

Mechanism of hepatic inflammation during hepatitis C virus infection

Amina Abduletif Negash

A dissertation

Submitted in partial fulfillment of the

Requirement for the degree of

Doctor of Philosophy

University of Washington

2013

Reading Committee:

Dr. Stephen Polyak

Dr. Edward Clark

Dr. Michael Gale Jr.

Program Authorized to Offer Degree:

Department of Immunology

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Amina Abduletif Negash

University of Washington

Abstract

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Amina Abduletif Negash

Chair of the Supervisory Committee:

Professor Michael Gale Jr. PhD

Department of Immunology

Chronic hepatitis C virus (HCV) infection is a leading cause of liver disease. Liver inflammation underlies infection-induced fibrosis, cirrhosis and liver cancer, but the processes that promote hepatic inflammation by HCV are not defined. We used a systems biology analysis together with multiple lines of evidence to demonstrate that interleukin-1 β (IL-1 β) production by intrahepatic macrophages confers liver inflammation through HCV-induced inflammasome signaling. Chronic hepatitis C patients exhibited elevated levels of serum IL-1 β compared to healthy controls. Immunohistochemical analysis of healthy control and chronic hepatitis C liver sections revealed that Kupffer cells- resident hepatic macrophages -are the primary cellular source of hepatic IL-1 β during HCV infection. Accordingly, we found that both blood monocyte-derived primary human macrophages and Kupffer cells recovered from normal donor liver, produce IL-1 β after HCV exposure. Using the THP-1 macrophage cell-culture model, we found

that HCV drives a rapid but transient caspase-1 activation to stimulate IL-1 β processing and secretion. HCV can enter macrophages through non-CD81 mediated phagocytic uptake that is independent of productive infection. Viral RNA triggered the MyD88-mediated TLR7 signaling to induce IL-1 β mRNA expression. HCV uptake concomitantly induced a potassium efflux that activated the NLRP3 inflammasome for IL-1 β processing and secretion. RNA sequencing analysis comparing THP1 cells and liver sample from chronic hepatitis C virus infected patients revealed that viral engagement of the NLRP3 inflammasome stimulates IL-1 β production to drive proinflammatory cytokine, chemokine, and immune-regulatory gene expression networks linked with HCV disease severity. These studies identified intrahepatic IL-1 β production as a central feature of liver inflammation during HCV infection. Thus, strategies to suppress NLRP3 or IL-1 β activity could offer therapeutic approaches to reduce hepatic inflammation and mitigate disease.

Dedication

I dedicate this dissertation to
my wonderful family

Acknowledgments

My success in graduate school is not achieved single handedly instead it is the outcome of the endless support I received from many people to whom I am very grateful. I would like to start by thanking my supervisor professor Michael Gale Jr. for his mentorship and guidance throughout my training in his laboratory. I thank him for giving me the opportunity and freedom to explore and grow to become an independent scientist. I also would like to extend my thanks to my thesis supervisory committee who gave me instructive advice and suggestions that helped guide my project. I have enjoyed my stay in the lab and I do value the interactions with both the past and present members of the Gale laboratory. I am very pleased to have had known each individual in the laboratory and I do appreciate their willingness to discuss science, help and provide advice as necessary. I would like to thank the department of immunology as a whole for being a very cooperative environment and for providing the necessary training for my success in graduate school. In particular, I would like to thank Peggy McCune for being here to answer my many questions, clear my confusions and keep me on top of things. Lastly, but most importantly, I would like to thank my family: mom, Nuria, Mustafa, Iman, Faisal and husband Ahmed. My dear family, to be where I am right now was not possible without your support because you believed in me, helped me and always loved me. Thank you all from the bottom of my heart.

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List of Abbreviations

- APCs - Antigen presenting cells
- ASC - Apoptosis-associated speck-like protein containing a CARD
- BCR - B cell receptor
- CARD - Caspase activation and recruitment domain
- CLDN-I- Claudin-1
- CpG - deoxycytidylate-phosphate-deoxyguanylate
- DAA - Direct acting antiviral
- DAMP- Danger-associated molecular pattern
- DCs - Dendritic cells
- DPI - Diphenyleneiodonium
- EMCV- Encephalomyocarditis virus
- FCAS - Familial cold autoinflammatory syndrome
- FDR - False discovery rate
- FMF - Familial Mediterranean fever
- G-CSF - Granulocyte-colony stimulating factor
- Glyben- Glybenclamide
- GM-CSF- Granulocyte macrophage-colony stimulating factor
- HCC - Hepatocellular carcinoma
- HSCs - Hepatic stellate cells
- IFN-I - Type-I interferon
- IKKi - I- Kappa-B kinase i

IL-1 β - Interleukin-1beta
IRF - Interferon regulatory factor
ISG - Interferon stimulated gene
ISGF3 - Interferon stimulated gene factor 3
KCL - Potassium chloride
LDLR - Low-density lipoprotein receptor
LLR - Leucine-rich repeats
MAPK- Mitogen-activated protein kinase
MAVS- Mitochondrial antiviral signaling
MDA5 - Melanoma-associated gene 5
MHC - Major histocompatibility complex
MWS - Muckle-Wells syndrome
MxA - Myxovirus A
NACHT- Nucleotide-binding oligomerization domain
NAFLD- Non-alcoholic fatty liver disease
NF κ B - Nuclear factor kappa B
NK cells- Natural killer cells
NLR - Nod-like receptor
NLRP3- Nacht, LRR and PYD domains-containing protein 3
NOMID- Neonatal-onset multisystem inflammatory disease
OAS - oligoadenylate synthetase
p-IFN - Pegylated interferon
PAMP - Pathogen-associated molecular pattern

pDCs - Plasmacytoid dendritic cells
PolyIC - Polyinosinic-polycytidylic acid
PoPs - Pysin only protein
PRR - Pathogen recognition receptor
RIG-I - Retinoic acid-inducible gene-I
RNA-seq- Next generation RNA sequencing
ROS -Reactive oxygen species
RVN -Ribavirin
SNP - Single nucleotide polymorphism
SRBI - Scavenger receptor class B type 1
STAT1- Signal transducer and activator of transcription 1
TBK1 – Tank-binding kinase 1
TCR - T cell receptor
TH17 - T helper 17
TIR - Toll/IL-1 receptor
TJ -Tight junction
TLR - Toll-like receptor
TRAF6- TNF receptor-associated factor 6
TRIF - TIR domain-containing adaptor inducing IFN- β

Chapter One

Introduction

Mammalian immune response to virus infection

Pathogenic microorganisms have evolved various mechanisms of infection to cause morbidity and mortality. Infection by a pathogen such as a virus occurs in series of stages including for example, adherence to epithelium, local infection and penetration of the epithelium, local infection of a tissue and then clearance of the infection or persistence. The immune system is a unique and complex system that has anti-microbial mechanism in place to restrict infection at each stage. The most defining property of the immune system is its ability to discriminate foreign antigens from self, which enables the host to combat a diverse array of pathogenic agents and limit infection by viruses, bacteria and fungi. There are two components of the human immune system that are distinct yet rely on one another for optimal immunity. These two components are the innate and adaptive immune responses.

Innate immune responses

Innate immune responses are initiated within minutes to hours after recognizing a pathogen and constitute first line of defense during any infection. This recognition of a foreign pathogen as foreign or as non-self is mediated by germline-encoded receptors called pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), Nod-like receptors (NLR), C-type lectin receptors (CLRs) and cytosolic DNA sensors(1-3). Innate myeloid cells such as macrophages, neutrophils and dendritic cells (DCs) express these receptors and serve as surveillances to sample the local environment for pathogens. These cells are found within specific tissues and some of them

continually circulate throughout the periphery. Macrophages and neutrophils are phagocytes because they can engulf and kill pathogens upon activation. Innate immune activation is not pathogen-specific and does not generate long-lived immunologic memory responses. Instead, innate cells recognize evolutionarily conserved motifs found within the various components of pathogens referred to as pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs is mediated by PRRs, which leads to activation of various innate immune cells and initiation of inflammatory responses.

During an infection, activated macrophages and DCs produce a range of cytokines such as IL-1 β , TNF- α , IL-6 as well as chemokines such as CXCL8. Innate immune cell engagement of PAMPs can lead to increased cell surface expression of certain adhesion molecules such as α X β 2, α D β 2 and CD11c/CD18(4). The release of cytokines by innate immune cells activates the acute-phase inflammatory responses that include but are not limited to the activation of the complement system, phagocytosis, decreased pathogen replication, increased antigen presentation, and initiation of adaptive immune responses (5). Infection of cells by intracellular pathogens such as viruses induces type-I interferons (IFN-I) release from phagocytes and DCs(6). The two antiviral subtypes of IFN-I are IFN- α and IFN- β . IFN-I is an antiviral cytokine with many immunomodulatory activities(7). The receptors for IFN-I are ubiquitously expressed on all cell types (8-10). IFN-I can signal through an autocrine and a paracrine fashion to establish an “antiviral state” both within infected cells and within surrounding uninfected cells (figure 1-1). Interferon signaling through its receptor leads to STAT1 phosphorylation, the formation of ISGF3 transcription complex and expression of hundreds of interferon-stimulated genes (ISGs)(11). ISGs can have a direct antiviral action or modulate the cell environment to restrict virus replication and spread and thus establishing the “antiviral state”. Additionally, the initial

signaling through IFN-I receptor is important for signal amplification leading to enhanced expression of the IFN- α and IFN- α subtypes. Besides contributing to acute phase antiviral responses, IFN-I orchestrates the survival and differentiation of adaptive immune cells(7, 12). However, viruses such as hepatitis C virus (HCV) (13), West Nile virus (WNV) (14) and influenza virus (15) have evolved various mechanism to evade the antiviral response and/or block IFN-I production. This inhibition of IFN-I production has profound consequences, which in the case of HCV allows the virus to persist and cause chronic infection; whereas during WNV and influenza infections, lack of IFN-I activation and signaling leads to aberrant adaptive responses that allow virus escape within infected host.

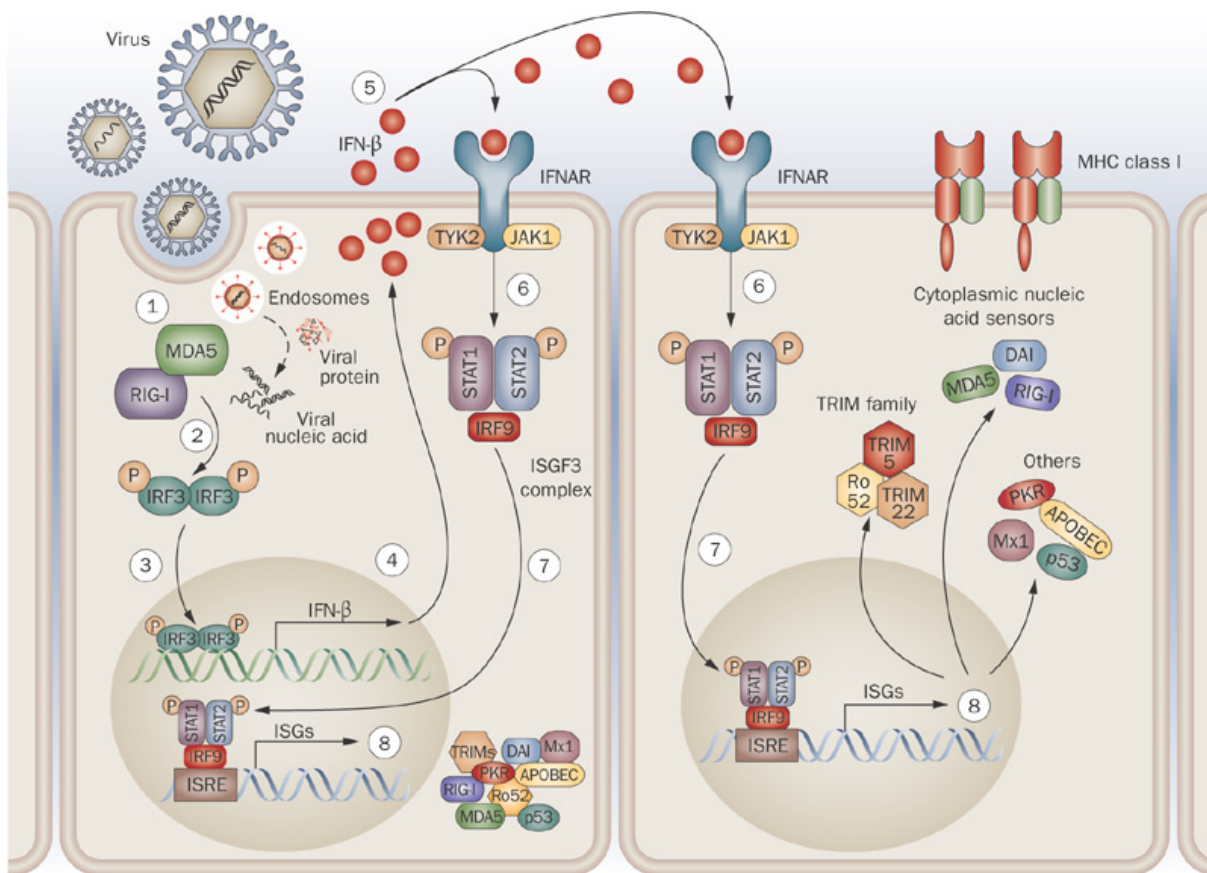


Figure 1-1 Interferon induction and response pathways. Infection with viruses leads to immune activation initiated by cytosolic PRRs recognition of viral nucleic acids. Once in the cytoplasm, viruses uncoat to expose both their protein and nucleic acids. (1) Viral nucleic acid detection is mediated by intracellular PRRs. (2) Engagement of cytosolic RNA sensing PRRs by viral nucleic acid activates downstream signaling pathway leading to the phosphorylation and dimerization of the transcription factor IRF3. (3) Dimerized IRF3 translocates to the nucleus and induces the expression of IFN- β . (4) IFN- β protein is secreted from virus-infected cell. (5) Secreted IFN- β binds to IFN receptor (IFNAR) to initiate signaling through the JAK-STAT pathway both in virus infected and uninfected adjacent cells. (6) IFN- β binding to IFNAR leads to JAK1 and Tyk2 recruitment and activation of STAT1 to form the ISGF3 transcription complex. (7) ISGF3 translocates to the nucleus, which binds the interferon stimulated responsive element (ISRE), to drive the expression of hundreds of interferon stimulated genes (ISGs). *Image adopted from: Hall JC, and Rosen A. Type I interferons: crucial participants in disease amplification in autoimmunity. Nat Rev Rheumatol. 2010;6(1):40-9.*

In addition to myeloid cells, an important innate cell that provides an immediate response during virus infection is the natural killer (NK) cells (16). NK cells have a cytolytic activity in which cytotoxic granules are released onto infected target cells to induce programmed cell death (17). NK cells are activated by IFN-I (18) or cytokines produced by macrophages such as IL-12. IL-12 induces IFN- γ production by NK cells, which serves to limit virus prior to the induction of T cell responses.

Virus recognition and induction of innate immunity

Virus sensing and recognition is triggered by the engagement of PRRs to specific motifs or PAMPs within viral structures that are essential for virus replication or growth (2). PRRs, are non-clonal receptors expressed in all cells including innate cells, and do not confer long-lived memory. PRRs, unlike T and B cell receptors, are not diverse in their pathogen specificity and when activated, they do not generate long-lived immunologic memory of the PAMPs that they recognize to mount immediate protection in case of re-exposure. Many PRRs show distinct abundance within cells and tissues, recognize distinct PAMPs and activate specific cellular

pathways that generate pathogen-tailored antimicrobial responses. There are four major families of PRRs: the TLRs, the RLRs, the NLRs, the CLRs and cytosolic DNA sensors (1, 3).

TLRs are type I integral membrane glycoproteins with extracellular domains containing varying numbers of leucine-rich repeats (LRRs) and cytoplasmic tails that contain a Toll/IL-1 receptor homology (TIR) domain (19, 20). Extracellular TLRs recognize pathogen-derived hydrophobic lipids and proteins whereas endosomal TLRs recognize nucleic acids. Thus far ten TLRs have been identified in human and thirteen in mice. TLR1, TLR2, TLR4, TLR5, TLR6 are expressed on cell surface whereas TLR3, TLR7, TLR8 and TLR9 are expressed in endosomes. Most TLRs require the recruitment of the adaptor protein myeloid differentiation factor 88 (MyD88) to the cytoplasmic tail to trigger signaling. TLR3 requires a domain called TIR-domain-containing adaptor inducing IFN- β (TRIF) and TLR4 can signal through both MyD88 and TRIF. Signaling through TLR pathways leads to the activation of key transcription factors including nuclear factor kappa B (NF κ B), mitogen-activated protein kinase (MAPK) and interferon regulatory factors (IRF 1, 3, 5, and 7) to drive the expression of interferons, inflammatory cytokines and cellular pathways modulatory factors (19, 20). TLR2 and TLR4 are highly expressed on macrophage cell surfaces whereas TLR9 and TLR7 are expressed in high abundance in plasmacytoid DCs (pDCs) (19).

TLR signaling is important during virus infections. TLR4 senses the fusion (F) protein of respiratory syncytial virus (RSV) (21, 22) whereas TLR2 senses RSV, measles virus, human cytomegalovirus and herpes simplex virus 1 (HSV-1) (23-26). Double-stranded RNA that is generated as replication intermediates during the life cycle of many viruses including mouse cytomegalovirus and HCV triggers TLR3 signaling (27, 28). TLR3-deficient mice, although resistant to WNV infection, demonstrate increased brain infection and encephalitis, suggesting

that WNV uses TLR3 to break the brain-blood barrier to cause infection of the brain (29). TLR9 recognizes unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) motifs found in many viruses including HSV-2 DNA (30, 31). TLR7/8, which are known to recognize single-stranded viral RNA (ssRNA), are important for controlling many RNA virus infections (32, 33).

RLRs and DNA sensors are important for detecting viral nucleic acid. The RLR family is composed of three DExD/H box helicases: retinoic acid-inducible gene I (RIG-I), melanoma-associated gene 5 (MDA5) and laboratory of genetics and physiology-2 (LGP-2) (34, 35). Both RIG-I and MDA-5 contain an N-terminal caspase activation and recruitment domain (CARD) domain followed by an RNA helicase domain and a C-terminal domain whereas LGP2 lacks the N-terminal CARD domain. RIG-I is maintained as a monomer and in an inactive state in resting cells by a repressor domain found within the C-terminus; but when RIG-I recognizes a virus infection or engages an RNA ligand, it undergoes a conformational change that promotes RIG-I oligomerization that exposes the CARD domain so that it can induce signaling(36). Both RIG-I and MDA5 require the adaptor protein mitochondrial antiviral signaling protein (MAVS) to propagate signaling upon virus recognition (36-38). Induction of type-I IFN production through the RLR pathway begins when RIG-I/MDA5 engages viral RNA leading to NF κ B activation and IRF-3 phosphorylation and dimerization. IRF3 phosphorylation is mediated by kinases I κ B kinase i (IKKi) and tank-binding kinase 1 (TBK1), which are associated in a complex with MAVS (39). Then activated IRF-3 and NF κ B translocate to the nucleus to drive the expression of IFN- β , other IRF3 target genes and NF κ B-induced genes (figure 1-2). Secreted IFN- β binds to its receptor to stimulate Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway to induce the expression of many ISGs including IFN α and IRF7. IRF7

induction is important to amplify the IFN response, which associates with itself to form a homodimer or with IRF3 to form a heterodimer, to drive gene expression (40).

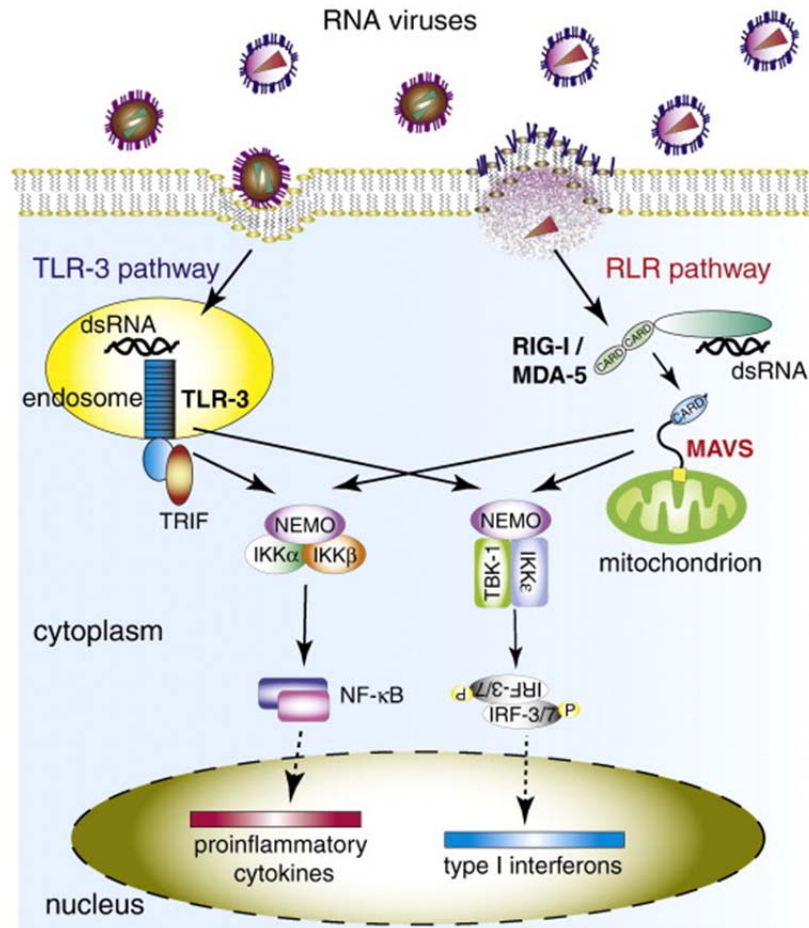


Figure 1-2 **Antiviral innate immune signaling during virus infection.** Viral nucleic acid is recognized by cytoplasmic RNA sensors such as RIG-I, MDA5 and/or TLR3. RIG-I/MDA5 engagement with an RNA ligand drives their activation. Signal transduction is propagated through MAVS. Activation of this pathway recruits kinases such as TBK1 and IKK that phosphorylate IRF3 to drive its dimerization, with itself or with IRF7, and translocation to the nucleus to drive type-1 interferons production and IRF3 target gene expression. Concurrently, NFκB is activated, which translocates to the nucleus to drive proinflammatory cytokine production. *Image adopted from: Koshiba T. Mitochondrial-mediated antiviral immunity. Biochim Biophys Acta. 2013;1833(1):225-32.*

The motifs, or PAMPs, within an invading RNA virus that are recognized by RIG-I and MDA5 are distinct (41, 42). RIG-I preferentially recognizes a 5'-triphosphorylated, uncapped short ssRNA. Since most host RNA are capped, RIG-I uses this to discriminate between viral and host derived RNA products (43). The motif recognized by MDA5 is less defined, but it is thought that MDA5 recognizes complex and long double-stranded RNA (dsRNA) structures. Based on this, RIG-I and MDA5 differ in the classes of viruses that they recognize. RIG-I recognizes HCV, vesicular stomatitis virus (VSV), measles (32, 39) while MDA5 recognizes encephalomyocarditis virus (EMCV) and mengo virus (44). DNA sensors are cytosolic PRRs that, as it is evident from their name, recognize DNA ligands. Several DNA sensors have been identified where their activation culminates in interferon and cytokine production through IRFs (45, 46). CLRs are PRRs expressed in DCs that recognize pathogen-derived carbohydrates such as mannose, fucose and glucan (3). CLRs recognition of carbohydrates induces NF κ B activation to trigger gene expression. NLRs are cytoplasmic sensors and they recognize intracellular pathogens. Unlike TLRs, RLRs and DNA sensors, NLRs activation triggers inflammatory IL-1 β and IL-18 production through the inflammasome pathway (discussed below) in response to viruses and other microbial-derived ligands (47).

Adaptive immune responses

While many infections by intracellular and extracellular pathogens can be controlled by the acute-phase responses initiated as part of innate immunity, these responses may not be sufficient to completely clear other infections and further cannot provide long-lived protection in case of re-exposure. Adaptive immunity provides a more pathogen-specific, fine-tuned and long-lived set of responses for a complete resolution of infection. Unlike innate immune responses, adaptive immunity develops slowly and typically takes a week or longer for the induction of

fully activated and potent and effective immune responses. A typical adaptive immune response to infection undergoes phases of expansion, contraction and maintenance (48, 49). Lymphocytes such as B and T cells make up the adaptive immune cell compartment and express receptors with a diverse repertoire of antigen specificities. For complete and proper lymphocyte activation three essential signals are required, which include priming, cell-to-cell co-stimulation and inflammatory signals produced by innate immune cells (50). The first priming signal, which is the activating signal, is provided by T cell-receptor (TCR) and/or B cell-receptor (BCR) recognition of pathogen-derived antigens. Professional antigen presenting cells (APCs) such as DCs process pathogen-derived antigens and present them loaded on major histocompatibility complexes (MHCs) to naïve T and B cells (51). TCR/BCR ligation with cognate antigen initiates a cascade of signal transduction pathways involved in cell survival, proliferation and differentiation. The second signal is provided by cell-cell contact through co-stimulatory molecules (52) such as CD28 (expressed on T cells) and the ligand for CD28, CD86 (expressed on APCs). Costimulatory signal is delivered to B cells through engagement of CD40 to DC40 ligand (CD40L). These costimulations provide a survival and proliferation signal to activated lymphocytes to prevent lymphocyte anergy, deletion and immune tolerance. The third and final signal is in the form of inflammatory cues (chemokines and cytokines) produced by innate immune cells, which promotes the survival, maintenance and differentiation of activated lymphocytes(53).

