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Ron receptor-dependent gene regulation of Kupffer cells during endotoxemia

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Abstract

Background—We have previously shown that Ron receptor tyrosine kinase signaling in macrophages, including Kupffer cells and alveolar macrophages, suppresses endotoxin-induced proinflammatory chemokine/cytokine production. Further, we have also identified genes from Ron replete and Ron deplete livers that were differentially expressed during the progression of liver inflammation associated with acute liver failure in mice by microarray analyses. While important genes and signaling pathways have been identified downstream of Ron signaling during progression of inflammation by this approach, the precise role that Ron receptor plays in regulating the transcriptional landscape in macrophages, and particular in isolated Kupffer cells, has still not been investigated.

Methods—Kupffer cells were isolated from wild-type (TK+/+) and Ron tyrosine kinase (TK−/−) deficient mice. *Ex vivo*, the cells were treated with lipopolysaccharide (LPS) in the presence or absence of the Ron ligand, Hepatocyte growth factor-like protein (HGFL). Microarray and qRT-PCR analyses were utilized to identify alterations in gene expression between genotypes.

Results—Microarray analyses identified genes expressed differentially in TK+/+ and TK−/− Kupffer cells basally as well as after HGFL and LPS treatment. Interestingly, our studies identified *Mefv*, a gene that codes for the anti-inflammatory protein pyrin, as an HGFL-stimulated Ron-dependent gene. Moreover, *Lcn2* (Lipocalin 2), a proinflammatory gene, which is induced by LPS, was significantly suppressed by HGFL treatment. Microarray results were validated by qRT-PCR studies on Kupffer cells treated with LPS and HGFL.

Conclusions—The studies herein suggest a novel mechanism whereby HGFL-induced Ron receptor activation promotes the expression of anti-inflammatory genes while inhibiting genes involved in inflammation with a net effect of diminished inflammation in macrophages.

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Ethical Approval: All mice were treated and euthanized in accordance with protocols approved by the Institutional Animal Care and Use Committee of University of Cincinnati.

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Keywords

Mst1R; Mefv; Lcn2; Met receptor; Kupffer cells; macrophages

INTRODUCTION

Innate immune cells including macrophages play a vital role in the progression of a variety of inflammatory diseases including acute liver failure (ALF), inflammatory bowel disease, rheumatoid arthritis, atherosclerosis and acute myocardial infarction which are a leading cause of mortality worldwide^(1, 2). Under normal conditions, inflammatory cytokine and chemokine production by macrophages is minimal⁽¹⁾. Studies have shown that macrophages are activated by inflammatory stimuli including bacterial endotoxin lipopolysaccharide (LPS) in these disease states and that activated macrophages secrete proinflammatory cytokines/chemokines, including TNF- α , IL-1 β , IL-12p40, IL-6, Ccl-2 and Cxcl-2 in a Toll like receptor (TLR) and NF- κ B dependant manner to propagate the inflammatory response^(1, 3). Attenuating innate immune system activation has been suggested as one of the potential therapies for inflammatory diseases^(1, 2). However, the detailed molecular mechanisms of innate immune cell activation in these inflammatory diseases are not completely understood. In the present study, using the model of resident liver macrophages (Kupffer cells), we have elucidated mechanisms associated with macrophage-mediated inflammation through microarray profiling.

The Ron receptor is a membrane spanning protein that belongs to the Met family of receptor tyrosine kinases. Ron is expressed on a variety of cell-types including epithelial cells and specialized macrophages such as osteoclasts, resident tissue macrophages (alveolar and peritoneal macrophages as well as Kupffer cells), but not on monocytes or circulating macrophages⁽⁴⁾. Ron expression in the liver starts at embryonic day 12.5 and immunohistochemical analyses have shown that Ron is expressed on embryonic as well as adult hepatocytes and Kupffer cells in both mouse and humans^(5, 6). Hepatocyte growth factor-like protein (HGFL) is the only known ligand for Ron⁽⁴⁾. Binding of HGFL to Ron receptor leads to activation of the receptor's intrinsic tyrosine kinase activity and binding of proteins containing the Src homology 2 and phosphotyrosine-binding domains. HGFL stimulated Ron activation leads to the induction of diverse downstream signaling cascades, which play important roles in a variety of biological processes such as inflammation and cancer⁽⁴⁾.

HGFL-induced Ron receptor activation in peritoneal macrophages induces spreading, chemotaxis and phagocytosis⁽⁷⁾. Using mice that lack the tyrosine kinase (TK) signaling domain of the Ron receptor (TK $-/-$ mice)⁽⁸⁾, our prior studies have demonstrated that TK $-/-$ mice have altered inflammatory responses compared to wild-type control (TK $+/+$) mice in various models of LPS-induced acute endotoxemia⁽⁸⁻¹⁰⁾. Specifically, we have shown Ron functions to limit the production of select proinflammatory cytokines and chemokines by attenuating LPS-induced NF- κ B signaling⁽¹¹⁻¹³⁾.

