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Cross-Talk Between Host Anti-Viral Innate Immune Responses and Metabolism

A dissertation submitted in partial satisfaction of the requirements  
for the degree Doctor of Philosophy in Microbiology, Immunology, and  
Molecular Genetics

by

Amir Ali Ghaffari

2013



## ABSTRACT OF DISSERTATION

Cross-Talk Between Host Anti-Viral Innate Immune Responses and Metabolism

By

Amir Ali Ghaffari

Doctor of Philosophy in Microbiology, Immunology, and Molecular Genetics

University of California, Los Angeles 2013

Professor Genhong Cheng, Chair

Viral infections can induce changes in host metabolism, either as a result of viral manipulations or host anti-viral responses. These metabolic changes in turn may result in various disorders. The liver, as the critical organ in maintaining metabolic homeostasis, is particularly susceptible to such changes during viral infections, and alterations in hepatic function can quickly result in severe metabolic imbalances. Viral infections can even contribute to liver failure by causing tissue damage through cytotoxicity and inflammation, as well as impaired drug metabolism. It is unclear to what extent these deleterious effects are due to viral manipulations or the host response. Here, we provide several mechanisms that illuminate the contribution of the host anti-viral responses to metabolic imbalances and tissue damage. First, we provide a mechanism by which activation of antiviral responses downregulates expression of nuclear hormone receptor retinoic X receptor- $\alpha$  (RXR $\alpha$ ) and its heterodimeric partner pregnane X receptor (PXR) in mice, resulting in suppression of

key downstream cytochrome P450 enzymes involved in metabolism of acetaminophen. Second, in addition to impairing drug metabolism, viral infections may result in liver damage by directly inducing cytopathic effects in infected cells, as well as by indirectly inducing host inflammatory responses that injure tissue. We have found that liver injury induced by mouse hepatitis virus is mediated by high-mobility group box 1 (HMGB1), a previously identified endogenous danger signal that can activate immune pathways. We further characterize an inhibitor of HMGB1, glycyrrhizic acid, as a hepatoprotective agent in both sterile and infectious challenges. Finally, in addition to effects on liver, we explore the cross-talk between anti-viral immune responses and cellular metabolism. Viruses rely on the metabolic network of the host cells for their replication, and while recent studies suggest that key genes in lipid metabolism are specifically modulated by viruses, it is unclear whether the host also modulates its own lipid metabolism to counter viral infection. We demonstrate that expression of fatty acid synthase (FASN), a critical gene in lipid metabolism, is regulated by the activation of innate immune pathways. Our findings offer novel mechanisms by which host-mediated downregulation of FASN can inhibit viral infections. Overall, these studies provide better understanding of the mechanisms underlying the cross-talk between innate immune pathways and metabolic imbalances.

The dissertation of Amir Ali Ghaffari is approved.

Jerzy Kupiec-Weglinski

Robert Modlin

Stephen Smale

Genhong Cheng, Committee Chair

University of California, Los Angeles

2013

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## VITA

### EDUCATION

- 09/2004 to 06/2005                      University of California, Irvine  
M.S., Biomedical Engineering, June 2005
- 09/2004 to 06/2005                      University of California, Irvine  
B.S., Biological Sciences, *Magna Cum Laude*, June 2004

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## **Chapter 1**

### **Introduction**

#### **Viral Infections and Metabolic Imbalances**

Metabolism (from Greek: μεταβολή *metabolē*, "change") refers to chemical transformations which provide energy in living organisms, allowing cells to grow and reproduce. Viruses, as non-living organisms, rely on the metabolic network of the host cell to provide energy and macromolecular precursors to fuel their replication. However, viral manipulations and host responses can lead to alterations in natural metabolic processes of host cells, resulting in various disorders. These metabolic diseases include defects in cholesterol metabolism, such as atherosclerosis, drug-related hepatotoxicity, bone metabolism defects, skin eruptions and diabetes<sup>1-5</sup>. Mechanisms by which viral infections lead to initiation and progression of various metabolic diseases remain unclear, though presumed to be through inflammation.

#### **A. Anti-Viral Innate Immune Responses**

The first line of defense against viral infections is the innate immune system. In response to invasion of a host by infectious agents, a wide range of immune signaling pathways are activated through immune surveillance mechanisms. This surveillance is carried out by pattern recognition receptors (PRRs), which recognize pathogen-specific, conserved molecular structures known as pathogen-associated molecular patterns (PAMPs). Several classes of PRRs, such as the Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and DNA receptors (cytosolic sensors for DNA), can detect various classes of PAMPs including proteins, lipids, carbohydrates and nucleic acids<sup>6,7</sup>. For instance, the lipopolysaccharide (LPS) found in cell walls of gram-negative bacteria leads to activation of TLR4. Some PRRs can specifically recognize viral PAMPs,

such as TLR3, localized in endosomes, which recognizes double-stranded RNA. Double-stranded RNA is also detected by cytosolic receptors such as Retinoic-acid-Inducible Gene I (RIG-I) and Melanoma Differentiation-Associated protein 5 (MDA-5)<sup>8,9</sup>.

In addition to PAMPs, cellular stress or injury can lead to the release of endogenous danger signals referred to as damage-associated molecular patterns (DAMPs), which can also activate PRRs. Sensing of PAMPs and DAMPs rapidly induces host immune responses, including inflammation, via the activation of complex signaling pathways which facilitate the eradication of pathogens. These pathways are mediated by various cytokines and chemokines, including the type I interferons (IFN), critical cytokines against viral infections. IFNs inhibit viral infections through induction of hundreds of IFN-stimulated genes (ISGs) with anti-viral effects. In macrophages, TLR3 and TLR4 activation leads to phosphorylation and activation of interferon regulatory transcription factor-3 (IRF3) through Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF). IRF3 is phosphorylated by the serine/threonine kinases, TANK binding kinase 1 (TBK1) or Inducible I $\kappa$ B kinase (IKKi) (54). In addition to TLR-TRIF dependent pathways, IRF3 is capable of being activated by cytoplasmic receptors, such as RIG-I and MDA5. Upon recognition of viral double stranded RNA, RIG-I and MDA5 can activate IRF3 and other transcription factors through the signaling adaptor molecule CARD adaptor inducing IFN-beta (CARDIF)<sup>10</sup>. IRF3 activation, in conjunction with NF- $\kappa$ B and AP-1, promotes transcription of Type I IFNs. While being the primary, or early, barrier to infectious agents, activation of innate immune pathways has been linked to various disorders such as autoimmune diseases as well as metabolic imbalances.



## **B. Viral Infections and Liver Metabolism**

The liver is the critical organ in the metabolism of a variety of substances, ranging from bile acids and fatty acids to steroids and drugs<sup>11</sup>. Thus, alterations in the overall homeostasis of hepatic metabolism in response to viral infections can quickly result in severe imbalances. Acute liver failure (ALF) is a life-threatening condition that results in altered metabolic disorder, coagulopathy, and alteration in mental status associated with high mortality<sup>11</sup>. Two leading causes of ALF are viral hepatitis and drug-induced hepatotoxicity, which can also result from impaired drug metabolism as a consequence of viral infection<sup>11</sup>. Altered states of drug metabolism were first noticed by physicians over 30 years ago when virally infected patients would exhibit new or pronounced adverse reactions to pharmacologic agents<sup>11,12</sup>. The effects of viral infections on liver metabolism are particularly striking in children, with viral infections often leading to severe adverse effects of drugs in young patients. For instance, Reye's Syndrome occurs primarily in children with aspirin (acetylsalicylic acid, ASA) therapy in the context of a viral infection, resulting in fatty liver degeneration and encephalopathy<sup>13</sup>. Not specific to any virus in particular, Reye's Syndrome has been linked to chickenpox, influenza A or B, adenoviruses, hepatitis A viruses, paramyxovirus, picornaviruses, reoviruses, herpesviruses, measles and varicella-zoster viruses<sup>14</sup>.

Furthermore, viruses can often lead to ALF even in the absence of drug-induced hepatotoxicity. In Asia, viral infections remain the most common cause of liver failure, with hepatitis B virus being an important cause of ALF in China<sup>12</sup>. Additionally, hepatitis C

virus infection is also an important cause of liver failure that can lead to hepatocellular carcinoma and fibrosis<sup>11</sup>. Viral infections remain an important contributor to metabolic disorders through direct injury of cells or induction of inflammation.

### *I. Viral infection and xenobiotic metabolism*

Exogenous or foreign substances, referred to as xenobiotics, are metabolized by the liver and usually modified to become more hydrophilic, allowing for easier excretion through urine or bile. Drug metabolism and excretion can involve up to three phases in the liver. During phase I, polar bodies are either introduced or unmasked by enzymes, including many cytochrome P450 family members (CYP450), through oxidation, reduction, hydrolysis, cyclization, or decyclization. This process can result in either activation or inactivation of a drug. During phase II, metabolites are conjugated with glycuronic acid, sulfonates, glutathione or amino acids by enzymes such as UDP-glucuronosyltransferase (UGT) isoforms, often important in drug detoxification. Finally, during phase III, the metabolites are transferred to the extracellular medium by transporters such as ATP-binding cassette (ABC) family members. Hepatic CYPs have been shown to be modulated during the response to infections and various inflammatory stimuli, resulting in altered drug metabolism and adverse effects<sup>15</sup>. Inflammatory cytokines including TNF $\alpha$ , IL1- $\beta$ , IL-6 and IFNs have all been shown to modulate particular sets of CYP450 enzymes. This selectivity is clinically important and implies the existence of multiple mechanisms for CYP450 regulation. Further, depending upon the nature of the drugs, nuclear hormone receptors and their target genes in the phase I, II and III drug metabolism systems can

differentially affect the accumulation of drug metabolites, leading to increases or decreases in toxicity.

Two well studied examples are the metabolism of ASA and acetaminophen (APAP). We previously demonstrated a novel mechanism by which viral infection leads to transcriptional down-regulation of nuclear hormone receptor retinoid X receptor  $\alpha$  (RXR $\alpha$ ) and its downstream CYP450 enzymes required for ASA metabolism<sup>16</sup>. RXR $\alpha$  forms heterodimers with various nuclear receptors including pregnane X receptor (PXR) and constitutive androstane receptor (CAR), which can regulate many CYP450 family members<sup>17-19</sup>. Downregulation of RXR $\alpha$  through IRF3 activation leads to decreased ASA clearance and enhanced liver toxicity. Given these adverse effects of ASA in patients with viral infections, APAP is often the first line of therapy to manage pain in children. To better understand the implications of this use of APAP, we evaluate the effects of crosstalk between nuclear hormone receptors and anti-viral pathways on metabolism and toxicity of APAP. During normal metabolism of APAP, bioactivation by CYP450 family members, CYP3A11/CYP3A4, CYP1A2 and CYP2E1, transforms APAP into N-acetyl-*p*-benzoquinone-imine (NAPQI)<sup>20</sup>. NAPQI is a highly reactive toxic intermediate that normally is conjugated with glutathione (GSH) by glutathione S-transferase (GST) enzymes, thereby creating a more hydrophilic form that is easily excreted. When APAP's metabolic pathway becomes saturated, NAPQI forms faster than it can be conjugated to GSH, allowing it to bind to nucleophilic cellular macromolecules and thus causing cell death and toxicity<sup>20</sup>. Mice with hepatocyte-specific ablation of RXR $\alpha$  have been demonstrated to be able to tolerate significantly higher dosages of APAP than wildtype mice without

development of hepatotoxicity<sup>17</sup>. In **Chapter 2**, we assess the effects of activation of anti-viral pathways on APAP metabolism and toxicity by identifying the mechanism underlying modulation of RXR/PXR and their downstream CYP enzymes involved in APAP metabolism.

## *II. Mechanisms of hepatic injury induced by viral infections*

In addition to altering CYP450 enzymes and hepatic drug metabolism, activation of innate immune responses can also contribute to liver injury. PRRs are activated in response to infection, triggering anti-viral responses and recruiting immune cells such as neutrophils and macrophages, which can lead to secondary inflammatory tissue damage. In addition to viral PAMPs, endogenous DAMPs can activate PRRs and induce enhanced inflammation and tissue injury. Induction of innate immune receptors in sterile liver injury models (i.e. APAP-induced hepatotoxicity) has been attributed to the release of endogenous DAMPs<sup>21-23</sup>; however, the involvement of DAMPs in mediating virus-induced hepatic injury and ALF remains unclear<sup>24</sup>. One DAMP of particular interest is high-mobility group box 1 (HMGB1), a nuclear nonhistone chromatin-binding protein involved in the regulation of gene transcription. In addition to its nuclear role, HMGB1 is passively released from damaged or necrotic tissue cells and is actively secreted by inflammatory cells in response to stress<sup>25,31</sup>, activating PRRs such as TLR2, TLR4, as well as receptor for advanced glycation end products (RAGE)<sup>25,26</sup>. Viral infections including West Nile virus, Dengue virus and HIV have been linked to elevated HMGB1 serum levels<sup>27-30</sup>. Moreover, HMGB1 has been shown to contribute to pathogenesis of various inflammatory diseases, including

sepsis, lupus, and various tissue injuries; however, its role in acute viral hepatitis and injury is not clear<sup>26</sup>. In **Chapter 3** we address whether viral hepatic injury is mainly caused by direct cytopathic effects of the virus or by secondary injury induced by released DAMPs.

Additionally, there are limited tools in managing ALF<sup>32</sup>. Patients presenting with symptoms of ALF require immediate intervention, and current treatment for ALF induced by APAP overdose is intravenous or oral N-acetylcysteine (NAC) therapy that must occur within the first 10 hours of APAP ingestion in order to be effective<sup>33</sup>. However, Liver transplantation is the only therapy currently proven to improve patient outcome, although liver transplantation success is affected by hepatic ischemia–reperfusion (I/R) injury, which leads to liver dysfunction and failure<sup>34</sup>. In **Chapter 3**, we study the effectiveness of a licorice extract used commonly in Traditional Chinese Medicine as an inhibitor of HMGB1 cytokine activity in suppressing liver injury induced by APAP, I/R as well as viral infections.

### **C. Viral Infections and Cellular Metabolism**

In addition to drug metabolism, viral infection can alter the cellular metabolism of the host, including changes in lipid and glucose metabolism. Recent studies characterize different viruses that have evolved to alter the host's metabolism in the favor of their replication. For instance, human cytomegalovirus (HCMV) infection upregulates fatty acid biosynthesis and glycolysis, while HSV-1 appears to inhibit glycolysis<sup>35</sup>. However, it is not

clear whether or how cells alter their metabolism as a part of innate immune responses during infections.

### *I. Lipid metabolism and viral infections*

There are examples of viral proteins that upregulate specific lipid metabolic enzymes. Fatty acid synthase (FASN) expression is induced by the EBV immediate-early protein BRLF1 and is required for lytic viral gene expression<sup>36</sup>. Additionally, recent studies suggest that both Kaposi Sarcoma Herpes virus (KSHV) and West Nile virus induce lipogenesis<sup>37,38</sup>. These findings suggest not only that lipid metabolism plays an important role in viral infections, but also some viruses may have gained the ability to modulate specific host metabolic genes. However, it is unclear whether the host also has the ability to modulate its own lipid metabolism as a mechanism against viral infections.

One metabolic disorder linked to activation of immune pathways is atherosclerosis, in which activation of PRRs including TLR4 and TLR2 are thought to contribute to the formation of foam cells, macrophages with increased lipid content. In fact, administration of LPS in mice has been shown to induce lipogenesis in peritoneal macrophages<sup>40</sup>. However, the molecular mechanism by which innate immune pathways can modulate cellular lipid metabolism is not well characterized. Thus, in **Chapter 4**, we discuss how activation of different innate immune signaling pathways can alter lipid metabolism. Furthermore, we assess the effects of such regulation upon anti-viral as well as anti-bacterial responses.

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## **Chapter 2**

### **PolyI:C Suppresses APAP-Induced Hepatotoxicity Independent of Type I IFNs and TLR3**

# Polyinosinic-Polycytidylic Acid Suppresses Acetaminophen-Induced Hepatotoxicity Independent of Type I Interferons and Toll-Like Receptor 3

Amir A. Ghaffari,<sup>1\*</sup> Edward K. Chow,<sup>2\*</sup> Shankar S. Iyer,<sup>2</sup> Jane C. Deng,<sup>3</sup> and Genhong Cheng<sup>1,2</sup>

Viral infections are often linked to altered drug metabolism in patients; however, the underlying molecular mechanisms remain unclear. Here we describe a mechanism by which activation of antiviral responses by the synthetic double-stranded RNA ligand, polyinosinic-polycytidylic acid (polyI:C), leads to decreased acetaminophen (APAP) metabolism and hepatotoxicity. PolyI:C administration down-regulates expression of retinoic X receptor- $\alpha$  (RXR $\alpha$ ) as well as its heterodimeric partner pregnane X receptor (PXR) in mice. This down-regulation results in suppression of downstream cytochrome P450 enzymes involved in conversion of APAP to its toxic metabolite. Although the effects of polyI:C on drug metabolism are often attributed to interferon production, we report that polyI:C can decrease APAP metabolism in the absence of the type I interferon receptor. Furthermore, we demonstrate that polyI:C can attenuate APAP metabolism through both its membrane-bound receptor, Toll-like receptor 3 (TLR3), as well as cytoplasmic receptors. **Conclusion:** This is the first study to illustrate that *in vivo* administration of polyI:C affects drug metabolism independent of type I interferon production or in the absence of TLR3 through crosstalk between nuclear receptors and antiviral responses. (HEPATOLOGY 2011;53:2042-2052)

Altered states of drug metabolism were first noticed by physicians over 30 years ago when virally infected patients would exhibit new or pronounced adverse reactions to pharmacologic agents.<sup>1</sup> Such perturbations in drug metabolism have been linked to the effects of infections or inflammatory stimuli on altering the activities and expression of various hepatic cytochrome P450 (CYP) enzymes.<sup>2,3</sup> CYPs are responsible for pharmacological activation or inactivation of many drugs as well as their clearance from the circulation.<sup>4</sup> In light of the considerable progress in our understanding of host antiviral innate

immune responses and pathways during the last decade, it is surprising that little recent research has been conducted on the mechanisms by which CYP enzymes are modulated during viral infections.

Hepatic CYPs have been shown to be affected differently in response to various inflammatory stimuli.<sup>4</sup> This selectivity is important clinically and implies the existence of multiple mechanisms for CYP regulation. We previously demonstrated a novel mechanism by which viral infection leads to transcriptional down-regulation of nuclear hormone receptor retinoid X receptor  $\alpha$  (RXR $\alpha$ ) and its downstream CYP enzymes

*Abbreviations:* ALT, alanine transaminase; APAP, acetaminophen; ASA, aspirin; CAR, constitutive androstane receptor; CYP, cytochrome P450; IFN, Type I interferon; IFNAR, Type I interferon receptor-1; IL-1, interleukin-1; MDA5, melanoma-differentiation-associated gene 5; NAPQI, N-acetyl-p-benzoquinone imine; PCN, pregnenolone 16 $\alpha$ -carbonitrile; PolyI:C, polyinosinic-polycytidylic acid; PXR, pregnane X receptor; RIG-I, retinoic acid-inducible gene-I; RXR  $\alpha$ , retinoid X receptor  $\alpha$ ; SXR, steroid xenobiotic receptor; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; TLR3, Toll-like receptor 3; VSV, vesicular stomatitis virus.

From the <sup>1</sup>Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, CA; <sup>2</sup>Molecular Biology Institute, University of California, Los Angeles, CA; <sup>3</sup>Division of Pulmonary and Critical Care Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA.

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\*These authors contributed equally to this work.

Address reprint requests to: Genhong Cheng, Ph.D., Professor, Department of Microbiology, Immunology & Molecular Genetics, University of California Los Angeles, 615 Charles Young Dr S., 210A BSRB, Los Angeles, CA 90095. E-mail: gcheng@mednet.ucla.edu; fax: (310) 206-3865.

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required for aspirin (ASA) metabolism.<sup>5</sup> Our results provided an explanation for how ASA consumption can cause Reye's syndrome, a condition where children with viral infections develop hepatotoxicity and neurological side effects.<sup>6</sup> Given these adverse effects of ASA in patients with viral infections, acetaminophen (APAP) is often the first line of therapy to manage pain in children.<sup>7</sup> In this study we evaluate the effects of crosstalk between nuclear hormone receptors and antiviral pathways on metabolism and toxicity of APAP.

APAP-induced toxicity is the leading cause of acute hepatic failure in the United States and many other developed countries worldwide.<sup>8</sup> APAP is metabolized by CYP family members CYP1A2, CYP2E1, and CYP3A11 (murine homolog of human CYP3A4) into N-acetyl-p-benzoquinone-imine (NAPQI) in mice.<sup>9,10</sup> NAPQI is a highly reactive intermediate that is normally conjugated to glutathione by glutathione S-transferase enzymes in order to become more excretable.<sup>11</sup> Accumulation of NAPQI causes cell death and toxicity through covalent binding to cysteine groups on proteins and formation of APAP-protein adducts. The generation of these APAP-protein adducts has been correlated with hepatotoxicity through oxidation of NAPQI-conjugated proteins.<sup>12</sup>

Among the CYP enzymes involved in NAPQI generation, CYP3A isoforms and CYP1A2 are subject to regulation by nuclear hormone receptors.<sup>13</sup> Nuclear hormone receptors are transcription factors that are activated by a wide range of molecules, such as lipids, cholesterol, bile acids, and xenobiotics. RXR $\alpha$  is an important nuclear hormone receptor and acts as a heterodimer with other nuclear hormone receptors such as pregnane X receptor (PXR) and constitutive androstane receptor (CAR).<sup>14</sup> RXR/PXR heterodimer is an important regulator of CYP3A isoforms; however, the involvement of this complex in transcriptional regulation of CYP1A2 is not well established. CYP1A2 is mainly regulated by aryl hydrocarbon receptor; however, PXR-deficient mice and hepatocyte RXR $\alpha$ -deficient mice express lower hepatic messenger RNA (mRNA) levels of CYP1A2 and CYP3A11 compared to wildtype mice, particularly after APAP administration.<sup>15-17</sup> Consequently, these knockout mice are resistant to APAP-induced hepatotoxicity.<sup>15,17</sup> Thus, any changes in the expression of these nuclear hormone receptors in response to activation of antiviral pathways could potentially alter APAP-induced toxicity through modulation of NAPQI generation.

Because viral infections can lead to significant induction of type I interferons (IFN), many groups have used IFN or IFN-inducing agents to study the

impact of activation of antiviral responses on drug metabolism.<sup>18</sup> One such agent is polyinosinic-polycytidylic acid (polyI:C), a viral double-stranded RNA (dsRNA) mimetic, which has been shown to impair drug metabolism.<sup>19</sup> Although the effects of polyI:C on drug metabolism have been ascribed to its ability to induce IFN, there has not been a conclusive study supporting this hypothesis. PolyI:C does induce other cytokines such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin-1 (IL-1) that could affect activity or expression of CYPs. IFNs as well as TNF- $\alpha$  and IL-1 have all been shown to alter drug metabolism when administered in patients or in animal models.<sup>4,20</sup>

Additionally, viral dsRNA and polyI:C are sensed by the endosomal receptor, Toll-like receptor (TLR3), as well as recently discovered cytoplasmic receptors, such as RNA helicase retinoic acid-inducible gene-I (RIG-I).<sup>21</sup> These receptors have cell-type and tissue-specific roles in sensing polyI:C; however, it has not been characterized which receptors are involved in mediating the effects of polyI:C on hepatic drug metabolism.<sup>22</sup>

Here we used polyI:C and vesicular stomatitis virus (VSV), a dsRNA virus, to study how activation of antiviral responses can modulate APAP metabolism and hepatotoxicity. We provide a mechanism by which *in vivo* administration of polyI:C suppresses APAP-induced hepatotoxicity independent of IFN production or in the absence of TLR3 through transcriptional down-regulation of RXR $\alpha$  and PXR and their downstream CYPs.

## Materials and Methods

**Animal Experiments.** Age-matched 6 to 9-week-old male mice were used for all experiments. Sources for the different strains of mice can be found in the Supporting Material. All experiments were performed in accordance with guidelines from the University of California, Los Angeles Institutional Animal Care and Use Committee. For ASA-induced toxicities, mice were given ASA (180 mg/L, Sigma-Aldrich) in drinking water for 5 days. For APAP hepatotoxicity studies, mice were fasted for 18 hours and then administered vehicle (normal saline, 0.9% NaCl) or APAP (175-600 mg/kg, Sigma-Aldrich) by intraperitoneal injections (i.p.). For serum and histological studies, mice were sacrificed at 6-7 hours post-APAP administration and serum and liver samples were retrieved. For polyI:C and VSV studies, mice were injected with saline or polyI:C (100  $\mu$ g, Invivogen) or VSV (2.5e7 plaque-forming units [pfu] i.p. 24 hours prior to APAP treatment. Green fluorescent protein

(GFP)-tagged VSV was a kind gift from G. Barber (University of Miami, Miami, FL).

To study the effects of pregnenolone 16 $\alpha$ -carbonitrile (PCN) on APAP treatment, mice were injected (i.p.) with PCN (75 mg/kg, Sigma-Aldrich) or control (1% DMSO, corn oil) 24 hours prior to APAP treatment. To study the effects of ethanol (EtOH) on APAP treatment, mice were given 20% EtOH (Gold Shield Chemical) in water ad libitum for 5 days prior to APAP administration. PolyI:C treatment for these experiments occurred at days 3 and 5. Serum alanine aminotransferase (ALT) levels were determined using the manufacturer's protocol (TECO Diagnostics).

**Histology and Immunohistochemistry Analysis.** For hematoxylin and eosin (H&E) staining, liver samples were fixed in formalin for 48 hours. H&E staining was performed at the UCLA Tissue Procurement Core Laboratory (TPCL). APAP-protein adduct staining was done using anti-APAP rabbit antibodies (Biogenesis/AbDserotec) as described.<sup>23</sup>

**Reverse-Transcription Polymerase Chain Reaction (PCR) Analysis.** For quantitative real-time PCR (Q-PCR), total liver RNA was isolated and cDNA was synthesized according to the manufacturer's protocol: Trizol (RNA) and Bio-Rad iScript (cDNA). PCR reactions were set up using Quantise Master Mix (2X SensiMix SYBR and Fluorescein).

## Results

**VSV Infection Suppresses APAP-Induced Hepatotoxicity.** Mice were given ASA (180 mg/L) in their drinking water for 5 days. Mice that were infected with VSV (2.5e7 pfu) at day 1 experienced higher ASA-induced hepatotoxicity compared to uninfected mice. This is evidenced by higher levels of serum ALT in mice treated with VSV and ASA compared to the ASA only group (Fig. 1A). Histological analysis of livers from VSV-infected mice further evidenced hepatic injury as indicated by increased fat (steatosis) on oil-red staining (data not shown) as well as H&E staining sections (Fig. 1B).

Mice were infected with VSV (2.5e7 pfu) and toxic doses of APAP (350 mg/kg) were administered 24 hours later to VSV-infected mice and uninfected controls, and serum ALT and histological evaluation of liver tissue were used as markers for hepatotoxicity. Mice that were infected with VSV (2.5e7 pfu) 24 hours prior to receiving APAP (350 mg/kg) had lower levels of serum ALT compared to uninfected controls 6 hours after APAP administration (Fig. 1C). Furthermore, histological evaluation of livers from VSV-infected mice did not reveal necrosis in contrast to

mice treated with APAP alone (Fig. 1D). These data demonstrate that concomitant VSV infection suppressed APAP-induced hepatotoxicity, which is opposite to what we observed with ASA.

**PolyI:C Pretreatment Suppresses APAP-Induced Hepatotoxicity.** In order to determine whether our observations are applicable to viruses other than VSV, mice were pretreated with polyI:C for 24 hours prior to APAP administration. APAP (600 mg/kg) was administered with or without 24-hour polyI:C pretreatment and the weight and body temperature of animals were monitored for 5 days. As seen in Fig. 2A, mice that received polyI:C had a higher survival rate than those given APAP alone. Mice pretreated with polyI:C exhibited lower serum ALT levels compared to untreated controls in response to 6 hours of APAP (350 mg/kg) treatment (Fig. 2B) and evidenced fewer necrotic foci on histological analysis (Fig. 2C). Administration of polyI:C 1 hour before APAP treatment did not have any effects on APAP-induced injury (data not shown).

**PolyI:C-Mediated Repression of Nuclear Hormone Receptors and Downstream APAP-Metabolizing Genes.** CYP enzymes that contribute to the metabolism of APAP to NAPQI, such as CYP3A11 and CYP1A2, have been identified as targets of the nuclear hormone receptors RXR $\alpha$ , PXR.<sup>15,24,25</sup> Because we previously demonstrated that the innate immune response to dsRNA inhibits RXR $\alpha$  expression, we assessed the effects of polyI:C treatment on these nuclear hormone receptors as well as their downstream CYPs involved in APAP-mediated toxicity. Following i.p. injections of polyI:C, both hepatic RXR $\alpha$  and PXR expressions were down-regulated at 24 hours (Fig. 3A). Similarly, the mRNA expression levels of CYP3A11 and CYP1A2 were also suppressed after polyI:C treatment, whereas CYP2E1 mRNA levels were not altered significantly (Fig. 3B, Supporting Fig. 1).