Activated B cells differentiate into immunoglobulin producing plasma cells or long-lived memory cells. B cell-mediated antiviral response is important, which involves the production of high affinity virus-neutralizing antibodies that are important for immunity. Antibodies can directly coat a virus particle preventing its interaction with a cellular receptor and thus

abrogating infection (54, 55). T cell-mediated immunity is often called the cell-mediated immune response. Activated T cells proliferate and differentiate into effector or memory T cells. Differentiated T cells migrate to the site of infection to exert their effector function. T lymphocytes are divided into CD4⁺ and CD8⁺ T cells. CD4⁺ T cells target pathogens that persist in macrophages, parasites and extracellular bacteria. CD4⁺ T cells main effector function is to activate infected macrophages, provide help to B and CD8⁺ T cells, enhance neutrophil responses and in some cases suppress T cell response (56-59). Depending on the type of infection CD4⁺ T cells can differentiate into pathogen-tailored effector cells including regulatory T cells (T-regs). T-reg cells main function is to suppress the activity and function of activated effector cells (CD4⁺ and CD8⁺ T cells) after resolution of an infection to prevent immune cell-mediated immunopathology and to inhibit autoreactive T and B cells to prevent autoimmunity (60). CD8⁺ T cells target intracellular pathogens such as viruses. CD8⁺ T cells main effector function is cytolytic killing of virus-infected cells. CD8⁺-mediated antiviral response is critical to fight and eliminate various viral infections including measles (61), borna virus (62) and herpesviruses (63). Antiviral CD8⁺ T cells prevent virus propagation, limit chronic viral infection and confer long-lived immunity. Induction of cell death in target cells is mediated by releasing granules such as granzyme and perforin. In addition, cytotoxic CD8⁺ T cells can produce cytokines such as IFN- γ (64, 65) and TNF- α . IFN- γ is important to directly inhibit viral replication and to increase MHC class-I up-regulation.

Proper innate immune triggering is essential during a virus infection for optimal adaptive immune induction to prevent hosts from succumbing to infection, to limit virus spread and to restrict virus persistence. Innate immune cells are critical for the initial activation of antiviral CD8⁺ T cells, maintenance of their survival and shaping their differentiation and effector

function. Virus infection-induced cytokines such as IFN-I, IL-12, IL-1 and IL-18 produced by activated innate immune cells promotes CD8⁺ T cells survival, persistent and differentiation. IFN-I and IL-12 are important cytokines to trigger transcriptional changes within naïve CD8⁺ T cells to drive their differentiation into short-lived cytolytic cells (66-69). IL-1 β induces IL-6 production by monocytes (70), which enhances the cytolytic activity of effector T cells (71). IL-18 is produced by macrophages and DCs and it is known as an IFN- γ enhancing cytokine because IL-18 induces IL-12, which is important for IFN- γ production (72). A very essential CD8⁺ T cells survival signal comes from IL-2 whereas IL-7 and IL-15 are important for maintaining memory CD8⁺ T cells (73). While certain cytokines are important to impart survival and differentiation signals to CD8⁺ T cells, some cytokines such as IL-10 and TGF- β deliver a suppressive signal. For example, IL-10 has an anti-proliferative activity (74), which prevents CD8⁺ T cell expansion by reducing the ability of macrophages to present antigens and produce inflammatory factors. TGF- β has an immunomodulatory activity to prevent infection-induced immunopathology by tightly controlling CD8⁺ T cell proliferation and effector function (75). Certain viruses such as HCV, human immunodeficiency virus (HIV), hepatitis B virus (HBV) and Epstein-Barr virus increase IL-10 production to dampen CD8⁺ T cell responses (76-78), which may contribute to virus escape leading to chronic infection. Other viruses hijack the immunomodulatory activity of TGF- β to promote pathology and virus persistence. Thus, virus infection triggers complex immune responses whereby proper innate immune induction is critical to promote the development of adaptive immunity, which ultimately restricts and clears an infection and generates long-lasting immunological memory.

Inflammasomes in immunity and human disease

Interleukin-1

Interleukin-1 (IL-1) is an acute phase protein that is produced in response to infection. IL-1 is a pleiotropic and potent inflammatory cytokine. In 1985, Cameron and March first cloned the human IL-1 gene and in their studies delineated the presence of two distinct forms of IL-1 (79, 80). These two forms of IL-1 are now known as IL-1 α and IL-1 β . The two genes *IL-1A* and *IL-1B* encode IL-1 α and IL-1 β , respectively. Both forms of IL-1 share the same IL-1 receptor type 1 (IL-1R1), which is expressed on all cell types (81-84). Monocytes, macrophages and other phagocytes are the major producers of IL-1. For IL-1 signaling a tertiary complex composed of IL-1, IL-1R1 and the IL-1 receptor accessory protein (IL-1Racp) is required. Signaling is propagated through MyD88, IL-1 receptor-associated kinase, and TRAF6 to induce, for example, NF κ B activation (32).

IL-1 affects a broad spectrum of biological functions in different tissues. For instance, IL-1 modulates metabolic processes such as insulin expression (85, 86). IL-1 further triggers physiological changes such as fever, decrease in appetite and increase in slow wave sleep (87-89). Most importantly, IL-1 is a master cytokine in inflammation and it regulates diverse cellular processes during both acute and chronic infections. It induces potent inflammatory molecules such as cox2, nitric oxide and TNF- α (90, 91). In addition, IL-1 contributes to myeloid cell differentiation by increasing the expression of growth factors such as G-CSF and GM-CSF. IL-1 signaling also increases neutrophil recruitment and enhances phagocytosis. Within the immune system, IL-1 controls the activation of CD4⁺ T cells, B cells and NK cells, it directs the differentiation of T helper 17 (Th17) cells, and further promotes DC maturation, and it enhances adjuvant activity (reviewed in (91)).

Inflammasome pathway activation

The two forms of IL-1 are differentially regulated in the body. While IL-1 α is constitutively expressed in the basal state, IL-1 β is regulated both at the transcriptional and post-translational levels. Processing of IL-1 β is achieved by intracellular aspartate specific cysteine protease caspase-1 (92-94). Caspase-1 is a cytosolic protease that typically exists as an inactive zymogen. Upon activation, caspase-1 precursor (~45kd) undergoes auto-proteolytic cleavage into 10 and 20kd subunits to produce the active protease. IL-1 β precursor is ~31kd that is processed by caspase-1 into the active 17kd subunit. The production of bioactive IL-1 β is a tightly regulated process, which was originally described in 2002 by Jurg Tschopp and his colleagues as a signaling pathway involving caspase-1 and as the formation of what they termed the “inflammasome complex” (95). The inflammasome complex is a cytoplasmic oligomerizing signaling platform containing an NLR, adaptor proteins and caspase-1. Each inflammasome is defined by the NLR involved. The NLRs have a tripartite structural arrangement, which serves as a scaffolding platform for the assembly of the inflammation complex. NLRs have an N-terminal CARD or PYRIN domain (PYD), a central nucleotide-binding oligomerization domain (also known as NACHT domain), and C-terminal leucine-rich repeat (LRR) (96). NLR activation by infection-derived PAMPs or danger-associated molecular patterns (DAMPs) triggers their oligomerization and recruitment of the effector caspase-1. PYRIN containing NLRs require the adaptor protein, apoptosis-associated speck-like protein containing CARD (ASC), which has a PYRIN and CARD domains, to link the PYRIN containing NLR to caspase-1 and thereby drive caspase-1 activation and subsequent IL-1 β and IL-18 maturation and secretion (32, 97). Induction of this pathway requires two signals (illustrated in figure 1-3): signal-one, which requires PAMP activation of PPRs to drive gene transcription and inactive pro-forms, proIL-1 β

or proIL-18, protein production. This is followed by signal two, which is activated by reactive oxygen species or danger signals including ATP or cathepsin B, to trigger NLR activation, which mediates caspase-1 cleavage of proIL-1 β and proIL-18 to produce the mature, secreted forms of each cytokine.

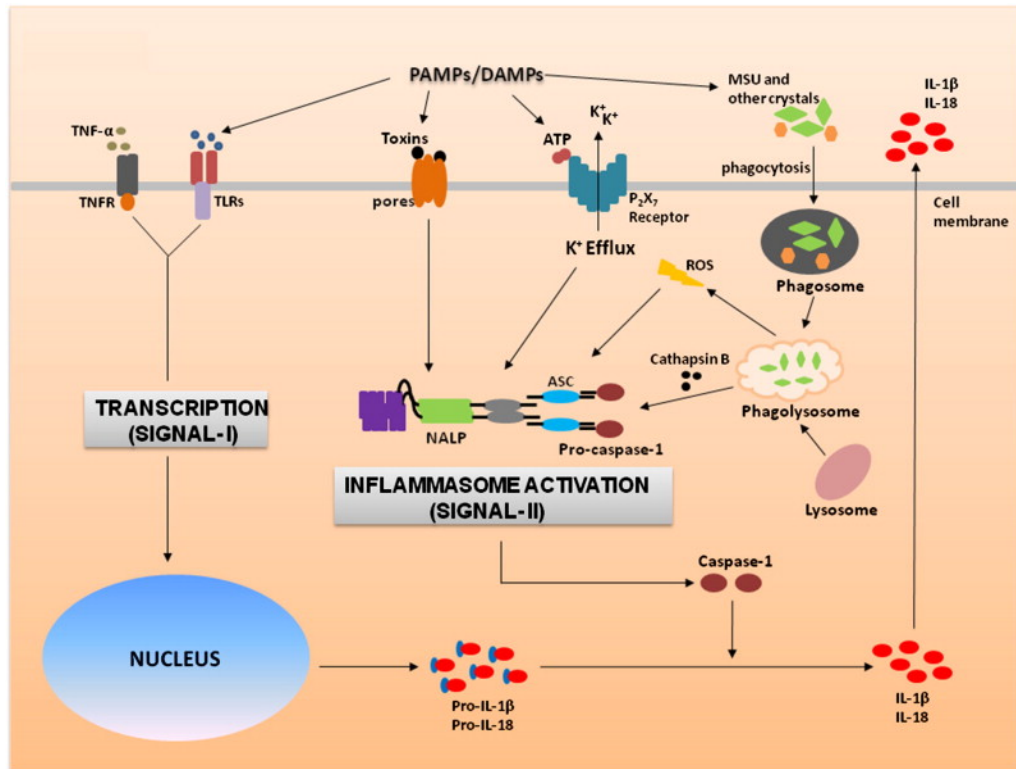


Figure 1-3 Inflammasome-signaling platform. Inflammasome signaling complex is composed of an NLR, adaptor protein ASC and caspase-1. Activation of inflammasome requires two signals: signal-one (priming step) and signal-two. The priming step of inflammasome pathway is initiated by a PRR recognizing a PAMP or DAMP. PRR-PAMP/DAMP interaction initiates signal transduction pathway that culminate in gene expression and inactive proIL-1 β /proIL-18 production. Signal-two activates caspase-1 via the multiprotein inflammasome complex. Diverse NLRP activators such as ATP, uric acid, cathapsin B, and reactive oxygen species drive NLRP/NALP oligomerization and recruitment of ASC and pro-caspel1. The association of these proteins leads to active caspase-1 production, which cleaves and processes proIL-1 β and proIL-18 into bioactive secreted cytokine. Image adopted from: Ouyang X, Ghani A, and Mehal WZ. Inflammasome biology in fibrogenesis. *Biochim Biophys Acta*. 2013;1832(7):979-88.

To date, several inflammasomes have been described including the NLRP1, NLRP3, NLRC4, NLRP6 and AIM2 inflammasomes with each triggered by a distinct stimulus. The NLRP3, RIG-I and AIM2 inflammasomes are triggered in response to virus infection (summarized in Table 1-1). Given the importance of inflammasomes in immunity, many viruses encode immune evasion proteins to block this pathway (reviewed in (98)). For example, poxviruses and herpesviruses encode proteins that inhibit oligomerization of the NLRP3 protein or ASC recruitment. Also Kaposi sarcoma herpesvirus (KSHV) encodes a viral protein Orf3 that is structurally homologous to NLRP1 and prevents IL-1 β production. Influenza virus encodes protein that blocks caspase-1 activation while member of the orthopoxviruses antagonize the inflammasome pathway by directly neutralizing IL-1 β /IL-18 activity (98) .

Inflammasomes	Signaling adaptor proteins	Viruses
NLRP3	ASC	Sendai virus(99), Hepatitis C virus(100), WNV(101), Influenza virus(102), Vaccinia virus(103) and Encephalomyocarditis virus(99, 104), Adeno virus(105)
RIG-I	MAVS/CARD9	Vesicular stomatitis virus(106)
AIM2	ASC	Vaccinia virus and mouse cytomegalovirus(107, 108)

Table 1-1 Virus responsive inflammasomes.

The role of inflammasome regulated IL-1 β in human diseases

IL-1 β plays an important role during infection-induced inflammatory responses. Aberrant IL-1 β production is associated with pathology in many inflammatory disorders (reviewed in (91 and 109)). IL-1 β has been found as a key pathologic factor in hereditary systemic autoinflammatory diseases such as Familial Mediterranean fever (FMF) and cryopyrin-

associated periodic syndrome (CAPS). FMF is caused by a mutation in the MEFV gene, which codes for pyrin. Ineffective pyrin protein causes overproduction of IL-1 leading to lifelong fever and pain in the abdomen. CAPS is a condition caused by an autosomal-dominant mutation in the cryopyrin (NLRP3, also known NALP3) gene. Altered NLRP3 protein activity leads to uncontrolled caspase-1 activation and IL-1 β production. In both type-1 and type-2 diabetes IL-1 β has shown to be toxic to pancreatic β -cells (insulin producing cells), and therapies that block IL-1 signaling can alleviate disease. Gout is an inflammatory disease causing joint pain due to accumulation of uric acid. *In vitro* studies have demonstrated that uric acid activates the NLRP3 inflammasome and IL-1 β production, suggesting that IL-1 β may play an important role in gout pathogenesis. Moreover, patients with osteoarthritis and rheumatoid arthritis (inflammation of joint or bone) who are treated with anakinra (an anti-IL-1 receptor antagonist) show reduced inflammation, suggesting that IL-1 β may be involved in the pathogenesis of these diseases (109, 110). IL-1 β has been further implicated in chronic inflammatory diseases such as idiopathic recurrent pericarditis (reviewed in 89 and 108). In many of these inflammatory diseases amelioration of disease and better clinical outcomes have been achieved with therapies such as anakinra, anti-IL-1 β antibody or a soluble IL-1 decoy receptor or soluble IL-1 decoy receptor that neutralize IL-1 activity (109).

Hepatitis C virus epidemiology, biology and pathogenesis

Global burden and disease course

Hepatitis C virus (HCV) is a global health problem. HCV is a common blood-borne pathogen that causes liver inflammation, cirrhosis, liver cancer and death (111). It is estimated that two to three million people are newly infected each year, of those 170 million people are chronically infected and at risk of developing liver diseases: of the chronically infected

individuals 5% of them die of HCV-related illnesses (112-114). The HCV genome exhibits a high degree of sequence diversity and there are six HCV genotypes and more than 83 subtypes (115). Genotypes 1, 2 and 3 are spread throughout the world, with genotype-1 being mostly common in the United States. Genotypes 4 and 5 are prevalent in the Middle East and Africa whereas genotype 6 is common in Southeast Asian countries. The most common route of HCV transmission is exposure to infected blood including blood transfusion, sharing injection needles among intravenous drug users or occupation related exposures (116). HCV infection can produce an inflammatory disease: acute infection often progresses to a chronic infection, which is characterized by persistent hepatic inflammation (117, 118). Most importantly, persistent liver inflammation is thought to serve as a platform for progressive liver injury and onset of liver cirrhosis and liver cancer. Liver fibrosis is characterized by the accumulation of tough, fibrous scar tissue in the liver. Over the course of 25-30 years of chronic HCV infection and inflammation, increased deposition of scar tissue leads to changes in the architecture of the liver, development of nodules and altered liver tissue. Extensive cirrhosis impairs liver function leading to liver failure and cancer (hepatocellular carcinoma (HCC)).

HCV biology

HCV belongs to the hepacivirus genus and is a member of the *flaviviridea* family. It is an envelope virus with one copy of a positive-sense, single-stranded RNA genome. In 1989, HCV was first identified as a blood transfusion-associated, non-A and non-B causative agent of hepatitis (119). HCV RNA is 9.6 kilo base (kb) long and it is flanked by highly conserved regions, the 3' and 5' untranslated regions (UTRs). HCV is a hepatotropic virus that replicates primarily in hepatocytes. HCV entry requires complex interaction with several host cellular receptors including the low-density lipoprotein receptor (LDLR), the tetraspanin CD81,

scavenger receptor class B type 1 (SR-B1) and the tight junction (TJ) proteins claudin (CLDN-1) and occludin (120-127). Initial viral attachment on hepatocytes is mediated by HCV virion interaction with the low affinity LDLR, which promotes high affinity interactions of HCV virions with SR-B1 and subsequent binding to CD81 (123, 126). This interaction facilitates HCV virion translocation and association with tight junction proteins. This sequential and complex interaction with cellular receptors mediates HCV internalization through clathrin-mediated endocytosis (128). Once HCV particles are released into the cytoplasm, viral RNA is exposed and HCV replication takes place. HCV replicates its RNA using the error-prone, virally encoded RNA-dependent RNA polymerase, which generates sequence diverse subpopulations also known as quasispecies. Besides viral factors, many host cellular factors regulate viral replication including enzymes such as phosphatidylinositol 4-kinase III a (PI4Ka), microRNAs such as miR-122 (miR-122) and cellular chaperones such as cyclophilin A (cypA) (129-131). The HCV long stranded RNA is translated into a single polyprotein of approximately 3000 amino acids in length, which is then cleaved by both host and viral proteases to yield three structural proteins (core, envelope-1 (E1) and envelope-2 (E-2)) and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (see figure 1-4).

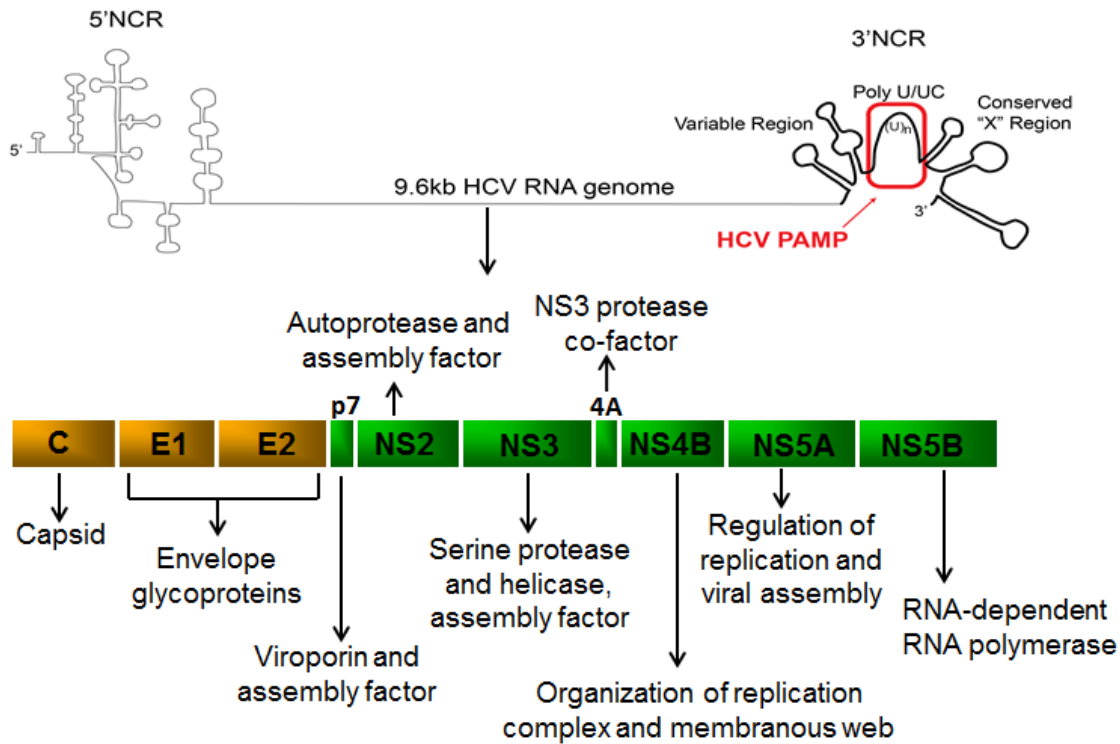


Figure 1-4 schematic presentation of HCV genome and proteins. HCV RNA is translated into a long single-polyprotein, which is processed to produce viral structural proteins shown in yellow and nonstructural proteins shown in green. Viral proteins are involved in viral replication or viral protein processing or host cellular pathways antagonism. HCV protease NS3 and the cofactor NS4A are involved in viral polyprotein processing. The HCV core makes up the capsid and it is important for HCV virion assembly. HCV NS5B is the virus encoded RNA-dependent RNA polymerase. NS5A, NS3/4A are involved in IFN pathway antagonism. p7 is an ion channel and E1 and E2 glycoproteins make up the viral envelope. *Image adopted and modified from: (1) Moradpour D, Penin F, and Rice CM. Replication of hepatitis C virus. Nat Rev Microbiol. 2007;5(6):453-63. (2) Saito T, Owen DM, Jiang F, Marcotrigiano J, and Gale M. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. Nature. 2008;454(7203):523-7.*

After mature viral proteins are produced, viral assembly and infectious virion release take place. Assembly of infectious particle is thought to take place in the lipid-droplet (LD) structures on the viral core protein. Some of the cellular factors that are involved in virion assembly and release include clathrin adaptor AP2M1, group IVA phospholipase A2 (PLAG4A), apolipoprotein E (apo E) and diacylglycerol acyltransferase-I (DGAT-I) (reviewed in (127)).

Immune responses to HCV and viral pathogenesis

HCV infection elicits immune activation and responses. This was evident by studies in chimpanzees that showed IFN-I and IFN-stimulated genes such as ISG-56, 2',5'-oligoadenylate synthetase (OAS)1 and 2 and myxovirus resistance protein A (MxA) up-regulation two days post infection (132). In addition, our laboratory and others have shown that HCV can trigger IFN-I signaling through the RIG-I pathway minutes to hours post infection (39). However, HCV employs diverse mechanisms to antagonize host antiviral pathways to escape immune detection and cause persistent infection. HCV through the action of its NS3/4A protease, which cleaves MAVS from membranes, blocks IFN-I induction (133). It has been proposed that the HCV polyprotein may block the JAK-STAT signaling pathway by inducing the expression of protein phosphatase 2A (PP2A), and thereby preventing ISG expression (134). Additionally, the HCV core alone has been shown to block the JAK/STAT pathway by directly binding to STAT1 to prevent STAT1 phosphorylation leading to the attenuation of STAT1 activity and induction of antiviral ISGs (135-137). Furthermore, the HCV core can induce the expression of suppressor of cytokine signaling-3 (SOCS3), which imposes negative regulation on the JAK-STAT pathway, leading to reduced STAT1 activity and inhibition of ISGs induction (138).

DCs, NK cells and myeloid cells (macrophages and monocytes) mediate the early anti-HCV responses. NK cells are enriched in the liver (139) and their cytolytic activity is important during HCV infection. It has been reported that individuals who are homozygous for the inhibitory NK cell receptor HLA-4 and KIR2DL3 resolve HCV infection (140, 141). This underscores the importance of NK cells in mediating anti-HCV immunity. Furthermore, at the acute stage of HCV infection NK cells up-regulate the activating receptor NKG2D, show enhanced IFN- γ production, degranulation and cytotoxicity (142). However, NK cells from

chronically infected patients show enhanced expression of the inhibitory receptors. Moreover, the lack of IFN production, due to viral antagonism at the initial stages of infection may contribute to the ability of the virus to escape killing by NK cells, which require IFN-I for proper activation (18). Furthermore, engagement of HCV-E2 with CD81 on NK cells delivers an inhibitory signals leading to less cytotoxic activity and low IFN- γ production (143). While DCs show normal activation and function at the acute stage of infection, their function is compromised at the chronic phase. For instance, viral proteins such as the HCV core interfere with antigen presentation and cytokine (IL-12) production in DCs (144).

Cell-mediated anti-HCV immunity is critical to clear HCV infections (145-147). HCV infection resolving immune responses during the acute phase are characterized by sustained T cell response targeting multiple viral epitope with intrahepatic IFN- γ production. During chronic HCV infection CD8⁺ T cells show impaired functionality. This impaired CD8⁺ T cell function during chronic HCV infection is influenced by T cell epitope escape mutations, the host's immune cell repertoire, incomplete differentiation of effector and memory T cells, and immune cell exhaustion. In individuals with chronic HCV infection, small number of HCV-specific T cells with narrow epitope specificities can be detected; this may contribute to the emergence of escape mutation variants and a low ability to combat the emerged mutated viruses. In addition, impaired CD4⁺ T cell differentiation and a shift from IFN- γ into IL-17, IL-22 and IL-21 producing CD4⁺ cells may contribute to liver injury as IL-17 has been shown to cause liver injury in HBV (148) patients. T-reg-derived cytokine, IL-10, which has been shown to be produced at high levels in HCV patients (77), could dampen T cell responses leading to virus escape and persistence within a host.

