The role of liver sinusoidal endothelial cells in HCV infection

Papić, Neven

UNIVERSITY OF ZAGREB
SCHOOL OF MEDICINE

Neven Papić

The role of liver sinusoidal endothelial cells in HCV infection

DISSERTATION

Zagreb, 2015.

UNIVERSITY OF ZAGREB
SCHOOL OF MEDICINE

Neven Papić

The role of liver sinusoidal endothelial cells in HCV infection

DISSERTATION

Zagreb, 2015.

This dissertation was made at the Department for Viral Hepatitis, University Hospital for Infectious Diseases, Zagreb, Croatia and at the Division of Gastroenterology, Hepatology and Nutrition, University of Utah, School of Medicine, Salt Lake City, Utah.

Mentors: Prof. Adriana Vince,
Prof. Curt Hagedorn

I would like to acknowledge everyone who has assisted me throughout my doctoral studies over the years. First and foremost I want to thank my mentors and friends prof. Adriana Vince and prof. Curt Hagedorn who were abundantly helpful and offered invaluable assistance, support and guidance. They have been instrumental in the successful completion of this project. I am forever indebted to my parents and friends for their understanding, endless patience and encouragement when it was most required.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

7-AAD	7-aminoactinomycin D
A20	tumor necrosis factor, alpha-induced protein 3
A2M	alpha-2-macroglobulin
ACAN	aggrecan
ACE	angiotensin I converting enzyme
ACP5	acid phosphatase 5, tartrate resistant
ACTB	actin, beta
ALCAM	activated leukocyte cell adhesion molecule
ANAPC1	anaphase promoting complex subunit 1
ANG2	angiogenin 2
ANGPT2	angiopoietin 2
AP1	activator protein 1 transcription factor
AP4E1	adaptor-related protein complex 4, epsilon 1 subunit
APC	antigen-presenting cells
apoB	apolipoprotein B
apoE	apolipoprotein E
AQP1	aquaporin 1 (Colton blood group)
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related
BAD	BCL2-associated agonist of cell death
BMP	bone morphogenetic proteins
BMPER	BMP binding endothelial regulator
BUB1	BUB1 mitotic checkpoint serine/threonine kinase
C1QTNF1	C1q and tumor necrosis factor related protein 1
CCL-5	chemokine (C-C motif) ligand 5
CCNE1	cyclin E1
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CD81	Cluster of Differentiation 81
CDC7	cell division cycle 7
cDNA	complementary DNA
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta
CHC	Chronic hepatitis C
CHEK1	checkpoint kinase 1
CLDN1	claudin 1
CLTC	clathrin, heavy chain
CMA1	chymase 1, mast cell
COL4A1	collagen, type IV, alpha 1
CTGF	connective tissue growth factor
CXCL-10	chemokine (C-X-C motif) ligand 10
CYBA	cytochrome b-245, alpha polypeptide
CYLD	cylindromatosis (turban tumor syndrome)

CYPA	cytochrome P450
DACH1	dachshund family transcription factor 1
DAPI	4',6-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC	dendritic cells
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DEG	diferentially expressed genes
DGAT1	diacylglycerol O-acyltransferase 1
DMEM	Dulbecco's modified Eagle's medium
DNM1	dynamin 1
dsRNA	Double-stranded RNA
EGFR	epidermal growth factor receptor
ER	endoplasmatic reticulum
ETV7	ets variant 7
FBS	fetal bovine serum
FDR	false discovery rate
FFU	focus forming units
FGF5	fibroblast growth factor 5
FOSL1	FOS-like antigen 1
FOXP4	forkhead box P4
FUT1	fucosyltransferase 1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GJA4	gap junction protein, alpha 4, 37kDa
GLIS3	GLIS family zinc finger 3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	Hepatitis C virus
HCV E1/2	HCV envelope protein
HDAC9	histone deacetylase 9
HES1	hes family bHLH transcription factor 1
HEYL	hes-related family bHLH transcription factor with YRPW motif-like
HIF3A	hypoxia inducible factor 3, alpha subunit
HIV	human immunodeficiency virus
HPX	hemopexin
HS	head spot (gene)
HSC	hepatic stellate cells
HSPB1	heat shock 27kDa protein 1
Huh7.5	hepatoma cell line 7.5
HUVEC	Human umbilical vein endothelial cells
ICAM	intercellular adhesion molecule 1
IFN	interferon
IFNγ	interferon gamma
IGB	Integrated Genome Browser
IGF2	insulin-like growth factor 2 (somatomedin A)
IL-10	Interleukin 10
IL-1b	Interleukin 1b
IL-2	interleukin 2
IPS-1	mitochondrial antiviral signaling protein

IRES	Internal Ribosome Entry Site
IRF3	Interferon regulatory factor 3
IRF7	interferon regulatory factor 7
ISG	interferon-stimulated genes
ISG20	Interferon stimulated gene 20
ITGAV	integrin, alpha V
ITGB2	integrin, beta 2
JAK	Janus kinase
JFH-1	Japanese fulminant hepatitis 1
KC	Kupffer cells
KEGG	Kyoto Encyclopedia of Genes and Genomes
KLF15	Kruppel-like factor 15
KLHDC7B	kelch domain containing 7B
L-SIGN	C-type lectin domain family 4, member M
LAMB2	laminin, beta 2
LDL-R	Low-density lipoprotein receptor
LGALS9	lectin, galactoside-binding, soluble, 9
LIF	leukemia inhibitory factor
LPS	Lipopolysaccharide
LSEC	liver sinusoidal endothelial cells
LTBP1	latent transforming growth factor beta binding protein 1
MAD2	mitotic spindle checkpoint protein
MAFB	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B
MAPK	mitogen-activated protein kinase
MCM	minichromosome maintenance complex
MDA5	Melanoma Differentiation-Associated protein 5
MDF1	antigen identified by monoclonal antibody A-3A4
MIP1B	or CCL4, chemokine (C-C motif) ligand 4
miR122	microRNA 122
MMP	matrix metallopproteinases
MOCK	negative control, infection of cells with empty vector
MOI	multiplicity of infection
mRNA	Messenger RNA
MX1	myxovirus (influenza virus) resistance 1
MXD4	MAX dimerization protein 4
MYC	v-myc avian myelocytomatosis viral oncogene homolog
MyD88	myeloid differentiation primary response 88
MYL	promyelocytic leukemia gene
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NKT	natural killer T-cells
NLRP3	NLR family, pyrin domain containing 3
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
NOLC1	nucleolar and coiled-body phosphoprotein 1
NOS3	nitric oxide synthase 3
NOSTRIN	nitric oxide synthase trafficking
NPC1L1	Niemann-Pick disease, type C1, gene -like 1
NS	Non structural

NT5E	5'-nucleotidase, ecto (CD73)
NUPR1	nuclear protein, transcriptional regulator, 1
OAS2	2'-5'-oligoadenylate synthetase 2
ORF	Open Reading Frame
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PD-L1	programmed cell death ligand 1
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKC	protein kinase C
PLCG2	phospholipase C, gamma 2
PLXNA1	plexin A1
PMA	phorbol 12-myristate 13-acetate
Pol II	RNA polymerase II
POU2F2	POU class 2 homeobox 2
PPARD	peroxisome proliferator-activated receptor delta
PRR	Pattern recognition receptor
PTPN11	protein tyrosine phosphatase, non-receptor type 11
RAC2	ras-related C3 botulinum toxin substrate 2
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RAS	renin-angiotensin system
RELb	v-rel avian reticuloendotheliosis viral oncogene homolog B
RGC32	response gene to complement 32 protein
RIG-I	retinoic acid-inducible gene 1
RNA	Ribonucleic acid
RNA-seq	next generation RNA sequencing
RNF125	ring finger protein 125, E3 ubiquitin protein ligase
ROCK2	Rho-associated, coiled-coil containing protein kinase 2
RPKM	Reads Per Kilobase per Million mapped reads
RUNX1	runt-related transcription factor 1
SALL2	spalt-like transcription factor 2
SARM	sterile alpha and TIR motif containing 1
SATB1	SATB homeobox 1
SELP	selectin P
SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1
SHC2	SHC (Src homology 2 domain containing) transforming protein 2
SIGIRR	single immunoglobulin and toll-interleukin 1 receptor (TIR) domain
siRNA	Small interfering RNA
SMOC1	SPARC related modular calcium binding 1
SOCS3	suppressor of cytokine signaling 3
SR-BI	scavenger receptor B type 1
STAT	signal-transducer and activator of transcription protein
TANK	TRAF family member-associated NFKB activator
TF	Transcriptional factors
TGFb	transforming growth factor, beta
Th1	T helper cells 1
THBD	thrombomodulin

THBS2	thrombospondin 2
THP-1	human acute monocytic leukemia cell line
TLR	Toll-like receptors
TNFa	tumor necrosis factor alpha
TNXB	tenascin XB
TRAF1	TNF receptor-associated factor 1
TTLL3	tubulin tyrosine ligase-like family, member 3
UTR	untranslated regions
VCAM1	vascular cell adhesion molecule 1
VEGFC	vascular endothelial growth factor C
WHO	World Health Organization
ZBTB16	zinc finger and BTB domain containing 16
ZFP36	or TTPZFP36 ring finger protein

1.

INTRODUCTION

Hepatitis C virus (HCV), a single stranded positive RNA virus of the *Flaviviridae* family, chronically infects about 3% of the world's population and often results in cirrhosis or liver cancer, thus representing a considerable public-health problem. (1-3) According to the WHO, two-thirds of liver transplants are linked to HCV infection.(3) Acute infections and less-advanced stages of chronic disease are usually clinically silent; therefore the majority of patients are unaware of their infection.(2) This results that HCV is frequently diagnosed in a late stage when therapeutic options and probability of cure are already limited. Unfortunately, almost 75% of HCV deaths occurred among adults between the ages of 45 and 64 that exceeds the mortality rate of HIV.(2, 4)

Although the HCV was cloned in 1989, the lack of adequate *in vitro* models has hampered our understanding of the disease pathogenesis and development of new therapeutic approaches. The HCV research gains momentum in 2005 when the first cell culture model supporting complete HCV replication was established.(5, 6) The replicon was a HCV genotype 2a clone isolated from Japanese patient with fulminant hepatitis (clone JFH-1).(5, 6) At the same time, progress in whole-genome sequencing technologies has opened a new era in genomics.(7) This represented a major breakthrough in the HCV field and enabled a broad range of fundamental and applied studies that lead to the development of new HCV disease models.

1.1. HCV VIROLOGY AND LIFE CYCLE

The HCV is a small (55–65 nm in size), enveloped, single-stranded, positive sense RNA virus.(8) There are at least 6 HCV genotypes with numerous subtypes. HCV genome contains 5' and 3' UTRs (Untranslated Regions) including control elements required for translation and replication.(8) The HCV 5′ UTR is the most conserved region of the genome; it is not capped and forms a complex secondary RNA structure, the Internal Ribosome Entry Site (IRES) that mediates direct binding of ribosomal subunits and cellular factors and subsequent translation. The HCV 3′ UTR is relatively short, less structured and contains a poly-uridyl tract that varies in length. The HCV ORF (Open Reading Frame) encodes a single polyprotein (3,010-3,011 amino acids), which is processed into 11 proteins; 3 structural (capsid protein C, E1, E2), a small protein p7 and 6 non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins.(8) The structural organization of HCV genome is schematically illustrated in Figure 1.

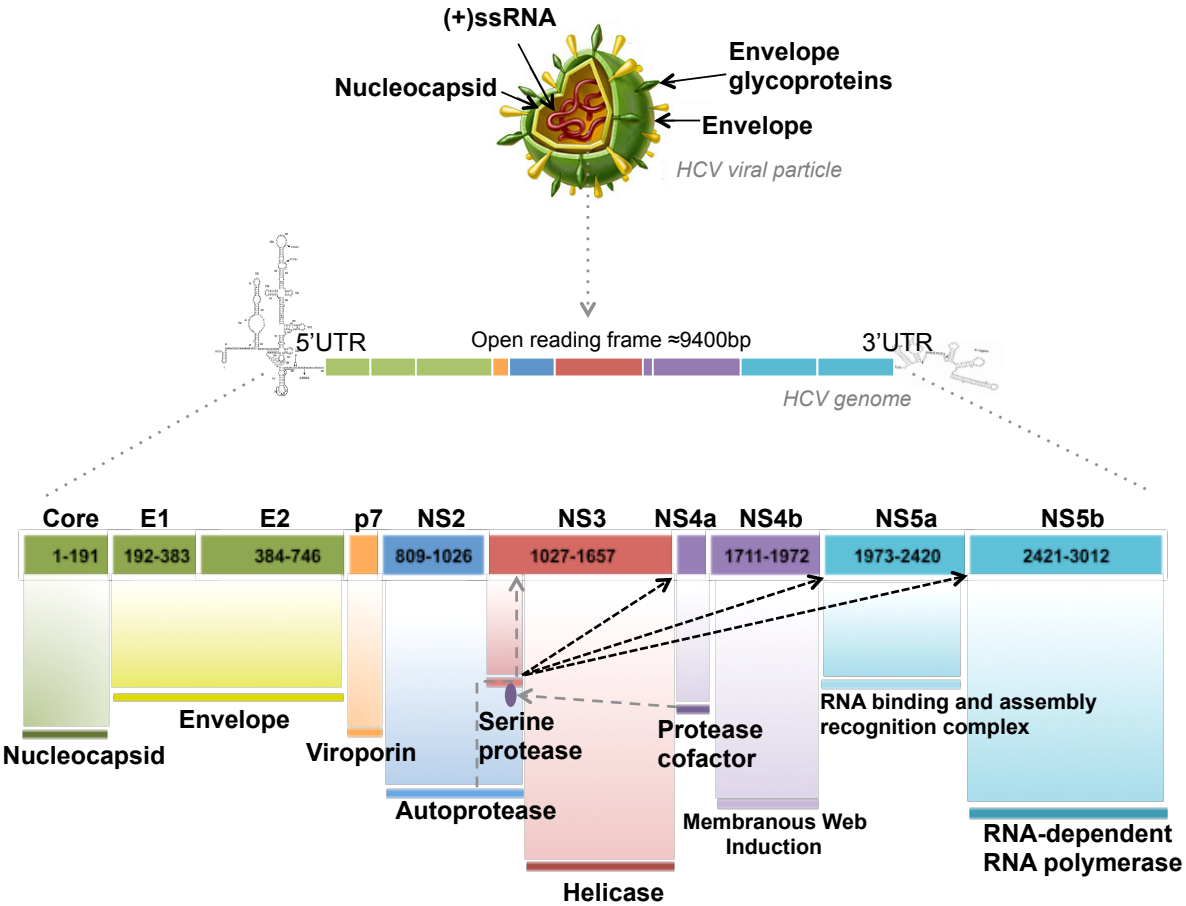


Figure 1. HCV viral particle structure (top), genome organization (middle) and polyprotein processing (bottom)

The complete life cycle of HCV happens in the cytoplasm of the host cell.(8) The binding of HCV into the host cell begins with receptor-mediated endocytosis. Several cell surface molecules have been proposed to mediate HCV binding and internalization (such as CD81, scavenger receptor B type I, SR-BI, low-density lipoprotein receptor, LDL-R, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin, DC-SIGN).(8) After attachment, the nucleocapsid is released into the cell cytoplasm as a result of a fusion process between viral and cellular membranes. When the virus is uncoated, it releases its positive-strand genomic RNA in the host's cytoplasm, where it serves as a messenger RNA for the synthesis of HCV polyprotein. The polyprotein is targeted to the endoplasmatic reticulum (ER), where is processed via host and viral proteases. The replication complex is assembled at the rearranged ER membrane (membranous web), where it directs the synthesis of intermediate negative-strand RNA, which is subsequently used as a template for the generation of positive-strand RNAs. Importantly, the HCV RNA polymerase has no proofreading, which results in a creation of numerous quasispecies variants. The new positive-sense HCV RNA is encapsidated with the structural proteins in the nucleocapsid, which is presumably enveloped by budding into the lumen of the ER. Finally, infectious virions are transported through the Golgi compartment to the plasma membrane and released to infect new cells. The HCV turnover rate can be quite high with replication ranging between 10^{10} to 10^{12} virions per day.(8)

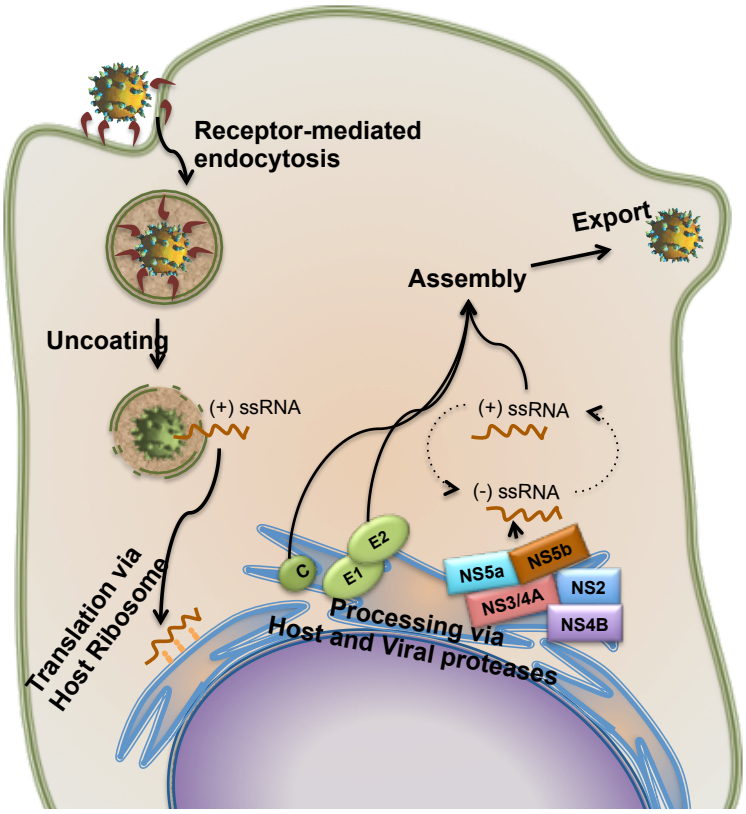


Figure 2. Hypothetical HCV life cycle.