One mechanism by which NAPQI mediates hepatotoxicity is through covalent binding with cysteine groups on proteins to form APAP-protein adducts.<sup>26</sup> Immunofluorescent analysis demonstrated that APAP-induced hepatotoxicity correlated with increased formation of APAP-protein adducts and NAPQI generation as indicated by the representative images (Fig. 3C) and the ImageJ analysis of different liver sections in each group (Supporting Fig. 2).<sup>23</sup> Liver sections of polyI:C pretreated mice did not exhibit APAP-protein adduct formation, suggesting decreased APAP metabolism. Decreased levels of APAP-protein adduct formation can potentially correlate with decreased NAPQI generation due to transcriptional suppression of CYP3A11 and CYP1A2 in response to polyI:C treatment. These findings suggest

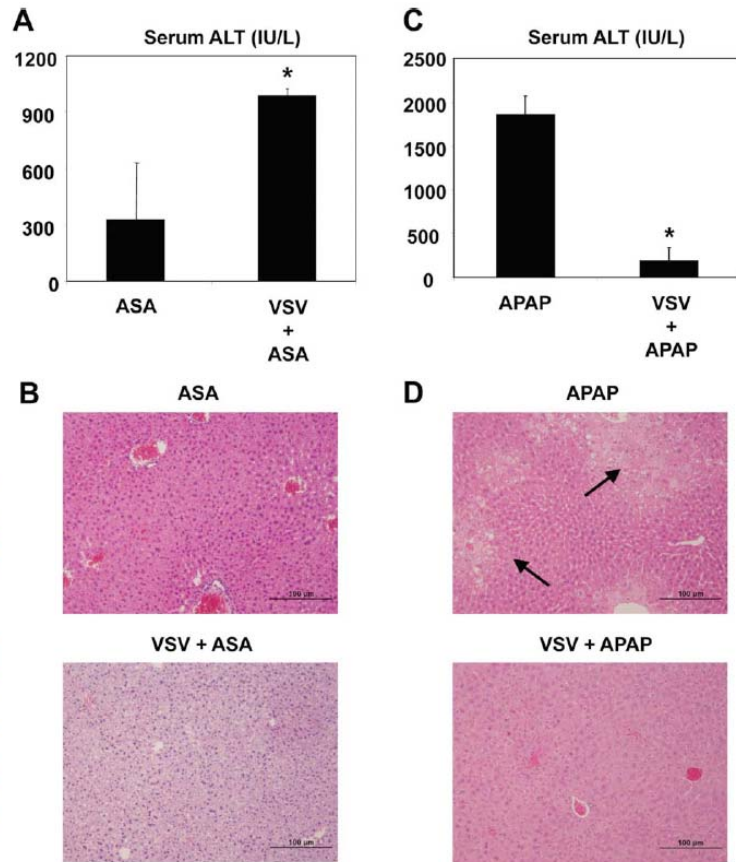


Fig. 1. Opposite effects of VSV infection on ASA and APAP-induced hepatotoxicity. (A,B) Mice were infected with VSV ( $2.5 \times 10^7$  pfu, i.p.) at day 1 and given ASA (180 mg/L) in drinking water for 5 days. At day 5 animals were euthanized and serum samples were collected and analyzed for serum ALT (A), and liver samples were fixed and stained with H&E (B). (C,D) For the APAP studies, mice were infected with VSV ( $2.5 \times 10^7$  pfu, i.p.) 24 hours prior to drug treatment. Animals were fasted for 18 hours before administration of APAP (350 mg/kg, i.p.). Serum samples were collected 6 hours after APAP administration for ALT analysis (C) and liver samples were fixed and stained with H&E (D). (n = 4, mean  $\pm$  standard deviation [SD], \* $P < 0.05$  compared to uninfected).

that the protective effect of polyI:C against APAP-mediated hepatotoxicity could result from the repression of nuclear hormone receptors and their target genes.

**PolyI:C Protects Against Synergistic Xenobiotic/APAP-Induced Hepatotoxicity.** Previous studies have demonstrated that the PXR/RXR $\alpha$  activator PCN can increase APAP-hepatotoxicity through induction of CYP3A11 and CYP1A2 in mice.<sup>27</sup> If polyI:C-mediated protection against APAP hepatotoxicity is caused through the repression of nuclear hormone receptors and their target CYP genes, then polyI:C should also be effective at protecting against nuclear hormone receptor enhanced APAP hepatotoxicity. Pretreatment of mice with PCN led to induction of CYP3A11, an effect which was suppressed in the presence of polyI:C (Fig. 4A). Consequently, PCN pretreatment greatly enhanced serum ALT levels following treatment with normally nontoxic levels of APAP (Fig. 4B). Administration of polyI:C abrogated APAP-induced hepatotoxicity which was enhanced by PCN. This was seen by

both serum ALT measurement and histology (Fig. 4B,E). Additionally, polyI:C administration protected mice against PCN-enhanced APAP lethality, further supporting the mechanism where polyI:C protection occurs through repression of nuclear hormone receptors and downstream CYPs (Fig. 4C).

Another example of hepatotoxicity from APAP in combination with CYP-inducing substances is APAP therapy following regular alcohol ingestion, which induces expression of CYP2E1 and CYP3A isoforms and enhances sensitivity to APAP.<sup>28,29</sup> Indeed, polyI:C was effective at preventing ethanol from potentiating APAP induction of serum ALT levels and hepatotoxicity (Fig. 4D,E).

**PolyI:C-Mediated Suppression of APAP-Induced Hepatotoxicity Is Independent of Type I IFN.** PolyI:C was first utilized to study the effects of viral infections on drug metabolism as an interferon inducing agent.<sup>19</sup> However, there has not been a conclusive study which addresses whether the effects of polyI:C



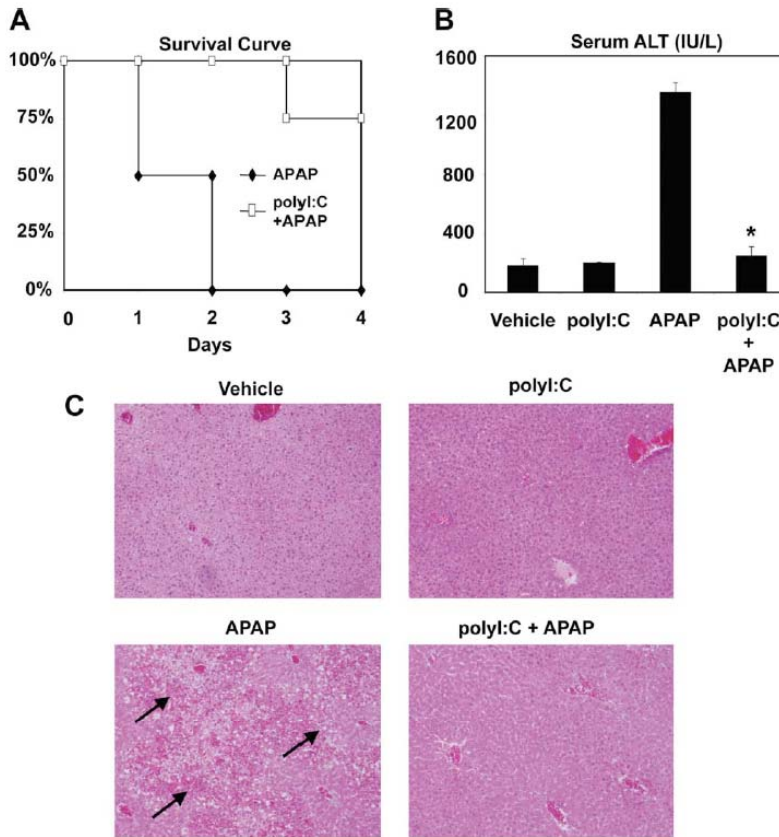


Fig. 2. PolyI:C protection against APAP-induced hepatotoxicity. (A) Wildtype ( $n = 8$ ) were treated with saline or polyI:C (100  $\mu\text{g}$ , i.p.) 24 hours prior to treatment with APAP (600 mg/kg, i.p.). Mice were followed for 5 days posttreatment. (B) Wildtype mice ( $n = 4$ ) were treated with saline or polyI:C (100  $\mu\text{g}$ , i.p.) 24 hours prior to treatment with APAP (350 mg/kg, i.p.). Six hours post-APAP treatment, mice were sacrificed and serum samples were collected and analyzed for serum ALT levels (TECO Diagnostic). (C) Liver samples were formalin-fixed and stained with H&E. Arrows indicate foci of necrosis ( $n = 4$ , mean  $\pm$  SD, \* $P < 0.05$  compared to polyI:C untreated).

on drug metabolism are truly dependent on IFN induction. In our model, polyI:C administration induced transcription of Type I IFNs such as IFN $\beta$  in the liver after 24 hours (Fig. 5A). Thus, we evaluated the contribution of IFN in polyI:C-mediated protection against APAP-induced hepatotoxicity in mice deficient in IFN signaling. Because IFN receptor-1 and IFN receptor-2 need to heterodimerize for effective IFN signaling, IFN signaling is absent in Type I interferon receptor-1 (IFNAR) deficient mice.<sup>30</sup> In our model, polyI:C was able to reduce RXR $\alpha$  and PXR mRNA levels and their downstream CYPs in IFNAR-deficient mice similar to wildtype mice after 24 hours (Fig. 5B, Supporting Fig. 3). Furthermore, in mice deficient for IFNAR, polyI:C was still able to attenuate APAP metabolism and toxicity (Fig. 5C). In order to confirm that polyI:C's protective effect against APAP toxicity in IFNAR deficient mice were through decreased metabolism, APAP adduct protein levels were measured. Liver sections of polyI:C pretreated wildtype and IFNAR deficient mice did not exhibit

APAP-protein adduct formation, suggesting decreased APAP metabolism (Fig. 5D).

We then analyzed two additional cytokines induced by polyI:C, TNF- $\alpha$  and IL-1, which have been shown to modulate CYP expression when administered in patients or animals.<sup>31</sup> However, both TNF- $\alpha$ -deficient mice (*Tnfa*<sup>-/-</sup>) and IL-1 receptor-deficient mice (*IL-1R*<sup>-/-</sup>) were protected by polyI:C against APAP-induced hepatotoxicity (Supporting Fig. 4).

**PolyI:C Can Attenuate APAP-Induced Hepatotoxicity Through Both Its Extracellular and Cytoplasmic Receptors.** TLR3 is the primary membrane-bound receptor for mediating the innate immune response to polyI:C.<sup>21</sup> In the absence of TLR3, APAP-induced hepatotoxicity was suppressed when mice were pretreated with polyI:C (Fig. 6B). This finding was confirmed using mice deficient in TRIF, the adaptor protein required for TLR3 signaling<sup>18</sup> (Fig. 6A). Moreover, mice lacking Cardif, the adaptor protein for cytoplasmic receptors of polyI:C, were also protected against APAP-induced hepatic injury<sup>18</sup> (Fig. 6C).

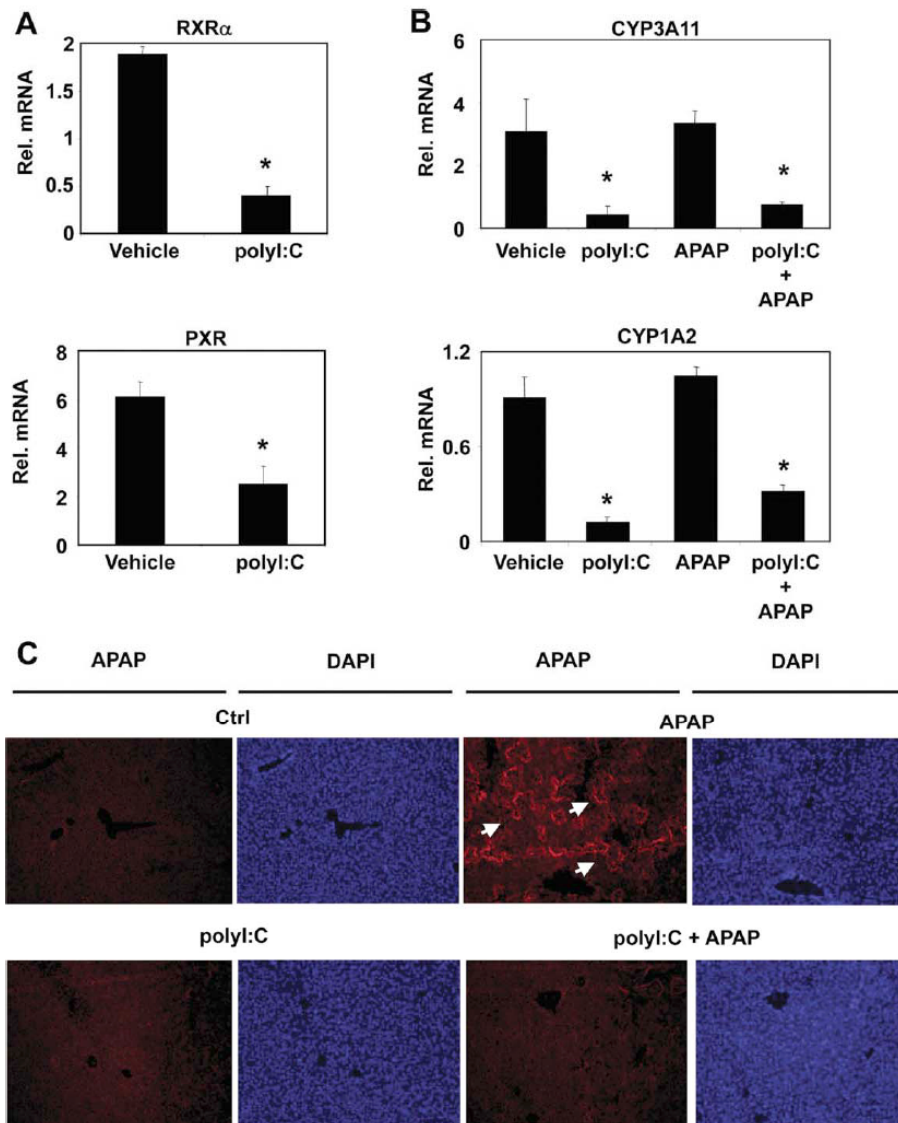


Fig. 3. PolyI:C decreases liver nuclear hormone receptors and their downstream genes and reduces APAP adduct protein formation. (A) Wild-type mice were treated with saline or polyI:C (100  $\mu$ g, i.p.). Twenty four hours posttreatment, liver RNA was isolated and analyzed by Q-PCR. (B,C) Wildtype mice were treated with saline or polyI:C (100  $\mu$ g, i.p.) 24 hours prior to treatment with APAP (350 mg/kg, i.p.). Six hours post-APAP treatment, mice were sacrificed and liver samples were collected. Liver RNA was isolated and analyzed by Q-PCR (B) and formalin-fixed liver samples were indirectly stained for APAP-bound proteins and the nucleus was identified using DAPI stain (C) ( $n = 4$ , mean  $\pm$  SD, \* $P < 0.05$  compared to polyI:C untreated).

However, polyI:C pretreatment in double knockout mice deficient in both Cardif and TLR3 failed to protect against APAP-induced hepatotoxicity (Fig. 6D). These findings suggest that membrane-bound and cytosolic receptors of polyI:C play complementary roles in this animal model.

## Discussion

There are many documented examples of impaired drug metabolism in patients with viral infections.<sup>1,2</sup> These effects have been attributed to modulation of CYP enzymes in response to activation of the innate

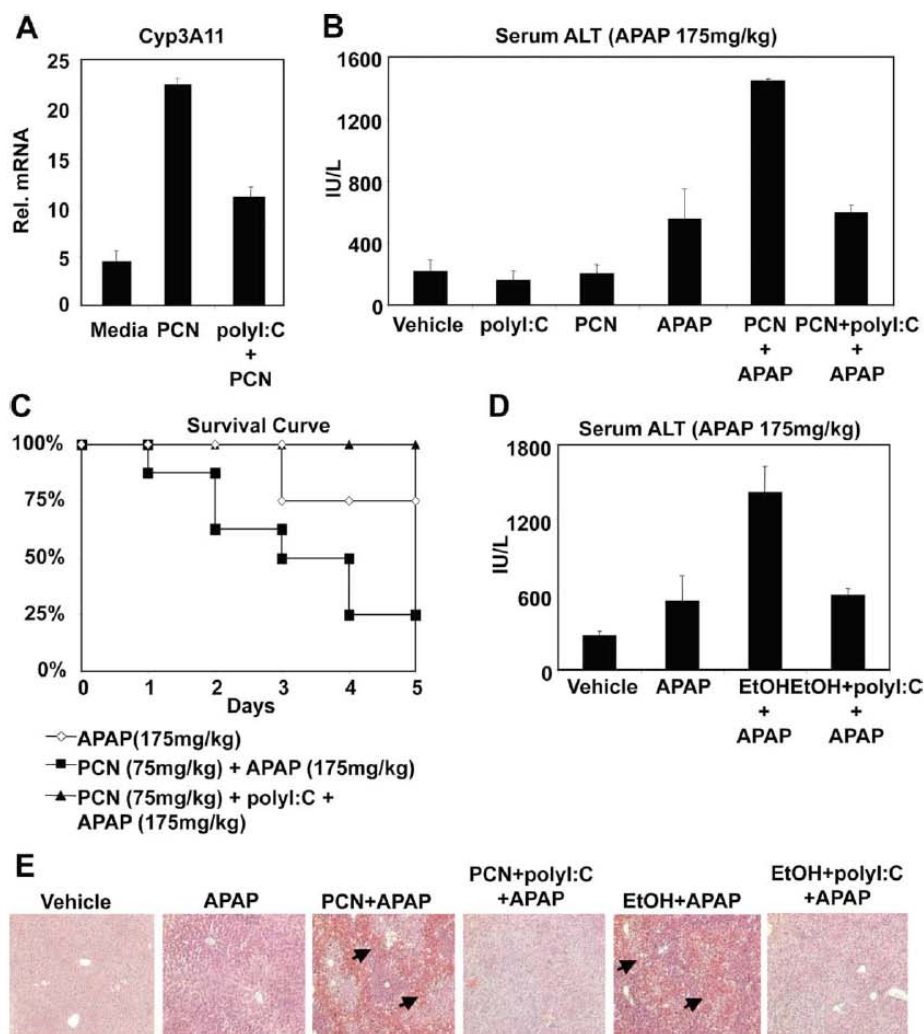


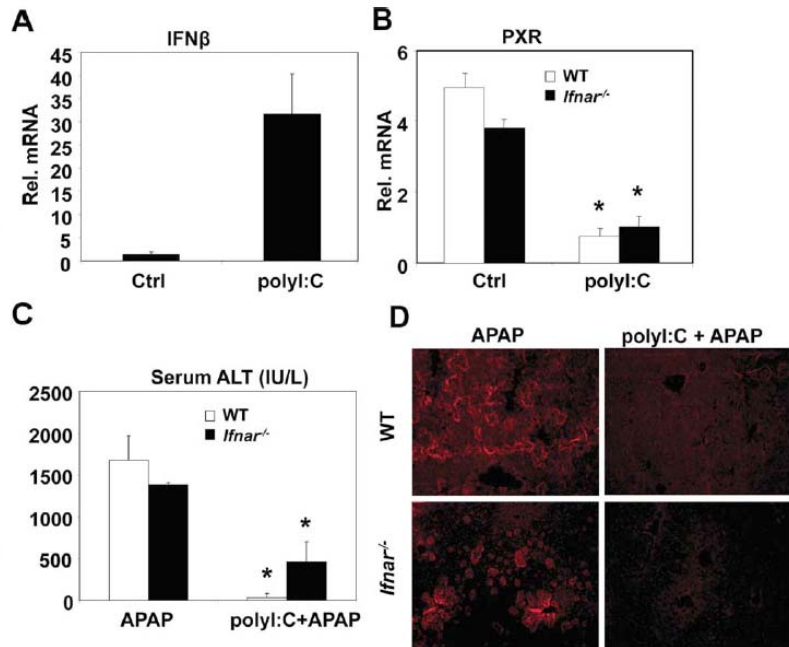
Fig. 4. PolyI:C protects against APAP-mediated hepatotoxicity promoted by PCN or EtOH. (A) Mice were treated with saline, PCN (75 mg/kg, i.p.), or PCN and polyI:C (100  $\mu$ g, i.p.). After 24 hours, liver RNA was isolated and analyzed by Q-PCR. (B) Mice were treated with saline, polyI:C (100  $\mu$ g, i.p.), or PCN (75 mg/kg, i.p.) 24 hours prior to treatment with APAP (175 mg/kg, i.p.) (n = 4, mean  $\pm$  SD, \*P < 0.05 compared to polyI:C untreated). (C) Mice (n = 8) were treated with saline or polyI:C (100  $\mu$ g, i.p.) and PCN (75 mg/kg, i.p.) 24 hours prior to administration of APAP (175 mg/kg, i.p.). Mice were followed for 5 days following treatment. (D) Alternatively, wildtype mice were given 20% EtOH *ad libitum* for 5 days, and saline or polyI:C were injected on days 3 and 5. Mice were fasted 18 hours prior to APAP treatment (175 mg/kg) on day 6. Six hours after APAP treatment, serum samples were isolated and analyzed for ALT levels. (E) Mice were treated as in (B,D). Six hours post-APAP treatment, mice were sacrificed and liver samples were isolated. Liver samples were formalin-fixed and stained with H&E. Arrows indicate foci of necrosis.

immune system.<sup>4</sup> Although the activity and expression levels of CYPs have been shown to be altered during viral infection or inflammatory states, the underlying molecular mechanisms are not well characterized.

Our previous work identified a potential mechanism of how innate immune activation can lead to enhanced

ASA-induced hepatotoxicity through down-regulation of CYP3A11, the CYP enzyme required for the clearance of the toxic intermediate of ASA.<sup>5</sup> PolyI:C stimulation can lead to transcriptional down-regulation of RXR $\alpha$  and subsequently decreasing the presence of RXR $\alpha$  on the PXR/RXR ER6 binding region on the

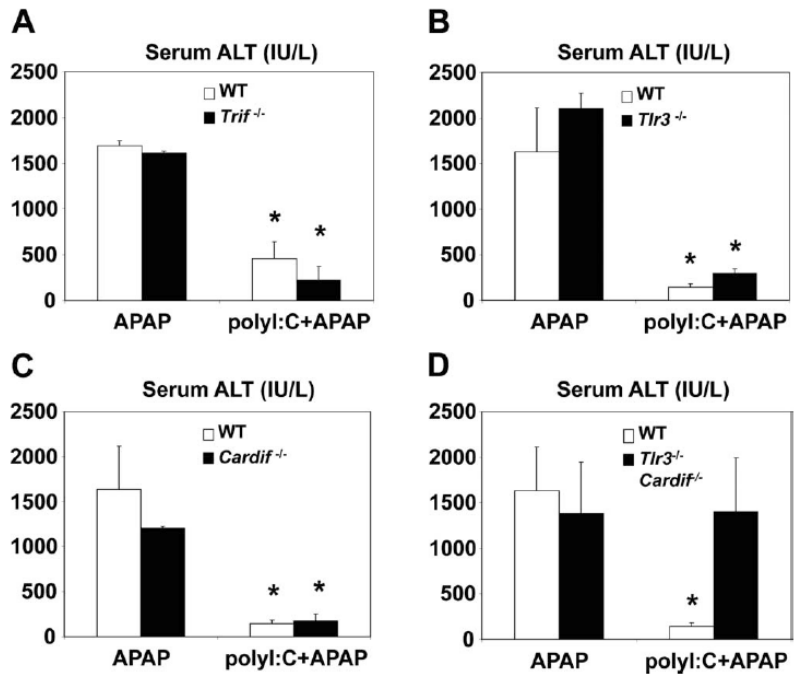
Fig. 5. Involvement of IFN and cytokines in polyI:C protection against APAP-induced hepatotoxicity. (A,B) Mice were treated with saline or polyI:C (100  $\mu$ g, i.p.). Twenty-four hours posttreatment, liver RNA was isolated and analyzed by Q-PCR (n = 4, mean  $\pm$  SD, \**P* < 0.05 compared to polyI:C untreated). (C,D) Wildtype and *Ifnar*<sup>-/-</sup> mice were treated with polyI:C and APAP as described above in Fig. 2B. Six hours after APAP administration, blood and liver samples were collected. Serum samples were analyzed for ALT levels (C) and formalin-fixed livers were indirectly stained for APAP-bound proteins (D) (n = 4, mean  $\pm$  SD, \**P* < 0.05 compared to polyI:C untreated).



promoter of *CYP3A4* (human homolog of *Cyp3a11*) in Huh7 cells.<sup>5</sup> Here we studied the effects of such crosstalk between antiviral responses and nuclear hor-

more receptors on the transcriptional regulation of CYPs involved in the metabolism and toxicity of another commonly used analgesic, APAP.

Fig. 6. PolyI:C can decrease APAP hepatotoxicity through either TLR3 or Cardif pathways. Wildtype, *Tlr3*<sup>-/-</sup>, *Tlr3*<sup>-/-</sup>, *Cardif*<sup>-/-</sup>, and *Tlr3/Cardif* double knockout mice were treated with polyI:C and APAP as described above. Six hours after APAP treatment, mice were sacrificed and serum samples were collected and analyzed for ALT levels (n = 4, mean  $\pm$  SD, \**P* < 0.05 compared to polyI:C untreated).



In this study we report that VSV infection as well as polyI:C pretreatment results in attenuated APAP-induced hepatotoxicity in mice. Early studies have also reported similar phenomena; however, the molecular mechanism by which such protection is mediated was never studied in detail.<sup>32</sup> Our findings suggest that this protection against APAP-induced toxicity can potentially be due to inhibition of nuclear hormone receptor-regulated metabolism, as we have shown that polyI:C suppresses expression of PXR, RXR $\alpha$ , and their target genes, CYP3A11 and CYP1A2. The transcription of the other CYP involved in APAP metabolism, CYP2E1, however, was not altered, as this gene is not downstream of any known nuclear hormone receptors.<sup>33</sup> As the result of these modulations, polyI:C pretreatment can potentially decrease generation of NAPQI, the toxic metabolite of APAP.

Given the key role of IFN in proper antiviral responses, we then set out to assess the involvement of IFN production in the suppression of APAP metabolism observed with polyI:C. The reported effects of polyI:C on drug metabolism were previously attributed to its ability to induce IFN.<sup>19</sup> Here we report that in IFNAR-deficient mice, polyI:C administration is still able to suppress expression of RXR $\alpha$ , PXR, and downstream CYPs. It is important to note that IFNAR-deficient mice were equally sensitive to APAP-induced hepatotoxicity as wildtype mice in our APAP model, in contrast to mice deficient in the Type II IFN receptor, which are protected against APAP-induced toxicity.<sup>34</sup> In other liver injury models, such as ischemia reperfusion injury, IFNAR-deficient mice are less susceptible to hepatic injuries.<sup>35</sup> This observation suggests that different innate immune pathways are activated during hepatic injuries induced by drugs (e.g., APAP) or ischemia reperfusion that could enhance tissue damage.

A recent study that can complement our findings also demonstrates suppressed APAP toxicity in mice infected with recombinant deficient adenoviruses, DNA viruses.<sup>36</sup> They suggest that polyI:C's protective effects are due to down-regulation of CYP2E1 and decreased generation of NAPQI. In our model, CYP2E1 mRNA levels are not altered after polyI:C treatment. One possible explanation is that replication deficient adenoviral infections can induce type II interferons, which have been shown to suppress CYP2E1 expression and activity in mice.<sup>37,38</sup> However, here we studied the effects of activation of antiviral pathways in response to dsRNA stimulants such as VSV and polyI:C, which do not lead to type II interferon induction.

Additionally, we evaluated the involvement of inflammatory cytokines induced by polyI:C in the metabolism and toxicity of APAP. Activation of innate immune cells during viral infections can lead to the release of TNF- $\alpha$  and IL-1.<sup>39</sup> Previous studies have demonstrated the effects of TNF- $\alpha$  or IL-1 treatment on CYPs, with activity and expression of different CYPs being suppressed or enhanced by either TNF- $\alpha$  or IL-1.<sup>4</sup> Thus, induction of these cytokines during viral infections could potentially explain the mechanism by which polyI:C pretreatment suppresses APAP-induced toxicity. However, our results illustrate that mice deficient in TNF- $\alpha$  or IL-1 receptors are still protected against APAP-induced hepatotoxicity after polyI:C pretreatment. There are other potential factors activated by polyI:C which may contribute to this protective phenotype that we did not explore. It has been suggested that activation of the p65 nuclear factor kappa B (NF- $\kappa$ B) subunit can result in the direct inhibition of RXR $\alpha$  DNA binding capabilities and thus repression of RXR $\alpha$ -regulated genes.<sup>40</sup> Although this proposed mechanism is interesting, the experimental support for this hypothesis is primarily found *in vitro*. Future *in vivo* experiments will provide greater insight into the role that NF- $\kappa$ B may play in repression of genes downstream of nuclear hormone receptors and innate immune response-mediated protection against APAP hepatotoxicity.