During chronic HCV infection, the hepatic microenvironment is a location (see figure 1-3) displaying a diverse set of cell-cell interaction, HCV-cell interactions and cellular pathways; it is a source of both inflammatory and immunomodulatory cytokines and chemokines (figure 1-3). The liver has unique anatomical structures for recruiting leukocytes, the endothelial cell lining hepatic sinusoid and the hepatic portal and central veins. Kupffer cells are the liver-resident macrophages, which are abundant in the liver and important for clearing dead cell debris, eliciting pathogen-induced inflammatory responses and involved in repairing infection-triggered liver injury. Pathogen recognition by intrahepatic Kupffer cells is known to trigger inflammatory IL-6, TNF- α and leukocyte chemoattractants such as CXCL8, CXCL1/2 and CCL3/4 (149), which are involved in neutrophils, monocytes and lymphocytes recruitment to the liver. Monocytes and neutrophils then secrete chemokines that further recruit other leukocytes into the hepatic environment. Liver infiltrating lymphocytes, on the other hand, produce inflammatory IFN- γ and exert cytolytic activity. Besides leukocytes, HCV infected the liver parenchymal cells- hepatocytes- produce CXCL10 (150) and TNF- α . As illustrated in figure 1-5 HCV sensing by hepatic cells, triggers complex immune activation and response. While HCV infection-induced production of inflammatory cytokines by intrahepatic myeloid cells and recruitment of cytolytic and IFN- γ producing T cells to the liver might seem characteristic of a protective and infection resolving inflammatory responses, these responses are persistently being triggered in the liver during chronic HCV infection. As a result, this creates a highly inflamed hepatic environment, attenuated innate immune induction and impaired T cell responses that support high viremia and ongoing liver injury and damage.

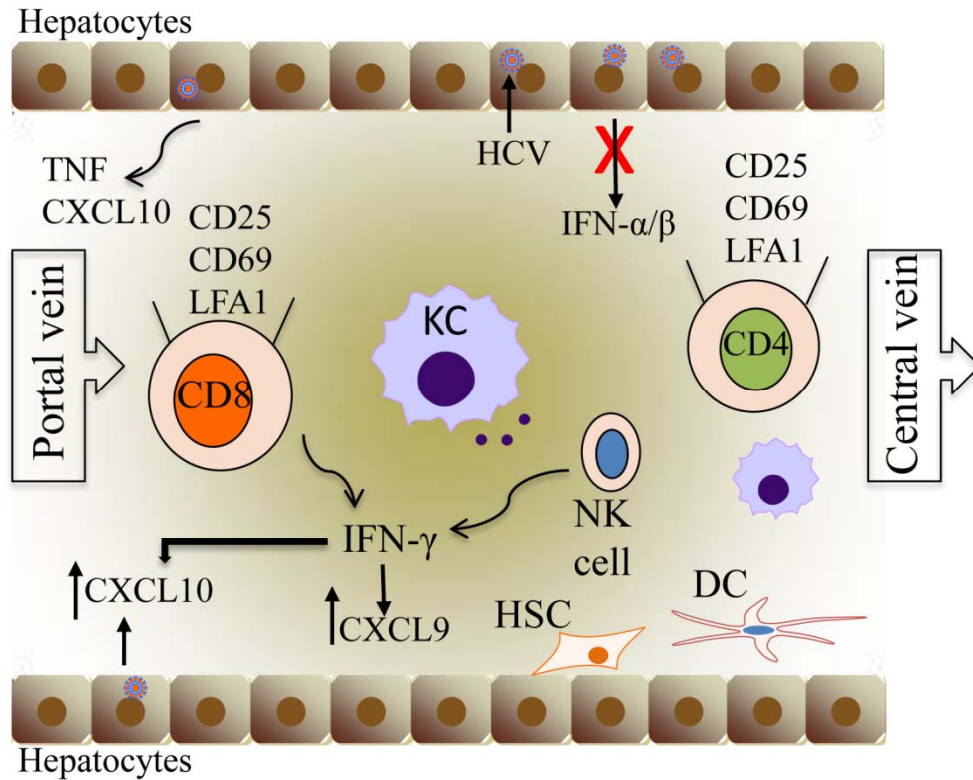


Figure 1-5. Hepatic microenvironment during chronic HCV infection. Blood enters the liver through the hepatic portal vein. It then travels through the sinusoid region and empties into the central vein. This blood brings with it pathogens such as HCV that infects hepatocytes and blocks IFN signaling in these cells. Within the sinusoid region HCV is recognized by liver-resident macrophages (Kupffer cells (KC)). HCV recognition leads to KC activation and the release of chemokines and proinflammatory cytokines, which activated anti-HCV immune responses and recruit lymphocytes and myeloid cells into the hepatic environment. Liver infiltrating lymphocytes (CD8⁺ and CD4⁺) T cells up-regulate CD25, CD69 and adhesion molecule LFA1. Activated CD8⁺ T cells release inflammatory IFN- γ . NK cells are activated and produce IFN- γ . DCs, on the other hand, produce inflammatory cytokines and participate in priming HCV-specific T lymphocytes. HCV infected hepatocytes produce CXCL10 and other inflammatory cytokines such as TNF. Hepatic stellate cells (HSCs) are activated directly by HCV or by inflammatory mediators produced in response to HCV infection, which may lead to their activation to become myofibroblast-like cells. *Image Adopted and modified from: Racanelli V, and Rehermann B. Hepatitis C virus infection: when silence is deception. Trends Immunol. 2003;24(8):456-64.*

HCV targeted therapies

IFN-based therapies are the current standard of care for treating chronic hepatitis C patients. A combination of pegylated interferon-alpha 2a/b (p-IFN) and ribavirin (RVN) is

routinely used to treat HCV patients. IFN is a potent inhibitor of HCV and successful treatment with p-IFN/RVN can achieve the so called a sustained virologic response, which is defined by undetectable level of HCV RNA 24 weeks after termination of therapy (151). Responses to therapy and outcomes of infection are influenced by many parameters; in particular, a patient's genetic make-up is thought to be an effective measure for predicting responsiveness to therapy (152). There is a strong association has been shown between the single nucleotide polymorphism (SNP) located on chromosome 19 within the IL-28B gene and the response to chronic HCV treatment. Besides patient's genomics, basal ISGs expression is thought to be another predictor of response to therapy. ISG expression is inversely correlated with response to treatment: up-regulation of ISGs pre-therapy is a predictor of poor responses to treatment and low ISG levels are a predictor of good responses to treatment (153). Virus genotype is another predictor of treatment outcome. Genotypes 1 and 4 viruses are less responsive to p-IFN/RVN treatment (50%) and genotypes 2 and 3 are more responsive (80%) to IFN treatment (151). Thus, IFN-based therapy is only partially effective. In addition to IFN/ribavirin therapy direct acting antiviral (DAA) drugs, which are NS3/4A protease inhibitors, telaprevir and boceprevir, are approved and are being administered in clinics (154-156). The DAA drugs show high efficacy when administered in combination with p-IFN/ribavirin (triple therapy). However, IFN-based treatment of chronic HCV infection is only partially effective and it is accompanied by unpleasant side effects including anaemia, depression and haemolysis.

Although extensive research have significantly contributed to our understanding of key HCV-host interactions, virus employed evasion mechanisms and enabled the development of new anti-HCV therapies, there is no effective vaccine to combat the global HCV epidemic and current therapies do not resolve infection in all patients with chronic HCV infection. As

discussed above, HCV infection elicits persistent inflammatory responses and creates very complex intrahepatic cell interactions. Thus, delineating the molecular mechanism of the distinct inflammatory pathways that are triggered within the HCV-infected liver and understanding how the interplay of these pathways influences the overall hepatic inflammation is important. In particular, IL-1 β as a key inflammatory mediator may influence HCV-induced hepatic inflammation, impact intrahepatic cell interactions and may play a central role in HCV pathogenesis. Although it is well known that IL-1 β is a key regulator of inflammation and prominent player in the pathogenesis of many inflammatory diseases, its role in HCV-driven hepatitis has never been investigated.

Chapter Two

Materials and Methods

Patient Samples

Chronic hepatitis C patient sera were obtained from patients infected with HCV genotype-1b who were not treated with anti-HCV therapy and exhibited liver inflammation with various stages of fibrosis. Liver biopsies for immunohistochemical staining analysis studies were from patients with chronic HCV-1b infection who had previously undergone standard of care IFN/ribavirin therapy and were classified as non-responders with mild liver disease. For RNA-seq studies, patient liver specimens were analyzed by RNA sequencing and included normal (unused donor liver; n=6), mild disease chronic hepatitis C (percutaneous liver biopsies with mild inflammation [Metavir grade 1-2] and no fibrosis; n=7) and severe disease chronic hepatitis C (liver explants from patients with chronic hepatitis C and cirrhosis undergoing liver transplantation; n=6). Biopsy specimens were from both men and women and were obtained under IRB approval. All samples were frozen in liquid nitrogen in RNAase free tubes immediately after collection and stored at -80° C until they were processed for RNA extraction and analysis.

For flow cytometric analyses, healthy human liver-associated mononuclear cells were collected from livers of living donors (n=2) after flushing the hepatic portal vein (hepatic artery) using cold (4°C) tissue preserving solution following removal of right liver lobes. Collection was performed according to the standard protocol preceding liver transplantation (157). Subsequently, liver resident/intrasinusoidal mononuclear cells were isolated by density gradient centrifugation on Ficoll-Hypaque. The CD14⁺ cells (negative for CD3, CD7, CD56, CD19 and

CD20) present in this cell population expressed CD68 and CD11b at levels similar to the CD14+ cells purified from homogenized livers and therefore are defined as “Kupffer cells” (158). Total purified cells from the liver wash-out were plated in a 96-well round bottom plate, treated with Brefeldin A (10 µg/ml) and stimulated for 6 hours with either LPS (1 µg/mL), HCV-containing (MOI 0.1) or mock supernatant. Cells were then surface stained for CD14 (clone M5E2, BD) and CD16 (clone 3G8, Biolegend) for monocytes, as well as for CD3 (clone HIT3a, Biolegend), CD7 (clone 4H9, eBioscience), CD56 (clone HCD56, Biolegend), CD19 (clone HIB19, Biolegend) and CD20 (clone 2H7, Biolegend) for the identification of specific cell population, i.e., T-NK-B cells. Fluorescence-intensity and size data from stained cells were acquired using a Becton Dickinson LSR II flow cytometer and analyzed using FACS Diva (BD) or FlowJo (Tree star) software.

Cell Culture

THP-1 cells were purchased from ATCC and grown in complete RPMI-1640 medium containing 10% fetal bovine serum, antibiotics, L-glutamine, pyruvate, and non-essential amino acid. THP-1 cells were differentiated by treatment with 20-40 nM of PMA overnight at 37°C. Huh7(159), Huh7.5, HEK293 and immortalized human hepatocytes (PH5CH8 (160) and IHH- a gift from Dr. R. Ray, St Louis University, St. Louis, MO.) were grown in Dulbecco's Modified Eagle's *Medium (DMEM)* supplemented with 10% fetal bovine serum, antibiotics, L-glutamine, pyruvate, non-essential amino acid and Hepes. Monocyte-derived macrophages were generated from peripheral blood mononuclear cells (PBMCs) obtained from healthy individuals under IRB approval as described (161). PBMCs were isolated as described previously (162) and

macrophages were cultured using plastic adherence as described elsewhere (161) and cultured in complete RPMI. All cells were incubated at 37C with 5% CO₂.

HCV polyU/UC RNA hydrodynamic injection

C57BL/6 wild type mice were injected with HCV polyU/UC RNA. 200ug of HCV polyU/UC was delivered hydrodynamically as described (41) via interaperitoneal injection. 6 to 24 hours post injection mice were sacrificed and perfused first with PBS and then with 4% paraformaldehyde for immunohistochemical analyses (IHC).

Propagation of HCV

An HCV JFH-1 genotype 2a infectious clone was produced from synthetic cDNA constructed from the published sequence of original JFH-1 clone (163). This virus, termed HCV-SJ (Synthetic JFH1) was then produced directly from in vitro-transcribed RNA transfected into Huh7.5 cells, and resulting virus was passaged to generate tissue-culture adapted virus. The culture-adapted HCV-SJ will be described in a subsequent report. For infection studies, HCV-SJ was grown in Huh7.5 cells, concentrated 100x using Centricon 100,000 MW cut-off filters (Millipore, Billerica, MA) and titered to determine focus forming units (ffu)/ml using a Huh7 cell-based FFU assay. Differentiated THP-1 cells were treated with HCV at a multiplicity of infection of 0.01-1 [moi=0.01-1 based on Huh7 focus forming units (ffu)]. We noted that preparations of HCV quantified by the FFU method contained both infectious and noninfectious particles, with the latter typically represented at 100 to 1000-fold excess over the former; thus noninfectious particles are likely to contribute to THP1 cell response to HCV (13, 126, 164). HCV virions were purified under a sucrose cushion by ultracentrifugation. Immortalized primary

hepatocytes and hepatoma cells were infected with HCV at an moi=0.1. Ultraviolet light inactivation was achieved using the spectrolinker UV crosslinker to irradiate HCV-containing cell culture media. The media containing UV-inactivated virus was then used to infect hepatocytes or to stimulate THP-1 cells.

Immunohistochemical staining and confocal microscopy

Slide-mounted paraffin-embedded biopsy tissue was deparaffinized, subjected to antigen retrieval, and stained with the indicated antibodies exactly as described previously (165). After staining, cover slips were mounted on slides using Prolong Gold mounting medium (Invitrogen). Stained tissues were visualized using a Nikon Eclipse TE2000 inverted microscope with the Nikon C1 laser scanning confocal module attached to a 10 mW Argon laser, 1 mW HeNe laser, and a 5 mW HeNe laser emitting light at 477 nm, 543 nm, and 633 nm wavelength, respectively. Confocal digital images were collected as 0.2 μ m optical sections and were processed using Nikon EZ-C1 Software v.3.40. Multiple images were collected for each sample analyzed. The primary antibodies used were anti-IL-1 β and anti-CD68.

For staining mouse liver tissue, livers were fixed in 4% paraformaldehyde and were paraffin embedded. All staining procedures were run on the Leica Bond automated immunostainer at the University of Washington histology and imaging core research laboratory; the following steps were performed: 1) slides were incubated for 10 minutes (min) at 65C and deparaffinized on the leica automated immunostainer; 2) antigen retrieval was performed using Bond HIER 1 (University of Washington) or HIER 2 (EDTA) for 10-20 min at 100C; 3) Leica bond peroxide block were incubated for 5 min at room temperature (RT); 4) primary antibody (anti-IL-1 β , 1:1000 from Prosci, Inc) was added diluted in Leica primary antibody diluent and

kept on for 15 min at RT; 5) Leica bond polymer DAB Refine was added for 8 minutes at room temperature. Step six, Leica Bond Mixed Refine (DAB) detection 2X was added and was incubated for 10 min at RT; 7) hematoxylin counterstain, manually performed for 10 seconds (sec) in Harris hematoxylin followed by two rinses with dionized water; and 8) clear to xyglene and mounting with synthetic resin mounting medium and 1.5 coverslip was performed.

RNA sequencing and transcriptomics analyses

For RNA sequencing (RNA-seq) and associated bioinformatics, total RNA was purified from cells or clinical biospecimens using Trizol (Invitrogen, USA) as described previously; and only RNA specimens yielding a Bioanalyzer (Agilent, USA) RNA integrity number (RIN) of ≥ 8.0 were used (166). cDNA libraries were prepared from poly(A) selected mRNA following Illumina RNA-seq protocols (167). Single 50 bp RNA-Seq reads were obtained using Illumina HiSeq 2000 protocols and analyzed following previous studies (168). A total of 20-29 million 50 bp reads were obtained for all chronic hepatitis C and control biospecimens and 16-26 million 50 bp reads for all THP-1 cell samples. Bioinformatics analysis of the RNA-seq data included adjustments for the depth of sequencing (168). Sequencing reads were aligned to the February 2009 human reference genome (GRCh37/hg19), with July 2011 updates, using Novoalign software. The USeqOverdispersedRegionScanSeqs (ORSS) application was used to count reads intersecting exons of each annotated gene and score them for differential expression in each sample. Scores were controlled for multiple testing and ranked by false discovery rate (FDR) and normalized ratio. Genes designated as significantly differentially expressed had an untransformed FDR of < 0.05 (< 5 false positives per 100 observations) and normalized change of ≥ 1.5 fold relative to controls. Hierarchical cluster analysis to produce heat maps of differentially

expressed genes was done with Cluster 3.0 software and visualized using Java TreeView (169).
 Red=increased expression and green=reduced expression.

***In vitro* transcription of HCV RNA**

In vitro transcribed full length HCV RNA (JFH-1), polyU/UC RNA, and X region RNA was prepared from linearized plasmid DNA encoding the synthetic JFH1 HCV 2a genome or specific subgenomic regions using Ambion Mega Script and purified free of DNA exactly as described (41).

Immunoblot analyses

Cell lysates were recovered from control or HCV-stimulated samples via cell lysis in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% Triton-x, 0.25% Na-deoxycholate, 150 mM NaCl, and 1mM EDTA). 20-30 µg of protein was subjected to 15% SDS-PAGE followed by immunoblot assay using the indicated antibodies as described (41). For HCV protein detection, serum A kindly provided by Dr. William Lee was used as in immunoblot to probe for viral proteins.

Protein name	Antibody name	Provider	Product number
IL-1β	Rabbit polyclonal anti-IL-1β	Cell signaling and ProSci, Inc	Cell signaling: 2021 ProSci: 49-460
IL-1β	Cleaved IL-1β (Asp116)	Cell signaling	#2022
IL-1β	Anti-human IL-1β /IL1F2	R & D systems	Catalog #: MAB201 Lot #: AWE08, clone 8516

Caspase-1	Caspase-1 p10 (C-20)	Santa Cruz. Biotechnology, Inc	sc-515
ASC	Anti-Human-ASC	MBL	D086-3, Clone: 23-4
NALP3	NALP3 (human), mAB (Nalpy3-b)	Enzo Life Sciences.	Catalog #: Alx-804- 819-C100 Lot #: L24439a
CD81	Purified mouse anti- human CD81	BD pharmingen	555675. Clone: JS-81
NALP1	NLRP1 antibody	Cell signaling	#4990
CD68	CD68 (H-255): SC-9139	Santa Cruz biotechnology, Inc.	Sc-9139
TLR2	Toll-like receptor 2 antibody	Cell signaling	#2229
TRIF	TRIF antibody	Cell signaling	#4596
TLR7	Toll-like receptor 7 antibody	Santa Cruz biotechnology, Inc.	Sc-57463
Isotype control	Mouse IgG1 (MOPC-21)	Sigma-Aldrich	M5284
MyD88	Anti-MyD88 antibody	Abcam	Ab2068-100 Lot #: 749200
MAVS	Anti-cardif, pAB (AT107)	Enzo Life Sciences	210-929-C100 Lot #: L24443

Table 2-1 Human antibodies used specific for the indicated genes

Enzyme-linked Immunosorbent Assay

IL-1 β ELISA was run according to the manufacture's protocol (Biolegend).

Quantitative Real Time PCR (qRT-PCR)

Total RNA was extracted from cultured cells using the Qiagen RNeasy mini kit. qRT-PCR was performed as previously described (170). Specific qRT-PCR primers for HCV were used; the probe contains 5' 6-FAM and 3' TAMRA-sp modification. RNA amplification was conducted from 50ng cDNA conducted in 25 μ l reaction mixture in optical 96-well plates. Relative RNA expression was quantified using SYBR GREEN (Applied biosystems) reaction mix. For SYBR GREEN the following conditions were performed: 30 min at 48C, 1 cycle at 95C for 10 min, 40 cycles at 95C for 15 sec and 1 min at 60C and dissociation curve (95C 1min, 65C 2 min and 65C-95C at 2/sec).

Gene name	Primer sequence
IL-1 β	Purchased from Qiagen. PPH00171B
Caspase-1	Purchased from Qiagen superarray PPH00105B
NLRP3	Purchased from Qiagen superarray PPH13170A
IFN- β 1	Purchased from Qiagen superarray PPH00384E
GAPDH	Purchased from Qiagen superarray PPH00150E
HCV taqman probe	238-267: 5' CCCGCAAGACTGCTAGCCGAGAGTGTTGG 3'
HCV taqman primers	Forward: 98-116:5'-GAGTGTCGTGCAGCCTCCA-3' Reverse: 313-294: 5'-CACTCGCAAGCACCTATCA-3'
TNF	Purchased from Qiagen superarray PPH00341F
IL-8	Forward: 5'-TTTTGCCAAGGAGTGCTAAAGA-3' Reverse: 5'-AACCTCTGCACCCAGTTTTTC-3'
CCL4	Purchased from Qiagen superarray PPH00563B

CCL5	Purchased from Qiagen superarray PPH00703B
CD40	Forward: 5'-CACCTGGAACAGAGAGACACA-3' Reverse: 5'-CCTCACTCGTACAGTGCCA-3'
CXCL1	Purchased from Qiagen superarray PPH00696C

Table 2-2 Quantitative real time polymerase-chain reaction (qRT-PCR) primer sequences. All primers amplify human genes.

Endotoxin testing

LPS contamination of cell extracts was tested by gel clot assay using *Limulus* Amoebocyte Lysate (LAL) Pyrotell from CAPE COD, Inc. according to manufacturer's recommendations.

RNA transfection

Differentiated THP-1 cells were transfected using the Mirus reagent following the company's protocol (Mirus).

Lentiviral shRNA transduction

Undifferentiated THP-1 cells were transduced with $1-3 \times 10^6$ lentiviral particles encoding non-targeting control or gene-specific shRNA in 48 well plates. 24hrs post transduction, selection media was added (RPMI containing puromycin at $1 \mu\text{g}/\text{ml}$). The cells were then maintained in selection media. For assessment of mRNA knockdown, THP1 cells were differentiated with PMA prior to use in experiments.

Statistical analysis

Statistical analyses were conducted using unpaired student *t-test* and GraphPad prism software.