Throughout its life cycle, HCV interacts with and hijacks variety of host-cell factors to enhance its own replication.(9) Previous gene-expression analysis on acutely HCV infected hepatoma cell lines have identified ≈1800 genes differentially expressed upon HCV infection, and many of these genes were subsequently reported to control different stages of HCV replication.(9, 10) Examples include *HS*, *CLDN1*, *EGFR* and *NPC1L1* gene products, necessary for efficient virus entry, liver specific microRNA *miR122* crucial for HCV translation, *PI4A* and *CypA* in formation of replication complex, or *apoB*, *apoE* and *DGAT1* involved in assembly and release of viral particles.(2, 8, 9)

1.2. NATURAL HISTORY OF HCV INFECTION

Upon infection, a large proportion of HCV-infected persons, ranging from 65%-85%, develop chronic HCV infection, and are at risk for advanced liver fibrosis, HCV-related extrahepatic complications, cirrhosis and HCC.(1, 2) An estimated 10%-15% of HCV-infected persons will advance to cirrhosis within the first 20 years.(1, 2)

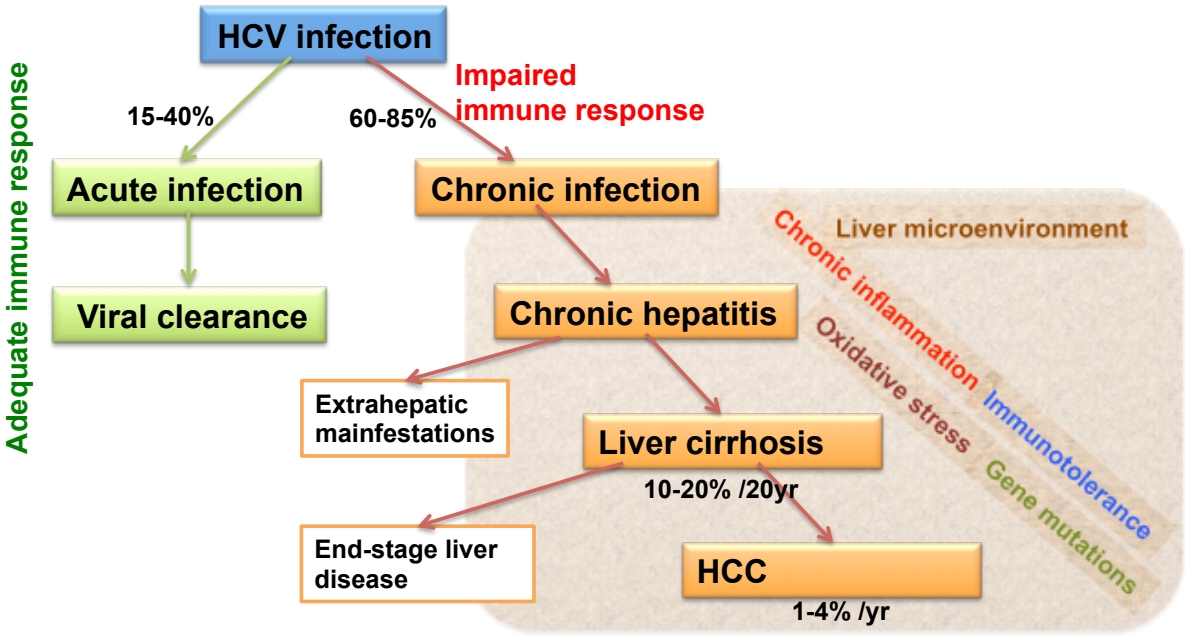


Figure 3. Natural history of HCV infection

1.2.1. IMMUNE RESPONSE TO HCV INFECTION

The mechanism by which HCV causes chronic infection in the majority of infected persons remains unclear. It is likely that HCV activates different mechanisms to evade host defences. Complexity of HCV replication provides partial explanation; it includes replication within enclosed structures (membranous web) that provide protection from the host's antiviral defenses; genetic diversity created by inaccurate replication that yields mutants resistant to the cell's antiviral strategies; and association of the virion with protective lipoproteins.(2, 9)

1.2.1.1. Innate immune response

Most literature has focused on the role of interference with endogenous interferon (IFN) system and the mechanism of disruption of the host cell ability to detect the virus and to respond to interferon. The recognition of RNA viral infection and subsequent activation of human antiviral defense mechanisms are accomplished primarily through signaling pathways leading to the production of type I interferons (IFN-α and -β).(11) These pathways are triggered by conserved viral pathogen associated molecular patterns (PAMPs) such as double-stranded RNA (dsRNA) and unmethylated CpG motifs (cytosine-guanine).(11) PAMPs are recognized by host cells through pathogen recognition receptor (PRR) proteins (such as Toll-like receptors, TLR, retinoic acid inducible gene-I (RIG-I), and melanoma differentiation associated gene 5), which play a major role in alerting cells that HCV is present and in activating antiviral pathways and genes.(11-13) In the settings of HCV infection, TLR-3 and RIG-I recognize dsRNA, and in turn function to activate transcription factors for host cell antiviral genes such as interferon regulatory factor-3 (IRF-3) and nuclear factor-κB (NF-κB).(14, 15) IRF-3 and NF-κB are localized to the nucleus where they promote expression of type I interferons. In turn, IFNs are secreted by viral-infected cells, bind to cell surface receptors of both the infected and adjacent uninfected cells, and activate transcription of hundreds of interferon-stimulated genes (ISGs), which ultimately function to limit viral replication by disrupting RNA transcription and translation.(16) Importantly, only a small percentage of these genes have been studied regarding their antiviral effects in cells. However, IFN-responses in the liver do not correlate with the outcome of infection, even though HCV replicons are highly sensitive to

type I IFNs *in vitro.* Furthermore, HCV is able to specifically inhibit these pathways at multiple points and to downregulate the expression or to inhibit functions of antiviral genes.(9, 15, 17)

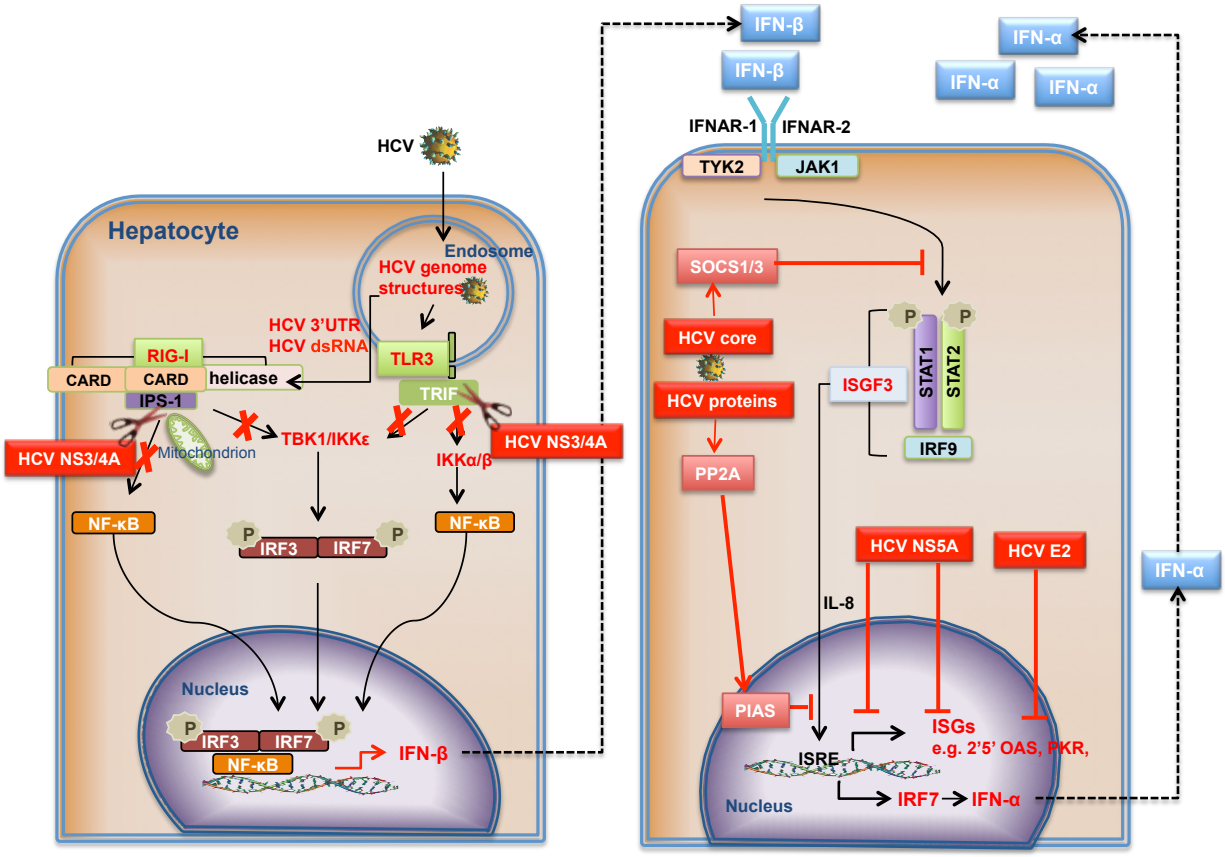


Figure 4. Activation/inhibition of interferon system in HCV infected hepatocytes

The HCV NS3-4A serine protease may block the phosphorylation and effector action of IRF3.(18) IPS-1 (interferon-beta promoter stimulator 1) is targeted and inactivated by NS3-4A.(14, 15) Also, the HCV core protein has been shown to induce the expression of SOCS3 (suppressor of cytokine signalling 3), which can suppress Jak (Janus kinase) – STAT (Signal Transducer and Activator of Transcription) signaling events and block the IFN-induced formation of ISGs.(19) Finally, HCV NS5A polymerase and envelope proteins directly inhibit the effector functions of different ISGs.(9, 20)

1.2.1.2. Adaptive immune response

Unlike acute resolving infection that is characterized by early and vigorous expansion of CD4+ and CD8+ T-lymphocytes, chronic infection is associated with delayed and weak adaptive responses, poor memory induction and deficiency of HCV specific cytotoxic T-lymphocytes.(21) However, the explanation of these phenomena remains obscure.

Mutations in antibody and T-cell epitopes have been shown for both HCV-infected humans and chimpanzees. In contrast to T-cell responses raised to other pathogens, HCV-specific T-cells are less differentiated and have impaired effector functions.(9) Finally, despite early and high viremia, HCV-specific T cells are not detectable in the liver within 1 month of experimental infection of chimpanzees, which might indicate impaired trafficking to the site of infection.(21)

Given the critical role of dendritic cells (DC) in priming T cell responses, both DC *in vitro* models and DCs from chronically infected patients have been extensively studied, with the idea that HCV-mediated inhibition of antigen-presenting functions could result in inefficient antiviral T-cell responses.(22-24) Conflicting evidence resulted from these studies; nevertheless, the ones that revealed a general subversion of both T-cells and DCs functions faced a problem that chronically infected individuals are not globally immunodeficient and have a relatively high level of endogenous IFNs.(22-24) Therefore, the hypothesis of selective impairment of local adaptive (and innate) immune responses at the site of the infection is favorable. Humoral immune responses appear late during infection or not at all, and they do not protect against re-infection.

1.2.2. INFLAMMATORY RESPONSE TO HCV INFECTION

Chronic inflammation is the major contributor of disease and is the basis of HCV pathogenesis.(20) However, the molecular mechanism(s) by which HCV confers hepatic inflammation are not defined. During chronic infection, a sustained production of chemokines drives persistent low-grade inflammation in the absence of immunocompetent Th1 response. In addition, HCV antagonizes the chemokine response to perturb infiltration of T-cell effector cells, thus allowing the constant

generation of virus particles. Meanwhile, continuous infiltration of the liver by a nonspecific inflammatory load causes collateral tissue damage and failure to eliminate the virus.(9, 20, 25)

To assess hepatic gene expression patterns and host response processes associated with liver disease in chronic HCV infection, a systems biology analysis of the host response to HCV infection that included high-throughput transcriptional profiling of human liver coupled with *in vitro* modeling of the HCV/host interface was conducted.(26, 27) This analysis identified a broad changes in inflammatory networks linked with fibrosis and severity of liver disease, as presented in Figure 5.

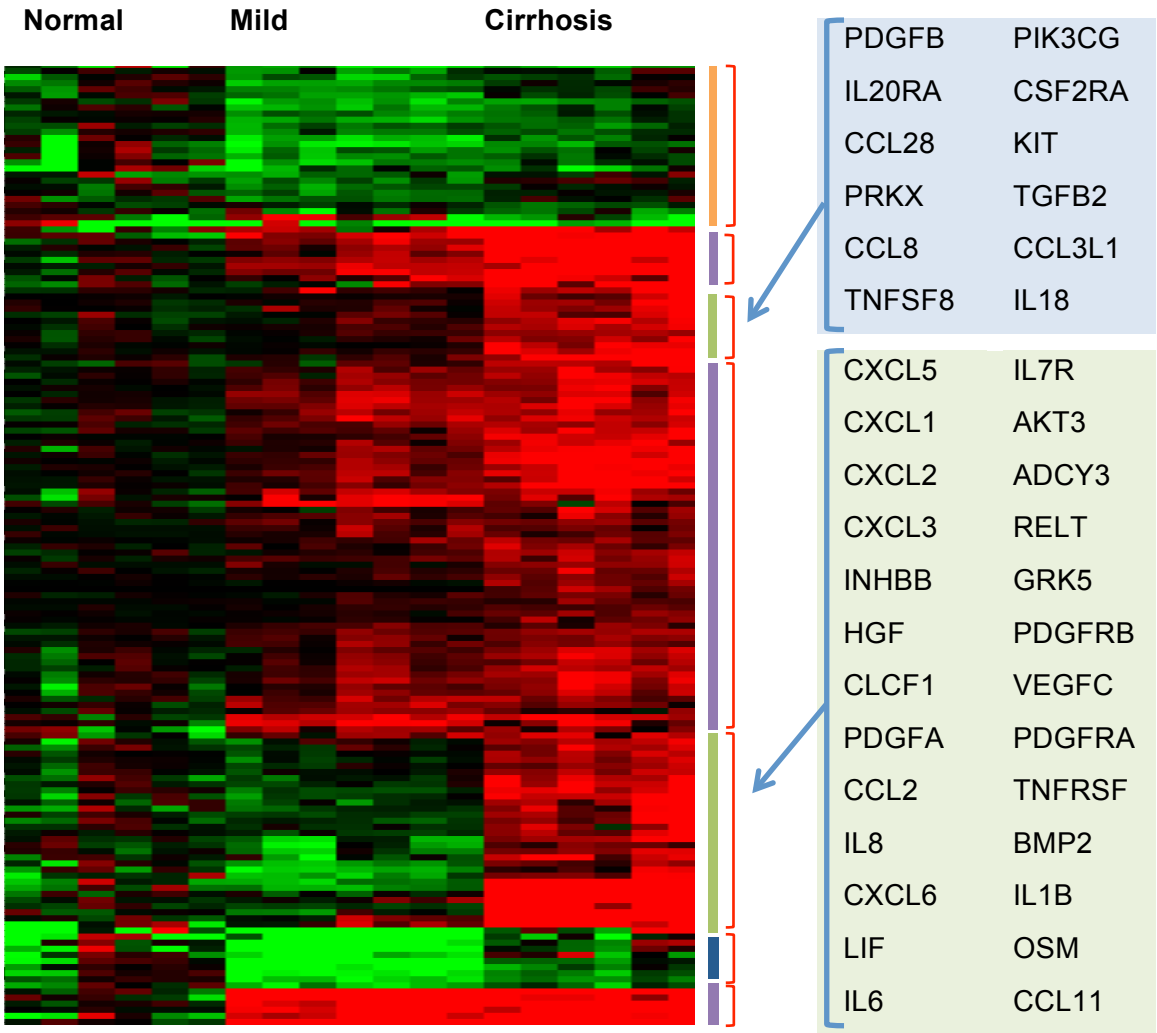


Figure 5. Hierarchical clustering of differentially expressed cytokine and chemokine genes in HCV infected livers identified major gene expression patterns that associated with liver disease.

Intrahepatic interleukin-1β (IL-1β) production was recently suggested as a central feature of liver inflammation during HCV infection and it was shown that Kupffer cells are the primary cellular source of hepatic IL-1β.(26, 27) IL-1β is considered as the key mediator of inflammatory response, and it is involved in a variety of cellular activities, including cell proliferation, growth, differentiation and apoptosis.(28) IL-1β activity is tightly controlled and requires two signals; “signal one” for production of IL-1β mRNA through NF-κB activation; “signal two”, the conversion of the inactive pro-IL-1β precursor, to the active cytokine through inflammasome signaling.(29) The mechanism of inflammasome activation in Kupffer cells includes activation of MyD88-mediated TLR7 signaling to induce IL-1β mRNA expression and a potassium efflux that activates the NLRP3 inflammasome for IL-1β processing and secretion.(26) These processes appear to drive the liver inflammation, and are closely correlated with liver fibrogenesis and oncogenesis.

