Recognition of Hepatitis C Virus RNA by Toll like Receptors 7 and 8: Implications for the Initiation of Innate Immune Response

Reconnaissance de l'ARN du virus de l'hépatite C par les récepteurs de type Toll 7 et 8 Implications dans l'initiation de la réponse immunitaire innée

By
Yuwei Zhang

Département de Microbiologie et immunologie
Faculté de Médecine

Thesis submitted to the Faculty of Medicine
to obtain the degree of Philosophiae Doctor (Ph.D.)
in Immunology

July 2014

Abstract

Hepatitis C virus (HCV), a positive single stranded RNA (ssRNA) virus that replicates in the liver, infects 200 million people worldwide, with approximately 80% of infected individuals ultimately suffering from chronic HCV infection. Antiviral therapies, including interferon and ribavirin, have improved considerably in recent years, but are effective in only about one-half of those treated, and are associated with significant side effects and toxicity.

Innate immune defenses are essential to control viral infection; the innate response is activated through recognition of viral macromolecular motifs known as pathogen-associated molecular patterns (PAMPs) that are recognized by a multitude of Pathogen recognition receptors (PRRs). Although immune activation induced by HCV RNA or proteins has been extensively studied, the detection of HCV by the innate immune system remains poorly understood. Despite activation of the immune response early after HCV infection *in vivo*, the persistent increase inpatient HCV viral load suggests the failure of the immune response to control HCV infection. A better understanding of the mechanisms of immune activation induced by HCV is crucial for development of effective strategies for HCV treatment.

Here we demonstrate in primary cell models that the HCV genome contained GU-rich RNA sequences that specifically trigger Toll-like receptors (TLR) 7/8, resulting in maturation of plasmacytoid dendritic cells (pDCs) and production of type I interferon (IFN), induction of inflammatory cytokines and chemokines in different antigen

presenting cells (APCs). Cytokines produced by monocytes and pDCs upon stimulation of HCV-derived ssRNA inhibited HCV production in an IFN-dependent manner, whereas cytokines produced by myeloid DCs (mDCs) and macrophages had no inhibitory effect on virus production, due to a defect in IFN induction by HCV ssRNA in these cells.

TLR7/8 stimulated cytokines also down-regulated the expression of the HCV receptor CD81 on Huh7.5 in an IFN-independent manner that may restrict HCV infection. However, although HCV receptors like CD81 were widely expressed on different subsets of lymphocytes, DCs and monocytes did not respond to HCV particles, and our data indicates that only macrophages sense HCV and produce inflammatory cytokines. The recognition of HCV by macrophages is related to the expression of DC-SIGN on macrophages, and results in TLR7/8 engagement. Similar to a TLR7/8 agonist, HCV stimulation induces inflammatory cytokine production (TNF-α, IL-8, IL-6 and IL-1b) by macrophages but does not stimulate interferon-beta production in macrophages, Congruously, TLR7/8 and HCV RNA mediated cytokine production in macrophages did not inhibit HCV replication.

Our results reveal that HCV RNA has the potential to trigger TLR7/8 in DC populations, and initiate an innate immune response against HCV infection that leads to an IFN-dependent suppression of viral replication. However, HCV is able to escape detection by monocytes and DCs, which produce type I IFN and suppress HCV replication when TLR7/8 is engaged. Macrophages possess the capacity to detect HCV, in part because of surface expression of DC-SIGN. In turn, macrophages produce inflammatory cytokines that nevertheless fail to inhibit HCV replication due to lack of IFN-beta production. Evasion of antiviral defense by HCV may explain the failure of innate immunity in

control of HCV infection. Moreover, inflammatory cytokine production by macrophages upon HCV stimulation *in vitro* suggests that activation of macrophages may contribute to inflammation in HCV infected individuals.

Key words: Hepatitis C virus, dendritic cells, interferon, single-strand RNA, TLR7, TLR8, CD81, DC-SIGN, macrophages

Résumé

Le virus de l'hépatite C (VHC) est un virus à ARN simple brin positif (ssARN) qui se replique dans le foie. Deux cents millions de personnes sont infectées par le virus dans le monde et environ 80% d'entre elles progresseront vers un stade chronique de l'infection. Les thérapies anti-virales actuelles comme l'interféron (IFN) ou la ribavirin sont de plus en plus utilisées mais ne sont efficaces que dans la moitié des individus traités et sont souvent accompagnées d'une toxicité ou d'effets secondaires indésirables.

Le système immunitaire inné est essentiel au contrôle des infections virales. Les réponses immunitaires innées sont activées suite à la reconnaissance par les Pathogen Recognition Receptors (PRRs), de motifs macromoléculaires dérivés du virus appelés Pathogen-Associated Molecular Patterns (PAMPs). Bien que l'activation du système immunitaire par l'ARN ou les protéines du VHC ait été largement étudiée, très peu de choses sont actuellement connues concernant la détection du virus par le système immunitaire inné. Et même si l'on peut très rapidement déceler des réponses immunes *in vivo* après infection par le VHC, l'augmentation progressive et continue de la charge virale met en évidence une incapacité du système immunitaire à contrôler l'infection virale. Une meilleure compréhension des mécanismes d'activation du système immunitaire par le VHC semble, par conséquent, essentielle au développement de stratégies antivirales plus efficaces.

Dans le présent travail nous montrons, dans un modèle de cellule primaire, que le génome ARN du VHC contient des séquences riches en GU capables de stimuler spécifiquement les récepteurs de type Toll (TLR) 7 et 8. Cette stimulation a pour conséquence la maturation des cellules dendritiques plasmacytoïdes (pDCs), le production d'interféron de type I (IFN) ainsi que l'induction de chémokines et cytokines inflammatoires par les différentes types de cellules présentatrices d'antigènes (APCs). Les cytokines produites après stimulation de monocytes ou de pDCs par ces séquences ssARN virales, inhibent la production du virus de façon dépendante de l'IFN. En revanche, les cytokines produites après stimulation de cellules dendritiques myéloïdes (mDCs) ou de macrophages par ces mêmes séquences n'ont pas d'effet inhibiteur sur la production virale car les séquences ssARN virales n'induisent pas la production d'IFN par ces cellules.

Les cytokines produites après stimulation des TLR 7/8 ont également pour effet de diminuer, de façon indépendante de l'IFN, l'expression du récepteur au VHC (CD81) sur la lignée cellulaire Huh7.5, ce qui pourrait avoir pour conséquence de restreindre l'infection par le VHC. Quoiqu'il en soit, même si les récepteurs au VHC comme le CD81 sont largement exprimés à la surface de différentes sous populations lymphocytaires, les DCs et les monocytes ne répondent pas aux VHC, Nos résultats indiquent que seuls les macrophages sont capables de reconnaître le VHC et de produire des cytokines inflammatoires en réponse à ce dernier. La reconnaissance du VHC par les macrophages est liée à l'expression membranaire de DC-SIGN et l'engagement des TLR 7/8 qui en résulte. Comme d'autres agonistes du TLR 7/8, le VHC stimule la production de cytokines inflammatoires (TNF-α, IL-8, IL-6 et IL-1b) mais n'induit pas la production d'interféron-beta par les macrophages. De manière attendue, la production de cytokines

par des macrophages stimulés par les ligands du TLR 7/8 ou les séquences ssARN virales n'inhibent pas la réplication virale.

Nos résultats mettent en évidence la capacité des séquences ssARN dérivées du VHC à stimuler les TLR 7/8 dans différentes populations de DC et à initier une réponse immunitaire innée qui aboutit à la suppression de la réplication virale de façon dépendante de l'IFN. Quoiqu'il en soit, le VHC est capable d'échapper à sa reconnaissance par les monocytes et les DCs qui ont le potentiel pour produire de l'IFN et inhiber la réplication virale après engagement des TLR 7/8. Les macrophages possèdent quant à eux la capacité de reconnaître le VHC grâce en partie à l'expression de DC-SIGN à leur surface, mais n'inhibent pas la réplication du virus car ils ne produisent pas d'IFN. L'échappement du VHC aux défenses antivirales pourrait ainsi expliquer l'échec du système immunitaire inné à contrôler l'infection par le VHC. De plus, la production de cytokines inflammatoires observée après stimulation *in vitro* des macrophages par le VHC suggère leur potentielle contribution dans l'inflammation que l'on retrouve chez les individus infectés par le VHC.

Mots clefs: virus de l'hépatite C, cellules dendritiques, interféron, ARN simple brin, TLR7, TLR8, CD81, DC-SIGN, macrophages

Table of Contents

LIST OF FIGURES

CONTRIBUTION OF AUTHORS

Chapter 2 HCV derived ssRNA induce innate immune response against HCV infection via TLR7 and TLR8

Yuwei Zhang, Elias Said, Abdel-Hakeem MS, Zhong He, Francesco A. Procopio, Rafick-Pierre Sekaly

Yuwei Zhang and Elias Said defined the criteria of GU-rich sequence and selected nine sequences from HCV genome. Yuwei Zhang performed screening of RNA sequences and TNF-α ELISA. Yuwei Zhang, with help of Zhong He, performed the luciferase assay. Elias Said performed DC separation and cytokine/chemokine dectection by Cytometric Bead Array with the help of Yuwei Zhang. Francesco A. Procopio optimized the method for HCV quantification with the help of Abdel-Hakeem MS. Yuwei Zhang and Zhong He performed viral production and infection *in vitro*. The manuscript and the figures were generated by Yuwei Zhang under the supervision of Rafick-Pierre Sekaly.

Chapter 3 Macrophages sense Hepatitis C virus to initiate innate immunity

Yuwei Zhang, Elias Said, Zhong He, Francesco A. Procopio, Rafick-Pierre Sekaly

Yuwei Zhang performed cell separation and macrophage generation. Yuwei Zhang performed TNF-α ELISA. Zhong He performed cytokine/chemokine dectection by Cytometric Bead assay. Francesco A. Procopio optimized the method for HCV quantification. Yuwei Zhang and Zhong He performed viral production and infection *in vitro*. Yuwei Zhang performed the measurement of gene expression and viral quantification by real-time PCR with the help of Francesco A. Procopio. Yuwei Zhang performed the phenotypic staining and flow cytometric analysis of innate immune cells and macrophages. The manuscript and the figures were generated by Yuwei Zhang, in collaboration with Elias Said, under the supervision of Rafick-Pierre Sekaly.

ABBREVIATIONS

APC: Antigen presenting cell

ARFP: Alternate reading frame protein

CBA: Cytometric Bead Array

CLDN1: Claudin 1

CTL: Cytotoxic T lymphocyte

DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3- Grabbing non-integrin

dsRNA: Double-strand RNA

Flu: Influenza virus

GM-CSF: Granulocyte-macrophage colony-stimulating factor

HCV: Hepatitis C virus

HCVcc: Cell culture-derived HCV

HCVpp: HCV pseudotype particles

HIV: Human immunodeficiency virus

HSC: Hepatic stellate cell

IFN: Interferon

IL-4: Interleukin 4

IRES: Internal ribosome entry site

IRF-3: Regulatory factor-3

ISGs: Interferon-stimulated genes

KC: Kupffer cell

LDLR: Low-density lipoprotein receptor

MDA-5: Melanoma Differentiation-Associated protein 5

mDC: Myeloid dendritic cell

MDDC: Monocyte-differentiated DC

MDM: Monocytes-derived macrophage

nAb: Neutralizing antibody

NK: Natural killer cell

NLP: Nucleocapsid-like particle

NS: Nonstructural protein

NTR: Non-translated region

PAMPs: pathogen-associated molecular patterns

PBMC: peripheral blood mononuclear cell

pDC: Plasmacytoid dendritic cell

PRRs: Pathogen recognition receptors

RIG-I: Retinoic acid-inducible gene-I

RLH: RIG-I-like RNA helicase

SRB1: Scavenger receptor class B-1

ssRNA: Single stranded RNA

Stat1: Signal transducer and activator of transcription 1

SV: Sendai virus

SVR: Sustained antiviral response

TLR: Trigger Toll-like receptors

VLP: Viral-like particles

ACKNOWLEDGEMENTS

First of all, I would like to express my sincerest appreciation to my supervisor, Dr. Rafick-Pierre Sékaly, who has provided me with the personal and financial resources for the completion of this work. With his expertise, extensive knowledge, patience, and attention, I have been able to learn and grow professionally. The experiences with him have been invaluable in shaping the researcher that I want to be.

I would like to express my gratitude to Dr. Elias Said for his valuable contribution through supervising and the numerous scientific discussions, as well as Dr. Naglaa Shoukry for her support. I also would like to thank PhD candidate Mohamed S. Abdel–Hakeem, Dr. Zhong He and Dr. Mohamed El-Far for their significant help with viral production and technical assistance. I also would like to thank Dr. Francesco Procopio for his help with optimization of real-time PCR. I also thank Dr. Jeff Ahlers, Dr. Yu Shi, Dr. John Hiscott, Dr. Joumana Zeidan and Dr. Petronela Ancuta for their editorial assistance.

The encouragement and love from my family and friends are very important to me, they have support me during my Ph.D. studies. I would not have been able to complete without their financial and emotional support.

CHAPTER 1

INTRODUCTION

1. Hepatitis C virus- prevalence

The hepatitis C virus, also known as HCV, is now a widespread public health problem that affects a global audience estimated to be 200 million individuals – equaling 3.3% of the world's population. Annually, the number of new reported cases of the hepatitis C infection reaches as high as 3 to 4 million (Perz, Armstrong et al. 2006, WHO 2011). The prevalence of HCV is comparatively lowest in Asia Pacific, Tropical Latin America and North America (<1.5%). According to a particular survey, the occurrence of HCV antibodies with respect people donating blood averages less than a meager one percent for the aforementioned region. On the flip side, the rates are incrementally higher as seen in South Asia, Southern Latin America and Europe (1.5%-3%). The occurrence of hepatitis C antibodies in Central and East Asia, North Africa/Middle East were reported around 3.8% (Mohd Hanafiah, Groeger et al. 2013). As far as many African countries were concerned though, alarming rates have been reported, reaching as high as 14.7% in Egypt (Mohamoud, Mumtaz et al. 2013).

HCV infection primarily takes place in the liver and causes hepatitis C (Choo, Weiner et al. 1990). The infection is usually asymptomatic in nature (Alberti, Noventa et al. 2002, Mendez-Sanchez, Ponciano-Rodriguez et al. 2005), however chronic infection can lead to scarring of the liver and subsequently lead to cirrhosis (Freeman, Dore et al. 2001). Progression of liver disease caused by HCV usually takes decades - As high as one-fifth of the people that are infected may go on to develop complications such as liver failure, cirrhosis or even hepatocellular carcinoma (Perz, Armstrong et al. 2006). Barring hepatocellular carcinoma, with respect to patients that develop cirrhosis or liver cancer, a

liver transplant may be required. HCV-induced liver inflammation and cirrhosis is considered as one of the major causes for liver transplants (Davis, Albright et al. 2003). Only 10% to 40% of HCV-infected individuals can be cured by spontaneous viral clearance, and 60% to 90% infected individuals develop chronic infection, depending on the genotype of the virus they are contracted (Gerlach, Diepolder et al. 2003, Lehmann, Meyer et al. 2004, Micallef, Kaldor et al. 2006). There is no effective vaccine to prevent the hepatitis C infection. A combination of peg-interferon and ribavirin is the current standard therapy.

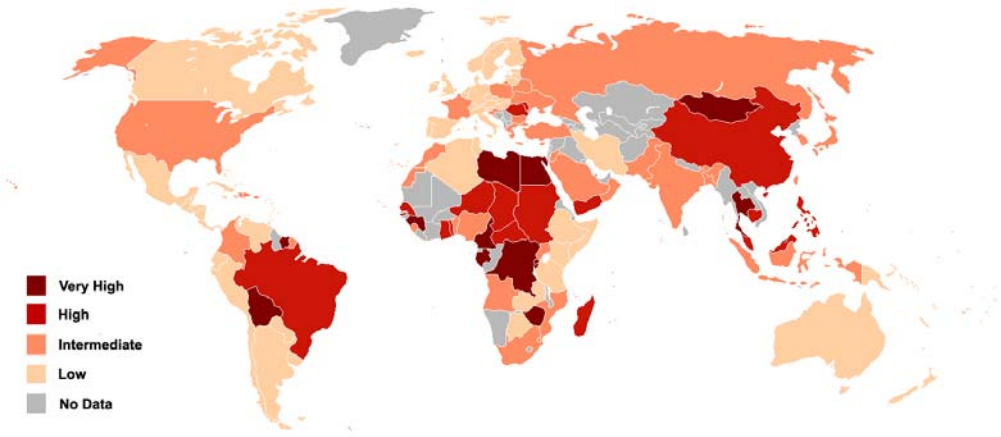


Figure 1. Geographic distribution of Hepatitis C prevalence

This image is originally from

http://commons.wikimedia.org/wiki/File:HCV_prevalence_1999.png

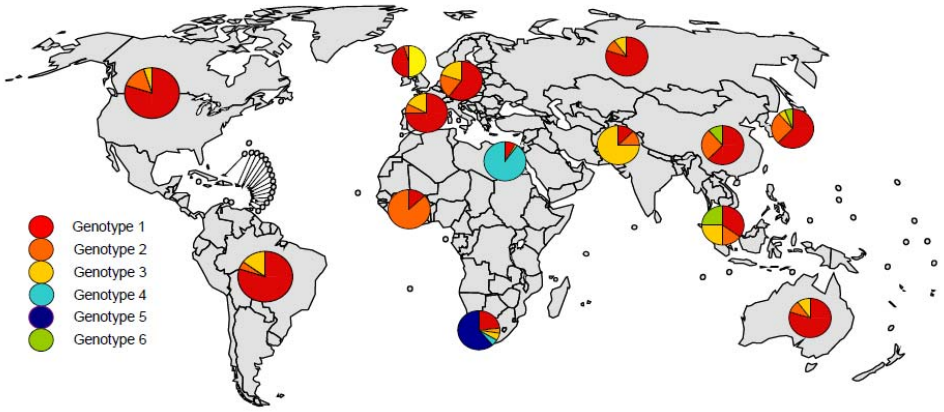


Figure 2. Global distribution of HCV genotypes

This image is originally from

http://www.who.int/vaccine_research/documents/ViralCancer7.pdf

2. Discovery of HCV

In the mid 1970s, a new hepatitis virus was recognized in blood transfusion recipients, identified as non-A, non-B hepatitis (Feinstone, Kapikian et al. 1975). A few distinctive characteristics aided in separating this new virus from HAV and HBV. Infection in chimpanzees produced chronic disease is characterized by inflammation and hepatocyte structural changes (Popper, Dienstag et al. 1980, Dienes, Popper et al. 1982). Complete identification of the new virus was hindered by difficulties in producing a cellular model that allowed viral replication (Lohmann, Korner et al. 1999). In 1987, Michael Houghton and Daniel Bradley collaborated to create a molecular cloning technique to sequence the viral genome (Choo, Kuo et al. 1989). Once HCV was identified, screening for it in blood transfusions became mandatory starting in 1990, which subsequently decreased the rate of infection (Kimber 1990, Weiner, Kuo et al.

1990). The highest transmission rates were initially from unsanitary injection drug use or unsterile needle use such as in unregulated body tattoo art and blood transfusions (Conry-Cantilena, VanRaden et al. 1996). Occupational exposure to HCV is and has been the most difficult type of transmission to control as most infections occur accidentally, even with proper precautions observed (Yazdanpanah, De Carli et al. 2005). Since blood screening started to be applied, there has been a decrease of HCV infection in developed countries. However, transmission of the virus in some areas is still growing despite advanced techniques to identify HCV in blood (Alter 2007, Martins, Narciso-Schiavon et al. 2011). Owing to the expensive nature of testing for the virus, underdeveloped countries are still struggling to control the spread of the virus (van der Poorten, Kenny et al. 2008). Still, continued HCV research has led to improved treatments against the virus.

3. HCV virology

Hepatitis C virus, classified as part of the *Flaviviridae* family, is predominantly a singularly stranded positive-sense RNA virus. This genome is almost 9.6 kilo-bases and has six different major genotypes, genotypes 1-6 (Okamoto 1995). HCV has a high propensity for genetic variations due to the error-prone nature of the RNA replicase (Bukh, Miller et al. 1995, Pawlotsky 1998). Therefore, HCV-infected individuals with certain genotypes and subtypes respond atypically to treatment (Manns, McHutchison et al. 2001), and the identity of HCV genotypes in infected individuals is important to the specific treatment regimen. Throughout the history of HCV therapy, treatment has been conventionally less effective—up to 30% to 50%—in patients harboring HCV genotype 1

compared to up to 80% effective clearance in genotypes 2 and 3 (Zein, Rakela et al. 1996, Neumann, Lam et al. 2000, Lehmann, Meyer et al. 2004). Thus, genotype 1 is associated with the development of chronic infection and is also the most prevalent genotype in the world (Fig 2).

3.1 HCV genome and proteins

HCV is a relatively small 50 nm enveloped RNA virus (Kaito, Watanabe et al. 1994). The genome, flanked by small 5' and 3' non-translated regions (NTRs), has one open reading frame that translates to a number of proteins of roughly 3000 residues (Choo, Richman et al. 1991). The 5' NTR region is known to contain an internal ribosomal entry site, denoted as IRES, and is considered extremely conserved among the various genotypes and subtypes. It is essential for RNA translation because it binds the 40S ribosome subunit, positioning it near the initiation site (Pestova, Shatsky et al. 1998, Friebe, Lohmann et al. 2001). The 3' NTR region with its three domains—6variable region, poly U region, and a conserved terminal domain—is highly inconsistent among the genotypes (Friebe and Bartenschlager 2002). Areas in the latter two regions are required for RNA replication.

The virus has ten proteins, three of which are structural proteins: core and envelope 1 and 2 (Takeuchi, Kubo et al. 1990). In between these non-structural as well as structural proteins happens to be a rather small yet integral membrane protein: p7 (Lin, Lindenbach et al. 1994). The nonstructural proteins are NS2 and NS3, NS4 (A and B) and NS5 (A and B). Barring NS2, the remaining nonstructural proteins are deemed to be an

essential requirement for replication (Brenndorfer, Karthe et al. 2009, Morikawa, Lange et al. 2011). Extensive research into each of these proteins has revealed specific roles for viral replication and survival. Alternatively, a frame shift may occur in the Core protein-coding region to produce an alternate reading frame protein (ARFP) (Branch, Stump et al. 2005).

The hepatitis C core protein is an RNA binding protein that is highly conserved in nature (Bukh, Purcell et al. 1994, Hitomi, McDonnell et al. 1995). They are also presumed to form the viral capsid. When expressed in several in vitro systems, the hepatitis C core protein is known to participate in the formation of NLPs. (Baumert, Ito et al. 1998, Blanchard, Brand et al. 2002). The two envelope glycoproteins, that are namely E1 and E2, are essential for viral entry and fusion (Bartosch, Dubuisson et al. 2003, Nielsen, Bassendine et al. 2004). E2 plays an important role during early stages of the infection. E2 interaction with either one or multiple receptor complex components is considered to be the root cause behind the initiation of viral attachment (Rosa, Campagnoli et al. 1996, Flint and McKeating 2000). p7 is predominantly a small, 63aa polypeptide, which is understood to be a fundamental membrane protein (Carrere-Kremer, Montpellier-Pala et al. 2002, Sakai, Claire et al. 2003).p7 appears to be essential for HCV infectivity (Sakai, Claire et al. 2003). NS2, along with the amino-terminal of the NS3 protein, along with the NS2-3 protease, splits the NS2 and NS3 (Grakoui, McCourt et al. 1993). NS3 is deemed to be a viral protein exhibiting multiple functionalities. NS4A plays a supporting role during the NS3 protease activity. NS3-4A has also been seen to exhibit added properties while interacting with the pathways of host cells as well as proteins, and resultantly may be considered as a vital factor during the lifecycle and

pathogenesis of infection (Bartenschlager, Lohmann et al. 1995, Lin, Thomson et al. 1995).

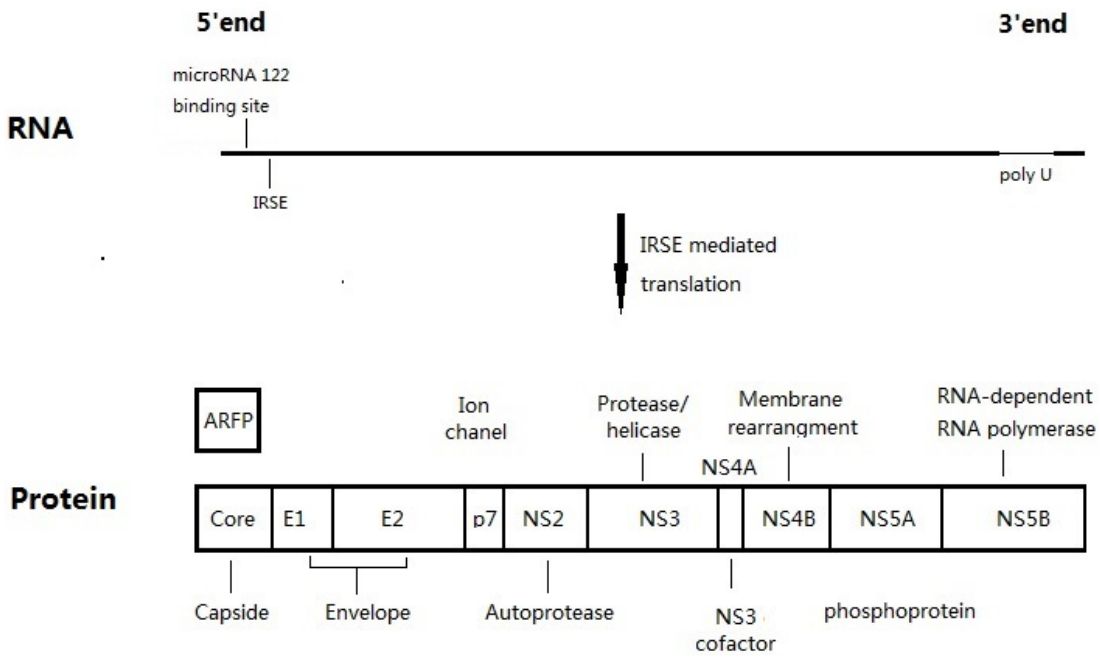


Figure 3. Genome and proteins of HCV virus

The 9.6 kb positive-strand RNA genome can be schematically depicted at the upper side of the figure. The RNA arrangements at the 5' and 3' ends of the hepatitis C genome are important inputs for translation and replication. The IRES-mediated translation produces a poly-protein precursor of almost 3000 amino acids. The host and viral proteases split poly-protein during and after translation to subsequently result in the 10 proteins depicted here. Adjacent to the colored boxes the representation of the known functionality of each protein is depicted. The translational frames shift may result in the production ARFP.

3.2 Viral life cycle

HCV infects and replicates in human hepatocytes. Chimpanzees are the only other animal model that will sustain replication (Bukh 2004). The HCV life cycle consists of five distinct steps: entry, protein translation, RNA replication, virion assembly, and virion release. The viral infection starts by binding of the envelope proteins to several host cellular receptors: CD81 (Pileri, Uematsu et al. 1998, Cormier, Tsamis et al. 2004), scavenger receptor class B-1 (SRB1) (Bartosch, Vitelli et al. 2003), glycosaminoglycans (Olenina, Kuzmina et al. 2005), low-density lipoprotein receptor (LDLR) (Monazahian, Bohme et al. 1999), and claudin-1 (Evans, von Hahn et al. 2007). But Albecka A et al showed recently that LDLR is not essential for infectious HCV particle entry (Albecka, Belouzard et al. 2011). Endocytosis of the virion occurs by clathrin mediation and acidification of the endosomal compartment (Blanchard, Belouzard et al. 2006). After entry, the viral and endosomal membrane fuse, allowing for the release of hepatitis C RNA into the cytoplasm. The translation of the viral polyprotein is dependent on the 5' NTR IRES of the positive single-stranded hepatitis C RNA (Otto and Puglisi 2004). The proteins—core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B—are manufactured in the cytoplasm and localized to the endoplasmic reticulum membrane where HCV negative-sense strand RNA production occurs (Liu, Aizaki et al. 2012). In the cytoplasm, while HCV proteins are translated from RNA, the proteins may interact with host factors to ensure viral survival. HCV proteins, in particular NS4B, mediate the formation of membranous webs postulated to be partial ER membranes, where further RNA replication from negative strand to positive strand occurs (Elazar, Liu et al. 2004). Close to the membranous webs, the RNA and structural proteins assemble into viral particles,

which bud into the ER or similar membrane compartments to exocytose out of the cell, leaving it intact (Bartenschlager, Penin et al. 2010, Lindenbach and Rice 2013).

4. Infectious HCV culture system *in vitro*

It has been very difficult to observe a complete infection cycle of HCV *in vitro* due to the absence of a hepatitis C clone with robust replication and cell line that is permissive for HCV infection. HCV replicon (self-replicating sub-genome with selection transfected into hepatoma cell lines) was able to provide significant insights regarding the functionality of viral RNA and proteins in infection (Lohmann, Korner et al. 1999, Blight, Kolykhalov et al. 2000). Replicon-based systems have been broadly used for the selection of anti-HCV drugs. Pseudo particles decorated with the HCV envelope GPs (HCVpp) allowed the study of steps in viral entry. However, the assembling and the release of infectious hepatitis C were not effectively summarized and restated by these experimental systems. Many attempts have already been made in the direction of developing a proper system for hepatitis C infection and replication in terms of a cell culture, however, as far as reports about the latter were concerned, the results were far from efficient. Fortunately, this situation took a change for the better with the establishment of a new, efficient culture model for hepatitis C infection. The infectious HCV system provides an important tool to study HCV infection, anti-HCV drug selection, and even HCV-related immune response. The first robust infectious clone, JFH1 strain, was isolated from a HCV host. Transfection of *in vitro* recorded full length JFH1 HCV RNA into Huh7 cells was able to replicate efficiently JFH-1 RNA along with

the secreting recombinant viral particles into the culture medium (Wakita, Pietschmann et al. 2005, Zhong, Gastaminza et al. 2005, Bukh and Purcell 2006). Significantly, the secreted viral particles were deemed to be infectious in the case of cultured cells and chimpanzees (Kato, Matsumura et al. 2007). It was also reported that cell culture-adapted mutations in JFH1 yield higher titers of infectious particles (Liu, Xiao et al. 2012). To expand the scope of this novel HCV infection system, several groups have constructed chimeric HCV genomes comprising JFH1-derived replicase proteins and structural proteins from heterologous HCV strains or other virus (Kylefjord, Danielsson et al. 2013, Li, Zhu et al. 2013, Lu, Tao et al. 2013), which might provide a useful tool for virological studies and evaluation of antiviral therapies.

Attempts to propagate HCV *in vitro* have proven difficult. Developments have led to establishment of several systems that support HCV replication *in vitro*, including hepatoma cell lines, primary or immortalized hepatocytes et al (Fournier, Sureau et al. 1998, Lohmann, Korner et al. 1999, Kanda, Basu et al. 2006, Gondeau, Briolotti et al. 2013). The rate of infection in the case of cultured cells was comparatively enhanced by using permissive cell lines, t but it is still far from perfect due to evidently low yields of the virus and minimal spread in cell culture (Sainz, Barretto et al. 2012). Expression of essential genes for HCV replication, such as microRNA122, may permit efficient HCV RNA replication and infectious virion production in hepatoma cell lines (Kambara, Fukuhara et al. 2011, Narbus, Israelow et al. 2011). The most permissive cell line for efficient RNA replication in vitro is the human hepatoma cell line Huh-7 and its clonal descendants (Blight, McKeating et al. 2002). Huh7.5, a hepatoma cell derived from HCV-harboring huh7 clones with prolonged treatment with alpha interferon (IFN-

alpha) to eliminate self-replicating subgenomic RNA within the cells. Huh7.5 appears highly permissive for HCV infection and replication. A single point mutation was observed in the dsRNA sensor RIG-I and deemed to positively correlate to seemingly increased permissiveness for the replication of HCV RNA (Sumpter, Loo et al. 2005). This observation illustrates that lack of antiviral restriction may substantially increase the permissiveness for HCV. Furthermore, although hepatocarcinoma cell lines have became important new virological tools to study the mechanisms of HCV infection, however, this experimental model remains distantly related to physiological and pathological conditions. It was reported recently that a new ex vivo model using human adult liver slices culture supports efficient replication of primary or culture hepatitis C virus isolates (Lagaye, Shen et al. 2012), this provides a powerful tool for studying the viral life cycle and dynamics of virus spread in native tissue.

5. HCV treatments and limitations

HCV therapy has improved considerably since the discovery of the virus in 1989 (Choo, Kuo et al. 1989). As far as chronic hepatitis C is concerned, the common course of treatment is with a glycoprotein commonly known as IFN-alpha, which is integral for the treatment since it accents the immune response against the virus (Carithers and Emerson 1997). Studies based on a long-term timeframe have suggested that a sustained antiviral response (SVR) shows an overall clearance of virus, and is associated with improved clinical outcomes and subsequent cure of the disease (Bizollon, Ahmed et al. 2003, Veldt, Heathcote et al. 2007, Pearlman and Traub 2011). Furthermore, the starting

of IFN monotherapy combined with recommendations of prg-IFN and ribavirin, there has been a noticeable increase in the number of patients achieving SVR (Jacobson, Gonzalez et al. 2005). IFN, although incompletely understood, acts as a direct anti-viral along with acting on the host's immune system, ribavirin on its own is unable to fully constrain the replication of HCV much expressively but enhances the anti-viral action of IFN (Chung, Gale et al. 2008).

However, the general response to therapy largely depends on the viral genotype and characteristics of the patient. Patients having different hepatitis C genotypes tend to react differently to IFN-alpha (Manns, McHutchison et al. 2001, Fried, Shiffman et al. 2002). This is largely due to the fact that the genotype is one of the strongest prognostic aspects of SVR (Zein, Rakela et al. 1996, Idrees and Riazuddin 2009). Genotype 1 is the viral strain that is most prevalent and most difficult to treat. More SVR was observed in patients suffering from hepatitis C genotype 2 or genotype 3 – more so when compared to HCV-infected hosts having genotype 1. Patients that suffer from HCV genotype 2 or 3 tend to show 75% SVR while people with the HCV genotype 1 have been reported to show 40% SVR (Veldt, Brouwer et al. 2003, Jeffers, Cassidy et al. 2004).

However, there are certain percentages that are not easy to treat. These may include people who do not respond to treatment when exposed to IFN-based therapies in the past, hosts that are suffering from severe liver fibrosis or cirrhosis, and individuals co-infected with HIV (Bourliere, Ouzan et al. , Poynard, Moussali et al. 1999, Jackson 2009, Lerias de Almeida, Alves de Mattos et al. 2010). There are however, no approved options for treatment in the case of people failing to respond to prior treatments. Conducted studies so far indicate that retreatment with extending the treatment duration can improve

SVR rates to 20% in non-responders (Goncales, Moma et al. 2010). Patients with HIV co-infection usually exhibit a delayed response, studies conducted on patients that are also infected with the HIV virus have exhibited SVR rates ranging from 17% to 62% (Carrat, Bani-Sadr et al. 2004, Torriani, Rodriguez-Torres et al. 2004). This could be down to a combination of two primary reasons – because of an increased viral load in the co-infected cases and the deficiency in the host's immune system.

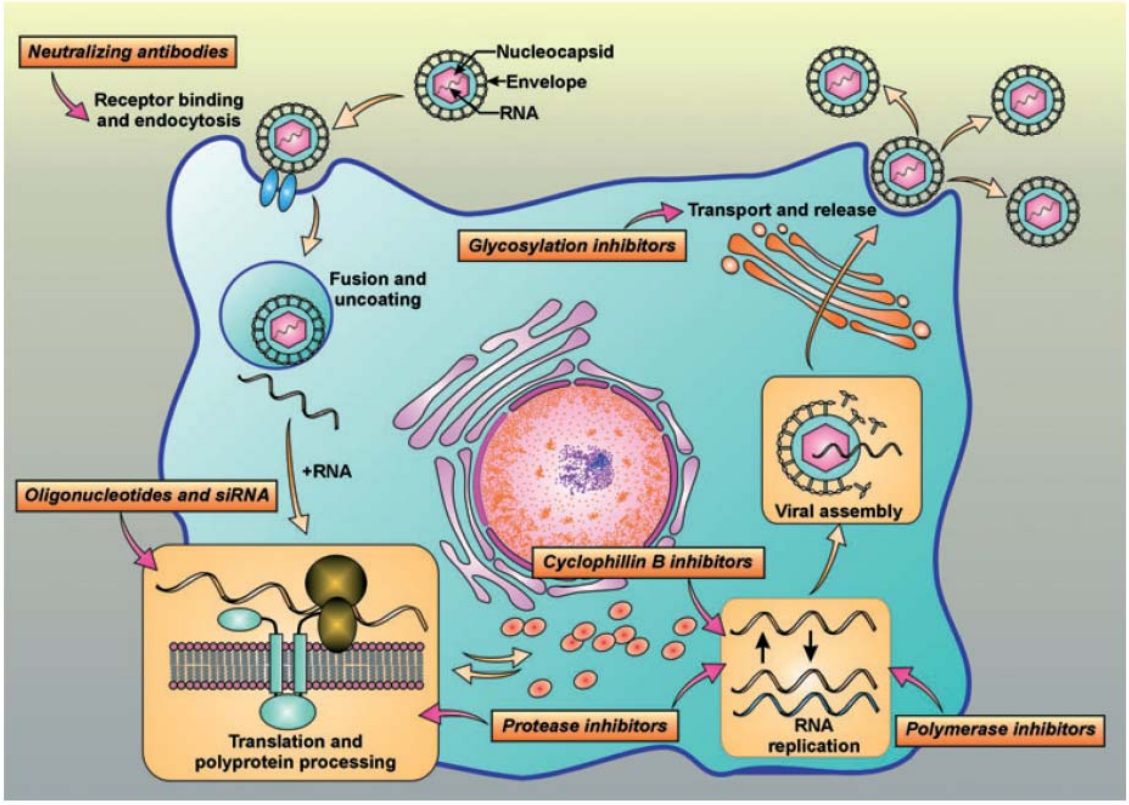


Figure 4 Hepatitis C virus (HCV) viral cycle and targets for drug development

The HCV lifecycle starts with virion attachment to its specific receptor. The HCV RNA genome serves as a template for viral replication and as a viral messenger RNA for viral production. It is translated into a polyprotein that is cleaved by proteases. Then, viral assembly occurs. Potentially, each step of the viral cycle is a target for drug development.

This image is originally from *Asselah T, Marcellin P. Liver Int. 2013 Feb,33*

Current interferon based therapies, while already marred with inadequate response rates, also have numerous side effects associated with them (McHutchison, Gordon et al. 1998, Manns, McHutchison et al. 2001). As a result, there has been growing focus on coming up with new interferon-free approaches that target various steps of the viral lifecycle as either replacements or further improvements for current strategies. This has resulted in developing several agents associated with targeting specific viral components (Stedman 2012). There have been major advancements during last several years with large numbers of ongoing trials with various direct-acting antivirals showing high potency, favorable tolerability profile, higher barrier to resistance, shortened treatment duration, all oral regimen, pan-genotypic (Asselah and Marcellin 2013). Presently there is increased emphasis in the development of more encouraging new agents such as polymerase inhibitors and protease. Moreover, there have been numerous compound inhibitors that have been designed successfully of the NS3 protease. They are subsequently an integral course of action spearheading pre-clinical and clinical development. BILN 2061 played a vital part in understanding the importance of these particular class of compounds, providing at least a decrease of 2 or 3 log10 in hepatitis C load within a timeframe of 48 hours (Lamarre, Anderson et al. 2003). Additionally, several NS5B polymerase compound inhibitors are also undergoing clinical development based on their encouraging results (Gane, Roberts et al. 2010, Le Pogam, Yan et al. 2012). In 2011, The U.S. Food and Drug Administration (FDA) approved Victrelis (boceprevir) and Incivek (telaprevir), known as inhibitor of HCV protease, for the treatment of hepatitis C. In late of 2013, FDA approved Olysio (simeprevir), a new therapy to treat chronic HCV infection. Most recently, a phase 2 clinical trial gave

encouraging result, treatment with Sofosbuvir and ledipasvir fixed-dose combination achieved over 95% SVR12 (sustained virological response 12 weeks after treatment) in patients with genotype 1 HCV infection, similar SVR12 was achieved even in those who previously had virological failure after receiving a protease inhibitor treatment (Lawitz, Poordad et al. 2013). The observation that hepatitis C virus (HCV) could only replicate in miR-122-positive hepatocytes led to the discovery that miR-122 is essential for HCV replication, and miR-122 is now one of the crucial host factors for anti-HCV therapy (Henke, Goergen et al. 2008, Lanford, Hildebrandt-Eriksen et al. 2013). Currently, the most advanced miR-122 targeting therapy is SPC3649 (miravirsen), a locked nucleic acid-modified oligonucleotide antagonizing miR-122, which is currently in phase 2 clinical trials for treatment of HCV infection (Janssen, Reesink et al. 2013).