Sequences

Procaspase-1: (NM_033292.2)

GCCATGGCCGACAAGGTCCTGAAGGAGAAGAGAAAGCTGTTTATCCGTTCCATGGG
TGAAGGTACAATAAATGGCTTACTGGATGAATTATTACAGACAAGGGTGCTGAACA
AGGAAGAGATGGAGAAAGTAAAACGTGAAAATGCTACAGTTATGGATAAGACCCG
AGCTTTGATTGACTCCGTTATTCCGAAAGGGGCACAGGCATGCCAAATTTGCATCAC
ATACATTTGTGAAGAAGACAGTTACCTGGCAGGGACGCTGGGACTCTCAGCAGATC
AAACATCTGGAAATTACCTTAATATGCAAGACTCTCAAGGAGTACTTTCTTCCTTTCC
AGCTCCTCAGGCAGTGCAGGACAACCCAGCTATGCCACATCCTCAGGCTCAGAAG
GGAATGTCAAGCTTTGCTCCCTAGAAGAAGCTCAAAGGATATGGAAACAAAAGTCG
GCAGAGATTTATCCAATAATGGACAAGTCAAGCCGCACACGTCTTGCTCTCATTATC
TGCAATGAAGAATTTGACAGTATTCTAGAGAAGTGGAGCTGAGGTTGACATCAC
AGGCATGACAATGCTGCTACAAAATCTGGGGTACAGCGTAGATGTGAAAAAAATC
TACTGCTTCGGACATGACTACAGAGCTGGAGGCATTTGCACACCCGCCAGAGCAC
AAGACCTCTGACAGCACGTTCCCTGGTGTTCATGTCTCATGGTATTCGGGAAGGCATT
TGTGGGAAGAAACACTCTGAGCAAGTCCCAGATATACTACAACCTCAATGCAATCTTT
AACATGTTGAATACCAAGAACTGCCCAAGTTTGAAGGACAAACCGAAGGTGATCAT
CATCCAGGCCTGCCGTGGTGACAGCCCTGGTGTGGTGTGGTTTAAAGATTCAGTAGG
AGTTTCTGGAAACCTATCTTTACCAACTACAGAAGAGTTTGAGGATGATGCTATTAA
GAAAGCCCACATAGAGAAGGATTTTATCGCTTTCTGCTCTTCCACACCAGATAATGT
TTCTTGAGACATCCCAATGGGCTCTGTTTTTATTGGAAGACTCATTGAACATAT
GCAAGAATATGCCTGTTCCCTGTGATGTGGAGGAAATTTTCCGCAAGGTTTCGATTTTC
ATTTGAGCAGCCAGATGGTAGAGCGCAGATGCCACCACTGAAAGAGTGACTTTGA
CAAGATGTTTCTACCTCTTCCCAGGACATTAATAAAGGAAACTGTATGAATGTCTG
TGGGCAGGAAGTGAAGAGATCCTTCTGTAAAGGTTTTTGGAATTATGTCTGCTGAAT
AATAAACTTTTTTGAATAATAAATCTGGTAG

ProIL-1 β : (NM_000576.2)

ACCAAACCTCTTCGAGGCACAAGGCACAACAGGCTGCTCTGGGATTCTCTTCAGCCA
ATCTTCATTGCTCAAGTGTCTGAAGCAGCCATGGCAGAAGTACCTGAGCTCGCCAGT
GAAATGATGGCTTATTACAGTGGCAATGAGGATGACTTGTCTTTGAAGCTGATGGC
CTAAACAGATGAAGTGCTCCTTCCAGGACCTGGACCTCTGCCCTCTGGATGGCGGC
ATCCAGCTACGAATCTCCGACCACCACTACAGCAAGGGCTTCAGGCAGGCCCGCGTC
AGTTGTTGTGGCCATGGACAAGCTGAGGAAGATGCTGGTTCCTGCCACAGACCTT
CCAGGAGAATGACCTGAGCACCTTCTTTCCCTTCATCTTTGAAGAAGAACCTATCTT
CTTCGACACATGGGATAACGAGGCTTATGTGCACGATGCACCTGTACGATCACTGAA
CTGCACGCTCCGGGACTCACAGCAAAAAAGCTTGGTGTGCTGGTCCATATGAACT
GAAAGCTCTCCACCTCCAGGGACAGGATATGGAGCAACAAGTGGTGTCTCCATGTC
CTTTGTACAAGGAGAAGAAAGTAATGACAAAATACCTGTGGCCTTGGGCCTCAAGG

AAAAGAATCTGTACCTGTCCTGCGTGTTGAAAGATGATAAGCCCACTCTACAGCTGG
AGAGTGTAGATCCCAAAAATTACCCAAAGAAGAAGATGGAAAAGCGATTTGTCTTC
ACAAGATAGAAATCAATAACAAGCTGGAATTTGAGTCTGCCAGTTCCCAACTG
GTACATCAGCACCTCTCAAGCAGAAAACATGCCCCTCCTGGGAGGGACCAAAG
GCGGCCAGGATATAACTGACTTCACCATGCAATTTGTGTCTTCCTAAAGAGAGCTGT
ACCCAGAGAGTCCTGTGCTGAATGTGGACTCAATCCCTAGGGCTGGCAGAAAGGGA
ACAGAAAGGTTTTTTGAGTACGGCTATAGCCTGGACTTTCCTGTTGTCTACACCAATG
CCCAACTGCCTGCCTTAGGGTAGTGCTAAGAGGATCTCCTGTCCATCAGCCAGGACA
GTCAGCTCTCTCCTTTCAGGGCCAATCCCCAGCCCTTTTGTGAGCCAGGCCTCTCTC
ACCTCTCCTACTCACTTAAAGCCCGCCTGACAGAAACCACGGCCACATTTGGTTCTA
AGAAACCCTCTGTCATTCGCTCCCACATTCTGATGAGCAACCGCTTCCCTATTTATTT
ATTTATTTGTTTGTGTTTATTTCATTGGTCTAATTTATTCAAAGGGGGCAAGAAGT
AGCAGTGTCTGTAAAAGAGCCTAGTTTTTAATAGCTATGGAATCAATTCAATTTGGA
CTGGTGTGCTCTCTTTAAATCAAGTCCTTTAATTAAGACTGAAAATATATAAGCTCA
GATTATTTAAATGGGAATATTTATAAATGAGCAAATATCATACTGTTCAATGGTTCT
GAAATAAACTTCACTGA

NLRP3 (NM_001079821.2)

ACCAAACCTCTTCGAGGCACAAGGCACAACAGGCTGCTCTGGGATTCTCTTCAGCCA
ATCTTCATTGCTCAAGTGTCTGAAGCAGCCATGGCAGAAGTACCTGAGCTCGCCAGT
GAAATGATGGCTTATTACAGTGGCAATGAGGATGACTTGTCTTTGAAGCTGATGGC
CCTAAACAGATGAAGTGTCTCCTTCCAGGACCTGGACCTCTGCCCTCTGGATGGCGGC
ATCCAGCTACGAATCTCCGACCACCACTACAGCAAGGGCTTCAGGCAGGCCGCGTC
AGTTGTTGTGGCCATGGACAAGCTGAGGAAGATGCTGGTTCCTGCCACAGACCTT
CCAGGAGAATGACCTGAGCACCTTCTTTCCCTTCATCTTTGAAGAAGAACCTATCTT
CTTCGACACATGGGATAACGAGGCTTATGTGCACGATGCACCTGTACGATCACTGAA
CTGCACGCTCCGGGACTCACAGCAAAAAGCTTGGTGATGTCTGGTCCATATGAACT
GAAAGCTCTCCACCTCCAGGGACAGGATATGGAGCAACAAGTGGTGTCTCCATGTC
CTTTGTACAAGGAGAAGAAAGTAATGACAAAATACCTGTGGCCTTGGGCCTCAAGG
AAAAGAATCTGTACCTGTCCTGCGTGTTGAAAGATGATAAGCCCACTCTACAGCTGG
AGAGTGTAGATCCCAAAAATTACCCAAAGAAGAAGATGGAAAAGCGATTTGTCTTC
ACAAGATAGAAATCAATAACAAGCTGGAATTTGAGTCTGCCAGTTCCCAACTG
GTACATCAGCACCTCTCAAGCAGAAAACATGCCCCTCCTGGGAGGGACCAAAG
GCGGCCAGGATATAACTGACTTCACCATGCAATTTGTGTCTTCCTAAAGAGAGCTGT
ACCCAGAGAGTCCTGTGCTGAATGTGGACTCAATCCCTAGGGCTGGCAGAAAGGGA
ACAGAAAGGTTTTTTGAGTACGGCTATAGCCTGGACTTTCCTGTTGTCTACACCAATG
CCCAACTGCCTGCCTTAGGGTAGTGCTAAGAGGATCTCCTGTCCATCAGCCAGGACA
GTCAGCTCTCTCCTTTCAGGGCCAATCCCCAGCCCTTTTGTGAGCCAGGCCTCTCTC
ACCTCTCCTACTCACTTAAAGCCCGCCTGACAGAAACCACGGCCACATTTGGTTCTA
AGAAACCCTCTGTCATTCGCTCCCACATTCTGATGAGCAACCGCTTCCCTATTTATTT
ATTTATTTGTTTGTGTTTATTTCATTGGTCTAATTTATTCAAAGGGGGCAAGAAGT
AGCAGTGTCTGTAAAAGAGCCTAGTTTTTAATAGCTATGGAATCAATTCAATTTGGA
CTGGTGTGCTCTCTTTAAATCAAGTCCTTTAATTAAGACTGAAAATATATAAGCTCA

GATTATTTAAATGGGAATATTTATAAATGAGCAAATATCATACTGTTCAATGGTTCT
GAAATAAACTTCACTGA

ASC: (NM_013258.3)

ATGGGGCGCGCGCGACGCCATCCTGGATGCGCTGGAGAACCTGACCGCCGAGGA
GCTCAAGAAGTTCAAGCTGAAGCTGCTGTCGGTGCCGCTGCGCGAGGGCTACGGGC
GCATCCCGCGGGGCGCGCTGCTGTCCATGGACGCCTTGGACCTACCGACAAGCTG
GTCAGCTTTTACCTGGAGACCTACGGCGCCGAGCTCACCGCTAACGTGCTGCGCGAC
ATGGGCCTGCAGGAGATGGCCGGGCGAGCTGCAGGCGGCCACGCACCAGGGCTCTGG
AGCCGCGCCAGCTGGGATCCAGGCCCTCCTCAGTCGGCAGCCAAGCCAGGCCTGC
ACTTTATAGACCAGCACCGGGCTGCGCTTATCGCGAGGGTCACAAACGTTGAGTGGC
TGCTGGATGCTCTGTACGGGAAGTCTTGACGGATGAGCAGTACCAGGCAGTGCGG
GCCGAGCCCACCAACCCAAGCAAGATGCGGAAGCTCTTCAGTTTACACCAGCCTG
GAACTGGACCTGCAAGGACTTGCTCCTCCAGGCCCTAAGGGAGTCCCAGTCCTACCT
GGTGGAGGACCTGGAGCGGAGCTGAGGCTCCTTCCAGCAAACTCCGGTCAGCCC
CTGGCAATCCCACCAAATCATCCTGAATCTGATCTTTTTATACAAATATACGAAA
GCCAGCTTGA

Primers

The genes of NLRP3 inflammasome components (proIL-1 β , procaspase-1, ASC and NLRP3) and HCV genotype-2a proteins (from pSJ plasmid (RRR)) were cloned into pEF-tak vector (RRR) to generate constructs expressing two flag tags at the N-terminus. The following PCR primers were used.

Gene name	Sequence
Procaspace-1	Forward: 5'-GCGCGCGGCCGCGGCCATGGCCGACAAGGTCCTGAAG-3' Reverse: 5'-GCGCGTTTTAAACCTACCAGATTTATTATTTCAAAAAG-3'
ProIL-1 β	Forward: 5'-GCGCGCGGCCGCGACCAAACCTCTTCGAGGCACAAGG-3' Reverse: 5'-GCGCGTTTTAAACTCAGTGAAGTTTATTTCAGAACCATTG-3'
NLRP3	Forward: 5'-GCGCGCGGCCGCGTGCCGTGTTCACTGCCTGGTATCT-3' Reverse: 5'-GCGCGTTTTAAACCTACCAAGAAGGCTCAAAGACGACG GT-3'

ASC	Forward: 5'-GCGCGCGGCCGCGATGGGGCGCGCGCGGACGCCATC-3' Reverse: 5'-GCGCGTTTAAACTCAAGCTGGCTTTTCGTATATTGTGTA-3'
HCV core	Forward: 5'-GCGC GCGGCCGC G ATGAGCACAAATCC-3' Reverse: 5'-GCGC GTTTAAAC CTAAGCAGAGACCGGAACG-3'
HCV E1	Forward: 5'-GCGC GCGGCCGC G GCCCAGGTGAAGAATACC-3' Reverse: 5'-GCGC GTTTAAAC CTACGCGTCCACCCCAGCG-3'
HCV E2	Forward: 5'-GCGC GCGGCCGC G GGCACCACCACC-3' Reverse: 5'-GCGC GTTTAAAC CTATTCGGCCTGGCCCAA-3'
HCV NS4A	Subcloned from a minigene containing NotI and PmeI sites 5'-GGTACCACCATGAGCACGTGGGTCTAGCTGGAGGAGTCCTGGCA GCCGTCGCCGCATATTGCCTGGCGACTGGATGCGTTTCCATCATCGGC CGCTTGCACGTCAACCAGCGAGTCGTCGTTGCGCCGGATAAGGAGGT CCTGTATGAGGCTTTTGTATGAGATGGAGGAATGCGATTATAAGGATG ATGATGATAAAGGTGATTATAAGGATGATGATGATAAATAGTAGGTT TAAAC-3'
HCV NS2	Forward: 5'-GCGC TTCGAA TATGACGCACCTGTGCAC-3' Reverse: 5'-GCGC GTTTAAAC CTAAAGGAGCTTCCACCC-3'
HCV NS3	Forward: 5'-GCGC GCGGCCGC G GCTCCCATCACTGCTTATGCC-3' Reverse: 5'-GCGC GTTTAAAC CTAGGTCATGACCTCAAGGTC-3'
HCV p7	Subcloned from a minigene containing KpnI and PmeI sites 5'-GCGGCCGCGGCAGCATTGGAGAAGTTGGTCGTCTTGCACGCTGCG AGTGCGGCTAACTGCCATGGCCTCCTATATTTTGGCATCTTCTTCGTG GCAGCTTGGCACATCAGGGGTCGGGTGGTCCCCTTGACCACCTATTGC

	CTCACTGGCCTATGGCCCTTCTGCCTACTGCTCATGGCACTGCCCCGG CAGGCTTATGCC GTTAAAC-3'
HCV NS4B	Forward: 5'-GCGC GCGGCCGC G GCCTCTAGGGCGGCTCTC-3' Reverse: 5'-GCGC GTTAAAC CTAGCATGGGATGGGGGCAGTC-3'
HCV NS5A	Forward: 5'-GCGC GCGGCCGC G TCCGGATCCTGGCTC-3' Reverse: 5'-GCGC GTTAAAC CTAGCAGCACACCGGTGGTATC-3'
HCV NS5B	Forward: 5'-GCGC GCGGCCGC G TCCATGTCATACTCC-3' Reverse: 5'-GCGC GTTAAAC CTACCGAGCGGGGAGTAG-3'
HCV NS3- 4A	Forward: 5'-GCGC GCGGCCGC G GCTCCCATCACTGCTTATGCC-3' Reverse: 5'-GCGC GTTAAAC CTAGCATTCCTCCATCTCATC-3'

Table 2-3 PCR primer sequences.

Other Materials

Mission lentiviral transduction particles (Non-targeting, caspase-1, NLRP3, NLRP1, ASC, MAVS, TLR7, TLR2, or MyD88-specific), adenosine 5'-triphosphate (ATP) disodium salt solution, Phorbol 12-Myristate 13-acetate, LPS, cytochalasin D, bafilomycin A, diphenyliodonium chloride and glybenclamide were each purchased from Sigma. Synthetic dsRNA Poly (I): Poly(C) was from GE Healthcare Life. TransIT-mRNA transfection kit was bought from Mirus and IL-1 β ELISA kit was purchased from Biolegend. For hydrodynamic injections, lipid-based *in vivo* transfection reagent was used from Altogen Biostystems.

Chapter Three

Hepatitis C virus-induced interleukin-1beta production by macrophages *in vivo* and *in vitro*

Introduction

HCV productively infects hepatocytes to induce liver inflammation and progressive tissue damage leading to fibrosis and cirrhosis. These processes underlie liver dysfunction and are thought to drive the onset of liver cancer (171). However, the molecular mechanism(s) by which HCV stimulates hepatic inflammation are not defined. Interleukin-1 β (IL-1 β) is a central component of the cytokine milieu that accompanies both acute and chronic inflammation and viral disease (91, 172). IL-1 β production is induced by cellular sensing of pathogen-associated molecular pattern (PAMP) motifs within microbial macromolecules and/or by metabolic products that accumulate from infection. Production of active IL-1 β requires two signals, “signal one” to activate NF- κ B in stimulated cells and induce IL-1 β mRNA expression, and “signal two” to activate a Nod-like receptor (NLR) to promote downstream caspase-1 cleavage and processing of proIL-1 β into a biologically active, secreted cytokine.

Virus infection has been shown to induce IL-1 β production through inflammasome signaling. In particular, *flavivirus* agents related to HCV, including West Nile virus (101) and Japanese encephalitis virus (173), trigger IL-1 β production through the NLRP3 inflammasome to induce inflammation and restrict infection. Although the breadth of IL-1 β -induced genes within the liver has not been defined, IL-1 β is thought to mediate its inflammatory actions by inducing the expression of proinflammatory genes, recruiting immune cells to the site of infection, and by modulating infiltrating cellular immune-effector actions. As a pleiotropic inflammatory factor,

IL-1 β has also been implicated in promoting tissue pathology and inducing the production of profibrogenic mediators (91, 109, 174), thereby underscoring its potential role in HCV disease.

As discussed in chapter one, the unique architecture of the liver and the high viremia (118) of HCV infection constantly expose resident hepatic cells and blood and liver-infiltrating myeloid cells to the virus. HCV evades hepatocytes-based innate antiviral responses by cleaving MAVS through NS3/4A protease action, which disables the production of type-1 interferon (13). Moreover, exposure of hepatic stellate cells to HCV, viral antigens, or inflammatory signals can induce their fibrogenic activity, which can lead to liver damage (175); whereas HCV exposure of hepatic myeloid cells, including resident macrophages/Kupffer cells and dendritic cells (176), induces cell activation. In particular, Kupffer cells, the intrahepatic macrophages, play critical roles in microbial surveillance in which constant phagocytic uptake of macromolecules from hepatic sinusoidal blood provides the means for liver-wide pathogen detection. Importantly, Kupffer cells can internalize HCV through a process of phagocytosis wherein internalized viral products can induce innate proinflammatory cytokines that serve to recruit immune cells to the site of infection, thus supporting immune-mediated liver damage characteristic of chronic hepatitis C. Based on this, I decided to investigate whether IL-1 β , a key proinflammatory cytokine and a product of the inflammasome pathway, contributes to HCV-induced hepatic inflammation. My studies presented here define an important mechanism of hepatic inflammation during HCV infection.

Results

HCV infection does not stimulate IL-1 β in hepatocytes

Since HCV replicates primarily in hepatocytes, we assessed whether hepatocytes

are a source of hepatic IL-1 β during HCV infection. To test this, we infected *in vitro* immortalized human hepatocytes (IHH and PH5CH8 cells) or hepatoma (Huh7) with HCV. Surprisingly, we found that upon either HCV exposure or infection neither immortalized primary human hepatocytes nor hepatoma cells expressed inducible IL-1 β mRNA (figure 3-1A top panel) or secreted appreciable levels of mature IL-1 β (figure 3-1 bottom panel). We know the conditions used elicit good infection and does not trigger cell death. We next hypothesized hepatocytes fail to produce IL-1 β upon HCV infection due to lack of inflammasome component expression such as caspase-1 and IL-1 β . We found hepatocyte cell lines express intact caspase-1 and weak basal IL-1 β expression (figure 3-1B) as detected by immunoblot analysis, suggesting that these hepatocyte cell lines might not express the necessary component for inflammasome assembly or exhibit a defect in IL-1 β secretion pathway.

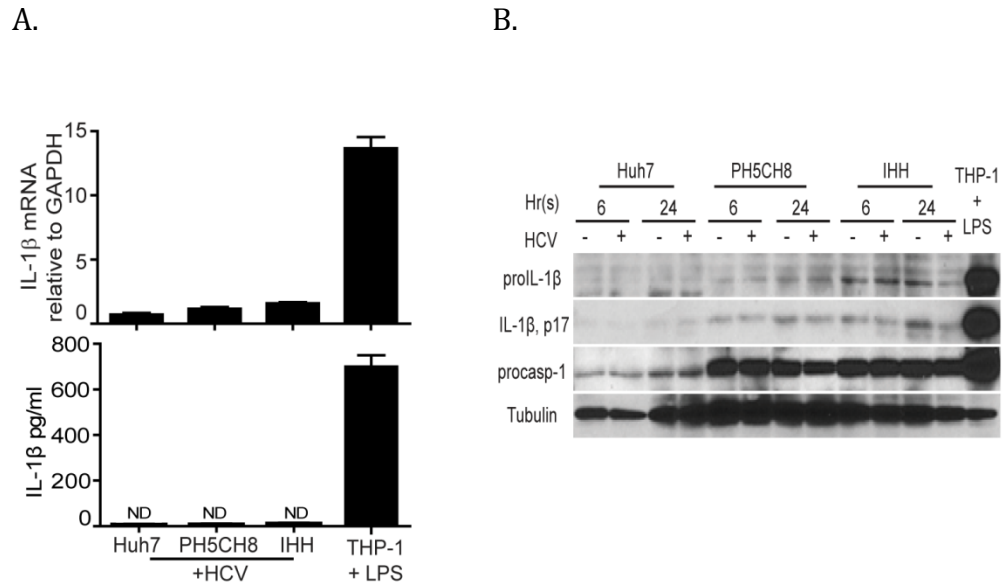
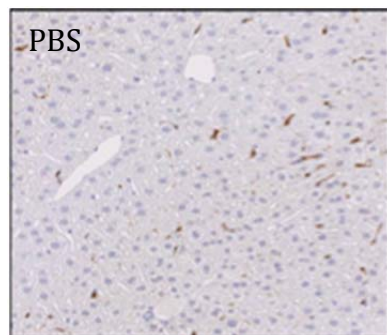


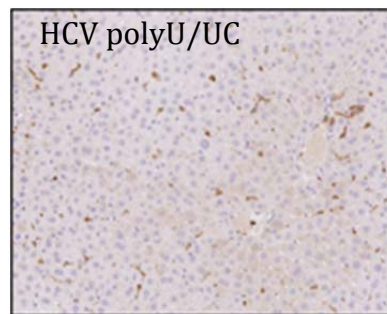
Figure 3-1 **HCV infection does not stimulate IL-1 β production in hepatocytes.** (A) Immortalized hepatocytes (IHH and PH5CH8) and hepatoma Huh7 cells were infected with HCV at moi of 0.1 then IL-1 β mRNA expression (upper panel) and protein secretion (lower panel). (B) Immunoblot examining the expression of inflammasome components in infected hepatocytes and hepatoma cells.

To test inflammasome induction *in vivo* we employed a hydrodynamic injection technique to deliver HCV polyU/UC RNA (PAMP) directly to the liver. Mice were injected i.p (intraperitoneal) by hydrodynamic injection with HCV polyU/UC and then mice liver sections were analyzed for the expression of IL-1 β by immunohistochemistry (figure 3-2 A-D). To differentiate between Kupffer cells/infiltrating macrophages and hepatocytes, we stained with an antibody specific for Mac2, a macrophage cell surface marker. This analysis in mice provided the first clue that HCV polyU/UC injection does drive IL-1 β expression. We found that after exposure to the HCV-derived PAMP, the expression of IL-1 β was only detectable in Mac2+ cells. In line with our *in vitro* observation (figure 3-1A and B), enriched IL-1 β expression not detected in hepatocytes, but rather was found in cells within the sinusoidal region of the liver (figure 3-1D), suggesting that *in vivo* macrophages, but not hepatocytes, might be a primary source of hepatic IL-1 β induced by HCV..

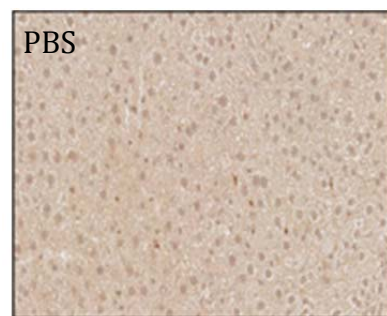
A.



B.



C.



D.

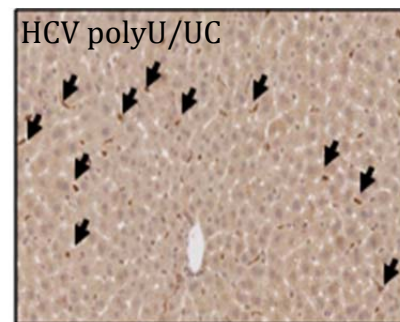


Figure 3-2 HCV polyU/UC RNA stimulates IL-1 β expression in mouse liver. HCV polyU/UC was hydro-dynamically injected into mice through I.P. Liver sections were analyzed by immunohistochemical staining for the expression of IL-1 β and mac2. (A) and (B) stained with anti-Mac antibody and show expression of mac2 in PBS or HCV polyU/UC injected mice. (C) and (D) stained with anti-IL-1 β and show expression of IL-1 β in PBS or HCV polyU/UC injected mice. The arrows show enriched expression of IL-1 β in macrophages.

IL-1 β expression is associated with severe liver disease

Since we did detect IL-1 β production in mouse liver post injection with HCV polyU/UC RNA, this provided the first clue that HCV-derived products can impart an inflammasome pathway activating signal within the hepatic environment to drive IL-1 β production. We then investigated whether HCV can impart such activating signal to drive hepatic IL-1 β production within livers of patients with chronic HCV infection. To address this question, we performed global transcriptome analysis using RNA-sequencing (RNA-seq) of RNA recovered from liver biopsies of normal controls (donor) or chronic HCV-infected livers of patients staged according to mild (mild inflammation and no fibrosis) or severe (cirrhosis undergoing liver transplantation) disease. This allowed us not only to assess the hepatic IL-1 β , but also enabled us to determine gene expression patterns and host response processes associated with liver disease in chronic HCV infection. The initial comparison of transcriptome profiles from control and HCV patient liver identified four major gene expression patterns that differentially associated with liver disease (figure 3-3 and Table 3-1). These analyses determined that the “cytokine/cytokine receptor interaction” and “chemokine signaling pathways” were the most highly represented groups with differentially expressed genes (>1.5-fold change and false discovery rate (FDR) 0.05) in the three liver biopsy sets examined. HCV patient livers displayed induced expression of a variety of immunomodulatory gene products known to be associated with inflammatory responses, including OSM, IL-6, IL-8, and TGF- β (Group 3, figure 3-3). Importantly, increased

expression of IL-1 β , a key proinflammatory cytokine, was generally associated with HCV infection and the onset of liver disease.