Finally, chronic ”low-level inflammation” is strongly interconnected with two other hallmarks of HCV infection, the immune tolerance and modulation of host immune responses that results in the virus eradication failure.(20, 25)

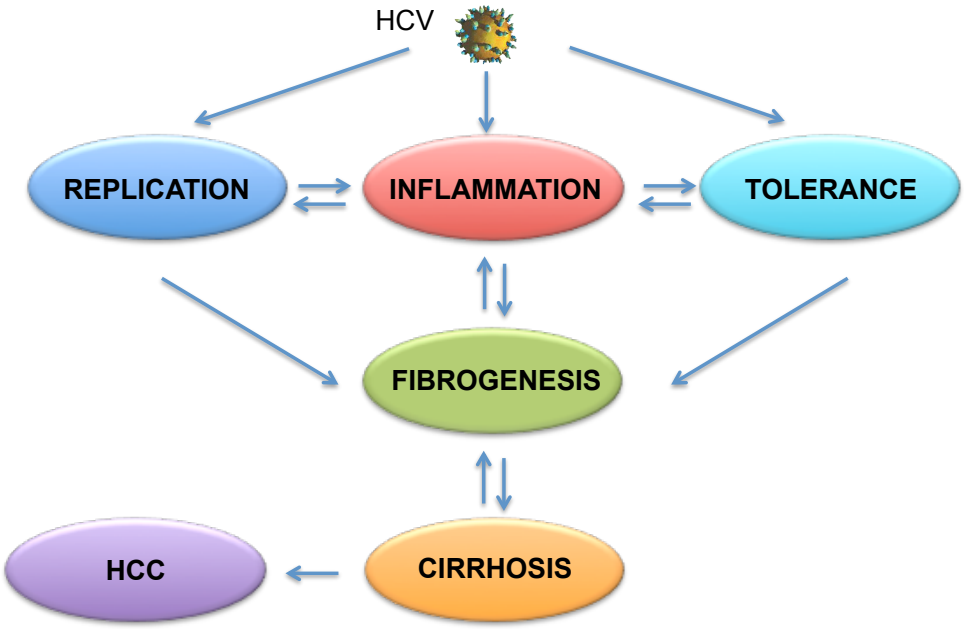


Figure 6. Proposed model of chronic hepatitis C pathogenesis

Mechanisms proposed to account for the inherent tolerogenicity of the liver have not yet been validated convincingly; they may be due to a combination of several biological properties that distinguish the liver from other parenchymal organs (*see below*). Although it was originally thought that HCV only infects hepatocytes and causes only inflammation of the liver, today there are accumulating evidences that HCV can enter and/or replicate in a variety of cells (B-cells, T-cells, monocytes, glia cells) and cause a variety of extrahepatic disorders (e.g. lymphoproliferative diseases).(30-40)

There is growing evidence that liver non-parenchymal cells, specifically liver sinusoidal endothelial cells (LSEC) and Kupffer cells (KC), may play key roles in regulating immune responses and facilitating tolerance induction.(41-43) Surprisingly, the biology of HCV, specifically regarding non-parenchymal liver cells, has been largely neglected in currently predominant molecular approach. Of note, it is likely that LSEC and KC play distinct roles in providing tolerant environment within the liver that favors HCV persistence and chronic inflammation.

1.3. THE ROLE OF THE LIVER NON-PARENCHYMAL CELLS IN HCV INFECTION

The liver is thought to be responsible for up to 500 separate functions, which reflects in its unique position with regard to the blood circulation and unique immuneregulatory properties.(44) The hepatic vascular bed is dually supplied; it receives venous blood from almost the entire gastrointestinal tract via the portal vein and from the systemic circulation via the hepatic artery.(44) These drain into the hepatic sinusoids, thus generating a mixed arterial-venous perfusion of the liver.(44) The sinusoids are lined with fenestrated endothelial cells (LSEC) and luminal Kupffer cells (KC).(44) Between LSEC and liver parenchyma lies the space of Disse, which contains hepatic stellate cells (HSC) and liver resident dendritic cells (DC).(44) In addition, the liver is enriched in natural killer (NK) cells and natural killer T (NKT) cells, which have traditionally been considered as key cellular components of the innate immune system.(44) (Table 1., Figure 1.)

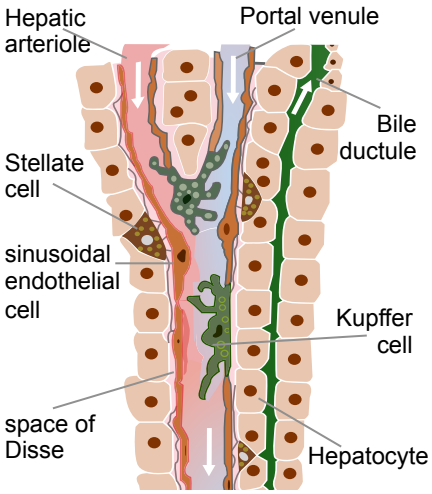


Table 1. Sinusoidal cell populations[1]

Hepatic cell population	% of volume	% of cells
Kupffer cells	2.1	15
Liver sinusoidal endothelial cells	2.8	19
Stellate cells	1.4	5-8
Liver associated lymphocytes	nd	nd
Hepatocytes	78	60
Dendritic cells	nd	nd

Figure 7. Illustration of liver sinusoids

Due to the distinctive microcirculation, liver is constantly exposed to the nutrients and endotoxins derived from the gut. While nutrients have to be extracted and metabolized, blood toxic waste products, endotoxins and bacterial degradation products have to be eliminated without provoking immune responses. This implies an existence of liver-specific, immuneregulatory mechanisms that lead to induction of tolerance.

LSEC are a morphologically distinct population of cells (due to the open fenestrations without a basement membrane) that form the lining of liver sinusoids.(44-46) Due to their position, LSEC are the first cells in contact with blood flow and serve to compartmentalize the vascular sinusoidal channels from the hepatic parenchyma.(44-46) However, LSEC are not simply barrier cells that restrict the access of bloodborne compounds to the liver parenchyma. LSEC have complex roles, including clearance of endotoxin, bacteria and other compounds, the regulation of inflammation, leukocyte recruitment and host immune responses to pathogens.(47, 48) Due to their extraordinary scavenger activity, expression of pattern recognition receptors (e.g. TLR3 and TLR7) and release of proinflammatory mediators (e.g. IL-1β, TNFα), LSEC do not depend on other immune cells in the initiation of inflammatory reactions.(49-51)

[1] According to ref. (44)

Liver non-parenchymal cells (KC and LSEC) are known to play a pivotal role in blood-borne virus clearance, yet recently, using modern techniques to repeat the vital stain clearance studies of early 20th century, the concept of sinusoidal scavenging has been amended.(52) These studies showed that LSEC take up and destroy the majority of blood-borne viruses, processing so quickly (minutes) and extensively (>90%) in ways specific to each virus.(53) However, the mechanism of impairment of LSEC clearance capacity during HCV infection remained unanswered.

Using a duck model of HBV infection, it was shown that LSEC rather than hepatocytes took up HBV and that infected hepatocytes were often observed in nearness of LSEC, suggesting the model of primary uptake into LSEC as a general mechanism by which blood borne agents are targeted to the liver.(52) There are growing evidences that this model applies for HCV infection also. It was found that HCV E1 and E2 bind L-SIGN (C-type lectin similar to DC-SIGN on dendritic cells), which further direct HCV particles in early lysosomal compartments.(54-57) These data lead us to the conclusion that rather than having the infection of LSECs, the bounded and internalized HCV escape host degradation mechanisms resulting in retained infectivity for an extended period of time, thus representing an efficient infectious reservoir for the underlying hepatocytes.

LSEC have been recognized as efficient antigen-presenting cells (APC).(51) In contrast to other APC, they induce tolerance rather than activation of immune response.(51, 58, 59) This is of particular relevance in the setting of HCV infection, where the lack of an effective immune response is implicated in the development of persistent infection and the immunological characteristics of chronic hepatitis. It is presumed that liver APCs can exist in a state of active tolerance and contribute to the tolerogenic liver environment by the continuous secretion of immunosuppressive cytokines, e.g. IL-10 and TGF-β.(60) The immune ignorance model assumes that after internalization, viral particles might be processed and presented to naive T cells. Interaction of naive T cells with antigen-presenting LSECs results in differentiation of T cells into regulatory T cells and impaired cytotoxic T lymphocytes that are unable to produce IL-2 or interferon-γ or to exhibit

cytotoxicity to infected cells and that undergo apoptosis.(60) This raises the question of whether the tolerogenic properties of the liver APCs contribute to the persistence of HCV.

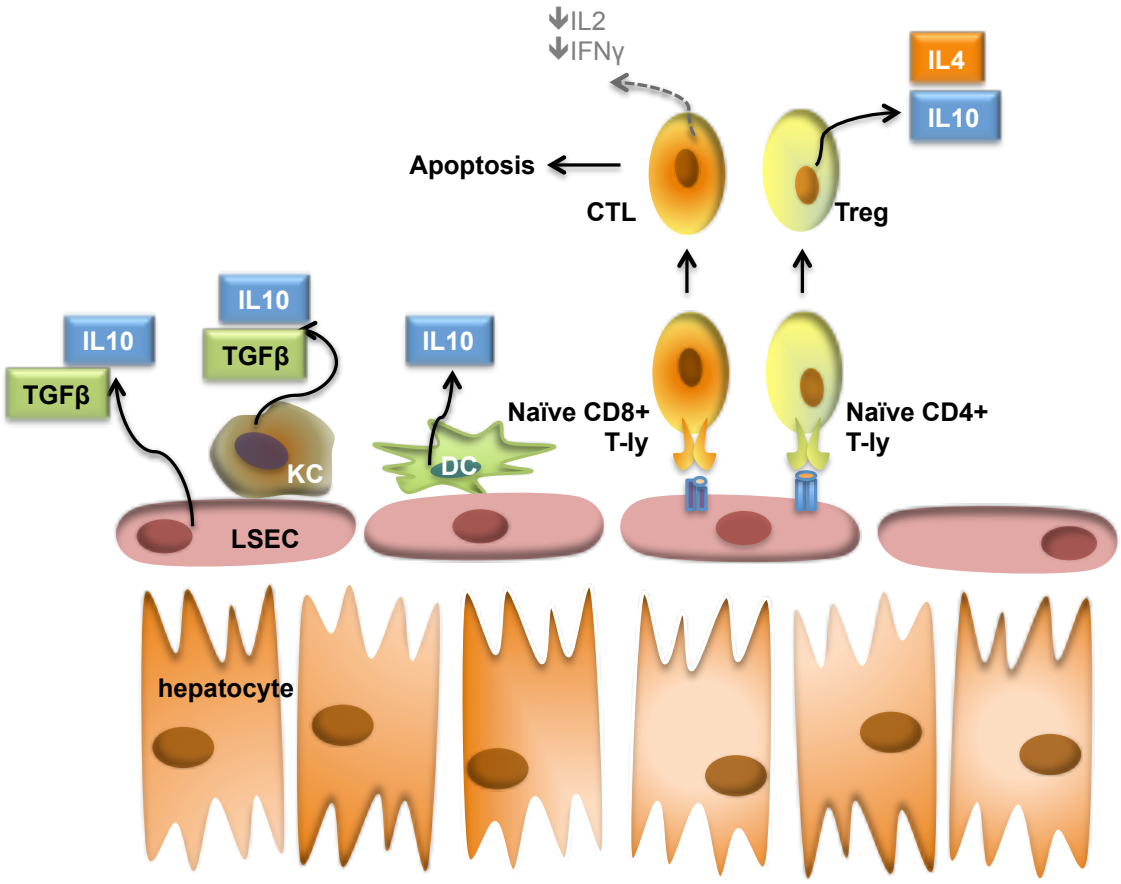


Figure 8. Hypothetical tolerogenic properties of liver non-parenchymal cells

1.4. SYSTEM BIOLOGY APPROACHES TO HCV INFECTION

Previously published gene expression analyses (mostly performed using microarray technology) have revealed new disease specific changes in gene expression, identified potential biomarkers of HCV infection and suggested a new mechanism of host cell-virus interaction that results in viral particle assembly, secretion and infectivity.(10, 61-64) However, the strategies by which HCV evades the surveillance of the host immune system and hijacks host cellular machinery for its own replication are not completely understood.

According to widely accepted model, binding/internalization of HCV particle results in virus recognition, activation of host signaling pathways that finally lead to activation/inhibition of transcriptional regulators resulting in the modulation of gene expression.

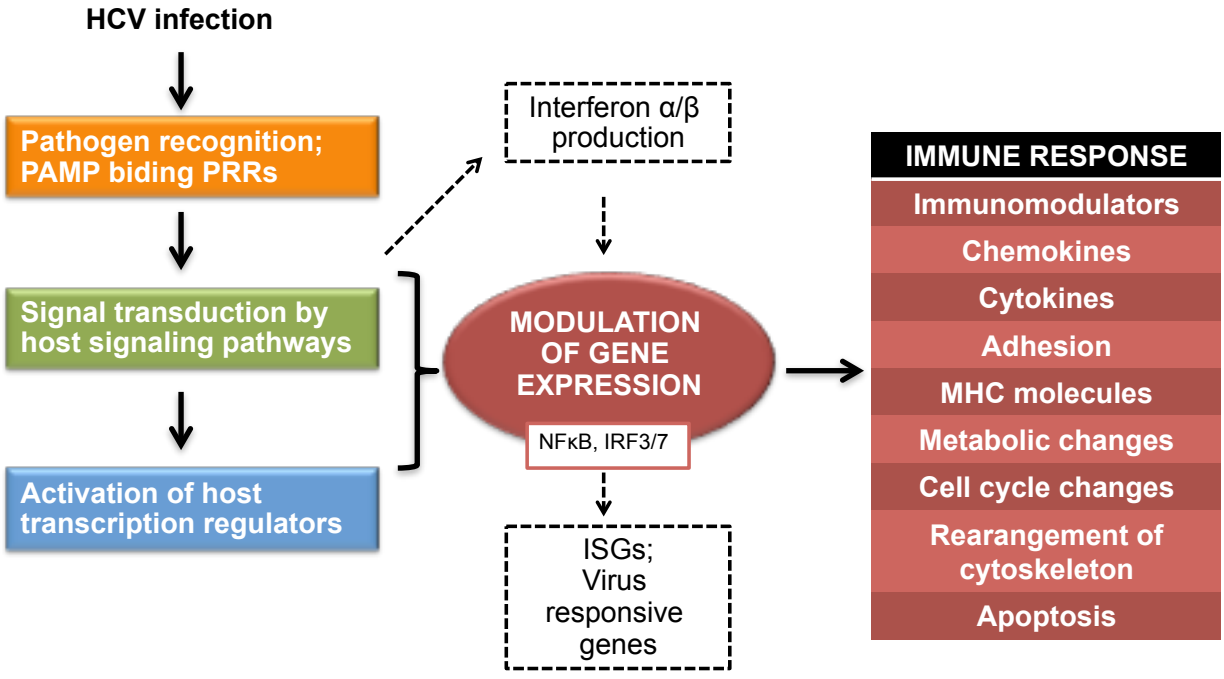


Figure 9. Model of HCV-innate immune response (detected by gene expression studies)

As previously described, the innate immune response relies on recognition of evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs), through pattern recognition receptors (PRRs).(11)

Upon PAMP recognition, PRRs signal to the host the presence of infection and trigger proinflammatory and antimicrobial responses by activating a multitude of intracellular signaling pathways, including adaptor molecules, kinases, and transcription factors.(11) PRR-induced signal transduction pathways ultimately result in the activation of gene expression and synthesis of a broad range of molecules, including cytokines, chemokines, cell adhesion molecules, which together orchestrate the early host response to infection and at the same time represent an important link to the adaptive immune response.(11-13) The outcome of virus-mediated PRR activation can range from an antiviral response that efficiently clears the infection to the establishment of a cellular environment that favors viral replication and spread.(9, 11-13, 15, 16, 23)

Next generation RNA sequencing technologies (RNAseq) have opened the door to more accurate gene expression studies in HCV infected clinical biospecimens and cultured cells.(7, 65) This technology has clear advantages over previous methods (e.g. microarray) and has revolutionized the manner in which transcriptomes are analyzed; it is not limited to detecting transcripts that correspond to existing genomic sequences; it can reveal the precise location of transcription boundaries; it has very low background signal; it does not have upper limit of quantification and has been shown to be highly accurate for quantifying expression levels, so the results show a high level of reproducibility.(7, 65)

Using a similar approach, we have described the first report of RNA sequencing analysis of 5' capped RNAs isolated from acutely HCV infected Huh 7.5 cells that have identified many new annotated and unannotated differentially expressed genes during acute HCV infection that were not identified in prior gene array analysis.(10) Among them, follow up siRNA studies of two newly identified, highly upregulated genes, fucosyltransferase 1 (*FUT1*) and kelch domain containing 7B (*KLHDC7B*), provided evidence that their expression during acute HCV infection is essential for the production of HCV RNA and infectious viral particles.(10) Furthermore, at least 38 cell pathways enriched post infection were identified; examples include MAPK signaling, adipocytokine signaling, TGFβ signaling, apoptosis, insulin signaling pathway, RIG-I like receptor signaling,

extracellular matrix (EMC)-receptor pathway, NOD-like receptor signaling and Notch signaling.(10)

A pilot study was performed to determine the drivers of inflammation in HCV infected liver; RNA-seq analysis of models of hepatocytes (Huh7 cells), Kupffer cells (THP1) and mild (fibrosis) and severe (cirrhosis) HCV+ liver biospeciments.(26, 27) In chronic hepatitis C liver >300 genes related to inflammation, chemotaxis and leukocyte activation were markedly increased compared to controls. Unlike RNA-seq analysis of macrophages that demonstrated a broad increase in IL-1β and NF-κB-responsive proinflammatory cytokines and chemokines, HCV did not induce a marked increase in such inflammatory signals in Huh7.5 cells.(10, 26, 27) Analysis of genes within these pathways showed increased IL-1β levels with increasing severity of liver disease and marked overlap in upregulated expression in macrophages exposed to HCV and liver specimens.(26, 27)

Almost all of the HCV related gene expression studies were performed in cell cultures of hepatoma cell lines or clinical liver biospecimens. Heterogeneous cellular composition of liver tissue (constituted by hepatocytes, Kupffer cells, stellate cells, extracellular matrix and infiltrated cells), and the obvious resulting complexity of liver transcriptome, doesn't provide insight into pathophysiological role of LSEC during acute or chronic HCV infection. To date, no gene expression study on hepatic sinusoidal endothelial cells has been published and a way of identifying endothelial specific pathways in HCV infection is needed to improve our understanding of HCV pathophysiology.