Various factors ranging from treatment related, host characteristics, viral related and disease related, may cause resistance to IFN therapy (Pawlotsky 2003, Chen, Borozan et al. 2005, Puig-Basagoiti, Forns et al. 2005). In addition to that, the apparent generation subsequent selection of resistant variants may lead to the virus escaping the anti-viral pressure that is exerted by HCV-targeting drugs. Verily, mutations pertaining to the polymerase and protease enzymes have been identified already (Tomei, Altamura et al. 2003, Lin, Lin et al. 2004, Lu, Pilot-Matias et al. 2004, De Francesco and Migliaccio 2005, Lin, Gates et al. 2005, Le Pogam, Jiang et al. 2006, Le Pogam, Kang et al. 2006, Tong, Chase et al. 2006, Koev, Dekhtyar et al. 2007). Mutations of drug-targeted viral protein occur rapidly, causing resistance to treatment and leading to treatment failure (Rong, Dahari et al. 2010, Pawlotsky 2011). Various mutations are known to exhibit either an increased or decreased level of resistance along with the changing of the overall

prevalence of individual mutations through the passage of time during the treatment stage. *In vitro* data further suggests a seemingly lowered probability entailing the cross resistance between a certain number of the diverse anti-HCV drugs (Tomei, Altamura et al. 2003, Lin, Lin et al. 2004, Lu, Pilot-Matias et al. 2004, De Francesco and Migliaccio 2005, Lin, Gates et al. 2005, Le Pogam, Jiang et al. 2006, Le Pogam, Kang et al. 2006, Tong, Chase et al. 2006, Koev, Dekhtyar et al. 2007). Adding to that, molecular biology seems to suggest no cross-resistance between protease and polymerase inhibitors (De Francesco and Migliaccio 2005). Based on those studies, it is advisable to use agents that show a low probability of cross-resistance to supplement the therapy, such as different inhibitors or an inhibitor plus pegIFN-alpha. Indeed, administrating telaprevir together with pegIFN-α2a or further combined with ribavirin seemed to prevent viral rebound (Sarrazin, Rouzier et al. 2007). Simeprevir has also shown to increase the rate of sustained virologic response among treatment-experienced patients with HCV genotype-1 infection (Zeuzem, Berg et al. 2013)

6. Acute, chronic infection and viral clearance

Acute hepatitis C infection usually occurs during the first six-month time-bracket following the onset of viremia. It is usually asymptomatic since merely a minority of patients (10%–15%) is known to report symptoms (Ozaras and Tahan 2009). The symptoms of acute hepatitis C infection include a noticeable decrease in appetite, increased fatigue, consistent abdominal pain, jaundice, repetitive itching, and symptoms similar to a case of flu. When the time these symptoms come forth, usually they are

related to a comparatively high rate of spontaneous clearance (Gerlach, Diepolder et al. 2003). Spontaneous viral clearance rates can be considerably varied —between 10% - 60% of HCV infected individuals (Lehmann, Meyer et al. 2004, Micallef, Kaldor et al. 2006, Santantonio, Medda et al. 2006). HCV is usually detectable in the blood by PCR within a timeframe ranging from one - three weeks after contracting the infection. Serum viral load tends to increase considerably during the early stages of infection without any clear-cut evidence of liver damage. The responses with respect to the cellular immune are usually evident first among circulating T cells 4 to 8 weeks after the beginning of infection (Thimme, Oldach et al. 2001, Woollard, Grakoui et al. 2003, Cox, Mosbruger et al. 2005). The start of cellular immunity is consistent with a spike in serum transaminases which may subsequently suggest a transient immune-associated liver injury (Thimme, Oldach et al. 2001).

Although spontaneous viral clearance occurs in a minority of patients during acute infection stage, recurring infections are quite common, and most patients along the way are seen to develop chronic hepatitis C (Micallef, Kaldor et al. 2006). Chronic HCV occurs when the infection bearing the hepatitis C virus persists for more than a period of six months. Clinically though, it is often asymptomatic in nature and is mostly discovered by accident due to elevated liver enzymes (Hoofnagle 2002). The natural course of chronic hepatitis C could be varied from person to person. While documented cases of hepatitis C usually suggest almost most people suffering from an inflammation on liver biopsy, the progression rate entailing liver fibrosis is considerably variable among patients. Within the untreated population, roughly 33.3% progress to liver cirrhosis within a period of 20 years while an additional one-third of the remaining portion have

been known to progress to cirrhosis within a period of 30 years (Poynard, Ratziu et al. 2000, Poynard, Ratziu et al. 2001, Ghany, Kleiner et al. 2003). Usually, factors adjudged to have a bearing on the rate of the hepatitis C disease's progress include classifications like age (the higher the age, the increased chances of rapid progression) (Poynard, Bedossa et al. 1997, Poynard, Ratziu et al. 2001), gender (men have been known to experience more rapid progression of the disease than women) (Wiese, Berr et al. 2000, Rodriguez-Torres, Rios-Bedoya et al. 2006), alcohol consumption (directly proportional) (Wiley, McCarthy et al. 1998), HIV co-infection (direct relation) (Rotman and Liang 2009, Konerman, Mehta et al. 2014), and fatty liver (positive correlation) (Younossi, McCullough et al. 2004, Younossi, Afendy et al. 2009, Younossi and McCullough 2009, Bhala, Angulo et al. 2011).

7. Immune response and HCV infection

Immune activation is associated with HCV infection. An immune response induced by HCV infection is characterized as rapid innate immune response and delayed adaptive immune response, regardless of the outcome of the disease progression (Shoukry, Grakoui et al. 2003, Dustin and Rice 2007). Innate immune activation was observed at the early stage after HCV infection in chimpanzees (Bigger, Guerra et al. 2004). Inflammatory cytokines also have been shown to be up-regulated in chronically HCV-infected individuals (Polyak, Khabar et al. 2001). Studies estimate that in the case of an infected chimpanzee at the peak of viremia, ten percent of the hepatocytes support

HCV replication at max (Bigger, Brasky et al. 2001). This goes on to suggest that an anti-viral state is induced quite early in the liver during HCV infection.

7.1 Innate immunity

HCV is usually recognized by pathogen recognition receptors (PRRs) which further goes on to initiate an innate immune response against HCV infection. Engagement of those receptors may activate and subsequently translocate latent IRF-3, further going on to activate the IFN-β promoter (Saito, Owen et al. 2008). Intrahepatic interferon signaling was observed and associated with HCV viremia, regardless of the outcome of the disease progression (Su, Pezacki et al. 2002, Thimme, Bukh et al. 2002). The cellular source of interferon is not certain however.

Several different cell types within the liver may take part in innate immune surveillance on HCV infection, including hepatocytes, Kupffer cells (KCs) that serve as resident liver macrophages, DCs and natural killer cells (NKs) (Crispe 2009). The first immune response to HCV infection was thought to be interferon induction in infected hepatocytes (Bigger, Guerra et al. 2004). Hypothetically, infected hepatocytes produce the first interferon, which acts on surrounding cells and amplifies interferon signals. Several studies showed that HCV infection induced IFN signaling in immortalized human hepatocytes (Kanda, Steele et al. 2007) or hepatoma cell lines (Lau, Fish et al. 2008). There is no direct evidence of interferon induction in hepatocytes of HCV patients *in vivo*, but intrahepatic interferon-induced genes were detected in infected chimpanzees (Lau, Fish et al. 2008). Type I IFNs production - as a reaction to hepatitis C infection - may lead to an antiviral state with respect to the surrounding cells. Plasmacytoid DCs

(pDCs) were reported to be activated by HCV-infected hepatocytes through cell-cell contact, resulting in producing type I IFN *in vitro* (Takahashi, Asabe et al. 2010). However, *in vivo* study has not supported pDCs as the source of intrahepatic interferon during HCV infection (Lau, Fish et al. 2008).

Kupffer cells form the highest percentage of tissue-resident macrophages in the liver (Laskin, Weinberger et al. 2001). Kupffer cells are responsible for the recruitment of CTLs and APCs to eliminate infected cells in the liver (Uwatoku, Suematsu et al. 2001), and they also carry out an important function in modulating inflammation in liver fibrosis development (Liu, Tao et al. 2010). NK cells are found to be in high quantity in the liver and are also essential early responders during HCV infection. NK cells facilitate lysis of infected cells and produce IFN-γ that control viral infection (Kokordelis, Kramer et al. 2013). NK cells may also contribute to maturation of DCs and bridge the gap between innate and adaptive immunity. The importance of NK cells in the resolution of HCV infection is revealed by the observation of genetic polymorphisms that affect the threshold of NK cell activation and influence the outcome of HCV infection (Khakoo, Thio et al. 2004).

7.2 Adaptive immunity

Controlling acute HCV infection is related to strong, broad directed, and most importantly a sustainable activation of hepatitis C-specific T cells (Thimme, Oldach et al. 2001, Thimme, Bukh et al. 2002, Flynn, Dore et al. 2013). The activation of T cell can be transiently observed at least, as hepatitis C is able to establish infection persistently (Ulsenheimer, Lucas et al. 2006). However, in the case of majority of the patients, cell-

mediated immunity is unable to eliminate hepatitis C infection completely (Guobuzaite, Chokshi et al. 2008). Even in the case of humans and chimpanzees, the chances of contracting reinfections through homologous and heterologous hepatitis C strains remain a plausible outcome. Protective immunity on the other hand can reduce the overall duration and extent of viremia in the case of repeated infection (Major, Mihalik et al. 2002, Mehta, Cox et al. 2002, Lanford, Guerra et al. 2004), but completely sterilizing immunity has still evaded proof.

Spontaneous resolution of hepatitis C is commonly linked with a strong and sustained T cell response that targets multiple hepatitis C epitopes at once and with intrahepatic production of IFN-γ (Cooper, Erickson et al. 1999, Lechner, Wong et al. 2000, Thimme, Oldach et al. 2001). HCV-specific CD4+ T cell activation is relatively easy to detect during the early stage of acute infection than in chronic infection (Lechner, Wong et al. 2000). Epitope-specific T cells can be identified when stained with MHC class I or class II tetramer peptide complexes. Tetramer-staining T cells may not be active in a functional capacity during acute or chronic infection. Indeed, studies conducted have shown that a significant percentage of hepatitis C-specific T cells have a "stunned" phenotype, even in the case of those hosts that go on to resolve infection (Lechner, Wong et al. 2000, Thimme, Oldach et al. 2001). Resolution of acute infection is associated with T cell recovery of an activated phenotype and the ability to produce IFN-γ. The broad patterns of $CD4^+$ and $CD8^+$ T cell specificities in individuals with resolved hepatitis C infection varies substantially with the rather limited amount of hepatitis C epitopes that are targeted by T cells with respect to people having chronic infection (Day, Lauer et al. 2002) (Lauer, Ouchi et al. 2002).

The role of humoral immunity to HCV is yet to be comprehended accurately. Seroconversion is seen to be a comparatively delayed phenomenon in the case primary hepatitis C infection (Netski, Mosbruger et al. 2005). The response of antibodies are not required for clearance of acute infection (Cooper, Erickson et al. 1999, Thimme, Bukh et al. 2002, Logvinoff, Major et al. 2004) and likely to diminish after spontaneous recovery (Takaki, Wiese et al. 2000). Studies suggest that broadly reactive neutralizing antibodies (nAbs) that target the viral envelope can be found in the serum of persistently infected hosts (Bartosch, Bukh et al. 2003, Logvinoff, Major et al. 2004, Meunier, Engle et al. 2005). However, the overall development of nAbs is substantially delayed in the case of acute infection (Logvinoff, Major et al. 2004, Netski, Mosbruger et al. 2005). In the event that patients remain persistently infected in spite of the presence of high titers of nAbs may subsequently indicates that nAbs do not facilitate sterilizing immunity, possibly because of continued viral mutation and antibody-dependent selection.

8. TLR7 and TLR8

Receptor-mediated detection of pathogen-derived nucleic acids helps to protect genomic nucleic acid in the event of foreign genetic material invasion. A number of pattern recognition receptors (PRRs) have been known to participate in the recognition of nucleic acid (Barbalat, Ewald et al. 2011). Generally speaking, PRRs can be classified into numerous families. The Toll-like receptor (TLR) family is known to consist of ten members. Moreover, viral RNA bearing certain traits can also be detected by members of the RIG-I-like RNA helicase (RLH) family, such as RIG-I and MDA-5. TLRs along with

the two RLH members namely RIG-I and MDA-5 can be differentiated with respect to their cellular localization, ligand specificity along with downstream signaling pathways (Thompson and Locarnini 2007, Thompson, Kaminski et al. 2011, O'Neill, Golenbock et al. 2013). Thus it suggests that host cells can employ several, non-redundant defense mechanisms in the detection of invading pathogens. While RIG-I and MDA-5 can be classified as cytosolic receptors (Barral, Sarkar et al. 2009), the four members of the TLR family (TLR3, TLR7, TLR8, and TLR9) involved in viral nucleic acid recognition are all present in the endosomal membrane (Lee and Barton 2014). TLR3 and MDA-5 recognize long double-stranded RNA (Liu, Botos et al. 2008, Wu, Peisley et al. 2013). Either single- or double-stranded RNA belonging to the triphosphate group at the 5' end was observed to be the ligand for RIG-I (Hornung, Ellegast et al. 2006), and also the ligand for TLR9 is DNA-containing CpG motifs (Rutz, Metzger et al. 2004). TLR7 and TLR8 detect single-strand RNA with certain motifs (Heil, Hemmi et al. 2004).

Sequence comparisons across vertebrate TLRs go on to reflect that TLR7 and TLR8 are the most closely related of the nucleic acid–sensing TLRs (Roach, Glusman et al. 2005, Mikami, Miyashita et al. 2012). In humans, remiquimod in known to stimulate TLR7 and TLR8, yet the related imidazoquinoline, imiquimod, predominantly activates TLR7, indicating considerable differences in ligand specificity and functions between the respective receptors (Gorden, Gorski et al. 2005, Larange, Antonios et al. 2009). As far as humans are concerned, TLR7 and TLR8 are able to aptly recognize distinct sequence motifs in ssRNA (Heil, Hemmi et al. 2004, Hornung, Guenthner-Biller et al. 2005, Forsbach, Nemorin et al. 2008). Expression of TLR7 and TLR8 is mainly limited to immune cell subsets. Moreover, in the human system, the expression of TLR7 is

restricted to PDCs and B cells. TLR8 is predominantly expressed in cells belonging to the myeloid lineage, such as monocytes, myeloid dendritic cells, and macrophages (Krug, Towarowski et al. 2001, Hornung, Rothenfusser et al. 2002). This differential expression is most likely to explain the distinct cytokine profiles influenced by TLR7 and TLR8 ligands in human cells (Gorden, Gorski et al. 2005). Whereas TLR7 and TLR8 are regulated by cytokines and can be induced upon cell activation, recent reports have indicated that NK cells and T cells also express these TLRs in some instances (Hart, Athie-Morales et al. 2005, Song, Zhuang et al. 2009).

When receptor/ligand interacts, TLR7 and TLR8 recruit the universal TLR adaptor protein MyD88 through its TIR domain (Hemmi, Kaisho et al. 2002, O'Neill and Bowie 2007). Instead, MyD88 recruits IRAK1 and IRAK4. IRAK4 then goes on to activate IRAK-1 by phosphorylation (Uematsu, Sato et al. 2005, Kim, Staschke et al. 2007). Both IRAK-1 and IRAK4 leave the MyD88-TLR complex and link up temporarily with TRAF6, which leads to its ubiquitination (Kawai, Sato et al. 2004). After ubiquitination, TRAF6 is able to form a complex with TAB2/TAB3/TAK1, inducing TAK1 activation (Besse, Lamothe et al. 2007). TAK1 then links up to the IKK complex which leads to the phosphorylation of IκB and the consequent nuclear localization of NF-κB (Adhikari, Xu et al. 2007). Activation of NF-κB induces the production of pro-inflammatory cytokines. Moreover, TLR7/8 can bind with MyD88 and activate IRAK1 and TRAF6. TRAF6mediates IRF-5 ubiquitination, which is important for IRF-5 nuclear translocation and interferon induction (Schoenemeyer, Barnes et al. 2005, Balkhi, Fitzgerald et al. 2008). Engagement of TLR7 or TLR8 may also activate IRF7 through activation of MyD88, BTK, and TRAF6 (Konno, Yamamoto et al. 2009). Formation of

the MyD88-TRAF6-IRF7 complex induces phosphorylation and IRF7 activation. Activation of IRF5 and IRF7 results in induction of type I interferon (Lazear, Lancaster et al. 2013).

9. Role of TLR7 in immune response against HCV infection

TLR7, which induces a strong type I interferon in pDCs and hepatocytes when it is engaged, has been expected to play an instrumental immunological role in HCV infection (Gibson, Lindh et al. 2002, Sagan and Sarnow 2010, Takahashi, Asabe et al. 2010). Indeed, TLR7 agonists have been shown remarkable antiviral activities by *in vitro* and *in vivo* studies. It was reported by several groups that TLR7 engagement initiated a strong innate immune response against HCV infection. TLR7 agonists have induced an antiviral response in hepatoma cell lines through both interferon-dependent and interferon-independent mechanisms (Lee, Wu et al. 2006). The potential of TLR7 agonist for hepatitis C therapy has been estimated (Lee, Wu et al. 2006, Thomas, Laxton et al. 2007), and the efficiency and safety of those compounds are under evaluation in clinical trials. The administration of TLR7 agonist results in significant interferon induction and suppression of viremia (up to 3 $\log_{10}$) (Horsmans, Berg et al. 2005, Bergmann, de Bruijne et al. 2011, Fidock, Souberbielle et al. 2011). Recently, Erilka et al. have proved the involvement of TLR7 in induction of interferon by HCV and in antiviral response against HCV infection. Knockdown of TLR7 in hepatoma cell lines abolished the induction of interferon-β. Recently Erilka et al. have proved the involvement of TLR7 in induction of interferon by HCV in cell lines that was highly permissive to HCV (Eksioglu, Zhu et al.

2011). However, the role of TLR7/8 in detection of HCV RNA by APCs remains unknown.

The potential implication of TLR7 in the innate immune response against HCV was postulated, nevertheless little is known about it and it is not fully demonstrated. In fact, pDCs have been shown to be able to be stimulated by infected Huh-7 cells in cell-culture by recognizing the viral RNA likely via a TLR7-dependent pathway (Python, Gerber et al. , Takahashi, Asabe et al. 2010). Moreover, the presence of a GU-rich fragment in HCV genome was shown to be detected by TLR7 (Zhang, Guo et al. 2009). Furthermore, the single nucleotide polymorphisms (SNPs) in TLR7 and TLR8 were associated with the magnitude of inflammation and fibrosis during HCV infection, with the response to IFN-α-based therapy and with the susceptibility to the infection with HCV (Schott, Witt et al. 2008, Wang, Eng et al. 2011). However, the role of TLR7 in HCV detection in APCs and the mechanisms leading to this detection are not known. Additionally, the role of TLR8 in the innate immune response against HCV is not known.

10. HCV detection by antigen-presenting cells

Antigen-presenting cells (APCs) are crucial for initiation of antiviral immunity. APCs sense viral components via PRRs to induce an innate immune response, they also capture viral antigens and present to naïve T cells to elicit an adaptive immune response. HCV-specific T cells were detected in HCV-infected chimpanzees and humans, regardless of the outcome of the disease progression, suggesting the capability of APCs for HCV uptake. However, although the immunogenicity of HCV components (RNA or proteins) have been shown, DCs appear “silent” when they encounter HCV. Many efforts

have been made to prove the immunogenicity of HCV particles, but the only common element in these studies is the unresponsiveness of DCs to HCV stimulation. Neither DC maturation nor induction of cytokines was observed (Shiina and Rehermann 2008, Liang, Russell et al. 2009, Zhang, Guo et al. 2009, Takahashi, Asabe et al. 2010). The mechanisms of DC tolerance to HCV are mysterious, and the causes that endow HCV with an "undetectable" character remain unknown. The HCV uptake capability of DCs still remains controversial. The internalization of HCV-like particles by DCs (Ludwig, Lekkerkerker et al. 2004, Barth, Ulsenheimer et al. 2005) and the absence of HCV in DCs from HCV infected-patients (Rollier, Drexhage et al. 2003, Longman, Talal et al. 2004) have both been reported. The presence of the HCV genome in blood cells, which includes DC, has been depicted in numerous studies through reverse transcription (RT)-PCR. However, the presence of viral RNA does not explain whether HCV enters cells or merely sticks to their surface. Recently, it has been shown that PBMCs are certainly not susceptible to HCV (Cormier, Tsamis et al. 2004), HCV in PBMCs from patients are mainly carried on the surface of cells (Natarajan, Kottilil et al. 2010). Freshly isolated plasmacytoisd DCs (pDCs) and myeloid DCs (mDCs) are not susceptible to pseudotype HCV, however, incubation with GM-CSF makes mDCs highly susceptible to pseudotype HCV (Kaimori, Kanto et al. 2004). In contrast, Monocyte-derived DCs (mDDCs) appear to have great capability for HCV uptake, which depends on DC-SIGN that is induced by GM-CSF/IL-4 (Ludwig, Lekkerkerker et al. 2004, Barth, Ulsenheimer et al. 2005).

The trail by which DCs are able to internalize antigens for presentation of T cells is receptor-mediated endocytosis, in which C-type lectins act in an important capacity. An increasing number of C-type lectins with specificity for mannosylated antigens have been

discovered to be expressed by DCs such as Langerin (Valladeau, Ravel et al. 2000), mannose receptor (Sallusto, Cella et al. 1995), DEC-205 (Kato, Neil et al. 1998), and DC-SIGN (Engering, Geijtenbeek et al. 2002). Studies suggest that DC-SIGN is understood to play an active in infection of DCs by the dengue virus (Navarro-Sanchez, Altmeyer et al. 2003, Tassaneetrithep, Burgess et al. 2003), another member of the *Flaviviridae* family. Recently, studies depicting recombinant HCV envelope glycoprotein 2 (E2) and HCV pseudotype particles (HCVpp's) have indicated that DC-SIGN and its liver-expressed homologue L-SIGN are vital receptors for HCV envelope glycoproteins E1 and E2 (Pohlmann, Zhang et al. 2003, Lozach, Amara et al. 2004, Ludwig, Lekkerkerker et al. 2004). Both HCV E1 and E2 are able to bind the same binding site on DC-SIGN as HIV and mycobacteria. Remarkably, internalized HCV virus-like particles were targeted towards non-lysosomal compartments within immature DCs in a not so dissimilar manner to that established for HIV-1 (Ludwig, Lekkerkerker et al. 2004), suggesting that HCV may target DC-SIGN to escape detection and facilitate viral dissemination.

HYPOTHESIS AND OBJECTIVES

Hypothesis:

Immune response induced by HCV infection is characterized as rapid innate immune response and delayed adaptive immune response (Dustin and Rice 2007), regardless of the outcome of the disease progression. Innate immune activation was observed at an early stage after HCV infection in chimpanzees (Bigger, Brasky et al. 2001). Antigen presenting cells (APCs) are deemed to be responsible for HCV detection, however, the mechanisms of immune activation induced by HCV infection has been difficult to clarify. It has been shown that HCV RNA is capable to induce anti-viral response (Saito, Owen et al. 2008, Zhang, Guo et al. 2009), nevertheless, the detection of HCV by APCs still remains elusive. Furthermore, the single nucleotide polymorphisms (SNPs) in TLR7 and TLR8 were associated with the magnitude of inflammation and fibrosis during HCV infection, with the response to interferon based therapy and with the susceptibility to the infection with HCV (Schott, Witt et al. 2008, Wang, Eng et al. 2011). However, the role of TLR7 and TLR8 in HCV detection by APCs and the mechanisms leading to this detection are still not known. We speculate that APCs has a unique way to detect HCV and TLR7/8 is involved in this process.

Objectives

1. Determine the capacity of HCV RNA to induce immune response. Is HCV RNA able to induce inflammatory cytokine? Dose HCV RNA contains GU-rich motifs which may be recognized by TLR7/8? Are those GU-rich sequences able to induce inflammatory cytokine and DC maturation?
2. Determine the role of TLR7 and TLR8 in HCV RNA detection. Does HCV RNA specifically trigger TLR7/8? Are those GU-rich sequence selected from HCV genome able to induce cytokines in similar profile as validated TLR7/8 agonist?
3. Determine the immune response induced by HCV RNA in different antigen presenting cells (APCs) and the anti-viral activity of such immune response. Does HCV RNA induce type I interferon? Do cytokines produced by APCs upon HCV RNA stimulation inhibit HCV replication?
4. Determine the role of different APCs in HCV detection and anti-HCV immune response. Are APCs able to respond to HCV particles? Is there any preference of APCs in HCV detection? Does HCV induce type I interferon in APCs? Is there any difference between the immune response induced by HCV RNA and particle? What are the mechanisms involved in HCV detection by APCs?

CHAPTER 2

HCV derived ssRNA induce innate immune response against HCV infection via TLR7 and TLR8

HCV derived ssRNA induce innate immune response against HCV infection via TLR7 and TLR8

Yuwei zhang[1-4], Elias Said[1-3], Abdel-Hakeem MS[1], Zhong He[1-4], Francesco A. Procopio[1-4], Rafick-Pierre Sekaly[1-6]

[1]Centre de recherche du centre Hospitalier de l'Université de Montréal (CRCHUM), Hôpital Saint-Luc, Québec H2X 1P1, Canada

[2]Laboratoire d'Immunologie, Département de Microbiologie et d'Immunologie, Université de Montréal, Montréal, Québec H3T 1J4, Canada

[3]Institut national de la Santé et de la Recherche médicale U743, Centre de Recherche, CHUM, Université de Montréal, Montréal, Québec H2X 1P1, Canada

[4]Vaccine and Gene Therapy Institute-Florida (VGTI-Fl), 11350 SW Village Pkwy, third floor. Port St. Lucie, Florida. USA, 34987.

[5]Departement of Microbiology and Immunology, McGill University, Montréal, Québec H3A 2B4, Canada

[6]Corresponding authors

Correspondence should be addressed to Rafick-Pierre Sekaly

COMPETING INTERESTS STATEMENT

The authors have declared that no conflict of interest exists.

Abstract

The hepatitis C virus (HCV) is a positive single-stranded RNA (ssRNA) virus that replicates in the liver and has infected 200 million people worldwide. Recognition of HCV-associated molecular patterns is crucial for initiation of innate immune response against HCV infection (Schoggins and Rice 2013). As a ssRNA virus, HCV RNA might be detected by Toll-like receptor 7 or 8, an endosomal/lysosomal RNA sensor, resulting in induction of type I interferon that limits infection. Although antiviral activities of the TLR7 agonist have been reported (Horsmans, Berg et al. 2005, Bergmann, de Bruijne et al. 2011), the role of TLRs in recognizing HCV and the induction of an immune response against HCV have not been well defined. In this study, we demonstrate that the HCV genome contains GU-rich sequences that can specifically trigger TLR7/8. The engagement of TLR7/8 by HCV-derived ssRNA results in the maturation of pDC and the production of type I interferon, and also leads to induction of inflammatory cytokines and chemokines in different APCs. Cytokines produced by monocytes and pDCs upon stimulation of HCV-derived ssRNA inhibit HCV production in an IFN-dependent manner, whereas HCV-ssRNA and cytokines produced by activated mDCs do not affect viral production due to lack of interferon signaling. HCV ssRNA-mediated cytokines also down-regulate CD81 expression on huh7.5 in an IFN-independent manner, which might inhibit HCV infection. Our results suggest that HCV RNA has the potential to trigger TLR7/8, which subsequently initiates an innate immune response against HCV infection.

Key words: HCV, single-strand RNA, TLR7, TLR8, dendritic cells, interferon, CD81

Introduction

The hepatitis C virus (HCV) is a worldwide public health problem affecting an estimated 200 million individuals (Lauer and Walker 2001). There are approximately 3 to 4 million new cases of HCV infection each year (WHO 2011). No effective anti-HCV vaccine currently exists. Only 20% of HCV-infected individuals can be cured by spontaneous viral clearance. Approximately 80% of infected individuals develop chronic infection (Manns, Foster et al. 2007). The progression of liver disease usually takes decades. Up to 20% of those infected may develop complications including cirrhosis, liver failure, or hepatocellular carcinoma (Afdhal 2004). Although antiviral therapies have improved considerably in recent years, they are associated with significant toxicity and are effective in only about one-half of those treated (Manns, Foster et al. 2007). Novel anti-HCV drugs targeting HCV proteins that are essential for HCV replication are under development. These drugs have achieved great effectiveness in HCV treatment (Asselah and Marcellin 2013). However, mutations of target viral proteins occur rapidly, causing resistance and failure of treatment (Rong, Dahari et al. 2010, Pawlotsky 2011). Thus, a better understanding of immunity against HCV is required to develop a solution for hepatitis C.

Innate immune defenses are essential to control viral infection through recognition of viral macromolecular motifs known as pathogen-associated molecular patterns (PAMPs). Pathogen recognition receptors (PRRs) recognize PAMPs and initiate innate immune responses (Gordon 2002, Janeway and Medzhitov 2002, Arpaia and Barton 2011). RNA viruses have the potential to be recognized by RNA sensors of immune cells that initiate immune responses against viral infection, i.e., influenza virus

(Diebold, Kaisho et al. 2004) and HIV detected by TLR7/8 (Heil, Hemmi et al. 2004). HCV is an enveloped positive single-stranded RNA virus that replicates in the liver. Several PRRs are involved in HCV recognition (Horner and Gale 2013). Recently, HCV RNA was reported to activate an innate immune response by activating the RIG-I pathway in hepatocytes (Saito, Owen et al. 2008). As a bridge from innate immunity to adaptive immunity, DCs uptake viral particles, then process and present antigens expressed by these particles to T-cells (Villadangos and Schnorrer 2007). DCs sense the presence of a virus by TLRs present in the endosomes-lysosome where RIG-I is not expressed (Thompson and Locarnini 2007). TLR3 and TLR7/8 are known RNA sensors in the endosomes-lysosome (Takeda, Kaisho et al. 2003). TLR3 recognizes double-strand RNA (dsRNA) (Alexopoulou, Holt et al. 2001). For HCV, dsRNA form exists only during viral replication. In the HCV-infected liver, TLR3 might be engaged by debris from nearby dying cells (Schulz, Diebold et al. 2005). TLR7/8 recognizes ssRNA in the endosomes-lysosome (Heil, Ahmad-Nejad et al. 2003). It is most likely that TLR7 or TLR8 senses HCV RNA in APCs. Hence, it is tempting to speculate that the HCV-genomic ssRNA could be recognized by TLR7 and/or TLR8 and contribute to immune activation during HCV infection. Here we investigate the immune response induced by HCV RNA and its effect on HCV infection.

Results

1. Identification of ssRNA sequences in HCV genome that stimulate innate immune responses

Like many viruses, HCV could be taken-up by DCs and processed through the endosomes-lysosome (Barth, Ulsenheimer et al. 2005). Several TLRs that can sense viral RNA are present in DCs endosomes-lysosome, such as TLR3, TLR7, and TLR8 (Thompson and Locarnini 2007). HCV RNA is potentially able to trigger those RNA sensors and initiate innate immunity. HCV is a single positive-strand RNA virus. Although several RNA sensors have been identified, TLR7 and TLR8, which are responsible for detection of single-strand RNA, are most likely the receptors sensing HCV RNA.

First, we investigated the capacity of HCV RNA sequences to stimulate the innate immune response. Extracted HCV RNA was fused to DOTAP and incubated with human PBMCs. TNF-α was measured at 24hrs by ELISA. As shown in Figure 1A, HCV RNA induces TNF-α production by PBMCs (400 pg/ml, $p<0.0001$), indicating that HCV RNA has the ability to trigger innate immunity. Indeed, based on the previous studies that have defined the G/U-rich motif as a primary structural feature recognized by TLR7 and TLR8 (Heil, Hemmi et al. 2004), we selected nine GU-rich ssRNA sequences (HCVL1 to HCVL9) that contain 65% G/U or more in the genomic RNA of HCV (Table 1) as potential ligands of TLR7 and 8. The ability of those GU-rich RNA to trigger TLR7/8 was evaluated. For this purpose, 10^6/ml PBMCs from healthy donors (N = 3) were stimulated with 7.5 μg/ml of each ligand for 24 hours, and TNF-α in the supernatant was

then measured by ELISA. Three of those GU-rich RNA sequences (HCVL1, HCVL4, and HCVL8) elicited TNF-α production (2300, 1800, 2100 and 3400 pg/ml for HCVL1, 4, 8 and RNA40 respectively, $p<0.0001$). However, six of those selected GU-rich RNA sequences were unable to induce cytokine production (Figure 1B). Those data suggest that certain GU-rich sequences, not all, may endow HCV RNA with stimulatory activity. In order to validate that the stimulation was induced by RNA and depended on sequences, we introduced RNase treatment and U-to-A replacement of RNA sequences. As we expected, the stimulatory activities of HCVL1, HCVL4, and HCVL8 were abolished by RNase treatment. RNA sequences with U-to-A replacement did not elicit TNF-α production (Figure 1.C), demonstrating that the immunogenicity of HCV-derived ssRNA requires the RNA containing the GU motif. In addition, blockage of TNF-α production was observed in the presence of IRS661, a specific inhibitor of TLR7/8 (Fig 1D). Our data further confirmed the specificity of the immune response induced by HCV ssRNA.

2. Specific recognition of HCV-derived ssRNA by TLR7 and TLR8

To further investigate the capacity and specificity of HCV-derived ssRNA in triggering TLR7 and TLR8, we first transfected HEK cells expressing TLR3, TLR7 or TLR8 (InvivoGen) with luciferase expression vectors pNifty2-luc, which allows luciferase production upon NF-κB activation. Eight hours later, cells were stimulated with HCVL1, HCVL4, or HCVL8 for 24hrs, and luciferase activity was measured. Our results showed an increase of the luciferase activity when TLR7- and TLR8-expressing cells were treated with these ssRNAs (4-, 2- and 3- fold increase for HCVL-1, 4 and 8 respectively in TLR7 expressing cells, and 15-, 6- and 3- fold increase respectively in

TLR8 expressing cells (Figure 2 B and C)). Luciferase activity did not change by using HEK cells expressing TLR3 that sense ssRNA (Figure 2). Our data clearly showed that three identified GU-rich sequences from the HCV genome act as specific ligands that activate TLR7 and TLR8.

3. Cytokine production and DC maturation upon ssRNA stimulation

In order to investigate the capacity of three identified RNA sequences on the induction of cytokines by APCs (including monocyte, mDCs and pDCs), DCs were stimulated with 7.5 μg/ml of each identified ssRNA for 24 hours. Production of inflammatory cytokines and chemokines were monitored by Cytometric Bead Array (CBA). The results showed that HCV-derived ssRNAs strongly induce inflammatory cytokines (IL-1β, IL-6, IL-10, TNF-α, and IL-12p70) and chemokines (CXCL9 and CXCL10) (Figure 3). Vast amounts of TNF-α and IL-6 were induced in monocyte, mDC, and pDC upon stimulation. CXCL9 and CXCL10 were mainly produced by pDCs. IL-1β, IL-12, and IL-10 were produced by monocytes and mDCs but barely detected in the supernatant of stimulated pDCs.

As a major source of type I interferon, pDC is always the hotspot for the study of innate immunity against HCV infection. Here we also measured IFN-α and IFN-β production by pDCs with HCV RNA stimulation (as described above). Moreover, pDC maturation upon stimulation with HCV ssRNA was detected by flow cytometry. As expected, HCV ssRNA acted as Cl097, an agonist for TLR7 and TLR8. pDCs produced vast amounts of IFN-α (1600, 1500 and 1700 pg/ml respectively, $p<0.0001$) and IFN-α (1300, 1000 and 2250 pg/ml respectively, $p<0.0001$) upon ssRNA stimulation (Figure

4A). Maturation markers CD80 and CD86 were also significantly up-regulated (Figure 4B). Significantly, the RNase treatment or the introduction of U-to-A mutations led to the abrogation of their stimulatory effect on pDCs, also confirming the specificity of the responses.

4. Effect of HCV-derived ssRNA-induced cytokines on HCV infection

It has been reported that TLR7 agonists induce type I interferon to suppress HCV replication (Bergmann, de Bruijne et al. 2011). Here we examined the antiviral activity of HCV-ssRNA-mediated cytokines. Purified monocytes, mDC, and pDC were stimulated with identified HCV ssRNA for 24hrs. Cell-free supernatants were harvested and added to HCV infected huh7.5 cells. Viral production was measured by real-time PCR at day 3. The results showed that the number of HCV in the supernatant was significantly decreased (2 to 3 $\log_{10}$) in the presence of HCV ssRNA-induced cytokines. However, surprisingly, only supernatants from ssRNA-stimulated monocytes and pDC had antiviral activities that suppressed viral production in HCV-infected huh7.5 cells. A similar effect was not observed on supernatants from stimulated mDCs (Figure 5 A).

Since type I interferon was considered a main factor of TLR7-medicated cytokines that inhibit HCV replication, a lack of antiviral activity of mDCs may be caused by the absence of type I interferon in the supernatant of stimulated mDCs. Thus, we examined the interferon activity of supernatants from HCV ssRNA-stimulated monocytes, mDC, and pDC. Interferon activity was determined by induction of pStat1 Y701 phosphorylation. PBMCs were incubated with supernatants from HCV ssRNA-stimulated monocytes, mDC, and pDC respectively, after 15min incubation. Stat1 Y701

phosphorylation in lymphocytes was measured by flow cytometry. Consistent with the antiviral activity assay, only supernatants from ssRNA-stimulated monocytes and pDC induced Stat1 Y701 phosphorylation (Figure 6). This indicates TLR7/8 engagement induced production of type I interferon in monocytes and pDCs but not in mDCs. To confirm that suppression of viral production is mediated by type I interferon, an antibody against Interferon-Alpha/Beta R2 (IFNAR2) was used to block interferon signaling induced by HCV ssRNA-mediated cytokines. The results showed that IFNAR2-blocking antibodies restore HCV production in the presence of supernatants from stimulated monocytes or pDC (Figure 5B). They also revealed that HCV ssRNA-activated monocytes and pDC inhibit HCV production in an IFN-dependent manner.

5. HCV-derived ssRNA-mediated cytokines down-regulate CD81 expression on huh7.5

Several cell surface proteins have been suggested to mediate the binding of HCV to hepatocytes and/or to be critical for viral entry. Among the acknowledged receptors or co-receptors of HCV are CD81 (Pileri, Uematsu et al. 1998), scavenger receptor class B type I (SCARB1) (Scarselli, Ansuini et al. 2002), low-density lipoprotein receptor (LDLR) (Agnello, Abel et al. 1999), and the recently identified claudin-1 (CLDN1) (Evans, von Hahn et al. 2007). As a major receptor for HCV binding and viral entry, CD81 plays a critical role in HCV infection (McKeating, Zhang et al. 2004, Lindenbach, Evans et al. 2005). CD81-neutralizing antibodies can block HCV infection. It was reported that huh7.5 cells with a low level of CD81 expression are less permissive to

HCV (Zhang, Zhang et al. 2010). It has been shown that IFN-alpha down-regulates CD81 on PBMCs in patients with chronic hepatitis C (Kronenberger, Ruster et al. 2001). Thus, we examined whether TLR7/8 mediated cytokines modulate CD81 expression on huh7.5 cells. Huh7.5 cells were incubated with supernatant from TLR7/8-agonist-stimulated PBMCs. IFN-α was set as control. After 24hrs, CD81 expression on huh7.5 was measured by flow cytometry. Results showed that TLR7/8-mediated cytokines induced a significant down-regulation of CD81 expression (Figure7). Compared to CD81 expression levels on huh7.5 cells (MFI 5118±147) with supernatant from non-stimulated PBMCs, CD81 expression on huh7.5 with supernatant from ssRNA-stimulated PBMCs was remarkably decreased (MFI, HCVL1 3473±189, HCVL4 3354±192, HCVL8 3166±301, Cl097 3233±375, $P<0.0001$). Surprisingly, IFN-alpha treatment barely affected CD81 expression (MFI 4682±34), indicating that down-regulation of CD81 is mainly elicited by TLR7/8-mediated cytokines other than IFN-α.

6. HCV-derived ssRNA pretreatment impairs pDCs capacity to produce IFNα

IFN-α produced by pDC is thought to be an important immune response to control HCV infection. However, the ability of pDC to produce IFN-α upon *in vitro* stimulation is significantly reduced in HCV-infected individuals (Uno, Suginoshita et al. 2005, Dustin and Rice 2007). Recent studies have found that TLR activation in DC causes tolerance in response to further stimulation (Assier, Marin-Esteban et al. 2007). Therefore, we investigated the effect of pre-exposure to HCV-encoded ssRNA on IFN-α production by pDCs response to further stimulation. PBMCs were stimulated with HCV ssRNA HCVL1, HCVL4, and HCVL8 for 24h. Cells were washed and then re stimulated

with the same HCV ssRNA or Cl097 (TLR7/8 agonist). Brefeldin A was added 2h after stimulation. IFN-α production in pDC was detected at 6h by intracellular staining. Our results showed that ssRNA or Cl097 could barely induce IFN-α expression in pretreated pDC (Figure 8). Our results also showed that the pre-exposure of pDCs to HCV-derived ssRNA significantly impaired IFN-α expression upon re-stimulation with TLR7 and 8 ligands (i.e., HCV ssRNA or Cl097), suggesting that persistent TLR7/8 activation by viral RNA triggering induces pDC tolerance during HCV infection.