We also examined the induction of known hepatoprotective genes against APAP-induced hepatotoxicity. Heme oxygenase-1 (HO-1) and metallothionein have been shown to play protective roles against APAP toxicity; however, the role of iNOS remains controversial.<sup>41-43</sup> We found that polyI:C treatment of mice for 24 hours increased liver mRNA levels of HO-1, inducible nitric oxide synthase (iNOS), and metallothionein-2 (Mt-2) (Supporting Fig. 5). Even though decreased NAPQI formation can explain the protective effects of polyI:C against APAP toxicity, induction of these genes by polyI:C can also contribute to this phenotype.

Finally, we sought to identify which receptors were necessary to sense polyI:C in our animal model. Prior to 2005, the only known receptor class for polyI:C was TLR3.<sup>21</sup> We now know of another family of polyI:C receptors, retinoic acid-inducible gene I-like helicases (including RIG-I and melanoma-differentiation-associated gene 5 [MDA5]). Several studies have suggested that these receptors may function in a cell-type-specific manner to sense polyI:C or viral dsRNA. TLR3 has been shown to play an important role in sensing polyI:C in epithelial cells, whereas only playing

a minor role in dendritic cells.<sup>44</sup> In contrast, RIG-I and MDA5 play more important roles in sensing polyI:C in fibroblasts and dendritic cells in comparison to TLR3.<sup>45</sup> However, it is not clear whether these two families of receptors play redundant roles in sensing polyI:C in the liver.<sup>46</sup> Our data illustrate that polyI:C, when administered i.p., can suppress APAP-induced hepatotoxicity in the absence of TRIF or Cardif, the adaptor proteins required for signal transduction of TLR3 or RIG-I/MDA5, respectively.<sup>46</sup> This is the first study to report that polyI:C administration *in vivo* can exert physiological effects in the absence of TLR3 through Cardif-dependent receptors in the liver.

In summary, the results of this study suggest that activation of antiviral responses can alter drug metabolism through transcriptional down-regulation of CYP3A11 and CYP1A2 independent of IFN production. Understanding the factors that contribute to or alleviate drug toxicity is important for the proper use of drugs under various clinical cases, including the use of common analgesics to relieve pain or fever during viral infections. This study, in conjunction with our previous work, provides further evidence that the use of APAP may be safer in the context of a viral infection than ASA therapy. Furthermore, PolyI:C is now a Food and Drug Administration (FDA)-approved drug that is being evaluated as an anticancer therapeutic agent (e.g., ovarian and renal cancer) as well as for chronic fatigue syndrome and AZT-resistant HIV.<sup>47-49</sup> Thus, it is important to study potential uncharacterized adverse effects that could occur in virally infected patients or individuals receiving polyI:C, a function for which the animal model we have established can be used. Identification of molecular mechanisms of the crosstalk between innate immune responses and nuclear hormone receptor-regulated metabolism can provide insight into the biological consequences of various drug treatments during viral infections, allowing for safer and more accurate assessment of proper drug therapy.

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## Supporting Material

**Virus collection and quantification.** GFP-tagged VSV was a kind gift from G. Barber (University of Miami, Miami, FL). The virus was grown on nearly confluent Madin Darby canine kidney (MDCK) cells, infected at a multiplicity of infection of 0.001. Two days after infection, cell-free supernatant was ultracentrifuged at >100,000 g through a 25% sucrose cushion. The viral pellet was resuspended in PBS. Standard plaque assay was used to determine the number of plaque forming units (pfu). In brief, confluent monolayers of MDCK cells in 6- or 12-well plates were infected in duplicate with serial dilution of the viral stock with intermittent shaking for 1 hour. Subsequently, cells were overlaid with 1× MEM BSA containing 0.7% low-melting-point agar. Plaques were allowed to develop over 24–36 hours and counted after staining cells with crystal violet.

**Animals:** IFN Alpha Ro/o 129/Sv (*Ifnar*<sup>-/-</sup>) mice were from B&K Universal and were backcrossed with C57BL/6J mice (The Jackson Laboratory) for six generations. *Trif*<sup>*Lps2/Lps2*</sup> mutant mice were provided by Dr. B. Beutler (The Scripps Research Institute, San Diego, CA). *Tlr3*<sup>-/-</sup> mice were provided by Dr. S Akira (Osaka University, Japan), *Tnf-a*<sup>-/-</sup> mice were provided by T. Mak, (University of Toronto, Toronto, Canada), and *Cardif*<sup>-/-</sup> mice are from Dr. J Tschopp (University of Lausanne, Switzerland). *IL-1R*<sup>-/-</sup> (B6.129S7-*Il1r1*<sup>*tm1Imx*</sup>/J) and WT C57BL/6J control mice were obtained from Jackson Laboratories.



**Immunohistochemistry Assays:** Briefly, tissue sections were blocked with 3% non-immune goat serum in PBS-T for 1 hour. Following washing in PBS-T, tissue sections were treated with anti-APAP rabbit antibodies (1:100; Biogenesis/AbDserotec) for 3 hours, washed 3 times and incubated with Alexa fluor 568 goat anti-rabbit IgG antibody (1:60) for 1 hour. After washing, sections were counterstained and observed using an Olympus BX41 fluorescent microscope. The sections were then analyzed using ImageJ .

**Reagents:** Since LPS has been shown to modulate the activity of many CYPs, we tested for endotoxin contamination in different polyI:C products from Sigma and Invivogen. PolyI:C samples provided by Sigma, not Invivogen, were contaminated with endotoxins and could induce IFN signaling in TLR3 deficient macrophages through TLR4 activation

### Supplementary Figure Legends:

**Supplementary Figure 1.** Wild-type mice were treated with saline or polyI:C (100 $\mu$ g, i.p.). Twenty four hours post-treatment, liver RNA was isolated and analyzed by Q-PCR. (n=4, mean +/- SD, \* $P$ <0.05 compared to polyI:C untreated)

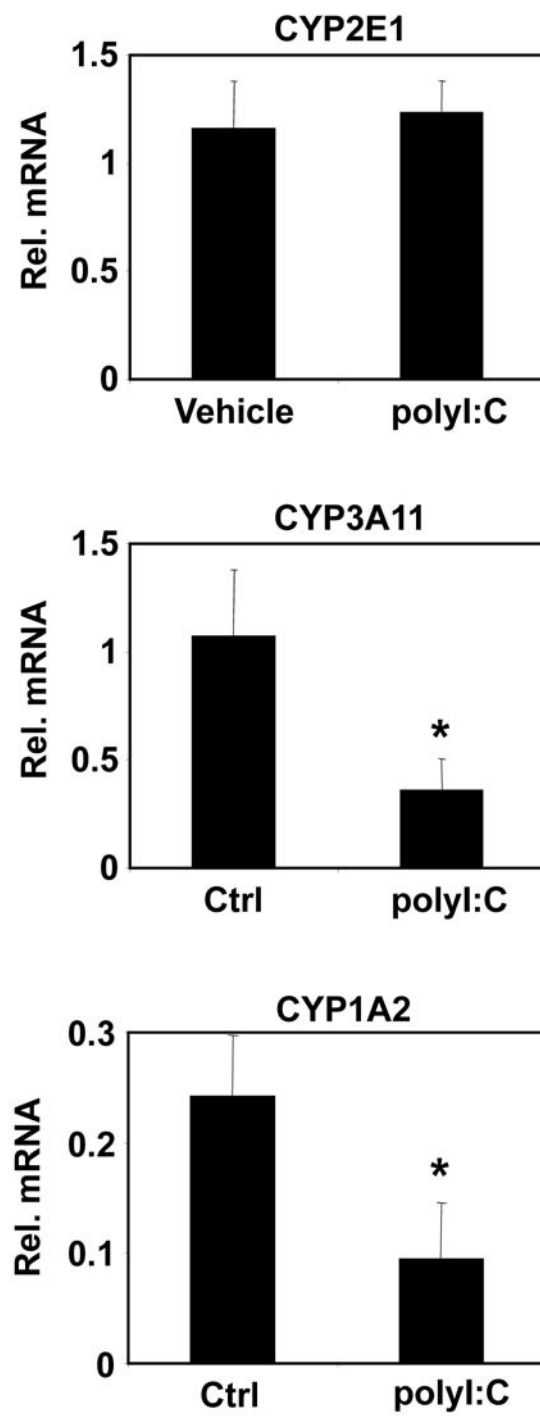
**Supplementary Figure 2.** Wild-type mice were treated with saline or polyI:C (100 $\mu$ g, i.p.) 24 hours prior to treatment with APAP (350mg/kg, ip). 6 hours post-APAP treatment, mice were sacrificed and liver samples were collected. Formalin fixed liver samples were indirectly stained for APAP-bound proteins, and the sections were analyzed using ImageJ. (n=4, mean +/- SD, \* $P$ <0.05 compared to polyI:C untreated)

**Supplementary Figure 3.** Wild-type and *Ifnar*<sup>-/-</sup> mice were treated with saline or polyI:C (100 $\mu$ g, i.p.). Twenty four hours post-treatment, liver RNA was isolated and analyzed by Q-PCR. (n=4, mean +/- SD, \* $P$ <0.05 compared to polyI:C untreated)

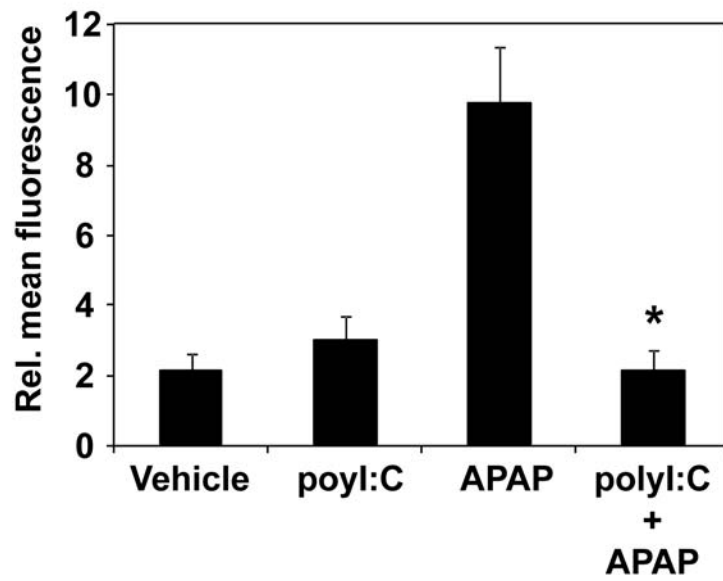
**Supplementary Figure 4.** Wild-type and *IL-1R*<sup>-/-</sup> and *Tnf- $\alpha$* <sup>-/-</sup> (n=4) were treated with saline or polyI:C (100 $\mu$ g, i.p.) 24 hours prior to treatment with APAP (350mg/kg, ip). 6 hours post-APAP treatment, mice were sacrificed and serum samples were collected and analyzed for serum ALT levels. (n=4, mean +/- SD, \* $P$ <0.05 compared to polyI:C untreated)

**Supplementary Figure 5.** Wild-type mice were treated with saline or polyI:C (100 $\mu$ g, i.p.). Twenty four hours post-treatment, liver RNA was isolated and analyzed by Q-PCR. (n=4, mean +/- SD, \* $P$ <0.05 compared to polyI:C untreated)

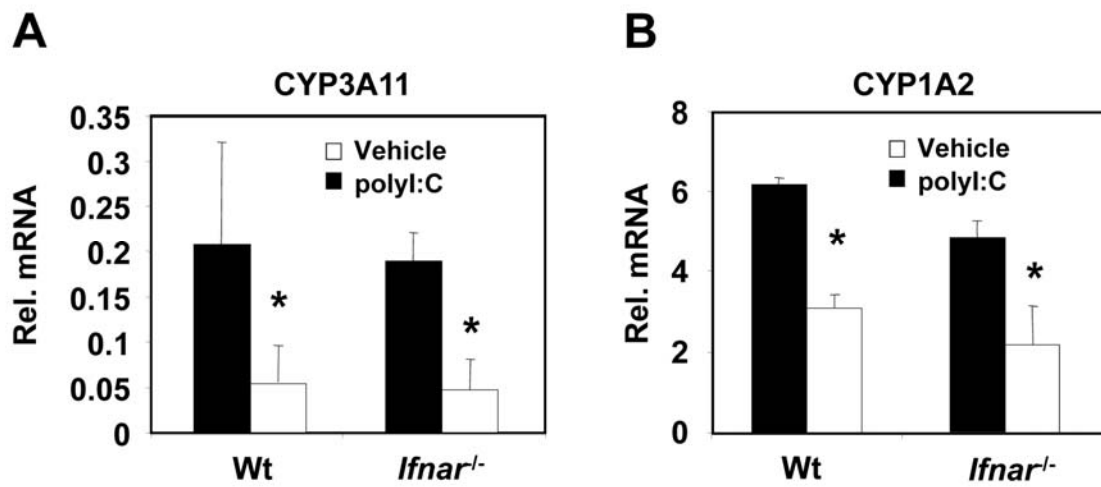
Supplementary Figure 2-1:



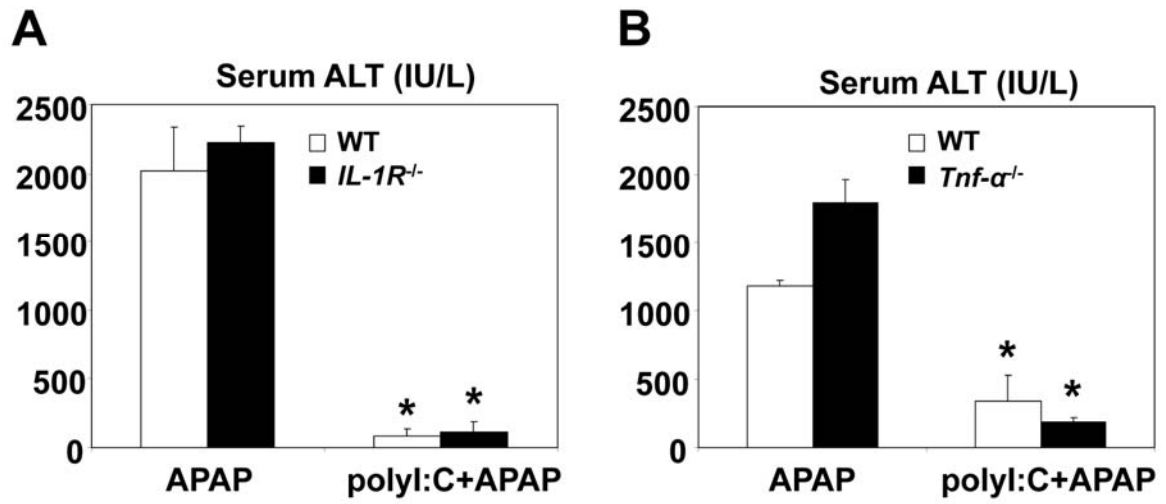
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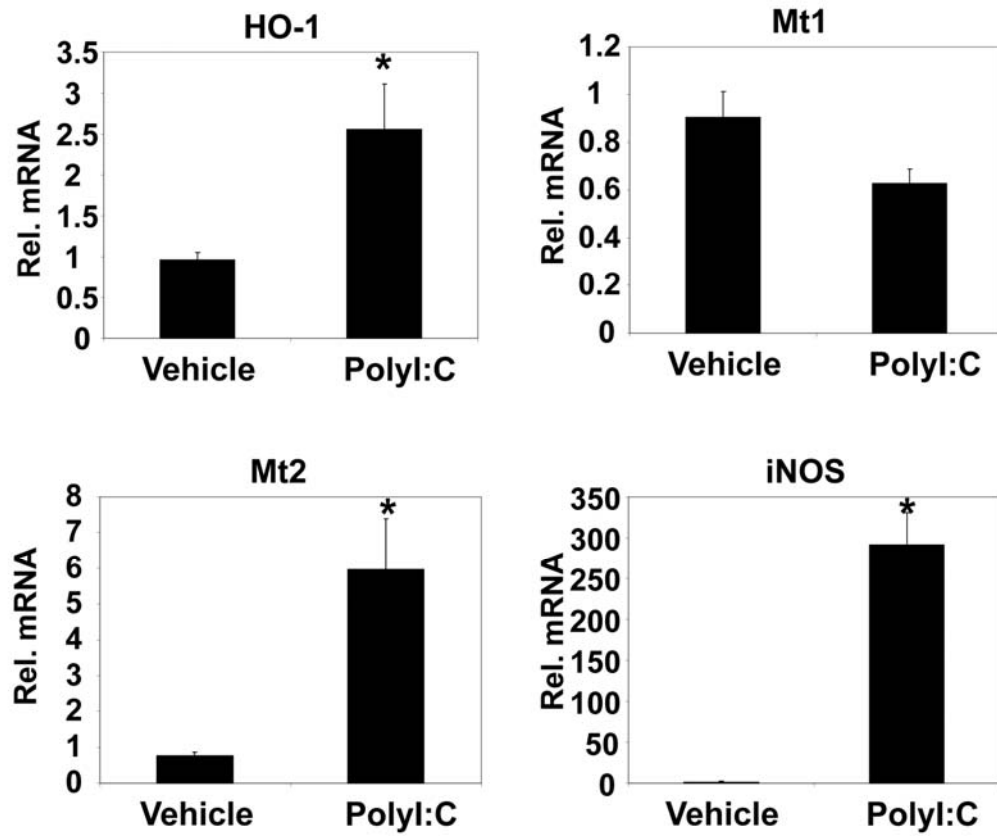
Supplementary Figure 2-3:



Supplementary Figure 2-4:



Supplementary Figure 2-5:





## **Chapter 3**

### **Hepatoprotective Characteristics of Glycyrrhizic Acid through Inhibition of HMGB1 Cytokine Activity**

## Introduction

Acute liver failure (ALF) is a life-threatening condition that results in altered metabolic disorder, coagulopathy, and alteration in mental status associated with high mortality<sup>1,2</sup>. In the U.S. as well as the U.K., acetaminophen (APAP), a widely used analgesic and antipyretic, is the most frequent etiology of ALF<sup>7,8</sup>. In Asia, viral infections remain the most common cause of liver failure with hepatitis B virus (HBV) being an important cause of ALF in China<sup>9</sup>. Patients presenting with symptoms of ALF need to be treated immediately; however, liver transplantation is the only therapy currently proven to improve patient outcome and the success of this approach is derogated by hepatic ischemia–reperfusion (I/R) injury, which leads to liver dysfunction and failure<sup>11,12</sup>. Thus, there is a pressing need for new approaches to prevent and manage liver injury and ALF.

Traditional Chinese Medicine (TCM) is a unique system of diagnosis and therapy with wide application in ancient and recent Chinese medical care. TCM relies mainly on herbal medicines, which are accepted conventionally for many years in Eastern medicine, but their molecular mechanism still need to be elucidated<sup>1,2</sup>. Interestingly, multiple herbal plants are present in majority of conventional TCM recipes<sup>3</sup>. One common herbal plant is licorice, which is included in many recipes including *Xiaoyao Powder*, a well known formula widely used to treat a wide range of diseases including liver and spleen imbalances. *Xiaoyao Powder* contains licorice or its bioactive extract, Glycyrrhizic Acid (GA), which has been shown to have anti-inflammatory effects in different systems<sup>4</sup>. However, the therapeutic reason for licorice in most TCM and the mechanisms of action for GA itself are not well understood.

Induction of innate immune receptors during sterile liver injury models (i.e. APAP-induced hepatotoxicity) has been attributed to the release of endogenous danger signals or damage-associated molecular patterns (DAMPs)<sup>13,14</sup>. DAMPs can activate pathogen recognition receptors (PRRs) and induce enhanced inflammation and tissue injury. One such DAMP is high-mobility group box 1 (HMGB1), a nuclear nonhistone chromatin-binding protein involved in the regulation of gene transcription<sup>15,16</sup>. In addition to its nuclear role, HMGB1 is passively released from damaged or necrotic tissue cells and is actively secreted by inflammatory cells in response to stress, activating PRRs such as toll-like receptor (TLR)-2, TLR4, as well as receptor for advanced glycation end products (RAGE)<sup>17,18</sup>. In fact, blocking HMGB1 activity using neutralizing antibody has been shown to be effective in protecting against APAP and I/R induced hepatic injury. However, the role of HMGB1 during viral infections remain unclear.

Recently, GA was reported to interact with BoxA of HMGB1 and inhibit its chemoattractant and mitogenic activities, while having little inhibitory effects on its intranuclear DNA-binding function<sup>19</sup>. While GA has been shown to inhibit mitogenic activity of HMGB1, it has not directly been shown previously whether GA affects HMGB1 cytokine activity and TLR activation. In this study, we examine whether the presence of GA in TCM such as *Xiaoyao Powder* provides hepatoprotective functions against potential toxicity of bioactive herbal ingredients. Furthermore, we explore the effectiveness of GA in protecting against hepatic injury in clinically important injury models including APAP-induced injury, I/R injury, and viral induced hepatic injury. We provide a potential mechanism in which GA acts as a strong hepatoprotective agent by inhibiting HMGB1

cytokine activity and suppressing induction of inflammatory genes known to mediate hepatic injury.

### **Results:**

**Role of GA as a hepatoprotective agent in TCM.** Licorice is one of the most frequently used compounds in TCM including *Xiaoyao Powder*; however, the mechanisms for its broad usage remain unclear. While *Xiaoyao Powder* usage appears to be safe and is commonly used without any reports of significant hepatotoxicity, one of its ingredients, *Radix bupleuri* (*Buplerurum Chinense* DC), has recently been associated with hepatotoxicity due to oxidative damage. Given its extensive use in many TCM formulas as well as its anti-inflammatory properties, we tested whether licorice is the ingredient in *Xiaoyao powder* that can suppress hepatotoxicity of ChaiHu. Indeed, long-term daily administration of TCM (I.G., 1g/kg) without licorice (TCM-GA) led to body weight loss compared to mice receiving TCM+GA (Figure 1A). Furthermore, serum ALT and AST levels were increased significantly after 21 days of injections in the TCM-GA group compared to control mice as well as the TCM+GA group (Figures 1B and 1C). Liver histology of these mice further indicates increased liver injury in the TCM-GA group (Figure 1D). Additionally, we examined the induction of IP-10, an inflammatory cytokine induced during liver injury. Removing GA from TCM formula leads to increased IP-10 release as indicated by ELISA data (Figure 1E). These findings suggest that licorice, or GA specifically, can serve as a hepatoprotective agent in *Xiaoyao Powder*.

**GA pretreatment suppresses APAP-induced hepatotoxicity and I/R hepatic injury.** Given our findings that GA can potentially be an important hepatoprotective agent in Eastern Medicine, we set out to determine the application of GA in Western Medicine.

APAP is the most commonly used analgesic and the number one cause of ALF in the US as well as UK. Here, we tested whether GA administration can potentially prevent hepatic injury induced by APAP. Mice were pretreated with GA (1mg, i.p.) for 90 min prior to APAP administration. APAP (600 mg/kg) was administered with or without GA pretreatment, and animal weight and body temperature were monitored for 14 days. As seen in Figure 2A, mice which received GA had a higher survival rate than those given APAP alone. Mice pretreated with GA exhibited lower serum ALT levels compared to un-treated controls in response to 6 hours of APAP (350 mg/kg) treatment (Figure 2B) and evidenced fewer necrotic foci on histological analysis (Figure 2C). Furthermore, GA treatment suppressed induction of pro-inflammatory cytokine gene expression, IP-10, in infected livers (Figure 2D). In addition to drug-induced toxicity, liver I/R is an important cause of hepatic injury and remains an important obstacle in liver transplantation, which is often the only solution in treating of ALF. Here, we tested the effectiveness of GA pretreatment in a mouse warm I/R hepatic injury, during which arterial and portal venous blood supply to the cephalad lobes is interrupted for 90 minutes followed by resumption of blood flow and reperfusion for 6 hours. Mice are then euthanized and liver and blood samples are collected. GA (1 mg, i.p.) pretreatment significantly abrogated I/R injury as indicated by decreased serum ALT levels (Figure 2E) and fewer necrotic foci on histological analysis (Figure 2G). Similar to the other models, IP-10 was induced as a result of I/R injury while GA treatment blocked the induction of IP-10 (Figure 2F).

**GA treatment effectively suppresses MHV-induced Acute Liver Injury and mortality.** Given our findings on hepatoprotive functions of GA in sterile inflammatory liver injury models, we set out to test the effectiveness of GA in alleviating hepatic injury

in a viral infection model. We have established acute lethal and sub-lethal Mouse Hepatitis Virus (MHV) infection mouse models using MHV-A59 strain (Supplementary Figure 2). MHV, a single-stranded RNA virus, is a coronavirus that causes epidemic murine illnesses characterized by numerous pathologies including enteritis, hepatitis, or encephalitis, dependent on the virus strain and genetic background of the mouse<sup>22-24</sup>. MHV-A59 strain is a moderately hepatotropic virus which may also cause moderate acute meningoencephalitis and chronic demyelination. Our survival studies suggest that administration of GA (20mg/kg, i.p.) on the day of infection and every other day after infection leads to increased survival rate of MHV infected mice (Figure 3A). However, GA treatment does not appear to affect MHV replication *in vivo* (Figures 3B). Despite having no effects on viral replication, GA treatment was able to decrease MHV-induced hepatotoxicity as mice receiving GA exhibited lower serum ALT levels compared to the control group and exhibited fewer necrotic foci on histological analysis (Figures 3C and 3D). Furthermore, we determined the gene expression of four pro-inflammatory cytokines including Interferon gamma-induced protein 10 (IP-10), Interleukin-6 (IL-6), Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and Interleukin1 $\beta$  (IL-1 $\beta$ ), which have been shown to be key players in the pathogenesis of hepatic injury in different models<sup>25-27</sup>. Our data suggest that GA treatment can suppress induction of these genes suggesting that GA's protective effects can be due to its anti-inflammatory properties (Figures 3E-H).

**GA Blocks HMGB1 cytokine activity and secondary inflammatory responses in MHV infected cells *in vitro*.** GA has a variety of pharmacological properties including anti-inflammatory as well as anti-viral activities<sup>28-30</sup>. Since GA treatment did not affect MHV infection rate *in vivo*, we explored the mechanisms by which GA can affect cellular

injury perhaps through its anti-inflammatory properties. GA was recently demonstrated to inhibit HMGB1 mitogenic activity. Interestingly, a significant amount of HMGB1 is released by murine hepatocyte BNL.CL2 cells infected with MHV (MOI = 0.5) (Figure 4A). Given that GA treatment prevented induction of inflammatory cytokines *in vivo* after MHV infection, we tested to see whether GA is able to inhibit induction of these genes by HMGB1. D2SC cells were co-incubated with HMGB1 (10 g/ml, HMGB1-FLAG protein was purified using ANTI-FLAG M2 Affinity Gel according to the manufacturer's instructions) and different amounts of GA for 6 hours. The culture supernatants were subsequently collected and analyzed for IL-6 and TNF- $\alpha$  production. HMGB1 stimulation leads to release of IL-6 as well as TNF- $\alpha$  which is inhibited by the presence of GA in the supernatant, suggesting that GA can block cytokine activity of HMGB1 (Figure 4B and C). Thus, we utilized anti-HMGB1 neutralizing anti-body as well as GA to examine whether blocking HMGB1 cytokine activity would abrogate induction of inflammatory cytokines in the context of MHV infection. The supernatant solution from MHV infected BNL.CL2 cells containing HMGB1 (48 h) (Figure 4A) was exposed to UV radiation for 30 minutes for virus inactivation and then added to RAW264.7 cells with either control, Anti-HMGB1 (final 10 $\mu$ g/ml) or GA solution (final 200 $\mu$ g/ml) for 1h. At 8h after incubation, the total RNA of stimulated cells was prepared and IP-10 mRNA expression was assessed using real-time PCR (Figure 4D). Additionally, TNF- $\alpha$  cytokine levels in the supernatants were measured. As hypothesized, UV inactivated supernatant of MHV infected cells was able to induce TNF $\alpha$  in RAW264.7 cells, and GA was able to suppress its induction as effective as HMGB1 neutralizing antibody (Figure 4E). Similar results were observed in induction of other cytokines such as TNF $\alpha$  and IP-10 (Supplementary Figure 4). Similar to HMGB1

antibody treatment *in vivo*, both GA and HMGB1 treatment *in vitro* are able to inhibit induction of IP-10 in MHV infected BNL.CL2 cells. (Figure 4F). In the presence of anti-HMGB1 antibody or GA treatment, virus titer was not affected at 48 hours post infection (Figure 4G), indicating that GA and HMGB1 antibody did not inhibit MHV replication *in vitro*.

**Suppression of mortality and severity of MHV-induced liver injury by HMGB1 neutralizing antibody.** Mice infected with MHV ( $1 \times 10^4$  PFU/mouse) have high levels of HMGB1 in their serum, suggesting that MHV infection causes release of HMGB1 into the serum either through active release by inflammatory cells or due to hepatocellular injury caused by viral replication. (Figure 5A). In order to examine whether HMGB1 mediates pathogenesis of MHV-induced lethality, mice were injected with control or HMGB1 neutralizing antibody (2.5 mg/kg, i.p.) after infection and every other day before euthanizing mice at 5 days post infection (dpi). With a lethal dose ( $8 \times 10^5$  PFU/mouse) of MHV infection, blocking HMGB1 cytokine activity using neutralizing antibody injection completely rescued mice (Figure 5B). Interestingly, the virus titers in the infected livers between the antibody-treated group and control group were not significantly different (Figure 5C), suggesting that neutralizing HMGB1 antibody treatment does not affect viral replication in the liver. However, the neutralizing antibody treated group expressed fewer indicators of liver injury as evidenced by decreased release of ALT as well as lack of histological injury signs (Figures 5D and 5E). These results indicate that HMGB1 plays an important role in mediating liver injury in our MHV infection model. Since we previously observed suppression of induction of inflammatory genes by GA (figure 3E-H), we investigated whether these effects are due to inhibition of HMGB1



cytokine activity. Inflammatory cytokines are induced in response to activation of pathogen recognition receptors. Our data illustrate that HMGB1 neutralizing antibody significantly suppresses transcriptional induction of IP-10, TNF- $\alpha$  and IL-6 in infected liver tissue (Fig.5F-H).