2.

HYPOTHESIS

HCV infection reprograms cellular gene expression of human liver sinusoidal endothelial cells that is implicated in the modulation of the inflammatory responses.

3.

AIMS AND PURPOSE OF THE RESEARCH

General Aim:

- To determine the whole Pol II transcriptome associated with immune response in LSECs triggered by HCV, and apply the data set toward understanding the molecular processes of the inflammatory response against HCV.

Specific Aims:

- To quantify the changing expression levels of transcripts during HCV infection and LPS treatment
- To define the transcriptome associated with innate and adaptive immune response activation after HCV infection and LPS treatment.
- To define endothelial specific pathways enriched after HCV infection and LPS treatment.
- To define host-viral interaction network in LSECs in order of better understanding the development of immunotolerant environment in the liver
- To identify changes in transcriptome that might modify fibrotic processes
- To identify new oncogene mechanism of HCV infection
- To compare changes in cellular transcriptomes of HCV infected LSECs with previous gene expression studies on Huh 7.5 and THP-1 cells.

4.

MATERIALS AND METHODOLOGY

4.1. MATHERIALS

4.1.1. LIVER SINUSOIDAL ENDOTHELIAL CELLS

Human liver sinusoidal cells isolated from three donors were obtained from ScienCell Company (California, USA). These cells were isolated from human liver, cryopreserved immediately after purification and delivered frozen. LSEC were characterized by immunofluorescent method with antibodies to vWF/Factor VIII and CD31 (P-CAM), and were negative for HIV-1, HBV, HCV, mycoplasma, bacteria, yeast and fungi.

Primary cultures were established in fibronectincoated flask/plates (2µg/cm^2) and cultured in endothelial cell selective media (ECM) (ScienCell, California, USA) at 37˚C in 5% CO_2. ECM consists of basal medium, 5% fetal bovine serum, endothelial cell growth supplement and penicillin/streptomycin solution. The medium was changed every three days until the culture was approximately 70% confluent. After that, medium was changed every day. Cells were subcultured when they were >90% confluent. For subculture, cells were harvested using the tryspin/trypsin neutralization solution, and reseeded in new fibronectin coated plates (5,000 cells/cm^2). All the experiments were performed between 2nd – 4th passages. All the experiments were done in triplicates and in three time points (8, 24 and 48 hours post HCV or LPS exposure; *see bellow*).

4.1.2. THP-1 MONOCYTE CELL LINE

THP-1 cells were purchased from ATCC and grown in complete RPMI-1640 medium containing 10% fetal bovine serum, antibiotics, L-glutamine, pyruvate, and non-essential amino acid. THP-1 cells were differentiated by treatment with 20–40nM of PMA overnight at 37°C.

4.1.3. HEPATOMA (Huh7.5) CELL LINE

The human hepatoma cell line, Huh 7.5, was maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, nonessential amino acids, and 10% fetal bovine serum (FBS) (Invitrogen) at 37˚C in 5% CO_2.

4.1.4. PRODUCTION OF INFECTIOUS HCV JFH-1 VIRUS

For infection studies, original HCV-JFH1 clone was used. Briefly, HCV-JFH-1 was transfected into Huh 7.5 cells, and the culture was maintained and sub-cultured every three days. Cell supernatants, containing infectious viral particles, were collected, concentrated and titrated to determine focus forming units (ffu)/ml using immunofluorescence assays with anti-NS5A Huh7 cell-based FFU assay.

4.1.5. HCV INFECTION STUDIES

LSEC were seeded at 50,000/well in 6-well plates (9.6cm^2 per well). After 48 hours of culture, the cells were infected with HCV at MOI of 2.5. The inoculums were incubated with cells for two hours at 37°C and then extensively washed with PBS and supplemented with fresh complete ECM. The cells were then continuously cultured to 8, 24, and 48 hours. Controls were MOCK infected (conditioned media) cells grown under identical conditions for the same time. These time points were selected based on previous microarray studies showing maximal changes in differential gene expression after 48 of acute JFH1 HCV infection. The 8-hour time point was chosen as an interval when few gene expression differences would be observed between HCV and mock-infected cells.

4.1.6. LIPOPOLYSACCHARIDE (LPS) AND CONTROL TESTING

Ultra-pure LPS from *E.coli* (a potent activator of inflammatory responses) was purchased (Invivogen, USA) and used as control. MOCK or negative control is conditioned media without the virus or LPS.

4.2. METHODS

4.2.1. RNA ISOLATION AND cDNA LIBRARY CONSTRUCTION

Total RNA from LSEC, THP1 and Huh 7.5 cells after acute HCV exposure was purified using Trizol (Invitrogen, USA), as described previously.(10, 64) RNA quality was assessed using a BioAnalyzer RNA 6000 Nano Chip (Aglient, USA) and only RNA specimens yielding a RNA integrity number (RIN) of ≥9.0 were used. The quantity of all RNA samples was measured by NanoDrop analysis. Illumina TruSeq RNA Sample Preparation Kit v2 with Oligo(dT) RNA selection was used for poly(A) RNAs selection and cDNA libraries construction.[2](10, 64) Poly(A) RNA were purified from 1µg of total RNA, and 100ng of cDNA library prep were used for RNA-seq.

4.2.2. NEXT-GENERATION RNA SEQUENCING

Single RNA-Seq reads were obtained using Illumina HiSeq 2000, 50-cycle protocols.[3] Clusters were prepared using the Illumina cluster station according to the manufacturer's instructions.

A total of 16–26 million 50 bp reads were obtained from LSEC cell samples (see section 4.3.).

4.2.3. REAL-TIME PCR VERIFICATION ASSAYS

Three additional primary cell cultures (isolated from different donors) were used for further analysis of differentially expressed genes at each time point. Total RNA from infected and control cells were extracted using TRIzol. First-strand cDNA was synthesized using Moloney Murine Leukemia Virus reverse transcriptase (SuperScript III; Invitrogen) with 20ng/ml of RNA at 55°C (60 min) with random hexamer primers. Each qPCR reaction was carried out in a 384-well optical plate (Roche Applied Science) in a 10µL reaction buffer containing LightCycler 480 Probes Master Mix (100mM Tris-HCl, 100mM KCl, 400mM of each dNTP (with

---

Available at:
[2] http://supportres.illumina.com/documents/documentation/chemistry_documentation/samplepreps_truseq/truseqrna/truseq-rna-sample-prep-v2-guide-15026495-f.pdf
[3] http://res.illumina.com/documents/products/datasheets/datasheet_hiseq2000.pdf

dUTP instead of dTTP), 64mM $MgCl_2$, FastStart Taq DNA Polymerase, 0.3 mM of each primer, 0.1mM hydrolysis probe and approximately 50ng of cDNA (done in triplicate)). Triplicate incubations without template were used as negative controls.

Thermal cycling was done in a Roche LightCycler 480 System (Roche Applied Science). The qPCR thermo cycling was 95°C for 5 min, 45 cycles at 95°C for 10 sec, 59°C for 30 sec and 72°C for 1 sec. The relative quantity of each RNA transcript was calculated with the comparative Ct (cycling threshold) method using the formula 2ΔCt. ΔCt represents the difference between target gene expression in mock-infected samples and target gene expression in HCV-infected samples. Reference genes (GAPDH and β-actin, ACTB) were used as controls and statistical significance was evaluated using the Mann-Whitney test.

4.2.4. IMMUNOFLUORESCENCE ASSAYS

For immunofluorescence assay, cells were extensively washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Fixed cells were blocked with 1% bovine serum albumin and 1% normal goat serum in PBS. HCV NS5A protein was detected in cells by incubation with an NS5A-specific monoclonal antibody and visualized with the secondary goat anti-mouse IgG conjugated with Alexa Fluor 594 fluorescein (Invitrogen, 1:1000 dilution). Cover slips were mounted onto slides with DAPI (Vector labs), and the HCV NS5A were visualized by fluorescence microscopy (Nikon E400). The viral titer is expressed as focus-forming units per milliliter of supernatant (ffu/ml), as determined by the average number of NS5A-positive foci detected by immunofluorescence for NS5A.

4.2.5. APOPTOSIS ASSAY

Annexin V-PE Apoptosis Kit (BD Biosciences Pharmingen, USA) allows fluorescent detection of Annexin V bound to apoptotic cells and quantitative determination by flow cytometry. The Annexin V labels phosphatidylserine sites on the membrane surface. The kit includes 7-Amino-actinomycin (7–AAD), a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. This combination allows the differentiation among early apoptotic cells (annexin V positive, 7-AAD negative), cells that are either in the end stage of

apoptosis, undergoing necrosis or are already dead (annexin V positive, 7-AAD positive), and viable cells (annexin V negative, 7-AAD negative).

For apoptosis experiments, LSEC were harvested and washed in cold phosphate-buffered saline (PBS). Cells were resuspended in 1X annexin-binding buffer to ≈1×10^6 cells/mL. 5µL Annexin V and 5µL 7-AAD was added to each 100 µL of cell suspension. After the 15min incubation period at room temperature, 400 µL 1X annexin-binding buffer was added and the stained cells were analyzed by flow cytometry (BD FACSCanto™, BD Biosciences, USA).

4.3. BIOINFORMATICS

RNA sequencing reads were aligned to the February 2009 human reference sequence genome (GRCh37/hg19, using the NovoAlign read aligner.(66, 67) Visualization tracks were prepared for each of the samples using the USeq ReadCoverage application; these tracks can be viewed using the Integrated Genome Browser (IGB).(67)

The DEseq (R/Bioconductor) application was used to identify differentially expressed genes. Bioinformatics analysis of the RNA-seq data includes adjustments for the depth of sequencing.(68, 69) DEseq application was used to count reads intersecting exons of each annotated gene and score them for differential expression in each sample. Scores were controlled for multiple testing and ranked by false discovery rate (FDR) and normalized ratio.(68, 69) Genes designated as significantly differentially expressed have an untransformed FDR of <0.05 (<5 false positives per 100 observations) and normalized change of ≥1.25 fold relative to controls.

DAVID[4] (The Database for Annotation, Visualization and Integrated Discovery), BioMart Software [5], and GeneOntology [6] were used to identify enriched

[4] http://david.abcc.ncifcrf.gov/
[5] http://www.ensembl.org/biomart/martview/e6d8b6deca5eb84e3ea3de14f3ee17c9

GeneOntology terms, KEGG pathways and relationship of changed genes with cellular processes. Network of enriched GO terms was created using the Cytoscype application[7]. Kegg pathways maps were created using the PathVisio software[8].

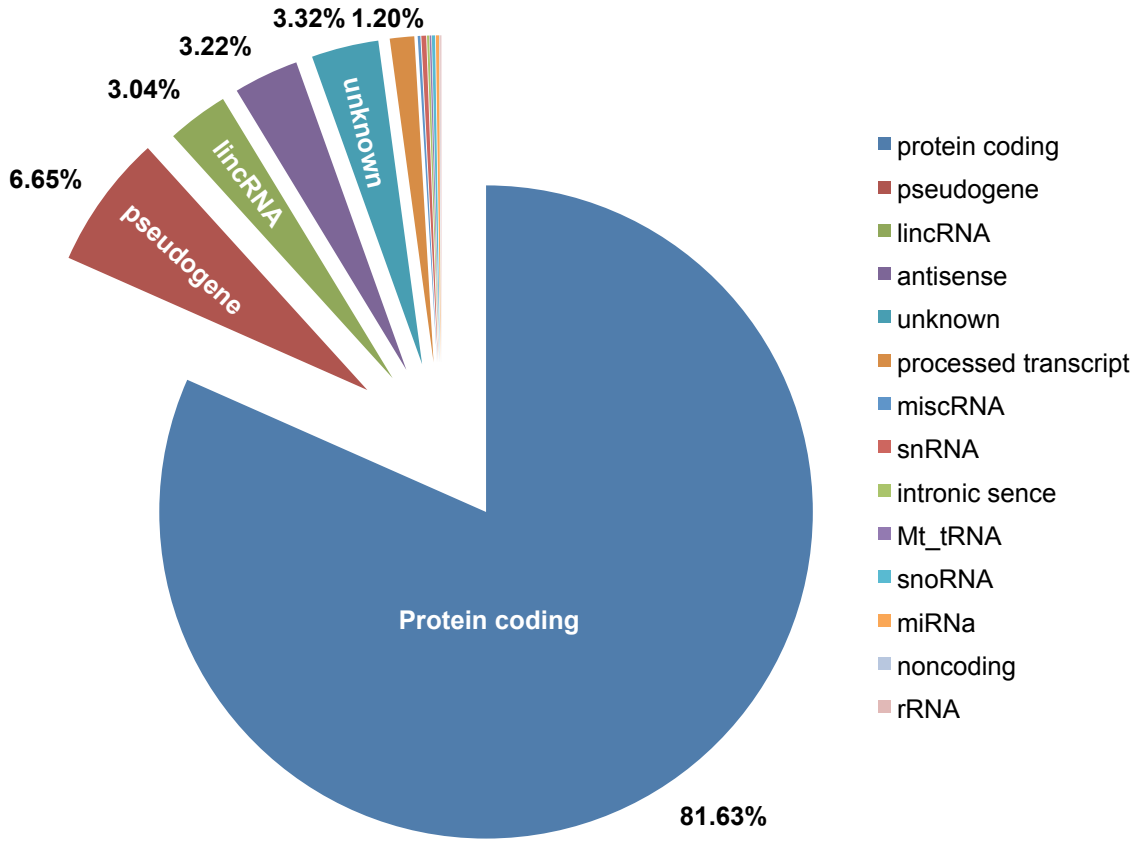


Figure 10. DESeq results. Classification of transcripts according to gene biotype.

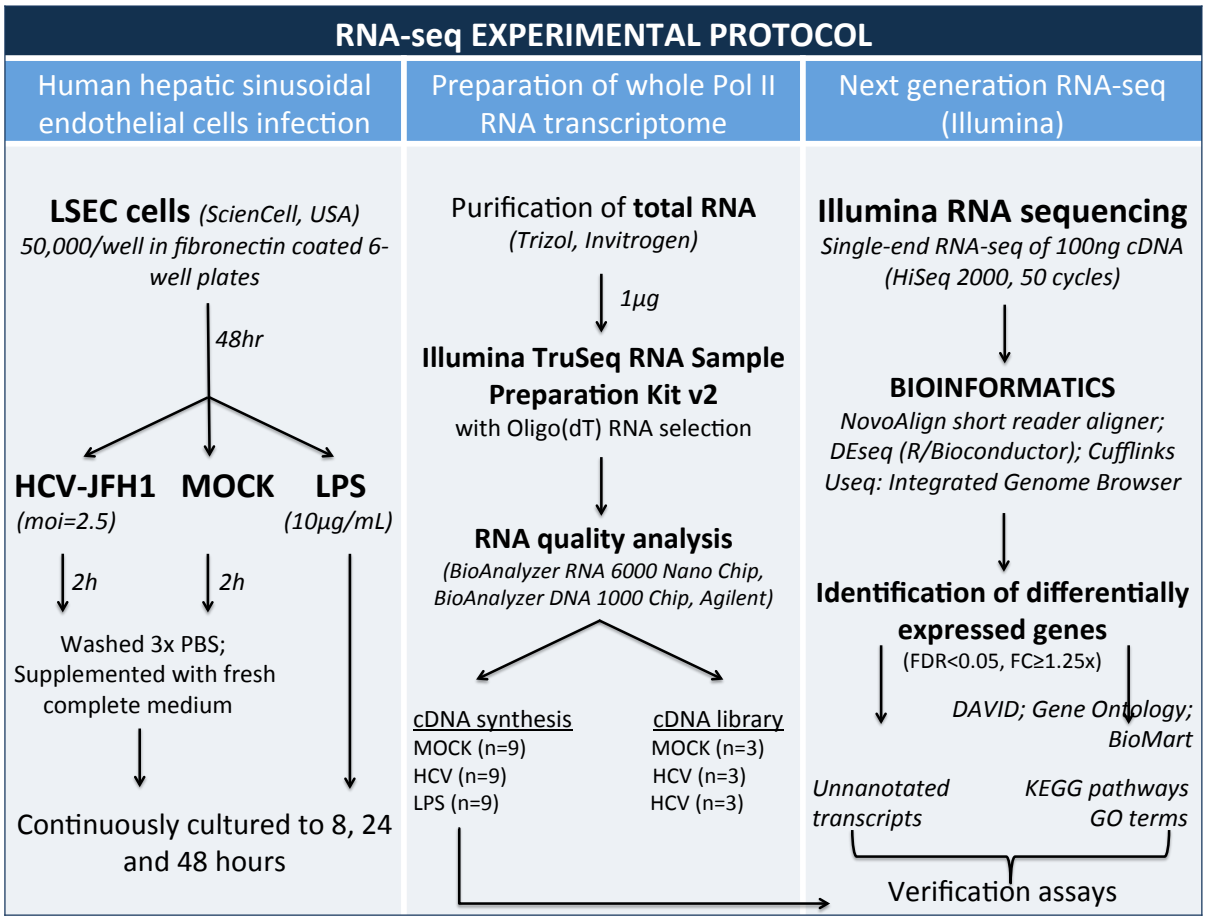
[6] http://www.geneontology.org/
[7] www.cytoscape.org/
[8] http://www.pathvisio.org/

4.4. RESEARCH PLAN

This study had 4 phases:

1. Establishment and infection of LSEC primary cultures
2. Isolation of total RNA, polyA selection, preparation of cDNA libraries for RNA-seq
3. RNA-seq
4. Bioinformatics

Research plan is shown in Diagram below:



5.