Discussion

PAMPs expressed by pathogens can be detected by PRRs, leading to the initiation of innate immune responses against infections (Mogensen 2009). As the HCV genome is a positive single-strand RNA, TLR7 and TLR8 very likely play an important role in the immune response against HCV. Here, we evaluated the immunostimulatory effect of HCV RNA and demonstrated that HCV RNA has the ability to stimulate immune cells and induce inflammatory cytokines. Three RNA sequences were identified as TLR7/8 agonists that induce TNF-α production by PBMCs and specifically trigger TLR7/8. The specificity was validated by TLR7/8 inhibitor, RNA mutants and TLR7 or TLR8 expressing cell lines. Those results revealed that HCV RNA can be recognized by TLR7/8 and has the potential to induce immune activation.

Most studies have focused on TLR7 signaling in pDC (Zhang, Guo et al. 2009), as pDC is considered the main source of IFN-α. Some studies have focused on the antiviral activity of the TLR7 agonist in hepatocytes (Lee, Wu et al. 2006), as they are the main target of HCV. However, HCV also can be captured by antigen-presenting cells, and viral RNA can be detected by TLR7/8 expressed in those cells (Barth, Ulsenheimer et al. 2005, Takahashi, Asabe et al. 2010). Here we examined the stimulatory effects of HCV ssRNA on monocytes, mDCs, and pDCs. Like the TLR7/8 agonist, HCV-derived ssRNA induced inflammatory cytokine production by different APCs subsets. The profile of cytokine production is quite different in monocytes, mDCs, and pDCs. The cytokine production preference may reveal the different roles of APCs in immune regulation and antiviral immunity. Furthermore, most of those cytokines are known to be up-regulated *in vivo*

during HCV infection, and some of them are correlated with disease progression, such as TNF-α and IL-8 were known as key factors in liver cirrhosis and resistance to interferon treatment (Polyak, Khabar et al. 2001).

Several agonists of Toll-like receptors have shown great antiviral activities, such as agonists of TLR3, TLR4, TLR7/8, and TLR9 (Doyle, Vaidya et al. 2002, Lee, Wu et al. 2006, Trapp, Derby et al. 2009, Ong, Wikstrom et al. 2013). Cytokines produced by activated PBMCs or hepatic stellate cells strongly inhibit HCV replication (Thomas, Laxton et al. 2007, Wang, Trippler et al. 2009). As a strong inducer of type I IFN, the TLR7 agonist has been broadly studied for its antiviral activities (Horsmans, Berg et al. 2005, Lee, Wu et al. 2006, Thomas, Laxton et al. 2007), and the potential of the TLR7 agonist for clinical therapy of hepatitis C has recently come under evaluation (Bergmann, de Bruijne et al. 2011). Here we showed that HCV ssRNA triggers TLR7/8 to initiate innate immunity against HCV in an IFN-dependent and independent manner (Figure 5B). First, we demonstrated that HCV RNA activated APCs and induced inflammatory cytokines. In accordance with previous studies, activated pDC has significant antiviral activity. HCV-ssRNA-induced cytokines produced by pDC significantly inhibited HCV production. Moreover, supernatants from ssRNA-stimulated monocytes that also suppressed viral production in a IFN dependent manner. The inhibitory effect can be abolished by neutralizing antibodies against IFN-α/β receptor 2. It indicates that not only pDCs but also monocytes contribute to innate immunity against HCV. Conversely, supernatants from HCV ssRNA-stimulated mDCs barely affect viral production since there is no IFN signaling induced by its supernatant. Those data confirm that interferon is

crucial for the control of HCV infection. TLR7/8-mediated cytokines inhibit HCV replication in an IFN-dependent manner.

CD81 is a main receptor for HCV infection. It was reported that IFN-α therapy down-regulated CD81 expression on PBMCs in HCV (Kronenberger, Ruster et al. 2001, Kronenberger, Herrmann et al. 2006). As we showed above, engagement of TLR7/8 by HCV ssRNA resulted in production of type I interferon. Thus, we also examined the effect of HCV-ssRNA-mediated cytokines on the expression of CD81 on huh7.5 cells. Our results revealed that HCV ssRNA-induced cytokines caused approximately 40% down-modulation of CD81 on huh7.5, but a similar phenomenon was not observed on huh7.5 with IFN-α treatment, which was expected to be a positive control. It is still unclear how IFN-α induced down-regulation of CD81 on PBMCs in HCV infected individuals. The differences between our observation and others are probably due to different mechanisms. CD81 is a widely expressed surface protein, and it is involved in cell activation. CD81 expression on PBMCs was increased in HCV-infected individuals (Kronenberger, Ruster et al. 2001, Kronenberger, Herrmann et al. 2006). Up-regulation of CD81 might be induced by viral infection or associated immune activation. IFN-α therapy suppressed HCV replication, resulting in the decrease of viral replication that turned immune activation toward normal levels. Less activation accompanied lower CD81 expression on PBMCs. Another possible mechanism is that IFN-α may affect CD81 expression by inducing degradation of CD81 directly, but it seems to work differently on various cell types (Kronenberger, Herrmann et al. 2006). In our study, changes of CD81 expression on huh7.5 are not due to HCV infection. IFN-α may have no effect on CD81 expression of huh7.5. Thus, IFN-α may not regulate CD81 expression in

the same way as it does on PBMCs *in vivo*. Cytokines induced by TLR7/8 probably decrease CD81 expression by regulation of CD81 transcription or induce CD81 degradation by proteins, such as PCSK9 (Labonte, Begley et al. 2009).

Two RNA sensors, TLR7 and RIG-I, have been broadly studied in innate immunity against HCV infection. RIG-I was thought to be critical in the detection of HCV since the deficient RIG-I was founded in huh7.5, a hepatoma cell line that is highly permissive to HCV (Sumpter, Loo et al. 2005, Saito, Owen et al. 2008). However, RIG-I is not indispensable for control of HCV replication (Feigelstock, Mihalik et al. 2010), both RIG-I and TLR7/8 could be involved in detection of HCV, even in hepatic cells (Lee, Wu et al. 2006). In this study, we showed that TLR7/8 also plays an important role in induction of innate immunity against HCV. TLR7/8 is expressed in endosomes/lysosomes, and RIG-I is expressed in cytoplasm (Thompson and Locarnini 2007). During the life cycle of HCV infection, viral RNA is released to cytoplasm after entry and uncoating, where RNA may be detected by RIG-I, leading to induction of an antiviral response (Saito, Owen et al. 2008). APCs capture viruses by different ways other than natural infection (Mercer and Greber 2013). HCV can bind to an antigen-capture receptor like DC-SIGN and be internalized into APCs (Ludwig, Lekkerkerker et al. 2004), which are involved in endosomes/lysosomes where viral RNA might be exposed to TLR7/8. Both RIG-I and TLR7/8 can sense HCV ssRNA, however, in certain cell types such as pDCs, detection of RNA viruses is totally dependent on TLR7 (Lee, Lund et al. 2007). Since pDCs is a major source of type I IFN, TLR7 is more important in the control of HCV infection. Furthermore, detection of HCV by TLR7/8 occurs before synthesis of viral protein starts. Nonstructural proteins of HCV possess the capability to

interact with adaptors of TLR or RIG-I pathways that strongly compromise signaling and initiation of a consequential response. So it seems TLR7/8 signaling is more efficient as it starts without interference of HCV proteins.

In conclusion, we reveal that HCV RNA contains GU-rich sequences and can specifically trigger TLR7/8. Immune activation induced by HCV RNA can suppress HCV production in an IFN-dependent manner. It can also down-regulate CD81 expression in an IFN-independent manner, which might decrease HCV infection. Thus, activation of TLR7/8 by HCV RNA may contribute to anti-HCV immune responses, however, repetitive stimulation by HCV RNA causes anergy of pDC.

Materials and methods

Cell lines and plasmid

Hepatoma cell huh7.5 (kindly provided by Dr. Charles Rice, Rockefeller University) and Hepatoma cell Huh7.5-20 producing JFH1 HCV (Kindly provide by Dr. T. Jake Liang, National Institutes of Health) were cultured in DMEM-based complete growth medium. 293HEK cells expressing TLR3, TLR7 or TLR8, NF-κB inducible plasmid pNiFty2-Luc were obtained from invivogen. pNiFty2-Luc contains report gene expression cassette that allows firefly luciferase production under control of NF-κB activation.

Cell isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated from leukapheresis following ficoll procedure. Monocytes were isolated from PBMCs by using human monocytes enrichment kit (STEMCELL Technologies Inc, Canada), purity was confirmed by staining with CD14-PB. pDCs were isolated from PBMCs by using Human Plasmacytoid DC Enrichment kit (STEMCELL Technologies Inc, Canada), and mDCs were isolated from PBMCs by using $CD1c^+$ Dendritic Cell Isolation Kit (Miltenyi Biotec Inc, USA), purity was confirmed by staining with Linage-Alexa700 (CD3, CD14, CD19, CD16), HLA-DR-APC-cy7, CD11c-APC and CD123-PE. Cells were cultured in RPMI1640 containing 10% human serum.

Stimulation of cells and detection of cytokines

HCV RNA was extracted from supernatant of HCV producing cell line huh7.5-20 by using QIAamp Viral RNA Mini Kit (QIAGEN, USA). HCV derived ssRNA containing more than 65% GU were selected and synthesized (Integrated DNA Technologies,

Canada). HCV RNA or HCV derived ssRNA were incubated with DOTAP for 15min. PBMCs, monocytes, mDC and pDC were stimulated with RNA-DOTAP complex for 24hrs. Supernatants were harvest and TNF alpha production was measured by Human TNF alpha ELISA Ready-SET, Type I Interferon was measured by Human Interferon alpha ELISA kit and Human Interferon Beta ELISA Kit (PBL Interferon Source, USA), inflammatory cytokines and chemokines were measured by Cytometric Bead Array (CBA) using Human Inflammatory Cytokine Kit and Human Chemokine Kit (BD biosciences, USA). RNA with U to A replacement and RNA with RNase A treatment were set as negative control.

Specificity of TLR7/8 engagement

5 x10^4 293HEK cells expressing TLR3, TLR7 or TLR8 were seeded in 96 well plate and cultured for overnight. Cells were transfected with pNiFty2-Luc when confluence reaches 80%. After 8hrs transfection, cells were stimulated with HCV ssRNA that fused to lipofectamine 2000 (invitrogen, USA), after 24hrs culture, cells were washed and lysis, luciferase activity was measured by Luciferase Assay System (promega, USA).

Viral production, infection and quantification

Cell line huh7.5-20 stably producing HCV was cultured in DMEM-based complete growth medium for 4 dyas, supernatant was harvested and filtered by 0.45um filter (sarstedt, Germany), viral load was quantified by real time PCR and viral stock was stored at -80 °C for further use. 2 x10^4 huh7.5 cells were seeded in 96 wells plate and cultured for overnight, then cells were incubated with virus at ratio 10 (RNA/cells), after 4hrs incubation cells were washed and refilled with fresh medium. For viral RNA quantification, viral RNA was extracted from supernatant and real time PCR was

performed by using QuantiFast SYBR Green RT-PCR Kit (QIAGEN, USA), forward primer 5'-CGGGAGAGCCATAGTGG-3', reverse primer 5'-AGTACCACAAGGCCTTT-3'. RNA standard was prepared by in vitro transcription using MEGAscript® T7 Kit (ambion, USA), quantity of RNA was determined by OD260 and RNA copies were calculated by formula from QIAGEN.

Suppression of viral production by HCV-ssRNA mediated cytokines

10^5 monocytes, mDC, or pDC were stimulated with HCV derived ssRNA for 24hrs, cells free supernatants were harvested. 2 x10^4 huh7.5 cells were seeded in 96 wells plate and cultured for overnight, cells were incubated with HCV for 4hrs and then washed, medium containing 10% of supernatant from non-stimulated or stimulated monocytes, mDCs or pDCs were added. Supernatants for infected huh7.5 were harvested at 72hrs and centrifuge at 1500rpm for 5min to remove debris. Viral load was quantified by real time PCR as described above. To show interferon dependent suppression, Human IFN-alpha/beta R2 MAb (Clone MMHAR-2, R&D Systems, USA) were added (10ug/ml) to HCV infected huh7.5 and incubated for 1h before adding supernatant from stimulated monocytes or pDCs.

DC maturation marker and CD81 expression by flow Cytometry

pDCs were stimulated with ssRNA HCVL1, HCVL4, HCVL8 and RNA mutants or RNase treated RNA for 24hrs, cells were stained with anti-CD80-FITC and anti-CD86-PE (bdbiosciences, USA), maturation marker CD80 and CD86 were monitored by flow cytometry. Huh7.5 cells were incubated with supernatants from stimulated PBMCs for 24hrs, cells were stained with anti-CD81-PE (bdbiosciences, USA) and detected by flow Cytometry.

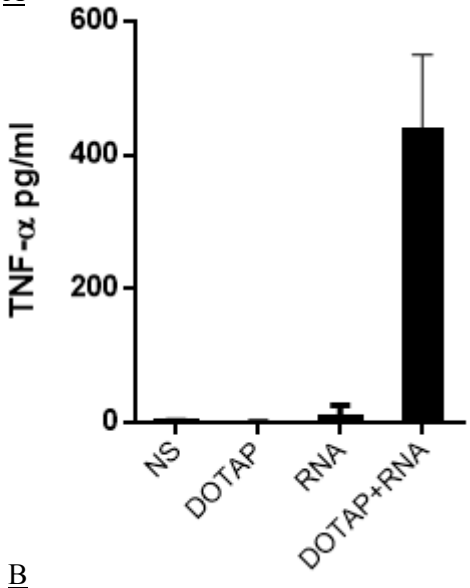
Statistical analysis

p-values were determined using two way paired student's *t* tests or Newman-Keuls one-way analysis. *p* –values less than 0.05 were considered statistically significant.

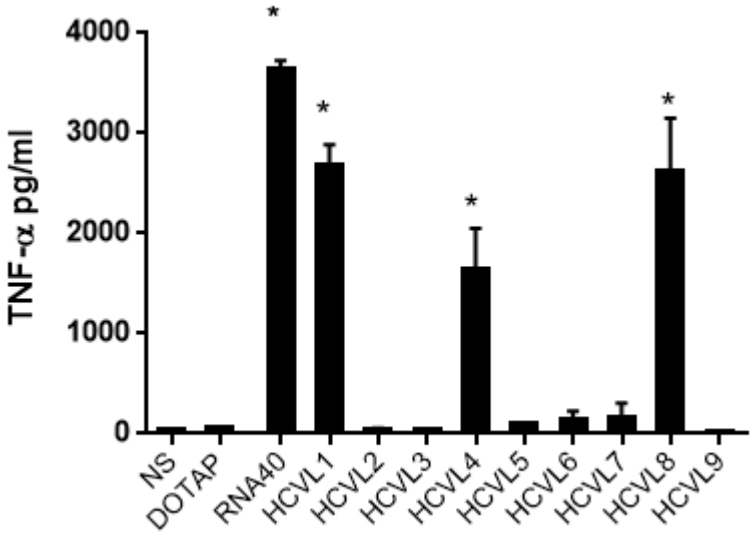
	Sequence	Region	Conservation
HCVL1	GUAGUGUUGGGUCGCGAAAGGCCUUGUGGU	5'UTR	genotype 3a and 1b
HCVL2	GUCAAGUUCCCGGGUGGCGGUCAGAUCGUUGGUGGAGUUUACUUGUUG	Core	genotype 1a and 1a/2a
HCVL3	UGUGCGGGUCUGUCUUUCUUGUUGGUCAACUGUUUACCUU	E1	genotype 1a, 2b, 2d, 6d
HCVL4	UUGUGGACGUGCAGUACUUGUACGGGGUAGGGU	E2	genotype 1a
HCVL5	GUCUUGUGUCCUUCCUCGUGUUCUUCUGCUUUGCGUGGUAUCUGAAGGGUAGGUGGGU	p7	genotype 1a and 1a/2a
HCVL6	GUCUUGUGUCCUUCCUCGUGUUCUUCUGCUUUGCGUGGU	p7	genotype 1a and 1a/2a
HCVL7	GUGGCCGCGUCGUGUGGCGGCGUUGUUCUUGUCGGGUU	NS2	genotype 1a and 1a/2a
HCVL8	GUUGUCGUCGUGUCGACCGA	NS3	genotype 1a and 1a/2a
HCVL9	GUGUGUGGCGACGACUUAGUCGUUAUCUGUGAAAGUG	NS5b	genotype 1a and 1a/2a

Table1 GU rich sequences in HCV genome

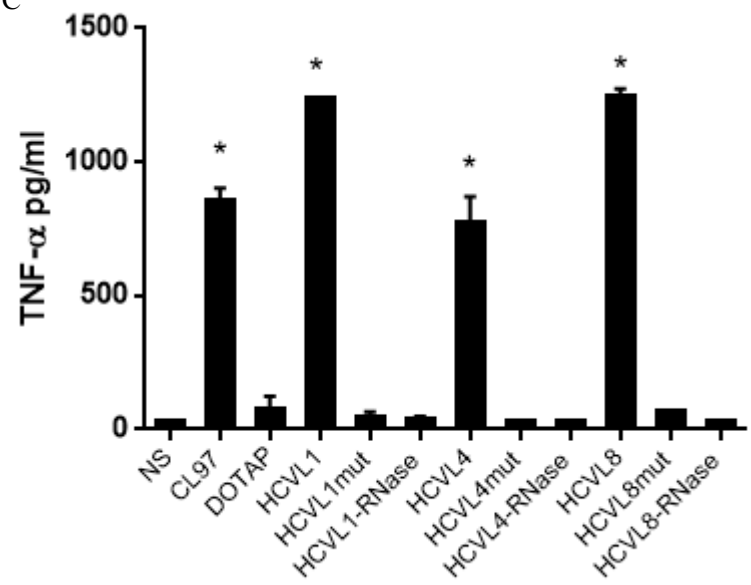
A


600
400
200
0
TNF-α pg/ml
NS
DOTAP
RNA
DOTAP+RNA

B


4000
3000
2000
1000
0
TNF-α pg/ml
*
*
*
*
NS
DOTAP
RNA40
HCVL1
HCVL2
HCVL3
HCVL4
HCVL5
HCVL6
HCVL7
HCVL8
HCVL9

C


1500
1000
500
0
TNF-α pg/ml
*
*
*
*
NS
CL97
DOTAP
HCVL1
HCVL1mut
HCVL1-RNase
HCVL4
HCVL4mut
HCVL4-RNase
HCVL8
HCVL8mut
HCVL8-RNase

D

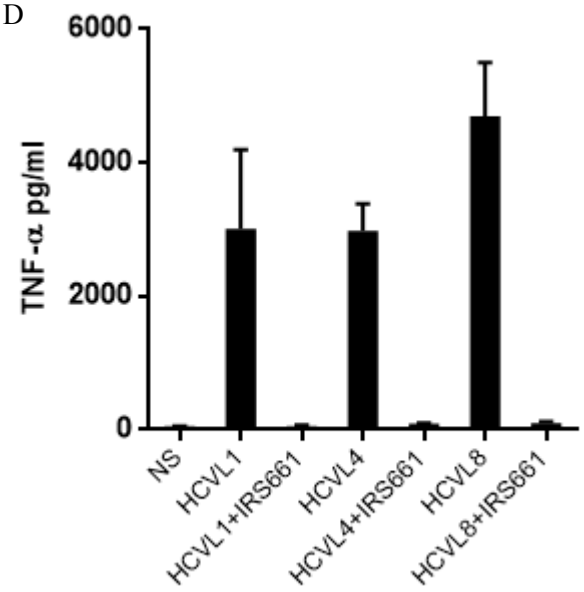

6000
4000
2000
0
TNF-α pg/ml
NS
HCVL1
HCVL1+IRS661
HCVL4
HCVL4+IRS661
HCVL8
HCVL8+IRS661

Figure 1: GU-rich sequences in HCV genome induce TNF-α production.

Identification of ssRNA from HCV genome that stimulates innate immune response

A. PBMCs were stimulated with DOTAP or extracted HCV RNA that was fused with DOTAP for 24hrs. B. Screening of single stranded RNA (ssRNA) sequences of HCV genome that are able to stimulate TNF-α production. 10^6/ml total PBMCs (N=3) were stimulated for 24 hours with DOTAP + different ssRNA at 7.5μg/ml. RNA-40 is used as a positive control. NS indicates non-stimulated. TNF-α levels were measured in the supernatant by ELISA. C. U to A mutant and RNase treatment show specificity of stimulation. D. PBMCs (N=3) were stimulated by indicated ssRNA with or without TLR7/8 inhibitor IRS661.

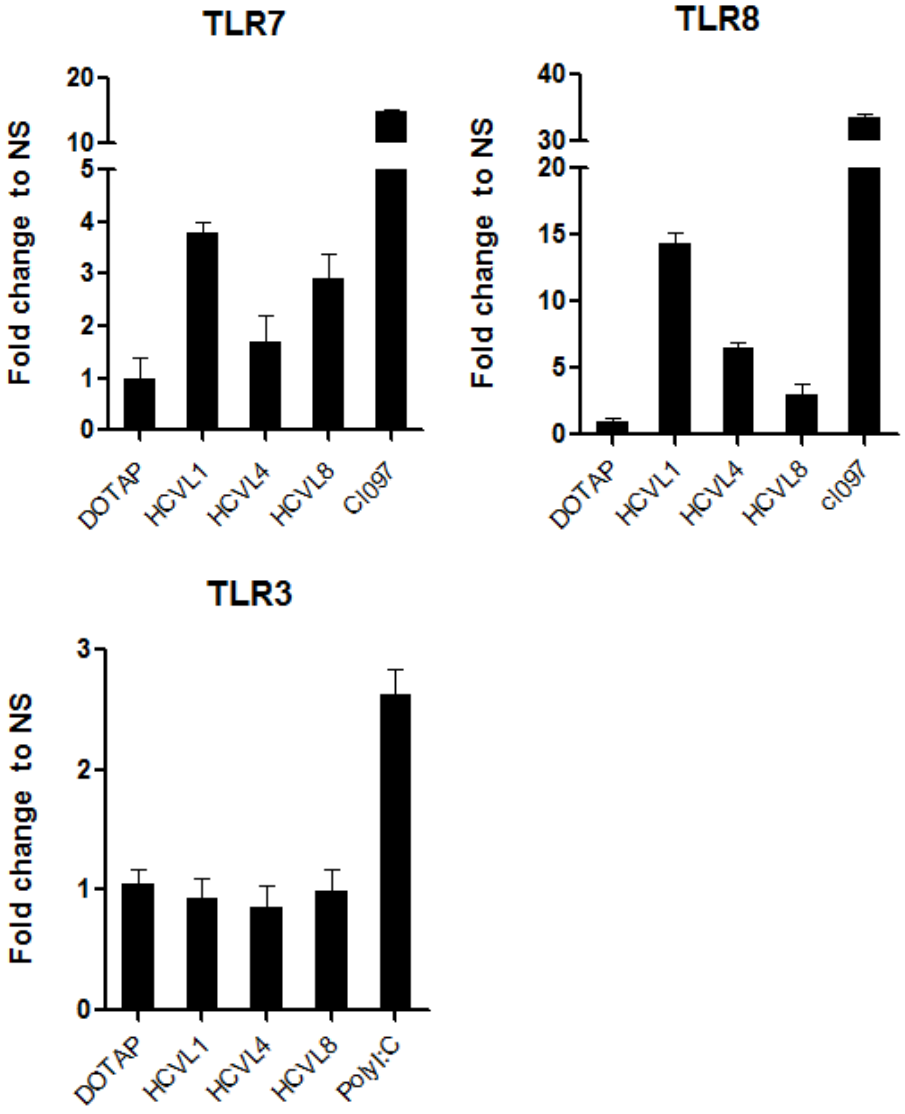

TLR7
Fold change to NS
20
10
5
4
3
2
1
0
DOTAP
HCVL1
HCVL4
HCVL8
Cl097
TLR8
Fold change to NS
40
30
20
15
10
5
0
DOTAP
HCVL1
HCVL4
HCVL8
cl097
TLR3
Fold change to NS
3
2
1
0
DOTAP
HCVL1
HCVL4
HCVL8
Poly I:C

Figure 2: Specificity of TLR7/8 triggering by HCV derived ssRNA

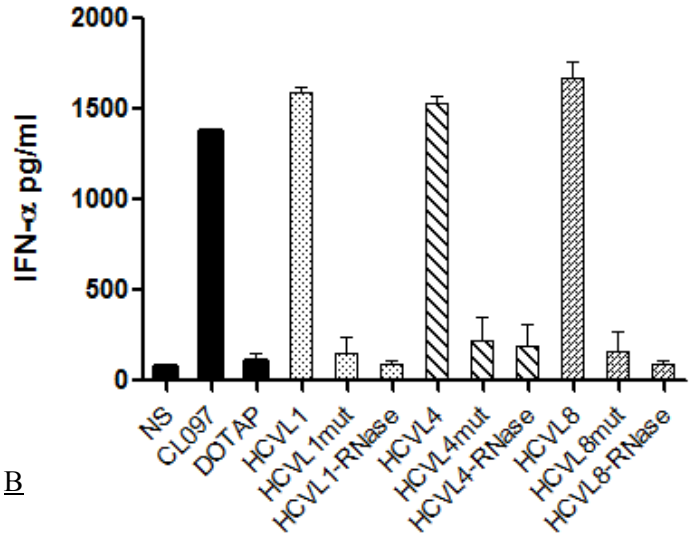
293 cells expressing TLR7, TLR8 and TLR3 were transfected with pNfity2-luc which allow luciferase expression upon NF-kB activation. Cells were stimulated with HCV derived ssRNA for 24h and the luciferase activity was measured. Results are expressed as a ratio to luciferase activity observed in stimulated *versus* non-stimulated cells.

Cell type	Ligand	TNF-α	IL-6	IL-1β	IL-10	IL-12	CXCL9	CXCL10
monocyte	NC	45	61	124	34	41	68	680
	cl097	8251	11884	5392	153	93	183	970
	DOTAP	54	112	133	34	39	73	817
	HCVL1	9344	12383	4927	75	157	408	1189
	HCVL1mut	48	961	196	41	37	66	681
	HCVL4	7252	12267	2803	205	66	360	1092
	HCVL4mut	53	205	131	65	44	62	679
	HCVL8	12483	12704	5540	223	176	355	1135
	HCVL8mut	276	3780	333	57	39	89	877
mDC	NC	287	4266	382	84	48	96	716
	cl097	9397	10443	6335	188	400	1122	877
	DOTAP	120	317	282	51	37	111	726
	HCVL1	3638	6354	3355	153	179	1427	1587
	HCVL1mut	59	391	302	55	37	92	680
	HCVL4	4060	8545	2115	80	200	1527	1483
	HCVL4mut	41	352	215	51	39	128	760
	HCVL8	6383	8528	3391	138	329	1432	1666
	HCVL8mut	79	433	261	53	43	91	743
pDC	NC	37	564	129	38	37	1087	1177
	cl097	1693	2257	224	60	74	13623	5653
	DOTAP	50	43	110	30	42	304	1899
	HCVL1	2155	2047	202	56	61	8349	6370
	HCVL1mut	40	47	109	33	41	362	2473
	HCVL4	1883	2370	183	68	48	9793	5862
	HCVL4mut	45	52	110	33	45	786	1662
	HCVL8	5476	2350	170	59	87	9309	6975
	HCVL8mut	58	55	109	35	38	328	2051

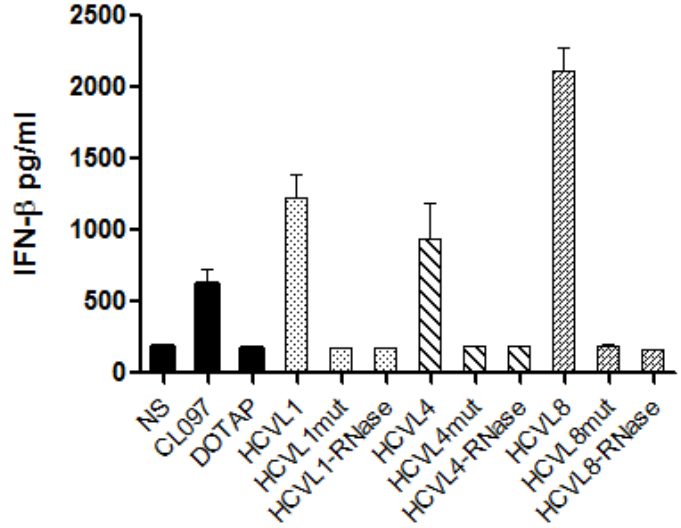
Figure 3: Inflammatory cytokines and chemokines production by monocytes, mDCs and pDCs.

10^5 purified monocytes, mDCs and pDCs were stimulated for 24 hours with different ssRNA at 7.5µg/ml. TNF-α, IL6, IL12, IL1b, CXCL9, CXCL10 and IL-10 levels were measured in the supernatant by Cytometric Bead Array (CBA). The mutated forms of HCVL1, HCVL4 and HCVL8 or treatment with RNAase were used as negative controls. NC indicates non-stimulated

A


IFN-α pg/ml
2000
1500
1000
500
0
NS
CL097
DOTAP
HCVL1
HCVL1mut
HCVL1-RNase
HCVL4
HCVL4mut
HCVL4-RNase
HCVL8
HCVL8mut
HCVL8-RNase

B


IFN-β pg/ml
2500
2000
1500
1000
500
0
NS
CL097
DOTAP
HCVL1
HCVL1mut
HCVL1-RNase
HCVL4
HCVL4mut
HCVL4-RNase
HCVL8
HCVL8mut
HCVL8-RNase

C

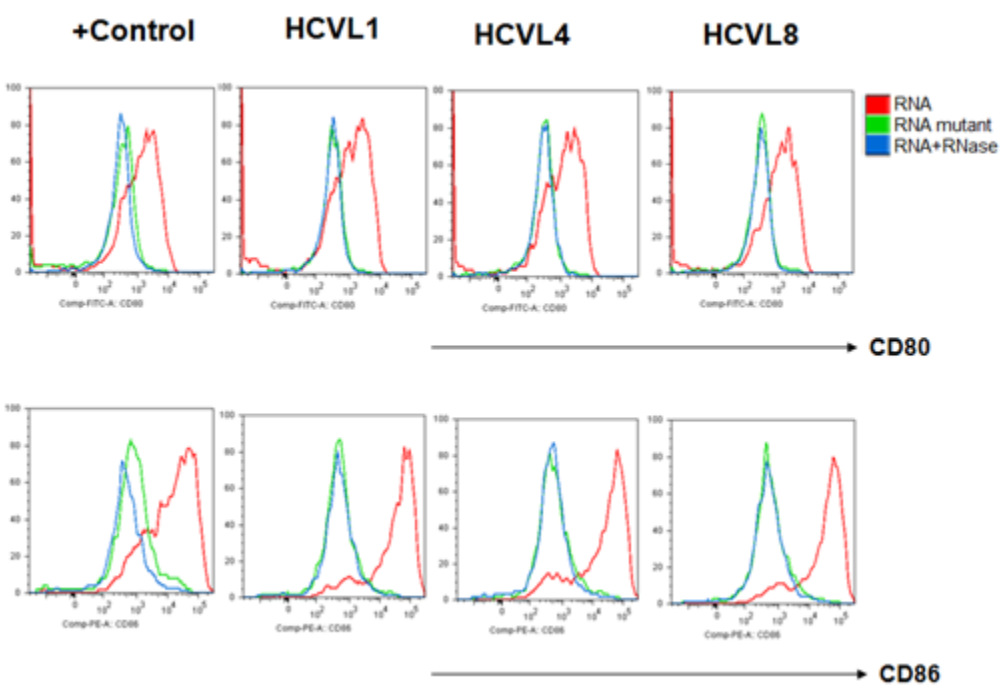
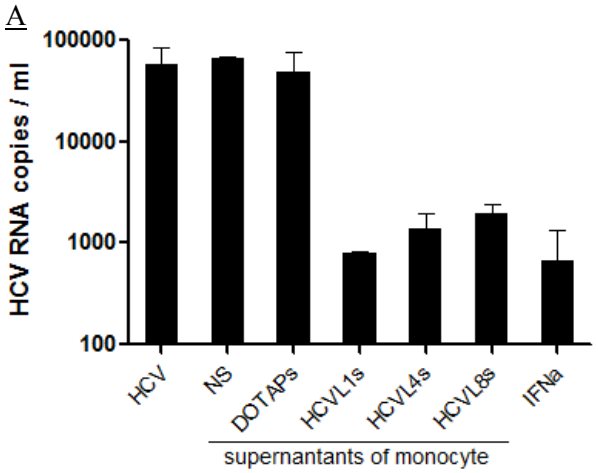
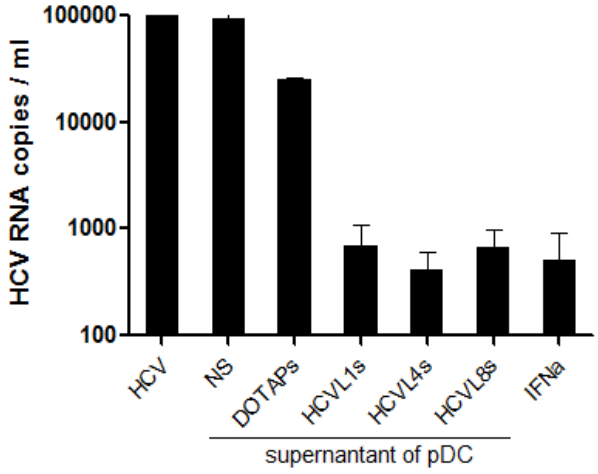

+Control
HCVL1
HCVL4
HCVL8
RNA
RNA mutant
RNA+RNase
CD80
CD86

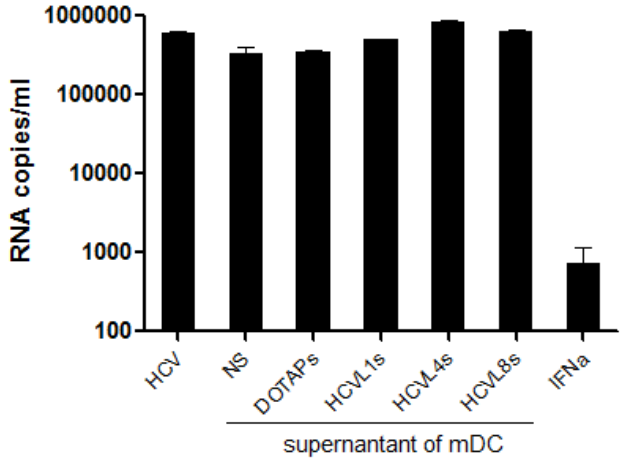
Figure 4: Maturation and type I IFN production of pDC

10^5 purified pDCs were stimulated for 24 hours with different ssRNA at 7.5µg/ml. The mutated forms of HCVL1, HCVL4 and HCVL8 or treatment with RNAse are used as negative controls. IFN-α (A) and IFN-β (B) production in the supernatant were measured by ELISA, DC maturation markers CD80 and CD86 (C) were detected by flow cytometry. NS indicates non-stimulated

A


HCV RNA copies / ml
100000
10000
1000
100
HCV
NS
DOTAPs
HCVL1s
HCVL4s
HCVL8s
IFNa
supernantants of monocyte


HCV RNA copies / ml
100000
10000
1000
100
HCV
NS
DOTAPs
HCVL1s
HCVL4s
HCVL8s
IFNa
supernantant of pDC


RNA copies/ml
1000000
100000
10000
1000
100
HCV
NS
DOTAPs
HCVL1s
HCVL4s
HCVL8s
IFNa
supernantant of mDC

B

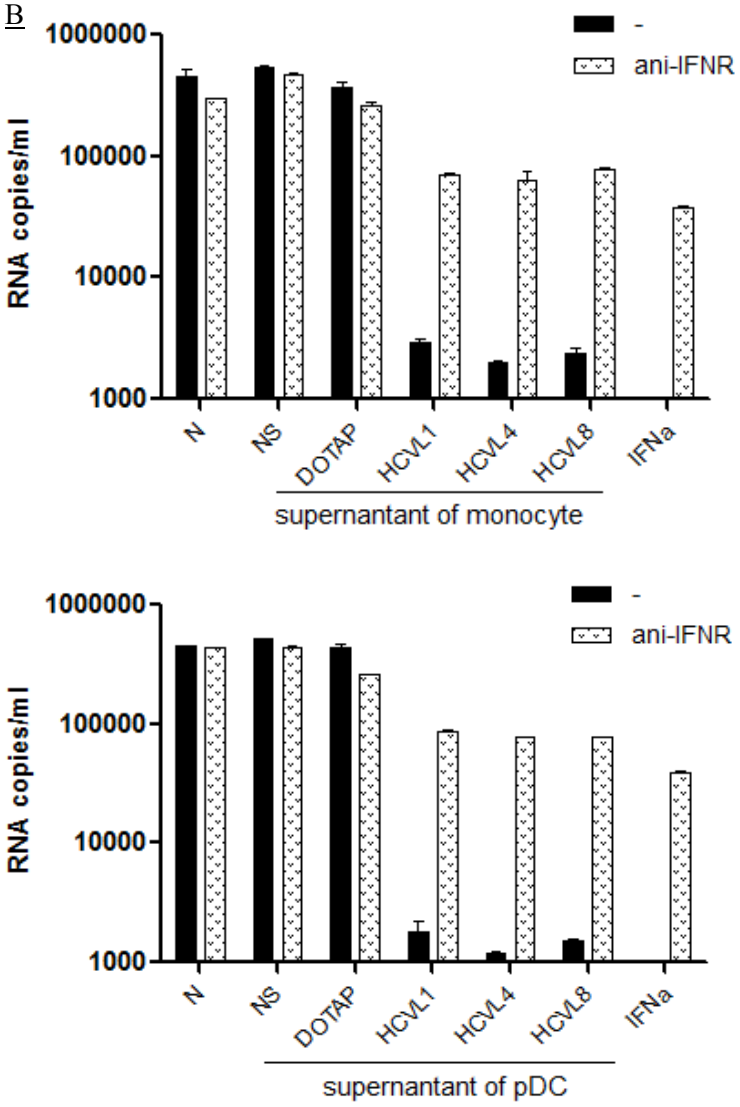

-
ani-IFNR
RNA copies/ml
1000000
100000
10000
1000
N
NS
DOTAP
HCVL1
HCVL4
HCVL8
IFNa
supernantant of monocyte
-
ani-IFNR
RNA copies/ml
1000000
100000
10000
1000
N
NS
DOTAP
HCVL1
HCVL4
HCVL8
IFNa
supernantant of pDC

Figure 5: HCV-ssRNA mediated cytokines inhibit HCV production in IFN-dependent manner.

A. 10^5 purified monocytes, mDC, and pDCs from healthy donors (N=3) were stimulated for 24 hours with 7.5 µg/ml of identified HCV ssRNA, cell-free supernatants were harvested. $2X10^4$ huh7.5 cells were infected by HCV ($2X\ 10^5$ copies). 4 hrs later, Huh 7.5 cells were washed, medium and supernatants from non-stimulated or stimulated monocytes, mDC, and pDCs were added. B. HCV infected huh7.5 cells were incubated with or without interferon receptor blocking antibody for 1h, supernatants form stimulated monocytes or pDCs were added. 3 days after infection, HCV RNA was extracted from the supernatants and quantified by real-time RT-PCR. NS indicates non-stimulated. N indicates non-treated cells, NS indicates supernatant from non-stimulated cells

SN of monocyte SN of pDC SN of mDC

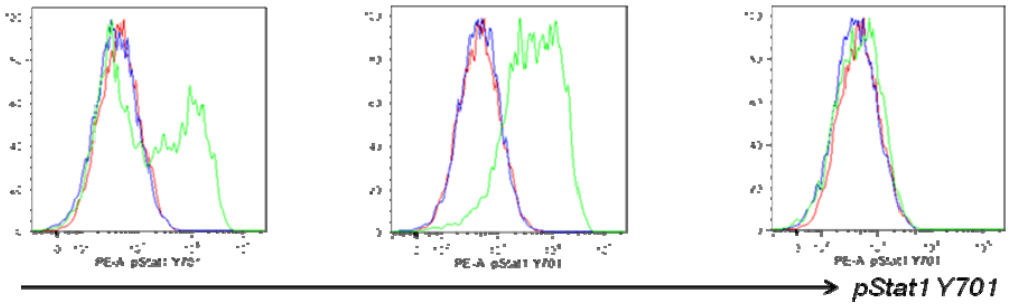


Figure 6: HCV-ssRNA mediated cytokines inhibit HCV production in IFN-dependent manner.

10^6 PBMCs were incubated with supernatants (SN) from HCV ssRNA stimulated monocytes, mDC, and pDC respectively, after 15min incubation, cells were fixed and stained with antibody against Y701 phosphorylated Stat1. Stat1 phosphorylation in lymphocytes was measured by flow Cytometry. Non-stimulated cells (blue), treatment with supernatant from non-stimulated PBMCs (red), treatment with supernatant from stimulated PBMCs (green).