**TLR4 signaling mediates hepatic injury induced by MHV infection.** TLR4 signaling has been indicated to contribute to tissue damage in both pathogen induced hepatic injury as well as sterile inflammatory injuries. (11). Given our findings that HMGB1, a known ligand of TLR4, plays an important role in MHV infection model, we examined whether TLR4 signaling is also critical in MHV infection. Interestingly, TLR4 KO mice are protected against lethal MHV infection compared to wild-type control mice while having similar viral titer (Figure 6A, B). Furthermore, TLR4 KO mice exhibited decreased signs of liver injury compared to control mice as indicated by lower serum ALT levels and absence of necrotic foci on histology (Figure 6C and D). Additionally, IP-10, TNF- $\alpha$  and IL-6 mRNA levels were not induced strongly in the TLR4 KO mice infected with MHV further suggesting that TLR4 is an important immune receptor in mediating inflammation during MHV infection (Figure 6E-G).

## Discussion

Licorice is a key ingredient in a wide variety of traditional herbal formulas in TCM, but it's unclear what molecular actions make the herb an effective treatment. GA, the main biologically active component of licorice, has been reported to have potential pharmacological properties such as anti-inflammatory, antiviral and immunomodulatory effects<sup>28,31,32</sup>. *Xiaoyao Powder* is a licorice-containing formula used for treatment of wide range of disorders including hepatic and splenic deficiencies. Here, we test formulations of *Xiaoyao powder* with and without GA. Surprisingly, while a standard formula of *Xiaoyao powder* is safe to use in mice, the formula without GA led to weight loss and increased serum liver enzyme levels, both indicators of hepatotoxicity. These data suggest that the inclusion of GA protects against hepatotoxicity induced by other ingredients in the herbal formula. Given this protective role for GA, we tested the effectiveness of the compound in suppressing APAP-induced hepatotoxicity, the most common cause of acute hepatitis and ALF in the US and UK. Indeed, GA pretreatment was able to completely protect against APAP toxicity in mice. We further found that GA is an effective hepatoprotective agent against Ischemia Reperfusion Injury as well as MHV induced hepatitis.

Although other studies also report hepatoprotective functions of GA, the underlying detailed mechanism by which GA improves the liver inflammatory response and pathology remained largely unknown. GA has been shown to have anti-inflammatory effects through different mechanisms such as antiradical activity, inhibitory effects on reactive oxygen species (ROS) and lipid peroxidation, with its anti-mitogenic effects by interacting with HMGB1 being discovered most recently. GA was recently reported to interact with BoxA of HMGB1 and inhibit its mitogenic activities, with little effect on its intranuclear DNA-

binding function <sup>19</sup>. Whether GA has a protective effect on viral liver injury and sterile liver inflammation mediated by HMGB1 had not previously explored.

HMGB1 has been indicated to play an important role in a wide range of diseases and injury models including sepsis as well as sterile inflammatory injury models. Thus, identifying inhibitors of cytokine activity of HMGB1 can have important clinical outcomes. Here we demonstrate that addition of GA in the media can block activation of inflammatory cytokines by HMGB1 such as TNFs well as IL-6 that suggests GA acts as a direct inhibitor of HMGB1 cytokine activity. HMGB1 has been indicated to play an important role in a wide range of diseases and injury models including sepsis as well as sterile inflammatory injury models. Thus, identifying inhibitors of cytokine activity of HMGB1 can have important clinical outcomes. Recent studies have shown that HMGB1 is a key player in liver injury induced by toxic metabolite of APAP, as mice pretreated with neutralizing HMGB1 have suppressed signs of liver injury <sup>33</sup>. Similarly, here we demonstrate that GA is effective in protecting mice from APAP-induced hepatotoxicity. Even when administering a high lethal dose of APAP, mice that were pretreated with GA were completely protected and there were no signs of liver hepatotoxicity. Additionally, GA treatment suppresses induction of inflammatory cytokines (e.g. IP-10) which have been shown to mediate APAP-induced hepatotoxicity. We also found similar results in the warm liver I/R model in which HMGB1 has been implicated to be a key mediator in liver injury <sup>12</sup>. Given that GA treatment protected mice against MHV hepatic injury, we explored whether HMGB1 could also play an important role in liver injury induced by MHV similar to liver APAP-induced toxicity and I/R injury.

Viral infections including West Nile virus, Dengue virus and HIV have been linked to elevated HMGB1 serum levels<sup>34-37</sup>. HMGB1 can be released passively by dying infected cells or actively by inflammatory cells in response to inflammatory cytokines or viral particles<sup>13,38</sup>. However, the contribution of HMGB1 in the pathogenesis of infection remains unclear. Inflammatory cytokines such as IP-10, IL-6, IL-1 $\beta$  and TNF $\alpha$  have been shown to play critical roles in mediating liver injury in different models including viral hepatitis<sup>12,26,39</sup>. Viral particles can directly activate PRRs, causing induction of these inflammatory cytokines. Furthermore, HMGB1 release during viral infection can activate TLR2, TLR4 and RAGE, known PRRs that have been shown to regulate transcription and release of inflammatory cytokines<sup>14,38</sup>.

Here, we have successfully established an acute MHV-A59 hepatitis model in mice to study the involvement of HMGB1 cytokine activity in the pathogenesis of hepatic injury caused by MHV. MHV infection leads to release of HMGB1 into the serum and blocking HMGB1 cytokine activity enhances the survival rate of infected mice. Interestingly, administration of the HMGB1 neutralizing antibody does not have any effects on the MHV infection rate, indicating that HMGB1 does not play a role in proper anti-viral responses against MHV. On the other hand, inhibiting HMGB1 cytokine activity significantly protects against MHV induced hepatic injury. Our data suggest that blocking HMGB1 cytokine activity prevents induction of inflammatory genes, indicating that HMGB1 is primarily responsible for induction of these known inflammatory cytokines mediating hepatic injury. These results are further confirmed *in vitro* using MHV infected murine hepatocyte BNL.CL2 cells. Additionally, we tested to see which of the PRRs activated by HMGB1 are important in pathogenesis of MHV hepatitis. We used mice deficient in TLR4,

a key PRR activated by HMGB1. Interestingly, TLR4 deficient mice were much more resistant to MHV and exhibited less signs of liver injury compared to control mice while having similar hepatic MHV viral titer. Together these data suggest that protecting mice from secondary inflammatory liver injury induced by HMGB1/TLR4 signaling can rescue mice infected with MHV regardless of the viral load.

Overall, our results highlight the importance of HMGB1 in mediating liver injury induced by viral infections such as MHV, prompting new studies to explore the use of HMGB1 cytokine inhibitors in the treatment of ALF. Furthermore, we report that GA can effectively inhibit HMGB1 cytokine activity *in vitro* and provide protection in models of APAP-induced hepatotoxicity and I/R liver injury, conditions in which HMGB1 has been indicated to play a critical role. Importantly, GA is a safe compound: in addition to its use as a therapeutic in TCM, GA has been used safely as an alternative herbal sweetener in candies and pharmaceuticals in worldwide. Thus, GA could potentially be developed as an effective drug targeting HMGB1 to combat hepatic injury in clinical settings.

## **Materials and Methods:**

**Cell culture.** Murine fibroblasts 17Cl1 cells, murine dendritic D2SC and embryonic hepatocyte cells BNL.CL2 were grown in Dulbecco's modified Eagle's medium (DMEM) contained 10% fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin in the presence of 5% CO<sub>2</sub> at 37°C. Murine macrophage RAW 264.7 cells ( ATCC) were cultured in RPMI medium 1640 supplemented with 10% FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin.

**Virus titer assay.** Wild type MHV-A59 virus stocks were produced in mouse 17Cl-1 cells. The titers in livers of infected mice and supernatants of cells were determined by plaque assay on the monolayer of 17Cl-1 cells. After 24 h incubation with serially diluted supernatants of tissue homogenates or supernatants, plaques were counted on the cell layers. All titrations were performed in duplicates, and the average plaque forming unit (PFU) per gram of liver tissue or per ml supernatants were calculated.

**Mouse experimental models.** Experimental female 6- to 8-week-old C57BL/6 mice (20g body weight) were purchased from Vitalriver ( Beijing, China) or Jackson laboratories. For the TLR4KO studies in Figure 6, 4- to 6-week-old C57BL/10ScNJ TLR4 KO and C57BL/10J WT mice (16~18g body weight) were purchased from National resource center for mutant mice (Nanjing, China). Mice were housed and studied under specific pathogen-free conditions and all experiments were performed in accordance with guidelines from the University of California, Los Angeles Institutional Animal Care and Use Committee. In TCM studies, TCM+GA composition included GA (11.4% of total weight) while GA was excluded in TCM-GA mixtures. The composition of exact ingredient of Xiaoyao Powder included Radix bupleuri, Angelica sinensis, Radix paeoniae

alba, Largehead atractylodes rhizome, Poria, Radix glycyrrhizae preparata, peppermint and fresh ginger.

Mice were given PBS, TCM+GA and TCM-GA solution (1g/kg, ig) daily for 21 days while monitoring for weight loss or mortality. Serum samples were collected on 5, 10 and 15 days post injection (dpi). Subsequently, animals were sacrificed and liver tissue and serum samples were collected 21 dpi. In the MHV infection model, mice were infected by i.p. injection of  $8 \times 10^5$  PFU/mouse (lethal) or  $1 \times 10^4$  PFU/mouse (sublethal) of MHV-A59 in 0.2 ml DMEM medium and monitored daily for weight loss or mortality. Animals were sacrificed and liver tissue and serum samples were collected 5 days post infection (dpi). For APAP studies, mice were fasted 18 hours prior to administration of APAP at the specified doses. We have used a warm hepatic I/R injury which we published previously<sup>20</sup>. Briefly, mice were injected with heparin (100  $\mu$ g/kg), and the arterial and portal venous blood supply to the cephalad lobes of the liver was interrupted using an atraumatic clip. The clip was removed after 90 minutes, initiating hepatic reperfusion. Mice were sacrificed after 6 h and blood and liver samples were collected. Three to 8 mice were used in each group, and each experiment as repeated at least three times.

**GA and anti-HMGB1 antibody and purified HMGB1 treatment.** For animal studies MHV infection model, GA was obtained from research and development center in Huzhou (CAS) in form of 18 $\beta$ -Glycyrrhetic acid and for the rest of the studies GA was provided by Sigma, USA in the form of glycyrrhizic acid ammonium salt. Two hours after viral infection, mice were administered with GA solution (20 mg/kg, i.p.) or an equivalent volume of PBS as control, and every other day for total of 3 times. Purified anti-HMGB1 monoclonal neutralizing antibody was provided by Dr. Jie Tang<sup>21</sup>. Two hours after MHV

viral infection, mice were administered with antibody dilution (2.5 mg/kg, i.p.) or an equivalent volume of PBS as control and every other day for total of 3 times. HMGB1-FLAG protein was purified using ANTI-FLAG M2 Affinity Gel according to the manufacturer's instructions.

**ALT activity assay.** Serum ALT levels were measured with a kit according to the manufacturer's instructions (biosino bio, Beijing or TECO Diagnostics, USA)

**Histological analysis.** Liver specimens were fixed in 10% neutral buffered formalin and liver paraffin-embedded sections (5µm thick) were stained with Hematoxylin and Eosin using standard techniques.

**Cytokine release assay *in vitro*.** The BNL.CL2 cells were infected by MHV-A59 with multiplicity of infection (MOI)=0.5, 1 hour later, the cells were treated with GA (1mg/10<sup>6</sup> cells) and anti-HMGB1 Ab (100µg/10<sup>6</sup> cells) for 15 minute and then added DMEM medium. IP-10 levels at different time points and virus titers on 48<sup>th</sup> hour were measured. The supernatant from MHV infected BNL.CL2 cell culture (48 h) UV radiated for 30 minutes to inactivate viruses. Anti-HMGB1 (final 10µg/ml) and GA solution (final 200µg/ml) incubated with same volume of HMGB1 supernatant solution for 1h at 37°C and then added mixed medium to RAW264.7 cells. On 8h after incubation, the total RNA of stimulated cells was prepared and IP-10 gene was assayed with real-time PCR. IP-10 and TNFα cytokine level in the supernatants after stimulation were also measured.

**Real time PCR.** Liver tissue or cultured cell total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions and the cDNA synthesized as described previously. Reverse transcribed mRNA expression of IP-10, IL-6, TNF-α, IL-1β and GAPDH gene was determined by real time PCR using SYBR Green Master Mix



(ABI, Foster City, CA). The primers for follow genes were listed: IP-10, 5'-CCAGTGAGAATGAGGGCCATA, 3'-TCGTGGCAATGATCTCAACAC; IL-6, 5'-CACAGAGGATAACCACTCCCAACA, 3'-TCCACGATTTCCCAGAGAACA; TNF- $\alpha$ , 5'-GGTGCCTATGTCTCAGCCTCTT, 3'-CGATCACCCCGAAGTTCAGTA; IL-1 $\beta$ , 5'-GAGCTGAAAGCTCTCCACCTCA, 3'-TCGTTGCTTGGTTCTCCTTGTAC; GAPDH, 5'-ACTCCACTCACGGCAAATTCA, 3'-CGCTCCTGGAAGATGGTG. The ABI Stepone plus Real-Time Thermal cycler was used to analyze the samples quantitatively under the following conditions: 95°C (5 min), 40 cycles of 95°C (20 s), 55°C (30 s), and 72°C (10 s). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or ribosomal protein L32 was used as an endogenous control for sample normalization.

**HMGB1 measurement.** Serum harvested from infected mice by MHV-A59 was used for detecting circulating levels of murine HMGB1 according to the manufacturer's instructions (Chondrex Systems, USA).

**Statistical Analysis.** All data results were expressed as mean  $\pm$  standard error of the mean. The difference between two groups was examined using Student's *t* test after analyzing the variance. Statistics were performed using GraphPad Pro. A *p* value of  $<0.05$  was considered statistically significant.

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## Figure Legends:

**Figure 3-1. Glycyrrhizic Acid (GA) in traditional Chinese medicine (TCM) solution alleviates liver injury during long-term injection.** C57BL/6 female mice were injected with PBS (control) or TCM+GA solution (*Xiaoyaosan*, containing GA) or TCM-GA solution (*Xiaoyaosan* containing no GA) every day (with dose of 1g/kg, i. g.) for 21 days. (A) Body weight changes (B) Serum ALT and (C) AST levels on 5,10,15 and 21 dpi. (D) Representative H&E staining of liver tissues on 21 dpi (magnification,×100). (E) IP-10 cytokine level in serum of different groups on 21 dpi. The data are represented as the means ± SEM; \* indicates  $P < 0.05$ , \*\* indicates  $P < 0.01$ ,\*\*\* indicates  $P < 0.001$ .

**Figure 3-2. Pretreatment with GA protects against I/R and Acetaminophen (APAP)-induced liver injury.** (A) Wildtype (n = 8) were treated with saline or GA (1mg, i.p.) 1.5 hours prior to treatment with APAP (600 mg/kg, i.p.). Mice were followed for 14 days post treatment. (B) Mice were treated with saline or GA ( i.p.) 1.5 hours prior to treatment with APAP (350 mg/kg, i.p.). Six hours post-APAP treatment, mice were sacrificed and serum samples were collected and analyzed for serum ALT levels. (C) Liver samples were formalin-fixed and stained with H&E. (D) RNA was isolated from liver samples and IP-10 mRNA expression was compared between different groups. (E) Wildtype (n = 5) were treated with saline or GA (1mg, i.p.) 1.5 hours prior to clamping hepatic artery for 90 min. Liver and blood samples were collected 6 hours after reperfusion. (F) serum ALTs were measured. (G) RNA was isolated from ischemic and non-ischemic liver samples and IP10 mRNA expression was compared between different groups (mean ± SD, \* $P < 0.05$ ). (C) Representative H&E staining of ischemic liver lobes.

**Figure 3-3. Protective effects of GA against MHV infection in mice by suppressing induction of inflammatory cytokines.** C57/BL6 mice were infected  $8 \times 10^5$  pfu/mouse (i.p.) for survival studies and  $1 \times 10^4$  pfu/mouse (i.p.) for acute infections. Two hours later, mice were injected with GA (20 mg/kg, i.p.) every other day (total 3 times). On 5th day post infection (dpi), mice were sacrificed and serum and liver samples were collected. (A) Effects of GA administration on the survival of MHV infected mice. (B) Virus titer of liver homogenates. (C) Serum ALT levels on 5 dpi; (D) Representative H&E staining of liver tissues on 5 dpi. The data are represented as the means  $\pm$  SEM \* indicates  $P < 0.05$

(E-H) RNA was isolated from Liver samples at day 5 and levels of inflammatory genes IP10, IL-6, TNF- $\alpha$  and IL-1 $\beta$  which have been shown to mediate liver injury were assessed using QPCR. The data are represented fold inductions compared to control treated group. (means  $\pm$  SEM, \*  $P < 0.05$ )

**Figure 3-4. GA inhibits immune activation triggered by HMGB1 *in vitro*.** (A) HMGB1 release assay in the media of BNL.CL2 hepatocyte cells after MHV infection (MOI = 0.5). (B-C) D2SC cells were co-incubated with HMGB1 (10 g/ml, HMGB1-FLAG protein was purified using ANTI-FLAG M2 Affinity Gel according to the manufacturer's instructions) and different amounts of GA for 6 hours. The culture supernatants were subsequently collected and analyzed for IL-6 and TNF- $\alpha$  production. (C) IP-10 level analysis in the presence of GA ( $1 \text{ mg}/10^6$  cells) or anti-HMGB1 Ab ( $100 \text{ }\mu\text{g}/10^6$  cells) in infected BNL.CL2 monolayer cells on 48th hour after incubation. (D) IP10 mRNA expression and (E) secreted TNF- $\alpha$  protein levels in RAW 264.7 cells stimulated by supernatant from infected BNL.CL2 (panel A) after UV inactivation treated with GA ( $0.3$



mg/10<sup>6</sup> RAW cells) or anti-HMGB1 Ab (15μg/10<sup>6</sup> RAW cells) 8h after stimulation. (F) IP-10 secretion in the media after infection with MHV in the presence of HMGB1 neutralizing Ab or GA. (G) Virus titer of infected BNL.CL2 treated with control, GA (1mg/10<sup>6</sup> cells) or anti-HMGB1 Ab (100μg/10<sup>6</sup> cells) The data are represented as the means±SEM. \* indicates P<0.05; \*\* indicates P<0.01.

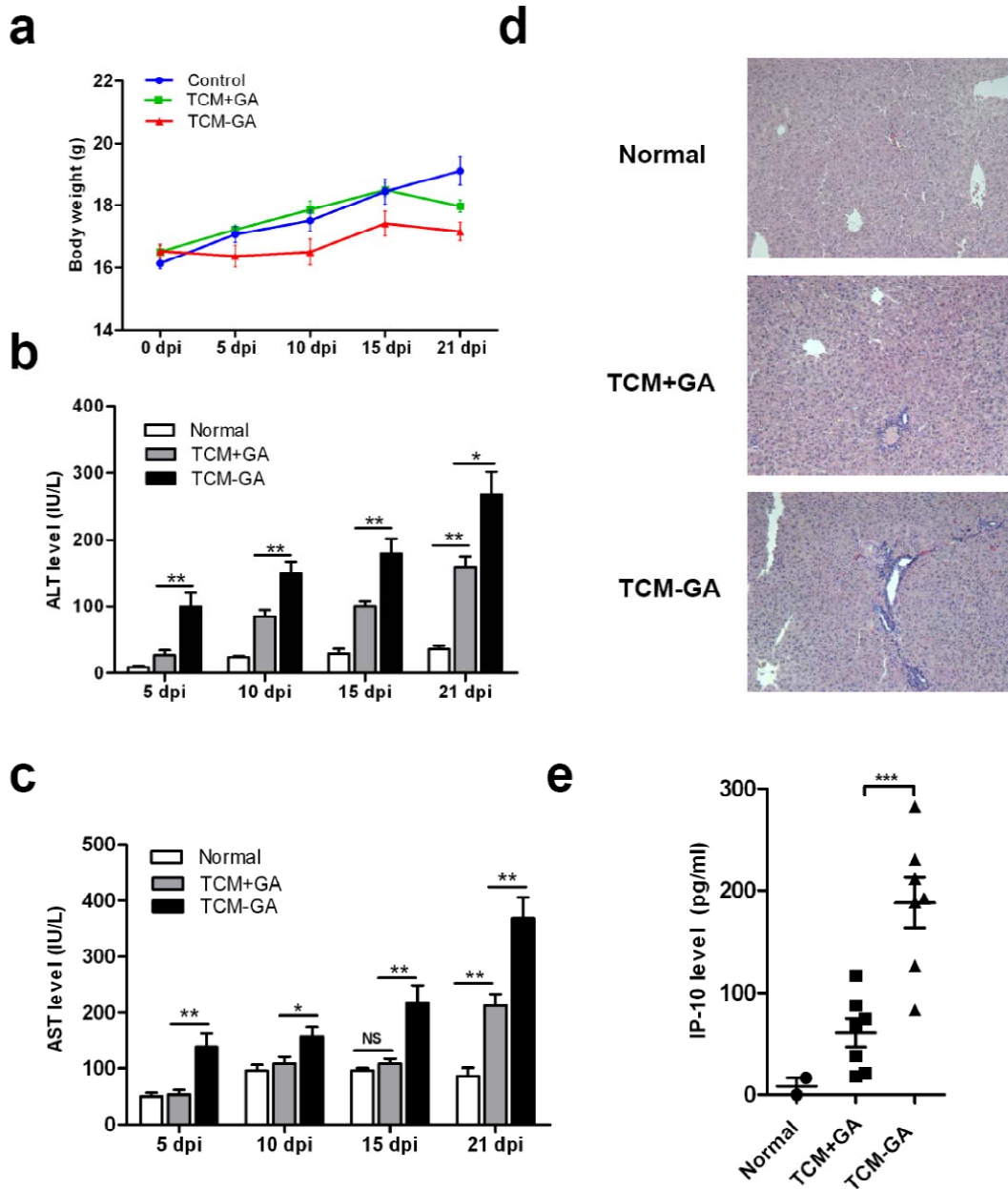
**Figure 3-5. Neutralizing HMGB1 antibody effectively alleviates liver injury induced by MHV-A59 infection.** C57/BL6 mice were infected i.p. 8×10<sup>5</sup> pfu/mouse for survival studies and 1×10<sup>4</sup> pfu/mouse for acute infections. Two hours after infection, animals were administered antibody (2.5mg/kg, i.p.) every other day (total 3 times) (A) serum HMGB1 levels following infection at 3 and 5 dpi. (B) Effects of HMGB1 neutralizing antibody on survival of MHV infected mice. (C) Virus titer of liver homogenates. (D) Serum ALT levels on 5 dpi; (E) Representative H&E staining of liver tissues on 5 dpi (magnification,×100). (F-H) RNA was isolated from Liver samples levels of inflammatory genes IP10, IL-6, TNF-α and IL-1β which have been shown to mediate liver injury were assessed using QPCR. The data are represented as the means ± SEM; \*\*\* indicates P<0.001.

**Figure 3-6: TLR4 signaling plays a key role in MHV induced hepatic injury.** C57BL6 wide type and TLR4 KO mice were infected similar as described previously. Two hours later, WT mice were injected with GA (20 mg/kg, i.p.) every other day (total 3 times). On 5th day post infection (dpi), the mice were sacrificed and serum and liver samples were collected (A) Effects of TLR4 molecular on survival of MHV infected mice. (B) Virus titer of liver homogenates. (C) Serum ALT levels on 5 dpi. (D) Representative H&E staining of liver tissues on 5 dpi (magnification,100×). (E-G) Liver IP-10, TNF-α and

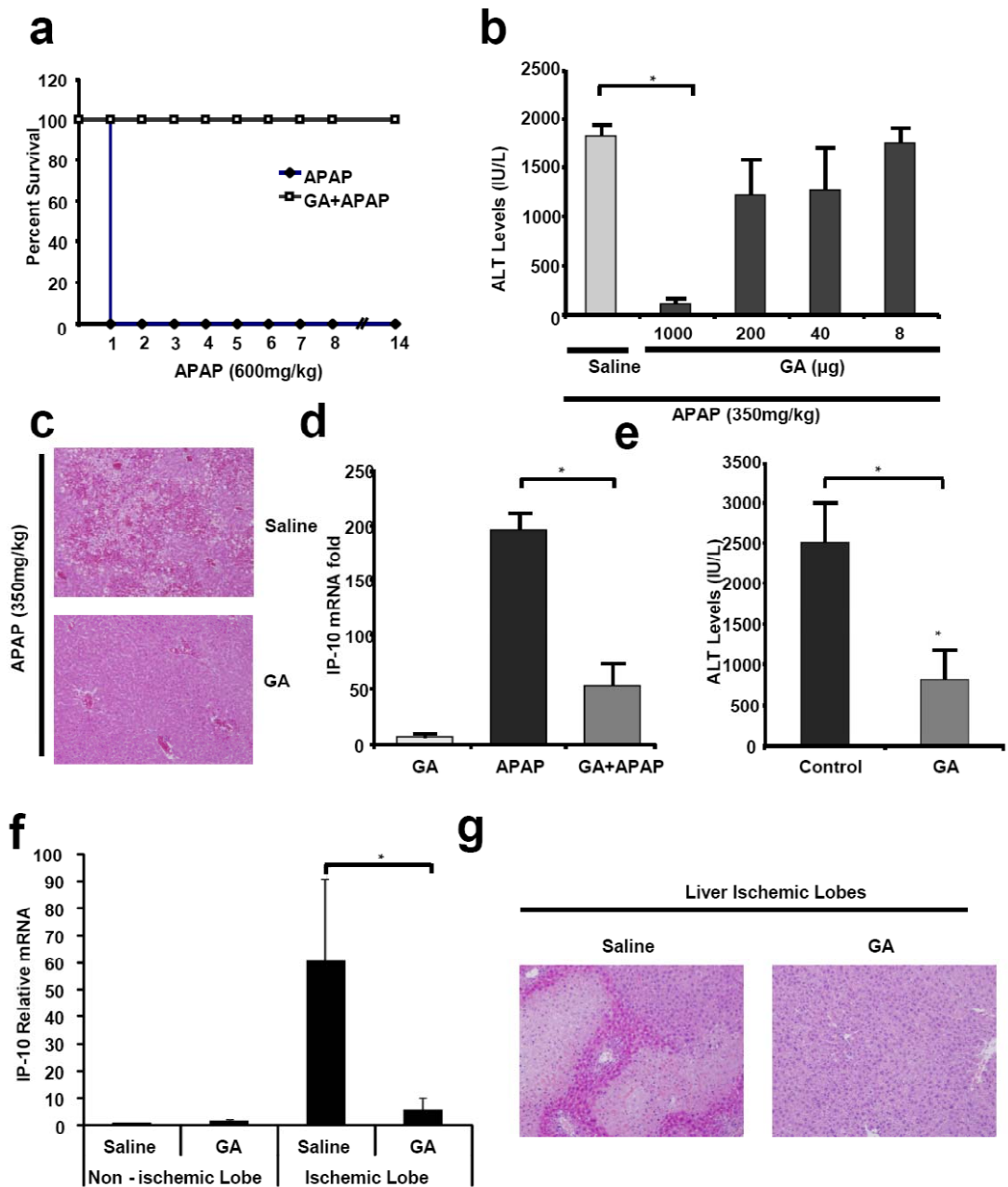
IL-6 mRNA expression in infected mice. The data are represented as the means $\pm$ SEM\* indicates  $P < 0.05$ , \*\* indicates  $P < 0.01$ .