RESULTS

5.1. HCV JFH-1 INFECTION OF HUMAN HEPATIC SINUSOIDAL ENDOTHELIAL CELLS

Primary cultures of human LSECs isolated from three donors were infected with HCV JFH-1 for 2 hours with a relatively high MOI of 2.5 virions/cell. As previously reported (57), we did not find a proof of HCV replication in primary human LSEC cultures, as measured by anti-NS5A immunostaining 48, 72 and 96 hours after HCV infection. Surprisingly for the cell system that doesn't support HCV replication, there was no decrease of cellular HCV RNA titer measured by qPCR at 24 or 48 hours as compared with 8 hours post infection (98%), suggesting that HCV RNA is stable in LSECs, at least for the highly conserved target sequence in the 5'UTR region of HCV genome that was used for qPCR amplification. These data lead us to the conclusion that rather than having the infection of LSEC, the bounded and internalized HCV escape host degradation mechanisms resulting in retained infectivity for an extended period of time, possibly representing an efficient infectious reservoir for the underlying hepatocytes.

Since it is possible that the long single strand HCV RNA molecule may fragment during the infection after such that the virus is no longer viable for infection, we designed an experiment to test if HCV retains its infectivity in LSEC. LSEC cell cultures were infected with HCV and cultured for 72 hours, extensively washed with PBS to remove the un-internalized HCV particles, trypsonised and co-cultured with Huh 7.5 cells. After 48 hours of cultivation, co-cultures were immunostained against HCV NS5A. While control Huh 7.5 cells infected with supernatants from HCV infected LSECs (from the first part of the experiment) did not show any immunofluorescence, LSEC-Huh 7.5 co-cultures were positive for HCV NS5A. Additional control, Huh 7.5 cells exposed to HCV particles incubated

in cell free medium at 37^{O}C for 72 hours showed a few NS5A positive colonies. This experiment cannot exclude infection from the bound, but not internalized virus (although cultures were extensively washed and trypsonised). Since LSECs supernatant didn't cause HCV replication in Huh7.5 cells, at least for the 6 cultures isolated from 2 donors that were tested, and in addition to qPCR data that showed HCV RNA stability, our results can suggest that HCV partly retains its infectivity in LSEC.

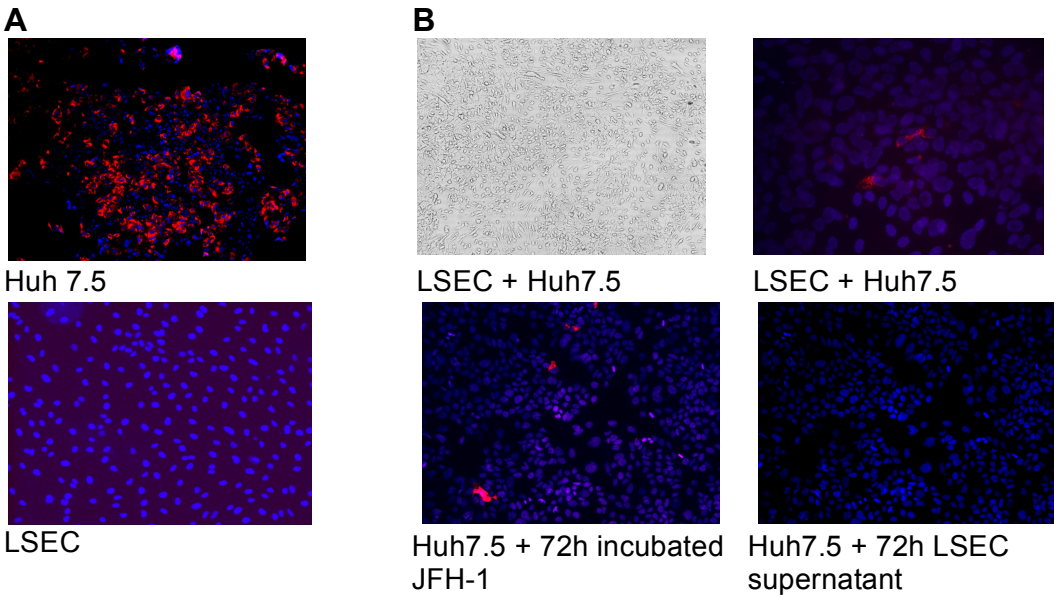


Figure 11. Panel A. HCV NS5A immunostaining of Huh7.5 and LSEC. **Panel B.** Infectivity of HCV-JFH1 in LSEC (*details in text*).

5.2. GLOBAL TRANSCRIPTIONAL RESPONSE TO HCV INFECTION AND LPS STIMULATION OF LSEC

The poly(A) selected RNAs from HCV infected, LPS stimulated and control cells were analyzed by next-generation RNA sequencing (RNA-seq) and data processed as described in *Methods*.

5.2.1. LPS INDUCED TRANSCRIPTIONAL RESPONSE

Lipopolysaccharide (LPS), a mayor structural component of the outer wall of all Gram-negative bacteria, has been widely used to describe the activation of host cell immune and inflammatory responses. Although the effects of LPS on hepatic metabolism and host immune response are extensively studied, the direct responses of human LSECs to LPS and the corresponding extent of gene expression changes have not been characterized using whole genome sequencing approach. RNA-seq showed that LPS stimulation promptly and dramatically changes gene expression. A total of 3949, 1912 and 1433 genes were differentially expressed at 8, 24 and 48 hours, respectively (fold change ≥1.25 and false discovery rate (FDR) >13), as presented in Figure 12.

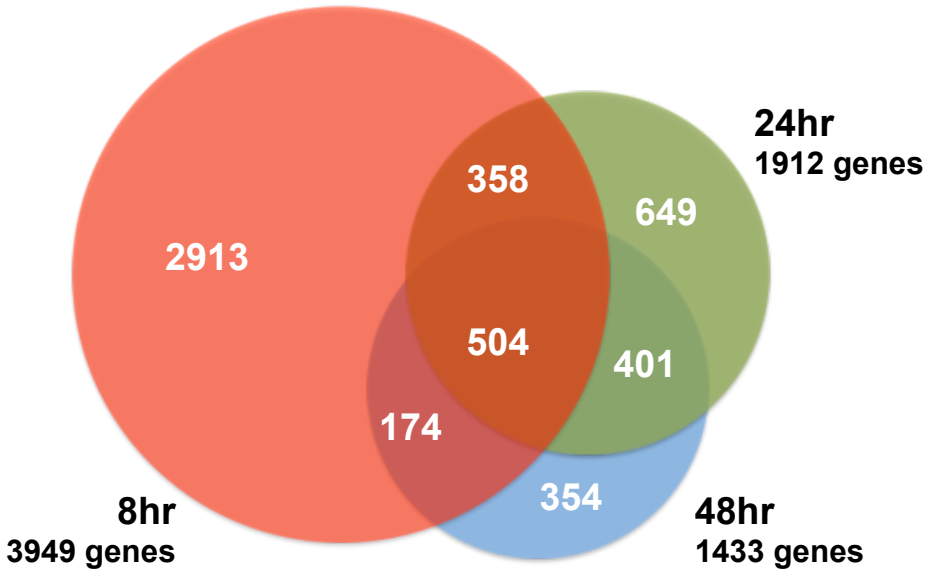


Figure 12. A Venn diagram depicting the number of genes that are differentially expressed at 8, 24 and 16 hours after LPS exposure is shown.

These data are unusual in comparison to other cell lines, in which number of differentially expressed genes usually gradually raises and rarely achieve similar number at 72 hours in comparison to only 8 hours of LPS stimulation of LSECs.(10, 62) Furthermore, the number of differentially expressed genes significantly dropped at 24 hours, and the same trend continued at 48 hours. This might suggest that in addition to exceptionally fast and potent activation of inflammatory responses, LSECs actively attenuate inflammatory response at the gene expression level.

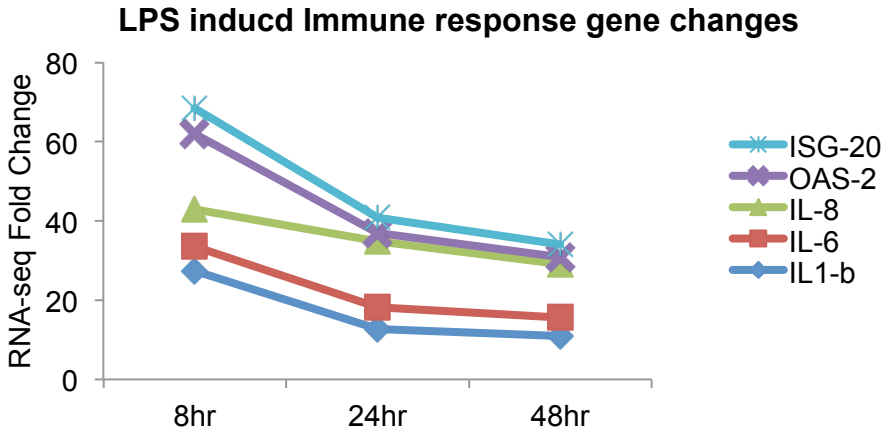


Figure 13. Time course of gene expression in LPS stimulated LSEC. Fold changes of selected inflammatory mediators are shown.

Indeed, the fold changes of proinflammatory genes decreased significantly from 8 to 48 hours (*IL-1β* 25.9- vs. 10.5-fold increase; *IL6* 6.1- vs. 4.6-; *TNFα* 12.8- vs. NS; *OAS2* 19.2- vs. 1.8-; *MX1* 43.1- vs. 2.1-; 8hr vs. 48hr fold increase). These changes were accompanied with upregulation of several negative regulators of transcription that were not expressed at 8 hours (encoded by *SALL2* 3.2-, *SATB1* 3.0-, *DACH1* 2.5-, GLIS3 2.3-fold increase at 48 hours) and decreased expression of positive regulators of transcription in comparison to 8 hours post stimulation (*FOSL1* 1.6 vs. -2.1; *IRF7* 6.5 vs. 1.5; *NOLC1* 1.3 vs. -1.3; *RUNX1* 1.2 vs. -1.4; *MYC* 1.0 vs. -1.3).

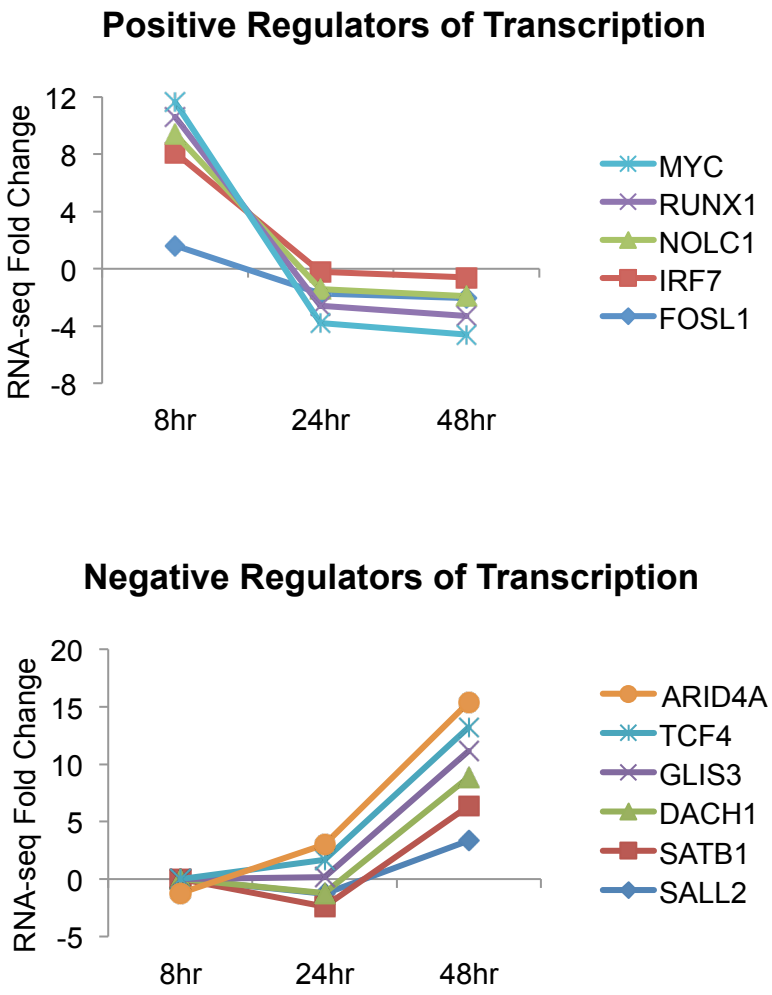


Figure 14. Time course of gene expression in LPS stimulated LSEC. Fold changes of positive (upper panel) and negative (bottom panel) of transcription in LPS stimulated cells are shown.

5.2.2. HCV INDUCED TRANSCRIPTIONAL RESPONSE

HCV infected LSECs showed entirely different pattern of gene expression. The total number of DEGs decreased significantly from 8 hours (754) to 24 hours (245) and then significantly rose again at 48 hours (2543) (Figure 14). The proportion of genes in common was much lower at 48 hours than at 8 or 24 hours, and the most of the gene changes were restricted to 8, 24, or 48 hours rather than persisting across time points. Additionally, in contrast to LPS stimulated LSECs, the majority of DEGs were observed at 48 hours after HCV infection. Presented kinetics of gene expression might suggest early *vs.* late response to HCV infection. This might be a reflection of HCV potential for chronicity, where by causing minimal impact upon entry into cells, HCV delays cellular changes in order to prevent the activation of immune response. Similar results were obtained from *in vitro* studies on hepatocyte models as in the *in vivo* studies of HCV infection in chimpanzees, where virus efficiently replicates in the absence of gene expression changes and activation of the immune response. Indeed, majority of changes in mRNAs related to immune response were restricted to 48 hours post HCV infection in our analysis.

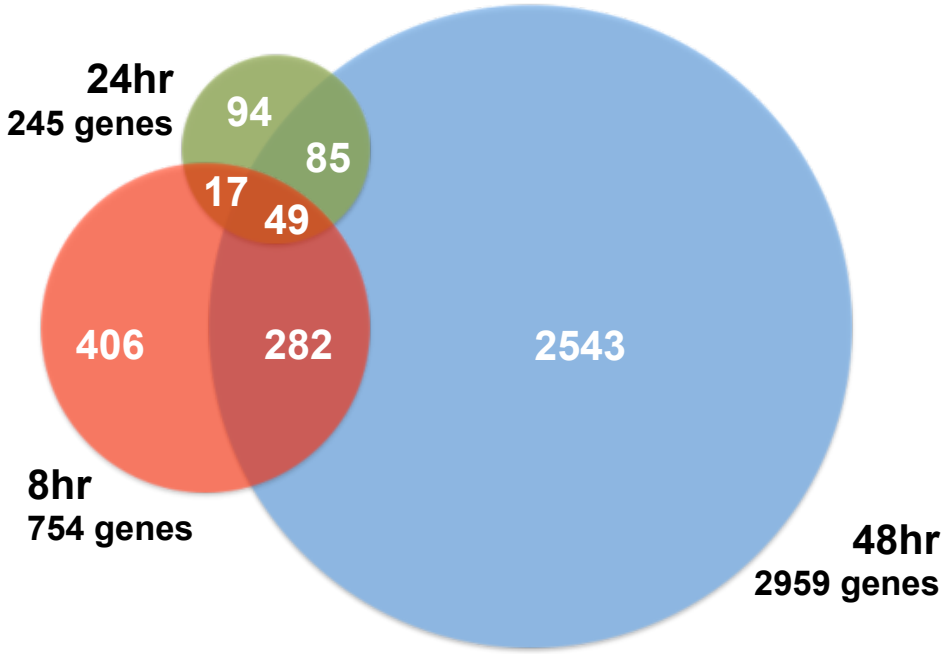


Figure 14. A Venn diagram depicting the number of genes that are differentially expressed at 8, 24 and 16 hours after HCV exposure is shown.