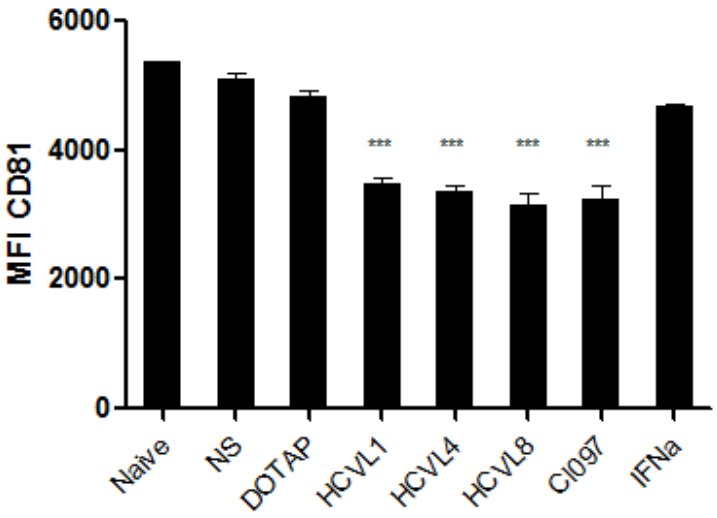
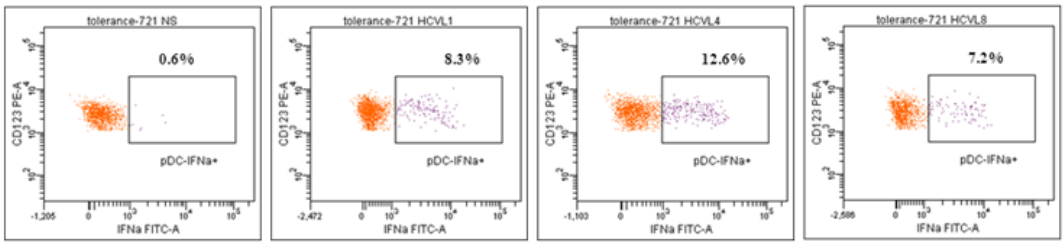

6000
4000
2000
0
MFI CD81
***
***
***
***
Naive
NS
DOTAP
HCVL1
HCVL4
HCVL8
Cl097
IFNa

Figure 7: HCV-derived ssRNA down-regulate CD81 expression on huh7.5.

10^5 PBMCs were stimulated with cl097 or HCV derived ssRNA for 24hrs, cell-free supernatants were added to huh7.5. After 24hrs incubation, CD81expression on huh7.5 were measured by flow Cytometry. "Naïve", non-treated cells

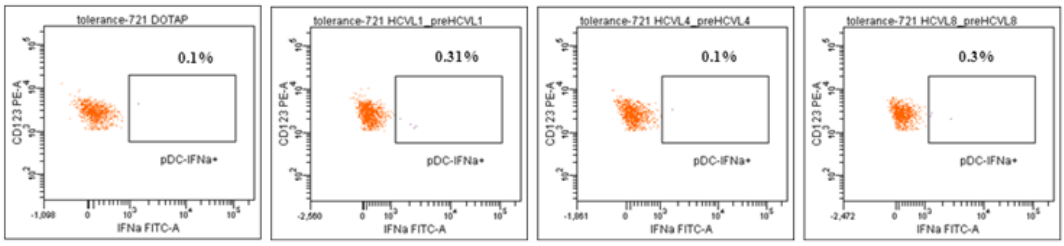
Pre-treatment: - - - -

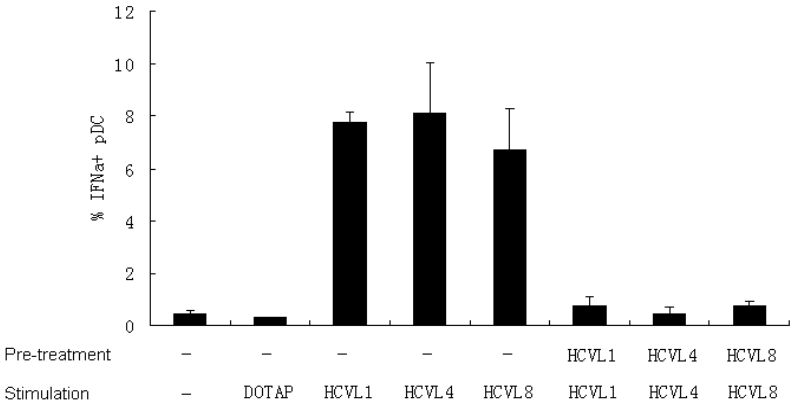
Re-stimulation: - *HCVL1* *HCVL4* *HCVL8*

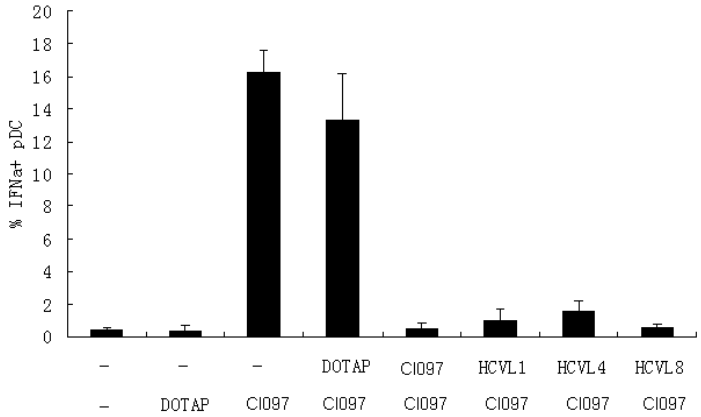


Pre-treatment: *DOTAP* *HCVL1* *HCVL4* *HCVL8*

Re-stimulation: *DOTAP* *HCVL1* *HCVL4* *HCVL8*







<u>Figure 8:</u> Impairment of IFN expression in pDC with HCV-derived ssRNA pre-treatment

A. PBMCs were stimulated with HCV ssRNA HCVL1, HCVL4 and HCVL8. B. PBMCs were stimulated with DOTAP, Cl097, HCVL1, HCVL4 and HCVL8. 24h later, cells were washed then re stimulated with indicated ssRNA or Cl097 (TLR7/8 agonist), brefeldin A was added 1h after stimulation. IFN expression in pDC was detected by intracellular staining ("-", non-stimulated cells).

Reference

Afdhal, N. H. (2004). "The natural history of hepatitis C." Semin Liver Dis**24 Suppl 2**: 3-8.

Agnello, V., G. Abel, et al. (1999). "Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor." Proc Natl Acad Sci U S A**96**(22): 12766-12771.

Assier, E., V. Marin-Esteban, et al. (2007). "TLR7/8 agonists impair monocyte-derived dendritic cell differentiation and maturation." J Leukoc Biol**81**(1): 221-228.

Barth, H., A. Ulsenheimer, et al. (2005). "Uptake and presentation of hepatitis C virus-like particles by human dendritic cells." Blood**105**(9): 3605-3614.

Bergmann, J. F., J. de Bruijne, et al. (2011). "Randomised clinical trial: anti-viral activity of ANA773, an oral inducer of endogenous interferons acting via TLR7, in chronic HCV." Aliment Pharmacol Ther**34**(4): 443-453.

Dustin, L. B. and C. M. Rice (2007). "Flying under the radar: the immunobiology of hepatitis C." Annu Rev Immunol**25**: 71-99.

Evans, M. J., T. von Hahn, et al. (2007). "Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry." Nature**446**(7137): 801-805.

Feigelstock, D. A., K. B. Mihalik, et al. (2010). "Increased susceptibility of Huh7 cells to HCV replication does not require mutations in RIG-I." Virol J**7**: 44.

Heil, F., H. Hemmi, et al. (2004). "Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8." Science**303**(5663): 1526-1529.

Horsmans, Y., T. Berg, et al. (2005). "Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic hepatitis C infection." Hepatology**42**(3): 724-731.

Kronenberger, B., E. Herrmann, et al. (2006). "Dynamics of CD81 expression on lymphocyte subsets during interferon-alpha-based antiviral treatment of patients with chronic hepatitis C." J Leukoc Biol**80**(2): 298-308.

Kronenberger, B., B. Ruster, et al. (2001). "Interferon alfa down-regulates CD81 in patients with chronic hepatitis C." Hepatology**33**(6): 1518-1526.

Labonte, P., S. Begley, et al. (2009). "PCSK9 impedes hepatitis C virus infection in vitro and modulates liver CD81 expression." Hepatology**50**(1): 17-24.

Lauer, G. M. and B. D. Walker (2001). "Hepatitis C virus infection." N Engl J Med**345**(1): 41-52.

Lee, H. K., J. M. Lund, et al. (2007). "Autophagy-dependent viral recognition by plasmacytoid dendritic cells." Science**315**(5817): 1398-1401.

Lee, J., C. C. Wu, et al. (2006). "Activation of anti-hepatitis C virus responses via Toll-like receptor 7." Proc Natl Acad Sci U S A**103**(6): 1828-1833.

Lindenbach, B. D., M. J. Evans, et al. (2005). "Complete replication of hepatitis C virus in cell culture." Science**309**(5734): 623-626.

Manns, M. P., G. R. Foster, et al. (2007). "The way forward in HCV treatment--finding the right path." Nat Rev Drug Discov**6**(12): 991-1000.

McKeating, J. A., L. Q. Zhang, et al. (2004). "Diverse hepatitis C virus glycoproteins mediate viral infection in a CD81-dependent manner." J Virol**78**(16): 8496-8505.

Pawlotsky, J. M. (2011). "Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus." Hepatology**53**(5): 1742-1751.

Pileri, P., Y. Uematsu, et al. (1998). "Binding of hepatitis C virus to CD81." Science**282**(5390): 938-941.

Polyak, S. J., K. S. Khabar, et al. (2001). "Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy." J Virol**75**(13): 6209-6211.

Rong, L., H. Dahari, et al. (2010). "Rapid emergence of protease inhibitor resistance in hepatitis C virus." Sci Transl Med**2**(30): 30ra32.

Saito, T., D. M. Owen, et al. (2008). "Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA." Nature**454**(7203): 523-527.

Scarselli, E., H. Ansuini, et al. (2002). "The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus." EMBO J**21**(19): 5017-5025.

Thomas, A., C. Laxton, et al. (2007). "Investigating Toll-like receptor agonists for potential to treat hepatitis C virus infection." Antimicrob Agents Chemother**51**(8): 2969-2978.

Uno, K., Y. Suginoshita, et al. (2005). "Impairment of IFN-alpha production capacity in patients with hepatitis C virus and the risk of the development of hepatocellular carcinoma." World J Gastroenterol**11**(46): 7330-7334.

Wang, B., M. Trippler, et al. (2009). "Toll-like receptor activated human and murine hepatic stellate cells are potent regulators of hepatitis C virus replication." J Hepatol**51**(6): 1037-1045.

Zhang, Y. L., Y. J. Guo, et al. (2009). "Hepatitis C virus single-stranded RNA induces innate immunity via Toll-like receptor 7." J Hepatol**51**(1): 29-38.

Zhang, Y. Y., B. H. Zhang, et al. (2010). "Novel function of CD81 in controlling hepatitis C virus replication." J Virol**84**(7): 3396-3407.

CHAPTER 3

Macrophages sense Hepatitis C virus to initiate innate immunity

Macrophages sense Hepatitis C virus to initiate innate immunity

Yuwei zhang[1-4], Elias Said[1-3], Zhong He[1-4],Francesco A. Procopio[1-4],Rafick-Pierre Sekaly[1-6]

[1]Centre de recherche du centre Hospitalier de l'Université de Montréal (CRCHUM), Hôpital Saint-Luc, Québec H2X 1P1, Canada

[2]Laboratoire d'Immunologie, Département de Microbiologie et d'Immunologie, Université de Montréal, Montréal, Québec H3T 1J4, Canada

[3]Institut national de la Santé et de la Recherche médicale U743, Centre de Recherche, CHUM, Université de Montréal, Montréal, Québec H2X 1P1, Canada

[4]Vaccine and Gene Therapy Institute-Florida (VGTI-Fl), 11350 SW Village Pkwy, third floor. Port St. Lucie, Florida. USA, 34987.

[5]Departement of Microbiology and Immunology, McGill University, Montréal, Québec H3A 2B4, Canada

[6]Corresponding authors

Correspondence should be addressed to Rafick-Pierre Sekaly

COMPETING INTERESTS STATEMENT

The authors have declared that no conflict of interest exists.

Abstract

Hepatitis C virus (HCV) is a positive single-stranded RNA (ssRNA) virus that replicates in the liver and has infected 200 million people worldwide. Approximately 80% of HCV-infected individuals become chronically infected. Although immune activation was observed after infection *in vivo*, persistently increased viral load suggests the failure of innate immunity to control ongoing infection. Immune activation induced by HCV components (proteins or RNA) have been studied, however, the detection of HCV by the innate immune system is still poorly understood. In this study, we demonstrate that HCV genomic RNA activates antigen-presenting cells (APCs). However, peripheral DCs and monocytes do not respond to JFH1-culture-derived HCV (HCVcc). Only macrophages sense HCV and produce inflammatory cytokines. HCV stimulation induces inflammatory cytokines production (TNF-α, IL-8, IL-6, and IL-1b). Interestingly, macrophages stimulated by HCV do not express interferon-beta and interferon-stimulated genes, which is very similar to TLR7/8 signaling that is characterized as deficient interferon induction in macrophages. TLR7/8 specific inhibitor IRS661 also provides evidence of involvement of TLR7/8 in HCV recognition. Furthermore, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) plays an important role in detection of HCV by macrophages. Cytokine production induced by HCV is compromised in the presence of a DC-SIGN-blocking antibody. However, monocytes-derived DC with higher expression of DC-SIGN does not respond to HCV stimulation, suggesting detection of HCV is cell-type-dependent, and DC-SIGN is not the only determinant in HCV detection. Our results reveal that macrophages have the ability to sense HCV and produce inflammatory cytokines but not IFN-α, suggesting that activation

of macrophages in HCV-infected individuals may contribute to inflammation but not control of viral infection.

Key words: HCV, innate immunity, Dendritic cells, inflammation, DC-SIGN, TLR7/8,Macrophages

Introduction

The hepatitis C virus, a member of the *Flaviviridae* family, is an ssRNA virus that causes hepatitis C and affects 200 million people worldwide (Lauer and Walker 2001). Extrahepatic disease is common and affects an estimated 40% of patients with chronic HCV infection (Mayo 2003). HCV infection is now the leading indication for liver transplantation (Brown 2005). HCV establishes persistent infection in the majority of those who contract it, despite the fact that it is recognized and targeted by innate, cellular, and humoral immune mechanisms. The high incidence of chronicity suggests that the virus has developed efficient mechanisms to escape host immune responses (Horner 2013).

HCV is recognized by innate virus-sensing mechanisms and induces a rapid immune response. Immune activation was observed in HCV-infected individuals (Su, Pezacki et al. 2002), but it is still unclear how HCV infection induces immune activation. The first defense was thought to be IFN-β produced by HCV-infected hepatocytes (Bigger, Guerra et al. 2004, Wieland, Makowska et al. 2013), however, it is still controversial whether HCV infection induces interferon production in hepatocytes (Cheng, Zhong et al. 2006, Kanda, Steele et al. 2007). Immune response induced by HCV infection is characterized as rapid innate immune response and delayed adaptive immune response (Dustin and Rice 2007), regardless of the outcome of the disease progression. Moreover, cellular immune responses are weak in chronically infected patients (Missale, Bertoni et al. 1996). Given the important roles of the innate response in initiation of adaptive immunity, impaired cellular response suggests compromised innate immunity

and inefficient T cell priming. Persistent increased viral load also implies that innate immunity fails to control HCV infection. Activation markers such as CD80, CD40, and CD86 on DCs and inflammatory cytokines have been found up-regulated in HCV-infected patients compared to healthy individuals (Liang, Russell et al. 2009). However, the same phenomenon was not observed on DCs that were stimulated with HCV *in vitro* (Shiina and Rehermann 2008). No cytokines were induced in DCs upon direct HCV stimulation (Liang, Russell et al. 2009, Zhang, Guo et al. 2009, Takahashi, Asabe et al. 2010). HCV non-structure proteins have been proposed as the cause of unresponsiveness. Indeed, overexpression of NS3, NS4, and NS5A strongly inhibit TLR-mediated cytokine production (Abe, Kaname et al. 2007). It was also reported that HCV particles inhibit TLR9-induced IFN-α production of pDCs. Interestingly, the inhibitory effect does not require DC infection or viral entry (Shiina and Rehermann 2008). However, HCV has no effect on cytokine production and DC maturation induced by an influenza virus or LPS (Shiina and Rehermann 2008), suggesting that HCV does not alter the capacity of DCs to respond to virus or microbe stimulation.

It remains unknown whether APCs are able to detect HCV and initiate an innate immune response or which type of APCs are responsible for HCV detection. There are abundant APCs in the liver where HCV infection mainly takes place. Kupffer cells (resident macrophages in the liver), myeloid DCs, and plasmacytoid DCs have been described to be involved in the innate immune response against viral infection (Crispe 2009). Several studies have suggested that HCV can be captured by DCs or DC-SIGN-expressing cells (Ludwig, Lekkerkerker et al. 2004, Barth, Ulsenheimer et al. 2005, Lambotin, Baumert et al. 2010). However, no solid evidence has demonstrated activation

of APCs by direct HCV stimulation, although immunostimulatory effects of HCV components have been reported (Dolganiuc, Oak et al. 2004, Chang, Dolganiuc et al. 2007, Zhang, Guo et al. 2009). Thus, in this study, we assessed the capacity of APCs to detect HCV and innate immune response induced by virus.

Results

1. Macrophages but not monocytes and DCs sense HCVcc

The capabilities of HCV proteins and RNA to activate innate immune cells have been reported (Dolganiuc, Oak et al. 2004, Zhang, Guo et al. 2009). Many groups have tried to investigate the immune response induced by HCV particles and the mechanism of HCV detection by antigen-presenting cells *in vitro*, but the immunostimulatory effect of HCV particles has not been observed (Liang, Russell et al. 2009, Zhang, Guo et al. 2009, Takahashi, Asabe et al. 2010). To verify if HCV has the ability to stimulate APCs, PBMCs were stimulated with HCVcc at different ratios, extracted viral RNA fused with DOTAP was set as positive control, and TNF-α in the supernatants were measured after 24hrs. Consistent with previous studies, HCV RNA induced TNF-α production (200 pg/ml, $p<0.0001$), but PBMCs showed no response to HCVcc stimulation (Figure 1A). Considering the low frequency of DCs in PBMCs and in order to identify which subsets are involved in HCV detection, we used purified monocytes, pan-DCs, and monocytes-derived macrophages (MDMs), and examined their responsiveness to HCVcc. Purified cells were incubated with HCVcc, and TNF-α in the supernatants were measured after 24hrs. Results showed that neither monocytes nor pan-DCs responded to HCVcc stimulation, and no TNF-α was induced in spite of their all responding to cl097, a commercial TLR7/8 agonist (Figures 1B, C). Surprisingly, TNF-α was significantly increased in macrophages upon HCVcc stimulation ($p=0.01$). MDMs generated from 7 out of 8 donors showed remarkable responsiveness to HCV (Figure1D). Part of the donors had higher responses than others. Overall, these data reveal that PBMCs,

monocytes, and DCs do not sense HCV. MDMs possess different characters and the ability to detect HCV particles leading to immune activation.

2. Induction of inflammatory cytokines by HCVcc stimulation

To show dose-dependent responses to HCVcc stimulation, MDMs from donors who had good responsiveness were incubated with HCVcc at different ratios as indicated. TNF-α production was monitored for 24hrs by ELISA. Results showed that HCVcc-induced TNF-α production in a dose-dependent manner (Figure 2A), TNF-α is still detectable when virus/cells at 2.5, EC50 of HCV stimulation is at a ratio of 20. MDMs and monocytes from 20 donors were stimulated with HCV at a ratio of 20. Results showed that MDMs from 19 donors out of 20 responded to HCV stimulation (average of 1500 pg/ml, $p< 0.0001$). TNF-α production induced by HCV stimulation varied among those 20 donors, indicating that the responsiveness of MDMs to HCV was different from one donor to another (Figure 2B). In contrast, no significant TNF-α induction was observed in monocytes upon HCV stimulation. Other inflammatory cytokines produced IL-6, IL-8, and IL-1 and were also found to be induced in MDMs by HCV (Figure 2C). No IL-10 production was detected.

3. HCVcc as well as TLR7/8 agonist do not trigger interferon pathway in macrophages

Interferon is well known as a key factor in controlling HCV infection. To investigate whether macrophages are involved in immunity against HCV infection, we monitored IFN-β and IFN-stimulated gene expression in macrophages after 3hrs incubation, with HCVcc, LPS, and cl097 set as positive control. Data showed a significant increase of TNF-α at 3hrs after HCVcc stimulation, as well as stimulation with LPS and cl097. Surprisingly, like TLR7/8 agonist, HCVcc stimulation did not induce interferon expression in macrophages. In contrast, up-regulation of IFN-β in macrophages with LPS stimulation demonstrated that MDMs were still capable of producing IFN-β (Figure 3A). Those results suggest that HCVcc most likely triggers TLR7/8 pathway, which is compromised in macrophages and characterized as a lack of IFN-β induction (Schroder, Spille et al. 2007). To confirm the deficiency of IFN-β, we measured the expression of IFN-stimulated genes at 16hrs after stimulation. Consistent with IFN, LPS-induced up-regulation of IFIT1, ISG15, and OAS1in macrophages, HCV stimulation led to an increase of TNF-α expression but not those IFN-stimulated genes (Fig3 B). It revealed that activation of macrophages by HCVcc stimulation does not involve an IFN signaling pathway.

4. Antiviral activity and responsiveness to HCVcc

Although macrophages are differentiated from monocytes, the responsiveness of macrophages to HCVcc appears completely different from that of monocytes. Among different APCs subsets, macrophages seem the only ones that detect HCV and initiate innate immunity, thus, it is alluring to understand the role of activated macrophages in

controlling viral infections. As shown above, HCVcc triggers TLR7/8 in macrophages. Hence, we investigated the antiviral activity of TLR7/8 agonist-activated macrophages and monocytes. Data showed that cl097 up-regulated TNF-α expression in both macrophages and monocytes, however, IFN-β expression was increased only in monocytes (Figure 4A). Supernatants from Cl097-stimulated macrophages and monocytes were added to HCV-infected huh7.5 cells. Consistent with IFN-β induction, results showed a significant decrease in viral production by HCVcc infected huh7.5 with supernatant from Cl097-stimulated monocytes but not macrophages (Figure 4B). Ironically, monocytes have the potential to suppress HCV production, but they are unable to detect HCV, macrophages can detect HCV, but they have no antiviral activity when TLR7/8 is triggered (Figure 4C).

5. RNA sensors involved in HCV recognition

Several PPRs have been identified as receptors to detect RNA viruses. TLR7/8 and TLR3 are known RNA sensors in endosomes that recognize ssRNA and dsRNA, respectively. Since HCV and TLR7/8 agonists induce similar responses in macrophages, and both induce TNF-α but do not activate IFN signaling, it is most likely that HCV triggers TLR7/8 in macrophages. Thus, we used TLR7/8 inhibitor IRS661 to validate if HCV stimulation goes through TLR7/8. In addition, chloroquine was used to validate the involvement of endosome processing. Results showed that around 50% of TNF-α production was inhibited by IRS661, and chloroquine completely abolished TNF-α production induced by HCV in macrophages (Figure 5A). To clarify the mechanism of

selective activation of macrophages by HCV, we examined the responsiveness of macrophages and monocytes to TLR7/8 and TLR3 agonists. Results showed that both macrophages and monocytes responded to Cl097 and poly I:C, but only macrophages produced TNF-α upon HCV stimulation (Figure 5B). Furthermore, both macrophages and monocytes respond to Sendai virus stimulation (Figure5C), it indicates that monocytes possess the capability to detect RNA viruses but still fail to respond to HCV stimulation. This suggests that differences in responsiveness of macrophages and monocytes to HCV might be due to mechanisms other than availability of RNA sensors.

6. DC-SIGN expression and responsiveness to HCV

One possible cause of the differences in responsiveness of macrophages and monocytes may be their ability to uptake a virus. Thus, we kept monocytes or macrophages in incubation with HCV for 4hrs. In order to measure those viruses actually entering cells, we used trypsin to remove virus binding on the surface. As shown in Figure 6, both macrophages and monocytes can bind HCV, however, HCV RNA becomes undetectable in monocytes after trypsin treatment. In contrast, although viral RNA was decreased in macrophages after trypsin treatment, most of the binding virus still remains (Figure 6A). These data indicate that HCVcc can bind to monocytes and macrophages, but internalization of a binding virus only occurs in macrophages, the virus binding to monocytes cannot enter cells.

Viral entry usually was mediated by specific receptors; therefore, we examined CD81 and DC-SIGN, two known receptors on APCs that interact with HCV. Results

showed that all the subsets of APCs expressed CD81, explaining how all the subsets had the ability to capture HCV. However, DC-SIGN expression was only detected on macrophages, not on monocytes and DCs (Figure 6B). These data strongly suggest that DC-SIGN might be the receptor that mediated the internalization of the virus captured by macrophages. To validate the role of DC-SIGN in uptake of HCV, we used a neutralizing antibody that can block the interaction of DC-SIGN with its ligand or HCV Envelope protein. Macrophages were incubated with HCV in the absence or presence of DC-SIGN-blocking antibody or isotype control. Compared to isotype and nontreated macrophages, TNF-α production by macrophages with DC-SIGN blocking was significantly decreased (up to 85%) (Figure 6C). This provides solid evidence for DC-SIGN-mediated internalization in macrophages.

7. Difference in responsiveness of DC-SIGN expressing cells to HCV

DC-SIGN was found on macrophages and residential DCs in tissue (Soilleux, Morris et al. 2002). As we have shown above, DC-SIGN plays a crucial role in the detection of HCV. This raises an interesting question as to whether DC-SIGN expressing DCs respond to HCV stimulation. Monocyte-differentiated DCs (MDDC), on which cells DC-SIGN was identified, express abundant DC-SIGN, if DC-SIGN is the determinant of HCV detection, MDDC is expected to be responsive to HCV stimulation. We stimulated MDDCs with HCVcc and measured TNF-α production after 24hrs as above. Surprisingly, MDDCs responded to TLR7/8 agonist but did not show responsiveness to HCVcc stimulation as expected (Figure 7B), in spite of the fact that DC-SIGN expression on

MDDCs was much higher than on macrophages (Figure 7A). To investigate if MDDCs have the capability to detect RNA virus and produce TNF-α, we used the Sendai virus, which is known as a strong activator of TLR7/8 in DCs. Unlike HCVcc, Sendai virus induced a large amount of TNF-α production by both macrophages and MDDCs (Figure 7B). This indicates that the non-responsiveness of MDDCs to HCVcc is not due to functional deficiency in the detection of RNA virus. HCV might escape detection by MDDCs.

Discussion

This study demonstrates that macrophages directly sense HCV and initiate an inflammatory response. Significantly, although HCV RNA triggers TNF-α production by PBMCs like other viral RNA, the mechanism of HCV detection appears to be different from that of other viruses like human immunodeficiency virus (HIV), influenza virus (flu), or Sendai virus (SV) that can directly stimulate different subsets of APCs. Here we have shown that HCV can be detected directly only by macrophages but not peripheral DCs and monocytes, in spite of all subsets expressing HCV receptor CD81 and capturing HCV. The unresponsiveness of monocytes to HCVcc may be due to the failure of the internalization of a binding virus.

It is very interesting that monocytes gain the ability to uptake and respond to HCV during the differentiation into macrophages (Figure 2B). The DC-SIGN has been reported to interact with HCV glycoproteins and was proposed as a receptor on APCs that mediate the uptake of HCV by DCs (Feng, Wang et al. 2004). Our results showed that DC-SIGNs selectively express on macrophages but not monocytes, pDCs, and mDCs, which is consistent with the selective responsiveness of macrophages to HCV. This strongly suggests that DC-SIGN-mediated endocytosis might be the mechanism of HCV detection by macrophages. Validation by DC-SIGN-blocking antibodies reveals that DC-SIGNs play a critical role in detection of HCV by macrophages. Peripheral DCs do not express DC-SIGN (Sun, Fernandez et al. 2009), but DC-SIGN is still detectable on a few of the peripheral myeloid cells and dermal DCs (Geijtenbeek, Krooshoop et al. 2000, Gringhuis, van der Vlist et al. 2010). However, DC-SIGN expression does not endow

DCs with a great ability to detect HCV. Although MDDCs are also differentiated from monocytes and express much higher DC-SIGN than macrophages, our results showed that MDDCs did not respond to HCVcc stimulation. This suggests that macrophages and DCs may capture and process a virus in different ways. It is controversial that DC-SIGN expression permits HCV entry into DCs (Ludwig, Lekkerkerker et al. 2004, Lai, Sun et al. 2006). Viruses can be detected by PRRs or escape detection, the key that determines detection or evasion might be the endosome-lysosome pathway it is involved in. It was reported that cells expressing DC-SIGN can capture HCV viral-like particles (VLP) and lead to internalization. However, viral protein is targeted to the early endosome but not the later endosome or lysosome in DC-SIGN-expressing THP-1 or MDDCs. In contrast to DCs, HCV VLP was targeted to lysosome in other DC-SIGN-expressing cell lines (Ludwig, Lekkerkerker et al. 2004). It was known that uncoating of HCV requires acidification (Coller, Berger et al. 2009). Thus, a virus might escape detection of PRRs and "hide" within DCs as it was targeted to a non-lysosomal compartment. The same mechanism was observed for HIV, and it was utilized by the virus for viral dissemination (Geijtenbeek and van Kooyk 2003). Moreover, another possible explanation for the unresponsiveness of MDDCs to HCV is that DC-SIGN expression does not permit HCV entry into MDDCs. It was reported that cell-surface expression of DC-SIGN failed to confer permissiveness to HCV pseudo-type infection (Hsu, Zhang et al. 2003). Furthermore, no HCV RNA was detected in MDDCs from persistently infected chimpanzees (Larsson, Babcock et al. 2004).

Here we have shown that activation of macrophages by HCV results in production of inflammatory cytokines, including TNF-α, IL-6, IL-8, and IL1b that have been

reported to be up-regulated in HCV-infected individuals. During chronic HCV infection, resident tissue macrophages like Kupffer cells (KCs) that express DC-SIGN (Schwartz, Alvarez et al. 2002) could be activated by HCV and become a major source of inflammatory cytokines, leading to recruitment of immune cells and liver damage. Cytokines such as TNF-α and IL-8 have been known as key factors that cause liver cirrhosis and resistance to interferon treatment. It has been shown that KCs activation and TNF-α induction are associated with hepatocellular apoptosis, inflammation, and the fibrosis process (Liu, Tao et al. 2010). It was reported that levels of IL-8 were significantly elevated in HCV-infected patients compared to levels in healthy subjects, and more importantly, levels of IL-8 were also significantly higher in patients who did not respond to IFN therapy than in patients who did respond to therapy (Polyak, Khabar et al. 2001). This suggests that HCV-induced cytokines by macrophages may be involved in antiviral resistance, persistence, and pathogenesis.

Type I interferon is considered as a key factor for the innate immune system to control HCV infection, as it has shown great antiviral activity and also has promising clinical records in hepatitis C therapy. The capability of immune cells to restrict viral replication also most likely depends on interferon induction (Knapp, Yee et al. 2003, Saito, Owen et al. 2008, Takahashi, Asabe et al. 2010). However, although HCV could be recognized by macrophages resulting in immune activation and production of inflammatory cytokines, up-regulation of interferon expression and related genes were not observed in activated macrophages, and cell-culture supernatant exhibits negligible antiviral activity (Figure 3, Figure 4). Lack of interferon induction upon HCV stimulation is not due to the inhibitory effect of HCV protein or the inability of HCV to stimulate

macrophages, since TNF-α expression was induced. HCV triggers TLR7/8 receptors (Figure 1D and Figure 2), which have alternative signaling in macrophages and are characterized by a lack of IFN-beta induction (Schroder, Spille et al. 2007). Interestingly, HCV seems to selectively stimulate macrophages that do not produce IFN when TLR7/8 is triggered, and it escapes detection by APCs which do produce type I IFN when TLR7/8 is engaged. This may partially explain the failure of the innate immunity to control viral infection and impaired adaptive immunity *in vivo*. Whereas type I IFN and IFN-induced genes are detected in the liver early during infection regardless of its outcome (Bigger, Brasky et al. 2001, Su, Pezacki et al. 2002, Thimme, Bukh et al. 2002, Wieland and Chisari 2005). This suggests that the existence of another mechanism besides direct detection of HCV by macrophages. The cellular source of IFN *in vivo* is not certain. Although hepatocytes have been proposed, it is controversial whether HCV infection induces IFN in hepatocytes (Mihm, Frese et al. 2004, Cheng, Zhong et al. 2006, Kanda, Steele et al. 2007). The absence of intrahepatic IFN induction was also observed *in vivo*. Another possibility is that liver pDCs sense HCV-infected hepatocytes and produce IFNs (Takahashi, Asabe et al. 2010). However, induction of Type I IFN and related genes in the liver does not determine the outcome of infection (Su, Pezacki et al. 2002), and most infected individuals develop chronic infection, implying that the contribution of antiviral immunity mediated by pDCs is limited. Many factors may cause failure of innate immunity: limited interaction of pDCs and HCV-infected hepatocytes, blockade of TLR and IFN pathways by HCV proteins, a tolerogenic environment formed by activated Kupffer cells, or infected hepatocytes.

Materials and methods

Cell lines

Hepatoma cell huh7.5 (kind gift from by Dr. Charles Rice, Rockefeller University) and Hepatoma cell Huh7.5-20 producing JFH1 HCV (Kindly provide by Dr. T. Jake Liang, National Institutes of Health) were cultured in DMEM-based complete growth medium.

Isolation monocytes, DCs and Preparation of macrophages, MDDCs

Human peripheral blood mononuclear cells (PBMCs) were isolated from leukapheresis following ficoll procedure. Monocytes were isolated from PBMCs by using human monocytes enrichment kit (STEMCELL Technologies Inc, Canada), purity was confirmed by staining with CD14-PB. Pan-DCs were isolated from PBMCs by using Human pan-DC pre-enrichment kit (STEMCELL Technologies Inc, Canada). To prepare macrophages, purified monocytes were cultured in X-vivo 15 medium (lonzabio, USA) for 6 days in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF, 50ng/ml, R&D systems, USA). To obtain MDDCs, purified monocytes were cultured in X-vivo 15 medium supplemented with GM-CSF (50ng/ml) and IL-4 (20ng/ml).

Viral production, quantification and concentration

Cell line huh7.5-20 stably producing HCV was cultured in DMEM-based complete growth medium for 4 days, supernatant was harvested and filtered by 0.45um filter (sarstedt, Germany). Viral RNA was extracted from supernatant by using QIAamp Viral RNA Mini Kit (QIAGEN, USA) and cDNAs were synthesized by SuperScript® VILO™ cDNA Synthesis Kit (invitrogen, USA). Real time PCR was performed to quantify viral

RNA by using LightCycler® 480 Probes Master (ROCHE, USA), forward primer 5'-CGGGAGAGCCATAGTGG-3', reverse primer 5'-AGTACCACAAGGCCTTT-3', probe 5'- /56-FAM/CTGCGGAACCGGTGAGTACAC/3IABlkFC (IDT, USA). RNA standard was prepared by in vitro transcription using MEGAscript® T7 Kit (ambion, USA), quantity of RNA was determined by OD260 and RNA copies were calculated by formula from QIAGEN. Culture medium of huh7.5 and huh7.5-20 were concentrated by passing Amicon ultra-15 centrifugal filter-100 (Millipore, USA), filters were sterilized with 70% alcohol before using.

Cell stimulation, cytokines production and gene expression

In vitro differentiated macrophages and isolated monocytes, pan-DCs were cultured in X-vivo 15 medium at 96 wells plate. Cells were incubated with HCVcc for 24hrs, supernatants were harvested after centrifuge, cytokine production was measured by Human TNF alpha ELISA Ready-SET, and inflammatory cytokines were measured by Cytometric Bead Array (CBA) by using Human Inflammatory Cytokine Kit (bdbiosciences, USA). TLR7/8 agonist cl097 and TLR3 agonist poly I:C were obtained from invivogen. For measurement of IFN and IFN-stimulated genes (IFIT1, OAS1 and IAS15), cells were stimulated with HCVcc for indicated time and washed with PBS, total RNA was extracted by RNeasy Mini Kit (QIAGEN, USA) and DNase treatment was performed using DNA-free kit (ambion, USA), cDNA synthesis and real-time PCR were performed as described above.

Flow Cytometry

Expression of CD81 and DC-SIGN were measured by flow Cytometry. PBMCs were stained with live/dead stain (invitrogen, USA), anti-CD14-Pacific blue, anti-Linage-

Alexa700 (CD3, CD19, CD16), anti-HLA-DR-APC-cy7, anti-CD11c-APC, anti-CD123-FITC, anti-CD81-PE (BDbioscience, USA) or anti-DC-SIGN-PE (R&D systems, USA) and analyzed on an LSR II (BD bioscience). CD81 and DC-SIGN expression were measured on monocytes ($CD14^{+}$), mDCs ($Lin^{-}CD14^{-}HLA\text{-}DR^{+}CD11c^{+}CD123^{-/dim}$) and pDCs ($Lin^{-}CD14^{-}HLA\text{-}DR^{+}CD11c^{-}CD123^{+}$). Macrophages and MDDCs were also stained with anti-CD81-PE or anti-DC-SIGN-PE to detect CD81 and DC-SIGN expression.

Uptake of HCVcc by macrophages

Macrophages and monocytes were incubated with HCVcc for 4hrs at 37°C, cells were intensively washed with PBS and then incubated with or without trypsin at 37°C (water bath) for 5mins. After intensive wash, cells were used for RNA extraction and quantification by real-time PCR.

Statistical analysis

p-values were determined using two way paired student's *t* tests. *p*–values less than 0.05 were considered statistically significant.

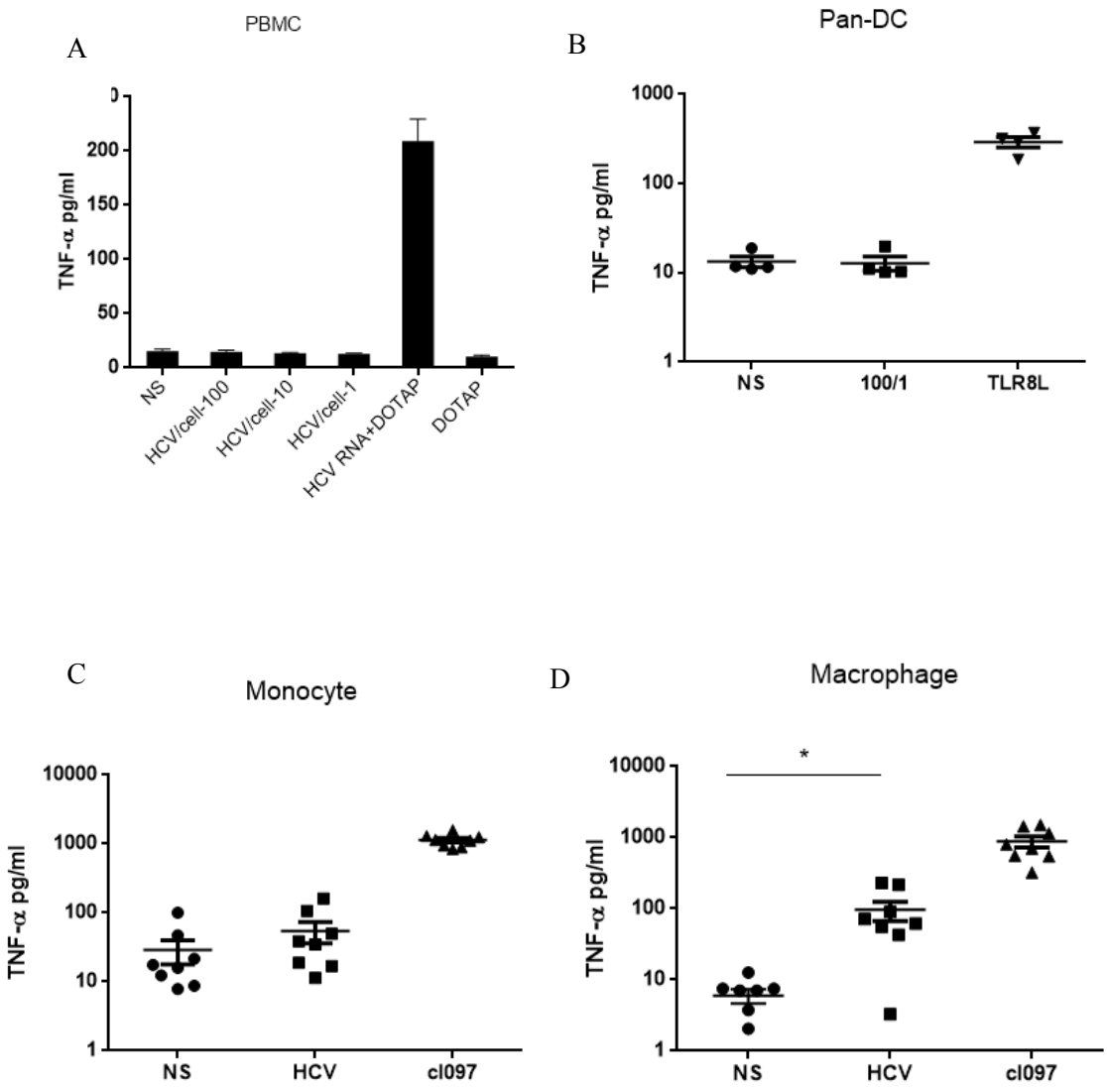

A
PBMC
TNF-α pg/ml
0
50
100
150
200
NS
HCV/cell-100
HCV/cell-10
HCV/cell-1
HCV RNA+DOTAP
DOTAP
B
Pan-DC
TNF-α pg/ml
1
10
100
1000
NS
100/1
TLR8L
C
Monocyte
TNF-α pg/ml
1
10
100
1000
10000
NS
HCV
cl097
D
Macrophage
*
TNF-α pg/ml
1
10
100
1000
10000
NS
HCV
cl097

Figure 1: Macrophages sense HCV.