**Figure 3-1. Glycyrrhizic Acid (GA) in traditional Chinese medicine (TCM)**

**solution alleviates liver injury during long-term injection.**



**Figure 3-2. Pretreatment with GA protects against I/R and Acetaminophen (APAP)-induced liver injury.**



**Figure 3-3. Protective effects of GA against MHV infection in mice by suppressing induction of inflammatory cytokines**

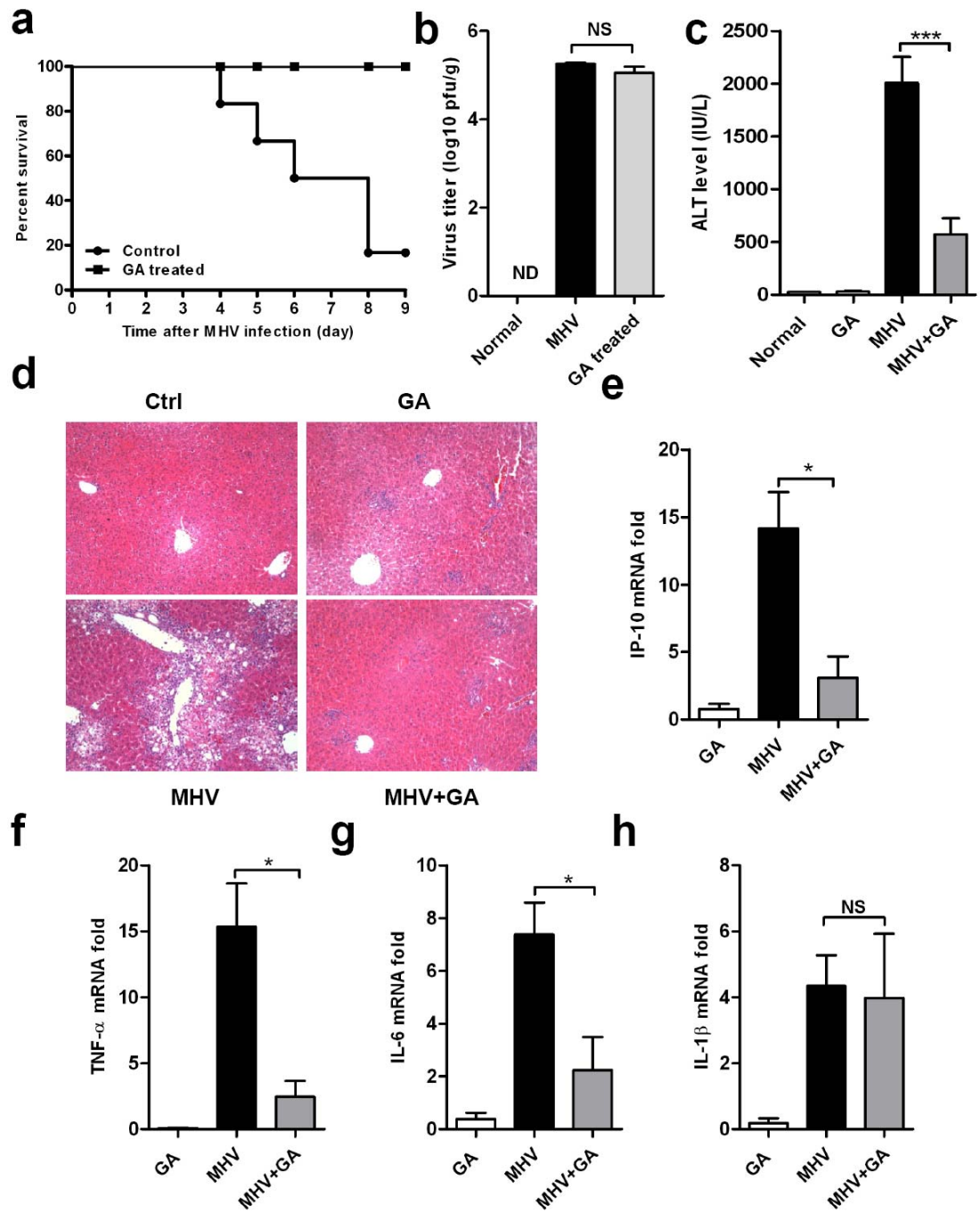
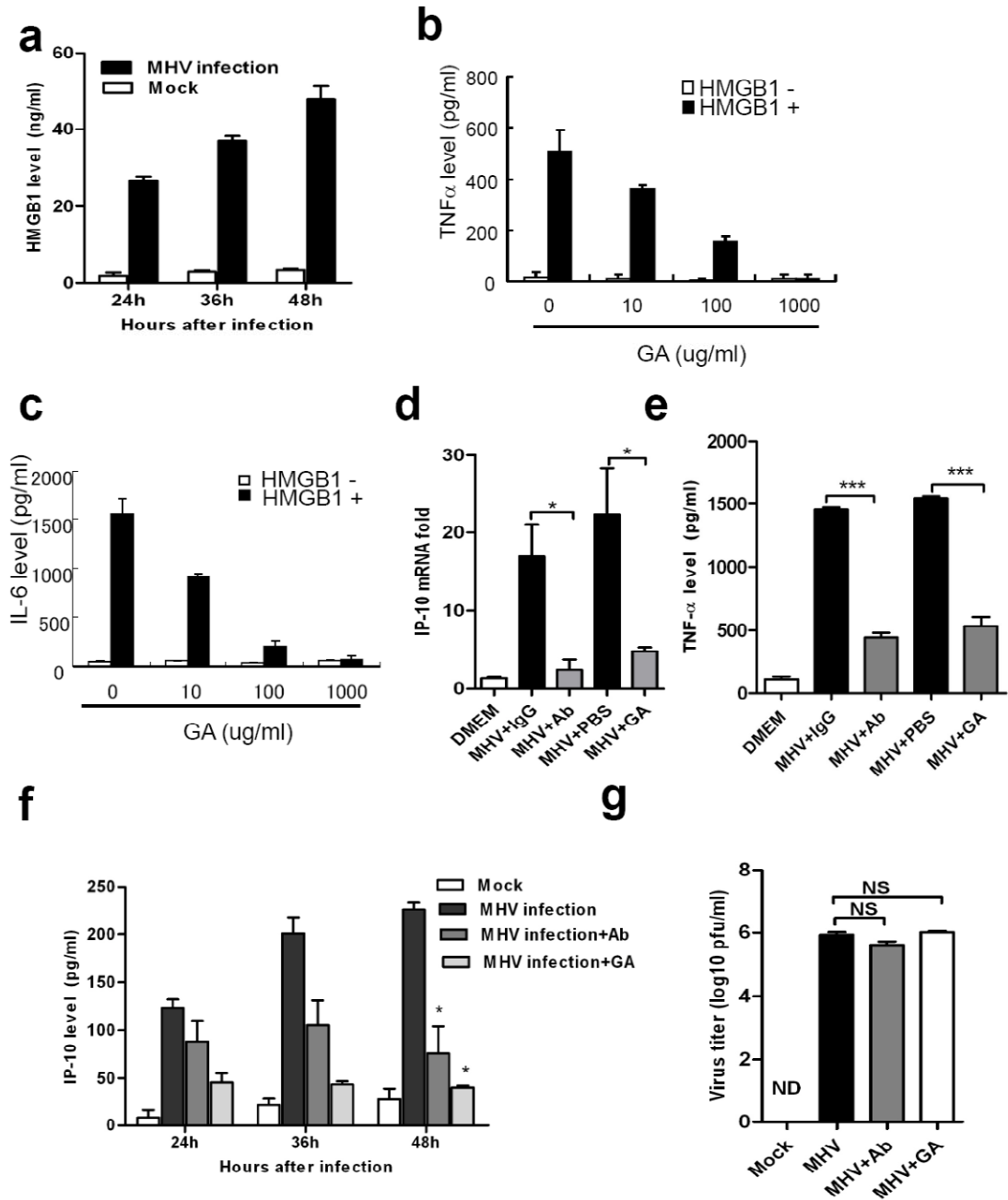


Figure 3-4. GA inhibits immune activation triggered by HMGB1 *in vitro*.



**Figure 3-5. Neutralizing HMGB1 antibody effectively alleviates liver injury induced by MHV-A59 infection.**

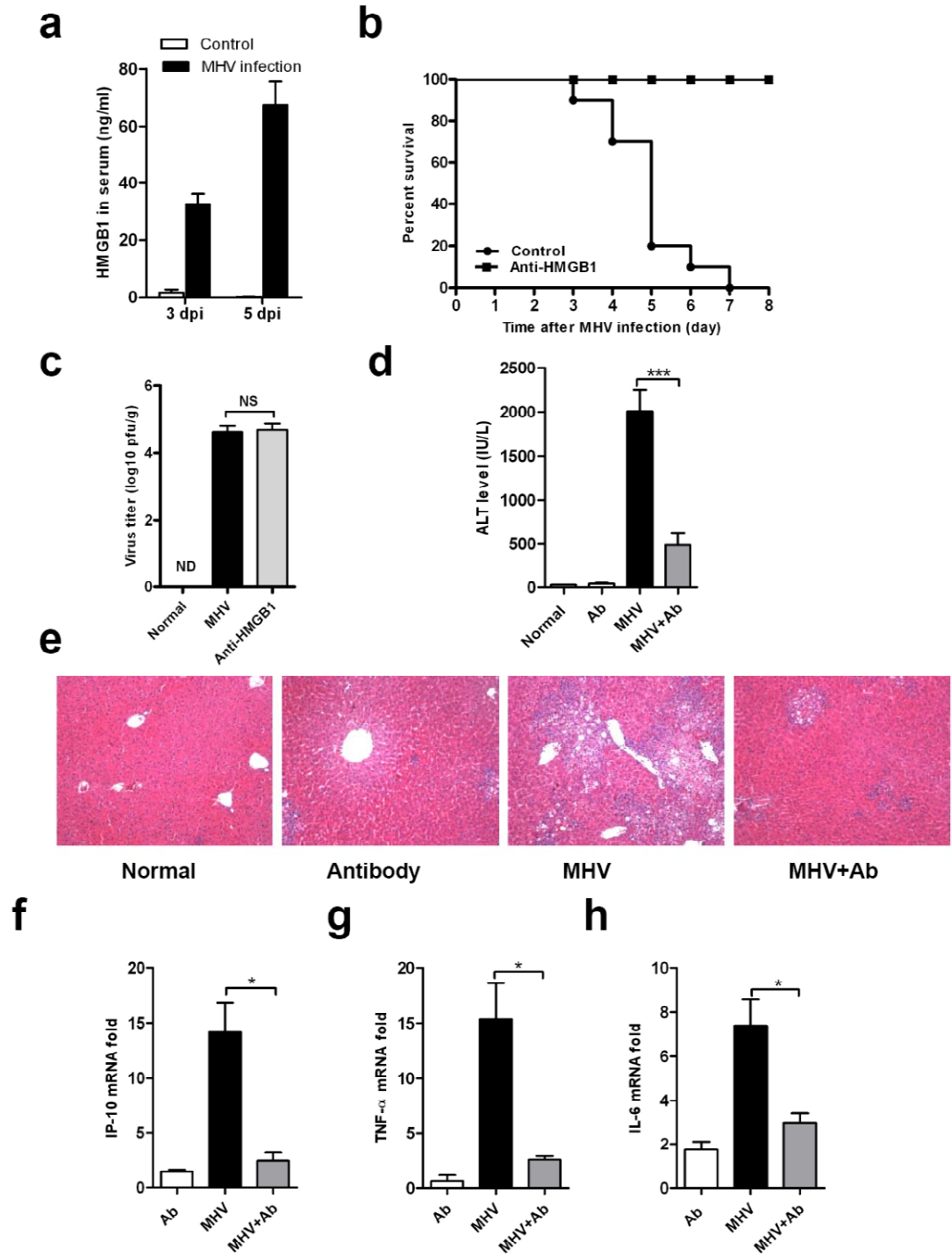
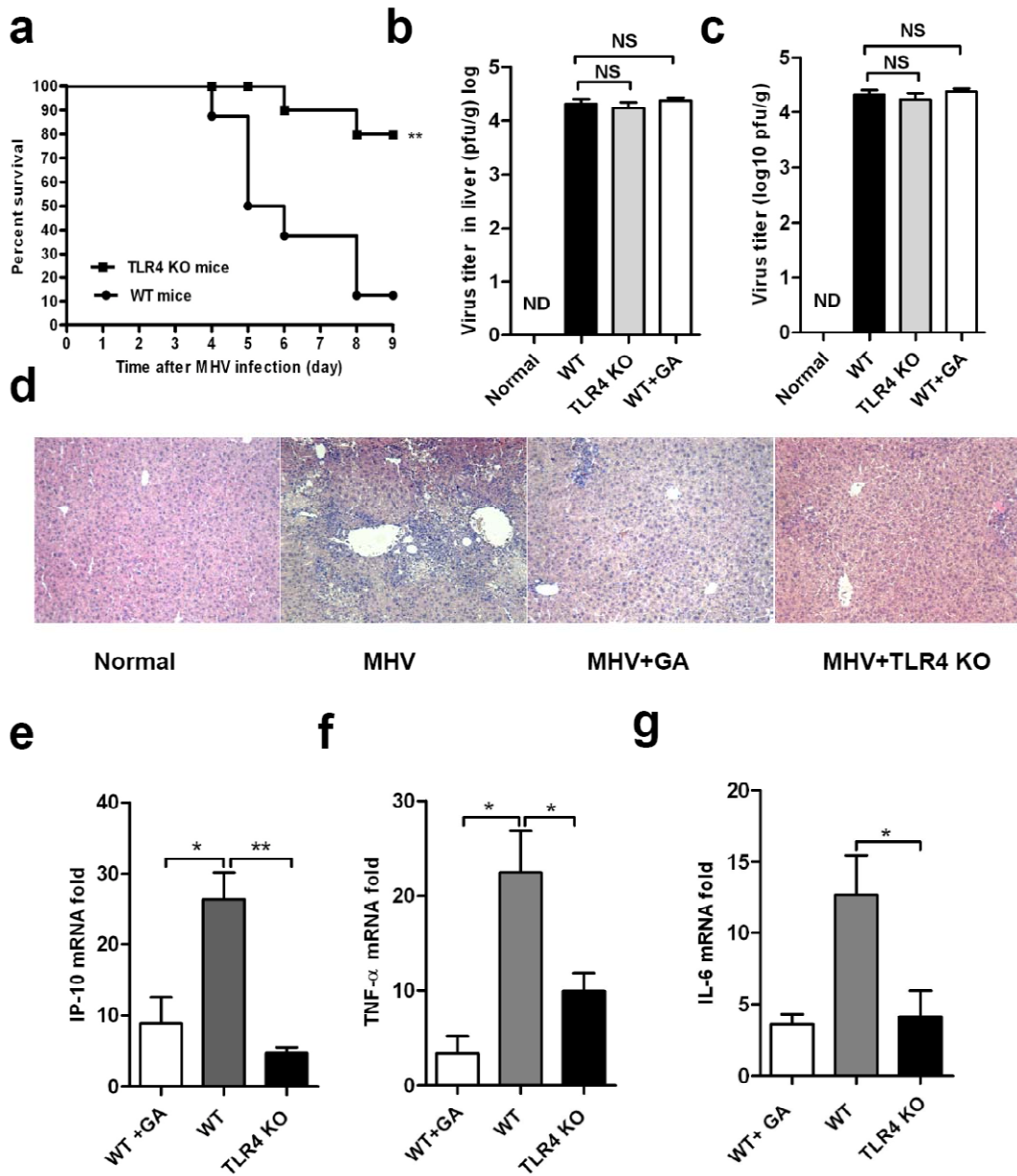
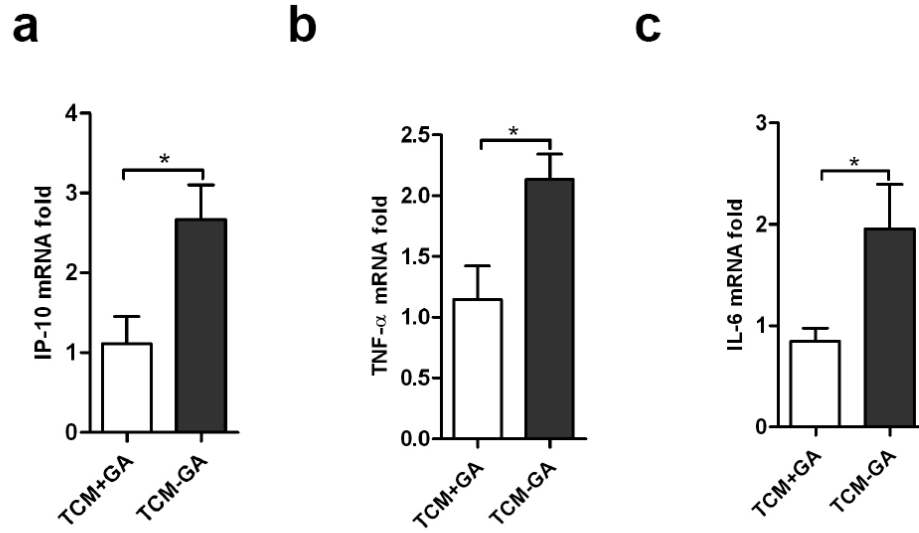


Figure 3-6: TLR4 signaling plays a key role in MHV induced hepatic injury.

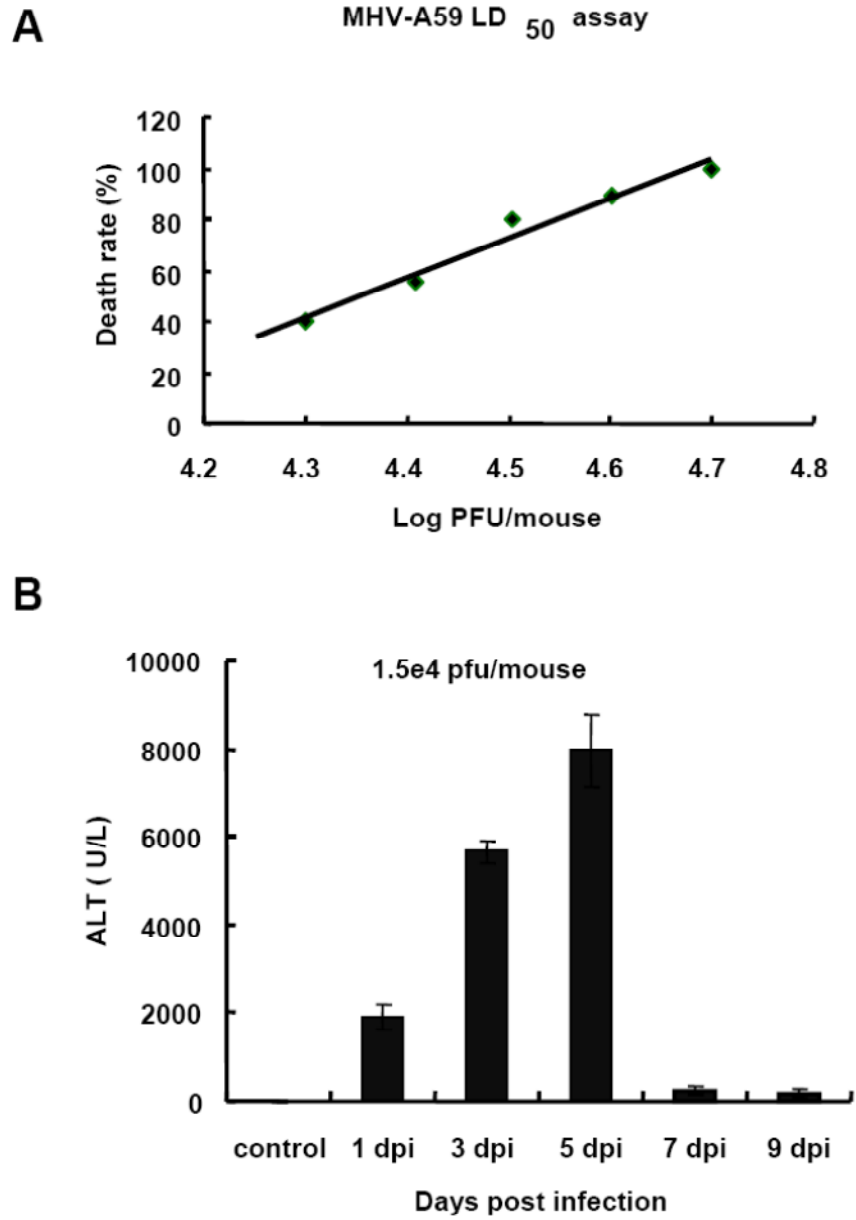




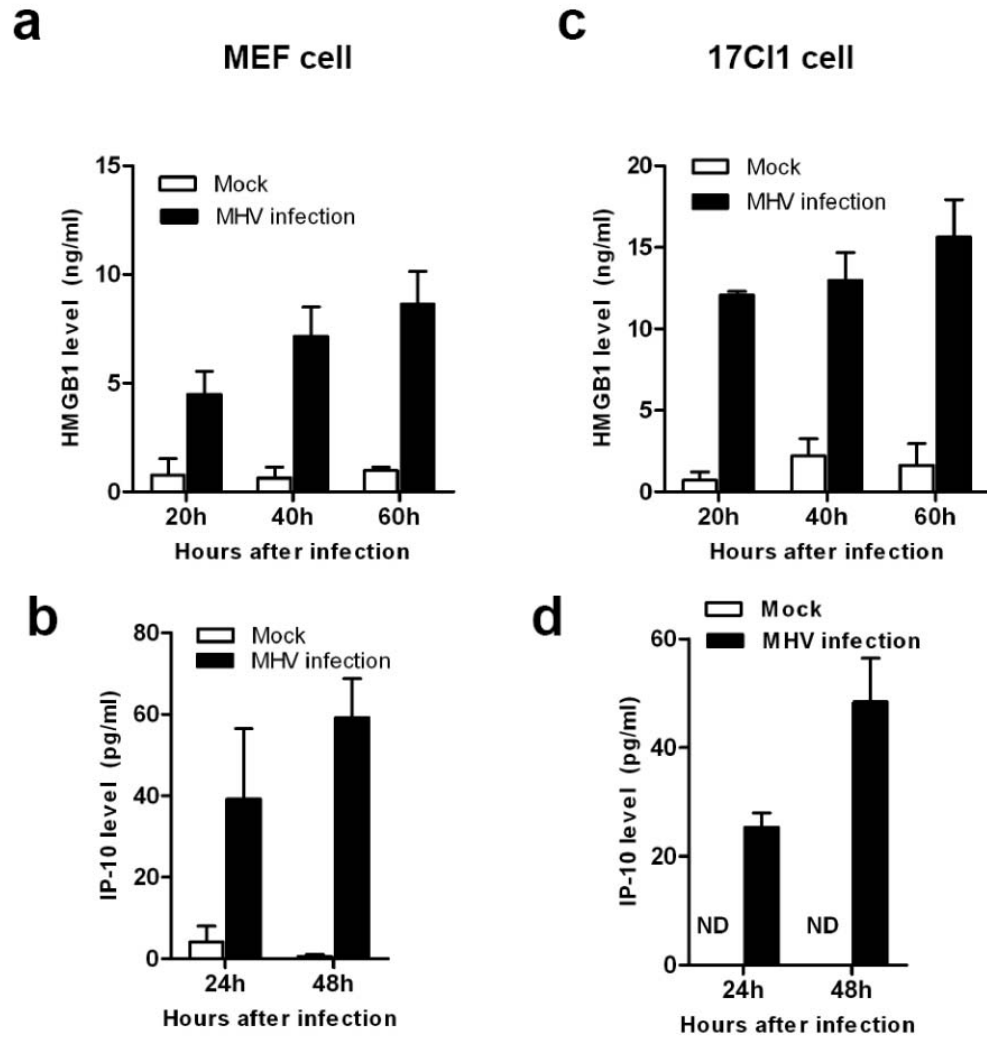
**Supplementary Figure 3-1. ( A)-(C) IP-10, TNF- $\alpha$  and IL-6 cytokine mRNA fold levels in liver of injected mice on 21 dpi.**



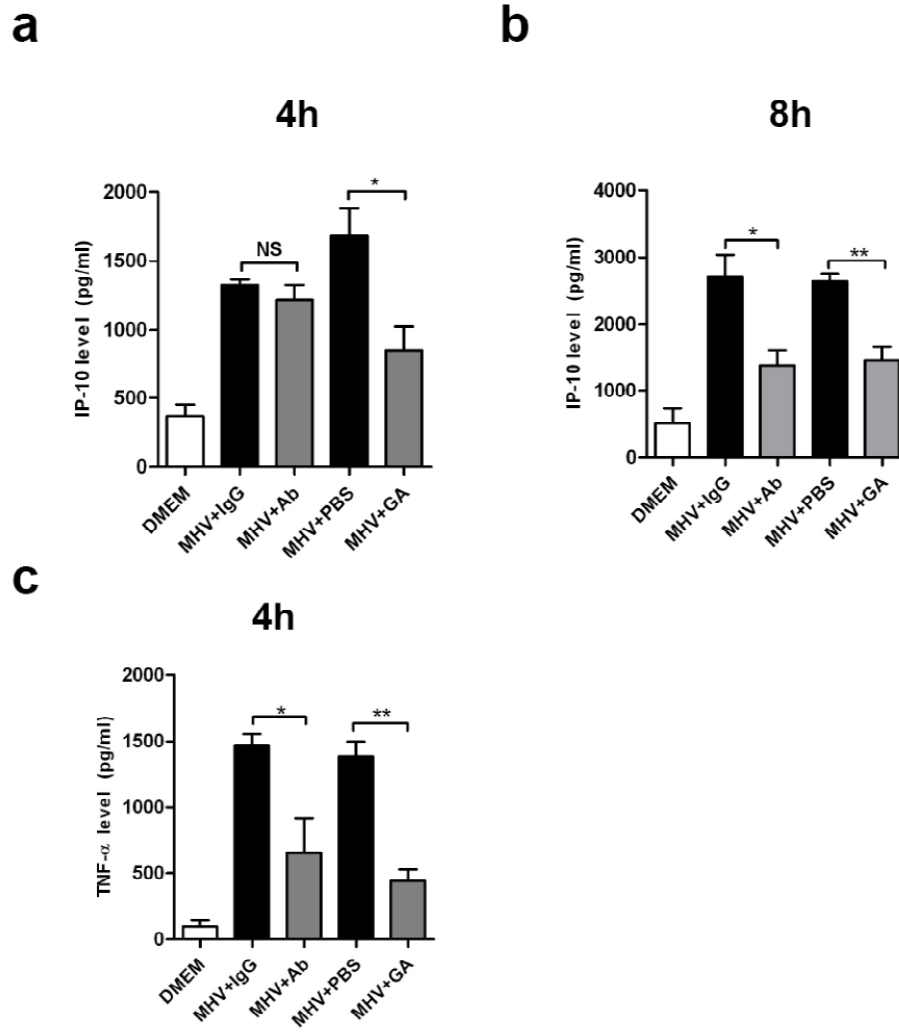
Supplementary Figure 3-2. A) MHV-A59 LD<sub>50</sub> assay (i.v. infections) B) ALT levels in MHV-A59 induced acute injury (1.5e4 pfu/mouse).



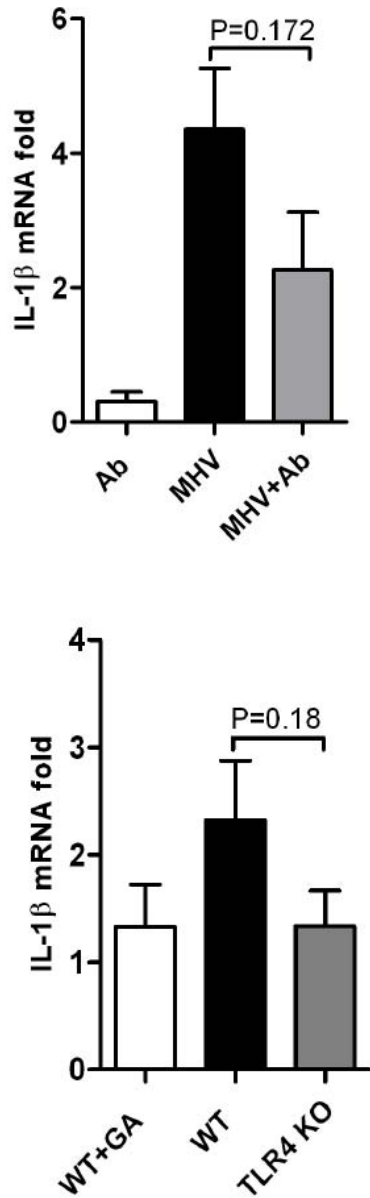
Supplementary Figure 3-3: The release assay of HMGB1 and IP-10 in the supernatants of MHV-infected MEF cells (a,b) and 17c11 cells (c,d) with ELISA.



**Supplementary Figure 3-4: The release assay of IP-10 (a,c) and TNF- $\alpha$ (b,d) cytokine in the supernatants of RAW264.7 cells on 4h and 8h after stimulation with inactivated and treated released HMGB1 solution.**



**Supplementary Figure 3-5: Fold Induction of IL-1 $\beta$ mRNA inflammatory genes from mice liver treated by anti-HMGB1 antibody (a) and in TLR4 KO mice (b) in response to MHV infection (5 dpi).**



## **Chapter 4**

**Fatty acid biosynthesis is an IFN-regulated pathway that plays opposite roles in anti-viral versus anti-bacterial responses**

## Introduction

Type I interferons (IFNs) were the first cytokines to be discovered, being named for their potent effects on viral “interference.” IFN is induced in response to activation of distinct innate immune receptors known as pathogen recognition receptors (PRR) which can detect viral nucleic acids as a pathogen association molecular patterns (PAMPs)<sup>1</sup>. Subsequently, a series of genes are induced downstream of type I IFN receptors (IFNAR) known as IFN inducible genes or ISGs<sup>2</sup>. Different ISGs have been identified to activate anti-viral responses through different mechanisms including amplification of IFN signaling, production of factors that directly inhibit viral replication at different cycles as well as activating adaptive immune responses. Interestingly, in addition to viral nucleic acids, bacterial components such as Lipopolysaccharides (LPS), a toll-like receptor 4 (TLR4) ligand, have also been shown to induce type I IFNs. However, the role of type I IFN signaling in anti-bacterial responses remains controversial. In fact, there are examples of negative effects of type I IFNs on clearance of some bacteria including *Listeria monocytogenes* and *Mycobacterium tuberculosis* (Mtb)<sup>3-7</sup>. Furthermore, activation of type I IFNs during viral infections such as influenza have been linked to increased susceptibility to secondary bacterial infections such as *Streptococcus pneumoniae*<sup>8</sup>. However, the mechanisms by which viral infections enhance bacterial infections remain poorly understood.