Approximately 57%, 51% and 48% of DEGs were downregulated at 8, 24 and 48 hours after HCV infection, respectively. Downregulation of genes is relatively uncommon event in gene expression analysis. This might suggest a different pattern of gene reprograming as a special characteristic of LSECs, where cells shut down their normal functions that can benefit or limit virus infectivity. Since, HCV does not replicate in LSECs, these changes should not correlate with HCV replication cycle, viral titer or presence of dsRNA, as in Huh 7.5 cells where the host transcriptional response corresponds closely to the levels of HCV replication, but rather suggest a different means of transcriptional activation. Furthermore, it was observed that viral attachment and entry into hepatocytes doesn't significantly impact host gene expression,(62) as was not the case in LSECs. Interestingly, in comparison to acutely infected Huh 7.5 cells, approximately 76% of changed transcripts were unique for HCV infected LSECs.

Interestingly, 920 genes overlapped between LPS and HCV exposed cells, representing 31%. However, while in LPS exposed cells the proinflammatory genes were highly expressed, the majority of these genes were downregulated in HCV infected LSEC.

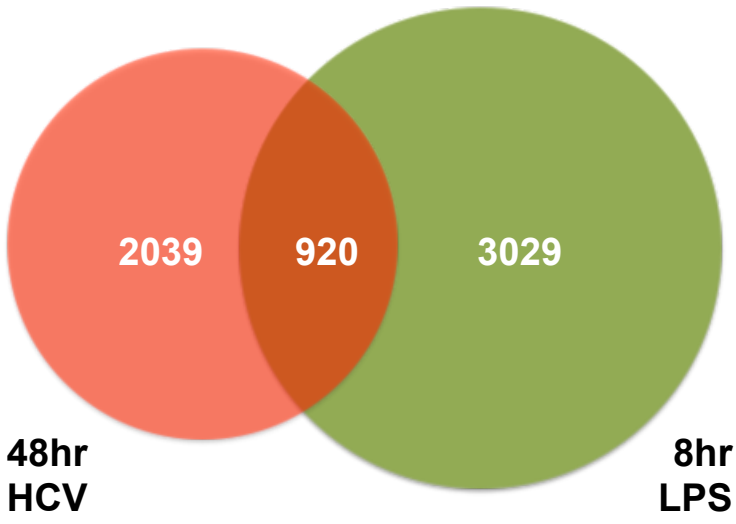


Figure 15. A Venn diagram depicting the number of genes that are differentially expressed at 48 hours after HCV infection and 8 hours after LPS exposure is shown. Genes that were significantly differentially expressed in both LPS and HCV exposed cells are shown in the overlapping regions.

Changes in gene expression revealed a broad and complex cellular transcriptional reprograming in wide range of gene functional categories and many of these DEGs haven't been previously associated with HCV infection. Gene ontology (GO) analysis was used to annotate the differentially expressed genes to their associated biological processes. All DEGs were subjected to GO term enrichment analysis that revealed 435 GO terms at the thresholds of 5 genes within the category and conservative FDR of <0.05 (at 48 hours time point). Notably, many of the differentially expressed genes belong to functional categories related with immune and inflammatory responses (103), cell adhesion (142), signaling (200), cell death (145), cell cycle (149) and cell growth/proliferation (153).

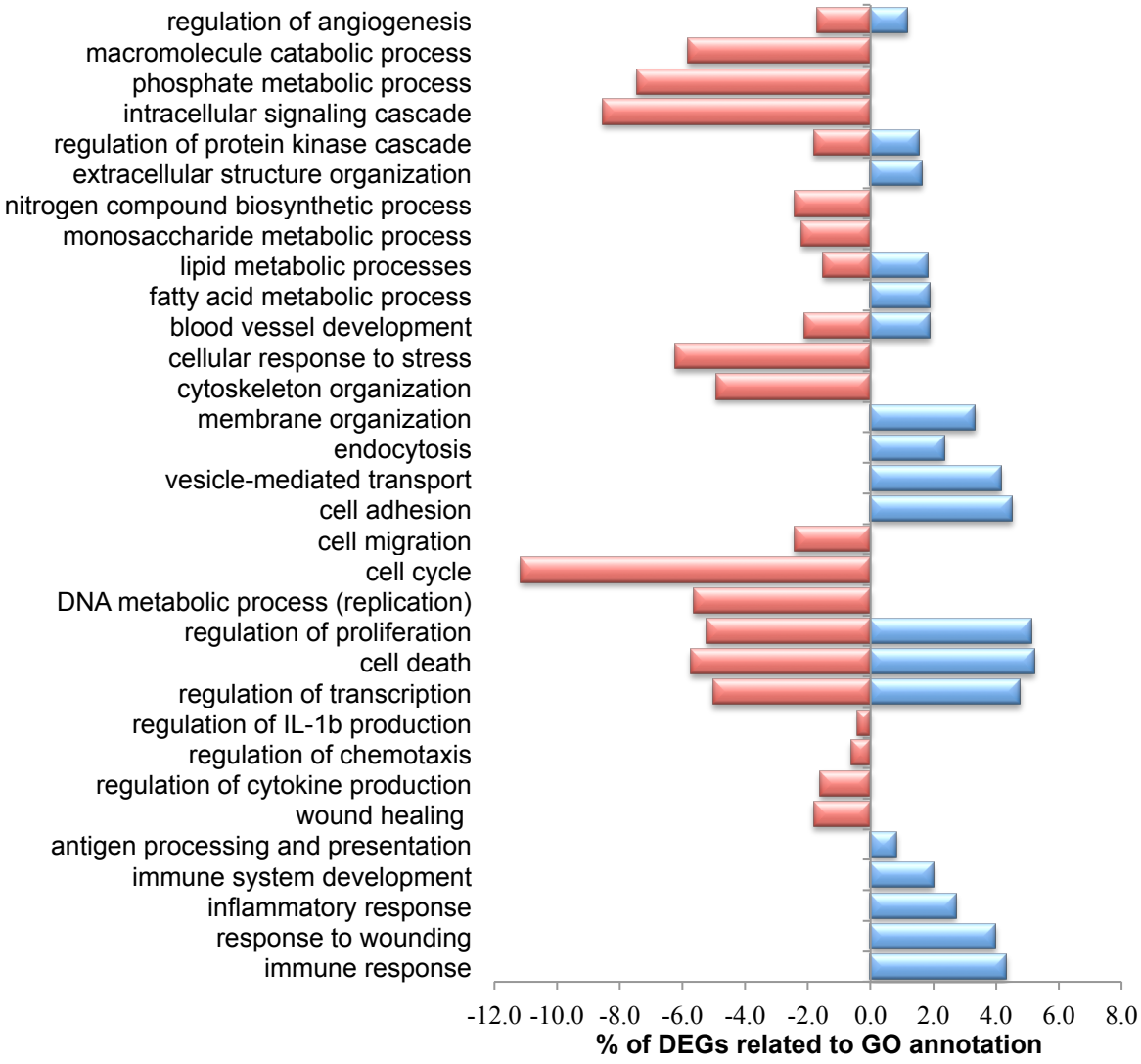


Figure 16. Gene ontology analysis of differentially expressed genes 48 hours after HCV infection.

TABLE 2. The 20 most highly upregulated genes after HCV exposure. Annotated genes that were significantly increased after HCV exposure, relative to MOCK treated cells, are presented in the order of their fold change are indicated.

Ensambl Gene ID	Gene Name	Gene Description	FDR	Fold Change
ENSG00000138315	OIT3	oncoprotein induced transcript 3	94	19.4
ENSG00000132514	CLEC10A	C-type lectin domain family 10, member A	44	12.9
ENSG00000134817	APLNR	apelin receptor	54	10.5
ENSG00000102575	ACP5	acid phosphatase 5, tartrate resistant	22	9.1
ENSG00000136011	STAB2	stabilin 2	16	9.0
ENSG00000240583	AQP1	aquaporin 1 (Colton blood group)	31	8.1
ENSG00000176046	NUPR1	nuclear protein, transcriptional regulator, 1	96	7.2
ENSG00000179914	ITLN1	intelectin 1 (galactofuranose binding)	28	7.0
ENSG00000113389	NPR3	natriuretic peptide receptor C/guanylate cyclase C	13	6.8
ENSG00000135409	AMHR2	anti-Mullerian hormone receptor, type II	17	6.6
ENSG00000175899	A2M	alpha-2-macroglobulin	70	6.2
ENSG00000163687	DNASE1L3	deoxyribonuclease I-like 3	47	6.0
ENSG00000182851	GPIHBP1	glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1	25	5.8
ENSG00000165810	BTNL9	butyrophilin-like 9	19	5.8
ENSG00000130600	H19	H19, imprinted maternally expressed transcript (non-protein coding)	50	5.8
ENSG00000104490	NCALD	neurocalcin delta	21	5.7
ENSG00000148357	HMCN2	hemicentin 2	33	5.6
ENSG00000055955	ITIH4	inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)	20	5.4
ENSG00000110876	SELPLG	selectin P ligand	65	5.4
ENSG00000137033	IL33	interleukin 33	18	5.2
ENSG00000117643	MAN1C1	mannosidase, alpha, class 1C, member 1	28	4.9

TABLE 3. The 20 most highly downregulated genes after HCV exposure. Annotated genes that were significantly increased after HCV exposure, relative to MOCK treated cells, are presented in the order of their fold change are indicated.

Ensambl ID	Gene Name	Gene Description	FDR	Fold Change
ENSG00000181634	TNFSF15	tumor necrosis factor (ligand) superfamily, member 15	16	-33.0
ENSG00000124721	DNAH8	dynein, axonemal, heavy chain 8	14	-6.4
ENSG00000183715	OPCML	opioid binding protein/cell adhesion molecule-like	14	-5.6
ENSG00000213694	S1PR3	sphingosine-1-phosphate receptor 3	19	-3.8
ENSG00000164251	F2RL1	coagulation factor II (thrombin) receptor-like 1	16	-3.8
ENSG00000152377	SPOCK1	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 1	14	-3.7
ENSG00000218336	ODZ3	odz, odd Oz/ten-m homolog 3 (Drosophila)	15	-3.3
ENSG00000105851	PIK3CG	phosphoinositide-3-kinase, catalytic, gamma polypeptide	40	-3.1
ENSG00000164176	EDIL3	EGF-like repeats and discoidin I-like domains 3	17	-3.0
ENSG00000152402	GUCY1A2	guanylate cyclase 1, soluble, alpha 2	94	-3.0
ENSG00000115008	IL1A	interleukin 1, alpha	13	-2.9
ENSG00000147082	CCNB3	cyclin B3	19	-2.9
ENSG00000135480	KRT7	keratin 7	20	-2.8
ENSG00000146147	MLIP	muscular LMNA-interacting protein	24	-2.8
ENSG00000240694	PNMA2	paraneoplastic antigen MA2	23	-2.7
ENSG00000180440	SERTM1	serine-rich and transmembrane domain containing 1	29	-2.7
ENSG00000106366	SERPINE1	serpin peptidase inhibitor, clade E, member 1	15	-2.7
ENSG00000198286	CARD11	caspase recruitment domain family, member 11	18	-2.7
ENSG00000163293	NIPAL1	NIPA-like domain containing 1	19	-2.6
ENSG00000092969	TGFB2	transforming growth factor, beta 2	24	-2.5
ENSG00000101670	LIPG	lipase, endothelial	23	-2.5

5.3. REGULATION OF PATHOGEN RECOGNITION RECEPTOR SIGNALING PATHWAYS IN HCV INFECTED LSEC AS COMPARED TO LPS STIMULATED LSEC

The innate immune response relies on recognition of evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs), through a limited number of germ line-encoded pattern recognition receptors (PRRs).(11) PRRs can be divided in membrane-bound receptors (Toll-like receptors, TLRs and C-type lectin receptors) and cytosolic PRRs (retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)).

We found constitutive expression of the wide repertoire of PRRs in LSEC, indicating efficient "sentinel" function. RNA-seq analysis has shown that LPS and HCV differentially regulates PRRs, adaptor molecules, and cascade of kinases that ultimately results in different biological outcomes. More than 400 DEGs in HCV infected LSECs were involved in the processes of intracellular signaling.

	HCV	LPS
Toll-like receptor pathway		
TLR3	ns	1.87
TLR4	-1.35	-1.82
CD14	2.69	2.36
C-type lectins		
MRC1	1.94	ns
MRC2	1.32	-1.29
CLEC12A	1.39	ns
CLEC12B	-1.60	-3.90
CLEC14A	1.39	-1.58
CLEC1A	1.52	1.68
CLEC2B	-1.39	-1.9
CLEC4M	1.67	ns
CLEC3B	4.05	ns

	HCV	LPS
Intracellular receptors		
RIG-I	-1.45	4.86
MDA5	1.37	11.08
LGP2	1.82	3.45
PK3	-1.3	1.25
NOD-like receptors		
NOD1	1.31	-1.29
NAIP	1.40	-1.43
NLRP1	ns	-1.83
NLRP12	1.26	ns
NLRX1	1.28	-1.55

TABLE 4. The list of differentially expressed PRRs in HCV and LPS exposed LSEC. Fold changes as compared to control are shown.

HCV infected LSECs downregulate intracellular sensors of dsRNA such as *RIG-I* (1.45-fold) and *PK3* (1.3-fold) but upregulate *MDA5* (1.4-fold) and *LGP2* (1.8-fold). Membrane bound *TLR4* that might sense HCV envelope glycoproteins, was downregulated 1.4-fold. Since LSECs constitutively express variety of mannose receptors, C-type lectins (CTLs), the upregulation of several CTLs was not a surprise (*CLEC3B* 4.1-; *MRC1* 1.9-; *CLEC1A* 1.5-; *CLEC14A* 1.4-; *MRC2* 1.3-fold increase). Interestingly, *CLEC4M* or L-SIGN that showed 1.7-fold increase binds HCV E1/E2 glycoproteins and probably has a role in intracellular HCV trafficking and escape from lysosome degradation.(54) This might suggest that HCV specifically upregulates L-SIGN receptor in order to increase uptake of the virus into the cells and prolong viral infectivity.

Several NOD-like receptors (NLRs) transcripts were upregulated in HCV infected LSECs (Table 4). During infection, viruses induce PRRs-dependent cytosolic accumulation of inactive IL-1β precursor that “primes” the cells and upon a second trigger that stimulates inflammasome multiprotein complex assembly, caspase-1 activation and pro-IL-1β cleavage, subsequently release the mature, biologically active IL-1β cytokine.(29) NLRs gained interest because of their key function in inflammasome activation. Since we did not observe upregulation of *IL1β* mRNA level in HCV infected cells (contrary to LPS stimulated LSECs), suggesting that HCV by itself doesn’t induce efficient TLR or RIG-I dependent cytokine transcription (lack of signal 1), we can speculate that upregulation of NLRs and several other inflammasome components might indicate activation of second signal necessary for and inflammasome proteins oligomerization and activation of caspase-1. However, in LPS treated LSECs, where *IL1β* was significantly upregulated, NLRs were downregulated while important genes involved in terminating PRRs responses, such as *A20* and *CYLD* where upregulated (4.4- and 1.5-fold increase, respectively). This might represent a negative feedback and attenuation of the inflammatory response to LPS, as we previously suggested.

The concept of PRR-signaling involves the recruitment of one or several adaptor molecules that activates downstream signal transduction pathways via phosphorylation, ubiquitination or protein-protein interactions. In closer look to the

downstream transcripts of PRR-signaling pathways after HCV exposure, many essential genes were significantly altered. Examples include:

- *SARM*, sterile alpha- and armadillo motif-containing protein, a negative regulator of TRIF-dependent TLR signaling,(70) was upregulated 1.5-fold in HCV infected LSECs as compared to control.
- *TRAM*, *TRAF1*, *TRAF3* and *TANK*, adaptor proteins of TLRs, were significantly downregulated after HCV infection (1.25-, 1.4-, 1.3-, 1.25-fold decrease, respectively).
- Single immunoglobulin IL-1 receptor-related molecule (*SIGIRR*) was upregulated 1.5-fold in HCV infected LSECs. *SIGIRR* is an Ig-like membrane protein critical for negative regulation of TLR1, 4, 5, and 9 and IL-1 -mediated immune responses.(71)
- *RNF125,* ring finger protein 125 (1.8-fold increase), induces ubiquitination and proteasomal degradation of RIG-I, MDA5 and IPS-1, which results in termination of IFN responses.(72)
- *ZFP36* (or *TTP*), zinc finger protein 36 homolog, a gene that promotes the rapid decay of various mRNAs, was 1.5-fold upregulated in HCV infected cells. *TTP* has been shown to promote destabilization of GM-CSF, TNFα and some CXC- chemokines through binding to the AU-rich RNA.(73)

This might suggest that PRRs signaling pathway and subsequent IFN- and inflammatory responses are inhibited on several different levels. Since RIG-I (and partly TLRs) signaling pathway is critical for the activation of the type I IFN-dependent antiviral innate immune response to HCV infection, its inhibition might be crucial in establishing virus-friendly environment within the liver. Importantly, while hepatocytes enable full HCV replication with production of non-structural (NS-) proteins that can block PRRs signaling, in LSEC a non-replicative HCV infection is sufficient to hamper RIG-I and TLR-pathways. Figure 17. shows enrichment of RIG-I pathway in HCV infected LSEC.