In the liver, the Ron receptor has been shown to suppress cytokine secretion from Kupffer cells and to inhibit hepatocyte survival in a murine model of liver inflammation, i.e.

ALF⁽¹³⁾. Further, microarray analysis of total liver RNA isolated from TK^{+/+} and TK^{-/-} mice in various stages of ALF identified gene expression patterns regulated by Ron⁽¹⁴⁾. While these studies were the first to note the consequences of Ron signaling during inflammatory liver disease progression, the genes that Ron regulates in specific liver cell types have not been investigated. Moreover, a major gap exists in our knowledge of the role of Ron in macrophage activation. The studies in this report utilized microarray analyses of Kupffer cells isolated from TK^{+/+} and TK^{-/-} mice stimulated with LPS and/or HGFL. Our analysis identified MEFV as a downstream effector of Ron, which may serve to dampen macrophage activation. In addition, our data also pinpointed Lcn2 as a proinflammatory gene that is induced upon Kupffer cell activation by LPS but negatively regulated by HGFL. In total, our analyses have identified several important downstream effectors of Ron signaling which have not been previously linked. These Ron-regulated genes provide important mechanistic insights into the pathways that Ron utilizes to regulate macrophage activation and have critical implications in regulating inflammatory diseases including ALF.

METHODS

Mice

Ron tyrosine kinase (TK) deficient mice (TK^{-/-} mice) were generated as previously described and backcrossed into a C57BL/6 background⁽⁸⁾. All mice were maintained under specific pathogen-free conditions and were treated and euthanized in accordance with protocols approved by the Institutional Animal Care and Use Committee of University of Cincinnati.

Kupffer Cell Isolation, Culture and Treatment

Kupffer cells were isolated from mice between 8 to 14 weeks of age and were cultured as previously described⁽¹⁵⁾. Kupffer cells were either not treated or treated with bacterial lipopolysaccharide (LPS) for 30 minutes or 1 hour (500 ng/ml) (*E. coli* serotype 0111:B4; Sigma, St. Louis, MO). Kupffer cells were also treated with HGFL (R&D systems, Minneapolis, MN) at 200 ng/ml for 1 hour or HGFL overnight (16–18 hours) followed by LPS stimulation for 30 minutes.

RNA Isolation and Microarray Analysis

Kupffer cells isolated from 2–3 separate livers were pooled together and from each pooled isolation, experiments were subsequently performed in duplicate. RNA was independently isolated from each duplicate and pooled in equal concentrations to represent one microarray (one lane of the heatmap). A second microarray was performed as noted and results from two microarrays for each treatment are compared in this report. As such, each condition is representative of 4–6 Kupffer cell isolations and quadruplicate experiments. Total RNA was isolated from Kupffer cells using TriZol Reagent (Invitrogen, Carlsbad, CA) as previously described^(13, 14). Isolated RNA was purified by sodium acetate precipitation and purified RNA was examined at the Cincinnati Children's Hospital Medical Center Affymetrix Core. RNA quality was assessed and quantified using an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). RNA was amplified using the WT-Ovation Pico System (NuGen, San Carlos, CA) and subjected to hybridization to the Mouse Gene 1.0 ST GeneChip array (Affymetrix,

Santa Clara, CA). The probe arrays were scanned using the Affymetrix GeneChip Scanner 3000 and GeneChip Operating Software 1v4 (Affymetrix, Santa Clara, CA).

Microarray Data Analysis

Gene expression changes from the microarray signals were determined using GeneSpring software (Silicon Genetics, Redwood City, CA). Gene lists from the microarray data were obtained based on a 1.5-fold-expression difference using the Welch t-test and 2-tailed Student's t-test with Benjamini and Hochberg false discovery rate with $P < 0.01$. Correlation of gene expression with numeric parameters was assessed using the Pearson's product-moment correlation coefficient test with P-value. Lists were filtered based on fold change and P-value. Statistical significance was determined using an unpaired 2-tail Welch t-test or the Mann-Whitney U test with Bonferroni's correction. 935 genes were obtained that were changed globally in all the groups comparing them to the TK+/+ untreated sample. Further analysis yielded treatment-specific comparisons as needed between groups. Clustered image maps for specific comparison groups were obtained using the CIMMiner program⁽¹⁶⁾. Gene lists generated for the specific treatment groups were uploaded on to the web-based PANTHER (Protein Analysis THrough Evolutionary Relationships) classification system software and functional annotation analyses were performed⁽¹⁷⁾.