In this study, we performed RNA sequencing (RNA-seq) analysis (also known as Whole Transcriptome Shotgun Sequencing) of wild-type and IFNAR deficient bone marrow derived macrophages (BMDMs) stimulated with lipid A, key component derivative of LPS,

to identify genes that are modulated by type I Interferon (IFN) in response to TLR4 signaling. In addition to looking at specific genes, we examined canonical pathways including metabolic processes that are modulated differentially in BMDMs isolated from WT and IFNAR KO mice stimulated with lipid A. Interestingly, a canonical pathway altered differentially in IFNAR KO compared to WT BMDMs in response to LipA stimulation was fatty acid biosynthesis mainly due to observed suppression of the two key genes in this pathway: Fatty acid synthase (*Fasn*) and acetyl-coA carboxylase (*Acaca*). LipA stimulation in WT macrophages led to decreased *Fasn* expression while induced *Fasn* expression in IFNAR ko BMDMs.

*Fasn* is the key enzyme catalyzing the *de novo* synthesis of fatty acids, and its induction by various viruses such as Hepatitis C Virus (HCV) and Epstein-Barr Virus (EBV) is required for viral cycle completion<sup>9,10</sup>. Serum FASN concentration has also been reported to be significantly increased in patients infected with HCV as well as Human immunodeficiency virus (HIV)<sup>11</sup>. Additionally, lipid metabolism has been linked to inflammasome activation, which is an important pathway in anti-bacterial responses<sup>12</sup>. Given these findings, we set out to investigate the regulation of *Fasn* in response to activation of innate immune pathways and its significance in proper anti-viral and anti-bacterial responses.



## Results:

BMDMs isolated from WT or IFNAR deficient mice were stimulated with lipid A (100ng/ml) for 2 and 6 hours. RNA samples were then harvested to make cDNA libraries prepared from poly(A) enriched transcripts for each treatment for RNAseq preparation and analyzed as described previously. Using ingenuity program, we assessed canonical pathways that are modulated differently in IFNAR KO vs WT BMDMs 6 hours after LipA stimulation (Figure 4-1A). Lipid metabolism regulation was found to be IFNAR dependent among which modulations of fatty acid biosynthesis and glycerophospholipid metabolism were statistically significant ( $p < 0.05$ ) (Fig 4-1B). In the fatty acid biosynthesis pathways defined in ingenuity, *Fasn* and *Acaca* appear to be induced in IFNAR KO BMDMs stimulated with LipA while suppressed in WT cells (Figure 4-1C). LipA is recognized by TLR4 which then can activate downstream genes through TIR-domain-containing adapter-inducing interferon- $\beta$  (Trif) and Myeloid differentiation primary response gene 88 (MyD88) adaptor molecules. While Trif leads to phosphorylation of IRF3 and induction of type I IFN, MyD88 activation does not induce type I IFN. Generally, Trif and MyD88 signaling are thought to be important in innate immune responses against viral and bacterial infections, respectively<sup>1</sup>. Thus, we set out to assess whether activation of Trif through TLR3 activation by viral RNA PAMPs can lead to suppression of *Fasn*. Indeed, Polyinosinic:polycytidylic acid (PolyI:C), a synthetic double stranded RNA and TLR3 agonist, led to downregulation of these genes in different macrophage cells including BMDMs, immortalized BMDMs (J2-BMMs), murine macrophage cell line RAW264.7 cells as well as mouse embryonic fibroblasts (MEFs) (Figure 4-2A-D). Moreover, polyI:C suppression of *Fasn* required new protein synthesis as cycloheximide treatment of cells

abrogated downregulation of *Fasn* by PolyI:C (Figure 4E). Indeed, the effects of polyI:C on *Fasn* regulation appear to be dependent of IFNAR and STAT1 signaling (Figure 4-2H-G). These findings suggest that suppression of *Fasn* in response to Trif activation requires type I IFN signaling.

Interestingly, in Trif KO BMDMs, LipA stimulation enhanced *Fasn* mRNA levels, suggesting MyD88 activation could upregulate *Fasn* transcription (Figure 4-3A). Thus, we investigated the effects of MyD88 signaling in the absence of Trif signaling on *Fasn* regulation. Unmethylated CpG oligonucleotide sequences (referred to as CpG) are recognized by TLR9 and subsequently can activate MyD88 in BMDMs without inducing type I IFN. As anticipated, MyD88 activation through CpG stimulation led to increased *Fasn* mRNA expression levels (Figure 4-B). We also confirmed that changes in *Fasn* mRNA levels correlated with Fasn protein levels using Western Analysis. LipA as well as IFN $\alpha$  stimulation resulted in decreased Fasn protein levels after 8 hours (Figure 4-3C). Finally, in order to mimic TLR4 stimulation, we activated both Trif and Myd88 pathways using polyI:C and CpG treatment. PolyI:C and CpG co-treatment led to decreased Fasn protein levels in WT BMDMs while PolyI:C treatment alone suppressed Fasn expression and CpG treatment increased Fasn expression (Figure 4-3D). Additionally, in IFNAR KO BMDMs, co-stimulation with both PolyI:C and CpG induced Fasn expression, further indicating that Trif activation abrogates induction of Fasn in response to MyD88 activation dependent of type I IFN signaling. Given that *Acaca* and *Fasn* appear to be modulated similarly (Figure 4-1E, F), we assessed the regulation of their upstream gene, SREBP1<sup>12</sup>. The dominant form of SREBP1 in macrophages, SREBP1a is indeed modulated similar to

Fasn as polyI:C stimulation suppresses its expression while CpG stimulation results in increased *Srebp1* levels (Figure 4-3E).

Previous studies have suggested that Fasn is induced during some infections including HCV and EBV<sup>9,13</sup>. Here, we tested other RNA viruses and DNA viruses to examine what effects they have on *Fasn* expression. Vesicular stomatitis virus (VSV) infection of BMDMs led to decreased *Fasn* mRNA levels. To test whether these effects were dependent on the recognition of viral PAMPs by the host, we utilized BMDMs isolated from mice deficient in both TLR3 as well as Cardif, the adaptor protein responsible for signaling of viral RNA receptors such as MDA5 and RIG-I. TLR3/Cardif double knock out (DK) BMDMs cannot recognize viral PAMPs and subsequently do not induce IFN and its downstream genes<sup>14</sup>. Since VSV infection does not lead to *Fasn* suppression in TLR3/Cardif DKO BMDMs, it suggests that *Fasn* modulation during VSV infection is the result of host's responses (Figure 4-4B). Additionally, infection of BMDMs with DNA viruses including murine gammaherpesvirus 68 (MHV68) as well as Herpes Simplex Virus-1 (HSV1) led to decreased *Fasn* levels in WT but not IFNAR ko cells (Figure 4-4C, D).

Given our data suggesting the desire of host to suppress Fasn levels during viral infections through type I IFN induction, we tested what effects Fasn overexpression or knockdown has on viral infections. Knockdown of FASN in HEK293 cells 24 hours prior to infection with VSV-GFP inhibited infection rate of VSV as evidenced by decreased number of cells expressing GFP (Figure 4-5B). On the other hand, overexpression of FASN led to

enhanced VSV infection in HEK293 cells (Figure 4-5C). Similarly, addition of palmitate, downstream product of FASN, 4 hours prior to infection led to increased VSV infection rate as well (Figure 4-5D). Additionally, overexpression and knock down of the gene upstream of FASN, SREBP1a, results in increased and decreased VSV infection in HEK293 cells, respectively (Figure 4-6). These findings implicate that Fasn downregulation by IFN is important for proper anti-viral responses against VSV infection.

We also used *C75*, a commonly used inhibitor of FASN, to study the effects of inhibiting FASN activity on viral infections. *C75* treatment of HEK293 cells leads to decreased infection of enveloped viruses such as VSV, MHV68, HSV-1 while having no effects on Adenovirus, a non-enveloped virus (Figure 4-7A-D). Moreover, daily local treatment of *C75* starting a day prior to vaginal HSV-1 infection inhibits infection rate of HSV-1 in mice. In control mice, HSV1 infection peaks at day 2 post infection in vaginal lavage collections; however, *C75* treatment abrogated this surge in viral titer (Figure 4-7E). Systemic *C75* treatment was also effective in decreasing MHV68 infection. Mice that received *C75* (5mg/kg, i.p.) starting a day prior to infection with MHV68 coexpressing luciferase (MHV-luc) cleared the virus by day 8 while control mice still exhibited high titers of MHV68 (Figure 4-7F). These results implicate that *C75* is a physiologically important antiviral agent.

To gain better insight on how decreased Fasn activity can lead to viral inhibition, we took advantage of tools available for VSV to study the effects of Fas inhibition on the viral lifecycle. We utilized the pseudotyped VSV $\Delta$ G-Luc reporter virus system that has the

receptor-binding G gene (VSV-G) replaced with a luciferase reporter gene permitting only a single-round of infection to study whether C75 has any effects on viral lifecycle processes from entry to protein synthesis<sup>15</sup>. Treatment of cells with C75 4 hour prior but not post infection inhibited VSVΔG-Luc expression indicating inhibition of viral replication by C75 occurs at early stages of lifecycle (Figure 4-8). One of the earliest steps of infection is attachment of the virus to the host cell's membrane. HEK293Ts were treated for 8 hr with ethanol vehicle control (EtOH), C75 and CPZ (10 μg/mL), an endocytosis inhibitor that would have no effect on binding. To measure binding, VSV (5MOI) was incubated with HEK293T at 4°C for 1 hr to allow for binding but not cell entry. After washing three times with cold PBS, quantification of VSV genomic RNA (gRNA) showed no statistical difference between C75 and controls.

We then set out the study another early step in viral life cycle: fusion. We took advantage VSV-G β-lactamase (βla) entry assay established previously using VSV-G pseudotyped onto viral-like particles made from the βla-Nipah virus matrix fusion protein, herein called VSV-G-βlaM<sup>16</sup>. As VSV-G fusion results in entry of βlaM in the cytoplasm; the βla activity cleaves the lipophilic fluorescence CCF2-AM substrate changing its color from green to blue allowing us to indirectly measure VSV-G mediated fusion<sup>16,17</sup>. We have previously successfully demonstrated that IFN treatment of HEK293Ts could cause a dose-dependent inhibition of viral entry using this assay<sup>18</sup>. C75 pretreatment of 293T cells inhibited VSV-G-βlaM fusion as indicated by decreased cleavage rate of CCF2-AM substrate suggesting inhibiting Fasn activity can result in decreased VSV fusion (Figure 4-8C-D).

In order to study whether C75 can inhibit fusion of other viruses, we used NiV fusion (F) and attachment (G) proteins to induce pH-independent cell-cell membrane fusion to form syncytias. Vero cells were transfected with NiV F and G for 5 hr and C75 or EtOH was added to cells. At 24 hours post transfection, cells were fixed and stained by Giemsa, grossly to assess syncytia formation. C75 treatment led to less syncytia formation and fewer nuclei per syncytia compared to control (Figure 4-9A-B). Also, to further confirm these results, we overexpressed human FASN as well as murine Fasn in these cells 24 hours prior to NiV F and G transfection. Overexpression of FASN leads to enhanced formation of syncytias (Figure 4-9C-D). These data demonstrate that inhibiting fatty acid synthesis modifies the cellular membrane to inhibit viral membrane fusion.

We and others have previously demonstrated that IFNAR ko mice are more resistant to *Listeria monocytogenes* infection, indicating that type I IFN may play a detrimental role in fighting against *Listeria* infection<sup>4</sup>. Here, we investigated whether Fasn as a type I IFN regulated gene plays opposite roles in anti-viral versus anti-bacterial responses. Fasn KO BMDMs appear to be more susceptible to *Listeria* infection compared to control cells as indicated by increased bacterial intracellular recovery at 7 hours post infection (hpi)(Figure 4-10A). Similarly, pretreatment of RAW264.7 cells and BMDMs with C75 (5mg/ml) also led to increased bacterial load at 6 hpi (Figure 4-10B-C). However, no differences were observed at 2hpi in BMDMs pretreated with C75 or vehicle suggesting that phagocytosis of bacteria by macrophages is not affected by C75 (Figure 4-10C). Overall, these findings indicate that C75 treatment suppresses the ability of macrophages to clear *Listeria* (Figure 4-10D). Recent studies suggest that autophagy, a tightly regulated process by which cells

degrade their own components, can be linked to lipid metabolism and play an important role during bacterial infection. Thus, we then examined whether induction of autophagy is affected by C75 pretreatment. Indeed, C75 pretreatment of BMDMs inhibited induction of autophagy by LPS, Mtb as well as Zymosan (TLR2 agonist) as indicated by decreased LC3 puncta formation (Figure 4-10E). Additionally, C75 pretreatment inhibited clearance of MtB following TLR ligand activation (Figure 4-10F).

## Discussion:

Viruses rely on the metabolic network of the host cell to provide energy and macromolecular precursors to fuel viral replication. In fact, recent studies characterize different viruses that have evolved to alter the host's metabolism in the favor of their replication. For instance, Human cytomegalovirus (HCMV) infection up-regulates fatty acid biosynthesis and glycolysis while HSV-1 appears to inhibit glycolysis<sup>19</sup>. There are also examples of viral proteins upregulating specific lipid metabolic enzymes. FASN expression is induced by the EBV immediate-early protein BRLF1 and is required for lytic viral gene expression<sup>9</sup>. Additionally, recent studies suggest that both Kaposi Sarcoma Herpes Virus (KSHV) and West Nile virus (WNV) induce lipogenesis<sup>20,21</sup>. These examples suggest that not only does lipid metabolism play an important role in viral infections, but also some viruses may have gained the ability to alter specific host metabolic genes. On the other hand, it is unclear whether the host also has the ability to modulate its own lipid metabolism as a mechanism against viral infections.

Through RNAseq analysis, we identified *Fasn* as an IFN-regulated gene potentially important during viral and bacterial infections. Activation of *Trif* signaling pathways through polyI:C or LipA leads to type I IFN induction which in turn downregulates *Fasn* transcription. However, MyD88 signaling in the absence of type I IFN signaling as in the case for TLR2 as well as TLR7 activation upregulates *Fasn* transcription. Furthermore, *Trif*-mediated suppression of *Fasn* takes precedent over its induction by MyD88 signaling as coactivation of *Trif* and MyD88 pathways leads to decreased *Fasn* levels.



In addition to *Fasn*, *Acaca* was also found to be modulated in a similar fashion prompting us to look at their common upstream regulator, SREBP1 which is encoded by *Srebf1*. Among the two isoforms of SREBP1, SREBP1a is the dominant form expressed in macrophages compared to SREBP1c<sup>12</sup>. Indeed, the gene coding for SREBP1a, *Srebf1a*, is modulated similarly to *Fasn* as polyI:C stimulations suppresses *Srebf1* levels while CpG stimulation results in elevated *Srebf1* levels. SREBP1 is known to be regulated by Liver X receptor (LXR) and Retinoid X-receptor (RXR) nuclear hormone receptors<sup>22,23</sup>. However, we previously demonstrated that downregulation of RXR through polyI:C is independent of type I IFN signaling<sup>24</sup> and since polyI:C suppression of SREBP1 and *Fasn* are dependant of type I IFN signaling, it is implicated that SREBP1a suppression by polyI:C is independent of LXR/RXR regulation. SREBP1 induction by CpG could be explained by a recent study identifying SP-1 binding sites of NF-κB in SREBP1a promoter region in BMDMs<sup>12</sup>. However, the authors in the same study demonstrate that LPS induces SREBP1a while our findings illustrate that SREBP1a, and its downstream genes including *Fasn* as well as *Acaca* are downregulated by LipA as well as PolyI:C. One potential explanation for the observed discrepancies in these results could be the conditions in which stimulations are performed. We noticed that stimulating macrophages after serum starvation or in 1% FBS without M-CSF results in slight induction of *Fasn* instead of suppression by LipA. Metabolic genes, including *Fasn*, are regulated tightly based on the presence of ample metabolites both extracellularly as well as intracellularly. Thus, in order to obtain physiologically relevant conditions, all stimulations were done in 10% FBS and M-CSF, the conditions which cells were acclimated in for at least 7 days.

Here, we propose that suppression of *Fasn* by type I IFN during viral infections plays an important role in anti-viral pathways as inhibition of Fasn activity results in decreased viral infection and viral membrane fusion. Although previously some viruses had been shown to induce Fasn expression, here we show that VSV, HSV1 as well as MHV68 infection lead to downregulation of Fasn due to host's responses through induction of type I IFN. In the context of HCV infection, it was previously demonstrated that active virus infection enhances Fasn expression, while UV inactivated HCV exposure leads to decreased of Fasn levels in Huh7 liver cells<sup>25</sup>. Our findings further support this as HCV, among other viruses, has evolved to inhibit type I IFN induction and signaling and the elevated FASN levels reflect the selective preference of HCV to induce lipid metabolism. However, when UV inactivated, HCV is no longer actively replicating in cells and the suppression of Fasn can be explained by the preference of the host to suppress fatty acid synthesis through type I IFN signaling pathways. While, C75 treatment has been reported to inhibit infection of viruses such as HCV, HCMV, and WNV which induce lipogenesis for completion of their viral cycle, here we offer a novel mechanism by which C75 treatment may broadly inhibit viral infection of enveloped viruses by inhibiting membrane fusion.

Viral infections have been linked to increased susceptibility to bacterial infections potentially due to opposite roles of type I IFN in anti-viral versus anti-bacterial host defense responses. In fact, a significant proportion of virally infected patients develop concomitant or secondary bacterial infection<sup>26</sup>. However, the mechanisms for how viral infections promote bacterial infections remains poorly understood. Type I IFN signaling has been shown to enhance infection rate of *Listeria* as well as *Mtb*<sup>3,7</sup>. Our findings identify Fasn as

an IFN-regulated gene that interferes with Listeria and Mtb clearance. Recently lipid metabolism in macrophages was linked to inflammasome activation and interleukin-1 $\beta$  (IL-1 $\beta$ ) secretion which have been illustrated to be important in Listeria and Mtb clearance<sup>12</sup>. However, we did not observe any changes in IL-1 $\beta$  release in C75 treated cells (Data not shown). Furthermore, BMDMs deficient in (Apoptosis-associated Speck-like Protein Containing a Caspase Recruitment Domain) ASC, an important adaptor in inflammasome activation, cleared Listeria similar to WT cells further implicating that our observed differences in Listeria infection *in vitro* were not due to changes in inflammasome activation (Data not shown). Recent studies suggest that autophagy can also be an important player in host responses against bacterial infections. Interestingly, C75 treatment inhibits autophagy induced by LPS and Mtb which in turn could explain its effects on decreased rate of clearance of Mtb in BMDMs. Although type I IFNs' effect on bacterial infections is likely complex, this study offers a novel mechanism by which anti-viral pathways through suppression of *Fasn* can promote bacterial infections.

Much attention has recently been given to developing FASN inhibitors as potential anticancer therapeutics. A wide variety of human malignancies, including carcinoma of the colon, prostate, ovary, endometrium and breast, express high levels of FASN. This has led to the development of novel FASN inhibitors as potential anticancer therapeutics without the major side effects of C75 such as anorexia and weight loss. Recent studies have demonstrated that inhibitors of FASN, including C75 and cerulenin, are able to inhibit growth of different tumors. The emergence of safer and more effective FASN inhibitors as anticancer therapeutics makes utilizing these drugs as anti-viral agents more feasible

clinically. Our data suggest that C75 treatment *in vivo* may be beneficial in blocking infection of enveloped viruses whose infection had not been linked to lipogenesis previously, prompting more studies to assess the effectiveness of C75 and the new generation of FASN inhibitors as potential broad anti-viral agents. On the other hand, our findings on the effects of C75 on increasing bacterial infection should be considered in patients undergoing treatment with C75 as anti-cancer agents.

Overall, our findings provide further evidence of the cross-talk between innate immune pathways and lipid metabolism. Here, we characterize Fasn as an IFN-regulated gene that can potentially play opposite roles in viral versus bacterial infection. Additionally, this study offers novel mechanisms by which type I IFN downregulation of Fasn can inhibit viral infections through decreased membrane fusion, while interfering with host's ability to clear bacteria potentially through inhibition of autophagy.

## Figure Legends:

**Figure 4-1. LipA stimulation results in opposite regulation of Fatty acid biosynthesis in WT and IFNAR ko BMDMs.** BMDMs isolated from WT or IFNAR deficient mice were stimulated with lipid A (100ng/ml) for 6 hours and RNA samples were isolated and prepared for RNAseq analysis. Using ingenuity program, canonical pathways which were modulated differently in IFNAR KO vs WT BMDMs were identified (A and B). Heat map of genes significantly modulated in fatty acid biosynthesis based on the RNAseq data (C). Verification of the results in (C) using qPCR analysis of BMDMs stimulated with LipA for 6 hours.

**Figure 4-2. PolyI:C downregulates Fasn through IFNAR/STAT1 pathways.** Fasn mRNA levels were determined using qPCR analysis in the following conditions: (A-C). BMDMs, J2-BMMs as well as RAW267.4 cells were treated with polyI:C (1ug/ml). (D) MEFs were transfected with polyI:C or control and Fasn levels determined using QPCR analysis after 8 hours. (E) 4 hr PolyI:C stimulation of BMDMs with or without 30min pretreatment with cycloheximide (0.5ug/ml). (F) Stimulation of BMDMs with different cytokines for 6 hours. (H-G) WT, IFNAR KO and STAT1 KO BMDMs were stimulated with polyI:C for 8 hours.

**Figure 4-3. Modulation of Fasn through Trif and MyD88 signaling pathways.** (A-B) Fasn mRNA levels were assessed using qPCR analysis in BMDMs stimulated with TLR ligands after 8 hours. (C-D) Fasn protein levels were determined using Western analysis in BMDMs stimulated with TLR ligands after 8 hours. (E) Srebp1a mRNA levels were assessed using qPCR analysis in BMDMs stimulated with TLR ligands after 4 hours.

**Figure 4-4. Effects of viral infections on Fasn levels in BMDMs.** (A-B) Fasn mRNA levels were determined using qPCR analysis in BMDMs stimulated with polyI:C or infected with VSV (MOI=1) after 8 hours. (C-D) BMDMs were infected with MHV68 or HSV1 at indicated MOIs and Fasn mRNA levels were measured using QPCR analysis.

**Figure 4-5. Effects of Fasn expression and activity on VSV infection.** HEK293T cells were transfected with siScrambl (control) or siFASN 24 hours prior to viral VSV infection (MOI=0.2). For overexpression studies, HEK293T cells were transfected with expression plasmids 36 hours before viral infection. Cells were treated with Palmitate/BSA or BSA alone (200uM) 12 hour before VSV infection. VSV infection was assessed indirectly by measuring %GFP expressing using FACS analysis 9 hpi.

**Figure 4-6. Effects of SREBP1 expression on VSV infection.** HEK293T cells were transfected with siScrambl (control) or siSREBP1 24 hours prior to viral VSV infection (MOI=0.2). For overexpression studies, HEK293T cells were transfected with expression plasmids 36 hours before viral infection. VSV infection was determined indirectly by measuring %GFP expressing using FACS analysis 9 hpi or using standard plaque assay 12hpi.

**Figure 4-7. Inhibition of VSV, MHV68 and HSV-1 by C75 treatment in HEK293 cells.** (A-D) HEK293 cell were treated with C75 at the specified doses for 12 hours before infections. VSV, MHV68 and HSV-1 viral titers 12 hpi were determined using standard pfu assays while Adenovirus infection rate was assessed indirectly by measuring %GFP expressing using FACS analysis 24 hpi. (E) Effects of daily local administration of C75 on HSV-1 vaginal infection. (D) Measuring luciferase activity in mice infected with

MHV68luc 8dpi with daily administration of C75 (5mg/kg, ip) or media starting 1 day before infection.

**Figure 4-8. C75 treatment effects early steps of VSV lifecycle by decreasing viral membrane fusion.** (A-B) Pre-treatment or post-treatment of C75 in 293HEK cells infected with replication deficient psuedo-type VSV-renilla luc. (C-D). HEK293 cells were pretreated with C75 at indicated doses for 12h before infection with pseudovirus encoding Nipam- $\beta$ -lactamase VSV-G ( $\beta$ la-VSV) for 1.5h.  $\beta$ -lactamase activity was measured by determining the rate of cleavage of CCF2 (green) to its cleaved form (Blue).

**Figure 4-9. Effects of modulation of Fasn activity on membrane fusion and Syncytia formation in Vero cells transfected with NiV F and G proteins.** (A-B) Vero cells were transfected with NiV F and G 5 hr before C75 or EtOH was added to cells. At 24 hours post transfection, cells were fixed and stained by Giemsa, grossly to assess syncytia formation. (C-D) FASN as well as murine Fasn were expressed in vero cells 24 hours prior to NiV F and G transfection and formation of syncytias was determined 24 hours after F and G transfection (Figure 4-9C-D).

**Figure 4-10. C75 treatment suppresses clearance of Listeria and Mtb.** (A) WT or FAS KO BMDMs were infected with Listeria (MOI=10) for 6 hours and cells were lysed and bacterial load was determined using a standard CFU assay. (B) RAW264.7 cells were treated with C75 (5ug/ml) 4 hours before listeria infection. (C) BMDMs were treated with C75 4 hours before Listeria infection. Cells were harvested and lysed at 2 and 7 hpi to assess CFU. (D) Percentage of phagocytosed bacterial being cleared in (C). (E) Measuring LC3 puncta per cells after TLR ligand stimulation or Mtb infection with or without pretreatment

of cells with FASN inhibitors C75 or EGCG. (F) Rate of clearance of Mtb induced by TLR ligand stimulations in the presence or absence of C57 or EGCG treatments.