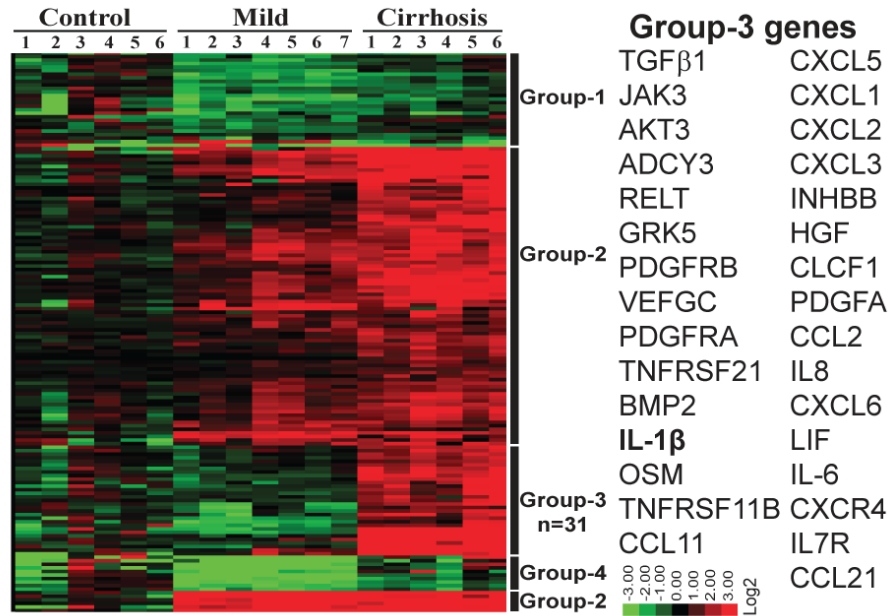


Figure 3-3 **Hierarchical clustering of differentially expressed genes.** RNA-seq analysis of liver specimens from control or HCV patients with mild (no fibrosis) and severe (cirrhosis) liver disease. Clustering analysis of a total of 158 differentially expressed genes (>1.5-fold change and FDR 0.05) in the cytokine-cytokine receptor and chemokine signaling pathways is shown. The expression of group-3 genes were increased only in patients with severe liver disease; Group-3 genes and the expression key are shown at the right (for full description, see Table 3-1). For analysis see methods.

Group-1	Group-2	Group-3	Group-4
IL-1R2	PDGFB	TGFB1	CCL20
BCAR1	IL20RA	JAK3	EPO
TNFRSF10A	CCL28	AKT3	TNFRSF12A
IFNGR1	TNFRSF19	ADCY3	TGFB3
IL1R1	CCL8	RELT	TNFSF14
PLCB1	TNFSF8	GRK5	OSMR
IKBKG	PRKX	PDGFRB	IL15RA
IL4R	PIK3CG	VEGFC	SHC1
NFKBIB	CSF2RA	PDGFRA	STAT3
LTBR	KIT	TNFRSF21	TNFRSF10D
ACVR1B	TGFB2	BMP2	
CHUK	CCL3L1	IL1 β	
FLT1	IL18	OSM	
CXCR1	LEPR	TNFRSF11B	
CSF3R	CX3CL1	CCL11	
CXCR2	CCL3	CXCL5	
CXCL16	CCL4L2	CXCL1	
INHBE	CCL4	CXCL2	
TNFSF11	CXCL12	CXCL3	
IL18RAP	CD27	INHBB	
IL18R1	IL2RG	HGF	
FOXO3	CCL18	CLCF1	
IL10	CCL24	PDGFA	
MAP2K1	TNFRSF17	CCL2	
IL1RAP	FLT3LG	IL8	
ADCY1	IL12RB1	CXCL6	
CXCL13	XCL1	LIF	
	CD40	IL6	
	XCL2	CXCR4	
	CXR3	IL7R	
	CCR2	CCL21	
	CCR5		
	FAS		
	IL3RA		
	EDA2R		
	TNFRSF18		
	IL10RA		
	CD40LG		
	ITK		
	TNFRSF4		
	TNFRSF25		
	TNFRSF13C		
	CCR4		
	IL12RB		
	STAT1		
	GHR		
	RASGRP2		
	IFNG		
	TNFSF4		
	CCL25		

	EDAR		
	CCL22		
	CCL17		
	CCR6		
	CTF1		
	PF4V1		
	GNB3		
	TIAM2		
	PLCB4		
	IL2		
	FLT3		
	CD70		
	TNFSF15		
	CCR10		
	TNFSF18		
	XCR1		
	IL21R		
	TNFRSF9		
	FASLG		
	DOCK2		
	PIK3CD		
	NCF1		
	CXCR6		
	ADCY7		
	PLCB2		
	RAC2		
	VAV1		
	PRLR		
	TSLP		
	CX3CR1		
	IL11RA		
	CXCL14		
	CCL16		
	CCL15		
	CXCL9		
	TNFSF10		
	CCL5		
	CXCL11		
	CCL19		
	CXCL10		

Table 3-1 **List of Differentially expressed genes.** Gene expression profile in liver biopsy specimens from chronic hepatitis C patients with mild (no fibrosis) and severe (cirrhosis) liver disease represented in (figure 3-3) Group-1 shows genes reduced in expression or “down-regulated” in association with HCV infection compared to control liver; group-2 shows genes with increased expression or “up-regulated” in HCV infection (mild and severe disease) compared to control liver; group-3 shows genes highly up-regulated in association with severe disease only compared to control liver; group-4 shows genes highly down-regulated in mild but not in severe disease patient livers.

Assessment of a subset of the genes expressed (Group 2 and Group 3, figure 3-3) in cells directly treated with IL-1 β confirmed that a range of proinflammatory cytokines, chemokines, and signaling factors could be directly induced by IL-1 β (figure 3-3). Thus, IL-1 β and IL-1 β -responsive proinflammatory cytokine expression is associated with HCV infection and liver disease severity.

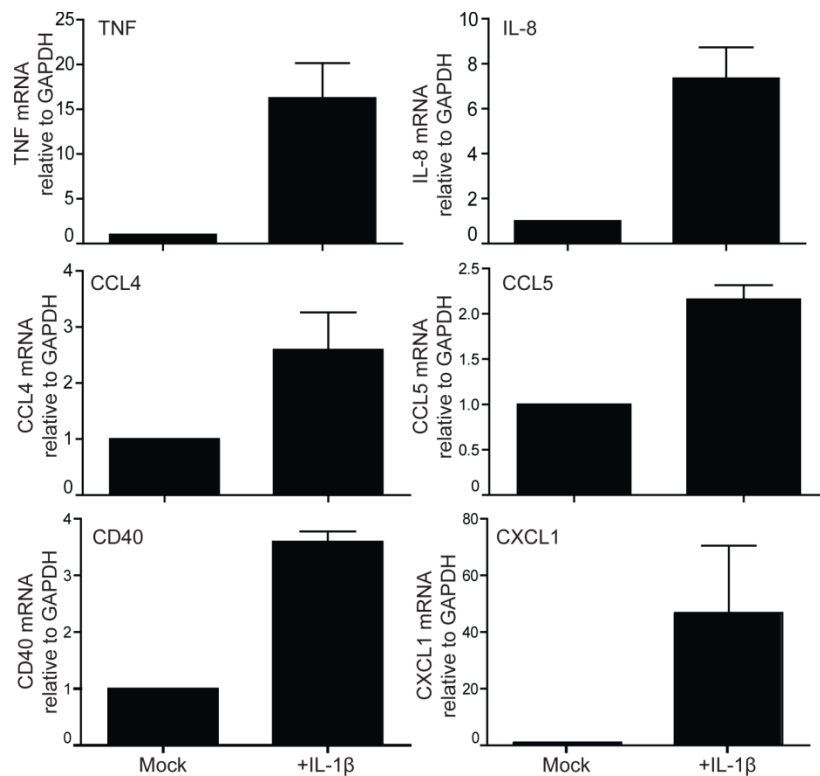


Figure 3-4 Validation of chemokine and cytokine gene expression induced by IL-1 β treatment of THP-1 cells. Differentiated THP-1 cells were treated with 100 ng/ml of recombinant IL-1 β for 6 or 24 hr. RNA was extracted and subjected to qRT-PCR analysis of gene expression.

To test whether circulating IL-1 β protein could be detected during chronic HCV infection, we evaluated serum levels of IL-1 β from both chronic hepatitis C patients and healthy controls. IL-1 β levels were significantly higher in individuals with chronic hepatitis C (figure 3-

5) and revealed a possible linkage of increasing IL-1 β with liver fibrosis, suggesting involvement of IL-1 β with HCV disease.

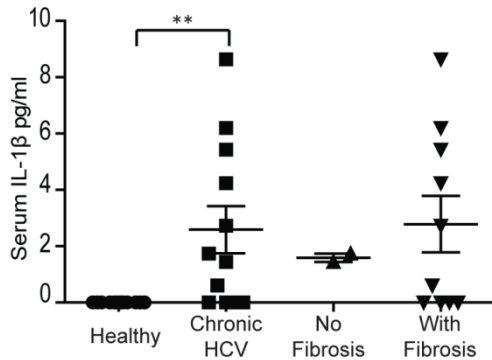


Figure 3-5 **IL-1 β levels in sera of healthy controls or hepatitis C patients.** Sera from chronic hepatitis C patients and healthy controls were obtained. Chronic hepatitis C patients were then stratified based on patients with fibrosis and no fibrosis. **** $P=0.0062$ by student t -test.**

Lastly, to identify the cellular source of hepatic IL-1 β during chronic HCV infection, we conducted confocal microscopy analyses of stained liver sections from normal donor livers or livers from patients with chronic hepatitis C infection. Liver sections were co-stained with anti-IL-1 β and anti-CD68, a surface marker present on macrophages such as the liver-resident myeloid Kupffer cells. We found that while CD68-negative cell populations, including hepatocytes, contained little or no IL-1 β , liver macrophages from chronic hepatitis C patients expressed IL-1 β , and CD68-positive cells were present at increased frequency compared with healthy controls (figure 3-6). Thus, IL-1 β associates with chronic HCV infection, and our observations identify Kupffer cells and/or infiltrating liver macrophages but not hepatocytes as a primary hepatic source of IL-1 β .

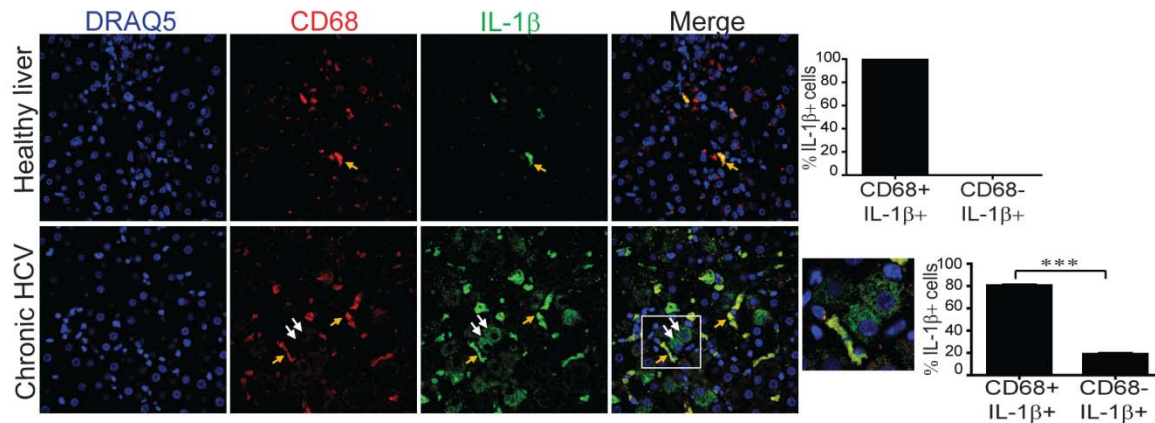


Figure 3-6 **Immunohistochemical staining and confocal microscopy analysis of healthy liver and chronic hepatitis C patient liver samples.** CD68 marks macrophages (Kupffer cells or infiltrating macrophages) (red), IL-1 β (green), and DRAQ5 (blue) stains the nuclei. A quantification plot of CD68+IL-1 β + cells and CD68-IL-1 β - of the total IL-1 β + cells is depicted from chronically infected (three patients,) and normal healthy liver samples. The area within the white box of the far right merged panel is enlarged and shown with cell frequency counts at right. *** $p < 0.0001$ by student's t-test. Arrows (white) indicate hepatocytes adjacent to CD68+/IL-1 β + Kupffer cells (yellow arrows).

HCV stimulates IL-1 β production in primary macrophages

Next, to assess the contribution of hepatic macrophages to the production of IL-1 β from HCV exposure, we first modeled HCV exposure of macrophages in culture using primary human blood monocyte-derived macrophages. Primary macrophages expressed IL-1 β mRNA and secreted IL-1 β upon exposure to HCV *ex vivo* (figure 3-7A). Neither Kupffer cells nor macrophages in general are known to be productively infected with HCV (177), although in the liver they are constantly exposed to the virus through liver sinusoidal blood flow; this suggested that exposure to HCV or its product might trigger production of IL-1 β by liver macrophages. To test this possibility, we collected mononuclear cells from saline wash-out of normal donor liver and treated the cells with conditioned cell culture media (mock) or media containing UV-inactivated HCV. Cells were then stained with specific antibodies to detect intracellular IL-1 β and monocyte/macrophage cell surface markers (CD14 and CD68). We found that all CD68+

cells recovered from donor liver also expressed CD14, thus defining them as hepatic macrophages (data not shown). Flow cytometric analysis of these cells demonstrated that HCV exposure induced IL-1 β production (figure 3-7B). Since the UV-inactivated HCV does not replicate (figure 3-7C and D), these observations suggest that hepatic macrophages can produce IL-1 β after HCV exposure in a manner independent of direct cell infection.

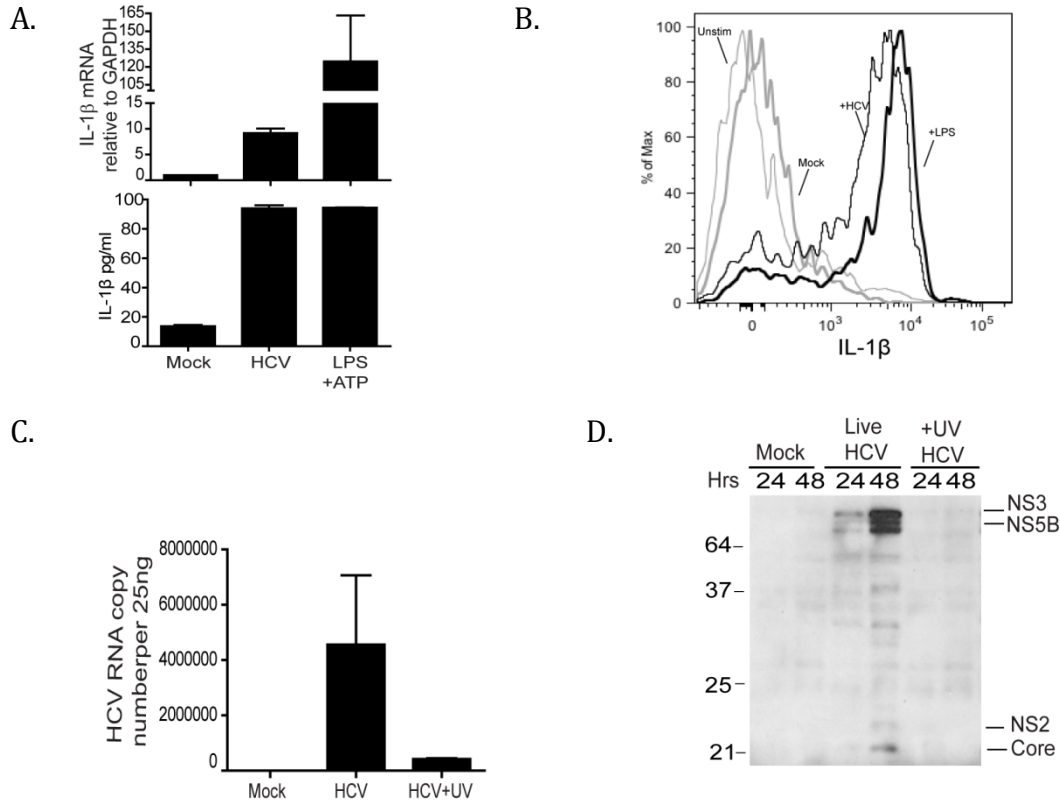


Figure 3-7 HCV-induced IL-1 β production in primary human macrophages. (A) IL-1 β mRNA expression (upper panel) and secreted IL-1 β protein levels (lower panel) from primary monocyte-derived macrophages of healthy human donor blood. Cells were mock-treated or treated with infectious HCV supernatant (moi=0.01 based on Huh7 focus forming units (ffu) or treated with 1 μ g/ml of LPS and ATP 5 mM (LPS/ATP; positive control). (B) Intracellular cytokine staining of treated CD14⁺ cells recovered from saline washout of healthy donor liver. Cells were left untreated (unstim) or were cultured with conditioned media (mock, negative control), LPS (positive control) or treated with UV-inactivated HCV (moi=0.01 based on Huh7 focus forming units (ffu)). Data are shown from one donor and are representative two experiments each of cells collected from two independent donors. In the analysis shown the frequency of IL-1 β -expressing cells was: unstim, 2.7%; mock, 6.4%; LPS, 76.5%; UV-HCV, 67.6%. Huh7.5 cells were infected with either viable or UV-inactivated HCV at moi =0.1. 48 hr later, the cells were harvested, and RNA and protein were extracted for qRT-PCR analysis (C) and viral protein abundance by immunoblot assay (D) antiserum from an HCV patient. Positions of viral proteins are indicated.

HCV-virion specific triggering of IL-1 β

To determine how HCV exposure could drive IL-1 β production from macrophages, using an *in vitro* model of macrophage exposure to HCV based on differentiated human THP-1 cells, we found that as in primary macrophages, exposure of THP-1 cells to HCV stimulated the expression of IL-1 β mRNA that peaked 3hr after virus exposure (figure 3-8A) leading to secretion of mature IL-1 β (figure 3-8B). THP-1 cell exposure to HCV also triggered a rapid, transient activation and cleavage of caspase-1, which coincided with the production (figure 3-8C) and secretion (figure 3-8D) of mature IL-1 β .

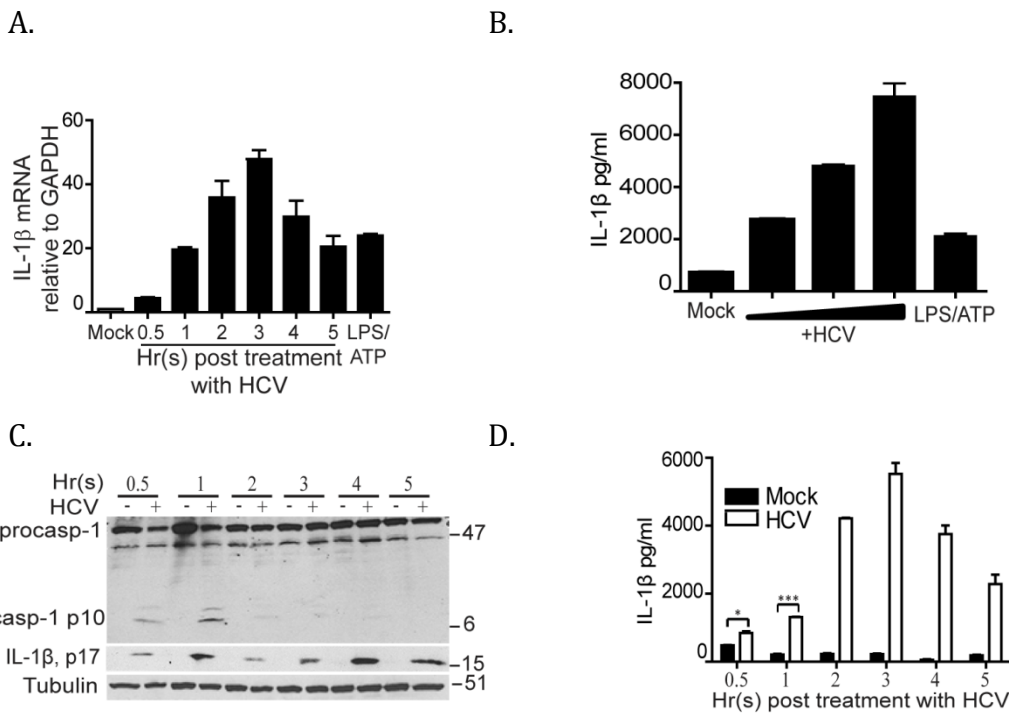


Figure 3-8 Analysis of inflammasome signal one and signal two activation in THP1 cells in response to HCV. (C) IL-1 β mRNA expression post exposure to HCV. (D) IL-1 β protein secretion after treatment with variable doses of HCV (moi= 0.001, 0.01 or 0.1 Huh7 ffu) or LPS/ATP at 1 μ g/ml for 24hr. (E) Immunoblot showing the kinetics of caspase-1 activation after HCV exposure. (F) Levels of secreted IL-1 β over a time course after HCV exposure (moi=0.01 based on Huh7 ffu).

To address the possibility that contaminants from the HCV preparation might trigger IL-1 β production and secretion, we purified HCV virions through a sucrose cushion prior to cell treatment. Both HCV-containing media and the resulting purified virions were infectious in Huh7 cells (figure 3-9A). We found that purified HCV virions stimulated IL-1 β secretion in a manner comparable to IL-1 β induction triggered by treatment of cells with HCV-containing supernatants (figure 3-9B), thus demonstrating the HCV virion-specific activity of IL-1 β production in macrophages.

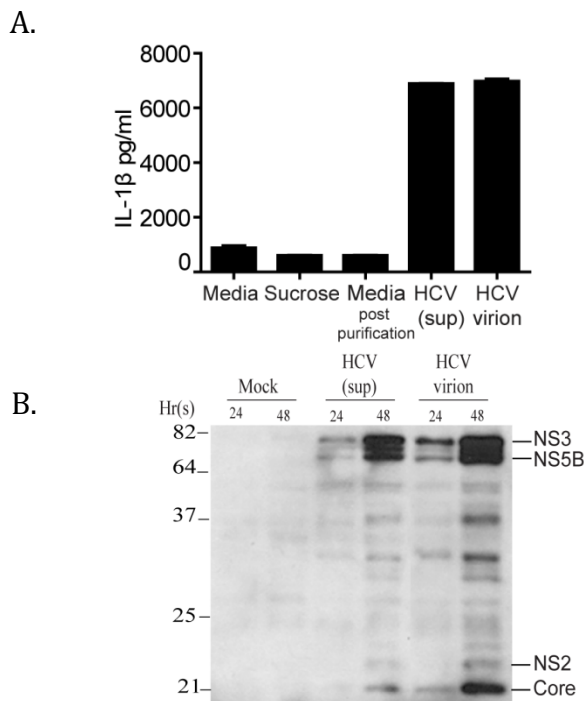
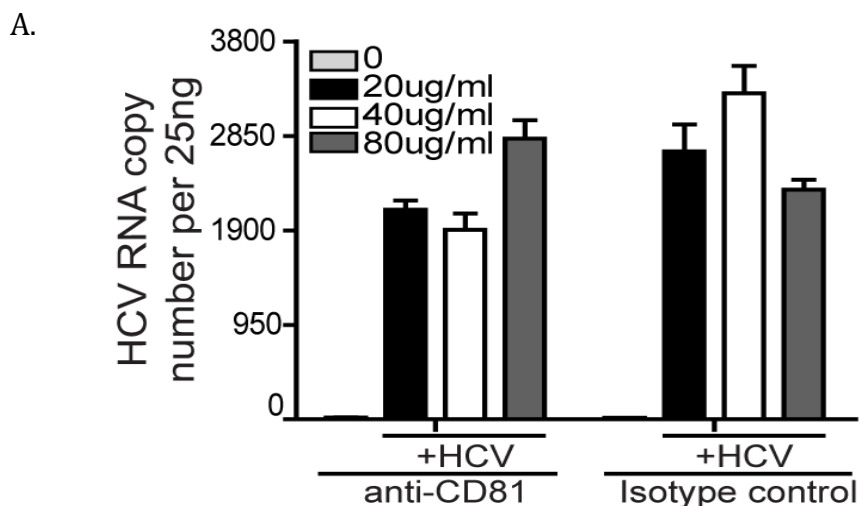


Figure 3-9 HCV-virion specific IL-1 β production. (A) IL-1 β levels secreted in THP-1 cells 24 hr after exposure to (left to right) cell culture media, sucrose solution, sucrose-purified culture media, infectious HCV supernatant or sucrose-purified HCV virions. (B) Immunoblot showing viral proteins in HCV-infected Huh7.5 cells. Cells were infected with HCV-containing supernatant (HCV sup) or purified HCV virion from sucrose gradient ultracentrifugation of infectious supernatant. Cells were infected with equivalent 0.1 focus forming units (ffu) of either infectious HCV sup or purified HCV virions for 1hr. 48 hr later, the cells were harvested and extracts were subjected to immunoblot analysis using HCV patient antiserum. Positions of viral proteins are indicated.