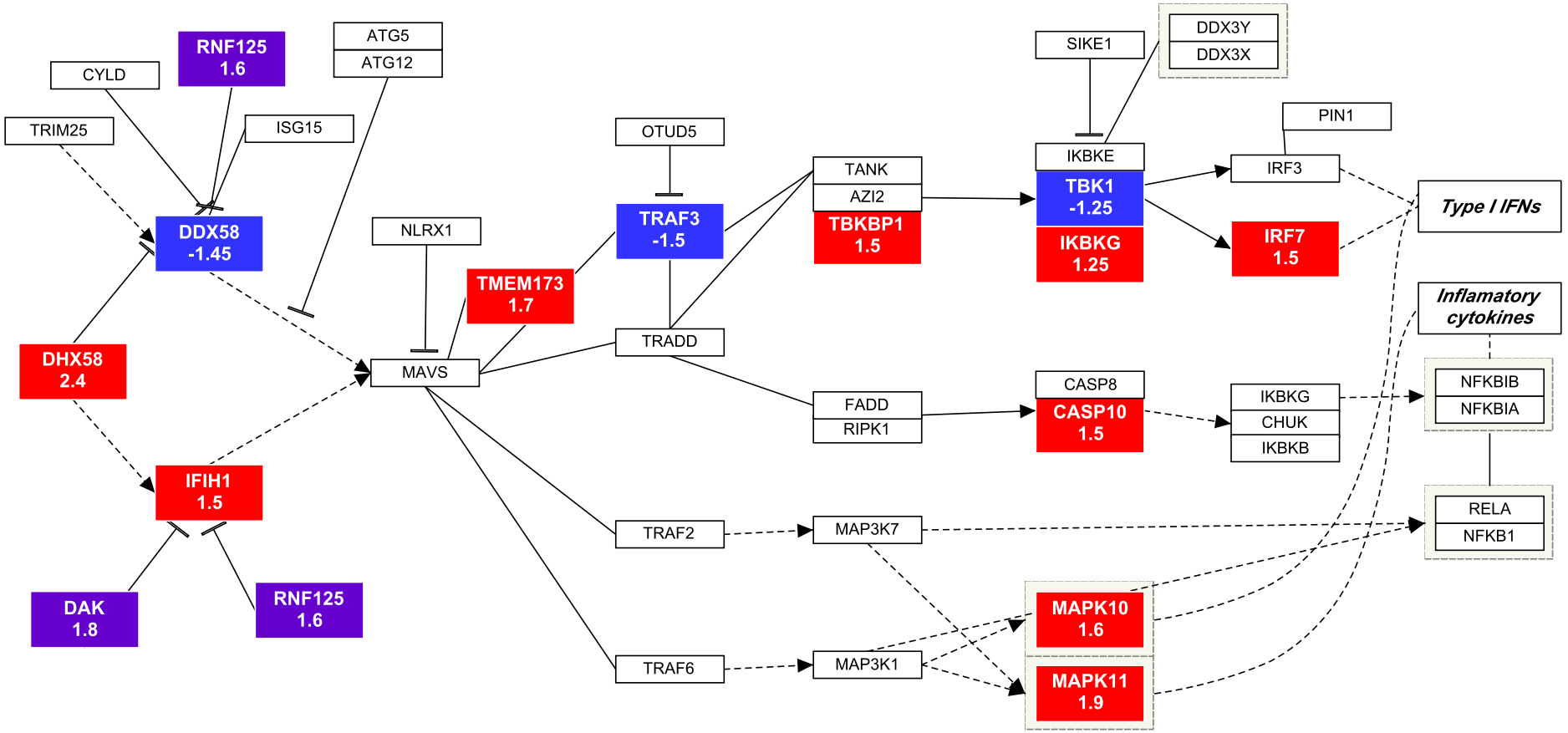


Figure 17. Enrichment of RIG-I signaling pathway in HCV infected LSEC.

5.4. REGULATION OF TRANSCRIPTION DRIVES ANTI-INFLAMMATORY RESPONSE TO HCV INFECTION

Transcription factors (TFs) are regulatory proteins with function to activate or inhibit transcription of DNA and are considered the most important and diverse mechanisms of gene regulation. For example, in order to initiate the innate immune and further activate and shape adaptive immune responses, signaling pathways induced by PRRs needs to activate transcription factors, most notably NF-κB, AP1 and IRF3/7 due to their capacity to stimulate the production of proinflammatory mediators, including cytokines and IFNs.

While LPS stimulated cells at 8 hours time point showed markedly upregulated transcriptional factors (such as *ETV7* 10.9-fold, *CEBPD* 10.6-fold, *POU2F2* 9.2-fold, *IRF7* 7.7-fold, *RELB* 5.8-fold, *NFKB2* 4.4-fold, *AP1* 2.2-fold increase) crucial for inflammatory response, HCV infected LSECs showed decreased approximately 20% of DEGs involved in regulation of transcription.

This includes:

- *HDAC9* (histone deacetylase 9) 3.4-fold decrease, a histone deacatylase that alters chromosome structure and affects transcription factor access to DNA;
- *EGR3* (early growth response 3) -2.8-fold, a critical determinant of VEGF signaling and monocyte adhesion;(74)
- *IRF6* (interferon regulatory factor 6) -2.0-fold, a member of IRF family involved in regulation of cell cycle, differentiation, adhesion;(75)
- *ZBTB16* (zinc finger and BTB domain containing 16) -1.6-fold decrease, a member of Krüppel-type transcription factors involved in cell cycle progression.(76)

In addition, 5% of DEGs that were increased are involved in negative regulation of transcription (such as *MXD4* and *HES1* both 1.4-fold; *MDF1* and *FOXP4* both 1.3-fold increase) at 8 hours infection. The list of 25 differentially expressed TFs with description of their physiological role is shown in Table 5.

The member of Maf family, v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (*MAFB*), as a basic leucine zipper transcriptional factor plays a role of transcriptional activator or repressor. It acts as a negative regulator of the expression of type I interferon genes and antagonizes anti-viral responses primary by blocking IRF3 and disrupting enhanceosome, a transcriptional system that regulates the IFN-β stimulated genes expression including the chemokines (e.g. CCL5 and CXCL10) suggesting a wide contribution in modulating antiviral response.(77, 78) RNA-seq analysis showed 4-fold increase at 48 hours HCV infection and 2-fold decrease at 24 hours in LPS treated cells which were confirmed by qPCR, as presented in Figure 18, Panel A and B.

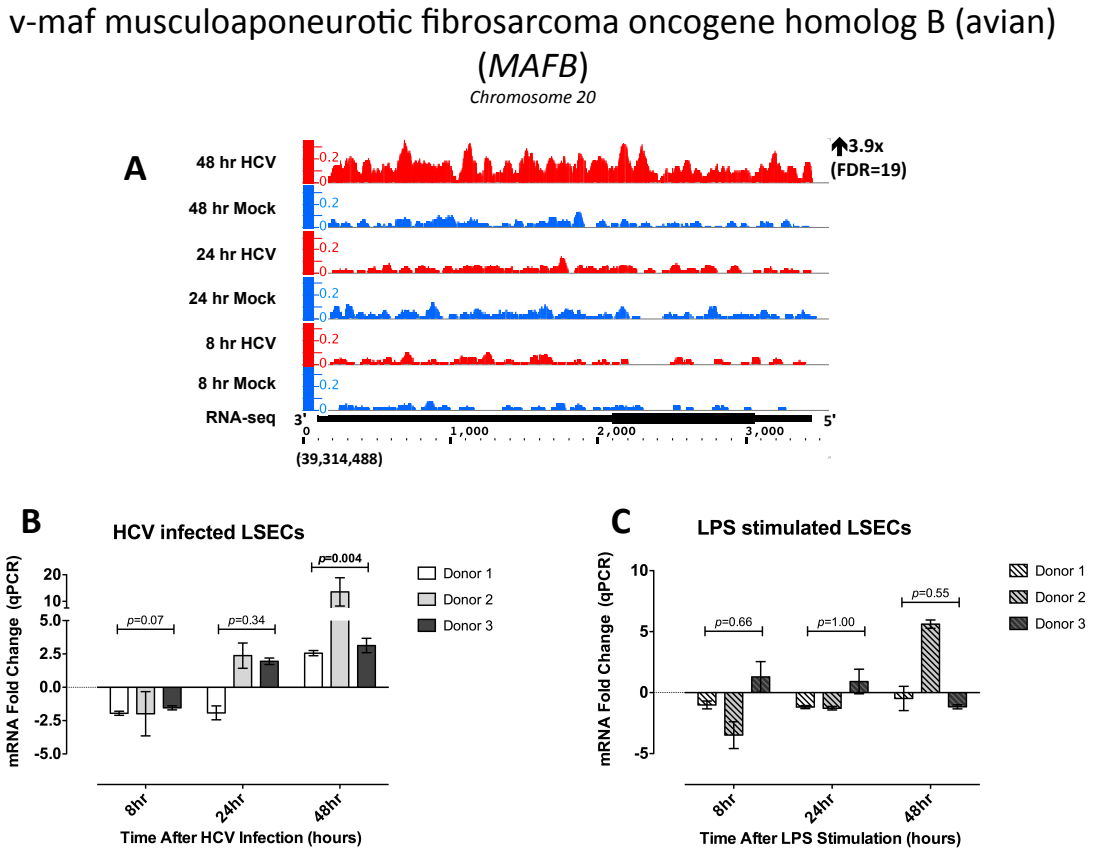


Figure 18. Increased expression of v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB) in HCV exposed LSEC. **Panel A.** Gene expression is displayed as the number of sequencing reads per kilobase of gene length per million reads (RPKM) using the Integrated Genome Browser (IGB). The y-axis represents RPKM, and x-axis represents chromosome location and gene structure, orientation and chromosomal location. **Panel B.** and **Panel C.** show qPCR verification analysis of 9 additional HCV+/- and LPS +/- samples.

TABLE 5. The 20 differentially expressed transcriptional regulators in HCV-exposed cells

Gene Name		FDR	Fold Change	Description[9]
NUPR1	nuclear protein, transcriptional regulator, 1	96	7.2	induces the expression of the anti-inflammatory genes
IL33	interleukin 33	18	5.2	negatively regulates proinflammatory NF-KB dependent transcription
HEYL	hairy/enhancer-of-split related with YRPW motif-like	16	4.5	transcriptional repressors and downstream effector of Notch signaling
MAFB	v-maf musculoaponeurotic fibrosarcoma homolog B	18	3.8	negative regulator of the expression of type I interferon genes
TXNIP	thioredoxin interacting protein	70	3.6	involved in activation of the NLRP3 inflammasome
KLF15	Kruppel-like factor 15	70	2.9	negatively regulates proinflammatory NF-KB dependent transcription
STAT4	signal transducer and activator of transcription 4	17	2.7	transcription activator
POU4F1	POU class 4 homeobox 1	17	2.6	regulates cell proliferation and survival, antiapoptotic role
HIF3A	hypoxia inducible factor 3, alpha subunit	17	2.5	negative regulator of hypoxia-inducible gene expression
IGF2	insulin-like growth factor 2 (somatomedin A)	58	2.4	involved in development and growth
SNAI2	snail homolog 2 (Drosophila)	17	2.4	transcriptional repressor , antiapoptotic activity
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	51	2.3	contributes to immunosuppression and inhibits phagocytosis
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	27	2.2	tumor supressor, negative regulator of cell proliferation
HSF4	heat shock transcription factor 4	28	2.2	negative regulation of DNA binding activity
CAMK4	calcium/calmodulin-dependent protein kinase IV	19	-2.1	regulates the activity of several transcription activators
RUNX1	runt-related transcription factor 1	20	-2.1	cell context-dependent transactivator or repressors
POLR3G	polymerase (RNA) III polypeptide G (32kD)	15	-2.1	DNA sensing pathway involving RNA polymerase III and RIG-I
RARB	retinoic acid receptor, beta	47	-2.3	limits growth of many cell types by regulating gene expression
MITF	microphthalmia-associated transcription factor	19	-2.3	promotes cancer and chronic inflammation
SFRP1	secreted frizzled-related protein 1	16	-2.4	regulation of Wnt pathway

[9] Gene description according to NCBI REFseq, available at http://www.ncbi.nlm.nih.gov/gene/

Nuclear protein transcriptional regulator 1 (*NURP1*) promotes the transcription of stress-regulated genes and the cellular growth in a way that helps the tissue to neutralize diverse injuries.(79, 80) It prevents autophagy, apoptosis and cell death.(79, 80) *NURP1* was upregulated 7.2-fold in HCV infected LSECs as compared to control. The upregulation of *NUPR1* in acute pancreatitis was correlated with the ability of the defense mechanism of the pancreatic cells by inducing the expression of the anti-inflammatory genes.(81)

Hypoxia inducible factor 3, alpha subunit *(HIF3A)*, a member of hypoxia induced transcription factors family that besides O_2-dependent activation pathways can also be induced by inflammatory stimulus, was 2.6-fold increased in HCV infected LSECs. In contrast to *HIF1A* that was upregulated after LPS stimulation; *HIF3A* does not potentiate inflammatory response, but rather negatively regulate hypoxia-inducible gene expression.(82)

CCAAT/enhancer binding protein delta (*CEBPD)* transcription factor that inhibits macrophage-mediated phagocytosis of damaged cells was 2.3-fold increased in HCV infection.(83) *CEBPD* positively regulates *PPARD*, which showed 1.5-fold increase in HCV infected LSECs. As a member of the peroxisome proliferator-activated receptor (PPAR) family it attenuates the increase of inflammatory cytokines and acute phase proteins such as IL-1β, IL-6 or TNF-α. Recent studies have reported that the anti-inflammatory effects of PPARs are mediated by negative transcriptional control of NF-KB.(84)

Several members of Ets variant (ETV-) transcriptional factors family were significantly downregulated in acutely infected LSECs (*ETV1* 1.9-; *ETV3L* 1.6-; *ETV5* 1.5-fold decrease). In vascular endothelium these transcription factors are highly induced after proinflammatory stimulation and by modulating expression of adhesion molecules (VCAM-1), chemokines (CCL-2) and matrix metalloproteinases (MPPs) contribute to the development of atherosclerosis.(85) In *ETV1* knockdown mouse a marked reduction in the recruitment of inflammatory cells, as the vascular remodeling were reported in a response to systemic inflammation. In addition to downregulation of mentioned transcripts in HCV infected LSECs, several downstream targets of *ETV1* were also downregulated,

such as *CCL2* 1.6; *VCAM1* 3.1-; *MMP1* 2.1-; *MMP10* 2.2-fold decrease 48hours after HCV infection.

The final effect of these expression changes might be attenuation instead of activation of the inflammatory and innate immune responses to promote anti-inflammatory microenvironment.

5.5. EXPRESSION OF CYTOKINES, PRO-INFLAMMATORY AND ANTI-INFLAMMATORY MOLECULES IN ACUTELY HCV INFECTED LSEC

As previously described, the ability of IFNs to confer an antiviral state depends on IFN mediated responses by JAK-STAT signaling pathway, resulting in broad expression of ISGs.(11, 21, 86) Unexpectedly, we didn't find differentially expressed type I IFNs in HCV infected LSECs. Furthermore, in comparison to LPS stimulated LSECs that produced a variety of ISGs, HCV infected LSECs did not show changes in ISGs (such as *OAS2*, *MX1*, *ISG15*, *ISG20*). While LPS stimulated cells induced a dramatic changes in inflammatory cytokines and chemokines expression, in HCV infected LSEC inflammatory signals were attenuated.

In exception of *CCL14* that activates, but does not induce chemotaxis of monocytes (2.4-fold increase), *CCL23* chemotactic for resting, but not for activated T-lymphocytes (3.3-fold increase), proangiogenic *CXCL16* that facilitate uptake of various antigens (2.0-fold increase) and *CXCL2* chemotactic for hematopoetic steam cells and neutrophils (2.4-fold increase), others members of chemokine's families known to play an important role in HCV pathogenesis and previously suggested to be produced by sinusoidal endothelium (e.g. *CXCR3*, *CCR5*, *CXCL10*, *CXCL9*, *CXCL11*, *CCL5*, *CCL3*, *CCL4*), were not differentially expressed. Moreover, *CCL2* a chemoatractant for monocytes, and *CXCL1*, a ligand for receptor highly expressed on hepatic stellate cells (HSC) that regulates

HSC activation and induces fibrogenesis, were significantly downregulated, 1.6- and 2.1-fold decrease, respectively (Table 6)

.