Quantitative Real Time (qRT)-PCR

Total RNA isolated from Kupffer cells after specified treatments was subjected to cDNA synthesis using the high capacity RNA to cDNA kit according to manufacturer's instructions (Applied Biosystems, Foster City, CA). qRT-PCR was performed on diluted cDNA using FastStart SYBR Green Master (Roche, Nutley, NJ). The genes analyzed and their corresponding primer sets are as follows: Egr-1 (5'-TCTTGGTGCCTTTTGTGTGAC-3'; 5'-CTCTTCCTCGTTTTTGCTCTC-3'), IL-6 (5'-TAGTCCTTCCTACCCCAATTTC-3'; 5'-TTGGTCCTTAGCCACTCCTTC-3'), Tnf- α (5'-CATCTTCTCAAAATTCGAGTGACAA-3'; 5'-TGGGAGTAGACAAGGTACAACCC-3'), Mevf (5'-TCATCTGCTAAACACCCTGGA-3'; 5'-GGGATCTTAGAGTGGCCCTTC-3'), Lcn2 (5'-TGGCCCTGAGTGTCATGTG-3'; 5'-CTCTTGCTAGCTCATAGATGGTGC-3') and β -glucuronidase (GusB) (5'-TTGAGAACTGGTATAAGACGCATCAG-3'; 5'-TCTGGTACTCCTCACTGAACATGC-3'). Expression levels were normalized to GusB as an internal control and relative gene expression results were reported in graphs. qRT-PCR analyses were performed at least twice with Kupffer cells pooled from 2 mice per isolation.

Cytokine Array

Conditioned media from Kupffer cells was collected basally (untreated) or following 30 minutes of LPS treatment or HGFL overnight pretreatment followed by 30 minutes of LPS treatment. Secreted cytokines were identified utilizing the mouse cytokine array panel A (R&D Systems) as described⁽¹³⁾.

Western Analyses

Western analyses were performed on isolated Kupffer cells as described previously⁽¹³⁾. The primary antibodies were Lcn2 (Abcam, Cambridge, MA), and Tubulin (Santa Cruz Biotechnology, Dallas, TX).

Statistical Analyses

Statistical significance for qRT-PCR and cytokine array was defined as $p < 0.05$ and determined by Student's t-test. Error bars represent SEM.

RESULTS

Microarray analyses of Ron proficient and Ron deficient Kupffer cells

We have previously shown that the Ron receptor tyrosine kinase is expressed on resident tissue macrophages including alveolar macrophages and Kupffer cells and that upon activation by its ligand, hepatocyte growth factor-like protein (HGFL), the Ron receptor diminishes the expression and secretion of select proinflammatory cytokines induced by lipopolysaccharide (LPS) exposure^(12, 13). However, a major gap exists in our knowledge of the genes that Ron modulates during macrophage activation. To elucidate the precise role that the Ron receptor plays in regulating the transcriptional landscape in macrophages, Kupffer cells were isolated from wild-type (TK+/+) and Ron tyrosine kinase deficient (TK-/-) mice and gene expression profiles were examined *ex vivo* in response to LPS, HGFL and the combination of LPS and HGFL. Following treatment, Kupffer cell RNA was isolated and subjected to microarray analysis. In an analysis of the data, 935 genes were identified that were significantly altered compared to the untreated TK+/+ Kupffer cells (Figure 1).

Basal gene expression changes between Ron proficient and Ron deficient Kupffer cells

Further data mining identified 24 genes that were differentially regulated between TK+/+ and TK-/- Kupffer cells basally, of which 9 genes were induced in TK-/- Kupffer cells while 15 genes were suppressed in TK-/- cells (Figure 2A). The web-based PANTHER⁽¹⁷⁾ gene function-organizing tool allowed grouping of these basally regulated genes into 13 overlapping biological process categories that included cellular processes, cell cycle, immune system processes and metabolic processes (Figure 2A). The genes that were basally upregulated in the TK-/- Kupffer cells included proinflammatory genes such as *Itgax* or *cd11c*, *ccl2*, *ccl22* and *tnfsf9* (*cd137l*). A number of anti-inflammatory genes, including IL-10, were decreased in untreated TK-/- Kupffer cells compared to TK+/+ cells. Thus, absence of functional a Ron receptor in Kupffer cells predisposes these cells to a proinflammatory phenotype by increasing the expression of select proinflammatory genes while decreasing the expression of anti-inflammatory genes.