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**Figure 4-1. LipA stimulation results in opposite regulation of Fatty acid biosynthesis n WT and IFNAR ko BMDMs.**

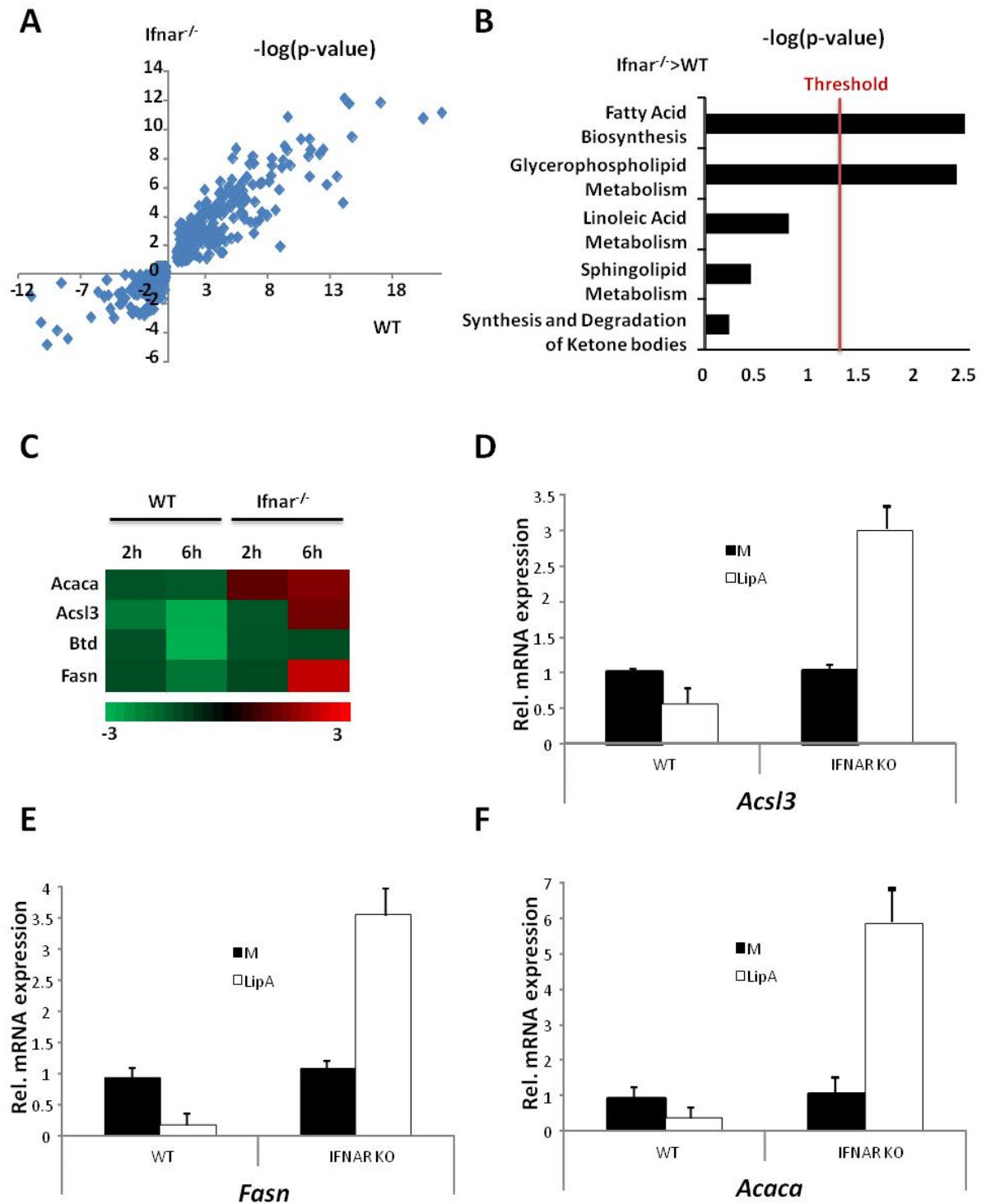


Figure 4-2. PolyI:C downregulates Fasn through IFNAR/STAT1 pathways

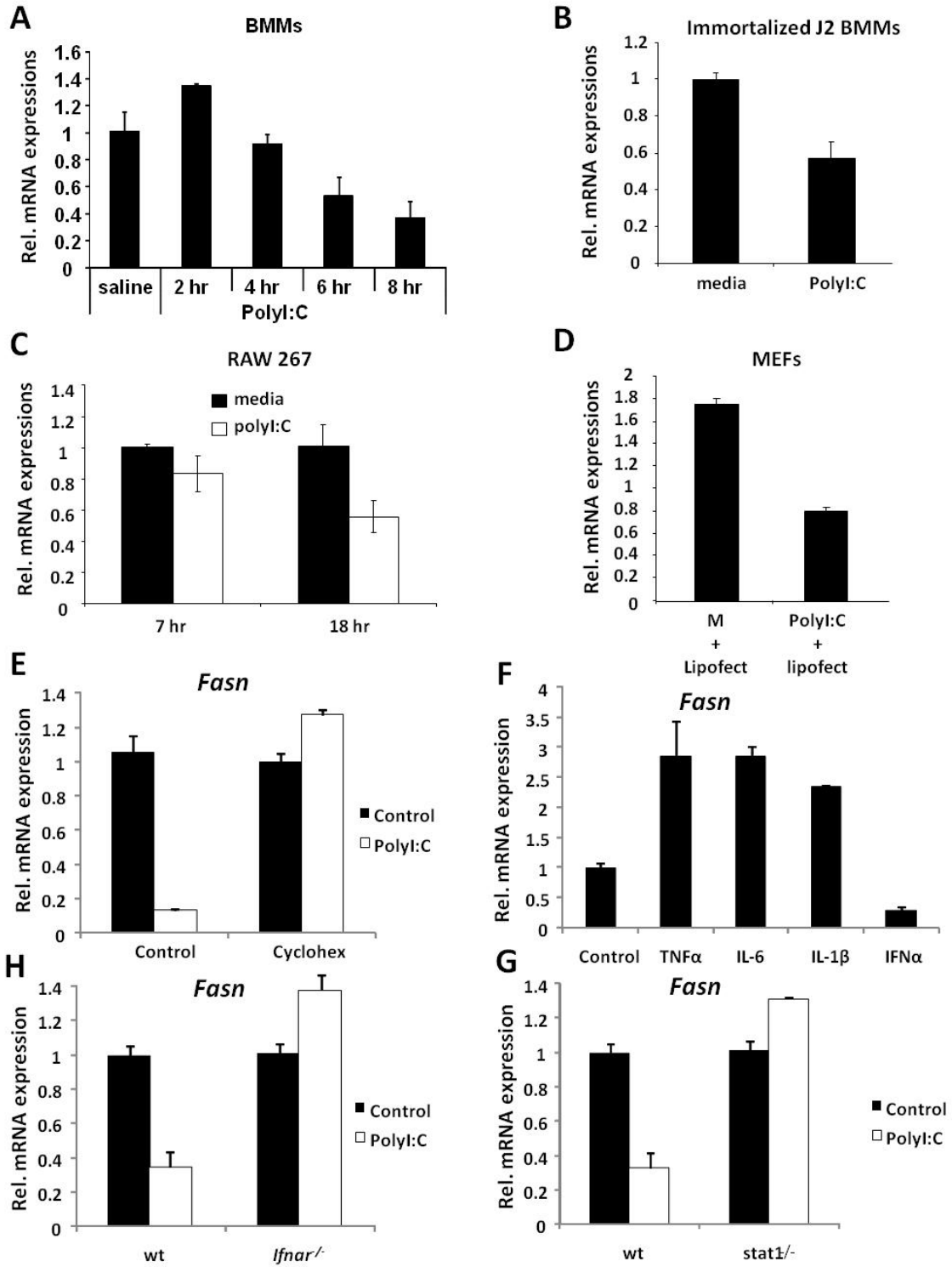


Figure 4-3. Modulation of *Fasn* through Trif and MyD88 signaling pathways

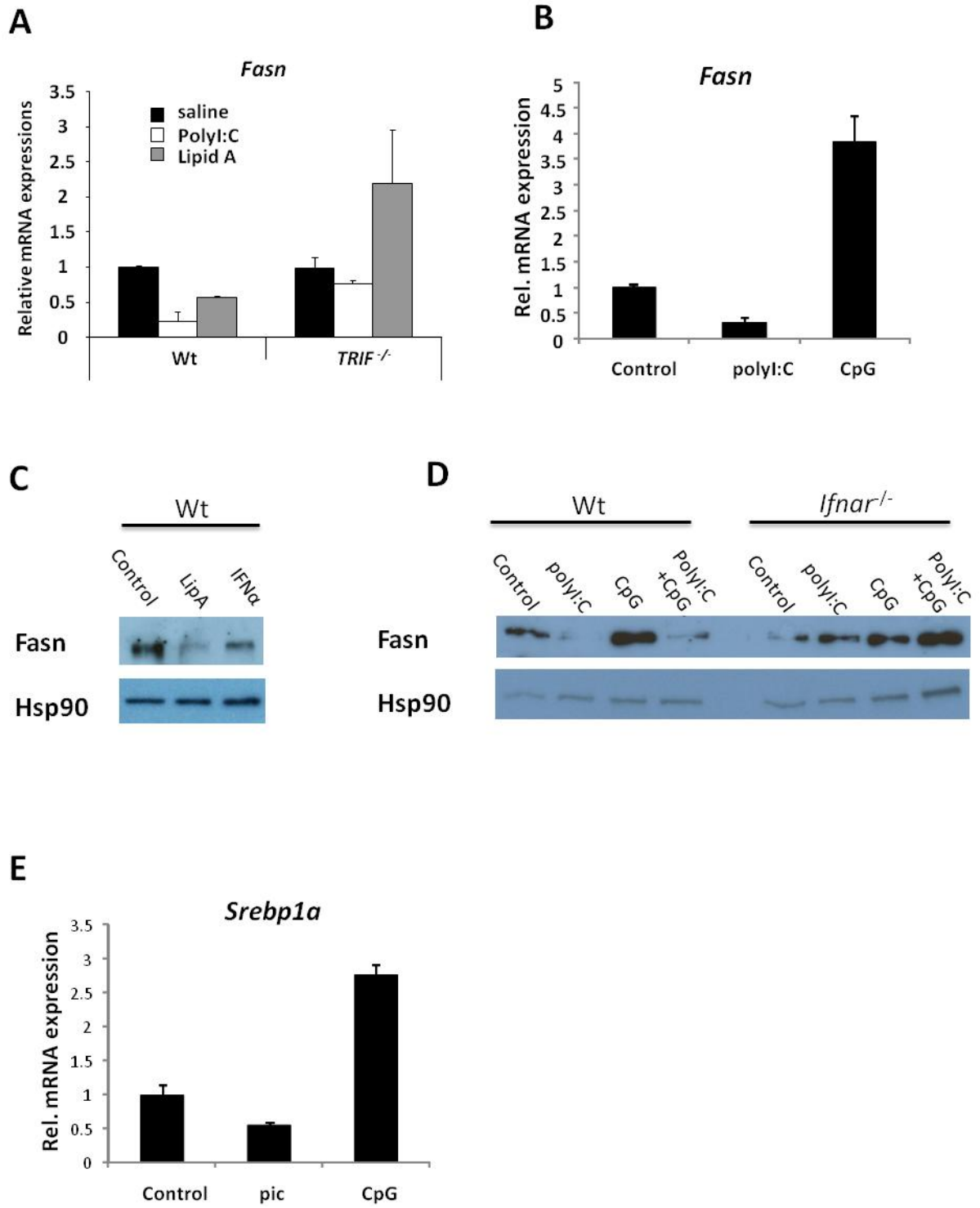
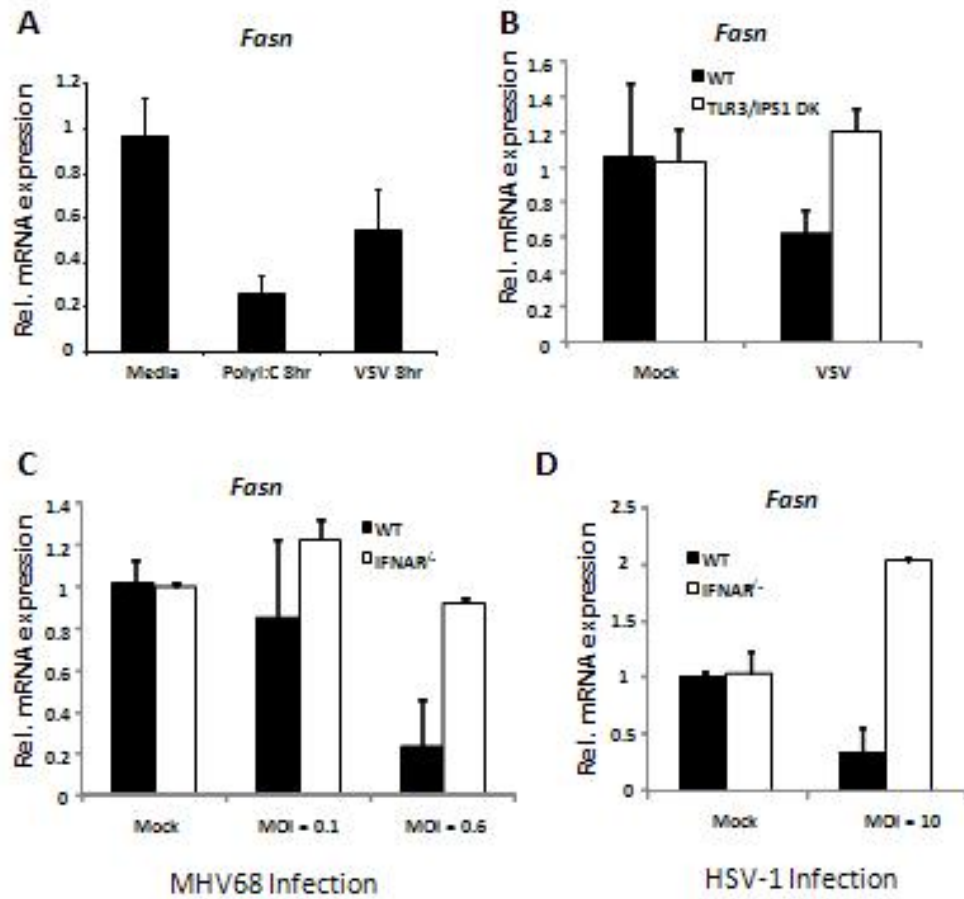


Figure 4-4. Effects of viral infections on *Fasn* levels in BMDMs.



**Figure 4-5. Effects of Fasn expression and activity on VSV infection**

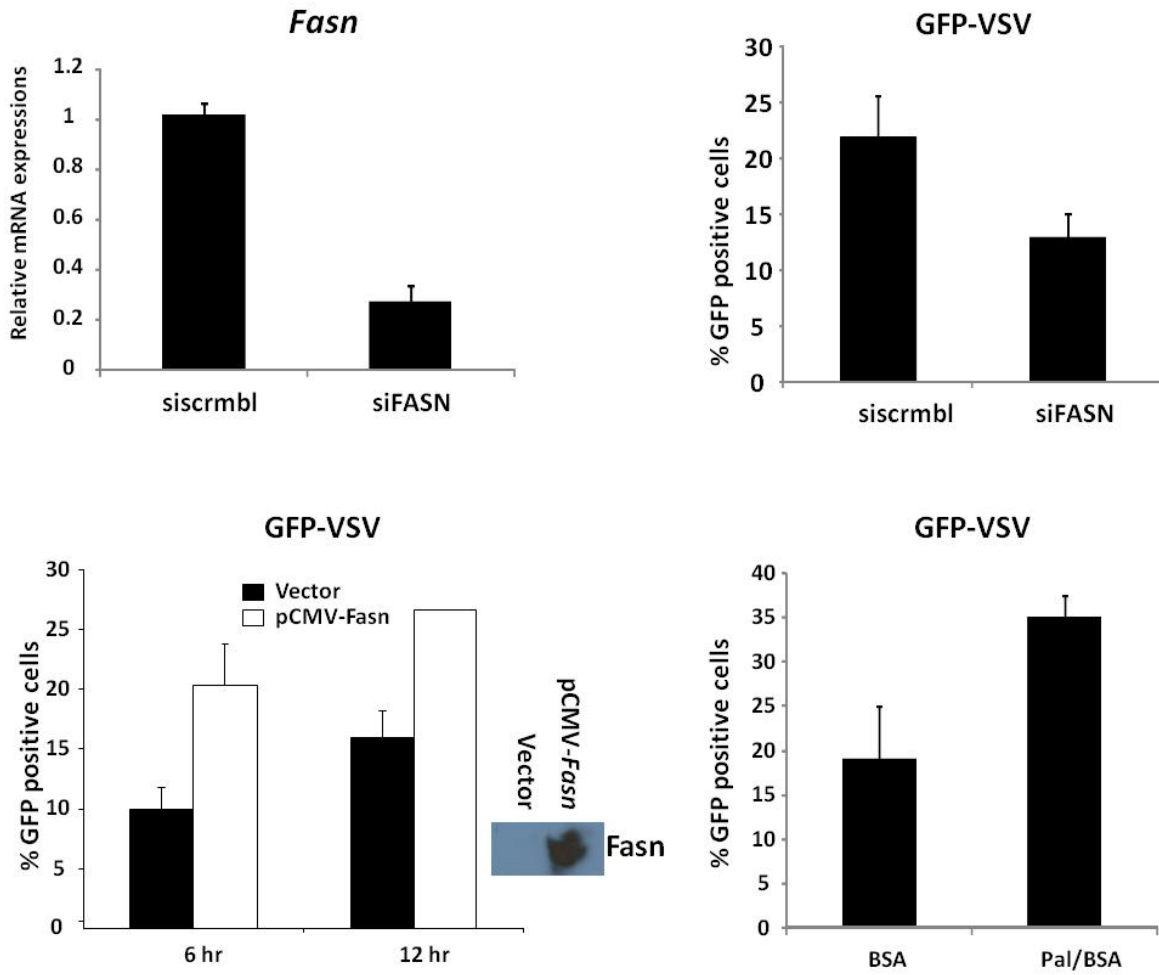




Figure 4-6. Effects of SREBP1 expression on VSV infection.

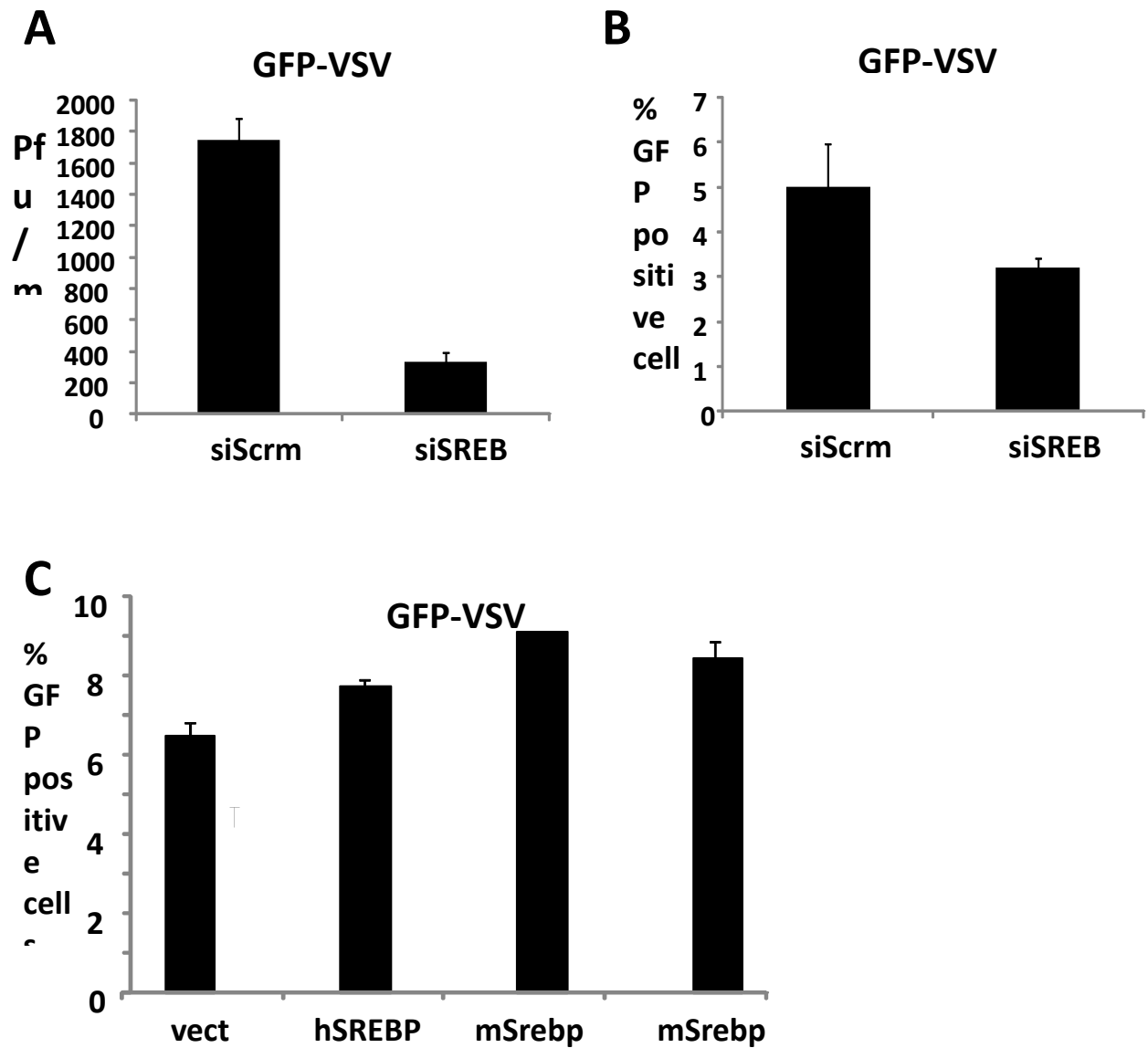
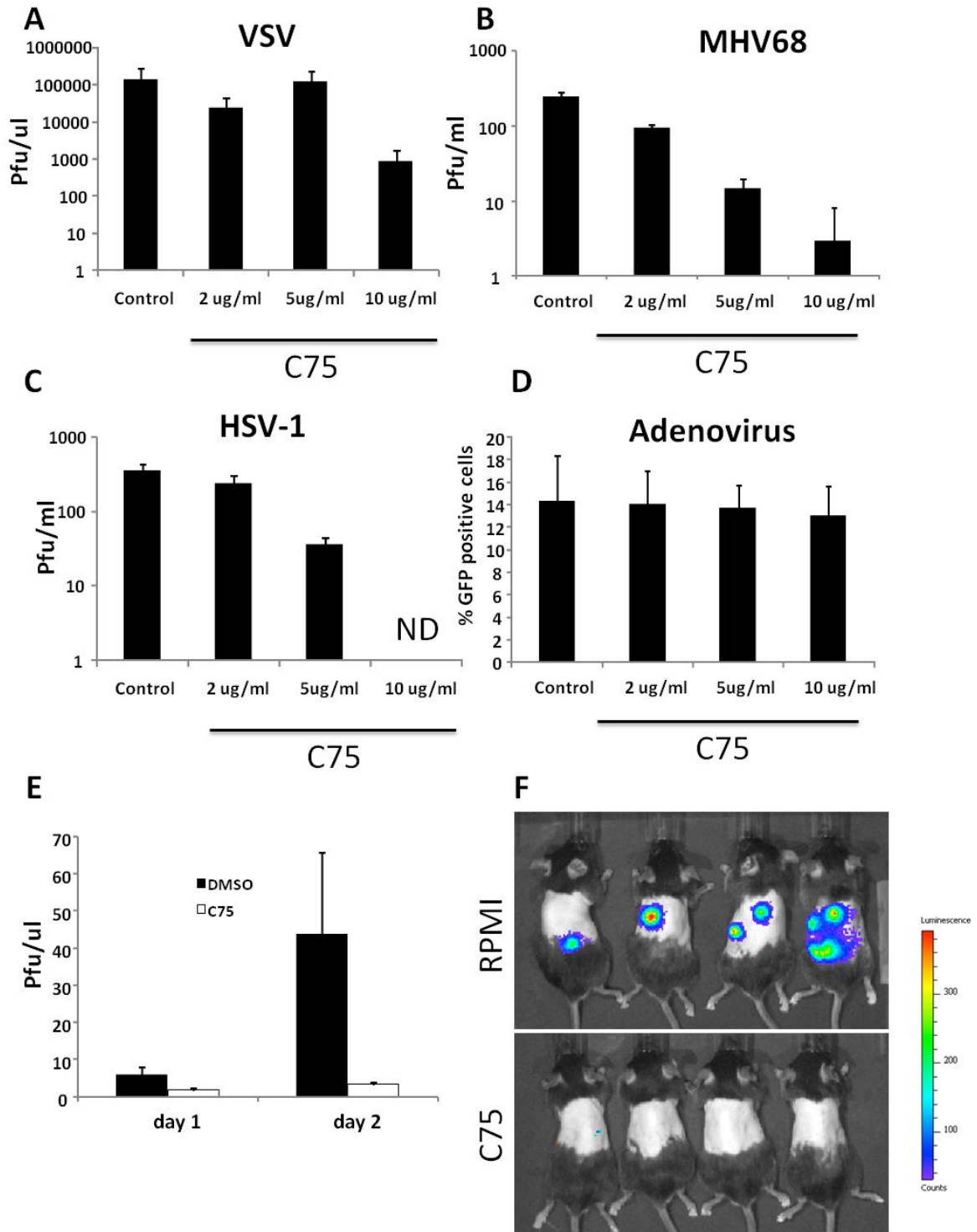
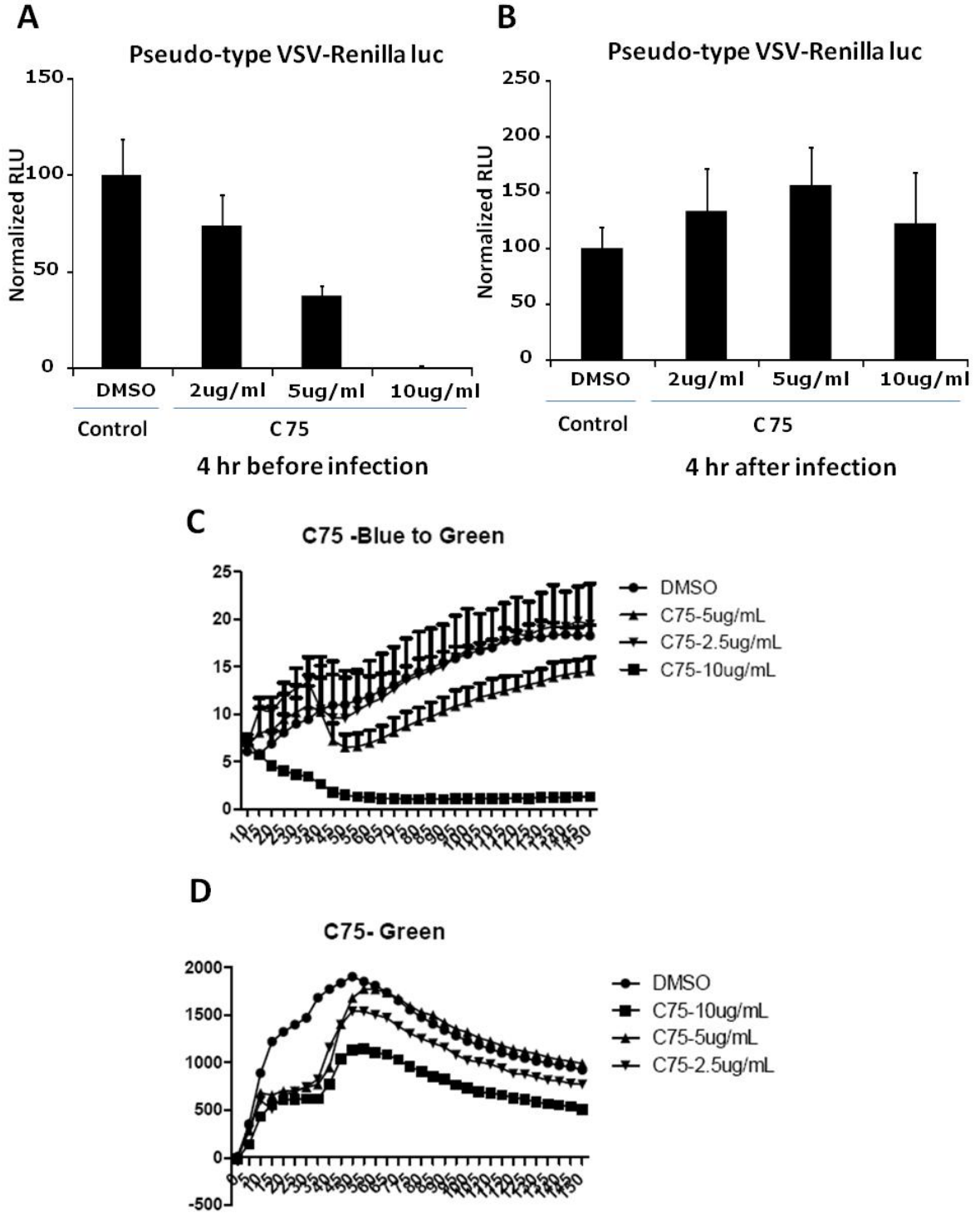


Figure 4-7. Inhibition of VSV, MHV68 and HSV-1 by C75 treatment in HEK293 cells.



