mechanism of lipopolysaccharide-induced SOCS-3 gene expression in macrophages and microglia. *J Immunol.* 2007; 179:5966–5976. [PubMed: 17947670]
35. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol.* 2007; 7:454–465. [PubMed: 17525754]
36. Rawlings JS, Rosler KM, Harrison DA. The JAK/STAT signaling pathway. *J Cell Sci.* 2004; 117:1281–1283. [PubMed: 15020666]
37. Strengell M, Lehtonen A, Matikainen S, Julkunen I. IL-21 enhances SOCS gene expression and inhibits LPS-induced cytokine production in human monocyte-derived dendritic cells. *J Leukoc Biol.* 2006; 79:1279–1285. [PubMed: 16551679]

38. Schmal H, Shanley TP, Jones ML, Friedl HP, Ward PA. Role for macrophage inflammatory protein-2 in lipopolysaccharide-induced lung injury in rats. *J Immunol.* 1996; 156:1963–1972. [PubMed: 8596051]
39. Shanley TP, Schmal H, Friedl HP, Jones ML, Ward PA. Role of macrophage inflammatory protein-1 alpha (MIP-1 alpha) in acute lung injury in rats. *J Immunol.* 1995; 154:4793–4802. [PubMed: 7722328]
40. Jaeschke H. Mechanisms of Liver Injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. *Am J Physiol Gastrointest Liver Physiol.* 2006; 290:G1083–1088. [PubMed: 16687579]
41. Michalopoulos GK, DeFrances MC. Liver regeneration. *Science.* 1997; 276:60–66. [PubMed: 9082986]
42. Wang K, Damjanov I, Wan YJ. The protective role of pregnane X receptor in lipopolysaccharide/D-galactosamine-induced acute liver injury. *Lab Invest.* 2010; 90:257–265. [PubMed: 19997066]
43. Kinjyo I, Hanada T, Inagaki-Ohara K, Mori H, Aki D, Ohishi M, et al. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity.* 2002; 17:583–591. [PubMed: 12433365]
44. Nakagawa R, Naka T, Tsutsui H, Fujimoto M, Kimura A, Abe T, et al. SOCS-1 participates in negative regulation of LPS responses. *Immunity.* 2002; 17:677–687. [PubMed: 12433373]
45. Jo D, Liu D, Yao S, Collins RD, Hawiger J. Intracellular protein therapy with SOCS3 inhibits inflammation and apoptosis. *Nat Med.* 2005; 11:892–898. [PubMed: 16007096]

Figure 1. 101 genes that are 1.5 fold changed in TK-/- livers compared to wild type (WT) livers basally

A) Gene Tree of all 101 dysregulated genes. **B)** Functional classification of a major subset of the genes based on biological process. Genes that were differentially expressed between untreated Ron TK-/- and WT mice were categorized using the DAVID GO term functional annotation tool. The number in parenthesis represents the total number of genes in those categories. **C)** Representative genes and their fold changes.

Figure 2. Gene expression changes in WT and TK-/- livers following 1 hour of endotoxin and GalN exposure

A) 312 genes whose expression was altered differently at 1hr after endotoxin exposure between WT and TK-/- livers. **B)** Cluster analysis of those 312 genes based on similarity of expression pattern. Utilizing a K-means algorithm from GeneSpring, six clustering groups were established to identify patterns of gene expression. The x-axis shows the genotypes and treatment times while the y-axis is the normalized intensity. **C)** 83 genes whose expression is altered similarly in both WT and TK-/- livers at 1 hr. **D)** Cluster analysis of 83 similarly regulated genes.

Figure 3. Suppressors of Cytokine Signaling are upregulated between 1 and 4 hrs in the TK-/- animals whereas in the WT animals their expression levels have returned to normal
 The fold change of SOCS 1, 2 and 3 expression was examined at 0 to 1 hours and at 1 to 4 hours following LPS/GalN treatment. SOCS expression levels were maintained at high levels in the TK-/- livers compared to the livers from WT mice at the later time points suggesting an enhanced down regulation of cytokine signaling in the TK-/- animals compared to controls.