LPS-responsive genes in Kupffer cells

In an analysis of LPS responsiveness, 43 genes in TK+/+ Kupffer cells were identified that were differentially regulated by LPS compared to unstimulated TK+/+ Kupffer cells. Thirty-six of these genes were induced after LPS treatment while 7 genes were suppressed. The PANTHER gene function-organizing tool allowed grouping of these genes into 13

overlapping biological process categories that included apoptosis, cell communication, cellular processes, cell cycle, developmental processes, immune system processes, metabolic processes and stimulus response (Figure 2B). Similarly, LPS treatment of the TK^{-/-} Kupffer cells altered the gene expression profiles of 56 genes, of which 41 genes were induced after LPS treatment while 15 genes were suppressed compared to unstimulated TK^{-/-} Kupffer cells. These genes were grouped into 11 overlapping biological process categories that included apoptosis, cell communication, cell cycle, cellular processes, developmental processes, immune system processes, metabolic processes and stimulus response (Figure 2C). Our data also supported the induction of a standard 'LPS-signature' as shown by previous studies^(3, 18, 19) and this included increases in Tnf- α , Cxcl10, Ccl4, Egr-1, IL-6, Socs3, Fos, Jun in TK^{+/+} as well as TK^{-/-} Kupffer cells after LPS treatment. Similarly, the Zinc finger proteins Znf760 and Zbtb1 as well as the phosphodiesterase enzyme Pde3b were suppressed in both genotypes following LPS treatment⁽¹⁹⁾.

HGFL responsive genes in Ron expressing Kupffer cells

HGFL treatment of TK^{+/+} Kupffer cells altered the expression of 13 genes, of which 12 genes were induced while 1 gene was suppressed. These 13 genes were grouped into 8 overlapping biological process categories that included apoptosis, cell communication, cellular processes, immune system processes, metabolic processes and stimulus response (Figure 2D). Genes induced by HGFL treatment included the proinflammatory genes Tnf- α and IL-6 as well as anti-inflammatory genes such as Mefv and Tnfaip3 (A20). The only gene that was suppressed in TK^{+/+} Kupffer cells after HGFL treatment was the inflammation modulator gene Lipocalin 2 (Lcn2). Lcn2 has been shown to be both proinflammatory as well as anti-inflammatory in a cell and context dependent manner^(20, 21). Thus, HGFL stimulation may initiate an anti-inflammatory response in Kupffer cells by altering the expression of anti-inflammatory genes as well as proinflammatory genes.

Differential gene expression changes between LPS-treated Ron proficient and Ron deficient Kupffer cells

The gene sets from LPS treated TK^{+/+} and TK^{-/-} Kupffer cells were further examined to identify genes that were differentially regulated between the genotypes in response to LPS exposure. Three gene sets were identified which included (i) LPS-induced genes in TK^{+/+} Kupffer cells (36 genes), (ii) LPS-induced genes in TK^{-/-} Kupffer cells (41 genes), and (iii) genes differentially regulated by LPS in TK^{-/-} vs. TK^{+/+} Kupffer cells (16 genes). Out of the 36 genes LPS-induced in TK^{+/+} and 41 in TK^{-/-} Kupffer cells after LPS treatment (Figure 2), 33 genes were similar in both the genotypes, while 3 genes (Fat3, JunB, 2310016C08Rik) were uniquely induced in TK^{+/+} Kupffer cells and 4 genes (Hist1h4c, Osm, AY078069, Prdm1) were uniquely induced in TK^{-/-} Kupffer cells after LPS treatment (Figure 3A). Ten genes were also identified that were suppressed in TK^{+/+} Kupffer cells but induced in TK^{-/-} Kupffer cells after LPS treatment. These genes include Fryl, Lypla2, Bcl2l1, LOC100042747, Tnfsf18, Saps1, LOC626000, Adpgk, Bak1 and Stx16 (Figure 3A).

Similar studies were performed with genes suppressed following LPS treatment. Three gene sets were identified that included (i) LPS-suppressed genes in TK^{+/+} Kupffer cells (7

genes), (ii) LPS-suppressed genes in TK^{-/-} Kupffer cells (15 genes) and (iii) genes differentially regulated by LPS in TK^{-/-} versus TK^{+/+} Kupffer cells (16 genes). Of the 7 genes suppressed in TK^{+/+} Kupffer cells and 15 in TK^{-/-} Kupffer cells (Figure 2), 2 genes were the same in both genotypes, while 5 genes (Fryl, 5430411C19Rik, LOC100042747, Stx16 and Gas5) were uniquely suppressed in TK^{+/+} Kupffer cells and 10 genes (Zfp422-rs1, 2700094F01Rik, Zbtb1, Yars2, Eif2c4, Pigp, Zfp87, LOC667203, Zfp672 and Guf1) were uniquely suppressed in TK^{-/-} Kupffer cells after LPS treatment (Figure 3B). Ten unique genes were also identified that were induced in TK^{+/+} Kupffer cells but suppressed in TK^{-/-} Kupffer cells after LPS treatment. These genes included Tmem101, Gstm5, Fat3, Med31, Hmcn1, Spaca1, Lsm8, Rpl221l, LOC668919 and Lcn2.