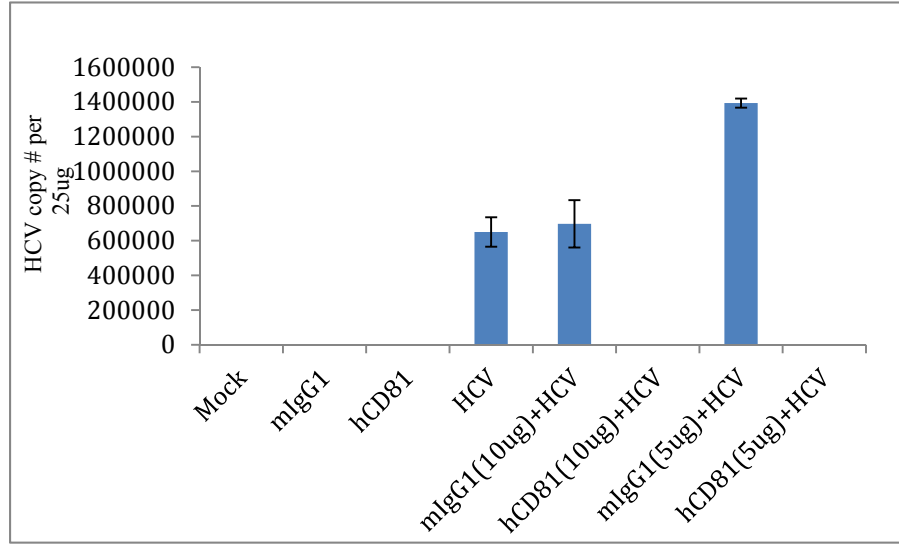
HCV-induced IL-1 β production requires phagocytosis and endosomal acidification

As myeloid cells do not support HCV replication (177), we investigated how HCV virions might trigger IL-1 β expression. HCV binds and enters hepatocytes through engagement of the cell surface CD81, followed by association with additional co-receptor molecules (178) (see chapter 1). However, we found that following virus exposure, HCV RNA was present in

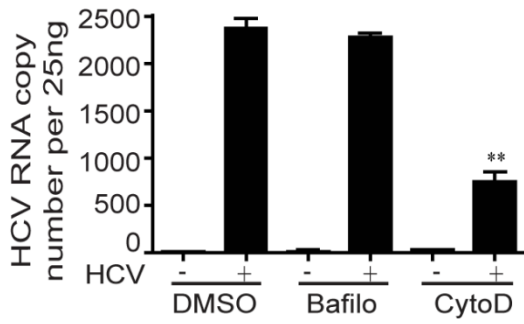
THP1 cells, entering through a process independent of the CD81 co-receptor, demonstrating that the virus is not entering THP-1 cells via an active entry mechanism (figure 3-10A). We have confirmed that the anti-CD81 dose used in THP-1 cells is high enough because inhibition of HCV entry is achieved in permissive Huh7 at a dose 8 fold lower (figure 3-10B). We therefore evaluated the role of phagocytosis in THP1 cell uptake of HCV by examining virus uptake after cell treatment with cytochalasin D (an inhibitor of phagocytosis) or the vacuolar type H⁺-ATPase inhibitor bafilomycin (which prevents endosome acidification). Exposure of THP-1 cells to HCV in the presence of cytochalasin D but not bafilomycin suppressed virus uptake as measured by intracellular accumulation of HCV RNA (figure 3-10C), while treatment of cells with either inhibitor caused a significant reduction in HCV-induced IL-1 β mRNA expression (figure 3-10D). We also found as in primary macrophages, that the IL-1 β -stimulatory activity of HCV in the THP-1 model did not require viral replication: UV-inactivated virus induced robust IL-1 β production from treated THP-1 cells as effectively as infectious HCV (figure 3-10E). Thus, both infection-independent phagocytic-mediated uptake of HCV virions as well as endosomal acidification are involved in IL-1 β stimulation in THP-1 cells and probably in macrophages.



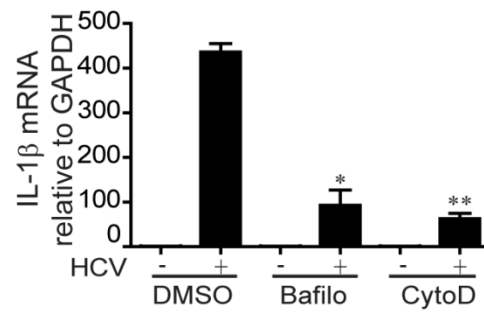
B.



C.



D.



E.

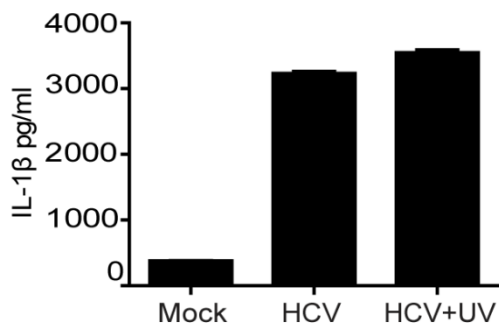


Figure 3-10 Phagocytosis is critical for HCV-induced IL-1 β production in macrophages. (A) HCV RNA in THP-1 cells. Differentiated THP-1 cells were pre-treated either with anti-CD81 or isotype control for 1 hour at the indicated concentrations. Cells were then washed and incubated with HCV (moi=0.01 based on Huh7 ffu) in the presence or absence of anti-CD81 or isotype control antibody at the same concentration. After 3 hours, cell lysates were harvested, and RNA was extracted and subjected to qRT-PCR analysis for determination of HCV RNA copy number as defined by qRT-PCR of standard HCV RNA template control. (B) similar to (A) Huh7 cells were pretreated either with isotype control (IgG) or anti-CD81 at 5ug and 10ug each for 1hr. Then HCV infection was performed in the presence of either CD81 or IgG. 48hr post infection HCV RNA was quantified by qRT-PCR.(C) Differentiated THP-1 cells were pretreated with bafilomycin (2.5 μ M) or cytochalasin D (10 μ M) for 1hr and then exposed to HCV in the presence or absence of continued drug treatment. Cell lysates were harvested 3hrs later, and RNA was extracted and subjected to qRT-PCR analysis for determination of HCV RNA copy number as defined by qRT-PCR of standard HCV RNA template control. (D) IL-1 β mRNA expression in pre-treated with DMSO (control), bafilomycin (2.5uM) or cytochalasin D (10 μ M) cells and exposed to media or infectious HCV supernatant (moi=0.01 Huh7 ffu). (E) Levels of secreted IL-1 β 24hr post treatment with conditioned media alone (mock) or treatment with live infectious HCV (HCV, moi=0.01) or UV-inactivated HCV (HCV-UV). *P-values*, *P=, **P=, and ***P= by student t-test.

HCV induces IL-1 β in macrophages by signaling through MyD88/TLR7

To determine how HCV triggers IL-1 β production in macrophages, we assessed the requirements for known PRRs in the activation of IL-1 β expression. We first examined the requirement for MAVS or myeloid differentiation primary response gene 88 (MyD88)-dependent innate immune signaling in IL-1 β expression. Whereas stable knockdown of MyD88 (figure 3-11A, B and C) abrogated HCV-induced IL-1 β mRNA induction and proIL-1 β production, knockdown of MAVS (figure 3-11A, B and D) had no effect on IL-1 β mRNA expression. In contrast to a role for MyD88 in activation of signal one of inflammasome activation and IL-1 β production, neither MyD88 nor MAVS were required for signal two that drives caspase-1 activation for processing IL-1 β into its active, secreted form (figure 3-11E).

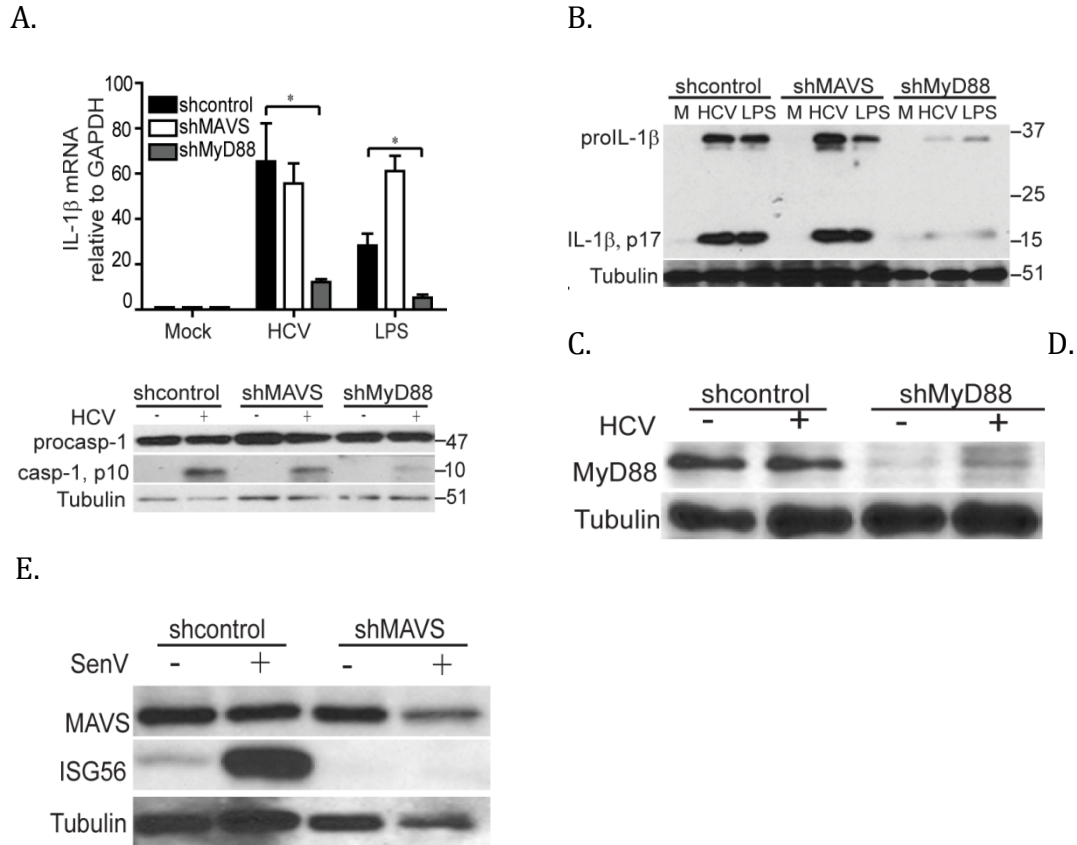


Figure 3-11 HCV requires signaling through MyD88 to stimulate IL-1 β induction. (A) THP1 cells stably expressing non-targeting shRNA (control) or shRNA specific to MAVS or MyD88 were mock treated with media alone, exposed to HCV (moi=0.01) or treated with LPS (1 μ g/ml) and IL-1 β expression assessed by qRT-PCR, $*P=0.0341$ and $*P=0.013$. (B) Immunoblot analysis of IL-1 β in THP-1 stably expressing the indicated shRNA. Cells were exposed to media alone (M) or infectious HCV supernatant for 6 hr. (C) Immunoblot of caspase-1 in THP-1 stably expressing the indicated shRNA. Immunoblot assay of MyD88 or MAVS abundance in THP1 cells expressing non-targeting control shRNA or shRNA specific to MyD88 (C) or MAVS (D). (E) Cells were infected with Sendai virus (SenV) for 24 hr prior to harvest. ISG56 and tubulin expression were respectively monitored as innate immune response gene and protein loading controls. This immunoblot confirms the functional knockdown of MAVS as ISG56 is a MAVS-dependent gene in this context and its production was completely abolished in shMAVS-expressing cells.

HCV RNA engages TLR7 to drive IL-1 β expression

Next, we sought to identify the HCV component(s) that trigger IL-1 β expression.

Previous studies have shown that macrophage exposure to viral products can trigger IL-1 β

production (179) and that HCV RNA itself is a potent PAMP that triggers host innate immune response programs (39, 41). We therefore evaluated the ability of the HCV RNA to trigger IL-1 β expression in THP1 cells. We found that transfected but not extracellular HCV RNA is sufficient to stimulate both IFN- β and IL-1 β mRNA expression (figure 3-12). By comparison, transfected synthetic double-stranded RNA, polyinosinic-polycytidylic acid (polyIC), also triggers strong IFN- β mRNA expression. However, despite comparable levels of IFN induced by both polyIC and HCV, HCV RNA was a much greater stimulant of IL-1 β , thus demonstrating that HCV RNA is a highly potent and specific agonist of IL-1 β mRNA induction.

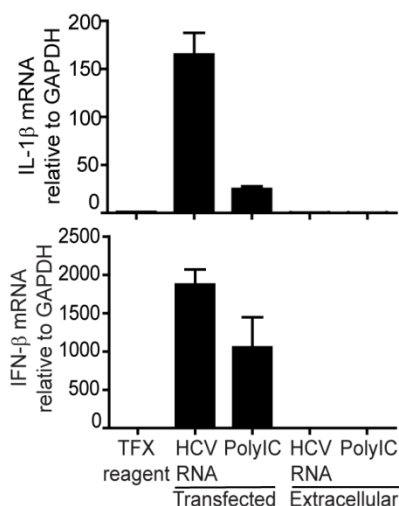


Figure 3-12 **HCV RNA alone stimulates IL-1 β mRNA induction.** IL-1 β (upper panel) and IFN- β (lower panel) mRNA expression in THP-1 post-treatment with transfection reagent alone (control) or transfected with full length HCV RNA or poly IC (transfected); or treated with media containing full length HCV RNA or poly IC (extracellular).

To define the HCV viral-RNA moiety and host PRR that drives IL-1 β mRNA induction, we stimulated cells with either the HCV RNA polyU/UC region (PAMP) or the non-stimulatory X-region of the viral RNA. The HCV RNA polyU/UC region is a uridine-rich motif within the 3' non-translated region of HCV genome that exhibits potent activation of type I IFN and has thus been defined as a viral PAMP for RIG-I activation (41, 180). In contrast, the HCV RNA X-region is located in the 3' non-translated region of the viral genome and lacks the ability to stimulate type I IFN (41). We found that the polyU/UC but not the X-region RNA motif was

sufficient to trigger IL-1 β mRNA expression when introduced into THP1 cells (figure 3-13A). Remarkably, IL-1 β and IFN- β mRNA were induced through MyD88 or MAVS-dependent signaling, respectively, in response to HCV RNA in macrophages (figure 3-13B), with both exhibiting dose dependence of polyU/UC motif concentration (figure 3-13C, and see figure 3-13A). Thus, the polyU/UC PAMP motif within the HCV RNA is a non-self ligand of PRR signaling through which MyD88-dependent signals impart IL-1 β mRNA expression while RIG-I/MAVS signaling drives IFN- β expression.

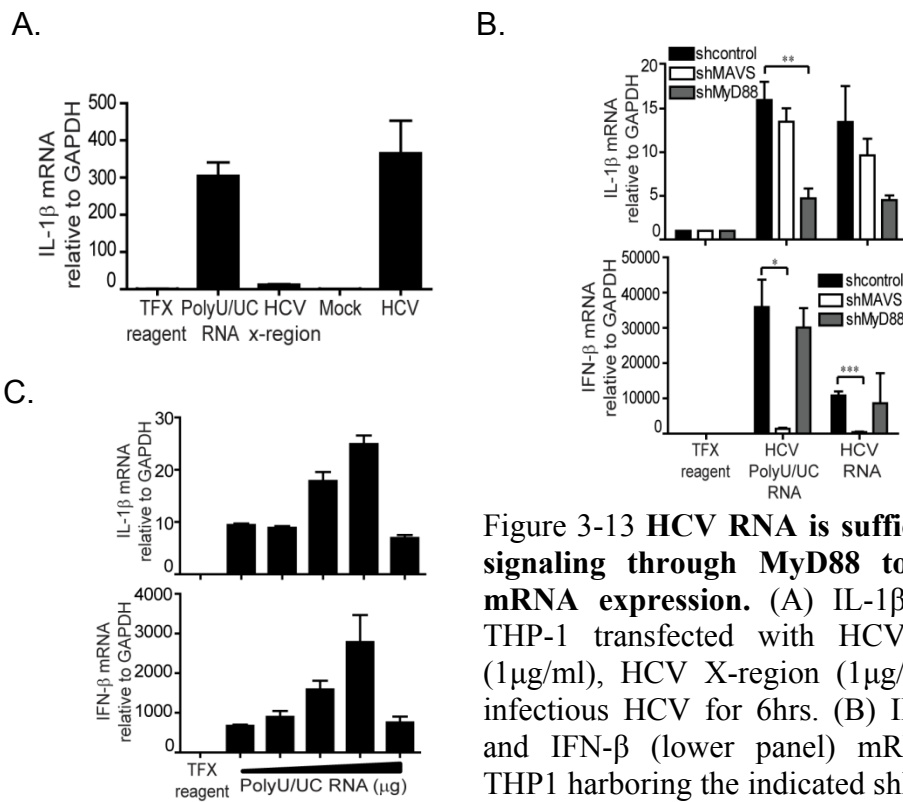


Figure 3-13 HCV RNA is sufficient and requires signaling through MyD88 to stimulate IL-1 β mRNA expression. (A) IL-1 β mRNA levels in THP-1 transfected with HCV polyU/UC RNA (1 μ g/ml), HCV X-region (1 μ g/ml) or exposed to infectious HCV for 6hrs. (B) IL-1 β (upper panel) and IFN- β (lower panel) mRNA expression in THP1 harboring the indicated shRNA, * $P=0.0115$, ** $P=0.0094$ and *** $P=0.0009$. (C) IL-1 β (upper panel) and IFN- β (lower panel) mRNA expression in THP-1 cells transfected with increasing doses (0.125, 0.25, 0.5, 1 and 2 μ g/ml) of HCV polyU/UC RNA.

We also assessed how MyD88 induction of IL-1 β is propagated in the context of HCV stimulation. Notably, TLR7 resides within endosomes and can recognize uridine-rich, single-stranded RNA as a PAMP to signal through MyD88 (181, 182). Moreover, TLR7 is abundantly

expressed in tissue macrophages and differentiated THP1 cells (20). As bafilomycin treatment abrogates IL-1 β expression induced by HCV (see figure 3-10C), signaling from endosomal TLRs is likely important for inflammasome triggering. We therefore examined the requirement for TLR7 in HCV RNA-induced IL-1 β mRNA expression. Knockdown of TLR7 expression significantly reduced signaling of IL-1 β mRNA expression triggered by cell exposure to HCV (figure 3-14A and B) or induced by HCV RNA (figure 3-14C). Thus, upon macrophage exposure to HCV “signal one” of inflammasome activation is mediated by TLR7 recognition of HCV RNA within endosomes to induce IL-1 β mRNA expression through MyD88-dependent signaling upon macrophage exposure to HCV.

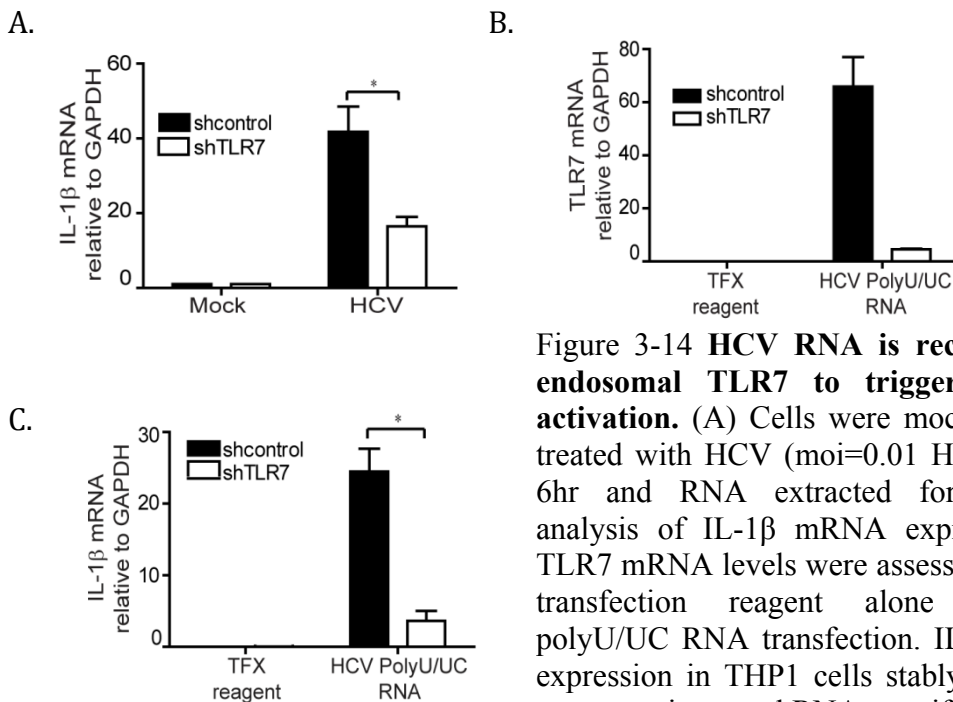


Figure 3-14 HCV RNA is recognized by endosomal TLR7 to trigger signalone activation. (A) Cells were mock-treated or treated with HCV (moi=0.01 Huh7 ffu) for 6hr and RNA extracted for qRT-PCR analysis of IL-1 β mRNA expression. (B) TLR7 mRNA levels were assessed 6 hr after transfection reagent alone or HCV polyU/UC RNA transfection. IL-1 β mRNA expression in THP1 cells stably expressing non-targeting or shRNA specific to TLR7, * $P=0.022$, by student’s t-test.

Discussion

These findings reveal that IL-1 β production and hepatic inflammation in HCV infection are linked and driven through virus-induced TLR7 and NLRP3 inflammasome signaling in liver

macrophages. These observations support a model of hepatic inflammation induced by phagocytic uptake of HCV by resident macrophages/Kupffer cells to trigger IL-1 β and drive expression of proinflammatory cytokines, with IL-1 β expression associating with liver disease in patients with chronic hepatitis C infection. Importantly, macrophages are not a tropic cell for productive HCV infection. Our studies demonstrate that IL-1 β is stimulated by HCV within hepatic macrophages in a manner that is independent of actual infection but mediated by phagocytic uptake of virus. As HCV circulates at high levels with monocytes in the bloodstream of patients with chronic hepatitis C, it is noteworthy that the phagocytic uptake of HCV was essential for induction of inflammasome activity in the macrophage model, and that undifferentiated monocytes do not phagocytose HCV. The requirement of phagocytic uptake of HCV for inflammasome stimulation may provide an important checkpoint against systemic IL-1 β production by blood monocytes under conditions of HCV viremia, thus limiting inflammatory signaling to differentiated tissue macrophages within the local hepatic environment. Indeed, phagocytic uptake of HCV and non-self-recognition of viral RNA is sufficient to trigger IL-1 β production from hepatic macrophages. Although RIG-I-deficient hepatoma cells have been shown to produce low levels of IL-1 β during HCV infection (183), we found that HCV infection of immune-competent hepatoma cells and primary hepatocytes does not trigger appreciable production of IL-1 β . These observations reveal an important regulatory feature of IL-1 β production from different cell sources, as hepatocytes comprise the liver parenchyma and are constantly exposed to blood-borne microbes like HCV where a wide release of IL-1 β could induce tissue toxicity. Rather, macrophages, including Kupffer cells, serve a specialized role to sample the local environment via phagocytosis and selectively render inflammatory signaling

upon pathogen identification, such as TLR7/MyD88-dependent signaling upon recognition of internalized HCV RNA.

These studies also reveal that in macrophages, HCV RNA is engaged by both RIG-I/MAVS-dependent and TLR7/MyD88-dependent PRR pathways to induce innate immune (IFN- β) and inflammatory (IL-1 β) signaling, respectively. In this respect, the intracellular compartmentalization of HCV upon macrophage uptake could expose virion components such as HCV RNA to endosomal TLR7 for PRR engagement (184). These observations also reflect a key difference from hepatocytes in which HCV RNA replication products of infection accumulate at endoplasmic reticulum membrane-associated cytosolic replication sites independent of endosomal TLRs but accessible by RIG-I (185), thus amenable to triggering IFN- β defenses but not IL-1 β production. Our RNA transfection experiments of THP-1 cells deliver HCV RNA into both endosomal and cytoplasmic compartments for signaling and reveal the dual potential of the viral RNA to be sensed by both RIG-I and TLR7 within a compartment-dependent context within macrophages. Thus, HCV products impart intracellular signaling and metabolic changes that drive IL-1 β production from macrophages.

Chapter Four

Hepatitis C virus triggers NLRP3 inflammasome activation

Introduction

A variety of viruses have been shown to activate the inflammasome pathway (172). To date, three virus-induced inflammasomes: the NLRP3, RIG-I and AIM2 inflammasomes (see table 1-1). In particular, the NLRP3 inflammasome is the most studied of all the virus-induced inflammasomes, and diverse RNA viruses such as influenza virus and the *flaviviruses* (West Nile virus and Japanese encephalitis virus) triggers its activation. NLRP3 is a PYRIN containing protein requiring the key inflammasome adaptor protein ASC to associate with procaspase-1 and mediate mature IL-1 β production (96).

In macrophages, activation of the NLRP3 inflammasome requires two signals, a priming step to induce not only IL-1 β mRNA induction but also to upregulate NLRP3 protein expression (186). A second signal, which accumulates during infection or pathogen encounter, is required to directly drive NLRP3 inflammasome activation. Although a direct NLRP3 binding to an activating factor has never been shown, it is thought that integration of diverse cellular signals induced by infection triggers NLRP3 inflammasome activation. These positive regulators of NLRP3 include potassium efflux, pore forming in cell membrane, calcium signaling, lysosome rupture, mitochondrial damage, increase production of reactive oxygen species and PKR signaling (187-193). After infection clearance and/or induction of T cell responses, inflammation must subside to prevent tissue injury. The NLRP3 inflammasome is negatively regulated by many cellular signals such as type-I interferon (194). Type-I interferon can down modulate the expression of IL-1 β through IL-10 (194). Furthermore, polyIC-induced IFN-I suppresses NLRP3

inflammasome activation by alum and *C.albicans* thus increasing susceptibility to fungal infection (195). Furthermore, T cells derived IFN- γ exerts an inhibitory effect on macrophages to dampen IL-1 expression in macrophages and monocytes (196). Also the TRIM family member TRIM20 (197), pyrin-only proteins (POPs) regulators of inflammasome (198) autophagy (199), and nitric oxide (200) are reported to negatively modulate NLRP3 activity. In addition, microRNAs such as miR-223 targets NLRP3 3'-UTR and prevents (201) NLRP3 protein accumulation. Virus derived factors such as orf3 and measles V protein (202, 203) inhibit NLRP3 activity.