(A) $2x10^4$ PBMCs were incubated with HCVcc, DOTAP or HCV RNA-DOTAP complex for 24 hours. (B, C, D) Purified pan-DCs, monocytes and monocytes-derived macrophages were incubated with HCVcc or TLR7/8 agonist Cl097 for 24 hours. TNF-α production were measured by ELISA. "NS", non-stimulated cells.

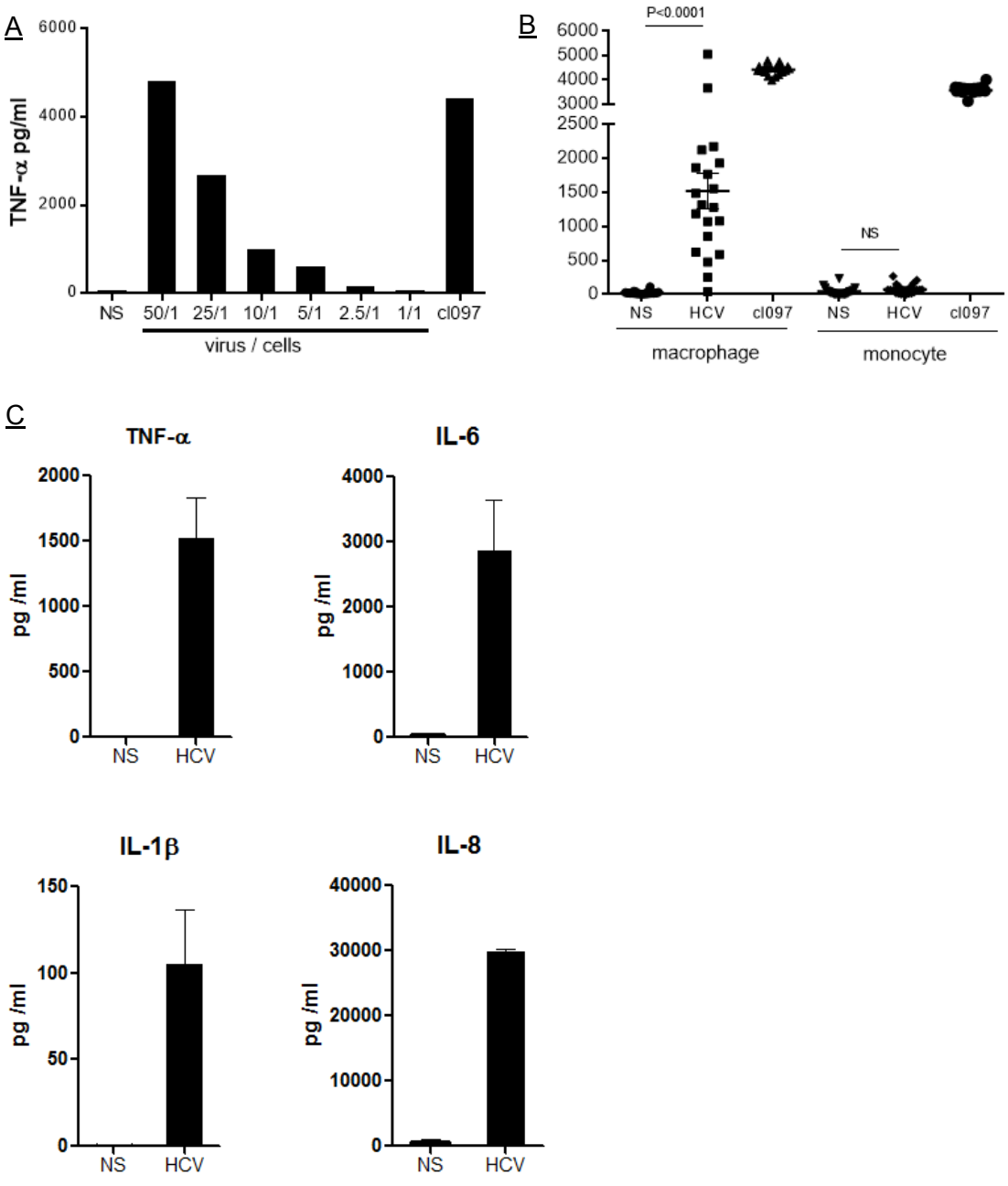
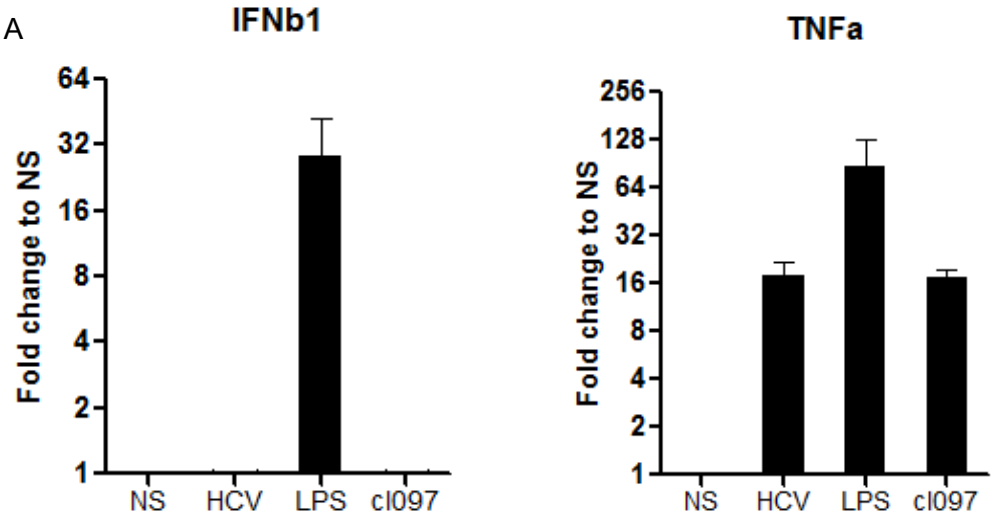

A
TNF-α pg/ml
6000
4000
2000
0
NS
50/1
25/1
10/1
5/1
2.5/1
1/1
cl097
virus / cells
B
P<0.0001
NS
NS
HCV
cl097
NS
HCV
cl097
macrophage
monocyte
C
TNF-α
IL-6
IL-1β
IL-8
pg /ml
NS
HCV

Figure 2: Dose-dependent activation and Inflammatory cytokines induction by HCVcc stimulation.

(A) 10^5 MDMs were stimulated with different ratio of HCVcc for 24h. (B) 10^5 MDMs or monocytes (n=20) were stimulated with HCVcc at ratio 20 for 24h. TNF-α production were measured by ELISA. (C) Inflammatory cytokines production by HCVcc stimulated MDMs were measured by CBA. NS indicates non-stimulated cells

A


IFNb1
Fold change to NS
64
32
16
8
4
2
1
NS
HCV
LPS
cl097
TNFa
Fold change to NS
256
128
64
32
16
8
4
2
1
NS
HCV
LPS
cl097

B

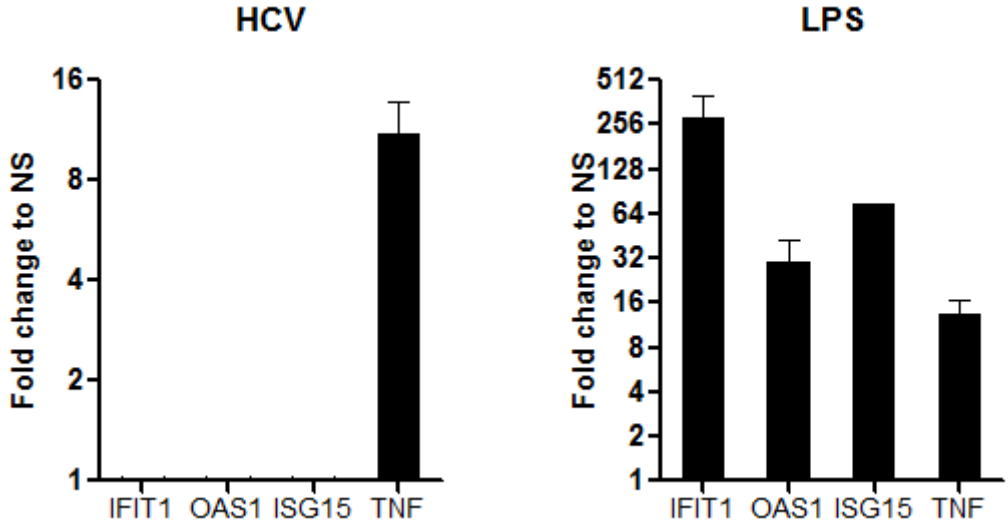

HCV
Fold change to NS
16
8
4
2
1
IFIT1
OAS1
ISG15
TNF
LPS
Fold change to NS
512
256
128
64
32
16
8
4
2
1
IFIT1
OAS1
ISG15
TNF

Figure 3: Expression of interferon and interferon-stimulated genes in macrophages upon HCV stimulation.

(A) 10^5 MDMs were stimulated with HCVcc, LPS or Cl097 for 3h, TNF-α and IFNb1 expression were measured by real time PCR. (B) 10^5 MDMs were stimulated with HCVcc or LPS for 16h, interferon-stimulated genes (IFIT1, OAS1, ISG15) and TNF-α were measured by real time PCR, results shown as fold change to gene expression in non-stimulated cells. "NS", non-stimulated cells.

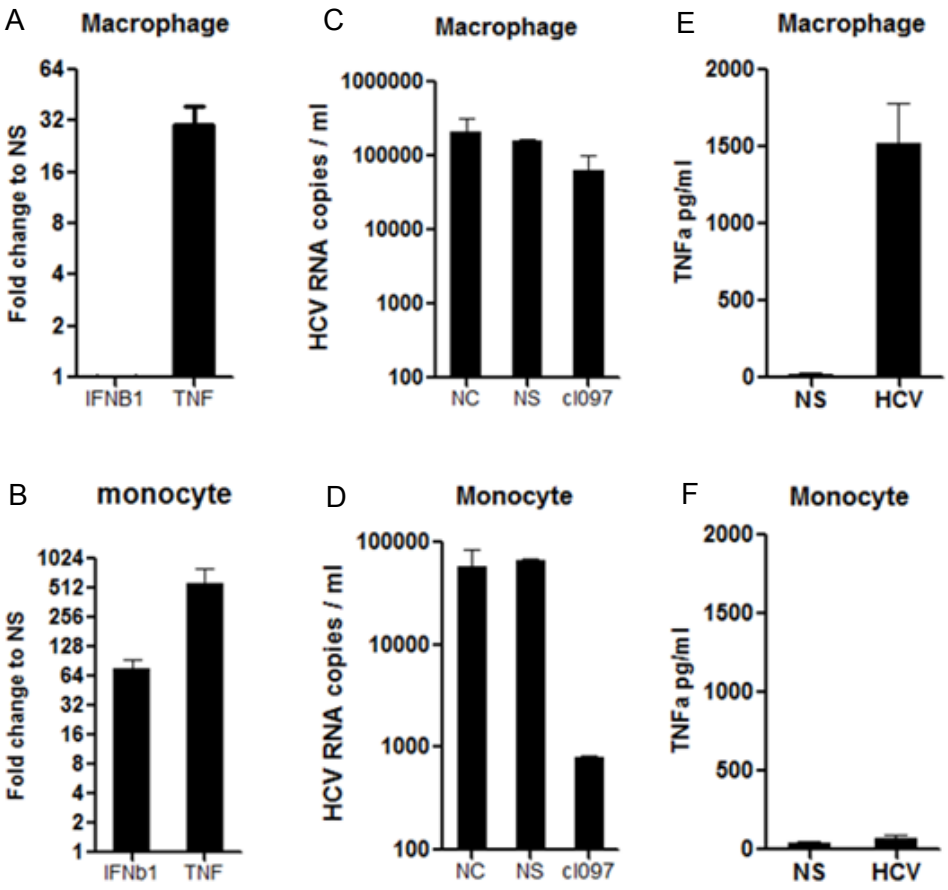
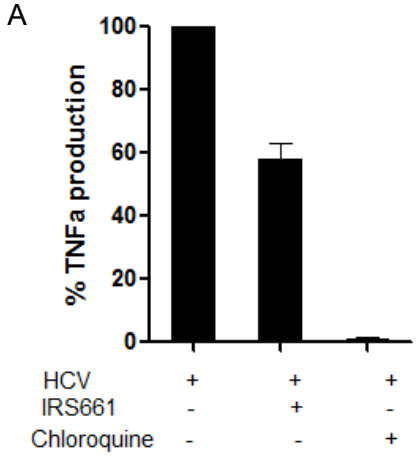

A
Macrophage
Fold change to NS
64
32
16
8
4
2
1
IFNB1
TNF
C
Macrophage
HCV RNA copies / ml
1000000
100000
10000
1000
100
NC
NS
cl097
E
Macrophage
TNFa pg/ml
2000
1500
1000
500
0
NS
HCV
B
monocyte
Fold change to NS
1024
512
256
128
64
32
16
8
4
2
1
IFNb1
TNF
D
Monocyte
HCV RNA copies / ml
100000
10000
1000
100
NC
NS
cl097
F
Monocyte
TNFa pg/ml
2000
1500
1000
500
0
NS
HCV

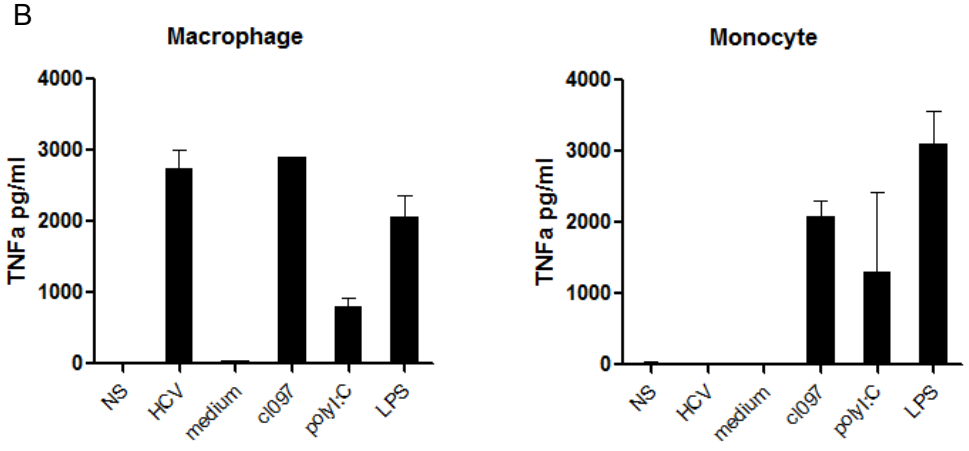
Figure 4: Antiviral activity and responsiveness to HCV.

Macrophages (A) or monocytes (B) were stimulated with cl097 for 3h. TNF and IFNb1 expression were measured by real time PCR. Supernatants from non-stimulated or cl097 stimulated macrophages (C) or monocytes (D) were added to HCV infected huh7.5, cells were washed and fresh medium were replaced, viral RNA in the supernatants were measured by real time PCR. macrophages (E) or monocytes (F) were incubated with HCVcc for 24h, TNF-α production in the supernatants were measured by ELISA. NS indicates non-stimulated cells

A


% TNFa production
100
80
60
40
20
0
HCV + + +
IRS661 - + -
Chloroquine - - +

B


Macrophage
TNFa pg/ml
4000
3000
2000
1000
0
NS
HCV
medium
cl097
polyI:C
LPS
Monocyte
TNFa pg/ml
4000
3000
2000
1000
0
NS
HCV
medium
cl097
polyI:C
LPS

C

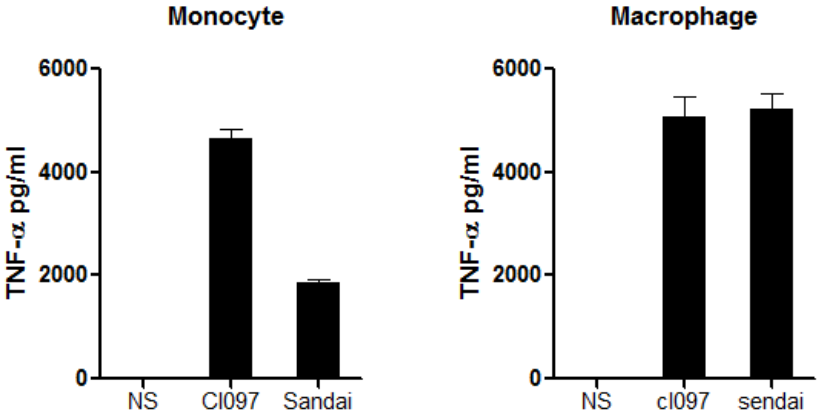
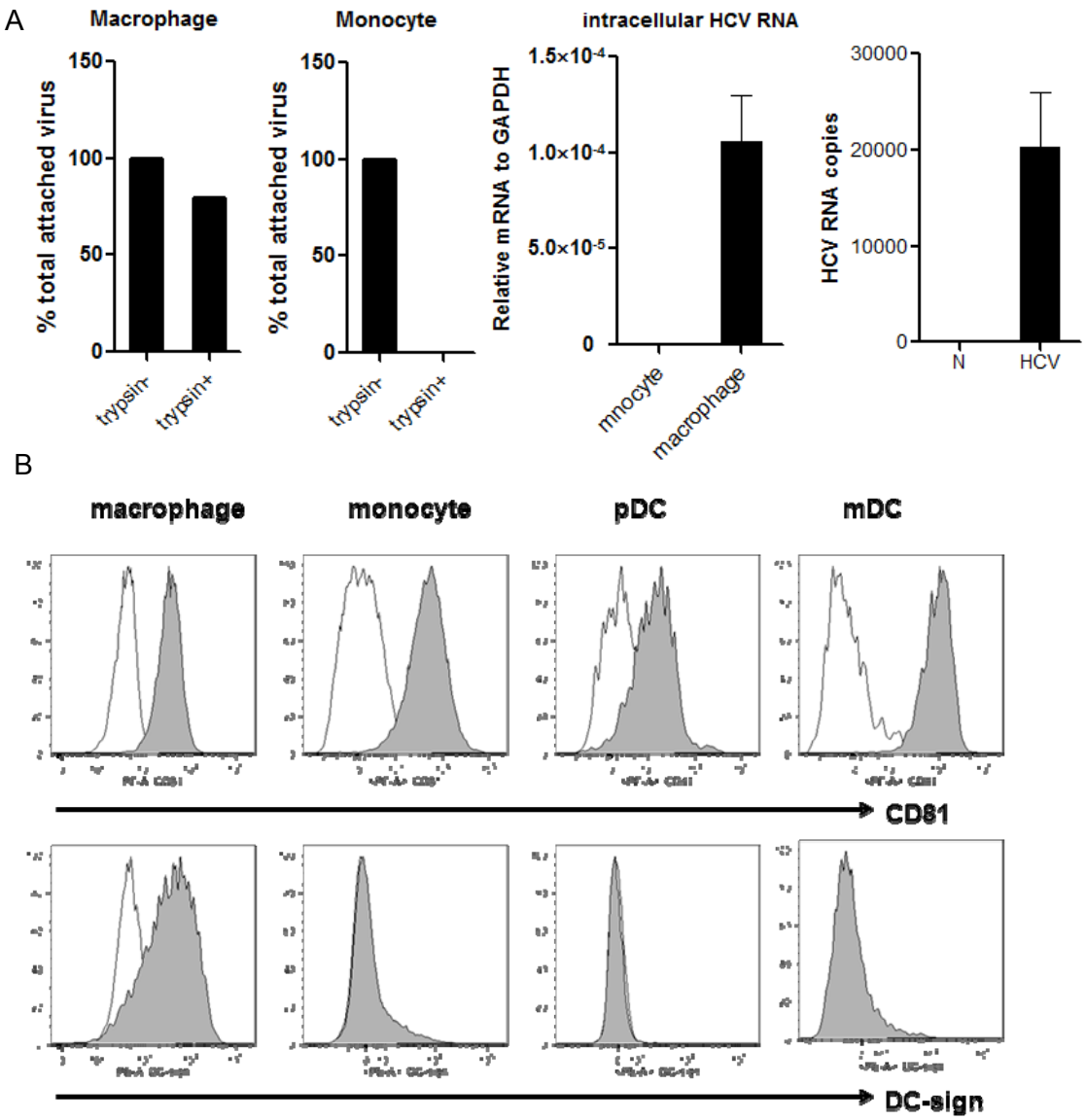
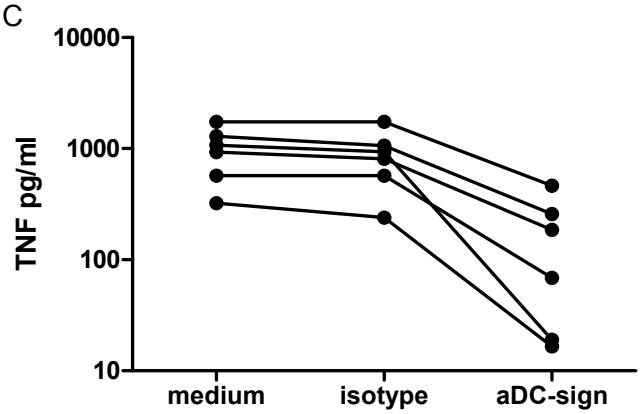

Monocyte
TNF-α pg/ml
6000
4000
2000
0
NS
Cl097
Sandai
Macrophage
TNF-α pg/ml
6000
4000
2000
0
NS
cl097
sendai

Figure 5: Pathway involved in HCV recognition.

(A) Macrophages were stimulated with HCVcc in the presence of TLR7/8 inhibitor IRS661 or chloroquine for 24h. (B) MDMs and monocytes were stimulated with HCVcc, medium (huh7.5 culture), cl097 (1ug/ml), polyI:C (30ug/ml) or LPS (1ng/ml) for 24h. TNF-α production were measured by ELISA. (C) 10^5 of macrophages and monocytes were stimulated with Sendai virus or CL097 for 24h. TNF-α production was measured by ELISA. "NS", non-stimulated cells


A
Macrophage
% total attached virus
150
100
50
0
trypsin-
trypsin+
Monocyte
% total attached virus
150
100
50
0
trypsin-
trypsin+
intracellular HCV RNA
Relative mRNA to GAPDH
1.5×10-4
1.0×10-4
5.0×10-5
0
mnocyte
macrophage
HCV RNA copies
30000
20000
10000
0
N
HCV
B
macrophage
monocyte
pDC
mDC
CD81
DC-sign

C


10000
1000
100
10
TNF pg/ml
medium
isotype
aDC-sign

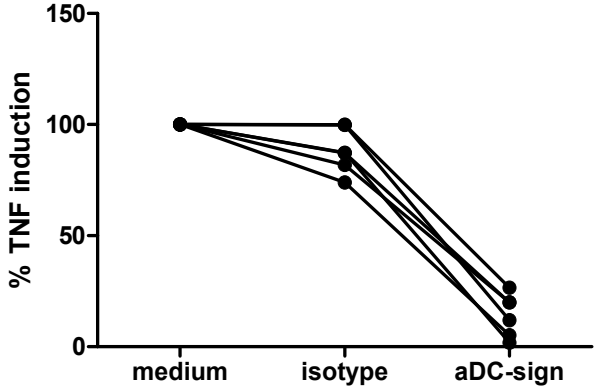
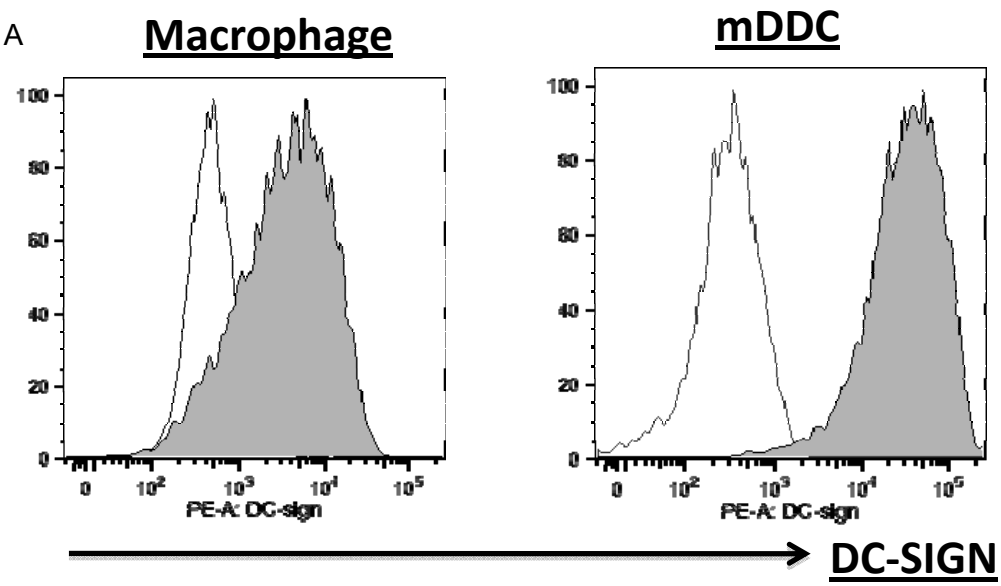

150
100
50
0
% TNF induction
medium
isotype
aDC-sign

Figure 6: DC-SIGN mediated uptake of HCV.

(A) Macrophage and monocytes were incubated with or without (N) HCV at 37 °C for 4h, cells were washed and treated with trypsin at 37 °C for 5min. After extensive wash, total RNA are extracted and viral RNA are measured by real-time PCR. Results were represented as percentage of viral RNA in cells without trypsin treatment. Viral RNA was normalized to GAPDH. (B) Macrophages or PBMCs were stained with isotype (open), anti-CD81 or anti-DC-SIGN (filled), CD81 and DC-SIGN expression on macrophages, monocytes, mDC and pDC by flow Cytometry. (C) Macrophages were incubated with medium, isotype control and DC-SIGN blocking antibody for 1 hour, then cells were stimulated with HCV for 24hrs, TNF-α production was measured by ELISA. Results were shown as concentration and percentage of TNF-α induced by HCV alone.

A


Macrophage
mDDC
100
80
60
40
20
0
0
10^2
10^3
10^4
10^5
PE-A: DC-sign
DC-SIGN

B

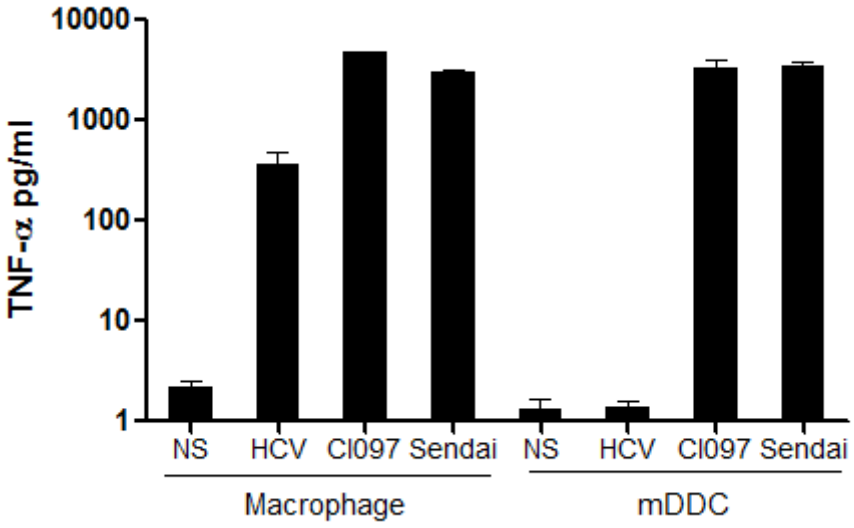

TNF-α pg/ml
10000
1000
100
10
1
NS
HCV
Cl097
Sendai
NS
HCV
Cl097
Sendai
Macrophage
mDDC

Figure 7: Responsiveness of macrophages and MDDCs to HCV.

Monocytes were cultured in the presence of GMCSF or GMCSF/IL4 respectively to generate macrophages and mDDCs, cells were harvested at day 6 and used for staining or stimulation. (A) DC-SIGN expression on macrophages and MDDCs were measured by flow cytometry. Cells were stained with isotype (open) or anti-DC-SIGN (filled) (B) 10^5 of macrophages and MDDCs were stimulated with HCV or CL097, or Sendai virus or CL097 for 24h. TNF-α production were measured by ELISA. NS indicates non-stimulated cells

Reference

Abe, T., Y. Kaname, et al. (2007). "Hepatitis C virus nonstructural protein 5A modulates the toll-like receptor-MyD88-dependent signaling pathway in macrophage cell lines." J Virol 81(17): 8953-8966.

Barth, H., A. Ulsenheimer, et al. (2005). "Uptake and presentation of hepatitis C virus-like particles by human dendritic cells." Blood 105(9): 3605-3614.

Bigger, C. B., K. M. Brasky, et al. (2001). "DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection." J Virol 75(15): 7059-7066.

Brown, R. S. (2005). "Hepatitis C and liver transplantation." Nature 436(7053): 973-978.

Cheng, G., J. Zhong, et al. (2006). "Inhibition of dsRNA-induced signaling in hepatitis C virus-infected cells by NS3 protease-dependent and -independent mechanisms." Proc Natl Acad Sci U S A 103(22): 8499-8504.

Dustin, L. B. and C. M. Rice (2007). "Flying under the radar: the immunobiology of hepatitis C." Annu Rev Immunol 25: 71-99.

Geijtenbeek, T. B., D. J. Krooshoop, et al. (2000). "DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking." Nat Immunol 1(4): 353-357.

Gringhuis, S. I., M. van der Vlist, et al. (2010). "HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells." Nat Immunol 11(5): 419-426.

Hsu, M., J. Zhang, et al. (2003). "Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles." Proc Natl Acad Sci U S A 100(12): 7271-7276.

Kanda, T., R. Steele, et al. (2007). "Hepatitis C virus infection induces the beta interferon signaling pathway in immortalized human hepatocytes." J Virol 81(22): 12375-12381.

Lambotin, M., T. F. Baumert, et al. (2010). "Distinct intracellular trafficking of hepatitis C virus in myeloid and plasmacytoid dendritic cells." J Virol 84(17): 8964-8969.

Larsson, M., E. Babcock, et al. (2004). "Lack of phenotypic and functional impairment in dendritic cells from chimpanzees chronically infected with hepatitis C virus." J Virol 78(12): 6151-6161.

Lauer, G. M. and B. D. Walker (2001). "Hepatitis C virus infection." N Engl J Med 345(1): 41-52.

Liang, H., R. S. Russell, et al. (2009). "Differential effects of hepatitis C virus JFH1 on human myeloid and plasmacytoid dendritic cells." J Virol 83(11): 5693-5707.

Liu, C., Q. Tao, et al. (2010). "Kupffer cells are associated with apoptosis, inflammation and fibrotic effects in hepatic fibrosis in rats." Lab Invest 90(12): 1805-1816.

Ludwig, I. S., A. N. Lekkerkerker, et al. (2004). "Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation." J Virol 78(15): 8322-8332.

Mayo, M. J. (2003). "Extrahepatic manifestations of hepatitis C infection." Am J Med Sci 325(3): 135-148.

Mihm, S., M. Frese, et al. (2004). "Interferon type I gene expression in chronic hepatitis C." Lab Invest 84(9): 1148-1159.

Missale, G., R. Bertoni, et al. (1996). "Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response." J Clin Invest 98(3): 706-714.

Polyak, S. J., K. S. Khabar, et al. (2001). "Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy." J Virol 75(13): 6209-6211.

Schroder, K., M. Spille, et al. (2007). "Differential effects of CpG DNA on IFN-beta induction and STAT1 activation in murine macrophages versus dendritic cells: alternatively activated STAT1 negatively regulates TLR signaling in macrophages." J Immunol 179(6): 3495-3503.

Schwartz, A. J., X. Alvarez, et al. (2002). "Distribution and immunophenotype of DC-SIGN-expressing cells in SIV-infected and uninfected macaques." AIDS Res Hum Retroviruses 18(14): 1021-1029.

Shiina, M. and B. Rehermann (2008). "Cell culture-produced hepatitis C virus impairs plasmacytoid dendritic cell function." Hepatology 47(2): 385-395.

Su, A. I., J. P. Pezacki, et al. (2002). "Genomic analysis of the host response to hepatitis C virus infection." Proc Natl Acad Sci U S A 99(24): 15669-15674.

Sun, P., S. Fernandez, et al. (2009). "Functional characterization of ex vivo blood myeloid and plasmacytoid dendritic cells after infection with dengue virus." Virology 383(2): 207-215.

Takahashi, K., S. Asabe, et al. (2010). "Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection." Proc Natl Acad Sci U S A 107(16): 7431-7436.

Thimme, R., J. Bukh, et al. (2002). "Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease." Proc Natl Acad Sci U S A 99(24): 15661-15668.

Wieland, S. F. and F. V. Chisari (2005). "Stealth and cunning: hepatitis B and hepatitis C viruses." J Virol 79(15): 9369-9380.

Zhang, Y. L., Y. J. Guo, et al. (2009). "Hepatitis C virus single-stranded RNA induces innate immunity via Toll-like receptor 7." J Hepatol 51(1): 29-38.

CHAPTER 4

DISCUSSION

1. Summary of findings

In this study, we revealed that HCV RNA possesses the capability to initiate innate immune responses via triggering TLR7/8, and that the HCV genome contains GU-rich sequences that can be recognized by TLR7/8. Three sequences in the HCV genome were identified as ligands of TLR7/8.

Stimulation with HCV-derived ssRNAs resulted in the maturation of pDC and the production of type I Interferon, HCV ssRNA also induced the production of inflammatory cytokines and chemokines by different APCs. Cytokines produced by stimulated monocytes and pDCs significantly inhibited HCV production (2 to 3 log decrease). Viral production could be restored by antibody against interferon-alpha/beta R2 (IFNAR2). Cytokines produced by activated mDCs barely affected viral production, which might be due to the absence of IFN signaling. Furthermore, HCV-RNA-mediated cytokines decreased CD81 expression on huh7.5. This down-regulation was not induced by type I IFN.

Moreover, we demonstrated that not only HCV RNA but also HCV virions can be detected by some APCs. However, among different APCs that we tested (monocytes, pan-DCs, macrophages, and MDDCs), only macrophages were able to respond to HCVcc. Incubation of macrophages with HCVcc resulted in production of inflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-8. Interestingly, stimulation of macrophages with HCVcc did not lead to induction of IFN and IFN-stimulated genes (IFIT1, OAS1, and ISG15). This phenomenon is very similar to TLR7/8 signaling in

macrophages. Involvement of TLR7/8 in HCV detection by macrophages was validated by using IRS661, a specific inhibitor of TLR7/8 (Barrat, Meeker et al. 2005).

We also showed that monocytes and macrophages captured HCV, but internalization occurred only in macrophages. Viral RNA was not detectable in monocytes after trypsin treatment. Although CD81 was broadly expressed on different peripheral APCs, DC-SIGN was detected only on macrophages. This strongly suggested that DC-SIGN plays an important role in HCV detection. The anti-DC-SIGN antibody, which blocks the interaction of DC-SIGN with HCV glycoprotein, validated the hypothesis that DC-SIGN is involved in HCV detection by macrophages. However, monocyte-differentiated DCs, which express very high levels of DC-SIGN, were unresponsive to HCV stimulation, suggesting that DC-SIGN expression is not the only determinant of the responsiveness to HCV.

2. Immunogenicity of HCV

Knowledge about the immunogenicity of HCV remains elusive. Although immune activation was observed in HCV-infected individuals (Bigger, Brasky et al. 2001), it has been very difficult to prove that HCV is immunogenic and able to activate APCs *in vitro* (Liang, Russell et al. 2009, Zhang, Guo et al. 2009, Takahashi, Asabe et al. 2010). The involvement of HCV proteins in both immune activation and immunosuppression was reported (Yao, Ray et al. 2001, Dolganiuc, Oak et al. 2004). It was shown that HCV proteins, core and NS3, trigger TLR2 to induce inflammatory activation (Dolganiuc, Oak et al. 2004), and TLR1 and TLR6 are involved in the process

of recognition of HCV Core and NS3 protein (Chang, Dolganiuc et al. 2007). In another study, TLR2 was shown to sense the hepatitis C virus core protein but not infectious virons (Hoffmann, Zeisel et al. 2009), suggesting that the intact structure of virons prevent recognition of core by TLR2. Core and NS3 proteins released from apoptotic hepatocytes may induce immune activation; HCV Core protein is detectable in the plasma of HCV-infected patients (Orito, Mizokami et al. 1996, Tanaka, Kiyosawa et al. 1996). On the contrary, both core and NS3 also exhibit strong inhibitory effects on the production of TLR-mediated cytokines (Tu, Pierce et al. 2010, Abe, Kaname et al. 2007). It is still unclear how HCV proteins contribute to activation of the innate immune system during HCV infection.

As an RNA virus, HCV RNA is an important PAMP that can be recognized by RNA sensors to initiate an innate immune response. Indeed, as we showed in Figure 1A in chapter 2, HCV RNA was capable to induce inflammatory cytokines production by PBMCs. Two RNA sensors, RIG-I and TLR7, have been proposed as the receptors that are responsible for detection of HCV RNA. Saito *et al.* reported that single-strand RNA of HCV triggers RIG-I to induce an IFN-dependent immune defense in hepatocytes, and a similar immune activation was observed *in vivo* with hydrodynamic RNA transfection in their study (Saito, Owen et al. 2008). However, in contrast to TLR7 and 8, RIG-I is expressed in the cytoplasmic compartments and not in the endosomes where HCV particles are likely to reside upon their uptake by APCs. Thus, TLR7/8 are most likely responsible for HCV detection. Moreover, the HCV genome contains GU-rich sequences, the essential motif for TLR7/8 recognition (Heil, Hemmi et al. 2004). Here, we proved that HCV RNA could trigger TLR7/8 in APCs (Figure 1D, Figure 2, chapter2). HCV-

derived GU-rich sequences were functional as TLR7/8 agonists to induce DC maturation and cytokine production (Figure 3, Figure 4). Interestingly, not all the GU-rich sequences we selected in the HCV genome can trigger TLR7/8. Actually, 6 out of 9 selected sequences failed to induce TNF-α production, despite we selected all sequences with the same criterions and similar GU/U density, which suggests that the presence of GU in the RNA sequence is not sufficient to be a ligand of TLR7/8, certain motif in GU-rich sequences is required for recognition by TLR7/8. However, GU is an indispensable character for RNA that triggers TLR7/8. Our results showed that U-to-A replacement of GU motifs completely abolished the stimulatory effects of those identified ssRNAs. Although many studies have tried to identify the precise motif for TLR7/8 recognition, they represent only part of the motifs that trigger TLR7/8 (Heil, Hemmi et al. 2004, Hornung, Guenthner-Biller et al. 2005, Forsbach, Nemorin et al. 2008). Anyhow, our study reveals that HCV RNA contains GU-rich sequences that trigger TLR7/8. Those GU-rich sequences endow HCV RNA with the capability to induce innate immune activation.

3. TLR7 and innate immunity against HCV infection

TLR7 agonists, which strongly induce type I interferon in pDCs, have been considered as potential candidates for HCV therapy. Several clinical trials have shown significant induction of type I interferon and viremia suppression by TLR7 agonists (Horsmans, Berg et al. 2005, Pockros, Guyader et al. 2007, Bergmann, de Bruijne et al. 2011). TLR7-mediated antiviral response was also observed in human hepatocyte lines

(Lee, Wu et al. 2006). All those studies demonstrated that TLR7 may play an important role in innate immunity against HCV infection. Viral RNA can be detected by TLR7 (Lund, Alexopoulou et al. 2004). It has been reported that pDCs could be activated by HCV-infected hepatocytes via TLR7 (Takahashi, Asabe et al. 2010). However, the ability of HCV RNA to trigger TLR7 and induce an immune response is still not well addressed. GU-rich sequences were identified as an essential motif to be recognized by TLR7 (Heil, Hemmi et al. 2004, Hornung, Guenthner-Biller et al. 2005). Based on previous findings, we selected GU-rich sequences from the HCV genome, and three sequences were identified as ligands of TLR7/8 (Figures 1 and 2, chapter 2). Our results showed that HCV ssRNA is an agonist of TLR7/8, specifically triggering TLR7 or TLR8 receptors, induction of DC maturation and type I interferon production, and induction of cytokine production profile in peripheral APCs (Figures 3 and 4, chapter 2). Moreover, similar to another TLR7 agonist, HCV ssRNA exhibited remarkable antiviral activity when it stimulates peripheral APCs. Indeed, HCV-ssRNA-induced cytokines significantly inhibited HCV production (Figure 5, chapter 2). This suggests HCV is able to induce antiviral response *via* TLR7/8 and restrict viral infection if APCs detect viral RNA and initiate immune response properly. However, as we showed, HCV particles escape triggering of DCs and monocytes, which can mount type I IFN and exhibit optimal anti-viral responses upon TLR7/8 activation. HCV Particles are able to activate macrophages through TLR7/8; however, these cells do not mount an anti-viral response against HCV.