Differential gene expression changes in Ron proficient Kupffer cells following LPS and HGFL treatment

The gene sets from LPS treated and HGFL plus LPS treated TK^{+/+} Kupffer cells were further analyzed to examine genes differentially regulated between treatments (Figure 3C). Of the genes induced by LPS, 5 genes were suppressed by the combination of HGFL and LPS treatment and included the proinflammatory genes Cxcl-10, IL-6 and Ccl4. This data is in agreement with our prior studies whereby Ron activation suppressed proinflammatory gene expression in macrophages, including Kupffer cells^(12, 13). The other 2 genes were Fat3 and Gbp5. There were 7 genes (A530040E14Rik, Fcgr4, Spry4, Lsm8, Rpl221l, C4b and Lcn2) that were suppressed after HGFL and LPS co-treatment. Three genes (Mefv, Itgax and Mbc2) were induced with HGFL plus LPS treatment. Mefv, an anti-inflammatory protein, was also induced after HGFL treatment alone in TK^{+/+} Kupffer cells. Fryl was induced by combined HGFL and LPS treatment but was suppressed by LPS alone. These microarray studies are consistent with prior studies, which have documented HGFL-induced Ron activation as a suppressor of LPS-mediated inflammation^(12, 13).

Validation of Microarray data

qRT-PCR was utilized to validate the microarray data (Figure 4). Egr-1, a LPS-induced transcription factor⁽¹³⁾, was dramatically upregulated following LPS treatment of both TK^{+/+} and TK^{-/-} Kupffer cells, 3-fold and 4-fold respectively, in the microarrays. qRT-PCR analysis on TK^{+/+} and TK^{-/-} Kupffer cells showed that Egr-1 mRNA was induced in both genotypes following LPS exposure (Figure 4A). Similarly, IL-6, an LPS-induced cytokine, was induced 2.57-fold and 1.73-fold in LPS-treated TK^{+/+} and TK^{-/-} Kupffer cells respectively in the microarray. qRT-PCR analyses showed an induction of IL-6 in TK^{+/+} and TK^{-/-} Kupffer cells (Figure 4B). HGFL induced expression of cytokines such as IL-6 (1.66-fold) and TNF- α (2.62-fold) in the microarray. qRT-PCR analysis showed IL-6 and TNF- α induced 3.01-fold and 2.91-fold respectively after HGFL treatment (Figure 4C). Microarray analysis also demonstrated an upregulation of LPS-induced IL-6 mRNA (2.57-fold), which was then diminished by HGFL and LPS dual treatment in TK^{+/+} Kupffer cells. qRT-PCR analysis showed an increase in IL-6 mRNA by LPS, which was diminished after HGFL plus LPS treatment (Figure 4D). Microarray analyses found that IL-10 was suppressed in TK^{-/-} Kupffer cells (0.64-fold) compared to TK^{+/+} Kupffer cells basally and that LPS induced IL-10 expression in Kupffer cells from both the genotypes (5.2-fold in TK^{+/+} Kupffer cells and 8.41-fold in TK^{-/-} Kupffer cells). Similarly, Cxcl10 mRNA was

induced 5.03-fold in TK^{+/+} Kupffer cells and 6.51-fold in TK^{-/-} Kupffer cells following LPS treatment, while Ccl4 mRNA was induced 2.86-fold in TK^{+/+} Kupffer cells and 2.29-fold in TK^{-/-} Kupffer cells after LPS treatment. As further validation of the microarray data, cytokine array analyses were performed on conditioned media from cultured Kupffer cells. As depicted in Figure 4E and 4F, no significant differences were observed in the secreted protein levels of IL-10 and Cxcl10 basally between genotypes. However, Ccl4 protein production was significantly higher in TK^{+/+} versus TK^{-/-} Kupffer cells (Figure 4E). After LPS-treatment, IL-10, Cxcl10 and Ccl4 proteins were secreted by TK^{+/+} and TK^{-/-} Kupffer cells and were 2.01-fold, 1.75-fold and 1.34-fold higher in the TK^{-/-} conditioned media compared to TK^{+/+} conditioned media (Figure 4F).