NLRP3 activity is tightly regulated and its aberrant activation has been associated with inflammatory diseases. Indeed, mutation within the NACHT domain of the NLRP3 protein are associated with three inflammatory disorders including Muckle-Wells syndrome (MWS), familial cold autoinflammatory syndrome (FACS) and neonatal-onset multisystem inflammatory disease (NOMID), all collectively termed cryopyrin-associated periodic syndromes (197, 204). Given that NLRP3 is induced during virus infection, associated with inflammatory disease and the fact that HCV stimulates IL-1 β production in macrophages, we investigated whether the NLRP3 inflammasome mediates HCV-induced IL-1 β maturation.

Results

NLRP3 inflammasome mediates HCV-induced IL-1 β maturation

To define the inflammasome responsible for IL-1 β processing triggered by HCV, we examined the requirement for specific NLR signaling in IL-1 β secretion. In particular, NLRP3, as mentioned above, drives proIL-1 β processing during virus infections. We first assessed IL-1 β production in THP-1 cells stably expressing non-targeting shRNA or shRNA targeting caspase-1 or NLRP3. IL-1 β mRNA expression was efficiently induced by HCV regardless whether

caspase-1 or NLRP3 were knocked down (figure 4-1A, B and C). However, processing of proIL-1 β to its active form in response to virus was completely abolished upon loss of caspase-1 or NLRP3 (figure 4-1D) implicating NLRP3 and caspase-1 in mediating HCV-induced IL-1 β maturation. Consistent with this observation, knocking down the expression ASC, the essential NLRP3 signaling-adaptor protein, similarly abrogated the processing of IL-1 β (figure 4-1D and E). Thus, HCV may induce the maturation of proIL-1 β in macrophages through activation of the NLRP3 inflammasome.

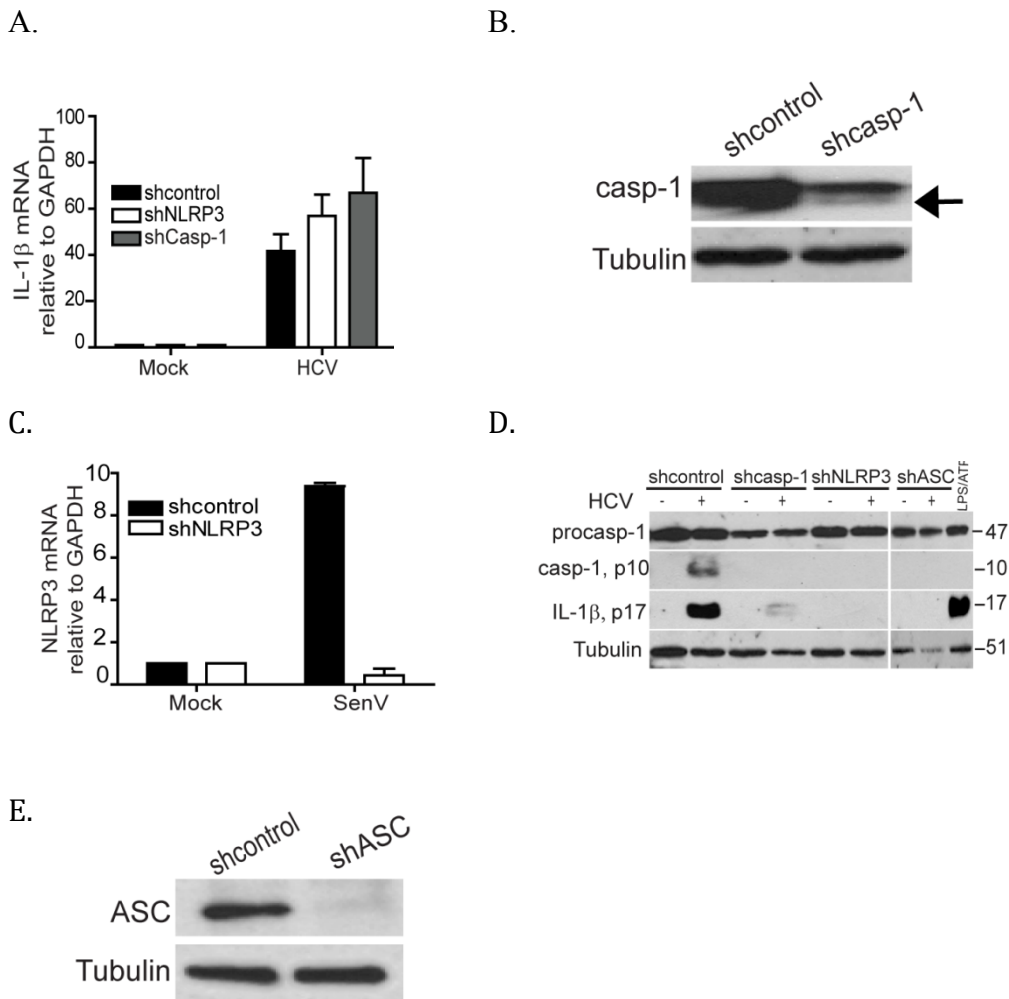


Figure 4-1 **HCV triggers NLRP3 inflammasome activation for mature IL- β production.** (A) IL- β mRNA expression in THP1 stably expressing non-targeting control shRNA or shRNA specific to NLRP3 or caspase-1. Immunoblot showing the levels of caspase-1 (B) and NLRP3 (C) in cells expressing specific knockdown shRNA in THP-1 cells transduced with lentiviral particles as compared to non-targeting control. (C) Immunoblot of caspase-1 and IL-1 β in THP1 stably expressing non-targeting control the indicated shRNA. (C) Immunoblot showing the levels of ASC in cells expressing specific knockdown shRNA in THP-1 cells.

HCV RNA is not sufficient to drive mature IL-1 β production

Comparison of proIL-1 β processing in cells stimulated with HCV RNA or HCV itself demonstrated that secretion of IL-1 β requires events triggered upon uptake of intact virus (figure 4-2) indicating that HCV must also induce the “signal two” that mediates full activation of the inflammasome leading to IL-1 β secretion. Macrophages exposed to HCV particle secreted mature IL-1 β . However, macrophages transfected with full length HCV (HCV RNA) or transfected with just the polyU/UC RNA was not able to produce mature. HCV RNA transfected macrophages are able to drive IL-1 β maturation only when a signal two agonist such as ATP was provided. These results suggest that HCV particle provide the necessary signals to trigger both signal one and signal two activation that drive mature IL-1 β production; whereas HCV RNA alone is not sufficient to stimulate signal two activation.

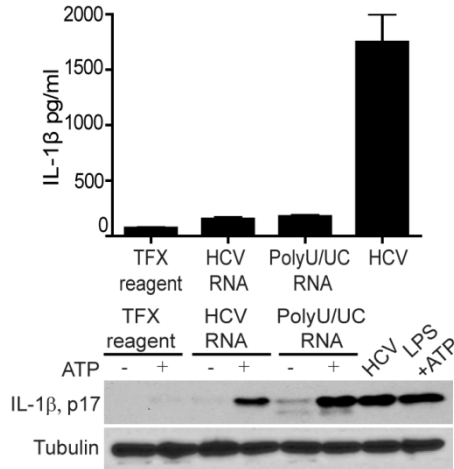


Figure 4-2 **HCV RNA is not sufficient to induce mature IL- β production.** Secreted IL- β protein levels (upper panel) and immunoblot analysis of IL-1 β (lower panel set) of THP-1 treated with transfection reagent or transfected with either with full length HCV RNA or polyU/UC RNA or exposed to HCV (moi=0.01).

Potassium efflux is required for HCV-induced mature IL-1 β production

HCV products have been shown to induce reactive oxygen species (ROS), while products of virus infection have been shown to drive sodium and potassium channel activities to mediate intracellular ion flux and pH change, each of which are implicated as cellular metabolic changes that induce the assembly of the NLRP3 inflammasome. To define the virus-induced metabolic changes that might impart NLRP3 inflammasome activation upon cellular uptake of HCV, we pretreated THP-1 cells with diphenyleneiodonium (DPI, ROS inhibitor) or glybenclamide (Glyben, inhibitor of potassium channels) and assessed processing of IL-1 β . While ROS inhibition only affected IL-1 β processing induced by HCV at high doses (figure 4-3A), IL-1 β maturation was blocked in a dose-dependent manner by glyben treatment of cells (figure 4-3B), implicating HCV-induced potassium efflux as a trigger of the NLRP3 inflammasome. While ROS inhibitor impacted mature IL-1 β production only at high doses, cell viability was greatly affected at the doses where inhibition observed. Thus the role of ROS in HCV-induced iNLRP3

activation is unclear. Glyben treatment of THP-1 cells, on the other hand, did not affect cell viability at the doses tested. Consistent with this notion, IL-1 β maturation was blocked when cells were cultured in isotonic media containing 100 mM KCL to prevent intracellular potassium depletion while culturing in isotonic NaCl media did not alter HCV-induced IL-1 β maturation (figure 4-3C). These results reveal a conserved process of virus-induced inflammatory signaling in which HCV induction of potassium efflux leads to NLRP3 inflammasome activation.

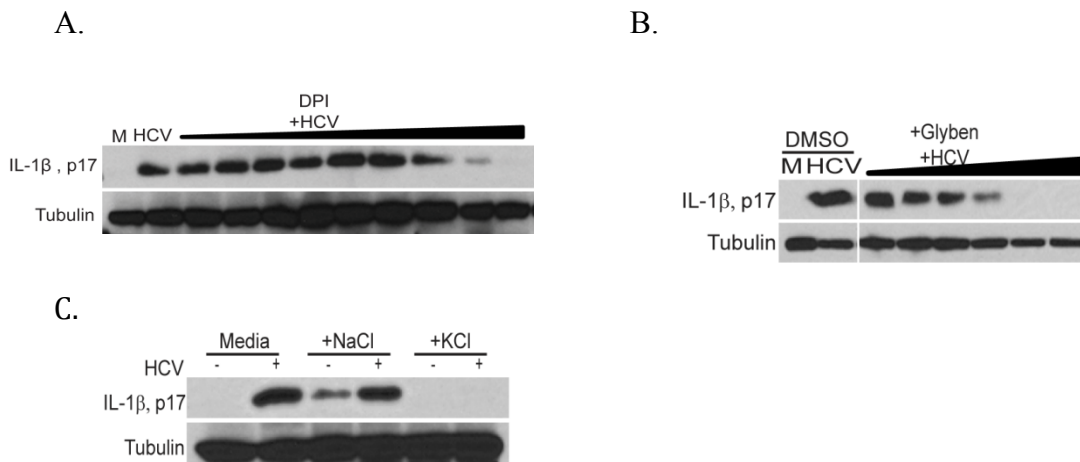


Figure 4-3 Potassium efflux is critical for HCV-induced NLRP3 activation. (A) Differentiated THP-1 cells were, from left to right, mock treated, treated with HCV (moi=0.01 based on Huh7 ffu) or pretreated with 0.3,0.6, 1.25, 2.5, 5, 10, 20, 40, 80 μ M Diphenyleneiodonium chloride (DPI) for 1hr followed by HCV treatment. 3 hr later cells were harvested and extracts subjected to immunoblot analysis for mature IL-1 β and tubulin. (B) THP-1 were pre-treated with DMSO (control) or with 6.25, 12.5, 25, 50, 100, 200 μ M of potassium channel inhibitor glybenclamide (Glyben) for 2hrs followed by mock treatment (M; control) or HCV (moi=0.01) exposure in the presence of glyben for an additional 1 hr. (C) IL-1 β p17 abundance in THP-1 cultured in normal media or in media containing NaCl (100 mM) or KCl (100 mM) for 1hr followed by mock-treatment (-) or exposure to HCV (moi=0.01) in the same media for an additional 1hr.

To ascertain how initial NLRP3 inflammasome signaling in macrophages propagates into a hepatic inflammatory response, we conducted a direct comparative RNA-seq analysis of gene expression datasets from chronic hepatitis C patients to the RNA-seq transcriptome of THP1

cells during an acute time course after exposure to HCV. The comparison identified a bioset of commonly induced genes in the two most highly represented pathways, cytokine-cytokine receptor signaling, and chemokine signaling, of which a subset of genes associated with severe liver disease in HCV patients, including IL-1 β and IL-1 β -responsive proinflammatory products (figure 4-4A, B and table 4-1). These observations imply that in patients with chronic hepatitis C, Kupffer cells and/or infiltrating liver macrophages produce IL-1 β , driving a hepatic response that includes the expression of a wide range of proinflammatory mediators of liver inflammation, fibrogenesis and disease.

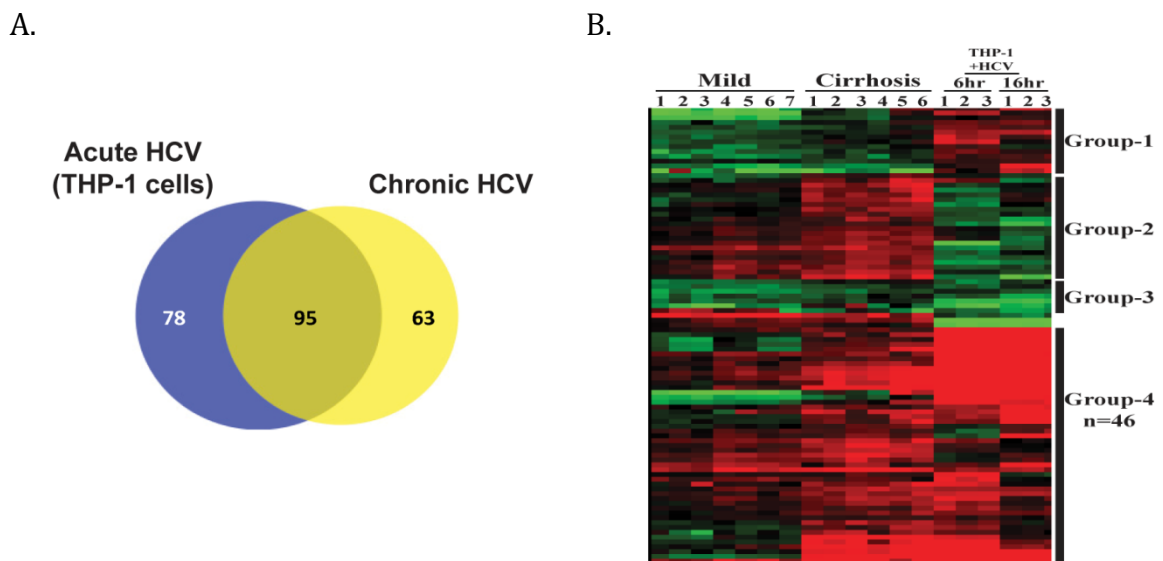


Figure 4-4 Comparison of gene expression profile of THP-1 cells and chronic hepatitis C patients liver. (A and B) RNA-seq analysis to directly compare the transcription profile of THP1 cells after acute HCV (moi=0.01) exposure for 6 and 16 hours and chronic hepatitis C patient liver staged by mild or severe disease. (A) The Venn diagram shows the number of differentially expressed genes (>1.5-fold change and FDR ,0.05) that were unique and common to THP-1 cells and chronic hepatitis C patient liver in the most highly represented KEGG pathways in both datasets, the cytokine-cytokine receptor signaling, and chemokine signaling pathways. (B) Hierarchical clustering analysis of differentially expressed genes common to both HCV-exposed THP-1 cells and chronic hepatitis C liver. Group-4 genes were expressed in both THP-1 cells and chronic hepatitis C liver (for full description, see Table 4-1). Group-4 genes and the expression key are shown at the right. See Methods for a description of bioinformatics analysis.

Group-1	Group-2	Group-3	Group-4
TNFRSF12A	HGF	INFR1	CCL22
TGFB3	BMP2	LTBR	IL1 β
OSMR	TGFB2	CXR1	CXCL2
BCAR1	CCL28	CSF3R	CXCL3
NFKBIB	PDGFRB	CXCR2	CXCL1
IL1R1	TNFRSF21	TNFSF14	CD40
IL18R1	TGFB1	ADCY1	TNFRSF9
IL10	KIT		TNFRSF18
TNFRSF10A	GRK5		CCL4
CXCL16	RASGRP2		CCL3
TNFRSF10D	FLT3		CCL4L2
MAP3K1	IL21R		CCL3L1
IL1R2	PIK3CD		IL7R
CXCL13	PRKX		CCL17
	ADCY7		CCL24
	IL11RA		OSM
	IL12RB1		CXCL5
	DOCK2		RELT
	PLCB2		TNFSF4
	CCR5		NCF1
	TNFRSF25		CXCL12
	CCL21		ITK
			CCR4
			IL2RG
			CXCR3
			CXCL14
			CCL5
			CD70
			TSLP
			PDGFB
			IL10RA
			IL3RA
			TNFRSF4
			TNFSF8
			TIAM2
			CSF2RA
			TNFSF15
			VEGFC
			TNFRSF11B
			INHBB
			CXCR4
			IL6
			LIF
			CXCL6
			IL8
			CCL19

Table 4-1 **List of gene expression.** Differentially expressed genes in chronic hepatitis C liver specimens with mild (no fibrosis) and severe (cirrhosis) disease or in THP-1 cells exposed to HCV represented in (Figure 4-1B). Group-1 shows genes up-regulated in HCV-exposed THP-1 cells only, as compared to mock-treated THP1 control cells; group-2 shows genes expressed in association with severe liver disease only but not in THP-1 cells; group-3 shows genes down-regulated in both hepatitis C liver and HCV-treated THP-1 cells; group-4 shows genes commonly expressed in both hepatitis C liver and HCV-treated THP-1 cells. Gene expression, as measured by RNA-seq analysis, in hepatitis C liver specimens was compared with control liver specimens. Gene expression in HCV-treated THP1 cells was compared with mock-treated THP1 cells.

Inflammasome component reconstituted HEK293 system

HCV RNA delivers a priming signal to induce IL-1 β mRNA expression, but alone fails to stimulate NLRP3 activation to trigger mature IL-1 β production (see figure 4-3A). This suggests that other viral factors might be involved in stimulating cellular signal such as potassium efflux (figure 4-3C). One possibility is that viral proteins synergize with viral RNA to trigger IL-1 β mRNA expression. The other possibility is that while viral RNA induces IL-1 β expression, viral proteins directly stimulate inflammasome activation. Our THP-1 cells culture-model express all the essential NLRP3 inflammasome components. To test viral protein role in THP-1 cells via transfection has been technically difficult. Therefore, we established an *in vitro* inflammasome reconstitution system in HEK293 cells. HEK293 cells do not expression caspase-1, IL-1 β , NLRP3 and ASC (PYCARD). When HEK293 cells are reconstituted with inflammasome components offers a useful tool to study the mechanism and biochemistry of inflammasome complex assembly and activation. To this end, flag-tagged constructs of each NLRP3 inflammasome component were made. Upon coexpression of these components, at high levels, the components associate and mature caspase-1 and IL-1 β (figure 4-5) can be detected.

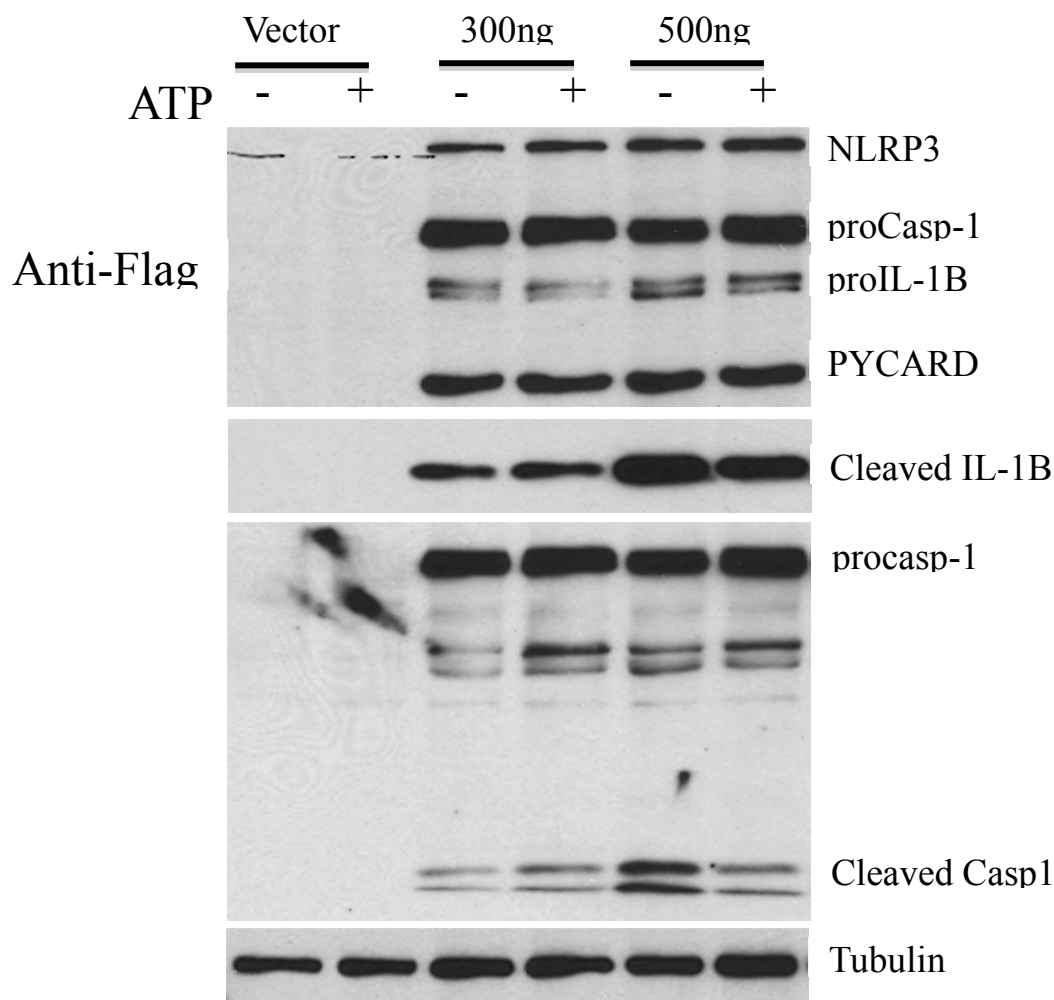


Figure 4-5 **NLRP3 inflammasome component reconstituted HEK293 system.** HEK293 cells were transfected with flag-tagged constructs expressing human flag-procaspase-1, flag-proIL-1 β , flag-ASC and flag-NLRP3 (NLRP3 components). These cells were seeded one day before transfection. All components are transfected simultaneously and then 24hr post transfection cells were collected. Cell lysates were prepared for immunoblot analysis. NLRP3 inflammasome components were detected with anti-flag antibody. Cleaved mature IL-1 β was detected with anti-IL-1 β specific antibody. Also procaspase-1 and cleaved forms were detected with caspase-1 specific antibody.

HCV-core protein affects mature IL-1 β production

To evaluate the contribution to stimulate mature IL-1 β in HEK293 system, we first cloned each of the viral protein to generate flag-tagged constructs. The goal was to cotransfect

the viral proteins with inflammasome components to assess IL-1 β cleavage. This system will allow evaluation if a viral protein can act individually or in concert to drive inflammasome activation. Immunoblot analyses (figure 4-6) demonstrated that the flag-tagged HCV protein expressing constructs were being expressed.

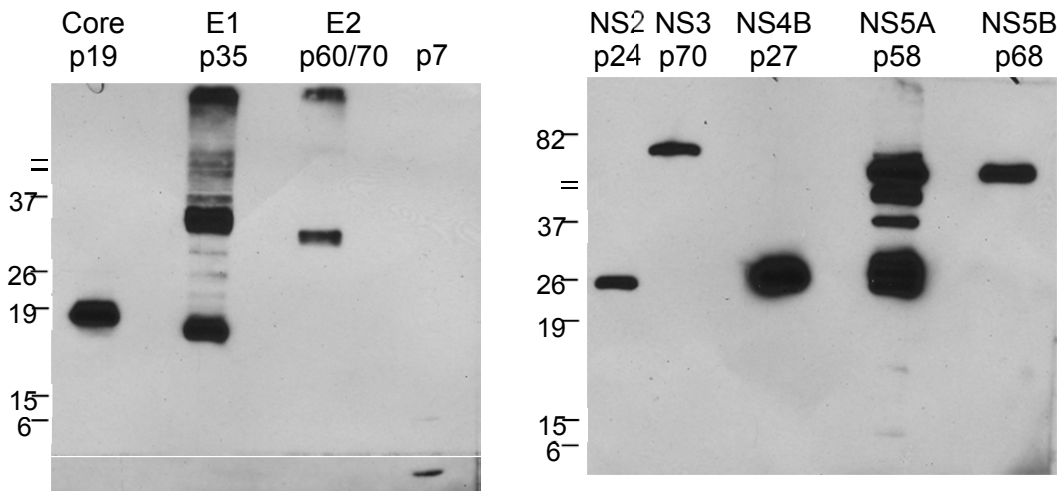


Figure 4-6 **Confirmation of Flag-tagged constructs expression.** HEK293T cells were transfected with each protein. Each viral protein is cloned into pEF-tak vector (RRR). All are flag-tagged. 24hr post transfection cell were collected. Cell lysates were prepared for immunoblot analysis. Shown is probing for Flag with anti-flag antibody to detect HCV structural and nonstructural proteins.