TLR7 plays an important role in anti-HCV defense in hepatocytes. However, this is not optimal as it was previously shown that decreased TLR7 expression and function in HCV-infected hepatocytes is a mechanism to prevent efficient innate immune responses

to HCV, thus highlighting the different layers of HCV escape mechanisms. A further level of escape mechanisms may arise from the fact that HCV Particles are able to activate macrophages, however these cells do not mount an anti-viral response against HCV when TLR7/8 are triggered. HCV also escape triggering of DCs and monocytes, two cellular subsets that can compensate and mount optimal responses.

4. Induction of innate immune response and inflammation in HCV infection

Immune response induced by HCV infection is characterized as rapid innate immune response and delayed adaptive immune response (Dustin and Rice 2007), regardless of the outcome of the disease progression. Innate immune activation was observed at an early stage after HCV infection in chimpanzees (Bigger, Brasky et al. 2001). Inflammatory cytokines also have been shown to be up-regulated in chronically HCV-infected individuals (Polyak, Khabar et al. 2001). This implies that the innate viral infection rapidly induce immune response. However, it has been difficult to clarify the mechanisms of immune activation induced by HCV infection, as immune activation in infected hepatocytes is obscure and DCs exhibit unresponsiveness to HCV *in vitro* (Bigger, Guerra et al. 2004, Wieland, Makowska et al. 2013). The sources of inflammation during HCV infection are not well defined. HCV can infect hepatocytes (Cormier, Tsamis et al. 2004), or it can be captured by liver sinusoidal endothelial cells (LSECs) through L-sign (Gardner, Durso et al. 2003, Cormier, Durso et al. 2004) or by resident DCs that express DC-SIGN (Ludwig, Lekkerkerker et al. 2004) and macrophages (Figure 6, chapter 3). HCV components (proteins Core and NS3, viral

RNA) were reported as possessing the capability to initiate innate immune activation (Dolganiuc, Oak et al. 2004, Zhang, Guo et al. 2009). As we demonstrated, HCV RNA can be detected by APCs and lead to production of inflammatory cytokines and chemokines (Figures 1, 3, chapter 2). The immune activation is mediated by TLR7/8 (Figure 2, chapter2). It also has been shown that HCV-infected hepatocytes activate pDCs through cell–cell contact, resulting in the production of type I IFN (Takahashi, Asabe et al. 2010).

Persistent HCV infections are typically associated with inflammatory response, which is related to HCV infection driving fibrogenesis, with increased deposition of extracellular matrix proteins leading to fibrotic scarring and ultimately cirrhosis (Tarao, Ohkawa et al. 2013). Activation of hepatic stellate cells (HSCs) is known to be important in this process and may be cytokine-driven, but the specific mechanisms are not very clear (Giannelli and Antonaci 2005). The sensing of HCV infection by the innate immune system likely contributes to these processes. As we showed, monocytes, mDCs and pDCs that were stimulated with HCV RNA produce abundant cytokines, such as TNF-α, IL-6, IL-12, IL-1b, and IL-10, and chemokines CXCL9 and CXCL10 (Figure 3, chapter 2). Immune activation induced by HCV RNA leads to production of inflammatory cytokines and chemokines that might promote the formation of an inflammatory environment in the liver, which are crucial for recruiting immune cells and activation of HSCs.

In this study, we demonstrated for the first time that HCV can be detected directly by APCs, but only macrophages ,not peripheral DCs, monocytes, and MDDCs, respond to HCV stimulation and produce inflammatory cytokines (Figures 1 and 2, chapter 3). Macrophages detect HCV particles directly and produce TNF-α, IL-1β, IL-6, and IL-8

(Figure 2, chapter3). This suggests that HCV-induced activation of macrophages in the liver (Kupffer cells) may contribute to the formation of the inflammatory environment, activation of HSCs, and further fibrosis. The release of chemokines (i.e., IL-8) is followed by recruitment of inflammatory cells, which further leads to tissue damage, leading to repeated cycles of inflammation and damage. TNF-α leads to altered expression of cell adhesion molecules on sinusoidal endothelial allowing the recruitment and sinusoidal transmigration of inflammatory cells (Bissell, Wang et al. 1995, Neubauer, Wilfling et al. 2000). Activated Kupffer cells and recruited inflammatory cells establish a special cytokine environment, which is of crucial importance in activation of HSCs (Grappone, Pinzani et al. 1999, Luo, Tang et al. 2005). The activated HSCs lose their ability to store fat and retinoids and produce cytokines, leading to an autocrine effect of the activation and proliferation of portal fibroblasts and myofibroblasts (Salmi, Adams et al. 1998). Thus, activation of macrophages induced by HCV might contribute to inflammation and promote disease progression.

5. Detection of HCV by antigen-presenting cells

Although the immunogenicity of HCV components (RNA or proteins) have been shown (Dolganiuc, Oak et al. 2004, Zhang, Guo et al. 2009), DCs appear “silent” when they encounter HCV (Liang, Russell et al. 2009, Zhang, Guo et al. 2009, Takahashi, Asabe et al. 2010). Many efforts have been made to prove the immunogenicity of HCV particles, but the only common element in those studies is the unresponsiveness of DCs to HCV stimulation. Neither DC maturation nor induction of cytokines was observed

(Shiina and Rehermann 2008, Liang, Russell et al. 2009, Zhang, Guo et al. 2009, Takahashi, Asabe et al. 2010). In concordance with previous findings, our results demonstrated that DCs, as well as monocytes, fail to respond to HCV stimulation (Figures 1B, C). HCV seems to utilize unique strategies to escape from DC detection, whereas other RNA viruses such as the influenza virus, Sendai virus, and HIV induce strong immune responses to DCs *in vitro* (Lee, Lund et al. 2007, Mandl, Barry et al. 2008).

The mechanisms of DC unresponsiveness to HCV are not known, and the causes that endow HCV with an "undetectable" characteristic still remain unidentified. Two possible mechanisms have been proposed and broadly studied: inhibitory effects of HCV proteins and inefficient uptake of HCV. HCV proteins such as core, NS3, and NS5A exhibited strong inhibitory effects on signaling of PRRs (Abe, Kaname et al. 2007, Tu, Pierce et al. 2010). Those proteins were thought to be the cause of the tolerance of DCs to viral stimulation. HCV particles were reported to have the same inhibitory effects on particular TLRs, such as TLR9, the mechanism is unclear (Shiina and Rehermann 2008). However, the inhibitory effects of HCV particles do not depend on expression of viral proteins since the inhibition does not require viral infection or entry of DCs (Shiina and Rehermann 2008). Moreover, HCV particles have no effect on cytokine induction and DC maturation induced by RNA virus or LPS (Shiina and Rehermann 2008), suggesting that HCV does not alter the capability of DCs to respond to virus or microbe stimulation. Similarly, the responsiveness of macrophages to HCV we observed in this study also implies that HCV virons do not generally shut down the function of PRRs. HCV still can be detected and induce an immune response.

The HCV uptake capability of DCs remains controversial. The internalization of HCV-like particles by DCs (Ludwig, Lekkerkerker et al. 2004, Barth, Ulsenheimer et al. 2005) and the absence of HCV in DCs from HCV-infected patients (Rollier, Drexhage et al. 2003, Longman, Talal et al. 2004) were both reported. Recently, it has been shown that PBMCs (Cormier, Tsamis et al. 2004) or peripheral DCs (Kaimori, Kanto et al. 2004) are certainly not susceptible to HCV infection. HCV particles in PBMCs from patients are predominantly carried on the surface of cells (Natarajan, Kottilil et al. 2010). Thus, many studies, together with our results, allow us to speculate that the unresponsiveness of DCs to HCV might be due to the incapability of viral uptake.

The process of HCV uptake by APCs is still unclear. Although peripheral DCs express very low levels of receptors that are essential for HCV infection on hepatocytes (such as CD81, SRBI, and CLDN1), HCV does not infect DCs (Kaimori, Kanto et al. 2004, Marukian, Jones et al. 2008). DC-SIGN, a Lectin that has been shown to interact with HCV glycoprotein, was proposed as the receptor for HCV uptake by DCs (Pohlmann, Zhang et al. 2003, Ludwig, Lekkerkerker et al. 2004). DC-SIGN has been found on resident DCs and macrophages in the lymph nodes and the liver (Schwartz, Alvarez et al. 2002, Lai, Sun et al. 2006, Gringhuis, van der Vlist et al. 2010). However, as we and others (Mengshol, Golden-Mason et al. 2009, Sun, Fernandez et al. 2009) have shown, DC-SIGN was not detected on peripheral DCs (Figure 6B, chapter 3), whereas it expressed on macrophages (Figure 6B, chapter 3). Interestingly, macrophages—the only subset of APCs expressing DC-SIGN among APCs we tested—were the only subset that responded to HCV stimulation (Figure 1D, chapter 3). This suggests that DC-SIGN is involved in HCV detection by macrophages. Indeed, the blockade of DC-SIGN inhibited

TNF-α production induced by HCV (Figure 6C, chapter 3). Thus, DC-SIGN does play a critical role in HCV detection. To investigate whether DC-SIGN is sufficient to endow APCs with the capability to sense HCV, we used monocyte-derived DCs (MDDCs), on which DC-SIGN was initially identified. DC-SIGN is highly expressed on MDDC (Figure 7A). As we showed (Figure 7B, chapter3), MDDCs unexpectedly did not respond to HCV stimulation, although those MDDCs exhibited strong responsiveness to other RNA viruses (Figure 7B, chapter 3). The mechanism that causes differences in responsiveness of macrophages and MDDCs to HCV is unknown. Both macrophages and MDDCs are derived from monocytes; the difference is that IL-4 is required for MDDCs differentiation. IL-4 induces a high level of DC-SIGN expression on DCs (Relloso, Puig-Kroger et al. 2002), however, it also may alter other properties of DCs (Montaner, da Silva et al. 1999). It was reported that myeloid DCs became susceptible to pseudo-type HCV with GM-CSF pretreatment, but GM-CSF-induced susceptibility was impaired in the presence of additional IL-4 (Kaimori, Kanto et al. 2004). It has also been shown that IL-4 could alter the endocytic activity in macrophages, leading to targeting of internalized particles to a non-lysosomal compartment (Montaner, da Silva et al. 1999), which was proposed as a viral strategy to escape detection by PPRs (Ludwig, Lekkerkerker et al. 2004).

6. Interferon and control of HCV infection

Type I interferon has been considered a key factor that mediates immune defense to control HCV infection. It has exhibited great antiviral activity and has good clinical

records in hepatitis C therapy (Dammacco, Tucci et al. 2010). Many studies have shown that establishment of immune defense in hepatoma cell lines or cytokine-mediated suppression of HCV replication totally depends on IFN induction. In accordance with previous studies, our results also showed that HCV RNA has the capability to induce cytokines that strongly inhibit HCV replication in an IFN-dependent manner (Figure 5, chapter2). mDCs (Figure 5,chapter 2) and macrophages (Figure 5, chapter 3) did not produce IFN upon HCV RNA stimulation and failed to restrict HCV replication.

Type I interferon and IFN-stimulated genes were rapidly induced in HCV-infected subjects, and the IFN response was correlated with the magnitude and duration of HCV infection. The source of interferon *in vivo* is still not well defined. We have shown that HCV RNA can induce IFN-β in pDCs or monocytes resulting in inhibition of HCV replication. Therefore, HCV RNA is able to induce optimal anti-viral immune responses by monocytes and pDCs; however, these cells are indeed compromised in their recognition of HCV particles (Figure 1, chapter 3). The mechanisms of unresponsiveness are still unknown. Our study demonstrated that macrophages possess the capacity to sense HCV (Figure 2, chapter 3). However, the immune response induced by HCV in macrophages lacks IFN production and IFN signaling (Figure 4, chapter 3), which are key mediators of viral inhibition. pDCs were proposed as the source of interferon. Recently, it has been shown that pDCs could be activated by HCV-infected hepatocytes via TLR7/8, resulting in production of type I interferon. Cell–cell contact is required in this process (Takahashi, Asabe et al. 2010). Infiltration of pDCs in patient liver tissues was observed, however, IFN-signaling was absent in intrahepatic pDCs. Also, IFN-stimulated gene (ISG-15)-expressing cells did not co-localize with pDCs (Lau, Fish et al. 2008). This

implies that pDCs may be not the major source of interferon in the liver of hepatitis C patients. In accordance with these observations, our results showed that HCV complete particles, but not viral RNA, do not induce activation of IFN-α/β producing cells (monocytes/pDCs), likely due to a blockage in the virus entry process, which might represent an escape mechanism of HCV from detection by innate immunity.

Intrahepatic interferon signaling was observed and associated with HCV viremia, regardless of the outcome of the disease progression (Su, Pezacki et al. 2002, Thimme, Bukh et al. 2002). This suggests that HCV induces type I interferon *in vivo*, but the infection is not controlled by it. Instead, up-regulation of IFN-stimulated genes has been defined as a predictor of non-responsiveness to interferon therapy (Sarasin-Filipowicz, Oakeley et al. 2008). The mechanisms of failure of intrahepatic interferon to control HCV infection are still mysterious. It is known that an innate immune response is impaired in the presence of HCV proteins (Abe, Kaname et al. 2007, Tu, Pierce et al. 2010). However, inhibitory effects of HCV proteins cannot explain the failure of infection control by interferon, since intrahepatic IFN-stimulated genes are detected (Sarasin-Filipowicz, Oakeley et al. 2008). Persistent HCV infections are typically associated with an inflammatory response that is characterized as an infiltration of immune cells and the formation of a cytokine environment in the liver (Bigger, Guerra et al. 2004, Blackard, Komurian-Pradel et al. 2006, Lau, Fish et al. 2008), which might alter the antiviral activity of interferon induced by HCV infection. It was reported that IL-8 inhibits the antiviral action of interferon (Khabar, Al-Zoghaibi et al. 1997). The mechanism is still unclear. Our results showed that HCV could activate macrophages and induce abundant IL-8 (Figure 2, chapter3). The largest population of resident macrophages (Kupffer cells)

is in the liver. Those macrophages could be activated by HCV and produce IL-8, which might compromise an IFN-mediated innate defense. Indeed, up-regulation of IL-8 was found in chronically HCV-infected individuals, and elevated levels of IL-8 in serum were associated with resistance to interferon therapy (Polyak, Khabar et al. 2001).

In conclusion, this study demonstrated that HCV RNA encodes for GU-rich sequences, which stimulate the production of inflammatory cytokines and chemokines by antigen presenting cells, upon triggering through TLR7 and TLR8. However, in contrast to pDCs, mDCs, monocytes and mDDCs, only macrophages can be stimulated by the complete HCV particles in a way that depends on endocytosis and partially involves TLR7 and TLR8, this activation is not optimal and does not mediate anti-viral responses due to lack of Type I interferon. This leads to the escape of HCV from the anti-viralresponse mediated by APCs. Our results help in understanding the mechanisms of an effective innate immune response to HCV, which is useful to the development of an anti-HCV vaccine and therapeutic interventions targeting TLR7, TLR8 and APCs to potentiate them for optimal antiviral response.

Perspectives

Our results demonstrate that *in vitro* differentiated Macrophages are able to detect HCV via TLR7/8; DC-SIGN is involved in this process. To understand the mechanism of HCV detection by macrophages and its contribution to pathogenesis of HCV infection, several questions remain to be answered.

1. Response or evasion

As we described above, macrophages are able to detect HCV and produce inflammatory cytokines, peripheral DCs and monocytes do not respond to HCV due to the obstacle of HCV uptake which likely caused by lack of DC-SIGN expression. It was reported that DC-SIGN mediates efficient HCV uptake (Ludwig, Lekkerkerker et al. 2004). Moreover, DC-SIGN is also detectable on tissue DCs and macrophages (Jameson, Baribaud et al. 2002, Soilleux, Morris et al. 2002), suggesting the possibility of HCV detection by DCs. However, as we showed, DC-SIGN expressing MDDCs do not respond to HCV stimulation. The differences between macrophages and MDDCs in HCV internalization may be the key to uncover the mechanism of HCV detection. Ludwig IS et al revealed that DC-SIGN mediated internalization is cell type dependent, the captured HCV can be targeted differently to intracellular compartments in different cells. Internalized HCV virus-like particles were targeted to non-lysosomal compartments within immature DCs, where they are protected from lysosomal degradation (Ludwig, Lekkerkerker et al. 2004). This may explain the un-responsiveness of MDDCs to HCV, as recognition of viral RNA by TLR7 takes place in lysosome (Lund, Alexopoulou et al. 2004). HCV may target DC-SIGN to escape immune surveillance. To verify this

hypothesis, localization of internalized HCV in macrophages and MDDCs need to be determined. Furthermore, we need to investigate the HCV responsiveness of tissue DCs and Kupffer cells.

2. Inflammation and resistance

Most interesting finding in this study is that HCV induce production of inflammatory cytokines but not interferon in macrophages. Since anti-HCV activity of interferon is dose dependent (Lam, Neumann et al. 1997), as the only APC we identified that respond to HCV stimulation, lack of interferon production may compromise the innate immune response against HCV infection. Furthermore, macrophages produce high level of IL-8 upon HCV stimulation (Chapter 3, Figure 2), a chemokine strongly inhibits interferon antiviral activity (Khabar, Al-Zoghaibi et al. 1997). It has been shown that elevated levels of interleukin-8 in serum are associated with HCV infection and resistance to interferon therapy; it implies that macrophages activated by HCV may contribute to pathogenesis of HCV infection. To determine the role of macrophages plays in HCV infection, we need to investigate the relationship of macrophage activation, inflammation and viral load.

Reference

Alter, M. J. (2007). "Epidemiology of hepatitis C virus infection." World J Gastroenterol**13**(17): 2436-2441.

Barth, H., A. Ulsenheimer, et al. (2005). "Uptake and presentation of hepatitis C virus-like particles by human dendritic cells." Blood**105**(9): 3605-3614.

Bartosch, B., J. Bukh, et al. (2003). "In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes." Proc Natl Acad Sci U S A**100**(24): 14199-14204.

Bergmann, J. F., J. de Bruijne, et al. (2011). "Randomised clinical trial: anti-viral activity of ANA773, an oral inducer of endogenous interferons acting via TLR7, in chronic HCV." Aliment Pharmacol Ther**34**(4): 443-453.

Bigger, C. B., K. M. Brasky, et al. (2001). "DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection." J Virol**75**(15): 7059-7066.

Branch, A. D., D. D. Stump, et al. (2005). "The hepatitis C virus alternate reading frame (ARF) and its family of novel products: the alternate reading frame protein/F-protein, the double-frameshift protein, and others." Semin Liver Dis**25**(1): 105-117.

Brenndorfer, E. D., J. Karthe, et al. (2009). "Nonstructural 3/4A protease of hepatitis C virus activates epithelial growth factor-induced signal transduction by cleavage of the T-cell protein tyrosine phosphatase." Hepatology**49**(6): 1810-1820.

Bukh, J., R. H. Miller, et al. (1995). "Biology and genetic heterogeneity of hepatitis C virus." Clin Exp Rheumatol**13 Suppl 13**: S3-7.

Carithers, R. L., Jr. and S. S. Emerson (1997). "Therapy of hepatitis C: meta-analysis of interferon alfa-2b trials." Hepatology**26**(3 Suppl 1): 83S-88S.

Carrat, F., F. Bani-Sadr, et al. (2004). "Pegylated interferon alfa-2b vs standard interferon alfa-2b, plus ribavirin, for chronic hepatitis C in HIV-infected patients: a randomized controlled trial." JAMA**292**(23): 2839-2848.

Chen, L., I. Borozan, et al. (2005). "Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection." Gastroenterology**128**(5): 1437-1444.

Choo, Q. L., G. Kuo, et al. (1989). "Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome." Science**244**(4902): 359-362.

Choo, Q. L., K. H. Richman, et al. (1991). "Genetic organization and diversity of the hepatitis C virus." Proc Natl Acad Sci U S A**88**(6): 2451-2455.

Conry-Cantilena, C., M. VanRaden, et al. (1996). "Routes of infection, viremia, and liver disease in blood donors found to have hepatitis C virus infection." N Engl J Med**334**(26): 1691-1696.

Cooper, S., A. L. Erickson, et al. (1999). "Analysis of a successful immune response against hepatitis C virus." Immunity**10**(4): 439-449.

Cormier, E. G., F. Tsamis, et al. (2004). "CD81 is an entry coreceptor for hepatitis C virus." Proc Natl Acad Sci U S A**101**(19): 7270-7274.

Cox, A. L., T. Mosbruger, et al. (2005). "Comprehensive analyses of CD8+ T cell responses during longitudinal study of acute human hepatitis C." Hepatology**42**(1): 104-112.

Day, C. L., G. M. Lauer, et al. (2002). "Broad specificity of virus-specific CD4+ T-helper-cell responses in resolved hepatitis C virus infection." J Virol**76**(24): 12584-12595.

De Francesco, R. and G. Migliaccio (2005). "Challenges and successes in developing new therapies for hepatitis C." Nature**436**(7053): 953-960.

Dienes, H. P., H. Popper, et al. (1982). "Histologic observations in human hepatitis non-A, non-B." Hepatology**2**(5): 562-571.

Dustin, L. B. and C. M. Rice (2007). "Flying under the radar: the immunobiology of hepatitis C." Annu Rev Immunol**25**: 71-99.

Eksioglu, E. A., H. Zhu, et al. (2011). "Characterization of HCV interactions with Toll-like receptors and RIG-I in liver cells." PLoS One**6**(6): e21186.

Engering, A., T. B. Geijtenbeek, et al. (2002). "The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells." J Immunol**168**(5): 2118-2126.

Feinstone, S. M., A. Z. Kapikian, et al. (1975). "Transfusion-associated hepatitis not due to viral hepatitis type A or B." N Engl J Med**292**(15): 767-770.

Fidock, M. D., B. E. Souberbielle, et al. (2011). "The innate immune response, clinical outcomes, and ex vivo HCV antiviral efficacy of a TLR7 agonist (PF-4878691)." Clin Pharmacol Ther**89**(6): 821-829.

Friebe, P. and R. Bartenschlager (2002). "Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication." J Virol**76**(11): 5326-5338.

Friebe, P., V. Lohmann, et al. (2001). "Sequences in the 5' nontranslated region of hepatitis C virus required for RNA replication." J Virol**75**(24): 12047-12057.

Goncales, F. L., Jr., C. A. Moma, et al. (2010). "Retreatment of hepatitis C patients with pegylated interferon combined with ribavirin in non-responders to interferon plus ribavirin. Is it different in real life?" BMC Infect Dis**10**: 212.

Horsmans, Y., T. Berg, et al. (2005). "Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic hepatitis C infection." Hepatology**42**(3): 724-731.

Idrees, M. and S. Riazuddin (2009). "A study of best positive predictors for sustained virologic response to interferon alpha plus ribavirin therapy in naive chronic hepatitis C patients." BMC Gastroenterol**9**: 5.

Jeffers, L. J., W. Cassidy, et al. (2004). "Peginterferon alfa-2a (40 kd) and ribavirin for black American patients with chronic HCV genotype 1." Hepatology**39**(6): 1702-1708.

Kaimori, A., T. Kanto, et al. (2004). "Pseudotype hepatitis C virus enters immature myeloid dendritic cells through the interaction with lectin." Virology**324**(1): 74-83.

Kaito, M., S. Watanabe, et al. (1994). "Hepatitis C virus particle detected by immunoelectron microscopic study." J Gen Virol**75 (Pt 7)**: 1755-1760.

Kanda, T., R. Steele, et al. (2007). "Hepatitis C virus infection induces the beta interferon signaling pathway in immortalized human hepatocytes." J Virol**81**(22): 12375-12381.

Kato, M., T. K. Neil, et al. (1998). "cDNA cloning of human DEC-205, a putative antigen-uptake receptor on dendritic cells." Immunogenetics**47**(6): 442-450.

Khakoo, S. I., C. L. Thio, et al. (2004). "HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection." Science**305**(5685): 872-874.

Kimber, R. J. (1990). "Screening of blood donations for hepatitis C virus antibody." Med J Aust**152**(11): 613.

Koev, G., T. Dekhtyar, et al. (2007). "Antiviral interactions of an HCV polymerase inhibitor with an HCV protease inhibitor or interferon in vitro." Antiviral Res**73**(1): 78-83.

Lamarre, D., P. C. Anderson, et al. (2003). "An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus." Nature**426**(6963): 186-189.

Lanford, R. E., B. Guerra, et al. (2004). "Cross-genotype immunity to hepatitis C virus." J Virol**78**(3): 1575-1581.

Lau, D. T., P. M. Fish, et al. (2008). "Interferon regulatory factor-3 activation, hepatic interferon-stimulated gene expression, and immune cell infiltration in hepatitis C virus patients." Hepatology**47**(3): 799-809.

Lauer, G. M., K. Ouchi, et al. (2002). "Comprehensive analysis of CD8(+)-T-cell responses against hepatitis C virus reveals multiple unpredicted specificities." J Virol**76**(12): 6104-6113.

Le Pogam, S., W. R. Jiang, et al. (2006). "In vitro selected Con1 subgenomic replicons resistant to 2'-C-methyl-cytidine or to R1479 show lack of cross resistance." Virology**351**(2): 349-359.

Le Pogam, S., H. Kang, et al. (2006). "Selection and characterization of replicon variants dually resistant to thumb- and palm-binding nonnucleoside polymerase inhibitors of the hepatitis C virus." J Virol**80**(12): 6146-6154.

Lechner, F., D. K. Wong, et al. (2000). "Analysis of successful immune responses in persons infected with hepatitis C virus." J Exp Med**191**(9): 1499-1512.

Lee, J., C. C. Wu, et al. (2006). "Activation of anti-hepatitis C virus responses via Toll-like receptor 7." Proc Natl Acad Sci U S A**103**(6): 1828-1833.

Lehmann, M., M. F. Meyer, et al. (2004). "High rate of spontaneous clearance of acute hepatitis C virus genotype 3 infection." J Med Virol**73**(3): 387-391.

Liang, H., R. S. Russell, et al. (2009). "Differential effects of hepatitis C virus JFH1 on human myeloid and plasmacytoid dendritic cells." J Virol**83**(11): 5693-5707.

Lin, C., C. A. Gates, et al. (2005). "In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061." J Biol Chem**280**(44): 36784-36791.

Lin, C., K. Lin, et al. (2004). "In vitro resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: structural analysis indicates different resistance mechanisms." J Biol Chem**279**(17): 17508-17514.

Lin, C., B. D. Lindenbach, et al. (1994). "Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini." J Virol**68**(8): 5063-5073.

Logvinoff, C., M. E. Major, et al. (2004). "Neutralizing antibody response during acute and chronic hepatitis C virus infection." Proc Natl Acad Sci U S A**101**(27): 10149-10154.

Lohmann, V., F. Korner, et al. (1999). "Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line." Science**285**(5424): 110-113.

Longman, R. S., A. H. Talal, et al. (2004). "Presence of functional dendritic cells in patients chronically infected with hepatitis C virus." Blood**103**(3): 1026-1029.

Lozach, P. Y., A. Amara, et al. (2004). "C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles." J Biol Chem**279**(31): 32035-32045.

Lu, L., T. J. Pilot-Matias, et al. (2004). "Mutations conferring resistance to a potent hepatitis C virus serine protease inhibitor in vitro." Antimicrob Agents Chemother**48**(6): 2260-2266.

Ludwig, I. S., A. N. Lekkerkerker, et al. (2004). "Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation." J Virol**78**(15): 8322-8332.

Major, M. E., K. Mihalik, et al. (2002). "Previously infected and recovered chimpanzees exhibit rapid responses that control hepatitis C virus replication upon rechallenge." J Virol**76**(13): 6586-6595.

Martins, T., J. L. Narciso-Schiavon, et al. (2011). "[Epidemiology of hepatitis C virus infection]." Rev Assoc Med Bras**57**(1): 107-112.

Mehta, S. H., A. Cox, et al. (2002). "Protection against persistence of hepatitis C." Lancet**359**(9316): 1478-1483.

Meunier, J. C., R. E. Engle, et al. (2005). "Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1." Proc Natl Acad Sci U S A**102**(12): 4560-4565.

Morikawa, K., C. M. Lange, et al. (2011). "Nonstructural protein 3-4A: the Swiss army knife of hepatitis C virus." J Viral Hepat**18**(5): 305-315.

Natarajan, V., S. Kottilil, et al. (2010). "HCV in peripheral blood mononuclear cells are predominantly carried on the surface of cells in HIV/HCV co-infected individuals." J Med Virol**82**(12): 2032-2037.

Navarro-Sanchez, E., R. Altmeyer, et al. (2003). "Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses." EMBO Rep**4**(7): 723-728.

Netski, D. M., T. Mosbruger, et al. (2005). "Humoral immune response in acute hepatitis C virus infection." Clin Infect Dis**41**(5): 667-675.

Neumann, A. U., N. P. Lam, et al. (2000). "Differences in viral dynamics between genotypes 1 and 2 of hepatitis C virus." J Infect Dis**182**(1): 28-35.

Okamoto, H. (1995). "[Classification of hepatitis C virus genotypes and its application to molecular epidemiology]." Nihon Rinsho**53 Suppl**(Pt 1): 71-79.

Ozaras, R. and V. Tahan (2009). "Acute hepatitis C: prevention and treatment." Expert Rev Anti Infect Ther**7**(3): 351-361.

Pawlotsky, J. M. (1998). "Genetic heterogeneity and properties of hepatitis C virus." Acta Gastroenterol Belg**61**(2): 189-191.

Pawlotsky, J. M. (2003). "The nature of interferon-alpha resistance in hepatitis C virus infection." Curr Opin Infect Dis**16**(6): 587-592.

Pawlotsky, J. M. (2011). "Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus." Hepatology**53**(5): 1742-1751.

Pawlotsky, J. M. and J. G. McHutchison (2004). "Hepatitis C. Development of new drugs and clinical trials: promises and pitfalls. Summary of an AASLD hepatitis single topic conference, Chicago, IL, February 27-March 1, 2003." Hepatology**39**(2): 554-567.

Pestova, T. V., I. N. Shatsky, et al. (1998). "A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs." Genes Dev**12**(1): 67-83.

Pohlmann, S., J. Zhang, et al. (2003). "Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR." J Virol**77**(7): 4070-4080.

Polyak, S. J., K. S. Khabar, et al. (2001). "Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy." J Virol**75**(13): 6209-6211.

Popper, H., J. L. Dienstag, et al. (1980). "The pathology of viral hepatitis in chimpanzees." Virchows Arch A Pathol Anat Histol**387**(1): 91-106.

Puig-Basagoiti, F., X. Forns, et al. (2005). "Dynamics of hepatitis C virus NS5A quasispecies during interferon and ribavirin therapy in responder and non-responder patients with genotype 1b chronic hepatitis C." J Gen Virol**86**(Pt 4): 1067-1075.

Rollier, C., J. A. Drexhage, et al. (2003). "Chronic hepatitis C virus infection established and maintained in chimpanzees independent of dendritic cell impairment." Hepatology**38**(4): 851-858.

Rong, L., H. Dahari, et al. (2010). "Rapid emergence of protease inhibitor resistance in hepatitis C virus." Sci Transl Med**2**(30): 30ra32.

Sallusto, F., M. Cella, et al. (1995). "Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products." J Exp Med**182**(2): 389-400.

Sarrazin, C., R. Rouzier, et al. (2007). "SCH 503034, a novel hepatitis C virus protease inhibitor, plus pegylated interferon alpha-2b for genotype 1 nonresponders." Gastroenterology**132**(4): 1270-1278.

Sen, G. C. (2001). "Viruses and interferons." Annu Rev Microbiol**55**: 255-281.

Shiina, M. and B. Rehermann (2008). "Cell culture-produced hepatitis C virus impairs plasmacytoid dendritic cell function." Hepatology**47**(2): 385-395.

Su, A. I., J. P. Pezacki, et al. (2002). "Genomic analysis of the host response to hepatitis C virus infection." Proc Natl Acad Sci U S A**99**(24): 15669-15674.

Sumpter, R., Jr., Y. M. Loo, et al. (2005). "Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I." J Virol**79**(5): 2689-2699.

Takahashi, K., S. Asabe, et al. (2010). "Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection." Proc Natl Acad Sci U S A**107**(16): 7431-7436.

Takaki, A., M. Wiese, et al. (2000). "Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C." Nat Med**6**(5): 578-582.

Takeuchi, K., Y. Kubo, et al. (1990). "The putative nucleocapsid and envelope protein genes of hepatitis C virus determined by comparison of the nucleotide sequences of two isolates derived from an experimentally infected chimpanzee and healthy human carriers." J Gen Virol**71 (Pt 12)**: 3027-3033.

Tassaneetrithep, B., T. H. Burgess, et al. (2003). "DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells." J Exp Med**197**(7): 823-829.

Thimme, R., J. Bukh, et al. (2002). "Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease." Proc Natl Acad Sci U S A**99**(24): 15661-15668.

Thimme, R., D. Oldach, et al. (2001). "Determinants of viral clearance and persistence during acute hepatitis C virus infection." J Exp Med**194**(10): 1395-1406.

Thomas, A., C. Laxton, et al. (2007). "Investigating Toll-like receptor agonists for potential to treat hepatitis C virus infection." Antimicrob Agents Chemother**51**(8): 2969-2978.

Tomei, L., S. Altamura, et al. (2003). "Mechanism of action and antiviral activity of benzimidazole-based allosteric inhibitors of the hepatitis C virus RNA-dependent RNA polymerase." J Virol**77**(24): 13225-13231.

Tong, X., R. Chase, et al. (2006). "Identification and analysis of fitness of resistance mutations against the HCV protease inhibitor SCH 503034." Antiviral Res**70**(2): 28-38.

Torriani, F. J., M. Rodriguez-Torres, et al. (2004). "Peginterferon Alfa-2a plus ribavirin for chronic hepatitis C virus infection in HIV-infected patients." N Engl J Med**351**(5): 438-450.

Valladeau, J., O. Ravel, et al. (2000). "Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules." Immunity**12**(1): 71-81.

van der Poorten, D., D. T. Kenny, et al. (2008). "Prevalence of and risk factors for hepatitis C in Aboriginal and non-Aboriginal adolescent offenders." Med J Aust**188**(10): 610-614.

Veldt, B. J., J. T. Brouwer, et al. (2003). "Retreatment of hepatitis C non-responsive to interferon. A placebo controlled randomized trial of ribavirin monotherapy versus combination therapy with Ribavirin and Interferon in 121 patients in the Benelux [ISRCTN53821378]." BMC Gastroenterol**3**: 24.

Woollard, D. J., A. Grakoui, et al. (2003). "Characterization of HCV-specific Patr class II restricted CD4+ T cell responses in an acutely infected chimpanzee." Hepatology**38**(5): 1297-1306.

Yazdanpanah, Y., G. De Carli, et al. (2005). "Risk factors for hepatitis C virus transmission to health care workers after occupational exposure: a European case-control study." Clin Infect Dis**41**(10): 1423-1430.

Zein, N. N., J. Rakela, et al. (1996). "Hepatitis C virus genotypes in the United States: epidemiology, pathogenicity, and response to interferon therapy. Collaborative Study Group." Ann Intern Med**125**(8): 634-639.

Zhang, Y. L., Y. J. Guo, et al. (2009). "Hepatitis C virus single-stranded RNA induces innate immunity via Toll-like receptor 7." J Hepatol51(1): 29-38.

Abe, T., Y. Kaname, I. Hamamoto, Y. Tsuda, X. Wen, S. Taguwa, K. Moriishi, O. Takeuchi, T. Kawai, T. Kanto, N. Hayashi, S. Akira and Y. Matsuura (2007). "Hepatitis C virus nonstructural protein 5A modulates the toll-like receptor-MyD88-dependent signaling pathway in macrophage cell lines." J Virol 81(17): 8953-8966.

Adhikari, A., M. Xu and Z. J. Chen (2007). "Ubiquitin-mediated activation of TAK1 and IKK." Oncogene 26(22): 3214-3226.

Afdhal, N. H. (2004). "The natural history of hepatitis C." Semin Liver Dis 24 Suppl 2: 3-8.

Agnello, V., G. Abel, M. Elfahal, G. B. Knight and Q. X. Zhang (1999). "Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor." Proc Natl Acad Sci U S A 96(22): 12766-12771.

Albecka, A., S. Belouzard, A. Op de Beeck, V. Descamps, L. Goueslain, J. Bertrand-Michel, F. Terce, G. Duverlie, Y. Rouille and J. Dubuisson (2011). "Role of low-density lipoprotein receptor in the hepatitis C virus life cycle." Hepatology 55(4): 998-1007.

Alberti, A., F. Noventa, L. Benvegnu, S. Boccato and A. Gatta (2002). "Prevalence of liver disease in a population of asymptomatic persons with hepatitis C virus infection." Ann Intern Med 137(12): 961-964.

Alexopoulou, L., A. C. Holt, R. Medzhitov and R. A. Flavell (2001). "Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3." Nature 413(6857): 732-738.

Alter, M. J. (2007). "Epidemiology of hepatitis C virus infection." World J Gastroenterol 13(17): 2436-2441.

Arpaia, N. and G. M. Barton (2011). "Toll-like receptors: key players in antiviral immunity." Curr Opin Virol 1(6): 447-454.

Asselah, T. and P. Marcellin (2013). "Interferon free therapy with direct acting antivirals for HCV." Liver Int 33 Suppl 1: 93-104.

Assier, E., V. Marin-Esteban, A. Haziot, E. Maggi, D. Charron and N. Mooney (2007). "TLR7/8 agonists impair monocyte-derived dendritic cell differentiation and maturation." J Leukoc Biol 81(1): 221-228.

Balkhi, M. Y., K. A. Fitzgerald and P. M. Pitha (2008). "Functional regulation of MyD88-activated interferon regulatory factor 5 by K63-linked polyubiquitination." Mol Cell Biol 28(24): 7296-7308.

Barbalat, R., S. E. Ewald, M. L. Mouchess and G. M. Barton (2011). "Nucleic acid recognition by the innate immune system." Annu Rev Immunol 29: 185-214.

Barral, P. M., D. Sarkar, Z. Z. Su, G. N. Barber, R. DeSalle, V. R. Racaniello and P. B. Fisher (2009). "Functions of the cytoplasmic RNA sensors RIG-I and MDA-5: key regulators of innate immunity." Pharmacol Ther 124(2): 219-234.

Barrat, F. J., T. Meeker, J. Gregorio, J. H. Chan, S. Uematsu, S. Akira, B. Chang, O. Duramad and R. L. Coffman (2005). "Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus." J Exp Med 202(8): 1131-1139.

Bartenschlager, R., V. Lohmann, T. Wilkinson and J. O. Koch (1995). "Complex formation between the NS3 serine-type proteinase of the hepatitis C virus and NS4A and its importance for polyprotein maturation." J Virol 69(12): 7519-7528.

Bartenschlager, R., F. Penin, V. Lohmann and P. Andre (2010). "Assembly of infectious hepatitis C virus particles." Trends Microbiol 19(2): 95-103.

Barth, H., A. Ulsenheimer, G. R. Pape, H. M. Diepolder, M. Hoffmann, C. Neumann-Haefelin, R. Thimme, P. Henneke, R. Klein, G. Paranhos-Baccala, E. Depla, T. J. Liang, H. E. Blum and T. F. Baumert (2005). "Uptake and presentation of hepatitis C virus-like particles by human dendritic cells." Blood 105(9): 3605-3614.

Bartosch, B., J. Bukh, J. C. Meunier, C. Granier, R. E. Engle, W. C. Blackwelder, S. U. Emerson, F. L. Cosset and R. H. Purcell (2003). "In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes." Proc Natl Acad Sci U S A 100(24): 14199-14204.

Bartosch, B., J. Dubuisson and F. L. Cosset (2003). "Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes." J Exp Med 197(5): 633-642.

Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia and F. L. Cosset (2003). "Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor." J Biol Chem 278(43): 41624-41630.

Baumert, T. F., S. Ito, D. T. Wong and T. J. Liang (1998). "Hepatitis C virus structural proteins assemble into viruslike particles in insect cells." J Virol 72(5): 3827-3836.

Bergmann, J. F., J. de Bruijne, D. M. Hotho, R. J. de Knegt, A. Boonstra, C. J. Weegink, A. A. van Vliet, J. van de Wetering, S. P. Fletcher, L. A. Bauman, M. Rahimy, J. R. Appleman, J. L. Freddo, H. L. Janssen and H. W. Reesink (2011). "Randomised clinical trial: anti-viral activity of ANA773, an oral inducer of endogenous interferons acting via TLR7, in chronic HCV." Aliment Pharmacol Ther 34(4): 443-453.

Besse, A., B. Lamothe, A. D. Campos, W. K. Webster, U. Maddineni, S. C. Lin, H. Wu and B. G. Darnay (2007). "TAK1-dependent signaling requires functional interaction with TAB2/TAB3." J Biol Chem 282(6): 3918-3928.

Bhala, N., P. Angulo, D. van der Poorten, E. Lee, J. M. Hui, G. Saracco, L. A. Adams, P. Charatcharoenwitthaya, J. H. Topping, E. Bugianesi, C. P. Day and J. George (2011). "The natural history of nonalcoholic fatty liver disease with advanced fibrosis or cirrhosis: an international collaborative study." Hepatology 54(4): 1208-1216.

Bigger, C. B., K. M. Brasky and R. E. Lanford (2001). "DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection." J Virol 75(15): 7059-7066.