Identification of Mefv (pyrin) and Lcn2 as novel downstream effectors of HGFL-induced Ron activation

Microarray analyses also documented an HGFL-dependent regulation of Kupffer cell genes including Mefv (pyrin), which was also consistently induced in TK^{+/+} Kupffer cells by qRT-PCR following HGFL treatment (Figure 5A). qRT-PCR for Lcn2 demonstrated an upregulation following LPS treatment, which was then diminished by HGFL and LPS dual treatment (Figure 5B). Similarly, protein levels of Lcn2 also showed an LPS-mediated induction, which was diminished by HGFL and LPS dual treatment in TK^{+/+} Kupffer cells (Figure 5C). Further, to test the effect of HGFL-mediated Ron activation on NLRP3 inflammasome activity, IL-1 β protein levels secreted by TK^{+/+} Kupffer cells following LPS and LPS-HGFL treatments were measured. IL-1 β secreted protein levels were significantly diminished by HGFL and LPS dual treatment compared to LPS alone in TK^{+/+} Kupffer cells (Figure 5D). Our data has identified two novel downstream effectors of Ron signaling. We have shown that HGFL-induced Ron activation in Kupffer cells induces Mefv (pyrin) and diminishes Lcn2 expression, which may promote the expression of anti-inflammatory genes while inhibiting genes involved in inflammation.

DISCUSSION

In the present study, we identified the gene expression profiles of TK^{+/+} and TK^{-/-} Kupffer cells under basal conditions, after exposure to LPS and after Ron activation by its ligand HGFL. This study is the first to identify Ron receptor-induced genes in primary macrophages. We identified genes that were basally different between TK^{+/+} and TK^{-/-} Kupffer cells and these genes included receptors, cytokines and inflammation modulators. LPS treatment of TK^{+/+} and TK^{-/-} Kupffer cells induced the 'LPS-signature' genes^(3, 18, 19), which included TNF- α , Cxcl10, Ccl4, Egr-1, IL-6, Socs3, Fos and Jun. Further, we identified genes differentially regulated between TK^{+/+} and TK^{-/-} Kupffer cells after LPS treatment. This gene pool included genes that were uniquely regulated in a genotype-specific fashion, as well as genes that were regulated in a similar direction following LPS treatment. HGFL-induced genes were also identified in TK^{+/+} Kupffer cells and included cytokines, receptors and inflammatory modulators. Our analyses also identified pyrin as a novel downstream effector of the Ron receptor to diminish LPS-induced inflammation.

We have shown that LPS treatment of macrophages, including Kupffer cells, induces the secretion of proinflammatory cytokines and chemokines and that this secretion is diminished with HGFL^(11, 13). Further, our previous data also demonstrated that this inhibition is associated with diminished activation of NF- κ B signaling^(11, 13). The exact molecular mechanisms by which the Ron receptor inhibits NF- κ B activation in macrophages are still not well understood. In the present study, we have identified Mefv (pyrin) as a gene downstream of HGFL-induced Ron activation in Kupffer cells, both by microarray as well as by qRT-PCR. Mefv (pyrin) mRNA was induced upon HGFL treatment of Kupffer cells and remained high through overnight treatment (Figure 5, and data not shown). Mefv gene encodes the protein called pyrin, which is an anti-inflammatory protein and inhibits NF- κ B activation⁽²²⁾. Mutations in the Mefv gene have been found to be associated with many auto-inflammatory diseases including Familial Mediterranean Fever (FMF) and Crohn's disease, which are characterized by exaggerated inflammation^(22, 23). Studies have shown that pyrin inhibits the processing and assembly of the NLRP3 inflammasome that promotes IL-1 β secretion. IL-1 β is a potent proinflammatory cytokine and is partly responsible for the inflammatory phenotype seen in FMF patients^(23, 24). The mutant forms of pyrin observed in Crohn's disease and FMF patients do not interact properly with their binding partners, which leads to the assembly of IL-1 β inflammasomes and hyper-inflammation⁽²³⁾. Moreover, mice expressing a pyrin mutant associated with FMF displayed hyper-inflammation upon LPS-induced endotoxemia compared to controls animals⁽²⁴⁾. Further, IL-1 β secreted protein levels were significantly diminished by HGFL and LPS dual treatment compared to LPS alone in TK+/+ Kupffer cells, suggesting a defect in inflammasome assembly in TK+/+ Kupffer cells upon Ron activation (Figure 5). Our studies suggest that pyrin is a novel downstream target of Ron receptor activation, which may play an important role to dampen the LPS-NF- κ B-induced proinflammatory response of Kupffer cells.

Very little is known about the transcriptional control of Mefv gene expression. Prior studies have identified a minimal promoter element as well as an enhancer region in the 5' region of the Mefv gene. These regions have binding sites for transcription factors that play a role in myeloid cell-specific cytokine gene expression and include c-myb, PU1, NF- κ B, C/EBP and AML⁽²³⁾. Although we have previously shown that Ron activation by HGFL induces NF- κ B signaling in various cancers of epithelial origin⁽⁴⁾, we did not observe NF- κ B activation (detected by phosphorylation of NF- κ B) in Kupffer cells after Ron activation by HGFL (data not shown). Thus far, there have been no reports of Ron activation and initiation of c-myb, PU1, C/EBP and AML-dependent transcription and the mechanisms by which pyrin is induced downstream of Ron activation in macrophages are still not known.