Figure 4. Increased expression and activation of JAK2 and STAT3 in TK^{-/-} liver and hepatocytes

A) Western analysis depicting increased JAK2 protein levels in TK^{-/-} livers 4 hours post LPS/GalN treatment. **B)** Densitometric quantification of Western blots for total JAK2 demonstrating a significant increase in JAK2 protein levels in TK^{-/-} livers. **C)** Immunohistochemistry of liver sections from WT and TK^{-/-} mice 4 hours post LPS/GalN treatment for STAT3 and JAK2 expression. Note the nuclear STAT3 localization in the TK^{-/-} hepatocytes compared to controls. Scale bar = 50 μ m. **D)** *Ex vivo* treatment of WT and TK^{-/-} hepatocytes with TNF- α and ActD demonstrates increased p-JAK2 and total JAK2 levels in the TK^{-/-} hepatocytes compared to controls.

Figure 5. SOCS3 is upregulated in TK-/- Kupffer cells more than WT cells after LPS stimulation

Quantitative real time PCR analysis of LPS-stimulated Kupffer cells for the induction of SOCS3. TK-/- Kupffer cells displayed 1.8-fold more SOCS3 expression after LPS stimulation for 30 minutes compared to WT Kupffer cells.

Figure 6. Working model

Based on our data, a working model for the hepato-protective phenotype observed in Ron TK^{-/-} livers of mice exposed to LPS/GalN was generated. We propose that high levels of SOCS genes during ALF seen in TK^{-/-} liver antagonize the LPS-induced expression and function of inflammatory cytokines/chemokines in Kupffer cells as well as antagonize the deleterious effect of cytokine signaling in the TK^{-/-} hepatocytes. High levels of SOCS, Bcl6 as well as higher NF-κB activity in TK^{-/-} livers may also contribute to hepato-protection.

Table 1

Differential cytokine, chemokine and transcriptional regulatory gene expression changes between 0 to 1 and 1 to 4 hours following LPS/GalN exposure.

Cytokines and Chemokines	0 hrs–1 hr		1 hr – 4hrs	
	WT	TK–/–	WT	TK–/–
Chemokine (C-C) ligand 2	8.7	4.4	NC	NC
Chemokine (C-C) ligand 3	14.7	8.6	NC	1.8
Chemokine (C-C) ligand 4	4.6	3.2	NC	NC
Chemokine (C-C) ligand 7	2.5	NC	4.6	1.9
Chemokine (C-C) ligand 5	NC	1.8	5.5	NC
Chemokine (C-C) ligand 19	NC	NC	NC	2.4
Chemokine (C-X-C) ligand 1	6.7	2.6	0.6	–1.7
Chemokine (C-X-C) ligand 2	63	31.6	–3.3	–2.0
Chemokine (C-X-C) ligand 9	NC	1.6	4.5	4.6
Chemokine (C-X-C) ligand 10	8.1	9.8	6.0	6.0
Chemokine (C-X-C) ligand 11	NC	NC	6.2	6.3
Chemokine (C-X-C) ligand 13	1.9	1.7	4.0	5.2
Colony stimulating factor 1	2.7	2	2.7	2.0
Interleukin 1 - alpha	5.9	8.8	NC	–3.3
Interleukin 1 - beta	15.2	12.3	–2.5	NC
Interleukin 6	47.9	42.6	–5.0	–10.0
Replication Initiator 1	–1.5	–2.2	NC	–1.7
Tumor necrosis factor- α	2.5	1.9	NC	NC

Transcriptional Regulation	0 hrs–1 hr		1 hr – 4hrs	
	WT	TK–/–	WT	TK–/–
B-cell CLL/lymphoma 6	1.9	NC	NC	NC
FBJ osteosarcoma oncogene	1.7	NC	NC	NC
Jun dimerization protein 2	4.3	3.2	NC	NC
Jun oncogene	–2.8	2.8	–2.5	–1.7
Jun-B oncogene	2.8	1.8	NC	NC
Spi-C transcription factor	2.4	3.8	1.6	3.1
Activating transcription factor 3	2.7	2.0	–1.7	2.0
Early growth response 2	5.0	3.0	–2.0	–1.7
Stat1	–1.7	NC	2.3	2.1
Stat2	NC	–1.5	NC	1.8
Jak2	NC	NC	1.9	1.9

Values are listed as the fold difference between time points for each genotype. NC indicates no change.