**Figure 4-8. C75 treatment effects early steps of VSV lifecycle by decreasing viral membrane fusion.**



**Figure 4-9. Effects of modulation of Fasn activity on membrane fusion and Syncyia formation in Vero cells transfected with NipV F and G proteins.**

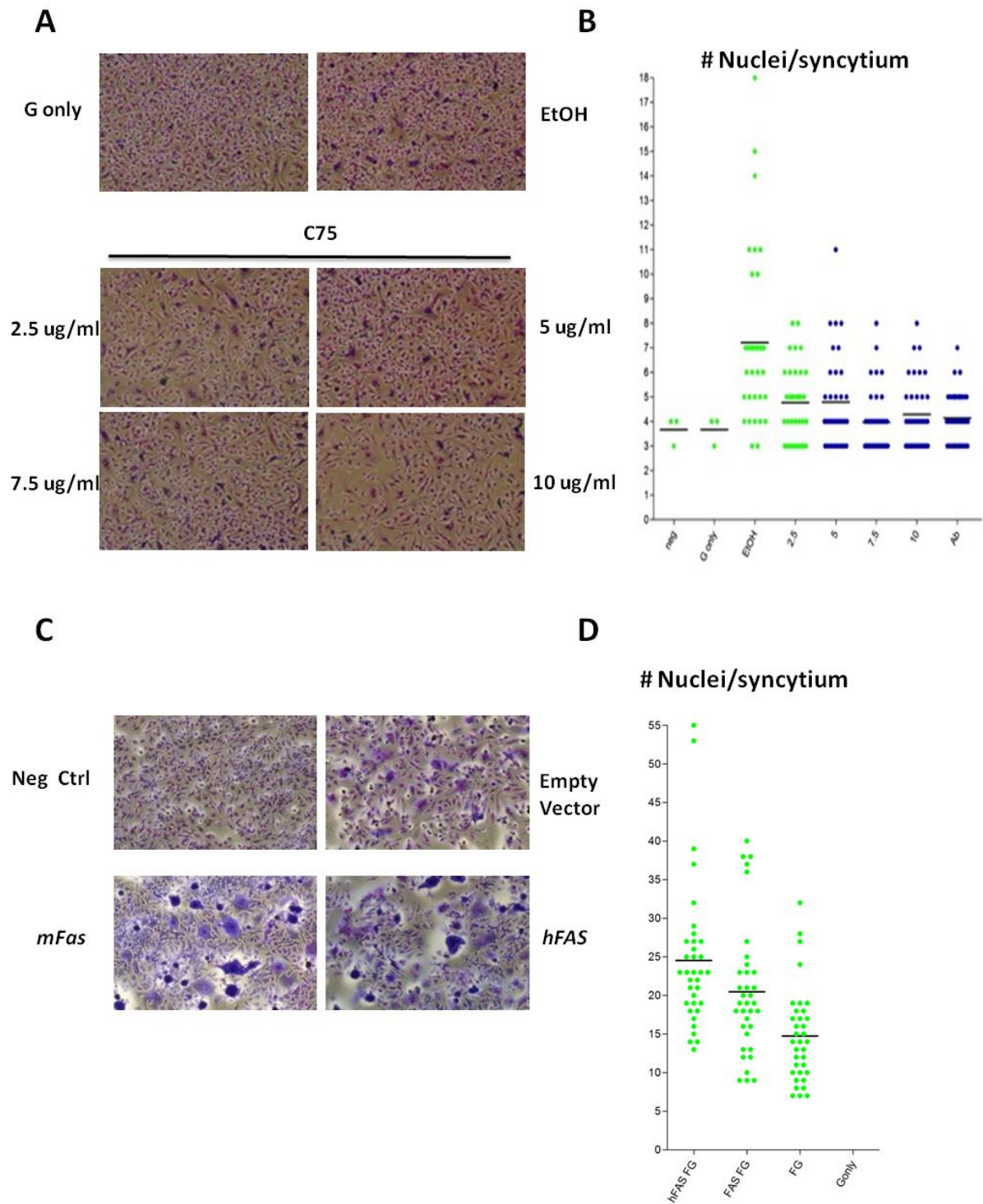
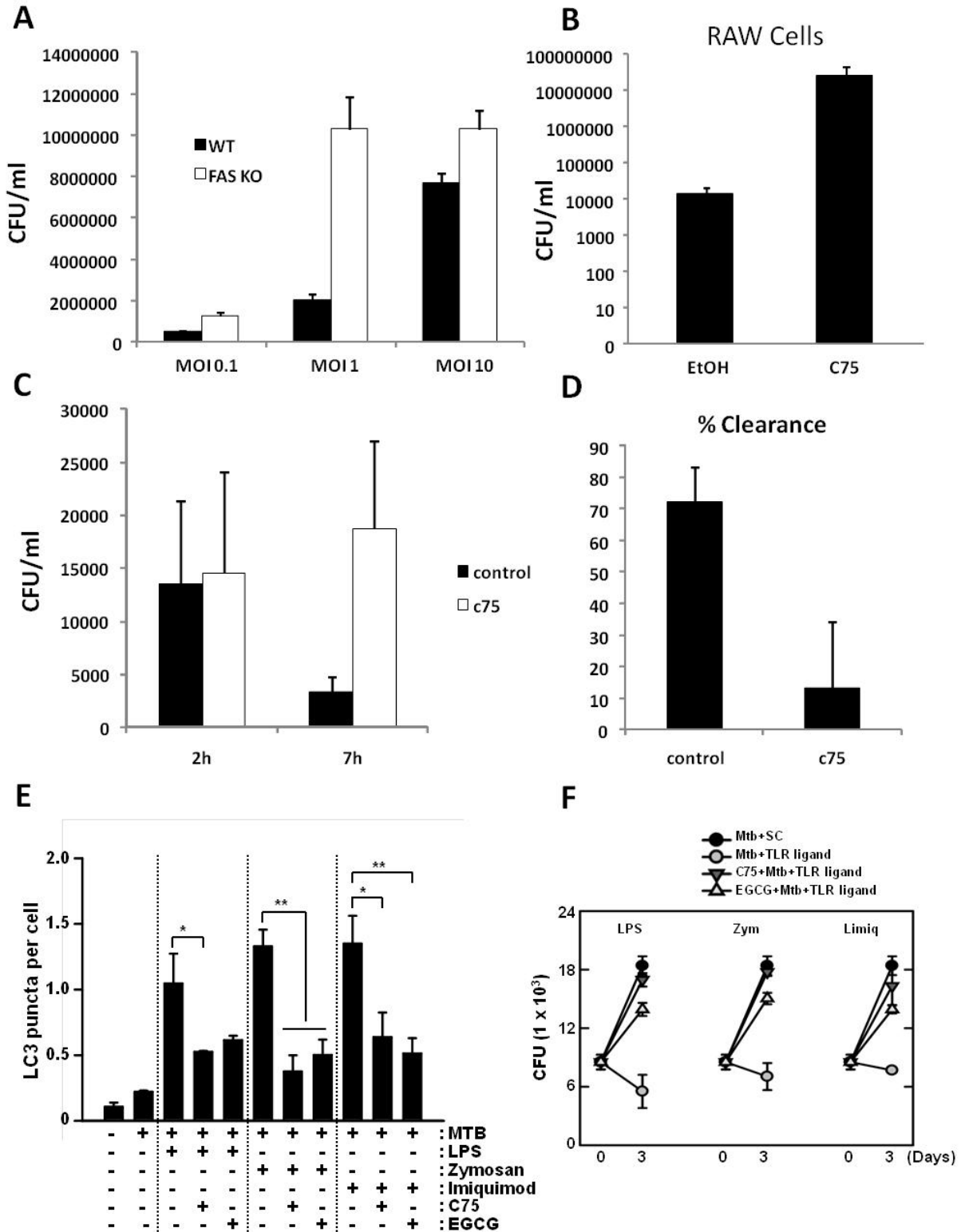
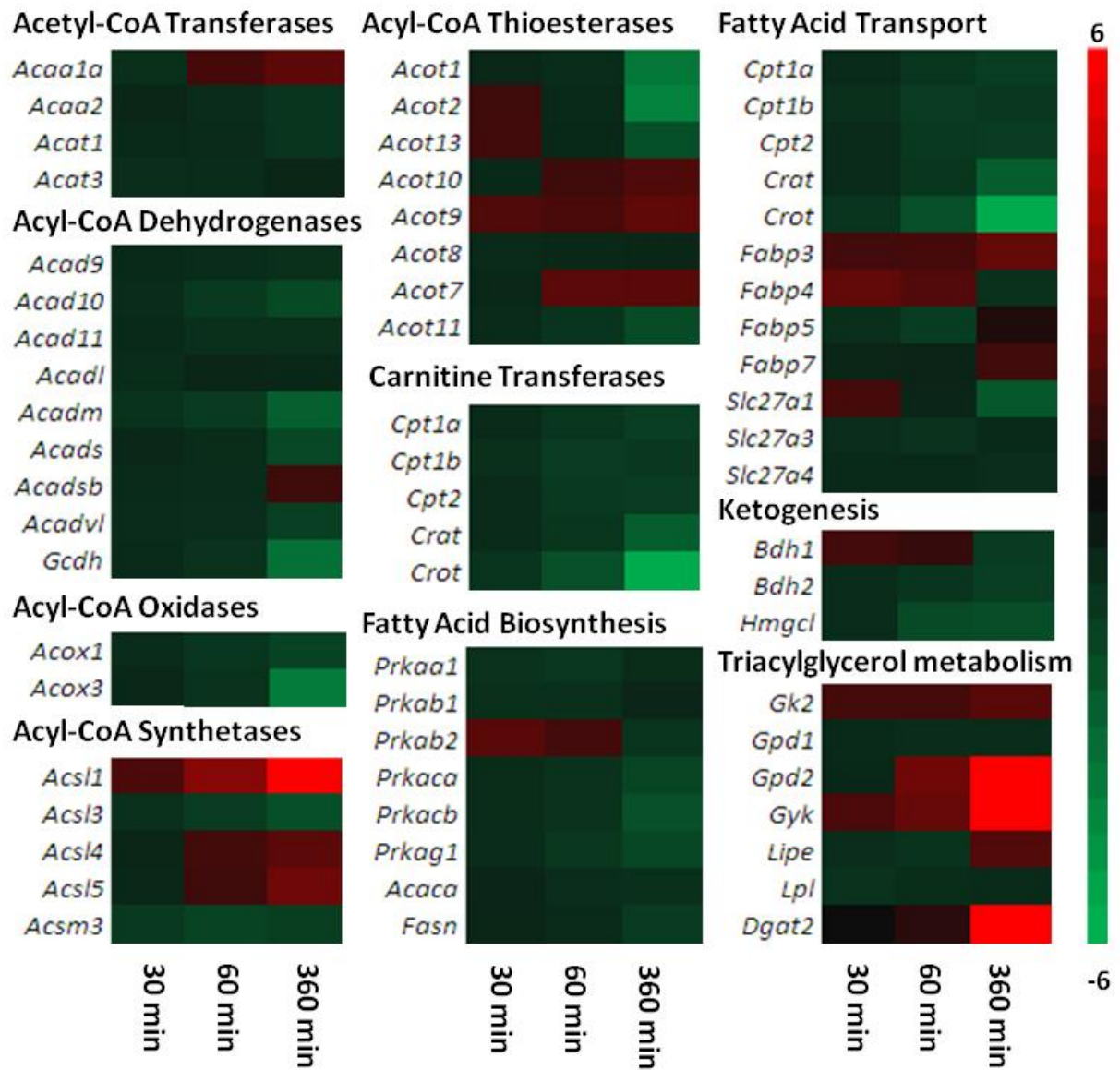


Figure 4-10. C75 treatment suppresses clearance of *Listeria* and *Mtb*.

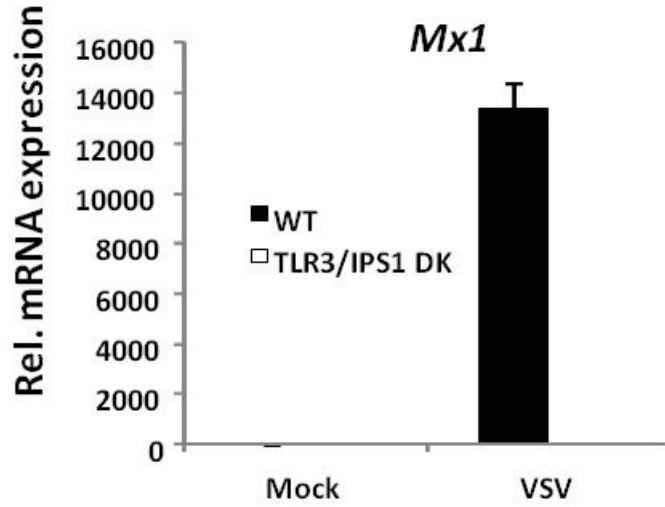


Supplementary Figure S4-1. Heat-map of lipid metabolic genes modulated in WT

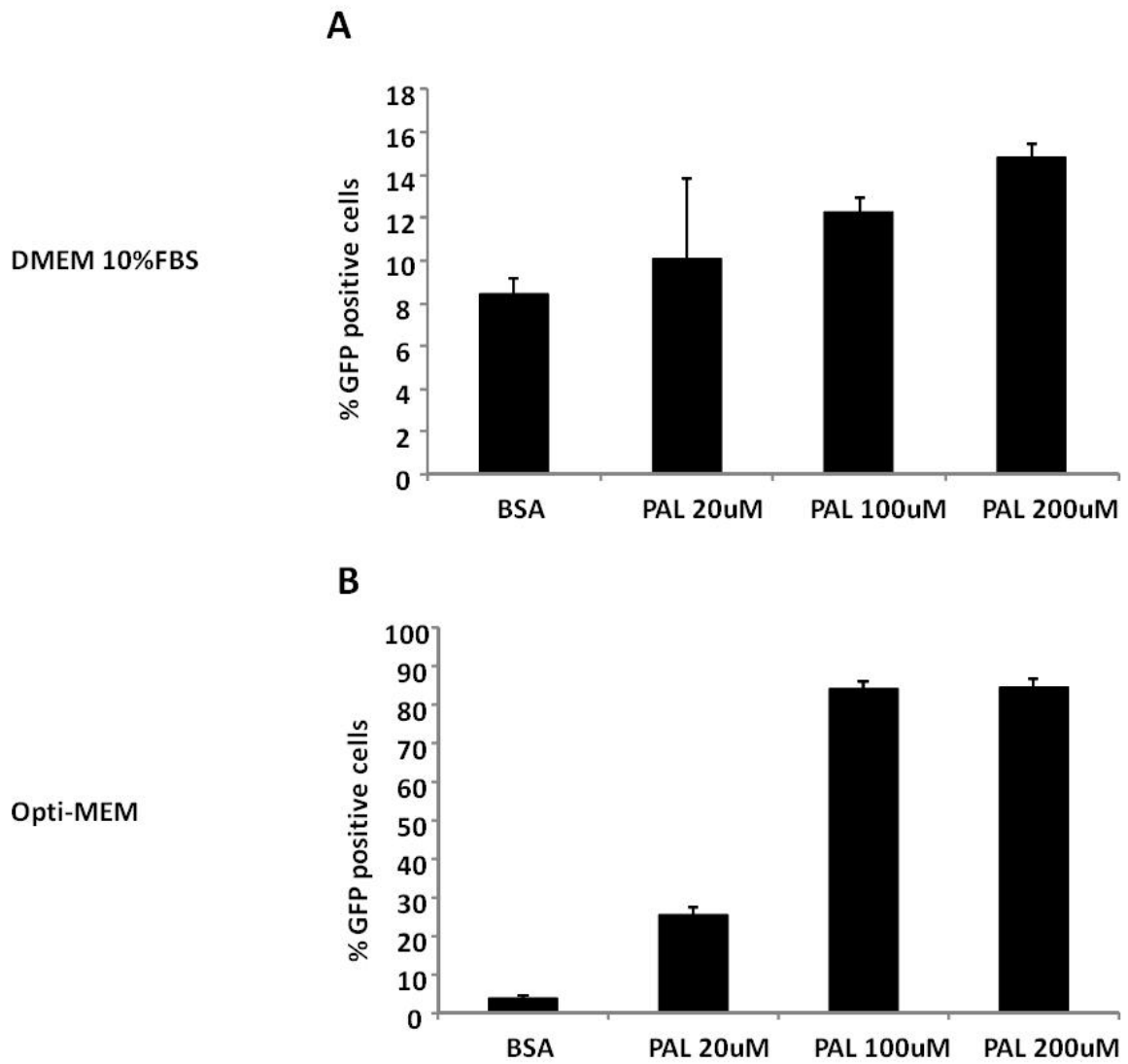
BMDMS 6 hours after LipA stimulations.



**Supplementary Figure S4-2. Induction of Mx1 in WT and TLR3/IPS1 Double KO BMDMs infected with VSV after 8 hours.**



**Supplementary Figure S4-3. Effects of addition of palmitate on VSV infection in 293HEK cells in regulate DMEM and 10%FBS compared to Opti-MEM (no lipids)**





## **Chapter 5**

### **Concluding Remarks and Future Perspectives**

Viruses, as non-living organisms, are dependent on the host cell's metabolic network for energy and macromolecular precursors to fuel their replication. In fact, viral infections often lead to altered states of metabolism in host cells causing various metabolic disorders. The two leading causes of acute liver failure (ALF) are viral hepatitis and drug-induced hepatotoxicity, which can be the consequence of impaired drug metabolism in patients with viral infections. Our findings in these studies elucidate the mechanisms by which viral infections can contribute to cellular and hepatic metabolic imbalances and liver injury.

There are many documented examples of impaired drug metabolism in patients with viral infections. While these effects have been attributed to modulation of CYP enzymes in response to activation of the innate immune system, the underlying molecular mechanisms are not well characterized. Our previous work identified a potential mechanism of how innate immune activation can lead to enhanced ASA-induced hepatotoxicity through down-regulation of CYP3A11, the CYP enzyme required for the clearance of the toxic intermediate of ASA. However, activation of anti-viral pathways through endoplasmic or cytosolic receptors for double stranded RNA by PolyI:C suppressed APAP toxicity in contrast to increased ASA toxicity observed previously. PolyI:C stimulation lead to transcriptional down-regulation of RXR $\alpha$  as well as PXR and their downstream CYP genes involved in conversion of APAP to its toxic metabolite, NAPQI. The results of the study described in **Chapter 2** suggest that activation of antiviral responses can alter APAP metabolism through transcriptional down-regulation of CYP3A11 and CYP1A2 independent of IFN production. Understanding the factors that contribute to or alleviate

drug toxicity is important for the proper use of drugs under various clinical cases, including the use of common analgesics to relieve pain or fever during viral infections. This study, in conjunction with our previous work, provides further evidence that the use of APAP may be safer in the context of a viral infection than ASA therapy. Additionally, the animal model that we have established can be used to study potential uncharacterized adverse effects that could occur in virally infected patients. Identification of molecular mechanisms of the crosstalk between innate immune responses and nuclear hormone receptor-regulated metabolism can provide insight into the biological consequences of various drug treatments during viral infections, allowing for safer and more accurate assessment of proper drug therapy.

In addition to drug-induced hepatotoxicity, viral hepatitis remains an important contributor of liver failure worldwide, and there is a pressing demand for new therapeutic targets to improve patient prognosis. Cell death and tissue injury during viral infections is thought to be caused by either direct cytopathic effects of viruses or inflammatory responses induced by PAMPs such as viral DNA or RNA. Here, we report that HMGB1, recently classified DAMP, is rapidly released and mainly responsible for triggering inflammation and hepatic injury in mice infected with MHV. Although blocking HMGB1 activity using neutralizing antibody did not affect MHV hepatic viral load, it promoted survival and suppressed MHV-induced liver injury. Similarly, we report that TLR4 KO survive better than WT mice infected with MHV further illustrating that MHV infection leads to tissue injury due to secondary inflammation and not as the consequence of deleterious cytopathic effects of the virus. Furthermore, administration of Glycyrrhizic

Acid (GA), a bioactive compound from licorice extract recently shown to block the cytokine activity of HMGB1, rescued mice infected with MHV and decreased liver injury. GA administration was also able to alleviate injury in other hepatic injury models such as acetaminophen-induced liver injury as well as ischemia reperfusion liver injury. This study demonstrates that HMGB1-mediated secondary inflammatory response is the common mechanism for tissue injuries induced by both sterile and infectious challenges, highlighting DAMPs as potential therapeutic targets for different types of liver injuries. Overall, our findings highlight the importance of HMGB1 in mediating liver injury induced by viral infections or sterile hepatic injury, prompting new studies to explore utilizing inhibitors of HMGB1 cytokine activity such as GA as an effective approach in treating patients at high risk of developing ALF. We hope that our findings in **Chapter 3** can lead to incorporation of GA as a hepatoprotective agent in western medicine. GA has been around in TCM for more than 2000 years and also has recently been used safely as an alternative herbal sweetener in candies and pharmaceuticals in western cultures extensively recently. As described in **chapter 2**, hepatotoxicity is an important adverse effect of many drugs, limiting their usage clinically. Perhaps, including GA as a mixture with different chemicals can compensate for the hepatotoxic properties of drugs which previously could have not been used clinically.

Lastly, we further illustrate the effects of the cross-talk between innate immunity and metabolism, a topic which has received much attention recently. It had been known for many decades that in cancer cells, metabolic pathways were altered to support increased proliferation rates. But, it was not until recently that the field began studying the underlying

mechanisms of this phenomenon in order to develop new therapeutic agents. Similarly, recent studies illustrate that during some viral infections, the cellular metabolism of the host can be altered, including changes in lipid and glucose metabolism. However, it is not clear whether/how cells alter their metabolism as part of proper innate immune responses during infections.

In **chapter 4**, we utilized RNA-seq analysis of wild-type IFNAR deficient bone marrow derived macrophages stimulated with lipid A to identify canonical pathway gene programs that are modulated by IFN. In addition to known IFN-inducible pathways, fatty acid biosynthesis was also found to be modulated in an IFN-dependent manner. A key enzyme in this pathway, FASN, was induced by Lipid A in IFNAR deficient macrophages while suppressed in wild-type macrophages. Activation of TLR3/TRIF pathway led to transcriptional down-regulation of *Fasn* through IFNAR/STAT1 pathway while activation of TLR9/MyD88 pathway up-regulated *Fasn* transcription. Additionally, inhibiting FASN activity using C75 had opposite effects on viral and bacterial infection rates. C75 pretreatment decreased infection of enveloped viruses such as MHV68, VSV, and HSV-1, while over-expression of FASN or pre-treatment with the downstream product of FASN, palmitic acid, enhanced the rate of VSV infection. We further studied the mechanisms by which FASN inhibition can lead to decreased viral infection. Our results indicate that decreased FASN activity could be linked to decreased viral membrane fusion. Interestingly, C75 pre-treatment enhanced *Listeria* infection by decreasing the ability of macrophages to clear phagocytized bacteria. Additionally, C75 treatment inhibited induction of autophagy by Mtb and LPS, suggesting a mechanism by which C75 treatment can block clearance of

bacteria in macrophages. These findings identify fatty acid biosynthesis as an IFN regulated pathway that can play opposite roles in anti-viral and anti-bacterial responses.

Our data suggest that IFN signaling results in suppression of FASN expression, however, FASN regulation is important in maintaining the energy balance in cells. FASN enzymatic activity results in generation of palmitate that can enter metabolic pathways including  $\beta$ -oxidation to generate ATP. Cells that have decreased fatty acid  $\beta$ -oxidation usually compensate by inducing glycolysis in order to meet their metabolic demands. However, it is not clear whether IFN signaling induces glycolysis in macrophages. Our preliminary results indicate that key genes in glycolysis including hexokinase 2 (HK2), glucose transporter 1 (Glut1 also known as Slc2a1), and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) are induced by LipA in WT BMDMs. However, only induction of PFKFB3 is dependant on IFNAR signaling (Figure 5-1). IFN $\alpha$  treatment of BMDMs also leads to increased *PFKFB3* levels (Figure 5-2). While a recent study indicates that glycolysis is linked to inflammasome activation in macrophages, the effects of induction of glycolysis through PFKFB3 expression in anti-viral pathways are not clear. As an IFN inducible gene, PFKFB3 induction could potentially play a role against viral infections. Interestingly, overexpression of PFKFB3 in 293HEK cells inhibits influenza infection (Figure 5-3). A potential explanation for these results could be the decrease in the pH of media observed after induction of glycolysis due to the release of lactic acid. Influenza viral fusion is a pH dependent process and low pH of the environment can lead to decreased viral infection rate. This hypothesis could be tested by increasing back the pH and determine the rate of influenza infection in these conditions after induction

of glycolysis. Further studies are required to delineate the pathways that lead to upregulation of these genes during viral infections, as well as the effects of regulation of glycolysis on viral replication. Chemical inhibitors of glycolysis in addition to siPFKFB3 reagents can be utilized to determine whether inhibition of glycolysis results in enhanced viral infection. Investigating the mechanisms by which glycolysis is induced during viral infections can provide better insight on the importance of the cross-talk between innate immune pathways and metabolism.

## **Figure Legends:**

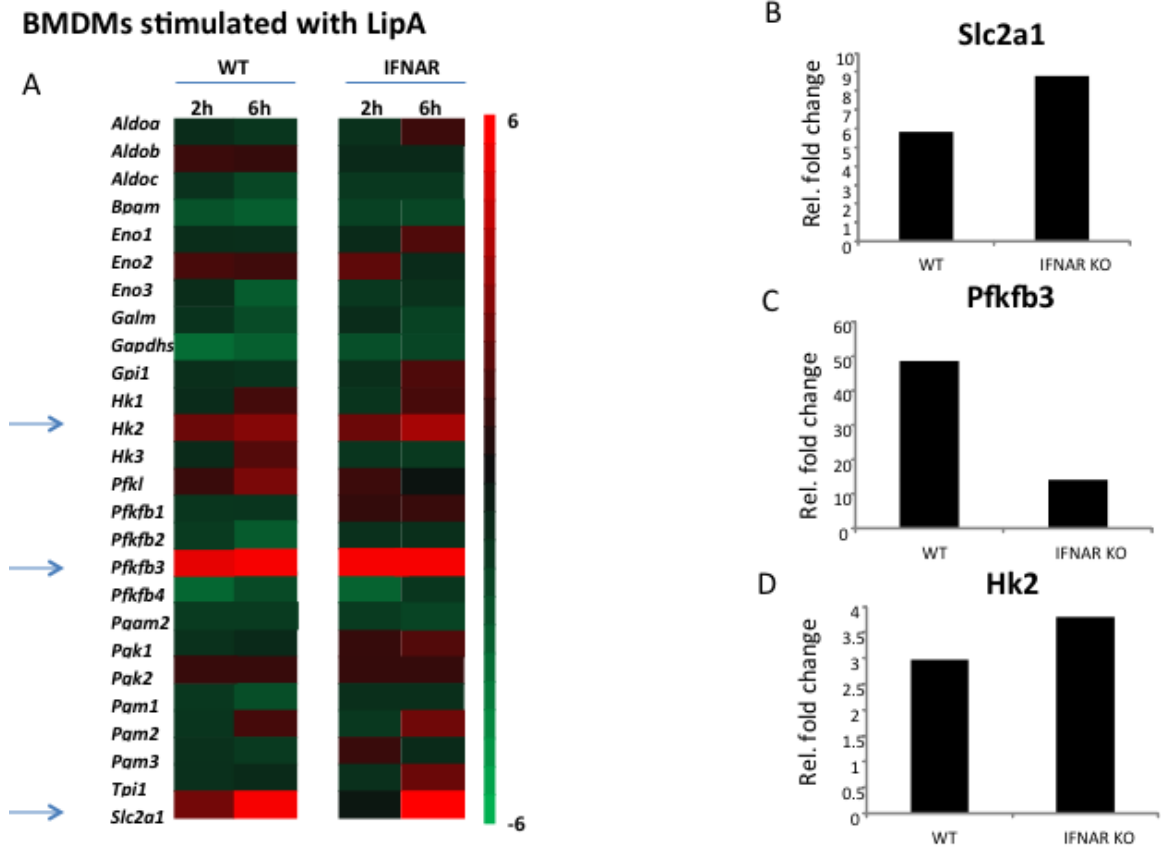
**Figure 5-1.** (A) Heat-map representing genes modulated in glycolysis in response to lipA stimulation in WT and IFNAR KO BMDMs in the RNAseq data described in Figure 4-1. (B-D) Bar graphs indicating the fold induction values of three key genes in glycolysis from the same dataset as in A at 6 hours.

**Figure 5-2.** BMDMs were stimulated with IFN $\alpha$  for 6 hours and mRNA levels of key glycolysis genes were assessed using QPCR analysis.

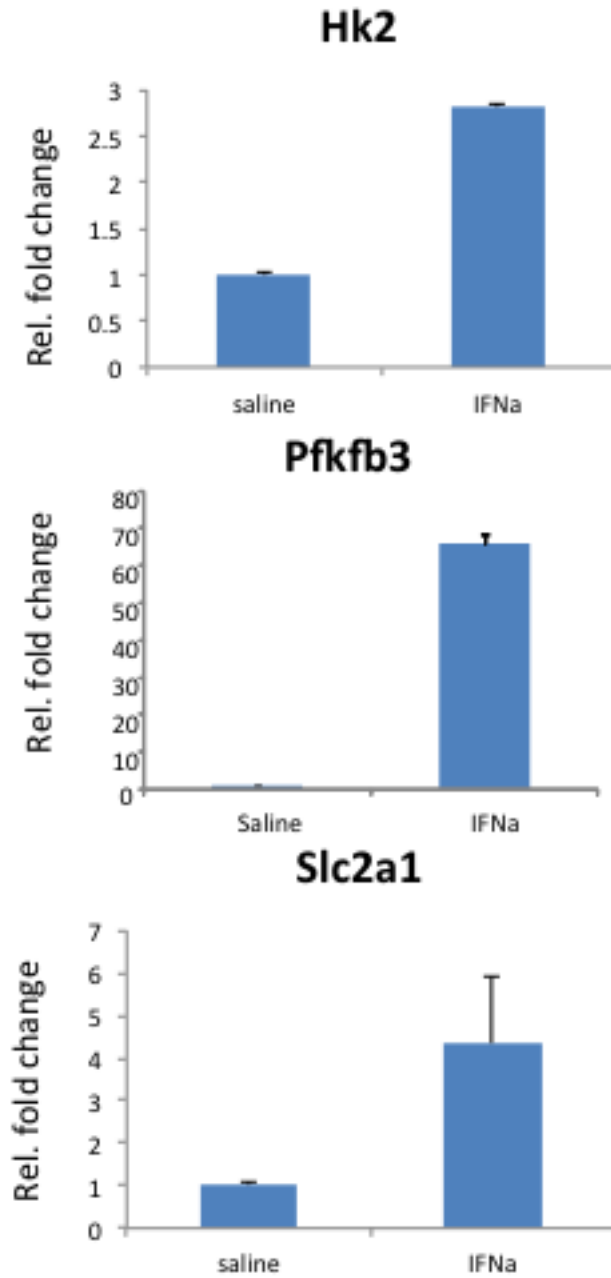
**Figure 5-3.** HEK293 cells were transfected with GFP (control) or PFKFB3 expression 24 hours prior to influenza infection. 24 hours post infection the amount of virus in the supernatant was determined using a reporter assay. The data are normalized to GFP (control) group.



Figure 5-1. Modulation of glycolysis genes in BMDMs stimulated with LipA.



**Figure 5-2. Induction of key glycolysis mRNA levels in BMDMs in response to type I IFN stimulation.**



**Figure 5-3. Overexpression of PFKFB3 decreases influenza infection rate in HEK293 cells.**