Lcn2 is an iron-trafficking protein, expressed on various cell types such as epithelial cells and macrophages. Lcn2 binds iron siderophores and limits ferric iron uptake by bacteria, resulting in diminished bacterial infection⁽²⁵⁾. Recent studies have identified both a pro as well as anti-inflammatory role for Lcn2. Lcn2 expression is induced in tissue resident macrophages including Kupffer cells and alveolar macrophages following LPS treatment *in vivo* in an NF- κ B dependent manner⁽²⁶⁾. Lcn2 has been shown to downregulate the inflammatory response and to protect the lung and liver from excessive damage induced by LPS. Lcn2 also binds and/or sequesters chemokines, thereby reducing their biological

activity and helps to diminish inflammation⁽²⁶⁾. Conversely, Lcn2 has been shown to be associated with various inflammatory states such as obesity and inflammatory bowel disease (IBD). Studies have shown increased expression of this protein in various murine models of IBD, especially colitis. Further, Lcn2 expression is also increased in human IBD, specifically ulcerative colitis patients⁽²⁷⁾. Studies with Lcn2 null mice have shed a light on understanding the role of this protein in inflammation. Lcn2 null mice showed lower inflammation and reduced tissue regeneration in models of spinal cord injury and ischemic reperfusion injury. Lcn2 null mice had diminished tissue infiltration of immune cells as well as decreased expression of proinflammatory cytokines including MIP-1 α , MCP-1, IL-1 and TNF- α in these studies^(28, 29). Thus, the majority of the data suggest that Lcn2 has a predominantly proinflammatory role. Accordingly, our data show that LPS induces Lcn2 expression in TK+/+ Kupffer cells and that HGFL is able to diminish the induction.

Microarray and qRT-PCR analyses demonstrated that a number of proinflammatory cytokines including IL-6 and TNF- α were moderately induced in Kupffer cells following Ron activation by HGFL. These levels were not as high as that observed by cells stimulated with LPS and were down to basal levels by 6 hours following HGFL treatment (data not shown). We have observed similar results in alveolar macrophages (data not shown). Further, it was shown that megakaryocytes express the Ron receptor and that activation of Ron on these cells by HGFL induces IL-6 production that helps in the maturation of megakaryocytes⁽³⁰⁾. Although HGFL induced Ron activation diminishes LPS-induced increases in inflammatory cytokine secretion by macrophages^(11, 13), the significance of moderate induction of the proinflammatory cytokines immediately after Ron activation is not known. IL-6 has been shown to act as an anti-inflammatory protein as well, especially during the acute phase response to inflammation by diminishing the levels of proinflammatory cytokines and increasing expression of anti-inflammatory proteins including IL-1Ra⁽³¹⁾. It may be possible that IL-6 induced by HGFL may actually act as an anti-inflammatory protein.

Based on our studies, we propose a working model by which the Ron receptor is able to suppress LPS-induced inflammation in Kupffer cells (Figure 6). We show that Ron activation in TK+/+ Kupffer cells induces pyrin (Mefv) expression by yet unknown mechanisms. We have previously shown that Ron activation in macrophages diminishes LPS-induced NF- κ B activation and induction of its downstream target genes^(11, 13). Pyrin, as described in earlier reports^(22–24), may act as an anti-inflammatory protein in Kupffer cells by diminishing NF- κ B activation as well as inflammasome assembly downstream of LPS-induced Kupffer cell activation. Further, HGFL-induced Ron signaling may inhibit Lcn2 (a proinflammatory protein) induction by LPS, which may further reduce Kupffer cell associated proinflammatory cytokine production as well as decrease immune cell infiltration to the area of injury.

Some inflammatory liver diseases such as chronic hepatitis C infection are characterized by increased urinary excretion of Lcn2⁽³²⁾. Unfortunately, the role of Ron receptor as well as pyrin, in hepatitis or other liver inflammatory diseases in humans is not known and needs further characterization. The negative regulation of LPS-induced inflammation by the Ron receptor in macrophages has opened avenues for the development of pharmacological

modulators of the pathway. The present study has gone a step further and identified the HGFL-Ron regulated genes in Kupffer cells as well as the differential gene expression profile in TK+/+ and TK-/- Kupffer cells after endotoxin exposure. The novel signaling mechanisms downstream of the Ron receptor are active in alveolar macrophage cell line MHS (data not shown) and may be active in other macrophages as well. These novel signaling pathways may be targets for therapeutic interventions in various macrophage-associated inflammatory diseases where the proper control of innate immune responses are critical. It has been suggested that targeting drugs and peptides specifically to Kupffer cells by liposomal and nanoparticle mediated drug delivery to decrease the secretion of proinflammatory mediators is one of the ways to limit the progression of inflammation in liver diseases⁽³³⁾. Based on this notion, it may be possible to develop molecular-targeted therapy to activate the Ron receptor by delivering its ligand HGFL, in its processed and active heterodimeric form, by liposomes or nanoparticles to Kupffer cells, which will help limit Kupffer cell mediated secretion of proinflammatory cytokines/chemokines and thus limit liver inflammation.