Within the incoming virion particle core (capsid), E1 and E2 are present. We hypothesized these proteins might be the viral components that macrophages encounter leading to the induction of cellular signaling pathways that drive NLRP3 activation. Indeed, co-transfection of HCV-core with inflammasome components drives mature IL-1 β production (figure 4-7) and this cleavage of IL-1 β depended on HCV-core protein dose.

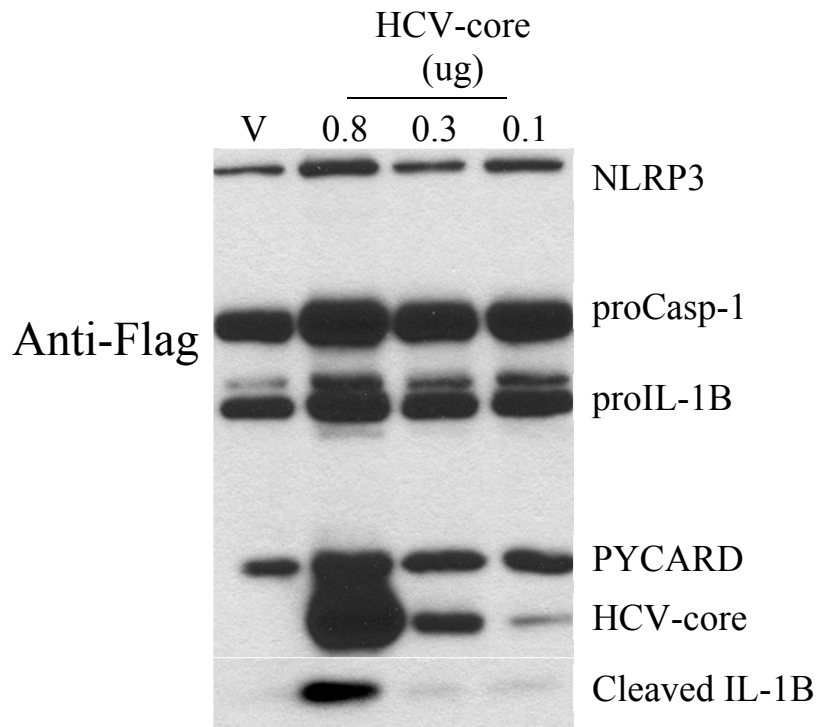


Figure 4-7 HCV-core protein affects mature IL-1 β production. HEK293 were co-transfected with NLRP3 inflammasome (procaspase-1, proIL-1 β , NLRP3 and ASC) and HCV core protein or vector control plasmids. All transfected constructs are flag-tagged. Decreasing levels of HCV core protein was transfected (0.8ug, 0.3ug and 0.1ug). Expression of NLRP3 inflammasome components was detected with anti-flag antibody. Cleaved IL-1 β was detected with IL-1 β specific antibody.

The HEK293 system exhibits some limitations: first, the expression levels of inflammasome components when macrophages are primed with inflammasome activating stimulus might not be as high and/or similar as the expression levels of ectopically expressed constructs. This in itself poses a problem since very high expression of each inflammasome component can drive the components to associate and generate a constitutively activated system. This problem can be overcome by fine-tuning the stoichiometric ratio of each component to achieve an inducible system. Indeed, as illustrated in figure 4-7, adjusting the stoichiometric ratio can produce an inducible system. Second, HEK293 might not express natural inhibitors of inflammasome since they do not have intact inflammasome pathway. Therefore, both the

positive and negative regulators of inflammasome pathway might be missing. Although the HEK293 system is not a perfect system and display some caveat, it serves as a screening tool to identify potential HCV protein inflammasome inducers whereby the finding obtained will be tested and validated in macrophages (both primary macrophages and THP-1 cells). I have mentioned above that transfecting THP-1 cells is technically challenging. This low efficiency of transfection in THP-1 cells can be circumvented using high throughput transfection techniques such as nucleofection. It has been shown that high transfection efficiency and good viability of THP-1 is achieved through nucleofection (205). Therefore, a potential viral protein will be nucleofected into primed THP-1 cells (to stimulate signal one) to assess “signal two” activation. Alternatively, THP-1 cells can be primed to activate “signal one”. Then to assess viral protein contribution, recombinant viral protein can be added to evaluate mature IL-1 β production. The other most effective way that can be used to overcome the low transfection efficiency of both THP-1 and primary macrophages is by generating lentiviral transduction particle for each HCV protein. Since THP-1 cells are efficiently transduced, the generated lentiviral particles will be transduced into primed THP-1 cells to evaluate mature IL-1 β production.

Discussion

The results presented here reveal that the NLRP3 inflammasome mediates the production of HCV-induced IL-1 β . HCV triggering of NLRP3 inflammasome required ASC and caspase-1. In addition, induction of potassium efflux is essential to stimulate IL-1 β production in HCV exposed macrophages. Further characterization revealed that viral RNA alone is not sufficient to drive mature IL-1 β /caspase-1 production suggesting that other viral factors such as viral proteins might contribute to inflammasome induction. To assess the requirement for viral protein in triggering inflammasome signal-two activation, we established HEK293 based *in vitro*

inflammasome reconstitution system. In this system, our initial characterization identifies that HCV core protein affects mature IL-1 β production in a dose dependent manner. HCV proteins, in particular HCV core, have been shown to stimulate ROS accumulation and regulate ion efflux. Therefore, HCV core might be the candidate viral protein that regulates inflammasome activation through modulation of inflammasome activating cellular potassium efflux. Moreover, the HCV p7 protein is a transmembrane cation channel and a member of the viroporin family whose actions can drive ion flux that could impart NLRP3 inflammasome activation during HCV infection(206). Thus, while HCV RNA triggers inflammasome “signal one” via TLR7, the transient exposure to p7, core and other HCV proteins may provide stimulus for “signal two”, including potassium flux that induces NLRP3 activation for IL-1 β maturation and secretion.

NLRP3 inflammasome activation and production of IL-1 β during infection by West Nile virus or Japanese encephalitis virus is essential for proper immune induction and virus control [11,12], NLRP3 activation of IL-1 β production by HCV associates markedly with immunopathogenesis from hepatic inflammation. This relationship is further evident by our RNA-seq analysis, which revealed that IL-1 β and IL-1 β -driven genes are associated with severe liver disease. Innate immune evasion supports chronic HCV infection and viremia that lends to macrophage uptake of HCV and signaling by TLR7 and the NLRP3 inflammasome. The linkage of these processes of innate immune evasion/persistent viral replication and viremia/ IL-1 β production and response thus mediates a cycle of chronic inflammatory stimulation that underlies liver disease in HCV infection.

Chapter Five

Final discussion

Summary

My studies presented here define with multiple lines of evidence the molecular mechanisms of hepatic inflammation induced by HCV infection. The current study reveals the induction of host NLRP3 inflammasome signaling pathway by HCV to stimulate hepatic inflammation. My studies identified that HCV infection of immune-competent hepatoma cells and primary hepatocytes does not trigger appreciable production of IL-1 β . Instead intrahepatic macrophages, which are not a tropic cell for productive HCV infection, produce IL-1 β in response to HCV in a manner that is independent of actual infection but mediated by phagocytic uptake of virus. Upon HCV uptake by macrophages, in endosomes the internalized virion genome is exposed to endosomal PRRs. This is evident by the *in vitro* characterization, which revealed that blockade of endosomal PRRs via bafilomycin treatment abrogates HCV-induced IL-1 β expression. Furthermore, we found *in vitro* HCV RNA is engaged by both RIG-I/MAVS-dependent and TLR7/MyD88-dependent PRR pathways to induce innate immune (IFN- β) and inflammatory (IL-1 β) signaling, respectively. Triggering of TLR7/MyD88 pathway stimulates IL-1 β mRNA expression and proIL-1 β protein production. HCV products are known to induce intracellular reactive oxygen species and ion flux, both of which trigger the NLRP3 inflammasome during virus infection, whereas potassium efflux is essential for inflammasome signaling by HCV. Based on these studies, the current working model depicted in (figure 5-1) demonstrates that the HCV-macrophage interface is key in understanding HCV-induced hepatic inflammation and disease.

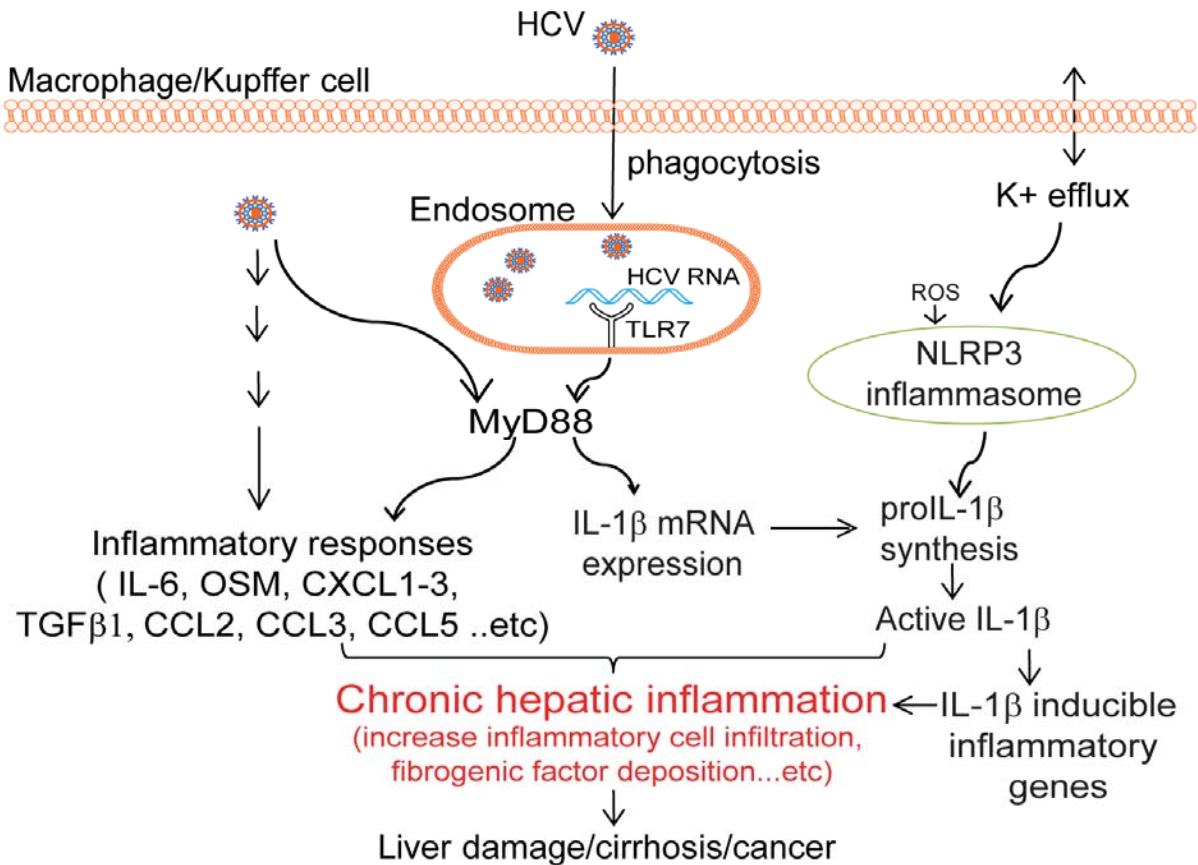


Figure 5-1 **Pathway depicting the process of HCV-induced IL-1 β by intrahepatic macrophages.** HCV sensing by macrophages leads to virus uptake via phagocytosis and into endosomal compartment. In endosomes, the viral genome (RNA engages TLR7 to drive IL-1 β mRNA expression through the adaptor protein MyD88. HCV-induced potassium efflux, on the other hand, triggers NLRP3 inflammasome activation and mature IL-1 β production. HCV sensing by macrophages is not limited to IL-1 β production and IL-1 β -induced genes, but also to other inflammatory genes such as IL-6, OSM, TGF- β ...etc, which together contribute to persistent liver inflammation.

The global transcriptome analyses by RNA-seq coupled with the *in vitro* characterization revealed an important correlation between inflammatory responses and severe liver disease.

HCV within infected livers induces the expression of genes known to play important role in inflammation such as IL-6, OSM and chemokines such as CCL1, CCL3. These inflammatory

responses triggered by HCV synergize with the induction and production of NLRP3-dependent IL-1 β , which drives the expression of other inflammatory genes, to augment the inflammatory state within the liver leading to persistent hepatic inflammation, which is characterized by increased cell recruitment, collagen deposition and persistent liver damage.

Future directions

Mechanism of NLRP3 activation by HCV

My studies have identified that HCV RNA triggers IL-1 β mRNA expression (signal-one activation). However, the viral factor that drives mature IL-1 β production (NLRP3 inflammasome activation) is remains to be identified. Induction of potassium efflux was important, but how this cellular process is triggered or what drives potassium efflux upon macrophage exposure to HCV needs to be elucidated. In the HEK293 *in vitro* inflammasome reconstitution system, I have identified that core could be a potential viral protein impacting mature IL-1 β production (see figure 4-3). While the contribution of other viral proteins needs to be determined, if core is the only viral protein modulating mature IL-1 β production, the future studies will focus on elucidating of how HCV core drives NLRP3 inflammasome activation in macrophages (both primary and THP-1 cells). First, we will determine if core modulates important cellular signaling pathways for NLRP3 activation, which include modulation of calcium signaling, potassium efflux and reactive oxygen species. The induction of these cellular signaling pathways will be determined through inhibitors specific for each pathway. Second, I will determine if HCV core affects NLRP3 oligomerization and complex formation. Third, I will determine if core drives NLRP3 activation through direct or indirect interaction with NLRP3 by co-immunoprecipitation. While a direct NLRP3 binding ligand has never been reported, the studies proposed here might offer novel finding in NLRP3 biology. I will determine if viral

protein-NLRP3 interaction is mediated indirectly by a cellular factor. Upon core transfection factors recruited to the NLRP3 inflammasome complex and NLRP3 interacting proteins can be identified by mass-spectrometry analysis. Lastly, if interaction is identified between core and NLRP3, the interaction motif within HCV core and NLRP3/or will be determined. These studies will provide more detailed insights of HCV regulation of inflammasome signaling pathway and provides novel findings in NLRP3 biology.

Decipher the exact role of IL-1 β in HCV infected liver

HCV-induced IL-1 β produced by intrahepatic macrophages might impose diverse actions within HCV infected liver. The potential IL-1 β role during HCV infection is illustrated in the diagram below.

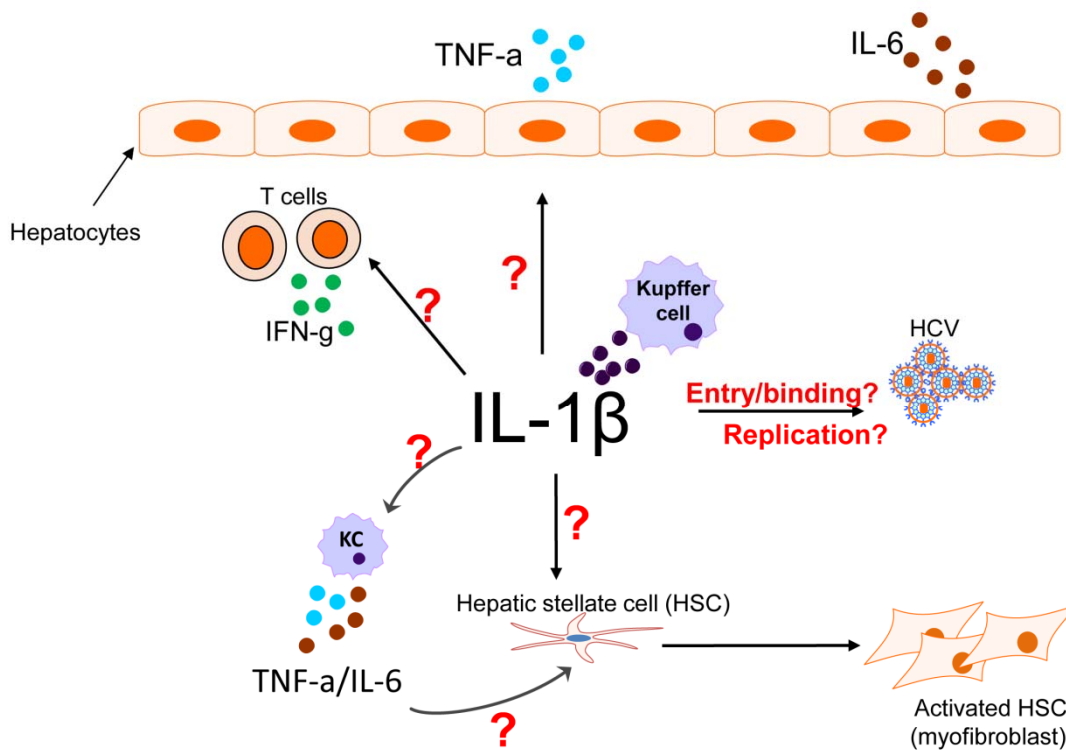


Figure 5-2 Diagram depicting potential activities of hepatic IL-1 β within HCV infected livers. IL-1 β can directly act on HCV to impact HCV entry, replication, translation and virion release. IL-1 β can induce factors that promote HCV replication and survival and propagation within the hepatic environment. It can also trigger the expression of other inflammatory genes from hepatic cells such as hepatocytes, KCs and liver infiltrating myeloid cells (neutrophils and monocytes). IL-1 β can modulate HSCs activity to acquire a myofibroblast-like phenotype leading to liver damage and fibrosis.

As it is depicted in (Figure 5-2), hepatic IL-1 β can have multiple activities within the HCV infected liver. One possibility, IL-1 β may act directly on HCV to impact its entry, replication, translation and egress from hepatocytes. Indeed, inflammatory cytokines such as TNF- α have been shown to modulate the expression of tight junction protein (207). Most importantly, IL-1 β treatment is reported to disrupt tight junction and down modulate the expression of tight junction proteins such as occludin thus IL-1 β may impact virus entry in hepatocytes. To this end, I will determine how treatment with IL-1 β impacts viral co-receptors expression both with mock and HCV infected cells. Hepatocytes have a unique structure when polarized and their infectivity with HCV *in vitro* has shown to decrease as they become more polarized. Therefore, IL-1 β in the liver may impact receptor expression and/or hepatocytes polarization status rendering the cells more permissible for HCV infection. Moreover, I will determine if IL-1 β impacts viral replication, translation and virion assembly. Both HCV infected and uninfected cells will be treated with recombinant IL-1 β and/or supernatant from HCV exposed macrophages then viral RNA replication and protein accumulation will be evaluated. The other possibility, IL-1 β may modulate inflammatory cytokines and chemokines production from hepatic cells.

As my studies revealed macrophage exposure to HCV triggers the induction of many inflammatory gene expression and IL-1 β -induced genes. In a similar way, IL-1 β may induce

inflammatory TNF- α and IL-6 from hepatocytes and hepatic macrophages. IL-1 β may also synergize with the local hepatic cytokine milieu to drive the differentiation of inflammatory Th17 cells. Simultaneously, IL-1 β can act with TGF- β and IL-10 to impose other immunomodulatory regulations. The last possibility, IL-1 β might influence liver fibrogenesis. Liver fibrosis is characterized by accumulation of extracellular matrix (ECM) following liver injury. The best-studied fibrogenic cells are hepatic stellate cells (HSCs), which orchestrate the deposition of ECM in normal and fibrotic livers when they acquire a myofibroblast phenotype. HSCs are activated in response to various stimuli and inflammatory signals produced by resident-Kupffer cells, which triggers HSCs activation into myofibroblast-like phenotype (175, 208). Thus, IL-1 β and IL-1 β -induced inflammatory cytokines may influence the activation of HSCs.

Implication and therapeutic applications

My studies presented here provide important advancement in our understanding of the mechanism of hepatic inflammation in HCV pathogenesis. My studies have identified IL-1 β as an important factor associated with severe liver disease. IL-1 β a key proinflammatory cytokine may serve an important role in inducing inflammatory gene expression, recruiting inflammatory cells, and exerting immunomodulatory functions within the liver leading to chronic hepatic inflammation that establishes an amenable platform for the onset of liver fibrosis and cirrhosis. My findings could also be applicable to other inflammatory liver diseases such as non-alcoholic fatty liver (NAFLD) and alcohol-induced liver disease (ALD) (209) and may offer some insights into the mechanism of hepatic inflammation associated with such diseases. NAFLD is a common form of liver disease that develops in individuals who do not intake alcohol at high quantities. It causes steatosis, steatohepatitis, fibrosis and cirrhosis. While steatosis is simple and non-

progressive, steatohapatitis is progressive leading to fibrosis and cirrhosis. ALD, on the other hand, is caused by high alcohol intake leading to liver damage due to accumulation of metabolites from alcohol degradation. Thus, similar inflammatory mechanism and inflammatory responses that are triggered during HCV-induced hepatic inflammation could be the underlying etiology in the pathogenicity of NAFLD and ALD.

The current therapy for hepatitis C patients is a triple therapy composed of pegylated interferon, ribavirin and the recently approved direct acting antiviral drugs (DAA), telaprevir or boceprevir. As I have discussed, response to therapy hinges on many factors including viral genotype, host genetic background, and basal ISG expression. Additionally, the cost for hepatitis C treatment is very expensive (210), which makes it not feasible to provide treatment at a broader scale to HCV infected individuals. Therefore, a more affordable means of treating HCV patients is needed. Although the new DAA drugs are being administered clinically and have shown good efficacy when combined with p-IFN and RVN, especially against hard to treat HCV genotype-1(211), there are concerns with these new direct acting drugs. First, in triple therapy both boceprevir and telaprevir show additional side effects. For instance, patients taking boceprevir have developed anemia, neutropenia and dysgeusia (altered taste sensation) whereas patients taking telaprevir are reported to develop anemia, rash, and anorectal discomfort(212, 213). Second, these new DAA drugs are hard to adhere to for patients because multiple doses must be administered per day(214). Third, DAA drugs pose the potential of developing antiviral resistance when used as monotherapy. Indeed, patients treated with telaprevir or boceprevir alone have developed drug resistant as early as 4 days after initiation of treatment (213, 215). Fourth, triple therapy shows restricted efficacy towards most HCV genotypes (reviewed in 213). Lastly, the potential of drug-drug interaction is another concern associated with using boceprevir and

telaprevir that may interfere with the efficacy of these drugs (216). For the reasons mentioned above, new second generation of DAA including inhibitors of HCV NS5A, polymerase NS5B and host factors targeting cyclophilin A and miR122 are underway that are thought to be more effective and have less of the concerns associated with the first generation of DAA drugs.

Prevention is the best way to fight the HCV epidemic globally, but currently there is no effective vaccine. The ideal effective vaccine is an immunogen that elicits a broad and effective B and T immunity that prevents T and B epitope escape mutations and overcome viral sequence diversity. However, the design of new vaccines has been hampered by the fact that HCV replication by the highly error-prone polymerase generates diverse sub-population or quasispecies. HCV glycoproteins E1 and E2 contain highly variable regions, the hyper variable region 1, that are constantly targeted by the host antibody responses (217, 218). Additionally, T cell-mediated response that targets the E proteins may also contribute to high variability within the E proteins. Furthermore, escape mutations in T cell epitope, already described during chronic HCV infection, are thought to limit the design of new vaccines (219). Besides viral sequence diversity, the lack of convenient experimental animal models to study HCV pathogenesis and to test vaccine candidates has hindered progress in the development of effective vaccines.

As I have alluded to earlier, the local ISG expression from hepatic myeloid cells can render a state of innate immune tolerance to attenuate the antiviral actions of IFN. Therefore, IFN treatment is only partially effective in suppressing HCV (220) and effective antiviral therapy may reduce liver inflammation. It has been revealed that the IFN response can antagonize inflammasome signaling (194) while IL-1 β can enhance the expression of specific ISGs to impart increased effectiveness of IFN actions (101). Thus, whereas effective antiviral therapy for HCV may actually reduce liver inflammation (221), this process could involve signaling

crosstalk between IFN and IL-1 β that overall enhances IFN actions. Currently, many anti-IL-1 drugs are being utilized in clinics to treat diverse inflammatory diseases. Anakinra, an IL-1RI antagonist, and riloncept, a soluble IL-1receptor that binds IL-1 β with higher affinity compared to IL-1 α , are approved therapies to treat rheumatoid arthritis (109, 222). In addition, canakinumab, neutralizing anti- IL-1 β IgG1 monoclonal antibody, is another approved drug to treat inflammation related diseases (223). Therefore, interventions that target IL-1 β or inflammasome components (224-226) could thus serve as therapeutic applications to mitigate HCV-induced hepatic inflammation and disease, particularly where antiviral agents have failed.

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