Bigger, C. B., B. Guerra, K. M. Brasky, G. Hubbard, M. R. Beard, B. A. Luxon, S. M. Lemon and R. E. Lanford (2004). "Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees." J Virol 78(24): 13779-13792.

Bissell, D. M., S. S. Wang, W. R. Jarnagin and F. J. Roll (1995). "Cell-specific expression of transforming growth factor-beta in rat liver. Evidence for autocrine regulation of hepatocyte proliferation." J Clin Invest 96(1): 447-455.

Bizollon, T., S. N. Ahmed, S. Radenne, M. Chevallier, P. Chevallier, P. Parvaz, S. Guichard, C. Ducerf, J. Baulieux, F. Zoulim and C. Trepo (2003). "Long term histological improvement and clearance of intrahepatic hepatitis C virus RNA following sustained response to interferon-ribavirin combination therapy in liver transplanted patients with hepatitis C virus recurrence." Gut 52(2): 283-287.

Blackard, J. T., F. Komurian-Pradel, M. Perret, M. Sodoyer, L. Smeaton, J. B. St Clair, S. Chapman, L. E. Taylor, G. Paranhos-Baccala and R. T. Chung (2006). "Intrahepatic cytokine expression is downregulated during HCV/HIV co-infection." J Med Virol 78(2): 202-207.

Blanchard, E., S. Belouzard, L. Goueslain, T. Wakita, J. Dubuisson, C. Wychowski and Y. Rouille (2006). "Hepatitis C virus entry depends on clathrin-mediated endocytosis." J Virol 80(14): 6964-6972.

Blanchard, E., D. Brand, S. Trassard, A. Goudeau and P. Roingeard (2002). "Hepatitis C virus-like particle morphogenesis." J Virol 76(8): 4073-4079.

Blight, K. J., A. A. Kolykhalov and C. M. Rice (2000). "Efficient initiation of HCV RNA replication in cell culture." Science 290(5498): 1972-1974.

Blight, K. J., J. A. McKeating and C. M. Rice (2002). "Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication." J Virol 76(24): 13001-13014.

Bourliere, M., D. Ouzan, M. Rosenheim, M. Doffoel, P. Marcellin, J. M. Pawlotsky, L. Salomon, F. Fagnani, S. Rouanet, A. Pinta and M. Vray "Pegylated interferon-alpha2a plus ribavirin for chronic hepatitis C in a real-life setting: the Hepatys French cohort (2003-2007)." Antivir Ther 17(1): 101-110.

Branch, A. D., D. D. Stump, J. A. Gutierrez, F. Eng and J. L. Walewski (2005). "The hepatitis C virus alternate reading frame (ARF) and its family of novel products: the alternate reading frame protein/F-protein, the double-frameshift protein, and others." Semin Liver Dis 25(1): 105-117.

Brenndorfer, E. D., J. Karthe, L. Frelin, P. Cebula, A. Erhardt, J. Schulte am Esch, H. Hengel, R. Bartenschlager, M. Sallberg, D. Haussinger and J. G. Bode (2009). "Nonstructural 3/4A

protease of hepatitis C virus activates epithelial growth factor-induced signal transduction by cleavage of the T-cell protein tyrosine phosphatase." Hepatology 49(6): 1810-1820.

Brown, R. S. (2005). "Hepatitis C and liver transplantation." Nature 436(7053): 973-978.

Bukh, J. (2004). "A critical role for the chimpanzee model in the study of hepatitis C." Hepatology 39(6): 1469-1475.

Bukh, J., R. H. Miller and R. H. Purcell (1995). "Biology and genetic heterogeneity of hepatitis C virus." Clin Exp Rheumatol 13 Suppl 13: S3-7.

Bukh, J. and R. H. Purcell (2006). "A milestone for hepatitis C virus research: a virus generated in cell culture is fully viable in vivo." Proc Natl Acad Sci U S A 103(10): 3500-3501.

Bukh, J., R. H. Purcell and R. H. Miller (1994). "Sequence analysis of the core gene of 14 hepatitis C virus genotypes." Proc Natl Acad Sci U S A 91(17): 8239-8243.

Carithers, R. L., Jr. and S. S. Emerson (1997). "Therapy of hepatitis C: meta-analysis of interferon alfa-2b trials." Hepatology 26(3 Suppl 1): 83S-88S.

Carrat, F., F. Bani-Sadr, S. Pol, E. Rosenthal, F. Lunel-Fabiani, A. Benzekri, P. Morand, C. Goujard, G. Pialoux, L. Piroth, D. Salmon-Ceron, C. Degott, P. Cacoub and C. Perronne (2004). "Pegylated interferon alfa-2b vs standard interferon alfa-2b, plus ribavirin, for chronic hepatitis C in HIV-infected patients: a randomized controlled trial." JAMA 292(23): 2839-2848.

Carrere-Kremer, S., C. Montpellier-Pala, L. Cocquerel, C. Wychowski, F. Penin and J. Dubuisson (2002). "Subcellular localization and topology of the p7 polypeptide of hepatitis C virus." J Virol 76(8): 3720-3730.

Chang, S., A. Dolganiuc and G. Szabo (2007). "Toll-like receptors 1 and 6 are involved in TLR2-mediated macrophage activation by hepatitis C virus core and NS3 proteins." J Leukoc Biol 82(3): 479-487.

Chen, L., I. Borozan, J. Feld, J. Sun, L. L. Tannis, C. Coltescu, J. Heathcote, A. M. Edwards and I. D. McGilvray (2005). "Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection." Gastroenterology 128(5): 1437-1444.

Cheng, G., J. Zhong and F. V. Chisari (2006). "Inhibition of dsRNA-induced signaling in hepatitis C virus-infected cells by NS3 protease-dependent and -independent mechanisms." Proc Natl Acad Sci U S A 103(22): 8499-8504.

Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley and M. Houghton (1989). "Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome." Science 244(4902): 359-362.

Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina-Selby, P. J. Barr and et al. (1991). "Genetic organization and diversity of the hepatitis C virus." Proc Natl Acad Sci U S A 88(6): 2451-2455.

Choo, Q. L., A. J. Weiner, L. R. Overby, G. Kuo, M. Houghton and D. W. Bradley (1990). "Hepatitis C virus: the major causative agent of viral non-A, non-B hepatitis." Br Med Bull 46(2): 423-441.

Chung, R. T., M. Gale, Jr., S. J. Polyak, S. M. Lemon, T. J. Liang and J. H. Hoofnagle (2008). "Mechanisms of action of interferon and ribavirin in chronic hepatitis C: Summary of a workshop." Hepatology 47(1): 306-320.

Coller, K. E., K. L. Berger, N. S. Heaton, J. D. Cooper, R. Yoon and G. Randall (2009). "RNA interference and single particle tracking analysis of hepatitis C virus endocytosis." PLoS Pathog 5(12): e1000702.

Conry-Cantilena, C., M. VanRaden, J. Gibble, J. Melpolder, A. O. Shakil, L. Viladomiu, L. Cheung, A. DiBisceglie, J. Hoofnagle, J. W. Shih and et al. (1996). "Routes of infection, viremia, and liver disease in blood donors found to have hepatitis C virus infection." N Engl J Med 334(26): 1691-1696.

Cooper, S., A. L. Erickson, E. J. Adams, J. Kansopon, A. J. Weiner, D. Y. Chien, M. Houghton, P. Parham and C. M. Walker (1999). "Analysis of a successful immune response against hepatitis C virus." Immunity 10(4): 439-449.

Cormier, E. G., R. J. Durso, F. Tsamis, L. Boussemart, C. Manix, W. C. Olson, J. P. Gardner and T. Dragic (2004). "L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus." Proc Natl Acad Sci U S A 101(39): 14067-14072.

Cormier, E. G., F. Tsamis, F. Kajumo, R. J. Durso, J. P. Gardner and T. Dragic (2004). "CD81 is an entry coreceptor for hepatitis C virus." Proc Natl Acad Sci U S A 101(19): 7270-7274.

Cox, A. L., T. Mosbruger, G. M. Lauer, D. Pardoll, D. L. Thomas and S. C. Ray (2005). "Comprehensive analyses of CD8+ T cell responses during longitudinal study of acute human hepatitis C." Hepatology 42(1): 104-112.

Crispe, I. N. (2009). "The liver as a lymphoid organ." Annu Rev Immunol 27: 147-163.

Dammacco, F., F. A. Tucci, G. Lauletta, P. Gatti, V. De Re, V. Conteduca, S. Sansonno, S. Russi, M. A. Mariggio, M. Chironna and D. Sansonno (2010). "Pegylated interferon-alpha, ribavirin, and rituximab combined therapy of hepatitis C virus-related mixed cryoglobulinemia: a long-term study." Blood 116(3): 343-353.

Davis, G. L., J. E. Albright, S. F. Cook and D. M. Rosenberg (2003). "Projecting future complications of chronic hepatitis C in the United States." Liver Transpl 9(4): 331-338.

Day, C. L., G. M. Lauer, G. K. Robbins, B. McGovern, A. G. Wurcel, R. T. Gandhi, R. T. Chung and B. D. Walker (2002). "Broad specificity of virus-specific CD4+ T-helper-cell responses in resolved hepatitis C virus infection." J Virol 76(24): 12584-12595.

De Francesco, R. and G. Migliaccio (2005). "Challenges and successes in developing new therapies for hepatitis C." Nature 436(7053): 953-960.

Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira and C. Reis e Sousa (2004). "Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA." Science 303(5663): 1529-1531.

Dienes, H. P., H. Popper, W. Arnold and H. Lobeck (1982). "Histologic observations in human hepatitis non-A, non-B." Hepatology 2(5): 562-571.

Dolganiuc, A., S. Oak, K. Kodys, D. T. Golenbock, R. W. Finberg, E. Kurt-Jones and G. Szabo (2004). "Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation." Gastroenterology 127(5): 1513-1524.

Doyle, S., S. Vaidya, R. O'Connell, H. Dadgostar, P. Dempsey, T. Wu, G. Rao, R. Sun, M. Haberland, R. Modlin and G. Cheng (2002). "IRF3 mediates a TLR3/TLR4-specific antiviral gene program." Immunity 17(3): 251-263.

Dustin, L. B. and C. M. Rice (2007). "Flying under the radar: the immunobiology of hepatitis C." Annu Rev Immunol 25: 71-99.

Eksioglu, E. A., H. Zhu, L. Bayouth, J. Bess, H. Y. Liu, D. R. Nelson and C. Liu (2011). "Characterization of HCV interactions with Toll-like receptors and RIG-I in liver cells." PLoS One 6(6): e21186.

Elazar, M., P. Liu, C. M. Rice and J. S. Glenn (2004). "An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication." J Virol 78(20): 11393-11400.

Engering, A., T. B. Geijtenbeek, S. J. van Vliet, M. Wijers, E. van Liempt, N. Demaurex, A. Lanzavecchia, J. Fransen, C. G. Figdor, V. Piguet and Y. van Kooyk (2002). "The dendritic cell-specific adhesion receptor DC-SIGN internalizes antigen for presentation to T cells." J Immunol 168(5): 2118-2126.

Evans, M. J., T. von Hahn, D. M. Tscherne, A. J. Syder, M. Panis, B. Wolk, T. Hatziioannou, J. A. McKeating, P. D. Bieniasz and C. M. Rice (2007). "Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry." Nature 446(7137): 801-805.

Feigelstock, D. A., K. B. Mihalik, G. Kaplan and S. M. Feinstone (2010). "Increased susceptibility of Huh7 cells to HCV replication does not require mutations in RIG-I." Virol J 7: 44.

Feinstone, S. M., A. Z. Kapikian, R. H. Purcell, H. J. Alter and P. V. Holland (1975). "Transfusion-associated hepatitis not due to viral hepatitis type A or B." N Engl J Med 292(15): 767-770.

Feng, Z. H., Q. C. Wang, Q. H. Nie, Z. S. Jia and Y. X. Zhou (2004). "DC-SIGN: binding receptor for HCV?" World J Gastroenterol 10(7): 925-929.

Fidock, M. D., B. E. Souberbielle, C. Laxton, J. Rawal, O. Delpuech-Adams, T. P. Corey, P. Colman, V. Kumar, J. B. Cheng, K. Wright, S. Srinivasan, K. Rana, C. Craig, N. Horscroft, M. Perros, M. Westby, R. Webster and E. van der Ryst (2011). "The innate immune response, clinical outcomes, and ex vivo HCV antiviral efficacy of a TLR7 agonist (PF-4878691)." Clin Pharmacol Ther 89(6): 821-829.

Flint, M. and J. A. McKeating (2000). "The role of the hepatitis C virus glycoproteins in infection." Rev Med Virol 10(2): 101-117.

Flynn, J. K., G. J. Dore, M. Hellard, B. Yeung, W. D. Rawlinson, P. A. White, J. M. Kaldor, A. R. Lloyd and R. A. Ffrench (2013). "Maintenance of Th1 hepatitis C virus (HCV)-specific responses in individuals with acute HCV who achieve sustained virological clearance after treatment." J Gastroenterol Hepatol 28(11): 1770-1781.

Forsbach, A., J. G. Nemorin, C. Montino, C. Muller, U. Samulowitz, A. P. Vicari, M. Jurk, G. K. Mutwiri, A. M. Krieg, G. B. Lipford and J. Vollmer (2008). "Identification of RNA sequence motifs stimulating sequence-specific TLR8-dependent immune responses." J Immunol 180(6): 3729-3738.

Fournier, C., C. Sureau, J. Coste, J. Ducos, G. Pageaux, D. Larrey, J. Domergue and P. Maurel (1998). "In vitro infection of adult normal human hepatocytes in primary culture by hepatitis C virus." J Gen Virol 79 (Pt 10): 2367-2374.

Freeman, A. J., G. J. Dore, M. G. Law, M. Thorpe, J. Von Overbeck, A. R. Lloyd, G. Marinos and J. M. Kaldor (2001). "Estimating progression to cirrhosis in chronic dhepatitis C virus infection." Hepatology 34(4 Pt 1): 809-816.

Friebe, P. and R. Bartenschlager (2002). "Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication." J Virol 76(11): 5326-5338.

Friebe, P., V. Lohmann, N. Krieger and R. Bartenschlager (2001). "Sequences in the 5' nontranslated region of hepatitis C virus required for RNA replication." J Virol 75(24): 12047-12057.

Fried, M. W., M. L. Shiffman, K. R. Reddy, C. Smith, G. Marinos, F. L. Goncales, Jr., D. Haussinger, M. Diago, G. Carosi, D. Dhumeaux, A. Craxi, A. Lin, J. Hoffman and J. Yu (2002). "Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection." N Engl J Med 347(13): 975-982.

Gane, E. J., S. K. Roberts, C. A. Stedman, P. W. Angus, B. Ritchie, R. Elston, D. Ipe, P. N. Morcos, L. Baher, I. Najera, T. Chu, U. Lopatin, M. M. Berrey, W. Bradford, M. Laughlin, N. S. Shulman and P. F. Smith (2010). "Oral combination therapy with a nucleoside polymerase inhibitor (RG7128) and danoprevir for chronic hepatitis C

genotype 1 infection (INFORM-1): a randomised, double-blind, placebo-controlled, dose-escalation trial." Lancet 376(9751): 1467-1475.

Gardner, J. P., R. J. Durso, R. R. Arrigale, G. P. Donovan, P. J. Maddon, T. Dragic and W. C. Olson (2003). "L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus." Proc Natl Acad Sci U S A 100(8): 4498-4503.

Geijtenbeek, T. B., D. J. Krooshoop, D. A. Bleijs, S. J. van Vliet, G. C. van Duijnhoven, V. Grabovsky, R. Alon, C. G. Figdor and Y. van Kooyk (2000). "DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking." Nat Immunol 1(4): 353-357.

Geijtenbeek, T. B. and Y. van Kooyk (2003). "DC-SIGN: a novel HIV receptor on DCs that mediates HIV-1 transmission." Curr Top Microbiol Immunol 276: 31-54.

Gerlach, J. T., H. M. Diepolder, R. Zachoval, N. H. Gruener, M. C. Jung, A. Ulsenheimer, W. W. Schraut, C. A. Schirren, M. Waechtler, M. Backmund and G. R. Pape (2003). "Acute hepatitis C: high rate of both spontaneous and treatment-induced viral clearance." Gastroenterology 125(1): 80-88.

Ghany, M. G., D. E. Kleiner, H. Alter, E. Doo, F. Khokar, K. Promrat, D. Herion, Y. Park, T. J. Liang and J. H. Hoofnagle (2003). "Progression of fibrosis in chronic hepatitis C." Gastroenterology 124(1): 97-104.

Giannelli, G. and S. Antonaci (2005). "Immunological and molecular aspects of liver fibrosis in chronic hepatitis C virus infection." Histol Histopathol 20(3): 939-944.

Gibson, S. J., J. M. Lindh, T. R. Riter, R. M. Gleason, L. M. Rogers, A. E. Fuller, J. L. Oesterich, K. B. Gorden, X. Qiu, S. W. McKane, R. J. Noelle, R. L. Miller, R. M. Kedl, P. Fitzgerald-Bocarsly, M. A. Tomai and J. P. Vasilakos (2002). "Plasmacytoid dendritic cells produce cytokines and mature in response to the TLR7 agonists, imiquimod and resiquimod." Cell Immunol 218(1-2): 74-86.

Goncales, F. L., Jr., C. A. Moma, A. G. Vigani, A. F. Angerami, E. S. Goncales, R. Tozzo, M. H. Pavan and N. S. Goncales (2010). "Retreatment of hepatitis C patients with pegylated interferon combined with ribavirin in non-responders to interferon plus ribavirin. Is it different in real life?" BMC Infect Dis 10: 212.

Gondeau, C., P. Briolotti, F. Razafy, C. Duret, P. A. Rubbo, F. Helle, T. Reme, M. P. Ripault, J. Ducos, J. M. Fabre, J. Ramos, E. I. Pecheur, D. Larrey, P. Maurel and M. Daujat-Chavanieu (2013). "In vitro infection of primary human hepatocytes by HCV-positive sera: insights on a highly relevant model." Gut.

Gorden, K. B., K. S. Gorski, S. J. Gibson, R. M. Kedl, W. C. Kieper, X. Qiu, M. A. Tomai, S. S. Alkan and J. P. Vasilakos (2005). "Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8." J Immunol 174(3): 1259-1268.

Gordon, S. (2002). "Pattern recognition receptors: doubling up for the innate immune response." Cell 111(7): 927-930.

Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone and C. M. Rice (1993). "A second hepatitis C virus-encoded proteinase." Proc Natl Acad Sci U S A 90(22): 10583-10587.

Grappone, C., M. Pinzani, M. Parola, G. Pellegrini, A. Caligiuri, R. DeFranco, F. Marra, H. Herbst, G. Alpini and S. Milani (1999). "Expression of platelet-derived growth factor in newly formed cholangiocytes during experimental biliary fibrosis in rats." J Hepatol 31(1): 100-109.

Gringhuis, S. I., M. van der Vlist, L. M. van den Berg, J. den Dunnen, M. Litjens and T. B. Geijtenbeek (2010). "HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells." Nat Immunol 11(5): 419-426.

Guobuzaite, A., S. Chokshi, L. Balciuniene, A. Voinic, A. Stikleryte, K. Zagminas, A. Ambrozaitis and N. Naoumov (2008). "Viral clearance or persistence after acute hepatitis C infection: interim results from a prospective study." Medicina (Kaunas) 44(7): 510-520.

Hart, O. M., V. Athie-Morales, G. M. O'Connor and C. M. Gardiner (2005). "TLR7/8-mediated activation of human NK cells results in accessory cell-dependent IFN-gamma production." J Immunol 175(3): 1636-1642.

Heil, F., P. Ahmad-Nejad, H. Hemmi, H. Hochrein, F. Ampenberger, T. Gellert, H. Dietrich, G. Lipford, K. Takeda, S. Akira, H. Wagner and S. Bauer (2003). "The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily." Eur J Immunol 33(11): 2987-2997.

Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner and S. Bauer (2004). "Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8." Science 303(5663): 1526-1529.

Hemmi, H., T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda and S. Akira (2002). "Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway." Nat Immunol 3(2): 196-200.

Henke, J. I., D. Goergen, J. Zheng, Y. Song, C. G. Schuttler, C. Fehr, C. Junemann and M. Niepmann (2008). "microRNA-122 stimulates translation of hepatitis C virus RNA." EMBO J 27(24): 3300-3310.

Hitomi, Y., W. M. McDonnell, A. A. Killeen and F. K. Askari (1995). "Sequence analysis of the hepatitis C virus (HCV) core gene suggests the core protein as an appropriate target for HCV vaccine strategies." J Viral Hepat 2(5): 235-241.

Hoffmann, M., M. B. Zeisel, N. Jilg, G. Paranhos-Baccala, F. Stoll-Keller, T. Wakita, P. Hafkemeyer, H. E. Blum, H. Barth, P. Henneke and T. F. Baumert (2009). "Toll-like receptor 2 senses hepatitis C virus core protein but not infectious viral particles." J Innate Immun 1(5): 446-454.

Hoofnagle, J. H. (2002). "Course and outcome of hepatitis C." Hepatology 36(5 Suppl 1): S21-29.

Horner, S. M. (2013). "Activation and Evasion of Antiviral Innate Immunity by Hepatitis C Virus." J Mol Biol.

Horner, S. M. and M. Gale, Jr. (2013). "Regulation of hepatic innate immunity by hepatitis C virus." Nat Med 19(7): 879-888.

Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K. K. Conzelmann, M. Schlee, S. Endres and G. Hartmann (2006). "5'-Triphosphate RNA is the ligand for RIG-I." Science 314(5801): 994-997.

Hornung, V., M. Guenthner-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, A. Noronha, M. Manoharan, S. Akira, A. de Fougerolles, S. Endres and G. Hartmann (2005). "Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7." Nat Med 11(3): 263-270.

Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres and G. Hartmann (2002). "Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides." J Immunol 168(9): 4531-4537.

Horsmans, Y., T. Berg, J. P. Desager, T. Mueller, E. Schott, S. P. Fletcher, K. R. Steffy, L. A. Bauman, B. M. Kerr and D. R. Averett (2005). "Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic hepatitis C infection." Hepatology 42(3): 724-731.

Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice and J. A. McKeating (2003). "Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles." Proc Natl Acad Sci U S A 100(12): 7271-7276.

Idrees, M. and S. Riazuddin (2009). "A study of best positive predictors for sustained virologic response to interferon alpha plus ribavirin therapy in naive chronic hepatitis C patients." BMC Gastroenterol 9: 5.

Jackson, D. B. (2009). "Clinical and economic impact of the nonresponder phenomenon--implications for systems based discovery." Drug Discov Today 14(7-8): 380-385.

Jacobson, I. M., S. A. Gonzalez, F. Ahmed, E. Lebovics, A. D. Min, H. C. Bodenheimer, Jr., S. P. Esposito, R. S. Brown, Jr., N. Brau, F. M. Klion, H. Tobias, E. J. Bini, N. Brodsky, M. A. Cerulli, A. Aytaman, P. W. Gardner, J. M. Geders, J. E. Spivack, M. G. Rahmin, D. H. Berman, J. Ehrlich, M. W. Russo, M. Chait, D. Rovner and B. R. Edlin (2005). "A randomized trial of pegylated interferon alpha-2b plus ribavirin in the retreatment of chronic hepatitis C." Am J Gastroenterol 100(11): 2453-2462.

Jameson, B., F. Baribaud, S. Pohlmann, D. Ghavimi, F. Mortari, R. W. Doms and A. Iwasaki (2002). "Expression of DC-SIGN by dendritic cells of intestinal and genital mucosae in humans and rhesus macaques." J Virol 76(4): 1866-1875.

Janeway, C. A., Jr. and R. Medzhitov (2002). "Innate immune recognition." Annu Rev Immunol 20: 197-216.

Janssen, H. L., H. W. Reesink, E. J. Lawitz, S. Zeuzem, M. Rodriguez-Torres, K. Patel, A. J. van der Meer, A. K. Patick, A. Chen, Y. Zhou, R. Persson, B. D. King, S. Kauppinen, A. A. Levin and M. R. Hodges (2013). "Treatment of HCV infection by targeting microRNA." N Engl J Med 368(18): 1685-1694.

Jeffers, L. J., W. Cassidy, C. D. Howell, S. Hu and K. R. Reddy (2004). "Peginterferon alfa-2a (40 kd) and ribavirin for black American patients with chronic HCV genotype 1." Hepatology 39(6): 1702-1708.

Kaimori, A., T. Kanto, C. Kwang Limn, Y. Komoda, C. Oki, M. Inoue, H. Miyatake, I. Itose, M. Sakakibara, T. Yakushijin, T. Takehara, Y. Matsuura and N. Hayashi (2004). "Pseudotype hepatitis C virus enters immature myeloid dendritic cells through the interaction with lectin." Virology 324(1): 74-83.

Kaito, M., S. Watanabe, K. Tsukiyama-Kohara, K. Yamaguchi, Y. Kobayashi, M. Konishi, M. Yokoi, S. Ishida, S. Suzuki and M. Kohara (1994). "Hepatitis C virus particle detected by immunoelectron microscopic study." J Gen Virol 75 (Pt 7): 1755-1760.

Kambara, H., T. Fukuhara, M. Shiokawa, C. Ono, Y. Ohara, W. Kamitani and Y. Matsuura (2011). "Establishment of a novel permissive cell line for the propagation of hepatitis C virus by expression of microRNA miR122." J Virol 86(3): 1382-1393.

Kanda, T., A. Basu, R. Steele, T. Wakita, J. S. Ryerse, R. Ray and R. B. Ray (2006). "Generation of infectious hepatitis C virus in immortalized human hepatocytes." J Virol 80(9): 4633-4639.

Kanda, T., R. Steele, R. Ray and R. B. Ray (2007). "Hepatitis C virus infection induces the beta interferon signaling pathway in immortalized human hepatocytes." J Virol 81(22): 12375-12381.

Kato, M., T. K. Neil, G. J. Clark, C. M. Morris, R. V. Sorg and D. N. Hart (1998). "cDNA cloning of human DEC-205, a putative antigen-uptake receptor on dendritic cells." Immunogenetics 47(6): 442-450.

Kato, T., T. Matsumura, T. Heller, S. Saito, R. K. Sapp, K. Murthy, T. Wakita and T. J. Liang (2007). "Production of infectious hepatitis C virus of various genotypes in cell cultures." J Virol 81(9): 4405-4411.

Kawai, T., S. Sato, K. J. Ishii, C. Coban, H. Hemmi, M. Yamamoto, K. Terai, M. Matsuda, J. Inoue, S. Uematsu, O. Takeuchi and S. Akira (2004). "Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6." Nat Immunol 5(10): 1061-1068.

Khabar, K. S., F. Al-Zoghaibi, M. N. Al-Ahdal, T. Murayama, M. Dhalla, N. Mukaida, M. Taha, S. T. Al-Sedairy, Y. Siddiqui, G. Kessie and K. Matsushima (1997). "The alpha chemokine, interleukin 8, inhibits the antiviral action of interferon alpha." J Exp Med 186(7): 1077-1085.

Khakoo, S. I., C. L. Thio, M. P. Martin, C. R. Brooks, X. Gao, J. Astemborski, J. Cheng, J. J. Goedert, D. Vlahov, M. Hilgartner, S. Cox, A. M. Little, G. J. Alexander, M. E. Cramp, S. J. O'Brien, W. M. Rosenberg, D. L. Thomas and M. Carrington (2004). "HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection." Science 305(5685): 872-874.

Kim, T. W., K. Staschke, K. Bulek, J. Yao, K. Peters, K. H. Oh, Y. Vandenburg, H. Xiao, W. Qian, T. Hamilton, B. Min, G. Sen, R. Gilmour and X. Li (2007). "A critical role for IRAK4 kinase activity in Toll-like receptor-mediated innate immunity." J Exp Med 204(5): 1025-1036.

Kimber, R. J. (1990). "Screening of blood donations for hepatitis C virus antibody." Med J Aust 152(11): 613.

Knapp, S., L. J. Yee, A. J. Frodsham, B. J. Hennig, S. Hellier, L. Zhang, M. Wright, M. Chiaramonte, M. Graves, H. C. Thomas, A. V. Hill and M. R. Thursz (2003). "Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of MxA, OAS-1 and PKR." Genes Immun 4(6): 411-419.

Koev, G., T. Dekhtyar, L. Han, P. Yan, T. I. Ng, C. T. Lin, H. Mo and A. Molla (2007). "Antiviral interactions of an HCV polymerase inhibitor with an HCV protease inhibitor or interferon in vitro." Antiviral Res 73(1): 78-83.

Kokordelis, P., B. Kramer, C. Korner, C. Boesecke, E. Voigt, P. Ingiliz, A. Glassner, M. Eisenhardt, F. Wolter, D. Kaczmarek, H. D. Nischalke, J. K. Rockstroh, U. Spengler and J. Nattermann (2013). "An effective IFN- mediated inhibition of HCV replication by NK cells is associated with spontaneous clearance of acute hepatitis C in HIV(+) patients." Hepatology.

Konerman, M. A., S. H. Mehta, C. G. Sutcliffe, T. Vu, Y. Higgins, M. S. Torbenson, R. D. Moore, D. L. Thomas and M. S. Sulkowski (2014). "Fibrosis progression in human

immunodeficiency virus/hepatitis C virus coinfected adults: Prospective analysis of 435 liver biopsy pairs." Hepatology.

Konno, H., T. Yamamoto, K. Yamazaki, J. Gohda, T. Akiyama, K. Semba, H. Goto, A. Kato, T. Yujiri, T. Imai, Y. Kawaguchi, B. Su, O. Takeuchi, S. Akira, Y. Tsunetsugu-Yokota and J. Inoue (2009). "TRAF6 establishes innate immune responses by activating NF-kappaB and IRF7 upon sensing cytosolic viral RNA and DNA." PLoS One 4(5): e5674.

Kronenberger, B., E. Herrmann, W. P. Hofmann, H. Wedemeyer, M. Sester, U. Mihm, T. Ghaliai, S. Zeuzem and C. Sarrazin (2006). "Dynamics of CD81 expression on lymphocyte subsets during interferon-alpha-based antiviral treatment of patients with chronic hepatitis C." J Leukoc Biol 80(2): 298-308.

Kronenberger, B., B. Ruster, R. Elez, S. Weber, A. Piiper, J. H. Lee, W. K. Roth and S. Zeuzem (2001). "Interferon alfa down-regulates CD81 in patients with chronic hepatitis C." Hepatology 33(6): 1518-1526.

Krug, A., A. Towarowski, S. Britsch, S. Rothenfusser, V. Hornung, R. Bals, T. Giese, H. Engelmann, S. Endres, A. M. Krieg and G. Hartmann (2001). "Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12." Eur J Immunol 31(10): 3026-3037.

Kylefjord, H., A. Danielsson, S. Sedig, O. Belda, D. Wiktelius, L. Vrang and P. Targett-Adams (2013). "Transient replication of a hepatitis C virus genotype 1b replicon chimera encoding NS5A-5B from genotype 3a." J Virol Methods 195: 156-163.

Labonte, P., S. Begley, C. Guevin, M. C. Asselin, N. Nassoury, G. Mayer, A. Prat and N. G. Seidah (2009). "PCSK9 impedes hepatitis C virus infection in vitro and modulates liver CD81 expression." Hepatology 50(1): 17-24.

Lagaye, S., H. Shen, B. Saunier, M. Nascimbeni, J. Gaston, P. Bourdoncle, L. Hannoun, P. P. Massault, A. Vallet-Pichard, V. Mallet and S. Pol (2012). "Efficient replication of primary or culture hepatitis C virus isolates in human liver slices: a relevant ex vivo model of liver infection." Hepatology 56(3): 861-872.

Lai, W. K., P. J. Sun, J. Zhang, A. Jennings, P. F. Lalor, S. Hubscher, J. A. McKeating and D. H. Adams (2006). "Expression of DC-SIGN and DC-SIGNR on human sinusoidal

endothelium: a role for capturing hepatitis C virus particles." Am J Pathol 169(1): 200-208.

Lam, N. P., A. U. Neumann, D. R. Gretch, T. E. Wiley, A. S. Perelson and T. J. Layden (1997). "Dose-dependent acute clearance of hepatitis C genotype 1 virus with interferon alfa." Hepatology 26(1): 226-231.

Lamarre, D., P. C. Anderson, M. Bailey, P. Beaulieu, G. Bolger, P. Bonneau, M. Bos, D. R. Cameron, M. Cartier, M. G. Cordingley, A. M. Faucher, N. Goudreau, S. H. Kawai, G. Kukolj, L. Lagace, S. R. LaPlante, H. Narjes, M. A. Poupart, J. Rancourt, R. E. Sentjens, R. St George, B. Simoneau, G. Steinmann, D. Thibeault, Y. S. Tsantrizos, S. M. Weldon, C. L. Yong and M. Llinas-Brunet (2003). "An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus." Nature 426(6963): 186-189.

Lambotin, M., T. F. Baumert and H. Barth (2010). "Distinct intracellular trafficking of hepatitis C virus in myeloid and plasmacytoid dendritic cells." J Virol 84(17): 8964-8969.

Lanford, R. E., B. Guerra, D. Chavez, C. Bigger, K. M. Brasky, X. H. Wang, S. C. Ray and D. L. Thomas (2004). "Cross-genotype immunity to hepatitis C virus." J Virol 78(3): 1575-1581.

Lanford, R. E., E. S. Hildebrandt-Eriksen, A. Petri, R. Persson, M. Lindow, M. E. Munk, S. Kauppinen and H. Orum (2013). "Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection." Science 327(5962): 198-201.

Larange, A., D. Antonios, M. Pallardy and S. Kerdine-Romer (2009). "TLR7 and TLR8 agonists trigger different signaling pathways for human dendritic cell maturation." J Leukoc Biol 85(4): 673-683.

Larsson, M., E. Babcock, A. Grakoui, N. Shoukry, G. Lauer, C. Rice, C. Walker and N. Bhardwaj (2004). "Lack of phenotypic and functional impairment in dendritic cells from chimpanzees chronically infected with hepatitis C virus." J Virol 78(12): 6151-6161.

Laskin, D. L., B. Weinberger and J. D. Laskin (2001). "Functional heterogeneity in liver and lung macrophages." J Leukoc Biol 70(2): 163-170.

Lau, D. T., P. M. Fish, M. Sinha, D. M. Owen, S. M. Lemon and M. Gale, Jr. (2008). "Interferon regulatory factor-3 activation, hepatic interferon-stimulated gene expression, and immune cell infiltration in hepatitis C virus patients." Hepatology 47(3): 799-809.

Lauer, G. M., K. Ouchi, R. T. Chung, T. N. Nguyen, C. L. Day, D. R. Purkis, M. Reiser, A. Y. Kim, M. Lucas, P. Klenerman and B. D. Walker (2002). "Comprehensive analysis of CD8(+)-T-cell responses against hepatitis C virus reveals multiple unpredicted specificities." J Virol 76(12): 6104-6113.

Lauer, G. M. and B. D. Walker (2001). "Hepatitis C virus infection." N Engl J Med 345(1): 41-52.

Lavillette, D., B. Bartosch, D. Nourrisson, G. Verney, F. L. Cosset, F. Penin and E. I. Pecheur (2006). "Hepatitis C virus glycoproteins mediate low pH-dependent membrane fusion with liposomes." J Biol Chem 281(7): 3909-3917.

Lawitz, E., F. F. Poordad, P. S. Pang, R. H. Hyland, X. Ding, H. Mo, W. T. Symonds, J. G. McHutchison and F. E. Membreno (2013). "Sofosbuvir and ledipasvir fixed-dose combination with and without ribavirin in treatment-naive and previously treated patients with genotype 1 hepatitis C virus infection (LONESTAR): an open-label, randomised, phase 2 trial." Lancet.

Lazear, H. M., A. Lancaster, C. Wilkins, M. S. Suthar, A. Huang, S. C. Vick, L. Clepper, L. Thackray, M. M. Brassil, H. W. Virgin, J. Nikolich-Zugich, A. V. Moses, M. Gale, Jr., K. Fruh and M. S. Diamond (2013). "IRF-3, IRF-5, and IRF-7 coordinately regulate the type I IFN response in myeloid dendritic cells downstream of MAVS signaling." PLoS Pathog 9(1): e1003118.

Le Pogam, S., W. R. Jiang, V. Leveque, S. Rajyaguru, H. Ma, H. Kang, S. Jiang, M. Singer, S. Ali, K. Klumpp, D. Smith, J. Symons, N. Cammack and I. Najera (2006). "In vitro selected Con1 subgenomic replicons resistant to 2'-C-methyl-cytidine or to R1479 show lack of cross resistance." Virology 351(2): 349-359.

Le Pogam, S., H. Kang, S. F. Harris, V. Leveque, A. M. Giannetti, S. Ali, W. R. Jiang, S. Rajyaguru, G. Tavares, C. Oshiro, T. Hendricks, K. Klumpp, J. Symons, M. F. Browner, N. Cammack and I. Najera (2006). "Selection and characterization of replicon variants

dually resistant to thumb- and palm-binding nonnucleoside polymerase inhibitors of the hepatitis C virus." J Virol 80(12): 6146-6154.

Le Pogam, S., J. M. Yan, M. Chhabra, M. Ilnicka, H. Kang, A. Kosaka, S. Ali, D. J. Chin, N. S. Shulman, P. Smith, K. Klumpp and I. Najera (2012). "Characterization of hepatitis C virus (HCV) quasispecies dynamics upon short-term dual therapy with the HCV NS5B nucleoside polymerase inhibitor mericitabine and the NS3/4 protease inhibitor danoprevir." Antimicrob Agents Chemother 56(11): 5494-5502.

Lechner, F., D. K. Wong, P. R. Dunbar, R. Chapman, R. T. Chung, P. Dohrenwend, G. Robbins, R. Phillips, P. Klenerman and B. D. Walker (2000). "Analysis of successful immune responses in persons infected with hepatitis C virus." J Exp Med 191(9): 1499-1512.

Lee, B. L. and G. M. Barton (2014). "Trafficking of endosomal Toll-like receptors." Trends Cell Biol.

Lee, H. K., J. M. Lund, B. Ramanathan, N. Mizushima and A. Iwasaki (2007). "Autophagy-dependent viral recognition by plasmacytoid dendritic cells." Science 315(5817): 1398-1401.

Lee, J., C. C. Wu, K. J. Lee, T. H. Chuang, K. Katakura, Y. T. Liu, M. Chan, R. Tawatao, M. Chung, C. Shen, H. B. Cottam, M. M. Lai, E. Raz and D. A. Carson (2006). "Activation of anti-hepatitis C virus responses via Toll-like receptor 7." Proc Natl Acad Sci U S A 103(6): 1828-1833.

Lehmann, M., M. F. Meyer, M. Monazahian, H. L. Tillmann, M. P. Manns and H. Wedemeyer (2004). "High rate of spontaneous clearance of acute hepatitis C virus genotype 3 infection." J Med Virol 73(3): 387-391.

Lerias de Almeida, P. R., A. Alves de Mattos and C. Valle Tovo (2010). "Sustained virological response according to the type of early virological response in HCV and HCV/HIV." Ann Hepatol 9(2): 150-155.

Li, T., S. Zhu, L. Shuai, Y. Xu, S. Yin, Y. Bian, Y. Wang, B. Zuo, W. Wang, S. Zhao, L. Zhang, J. Zhang, G. F. Gao, J. P. Allain and C. Li (2013). "Infection of common marmosets with hepatitis C virus/GB virus-B chimeras." Hepatology.

Liang, H., R. S. Russell, N. L. Yonkers, D. McDonald, B. Rodriguez, C. V. Harding and D. D. Anthony (2009). "Differential effects of hepatitis C virus JFH1 on human myeloid and plasmacytoid dendritic cells." J Virol 83(11): 5693-5707.

Lin, C., C. A. Gates, B. G. Rao, D. L. Brennan, J. R. Fulghum, Y. P. Luong, J. D. Frantz, K. Lin, S. Ma, Y. Y. Wei, R. B. Perni and A. D. Kwong (2005). "In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061." J Biol Chem 280(44): 36784-36791.

Lin, C., K. Lin, Y. P. Luong, B. G. Rao, Y. Y. Wei, D. L. Brennan, J. R. Fulghum, H. M. Hsiao, S. Ma, J. P. Maxwell, K. M. Cottrell, R. B. Perni, C. A. Gates and A. D. Kwong (2004). "In vitro resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: structural analysis indicates different resistance mechanisms." J Biol Chem 279(17): 17508-17514.

Lin, C., B. D. Lindenbach, B. M. Pragai, D. W. McCourt and C. M. Rice (1994). "Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini." J Virol 68(8): 5063-5073.

Lin, C., J. A. Thomson and C. M. Rice (1995). "A central region in the hepatitis C virus NS4A protein allows formation of an active NS3-NS4A serine proteinase complex in vivo and in vitro." J Virol 69(7): 4373-4380.

Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating and C. M. Rice (2005). "Complete replication of hepatitis C virus in cell culture." Science 309(5734): 623-626.

Lindenbach, B. D. and C. M. Rice (2013). "The ins and outs of hepatitis C virus entry and assembly." Nat Rev Microbiol 11(10): 688-700.

Liu, C., Q. Tao, M. Sun, J. Z. Wu, W. Yang, P. Jian, J. Peng, Y. Hu and P. Liu (2010). "Kupffer cells are associated with apoptosis, inflammation and fibrotic effects in hepatic fibrosis in rats." Lab Invest 90(12): 1805-1816.