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Figure 1. Global changes in gene expression in TK+/+ Kupffer cells compared to TK-/- cells prior to and after treatment with LPS and HGFL

TK+/+ and TK-/- Kupffer cells were untreated (-) or treated with LPS for 30 minutes, HGFL 60 minutes, or overnight HGFL followed by 30 minutes LPS in duplicate. RNA was isolated and subjected to microarray analysis. 935 genes were identified which changed significantly over untreated TK+/+ control RNA and are depicted in the heat map.

Figure 2. Gene expression changes in Kupffer cells after LPS and HGFL treatments
(A) Heat map of 24 genes differentially expressed between TK+/+ and TK-/- Kupffer cells in the absence of treatment (basal changes). **(B)** Heat map of 43 genes differentially expressed in TK+/+ Kupffer cells after LPS treatment for 30 minutes. **(C)** Heat map of 56 genes differentially expressed in TK-/- Kupffer cells after LPS treatment for 30 minutes. **(D)** Heat map of 13 genes differentially expressed in TK+/+ Kupffer cells after HGFL treatment for 1 hour. Gene ontology function of the web-based program, Panther, was used to classify the genes that were upregulated or down regulated basally or after each treatment into different biological processes categories **(A-D)**.

Figure 3. Differential gene expression in Kupffer cells with LPS and HGFL treatment

(A) Venn diagram representing genes induced after LPS treatment in TK-/- compared to TK+/+ Kupffer cells. Ten unique genes were induced in TK-/- but suppressed in TK+/+ Kupffer cells after LPS treatment. (B) Venn diagram representing genes suppressed after LPS treatment in TK-/- compared to TK+/+ Kupffer cells. Analyses identified 13 unique genes that were suppressed in TK-/- Kupffer cells but were induced in TK+/+ cells after LPS treatment. (C) Venn diagram representing genes changed in TK+/+ Kupffer cells after LPS and/or HGFL treatment. Three unique genes were induced in Kupffer cells after dual treatment. Five genes induced by LPS were suppressed by HGFL and one gene, which was suppressed by LPS treatment, was induced after HGFL and LPS treatment. Seven unique genes were suppressed by HGFL and LPS treatment. The biological processes categories are noted for the differential treatment groups (A-B).

Figure 4. Validation of microarray data

qRT-PCR analyses on TK+/+ and TK-/- Kupffer cells showed EGR-1 mRNA was induced 1.8 fold more in the TK-/- compared to TK+/+ Kupffer cells after 30 minutes of LPS treatment (A). IL-6 mRNA was induced 2.1 fold more in the TK+/+ compared to TK-/- Kupffer cells after LPS treatment (B). qRT-PCR analyses showed that HGFL treatment for 1 hour modestly increased IL-6 (3 fold) and TNF-α (2.9 fold) mRNA levels (C). LPS induced IL-6 (6.67 fold) mRNA levels were suppressed by overnight HGFL treatment followed by LPS treatment (3.31 fold) (D). Cytokine array of conditioned media from TK+/+ and TK-/- Kupffer cells showing basal (E) and LPS-stimulated cytokine protein levels (F). *denotes P<0.05.

Figure 5. HGFL treatment induces Mefv (pyrin) and suppresses Lcn2 in TK+/+ Kupffer cells RNA from Kupffer cells was obtained after HGFL and LPS treatment. qRT-PCR for Mefv (pyrin) (**A**) and Lcn2 (**B**) were performed under various treatment conditions. Western blot for Lcn2 protein expression in TK+/+ Kupffer cells after various treatment conditions (**C**). Secreted levels of IL-1 β in conditioned media from TK+/+ Kupffer cells after various treatment conditions (**D**). *denotes $P < 0.05$.

Figure 6. Working Model

Based on our data, we propose a role for pyrin as a downstream mediator of Ron signaling in inhibiting LPS-induced NF-κB mediated inflammation in Kupffer cells. Further, we propose that Ron signaling inhibits Lcn2 expression, which may act through multiple mechanisms to regulate inflammation.