Liu, H. M., H. Aizaki, K. Machida, J. H. Ou and M. M. Lai (2012). "Hepatitis C virus translation preferentially depends on active RNA replication." PLoS One 7(8): e43600.

Liu, L., I. Botos, Y. Wang, J. N. Leonard, J. Shiloach, D. M. Segal and D. R. Davies (2008). "Structural basis of toll-like receptor 3 signaling with double-stranded RNA." Science 320(5874): 379-381.

Liu, S., L. Xiao, C. Nelson and C. H. Hagedorn (2012). "A cell culture adapted HCV JFH1 variant that increases viral titers and permits the production of high titer infectious chimeric reporter viruses." PLoS One 7(9): e44965.

Logvinoff, C., M. E. Major, D. Oldach, S. Heyward, A. Talal, P. Balfe, S. M. Feinstone, H. Alter, C. M. Rice and J. A. McKeating (2004). "Neutralizing antibody response during acute and chronic hepatitis C virus infection." Proc Natl Acad Sci U S A 101(27): 10149-10154.

Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann and R. Bartenschlager (1999). "Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line." Science 285(5424): 110-113.

Longman, R. S., A. H. Talal, I. M. Jacobson, M. L. Albert and C. M. Rice (2004). "Presence of functional dendritic cells in patients chronically infected with hepatitis C virus." Blood 103(3): 1026-1029.

Lozach, P. Y., A. Amara, B. Bartosch, J. L. Virelizier, F. Arenzana-Seisdedos, F. L. Cosset and R. Altmeyer (2004). "C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles." J Biol Chem 279(31): 32035-32045.

Lu, J., W. Tao, R. Li, Y. Xiang, N. Zhang, X. Xiang, Q. Xie and J. Zhong (2013). "Construction and characterization of infectious hepatitis C virus chimera containing structural proteins directly from genotype 1b clinical isolates." Virology 443(1): 80-88.

Lu, L., T. J. Pilot-Matias, K. D. Stewart, J. T. Randolph, R. Pithawalla, W. He, P. P. Huang, L. L. Klein, H. Mo and A. Molla (2004). "Mutations conferring resistance to a potent hepatitis C virus serine protease inhibitor in vitro." Antimicrob Agents Chemother 48(6): 2260-2266.

Ludwig, I. S., A. N. Lekkerkerker, E. Depla, F. Bosman, R. J. Musters, S. Depraetere, Y. van Kooyk and T. B. Geijtenbeek (2004). "Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation." J Virol 78(15): 8322-8332.

Lund, J. M., L. Alexopoulou, A. Sato, M. Karow, N. C. Adams, N. W. Gale, A. Iwasaki and R. A. Flavell (2004). "Recognition of single-stranded RNA viruses by Toll-like receptor 7." Proc Natl Acad Sci U S A 101(15): 5598-5603.

Luo, B., L. Tang, Z. Wang, J. Zhang, Y. Ling, W. Feng, J. Z. Sun, C. R. Stockard, A. R. Frost, Y. F. Chen, W. E. Grizzle and M. B. Fallon (2005). "Cholangiocyte endothelin 1 and transforming growth factor beta1 production in rat experimental hepatopulmonary syndrome." Gastroenterology 129(2): 682-695.

Major, M. E., K. Mihalik, M. Puig, B. Rehermann, M. Nascimbeni, C. M. Rice and S. M. Feinstone (2002). "Previously infected and recovered chimpanzees exhibit rapid responses that control hepatitis C virus replication upon rechallenge." J Virol 76(13): 6586-6595.

Mandl, J. N., A. P. Barry, T. H. Vanderford, N. Kozyr, R. Chavan, S. Klucking, F. J. Barrat, R. L. Coffman, S. I. Staprans and M. B. Feinberg (2008). "Divergent TLR7 and TLR9 signaling and type I interferon production distinguish pathogenic and nonpathogenic AIDS virus infections." Nat Med 14(10): 1077-1087.

Manns, M. P., G. R. Foster, J. K. Rockstroh, S. Zeuzem, F. Zoulim and M. Houghton (2007). "The way forward in HCV treatment--finding the right path." Nat Rev Drug Discov 6(12): 991-1000.

Manns, M. P., J. G. McHutchison, S. C. Gordon, V. K. Rustgi, M. Shiffman, R. Reindollar, Z. D. Goodman, K. Koury, M. Ling and J. K. Albrecht (2001). "Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial." Lancet 358(9286): 958-965.

Martins, T., J. L. Narciso-Schiavon and L. Schiavon Lde (2011). "[Epidemiology of hepatitis C virus infection]." Rev Assoc Med Bras 57(1): 107-112.

Marukian, S., C. T. Jones, L. Andrus, M. J. Evans, K. D. Ritola, E. D. Charles, C. M. Rice and L. B. Dustin (2008). "Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells." Hepatology 48(6): 1843-1850.

Mayo, M. J. (2003). "Extrahepatic manifestations of hepatitis C infection." Am J Med Sci 325(3): 135-148.

McHutchison, J. G., S. C. Gordon, E. R. Schiff, M. L. Shiffman, W. M. Lee, V. K. Rustgi, Z. D. Goodman, M. H. Ling, S. Cort and J. K. Albrecht (1998). "Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group." N Engl J Med 339(21): 1485-1492.

McKeating, J. A., L. Q. Zhang, C. Logvinoff, M. Flint, J. Zhang, J. Yu, D. Butera, D. D. Ho, L. B. Dustin, C. M. Rice and P. Balfe (2004). "Diverse hepatitis C virus glycoproteins mediate viral infection in a CD81-dependent manner." J Virol 78(16): 8496-8505.

Mehta, S. H., A. Cox, D. R. Hoover, X. H. Wang, Q. Mao, S. Ray, S. A. Strathdee, D. Vlahov and D. L. Thomas (2002). "Protection against persistence of hepatitis C." Lancet 359(9316): 1478-1483.

Mendez-Sanchez, N., G. Ponciano-Rodriguez, N. C. Chavez-Tapia, D. Motola-Kuba, P. Almeda-Valdes, K. Sanchez-Lara, M. H. Ramos and M. Uribe (2005). "Prevalence of hepatitis C infection in a population of asymptomatic people in a checkup unit in Mexico city." Dig Dis Sci 50(4): 733-737.

Mengshol, J. A., L. Golden-Mason, N. Castelblanco, K. A. Im, S. M. Dillon, C. C. Wilson and H. R. Rosen (2009). "Impaired plasmacytoid dendritic cell maturation and differential chemotaxis in chronic hepatitis C virus: associations with antiviral treatment outcomes." Gut 58(7): 964-973.

Mercer, J. and U. F. Greber (2013). "Virus interactions with endocytic pathways in macrophages and dendritic cells." Trends Microbiol 21(8): 380-388.

Meunier, J. C., R. E. Engle, K. Faulk, M. Zhao, B. Bartosch, H. Alter, S. U. Emerson, F. L. Cosset, R. H. Purcell and J. Bukh (2005). "Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1." Proc Natl Acad Sci U S A 102(12): 4560-4565.

Micallef, J. M., J. M. Kaldor and G. J. Dore (2006). "Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies." J Viral Hepat 13(1): 34-41.

Mihm, S., M. Frese, V. Meier, P. Wietzke-Braun, J. G. Scharf, R. Bartenschlager and G. Ramadori (2004). "Interferon type I gene expression in chronic hepatitis C." Lab Invest 84(9): 1148-1159.

Mikami, T., H. Miyashita, S. Takatsuka, Y. Kuroki and N. Matsushima (2012). "Molecular evolution of vertebrate Toll-like receptors: evolutionary rate difference between their leucine-rich repeats and their TIR domains." Gene 503(2): 235-243.

Missale, G., R. Bertoni, V. Lamonaca, A. Valli, M. Massari, C. Mori, M. G. Rumi, M. Houghton, F. Fiaccadori and C. Ferrari (1996). "Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response." J Clin Invest 98(3): 706-714.

Mogensen, T. H. (2009). "Pathogen recognition and inflammatory signaling in innate immune defenses." Clin Microbiol Rev 22(2): 240-273, Table of Contents.

Mohamoud, Y. A., G. R. Mumtaz, S. Riome, D. Miller and L. J. Abu-Raddad (2013). "The epidemiology of hepatitis C virus in Egypt: a systematic review and data synthesis." BMC Infect Dis 13: 288.

Mohd Hanafiah, K., J. Groeger, A. D. Flaxman and S. T. Wiersma (2013). "Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence." Hepatology 57(4): 1333-1342.

Monazahian, M., I. Bohme, S. Bonk, A. Koch, C. Scholz, S. Grethe and R. Thomssen (1999). "Low density lipoprotein receptor as a candidate receptor for hepatitis C virus." J Med Virol 57(3): 223-229.

Montaner, L. J., R. P. da Silva, J. Sun, S. Sutterwala, M. Hollinshead, D. Vaux and S. Gordon (1999). "Type 1 and type 2 cytokine regulation of macrophage endocytosis: differential activation by IL-4/IL-13 as opposed to IFN-gamma or IL-10." J Immunol 162(8): 4606-4613.

Morikawa, K., C. M. Lange, J. Gouttenoire, E. Meylan, V. Brass, F. Penin and D. Moradpour (2011). "Nonstructural protein 3-4A: the Swiss army knife of hepatitis C virus." J Viral Hepat 18(5): 305-315.

Narbus, C. M., B. Israelow, M. Sourisseau, M. L. Michta, S. E. Hopcraft, G. M. Zeiner and M. J. Evans (2011). "HepG2 cells expressing microRNA miR-122 support the entire hepatitis C virus life cycle." J Virol 85(22): 12087-12092.

Natarajan, V., S. Kottilil, A. Hazen, J. Adelsberger, A. A. Murphy, M. A. Polis and J. A. Kovacs (2010). "HCV in peripheral blood mononuclear cells are predominantly carried on the surface of cells in HIV/HCV co-infected individuals." J Med Virol 82(12): 2032-2037.

Navarro-Sanchez, E., R. Altmeyer, A. Amara, O. Schwartz, F. Fieschi, J. L. Virelizier, F. Arenzana-Seisdedos and P. Despres (2003). "Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses." EMBO Rep 4(7): 723-728.

Netski, D. M., T. Mosbruger, E. Depla, G. Maertens, S. C. Ray, R. G. Hamilton, S. Roundtree, D. L. Thomas, J. McKeating and A. Cox (2005). "Humoral immune response in acute hepatitis C virus infection." Clin Infect Dis 41(5): 667-675.

Neubauer, K., T. Wilfling, A. Ritzel and G. Ramadori (2000). "Platelet-endothelial cell adhesion molecule-1 gene expression in liver sinusoidal endothelial cells during liver injury and repair." J Hepatol 32(6): 921-932.

Neumann, A. U., N. P. Lam, H. Dahari, M. Davidian, T. E. Wiley, B. P. Mika, A. S. Perelson and T. J. Layden (2000). "Differences in viral dynamics between genotypes 1 and 2 of hepatitis C virus." J Infect Dis 182(1): 28-35.

Nielsen, S. U., M. F. Bassendine, A. D. Burt, D. J. Bevitt and G. L. Toms (2004). "Characterization of the genome and structural proteins of hepatitis C virus resolved from infected human liver." J Gen Virol 85(Pt 6): 1497-1507.

O'Neill, L. A. and A. G. Bowie (2007). "The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling." Nat Rev Immunol 7(5): 353-364.

O'Neill, L. A., D. Golenbock and A. G. Bowie (2013). "The history of Toll-like receptors - redefining innate immunity." Nat Rev Immunol 13(6): 453-460.

Okamoto, H. (1995). "[Classification of hepatitis C virus genotypes and its application to molecular epidemiology]." Nihon Rinsho 53 Suppl(Pt 1): 71-79.

Olenina, L. V., T. I. Kuzmina, B. N. Sobolev, T. E. Kuraeva, E. F. Kolesanova and A. I. Archakov (2005). "Identification of glycosaminoglycan-binding sites within hepatitis C virus envelope glycoprotein E2*." J Viral Hepat 12(6): 584-593.

Ong, M. L., M. E. Wikstrom, P. Fleming, M. J. Estcourt, P. J. Hertzog, G. R. Hill, C. E. Andoniou and M. A. Degli-Esposti (2013). "CpG pretreatment enhances antiviral T-cell immunity against cytomegalovirus." Blood 122(1): 55-60.

Orito, E., M. Mizokami, T. Tanaka, J. Y. Lau, K. Suzuki, M. Yamauchi, Y. Ohta, A. Hasegawa, S. Tanaka and M. Kohara (1996). "Quantification of serum hepatitis C virus core protein level in patients chronically infected with different hepatitis C virus genotypes." Gut 39(6): 876-880.

Otto, G. A. and J. D. Puglisi (2004). "The pathway of HCV IRES-mediated translation initiation." Cell 119(3): 369-380.

Ozaras, R. and V. Tahan (2009). "Acute hepatitis C: prevention and treatment." Expert Rev Anti Infect Ther 7(3): 351-361.

Pawlotsky, J. M. (1998). "Genetic heterogeneity and properties of hepatitis C virus." Acta Gastroenterol Belg 61(2): 189-191.

Pawlotsky, J. M. (2003). "The nature of interferon-alpha resistance in hepatitis C virus infection." Curr Opin Infect Dis 16(6): 587-592.

Pawlotsky, J. M. (2011). "Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus." Hepatology 53(5): 1742-1751.

Pawlotsky, J. M. and J. G. McHutchison (2004). "Hepatitis C. Development of new drugs and clinical trials: promises and pitfalls. Summary of an AASLD hepatitis single topic conference, Chicago, IL, February 27-March 1, 2003." Hepatology 39(2): 554-567.

Pearlman, B. L. and N. Traub (2011). "Sustained virologic response to antiviral therapy for chronic hepatitis C virus infection: a cure and so much more." Clin Infect Dis 52(7): 889-900.

Perz, J. F., G. L. Armstrong, L. A. Farrington, Y. J. Hutin and B. P. Bell (2006). "The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide." J Hepatol 45(4): 529-538.

Pestova, T. V., I. N. Shatsky, S. P. Fletcher, R. J. Jackson and C. U. Hellen (1998). "A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon

during internal translation initiation of hepatitis C and classical swine fever virus RNAs." Genes Dev 12(1): 67-83.

Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi and S. Abrignani (1998). "Binding of hepatitis C virus to CD81." Science 282(5390): 938-941.

Pockros, P. J., D. Guyader, H. Patton, M. J. Tong, T. Wright, J. G. McHutchison and T. C. Meng (2007). "Oral resiquimod in chronic HCV infection: safety and efficacy in 2 placebo-controlled, double-blind phase IIa studies." J Hepatol 47(2): 174-182.

Pohlmann, S., J. Zhang, F. Baribaud, Z. Chen, G. J. Leslie, G. Lin, A. Granelli-Piperno, R. W. Doms, C. M. Rice and J. A. McKeating (2003). "Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR." J Virol 77(7): 4070-4080.

Polyak, S. J., K. S. Khabar, M. Rezeiq and D. R. Gretch (2001). "Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy." J Virol 75(13): 6209-6211.

Popper, H., J. L. Dienstag, S. M. Feinstone, H. J. Alter and R. H. Purcell (1980). "The pathology of viral hepatitis in chimpanzees." Virchows Arch A Pathol Anat Histol 387(1): 91-106.

Poynard, T., P. Bedossa and P. Opolon (1997). "Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups." Lancet 349(9055): 825-832.

Poynard, T., J. Moussali, V. Ratziu, C. Regimbeau and P. Opolon (1999). "Effects of interferon therapy in "non responder" patients with chronic hepatitis C." J Hepatol 31 Suppl 1: 178-183.

Poynard, T., V. Ratziu, Y. Benmanov, V. Di Martino, P. Bedossa and P. Opolon (2000). "Fibrosis in patients with chronic hepatitis C: detection and significance." Semin Liver Dis 20(1): 47-55.

Poynard, T., V. Ratziu, F. Charlotte, Z. Goodman, J. McHutchison and J. Albrecht (2001). "Rates and risk factors of liver fibrosis progression in patients with chronic hepatitis c." J Hepatol 34(5): 730-739.

Puig-Basagoiti, F., X. Forns, I. Furcic, S. Ampurdanes, M. Gimenez-Barcons, S. Franco, J. M. Sanchez-Tapias and J. C. Saiz (2005). "Dynamics of hepatitis C virus NS5A quasispecies during interferon and ribavirin therapy in responder and non-responder patients with genotype 1b chronic hepatitis C." J Gen Virol 86(Pt 4): 1067-1075.

Python, S., M. Gerber, R. Suter, N. Ruggli and A. Summerfield "Efficient sensing of infected cells in absence of virus particles by plasmacytoid dendritic cells is blocked by the viral ribonuclease E(rns.)." PLoS Pathog 9(6): e1003412.

Relloso, M., A. Puig-Kroger, O. M. Pello, J. L. Rodriguez-Fernandez, G. de la Rosa, N. Longo, J. Navarro, M. A. Munoz-Fernandez, P. Sanchez-Mateos and A. L. Corbi (2002). "DC-SIGN (CD209) expression is IL-4 dependent and is negatively regulated by IFN, TGF-beta, and anti-inflammatory agents." J Immunol 168(6): 2634-2643.

Roach, J. C., G. Glusman, L. Rowen, A. Kaur, M. K. Purcell, K. D. Smith, L. E. Hood and A. Aderem (2005). "The evolution of vertebrate Toll-like receptors." Proc Natl Acad Sci U S A 102(27): 9577-9582.

Rodriguez-Torres, M., C. F. Rios-Bedoya, J. Rodriguez-Orengo, A. Fernandez-Carbia, A. M. Marxuach-Cuetara, A. Lopez-Torres, R. Salgado-Mercado and N. Brau (2006). "Progression to cirrhosis in Latinos with chronic hepatitis C: differences in Puerto Ricans with and without human immunodeficiency virus coinfection and along gender." J Clin Gastroenterol 40(4): 358-366.

Rollier, C., J. A. Drexhage, B. E. Verstrepen, E. J. Verschoor, R. E. Bontrop, G. Koopman and J. L. Heeney (2003). "Chronic hepatitis C virus infection established and maintained in chimpanzees independent of dendritic cell impairment." Hepatology 38(4): 851-858.

Rong, L., H. Dahari, R. M. Ribeiro and A. S. Perelson (2010). "Rapid emergence of protease inhibitor resistance in hepatitis C virus." Sci Transl Med 2(30): 30ra32.

Rosa, D., S. Campagnoli, C. Moretto, E. Guenzi, L. Cousens, M. Chin, C. Dong, A. J. Weiner, J. Y. Lau, Q. L. Choo, D. Chien, P. Pileri, M. Houghton and S. Abrignani (1996). "A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells." Proc Natl Acad Sci U S A 93(5): 1759-1763.

Rotman, Y. and T. J. Liang (2009). "Coinfection with hepatitis C virus and human immunodeficiency virus: virological, immunological, and clinical outcomes." J Virol 83(15): 7366-7374.

Rutz, M., J. Metzger, T. Gellert, P. Luppa, G. B. Lipford, H. Wagner and S. Bauer (2004). "Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner." Eur J Immunol 34(9): 2541-2550.

Sagan, S. M. and P. Sarnow (2010). "Plasmacytoid dendritic cells as guardians in hepatitis C virus-infected liver." Proc Natl Acad Sci U S A 107(17): 7625-7626.

Sainz, B., Jr., N. Barretto, X. Yu, P. Corcoran and S. L. Uprichard (2012). "Permissiveness of human hepatoma cell lines for HCV infection." Virol J 9: 30.

Saito, T., D. M. Owen, F. Jiang, J. Marcotrigiano and M. Gale, Jr. (2008). "Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA." Nature 454(7203): 523-527.

Sakai, A., M. S. Claire, K. Faulk, S. Govindarajan, S. U. Emerson, R. H. Purcell and J. Bukh (2003). "The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences." Proc Natl Acad Sci U S A 100(20): 11646-11651.

Sallusto, F., M. Cella, C. Danieli and A. Lanzavecchia (1995). "Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products." J Exp Med 182(2): 389-400.

Salmi, M., D. Adams and S. Jalkanen (1998). "Cell adhesion and migration. IV. Lymphocyte trafficking in the intestine and liver." Am J Physiol 274(1 Pt 1): G1-6.

Santantonio, T., E. Medda, C. Ferrari, P. Fabris, G. Cariti, M. Massari, S. Babudieri, M. Toti, R. Francavilla, F. Ancarani, G. Antonucci, G. Scotto, V. Di Marco, G. Pastore and T. Stroffolini (2006). "Risk factors and outcome among a large patient cohort with community-acquired acute hepatitis C in Italy." Clin Infect Dis 43(9): 1154-1159.

Sarasin-Filipowicz, M., E. J. Oakeley, F. H. Duong, V. Christen, L. Terracciano, W. Filipowicz and M. H. Heim (2008). "Interferon signaling and treatment outcome in chronic hepatitis C." Proc Natl Acad Sci U S A 105(19): 7034-7039.

Sarrazin, C., R. Rouzier, F. Wagner, N. Forestier, D. Larrey, S. K. Gupta, M. Hussain, A. Shah, D. Cutler, J. Zhang and S. Zeuzem (2007). "SCH 503034, a novel hepatitis C virus protease inhibitor, plus pegylated interferon alpha-2b for genotype 1 nonresponders." Gastroenterology 132(4): 1270-1278.

Scarselli, E., H. Ansuini, R. Cerino, R. M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese and A. Vitelli (2002). "The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus." EMBO J 21(19): 5017-5025.

Schoenemeyer, A., B. J. Barnes, M. E. Mancl, E. Latz, N. Goutagny, P. M. Pitha, K. A. Fitzgerald and D. T. Golenbock (2005). "The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signaling." J Biol Chem 280(17): 17005-17012.

Schoggins, J. W. and C. M. Rice (2013). "Innate immune responses to hepatitis C virus." Curr Top Microbiol Immunol 369: 219-242.

Schott, E., H. Witt, K. Neumann, A. Bergk, J. Halangk, V. Weich, T. Muller, G. Puhl, B. Wiedenmann and T. Berg (2008). "Association of TLR7 single nucleotide polymorphisms with chronic HCV-infection and response to interferon-a-based therapy." J Viral Hepat 15(1): 71-78.

Schroder, K., M. Spille, A. Pilz, J. Lattin, K. A. Bode, K. M. Irvine, A. D. Burrows, T. Ravasi, H. Weighardt, K. J. Stacey, T. Decker, D. A. Hume, A. H. Dalpke and M. J. Sweet (2007). "Differential effects of CpG DNA on IFN-beta induction and STAT1 activation in murine macrophages versus dendritic cells: alternatively activated STAT1 negatively regulates TLR signaling in macrophages." J Immunol 179(6): 3495-3503.

Schulz, O., S. S. Diebold, M. Chen, T. I. Naslund, M. A. Nolte, L. Alexopoulou, Y. T. Azuma, R. A. Flavell, P. Liljestrom and C. Reis e Sousa (2005). "Toll-like receptor 3 promotes cross-priming to virus-infected cells." Nature 433(7028): 887-892.

Schwartz, A. J., X. Alvarez and A. A. Lackner (2002). "Distribution and immunophenotype of DC-SIGN-expressing cells in SIV-infected and uninfected macaques." AIDS Res Hum Retroviruses 18(14): 1021-1029.

Sen, G. C. (2001). "Viruses and interferons." Annu Rev Microbiol 55: 255-281.

Shiina, M. and B. Rehermann (2008). "Cell culture-produced hepatitis C virus impairs plasmacytoid dendritic cell function." Hepatology 47(2): 385-395.

Shoukry, N. H., A. Grakoui, M. Houghton, D. Y. Chien, J. Ghrayeb, K. A. Reimann and C. M. Walker (2003). "Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection." J Exp Med 197(12): 1645-1655.

Soilleux, E. J., L. S. Morris, G. Leslie, J. Chehimi, Q. Luo, E. Levroney, J. Trowsdale, L. J. Montaner, R. W. Doms, D. Weissman, N. Coleman and B. Lee (2002). "Constitutive and induced expression of DC-SIGN on dendritic cell and macrophage subpopulations in situ and in vitro." J Leukoc Biol 71(3): 445-457.

Song, Y., Y. Zhuang, S. Zhai, D. Huang, Y. Zhang, W. Kang, X. Li, Q. Liu, Q. Yu and Y. Sun (2009). "Increased expression of TLR7 in CD8(+) T cells leads to TLR7-mediated activation and accessory cell-dependent IFN-gamma production in HIV type 1 infection." AIDS Res Hum Retroviruses 25(12): 1287-1295.

Stedman, C. A. (2012). "Current prospects for interferon-free treatment of hepatitis C in 2012." J Gastroenterol Hepatol 28(1): 38-45.

Su, A. I., J. P. Pezacki, L. Wodicka, A. D. Brideau, L. Supekova, R. Thimme, S. Wieland, J. Bukh, R. H. Purcell, P. G. Schultz and F. V. Chisari (2002). "Genomic analysis of the host response to hepatitis C virus infection." Proc Natl Acad Sci U S A 99(24): 15669-15674.

Sumpter, R., Jr., Y. M. Loo, E. Foy, K. Li, M. Yoneyama, T. Fujita, S. M. Lemon and M. Gale, Jr. (2005). "Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I." J Virol 79(5): 2689-2699.

Sun, P., S. Fernandez, M. A. Marovich, D. R. Palmer, C. M. Celluzzi, K. Boonnak, Z. Liang, H. Subramanian, K. R. Porter, W. Sun and T. H. Burgess (2009). "Functional characterization of ex vivo blood myeloid and plasmacytoid dendritic cells after infection with dengue virus." Virology 383(2): 207-215.

Takahashi, K., S. Asabe, S. Wieland, U. Garaigorta, P. Gastaminza, M. Isogawa and F. V. Chisari (2010). "Plasmacytoid dendritic cells sense hepatitis C virus-infected cells, produce interferon, and inhibit infection." Proc Natl Acad Sci U S A 107(16): 7431-7436.

Takaki, A., M. Wiese, G. Maertens, E. Depla, U. Seifert, A. Liebetrau, J. L. Miller, M. P. Manns and B. Rehermann (2000). "Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C." Nat Med 6(5): 578-582.

Takeda, K., T. Kaisho and S. Akira (2003). "Toll-like receptors." Annu Rev Immunol 21: 335-376.

Takeuchi, K., Y. Kubo, S. Boonmar, Y. Watanabe, T. Katayama, Q. L. Choo, G. Kuo, M. Houghton, I. Saito and T. Miyamura (1990). "The putative nucleocapsid and envelope protein genes of hepatitis C virus determined by comparison of the nucleotide sequences of two isolates derived from an experimentally infected chimpanzee and healthy human carriers." J Gen Virol 71 (Pt 12): 3027-3033.

Tanaka, E., K. Kiyosawa, A. Matsumoto, T. Kashiwakuma, A. Hasegawa, H. Mori, O. Yanagihara and Y. Ohta (1996). "Serum levels of hepatitis C virus core protein in patients with chronic hepatitis C treated with interferon alfa." Hepatology 23(6): 1330-1333.

Tarao, K., S. Ohkawa, Y. Miyagi, S. Morinaga, K. Ohshige, N. Yamamoto, M. Ueno, S. Kobayashi, R. Kameda, S. Tamai, Y. Nakamura, K. Miyakawa, Y. Kameda and M. Okudaira (2013). "Inflammation in background cirrhosis evokes malignant progression in HCC development from HCV-associated liver cirrhosis." Scand J Gastroenterol 48(6): 729-735.

Tassaneetrithep, B., T. H. Burgess, A. Granelli-Piperno, C. Trumpfheller, J. Finke, W. Sun, M. A. Eller, K. Pattanapanyasat, S. Sarasombath, D. L. Birx, R. M. Steinman, S. Schlesinger and M. A. Marovich (2003). "DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells." J Exp Med 197(7): 823-829.

Thimme, R., J. Bukh, H. C. Spangenberg, S. Wieland, J. Pemberton, C. Steiger, S. Govindarajan, R. H. Purcell and F. V. Chisari (2002). "Viral and immunological determinants of

hepatitis C virus clearance, persistence, and disease." Proc Natl Acad Sci U S A 99(24): 15661-15668.

Thimme, R., D. Oldach, K. M. Chang, C. Steiger, S. C. Ray and F. V. Chisari (2001). "Determinants of viral clearance and persistence during acute hepatitis C virus infection." J Exp Med 194(10): 1395-1406.

Thomas, A., C. Laxton, J. Rodman, N. Myangar, N. Horscroft and T. Parkinson (2007). "Investigating Toll-like receptor agonists for potential to treat hepatitis C virus infection." Antimicrob Agents Chemother 51(8): 2969-2978.

Thompson, A. J. and S. A. Locarnini (2007). "Toll-like receptors, RIG-I-like RNA helicases and the antiviral innate immune response." Immunol Cell Biol 85(6): 435-445.

Thompson, M. R., J. J. Kaminski, E. A. Kurt-Jones and K. A. Fitzgerald (2011). "Pattern recognition receptors and the innate immune response to viral infection." Viruses 3(6): 920-940.

Tomei, L., S. Altamura, L. Bartholomew, A. Biroccio, A. Ceccacci, L. Pacini, F. Narjes, N. Gennari, M. Bisbocci, I. Incitti, L. Orsatti, S. Harper, I. Stansfield, M. Rowley, R. De Francesco and G. Migliaccio (2003). "Mechanism of action and antiviral activity of benzimidazole-based allosteric inhibitors of the hepatitis C virus RNA-dependent RNA polymerase." J Virol 77(24): 13225-13231.

Tong, X., R. Chase, A. Skelton, T. Chen, J. Wright-Minogue and B. A. Malcolm (2006). "Identification and analysis of fitness of resistance mutations against the HCV protease inhibitor SCH 503034." Antiviral Res 70(2): 28-38.

Torriani, F. J., M. Rodriguez-Torres, J. K. Rockstroh, E. Lissen, J. Gonzalez-Garcia, A. Lazzarin, G. Carosi, J. Sasadeusz, C. Katlama, J. Montaner, H. Sette, Jr., S. Passe, J. De Pamphilis, F. Duff, U. M. Schrenk and D. T. Dieterich (2004). "Peginterferon Alfa-2a plus ribavirin for chronic hepatitis C virus infection in HIV-infected patients." N Engl J Med 351(5): 438-450.

Trapp, S., N. R. Derby, R. Singer, A. Shaw, V. G. Williams, S. G. Turville, J. W. Bess, Jr., J. D. Lifson and M. Robbiani (2009). "Double-stranded RNA analog poly(I:C) inhibits human immunodeficiency virus amplification in dendritic cells via type I interferon-mediated activation of APOBEC3G." J Virol 83(2): 884-895.

Tu, Z., R. H. Pierce, J. Kurtis, Y. Kuroki, I. N. Crispe and M. S. Orloff (2010). "Hepatitis C virus core protein subverts the antiviral activities of human Kupffer cells." Gastroenterology 138(1): 305-314.

Uematsu, S., S. Sato, M. Yamamoto, T. Hirotani, H. Kato, F. Takeshita, M. Matsuda, C. Coban, K. J. Ishii, T. Kawai, O. Takeuchi and S. Akira (2005). "Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-{alpha} induction." J Exp Med 201(6): 915-923.

Ulsenheimer, A., M. Lucas, N. P. Seth, J. Tilman Gerlach, N. H. Gruener, A. Loughry, G. R. Pape, K. W. Wucherpfennig, H. M. Diepolder and P. Klenerman (2006). "Transient immunological control during acute hepatitis C virus infection: ex vivo analysis of helper T-cell responses." J Viral Hepat 13(10): 708-714.

Uno, K., Y. Suginoshita, K. Kakimi, F. Moriyasu, M. Hirosaki, T. Shirakawa and T. Kishida (2005). "Impairment of IFN-alpha production capacity in patients with hepatitis C virus and the risk of the development of hepatocellular carcinoma." World J Gastroenterol 11(46): 7330-7334.

Uwatoku, R., M. Suematsu, T. Ezaki, T. Saiki, M. Tsuiji, T. Irimura, N. Kawada, T. Suganuma, M. Naito, M. Ando and K. Matsuno (2001). "Kupffer cell-mediated recruitment of rat dendritic cells to the liver: roles of N-acetylgalactosamine-specific sugar receptors." Gastroenterology 121(6): 1460-1472.

Valladeau, J., O. Ravel, C. Dezutter-Dambuyant, K. Moore, M. Kleijmeer, Y. Liu, V. Duvert-Frances, C. Vincent, D. Schmitt, J. Davoust, C. Caux, S. Lebecque and S. Saeland (2000). "Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules." Immunity 12(1): 71-81.

van der Poorten, D., D. T. Kenny and J. George (2008). "Prevalence of and risk factors for hepatitis C in Aboriginal and non-Aboriginal adolescent offenders." Med J Aust 188(10): 610-614.

Veldt, B. J., J. T. Brouwer, M. Adler, F. Nevens, P. Michielsen, J. Delwaide, B. E. Hansen and S. W. Schalm (2003). "Retreatment of hepatitis C non-responsive to interferon. A placebo controlled randomized trial of ribavirin monotherapy versus combination therapy with

Ribavirin and Interferon in 121 patients in the Benelux [ISRCTN53821378]." BMC Gastroenterol 3: 24.

Veldt, B. J., E. J. Heathcote, H. Wedemeyer, J. Reichen, W. P. Hofmann, S. Zeuzem, M. P. Manns, B. E. Hansen, S. W. Schalm and H. L. Janssen (2007). "Sustained virologic response and clinical outcomes in patients with chronic hepatitis C and advanced fibrosis." Ann Intern Med 147(10): 677-684.

Villadangos, J. A. and P. Schnorrer (2007). "Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo." Nat Rev Immunol 7(7): 543-555.

Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager and T. J. Liang (2005). "Production of infectious hepatitis C virus in tissue culture from a cloned viral genome." Nat Med 11(7): 791-796.

Wang, B., M. Trippler, R. Pei, M. Lu, R. Broering, G. Gerken and J. F. Schlaak (2009). "Toll-like receptor activated human and murine hepatic stellate cells are potent regulators of hepatitis C virus replication." J Hepatol 51(6): 1037-1045.

Wang, C. H., H. L. Eng, K. H. Lin, C. H. Chang, C. A. Hsieh, Y. L. Lin and T. M. Lin (2011). "TLR7 and TLR8 gene variations and susceptibility to hepatitis C virus infection." PLoS One 6(10): e26235.

Weiner, A. J., G. Kuo, D. W. Bradley, F. Bonino, G. Saracco, C. Lee, J. Rosenblatt, Q. L. Choo and M. Houghton (1990). "Detection of hepatitis C viral sequences in non-A, non-B hepatitis." Lancet 335(8680): 1-3.

WHO, W. H. O. (2011). "Hepatitis C (Fact sheet N°164)."

Wieland, S., Z. Makowska, B. Campana, D. Calabrese, M. T. Dill, J. Chung, F. V. Chisari and M. H. Heim (2013). "Simultaneous detection of hepatitis C virus and interferon stimulated gene expression in infected human liver." Hepatology.

Wieland, S. F. and F. V. Chisari (2005). "Stealth and cunning: hepatitis B and hepatitis C viruses." J Virol 79(15): 9369-9380.

Wiese, M., F. Berr, M. Lafrenz, H. Porst and U. Oesen (2000). "Low frequency of cirrhosis in a hepatitis C (genotype 1b) single-source outbreak in germany: a 20-year multicenter study." Hepatology 32(1): 91-96.

Wiley, T. E., M. McCarthy, L. Breidi and T. J. Layden (1998). "Impact of alcohol on the histological and clinical progression of hepatitis C infection." Hepatology 28(3): 805-809.

Woollard, D. J., A. Grakoui, N. H. Shoukry, K. K. Murthy, K. J. Campbell and C. M. Walker (2003). "Characterization of HCV-specific Patr class II restricted CD4+ T cell responses in an acutely infected chimpanzee." Hepatology 38(5): 1297-1306.

Wu, B., A. Peisley, C. Richards, H. Yao, X. Zeng, C. Lin, F. Chu, T. Walz and S. Hur (2013). "Structural basis for dsRNA recognition, filament formation, and antiviral signal activation by MDA5." Cell 152(1-2): 276-289.

Yao, Z. Q., S. Ray, A. Eisen-Vandervelde, S. Waggoner and Y. S. Hahn (2001). "Hepatitis C virus: immunosuppression by complement regulatory pathway." Viral Immunol 14(4): 277-295.

Yazdanpanah, Y., G. De Carli, B. Migueres, F. Lot, M. Campins, C. Colombo, T. Thomas, S. Deuffic-Burban, M. H. Prevot, M. Domart, A. Tarantola, D. Abiteboul, P. Deny, S. Pol, J. C. Desenclos, V. Puro and E. Bouvet (2005). "Risk factors for hepatitis C virus transmission to health care workers after occupational exposure: a European case-control study." Clin Infect Dis 41(10): 1423-1430.

Younossi, Z. M., A. Afendy, M. Stepanova, N. Hossain, I. Younossi, K. Ankrah, T. Gramlich and A. Baranova (2009). "Gene expression profile associated with superimposed non-alcoholic fatty liver disease and hepatic fibrosis in patients with chronic hepatitis C." Liver Int 29(9): 1403-1412.

Younossi, Z. M. and A. J. McCullough (2009). "Metabolic syndrome, non-alcoholic fatty liver disease and hepatitis C virus: impact on disease progression and treatment response." Liver Int 29 Suppl 2: 3-12.

Younossi, Z. M., A. J. McCullough, J. P. Ong, D. S. Barnes, A. Post, A. Tavill, D. Bringman, L. M. Martin, J. Assmann, T. Gramlich, K. D. Mullen, R. O'Shea, W. D. Carey and R. Ferguson (2004). "Obesity and non-alcoholic fatty liver disease in chronic hepatitis C." J Clin Gastroenterol 38(8): 705-709.

Zein, N. N., J. Rakela, E. L. Krawitt, K. R. Reddy, T. Tominaga and D. H. Persing (1996). "Hepatitis C virus genotypes in the United States: epidemiology, pathogenicity, and response to interferon therapy. Collaborative Study Group." Ann Intern Med 125(8): 634-639.

Zeuzem, S., T. Berg, E. Gane, P. Ferenci, G. R. Foster, M. W. Fried, C. Hezode, G. M. Hirschfield, I. Jacobson, I. Nikitin, P. J. Pockros, F. Poordad, J. Scott, O. Lenz, M. Peeters, V. Sekar, G. De Smedt, R. Sinha and M. Beumont-Mauviel (2013). "Simeprevir Increases Rate of Sustained Virologic Response Among Treatment-Experienced Patients With HCV Genotype-1 Infection: A Phase IIb Trial." Gastroenterology.

Zhang, Y. L., Y. J. Guo, L. Bin and S. H. Sun (2009). "Hepatitis C virus single-stranded RNA induces innate immunity via Toll-like receptor 7." J Hepatol 51(1): 29-38.

Zhang, Y. Y., B. H. Zhang, K. Ishii and T. J. Liang (2010). "Novel function of CD81 in controlling hepatitis C virus replication." J Virol 84(7): 3396-3407.

Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita and F. V. Chisari (2005). "Robust hepatitis C virus infection in vitro." Proc Natl Acad Sci U S A 102(26): 9294-9299.

APPENDIX : JOURNAL PUBLICATIONS

1. Said EA, Dupuy FP, Trautmann L, Zhang Y, Shi Y, El-Far M, Hill BJ, Noto A, Ancuta P, Peretz Y, Fonseca SG, Van Grevenynghe J, Boulassel MR, Bruneau J,Shoukry NH, Routy JP, Douek DC, Haddad EK, Sekaly RP. Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection. *Nat Medicine. 2010 Apr,16(4):452-9*
2. Van Grevenynghe J1, Procopio FA, He Z, Chomont N, Riou C, Zhang Y, Gimmig S, Boucher G, Wilkinson P, Shi Y, Yassine-Diab B, Said EA, Trautmann L, El Far M, Balderas RS, Boulassel MR, Routy JP, Haddad EK, Sekaly RP. Transcriptional factor FOXO3a controls the persistence of memory CD4+ T cells during HIV infection. *Nature Medicine. 14, 266 - 274 (2008)*
3. Bernier A[1], Cleret-Buhot A[1], Zhang Y[1], Goulet JP, Monteiro P, Gosselin A, DaFonseca S, Wacleche VS, Jenabian MA, Routy JP, Tremblay C, Ancuta P. Transcriptional profiling reveals molecular signatures associated with HIV permissiveness in Th1Th17 cells and identifies peroxisome proliferator-activated receptor gamma as an intrinsic negative regulator of viral replication. *Retrovirology. 2013 Dec 21,10:160.*

Copyright Clearance

(Figure 4 in Chapter 1)

Licensee: yuwei zhang

License Number	3310970613640
License date	Jan 16, 2014
Licensed content publisher	John Wiley and Sons
Licensed content publication	Liver International
Licensed content title	Interferon free therapy with direct acting antivirals for HCV
Licensed copyright line	© 2012 John Wiley & Sons A/S
Licensed content author	Tarik Asselah,Patrick Marcellin
Licensed content date	Jan 3, 2013
Start page	93
End page	104
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Electronic
Portion	Figure/table
Number of figures/tables	1
Original Wiley figure/table number(s)	Figure 1
Will you be translating?	No