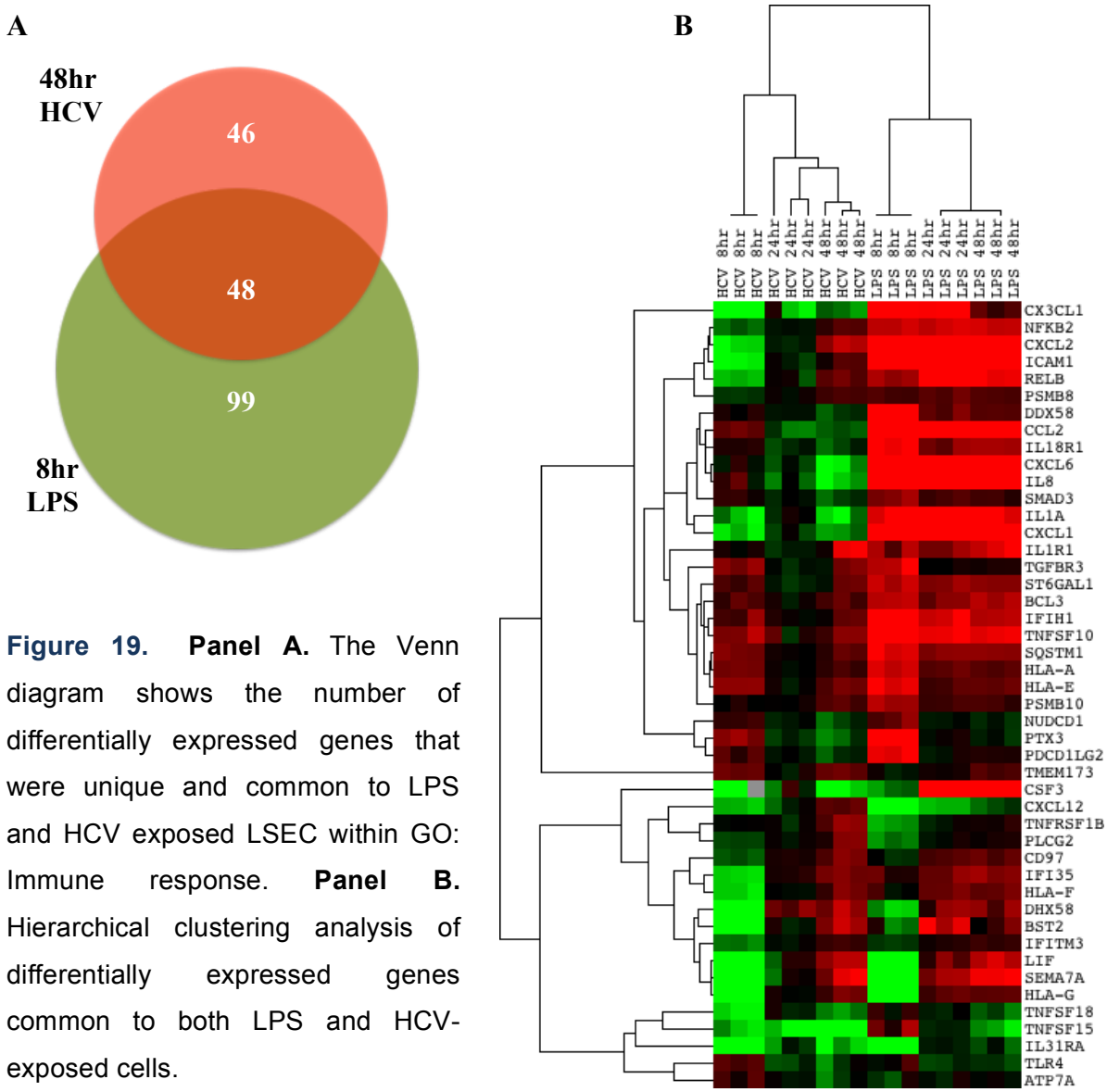


Figure 19. **Panel A.** The Venn diagram shows the number of differentially expressed genes that were unique and common to LPS and HCV exposed LSEC within GO: Immune response. **Panel B.** Hierarchical clustering analysis of differentially expressed genes common to both LPS and HCV-exposed cells.

TABLE 6. List of differentially expressed cytokines and their receptors in HCV infected LSEC

Ligand	Receptor	Function[3]
CXC subfamily		
CXCL1 (-2x) CXCL2 (2.7x)	IL8RB	Neutrophil chemoattractant
CXCL6 (-2.7x) IL8 (-2.9x)	IL8RA	Neutrophil chemoattractant
CXCL12 (1.6x)	CXCR4 (-1.4x)	Lymphocytes chemoattractant; neovascularisation
CXCL16 (2.2x)	CXCR6	Lymphocytes chemoattractant
CX3X subfamily		
CX3CL1 (-2x)	CX3CR1	Promotes strong adhesion of leukocytes to activated endothelial cells
CC subfamily		
CCL20	CCR6 (1.5x)	Lymphocytes chemoattractant
CCL2 (-1.6)	CCR2	Monocytes chemoattractant
CCL14 (2.4x) CCL23 (3.6x)	CCR1, CCR3	Activates monocytes; highly chemotactic for resting T-cells and monocytes
Hematopoietins		
IL6	IL6R (1.6x)	Anti-/pro- inflammatory
IL11	IL11RA (2.3x)	Induce acute phase proteins
LIF (2.3x),CNTF(1.3x)	LIFR (1.4x)	Anti-inflammatory
CSF3 (-3.3x)	CSF3R	Stimulates survival, proliferation, differentiation and function of neutrophils
LEP	LEPR (1.7x)	Susceptibility to Entamoeba histolytica infections?
IL3	IL3RA (2.6x)	Promotes endothelial cell motility and neovascularization
PDGF family		
PDGFC (-1.3x)	PDGFRB (2.4x)	Modulates endothelial proliferation and angiogenesis
HGF	MET (-1.5x)	“Program cell invasion”
EGF	EGFR (-2x)	Cell migration, adgesion, proliferation
VEGFC (-2x)	FLT4 (-1.3)	Angiogenesis, growth and survival
CSF1 (1.4x)	CSF1R	Stimulates survival, proliferation, differentiation and function of macrophages
KITLG (-1.3x)	KIT (-2x)	Survival, migration and tube formation of endothelium

TABLE 6. (*continued*) List of differentially expressed cytokines and their receptors in HCV infected LSEC

Ligand	Receptor	Function[10]
TNF family		
TNFSF10 (1.7x)	SF10C (-1.4x) SF10D (-1.4x)	Preferentially induces apoptosis in transformed and tumor cells, but does not appear to kill normal cells.
TNFSF11A	SF11AS (1.9x) SF25 (1.6x)	A dentritic cell survival factor and is involved in the regulation of T cell-dependent immune response. Involved in regulation of cell apoptosis
TNFSF12 (-1.5x)	SF12A (-1.5x)	Induces apoptosis, promote proliferation and migration of endothelial cells, and thus acts as a regulator of angiogenesis
TNF	SF1B (1.9)	Involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation
TNFSF14 (1.8x)	SF14 (1.9x)	Stimulate the proliferation of T cells, prevent TNFα-mediated apoptosis in primary hepatocyte
TNFSF4 -1.9x)	SF4	Involved in T-cell antigen-presenting cell interactions; This protein and its receptor are reported to directly mediate adhesion of activated T cells to vascular endothelial cells
TNFSF18 (-2x)	SF18	Important for interaction between T-lymphocytes and endothelial cells
TGF-beta family		
TGFB2 (-2.4x)	TGFBE1/2	Suppressive effects of interleukin-2 dependent T-cell growth
INHBB (1.6x)	ACVR	Role in cell growth and proliferation
BMP2 (1.5x)	BMPR2	Regulates cell growth and differentiation
IL1 family		
IL1A (-3.0x)	IL1R1 (2.5x)	A pleiotropic cytokine involved in various immune responses, inflammatory processes, and hematopoiesis
IL18	IL18R1 (-1.4x)	A proinflammatory cytokine
IL33 (6.3x)	IL33R	Drives production of Th2-associated cytokines

[10] List of genes according to KEGG pathway Cytokine-cytokine receptor interaction. Fold changes are shown in brackets (symbols in red are upregulated, and symbols in blue are downregulated).
Protein function is described according to NCBI REFseq, available at http://www.ncbi.nlm.nih.gov/gene/

Contrary to previously published upregulation of *IL8* in HCV core antigen infected human umbilical vein endothelial cells (HUVEC),(42) we noticed a significant decrease of *IL8* in LSECs (2.8-fold decrease). Furthermore, anti-inflammatory cytokine *IL10* and IL10 receptor *IL10RA* were 2-fold and 2.1- fold increased, respectively.

Meanwhile, several highly upregulated genes were linked with anti-inflammatory responses.

Interestingly, one of the highly upregulated genes was alpha-2-macroglobulin (*A2M*, 6.4-fold increase) that was downregulated in LPS treated cells (2.4-fold decrease). The role of this irreversible protease inhibitor has been shown to predict the outcome of inflammatory conditions such as sepsis.(87, 88) The numerous growth factors, cytokines and hormones bind to A2M, which can inhibits biological effects of some of them (e.g. TGF-beta, IL1, IL6).(88)

Highly upregulated member of metallophosphesterase superfamily, acid phosphatase 5, tartarate resistant (*ACP5*) showed 9.1-fold increase in HCV infected cells (but not in LPS stimulated), which was confirmed by qPCR analysis of additional samples (Figure 20, Panel A and B). ACP5 is enrolled in negative regulation of IL1β, IL12, TNFα production.(89) Interestingly, in humans ACP5 gene mutation was related with a bone dysplasia with autoimmune disorders and upregulation of type I interferon responses.(90) Consequently, the upregulation of *ACP5* might be a candidate gene for impaired IFN signatures in HCV infected LSECs.

IL1 family members are highly inflammatory cytokines, related with various pathologic effects, whose secretion is closely related with inflammasome activation.(29) Interestingly, *IL1A* was significantly downregulated, 3.0-fold decrease. However, unconventional member of IL-1 family, mainly expressed by cells of barrier tissues, interleukin 33 (*IL33*) was significantly upregulated, 5.2-fold, which was confirmed with qPCR analysis (Figure 21, Panel A and B).

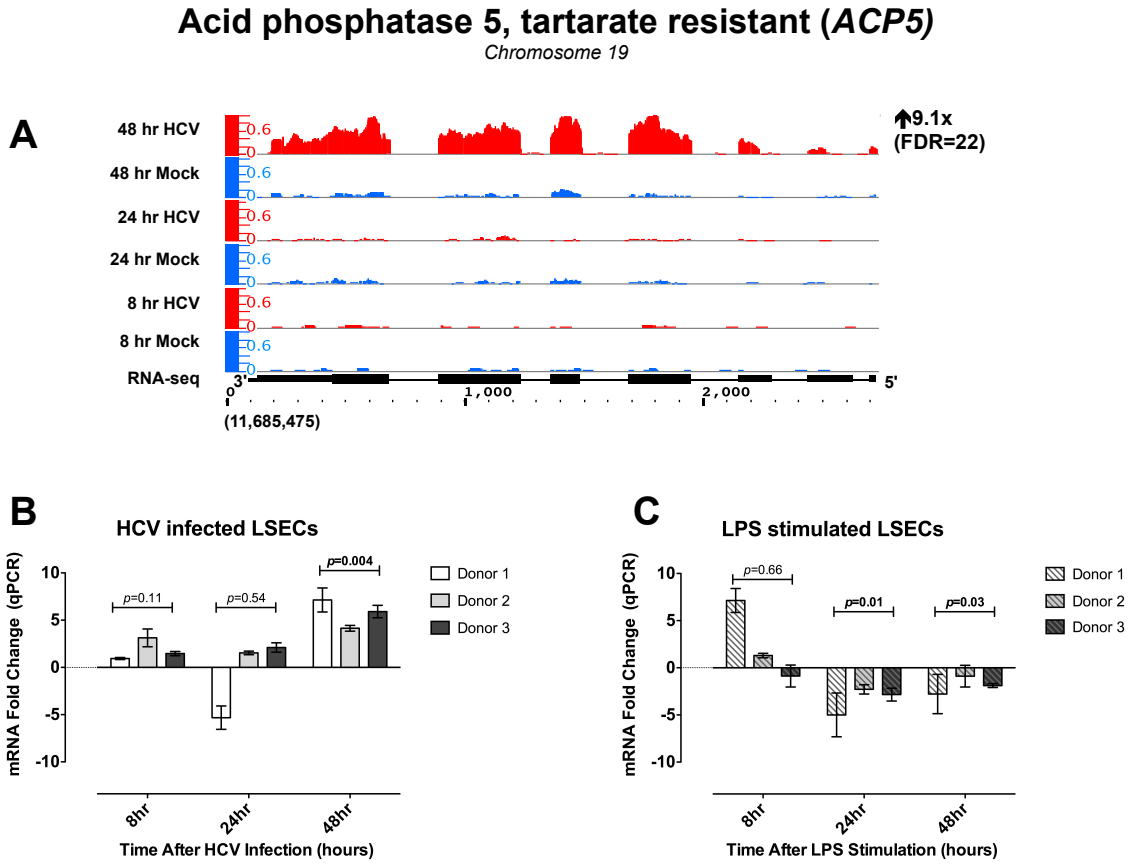


Figure 20. Increased expression of acid phosphatase 5, tartarate resistant (*ACP5*) in HCV exposed LSEC. **Panel A.** Gene expression is displayed as the number of sequencing reads per kilobase of gene length per million reads (RPKM) using the Integrated Genome Browser (IGB). The y-axis represents RPKM, and x-axis represents chromosome location and gene structure, orientation and chromosomal location. **Panel B.** and **Panel C.** show qPCR verification analysis of 9 additional HCV+/- and LPS +/-

IL33 induces expression of T_H2 cytokines and plays an important role in T_H2 cell mobilization.(91) Importantly, T_H2 cytokines (e.g. IL4, IL5 and IL13) induce fibrosis. IL33 also induces dendritic cells to upregulate expression of MHC class II molecules, and drives T_H2 lymphocyte development. However it can also promote the resolution of inflammatory responses, inhibit TLR signaling and cause reduction of systemic inflammatory responses by lowering levels of IL6, TNFα, and CXCL2 in contrast to other IL-1 members.(92) Interestingly, non-processed IL33 can translocate to the nucleus, where it interacts with transcription factor NFKB

and negatively regulates NFKB dependent transcription, consequently reducing cytokine-mediated inflammation.(93) Recently published papers showed higher blood IL33 levels in patients with chronic hepatitis C and IL33 intrahepatic expression was strongly associated with fibrosis.(94) In addition, we identified LSECs as a major source of *IL33*, as HCV infected TPH-1 or Huh 7.5 cells did not significantly expressed *IL33* transcript.

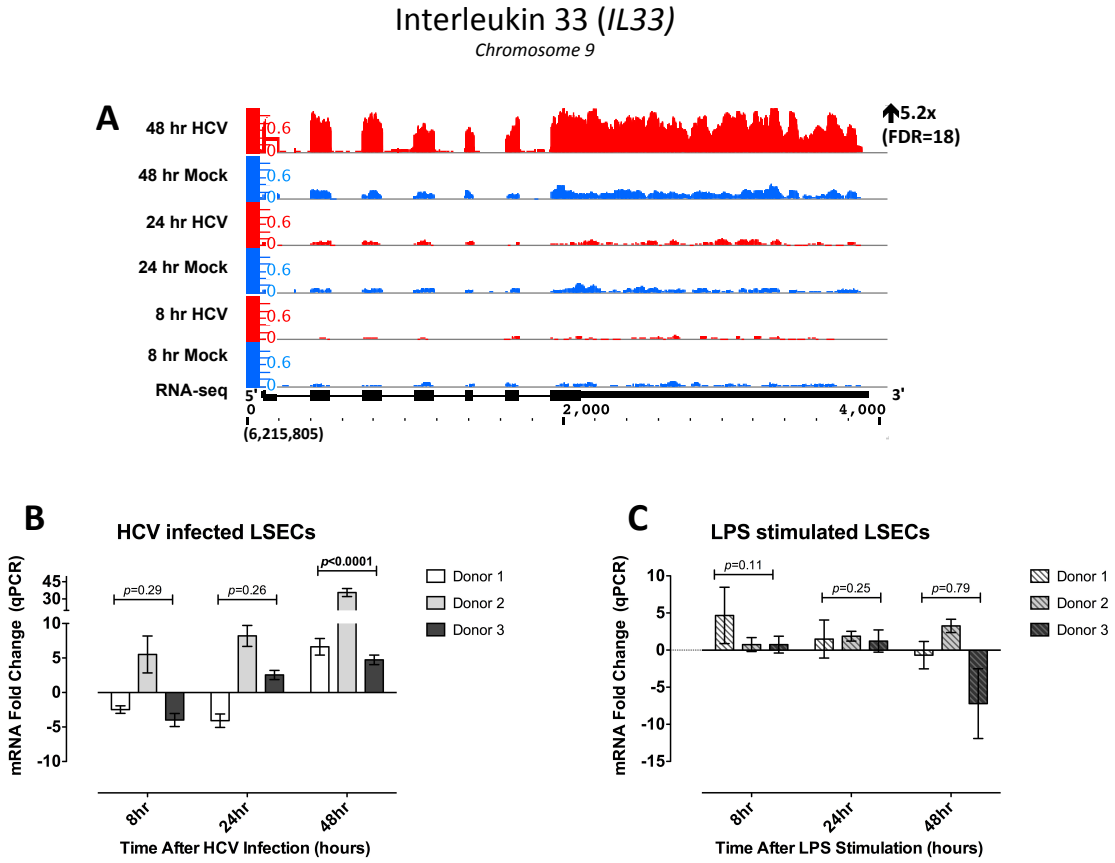


Figure 21. Increased expression of interleukin 33 (*IL33*) in HCV exposed LSEC. **Panel A.** Gene expression is displayed as the number of sequencing reads per kilobase of gene length per million reads (RPKM) using the Integrated Genome Browser (IGB). **Panel B.** and **Panel C.** show qPCR verification analysis of 9 additional HCV+/- and LPS +/- samples.

Bone morphogenetic proteins (BMPs) and BMP binding endothelial regulator (BMPER) are part of the TGF-β signaling pathway. Interestingly, *BMP2* showed 1.6-fold decrease, and *BMP4* 1.3-fold increase of mRNA and upregulation of these molecules were shown to lead to endothelial dysfunction in the absence of *BMPER* (BMP endothelial precursor cell-derived regulator), Figure 22.

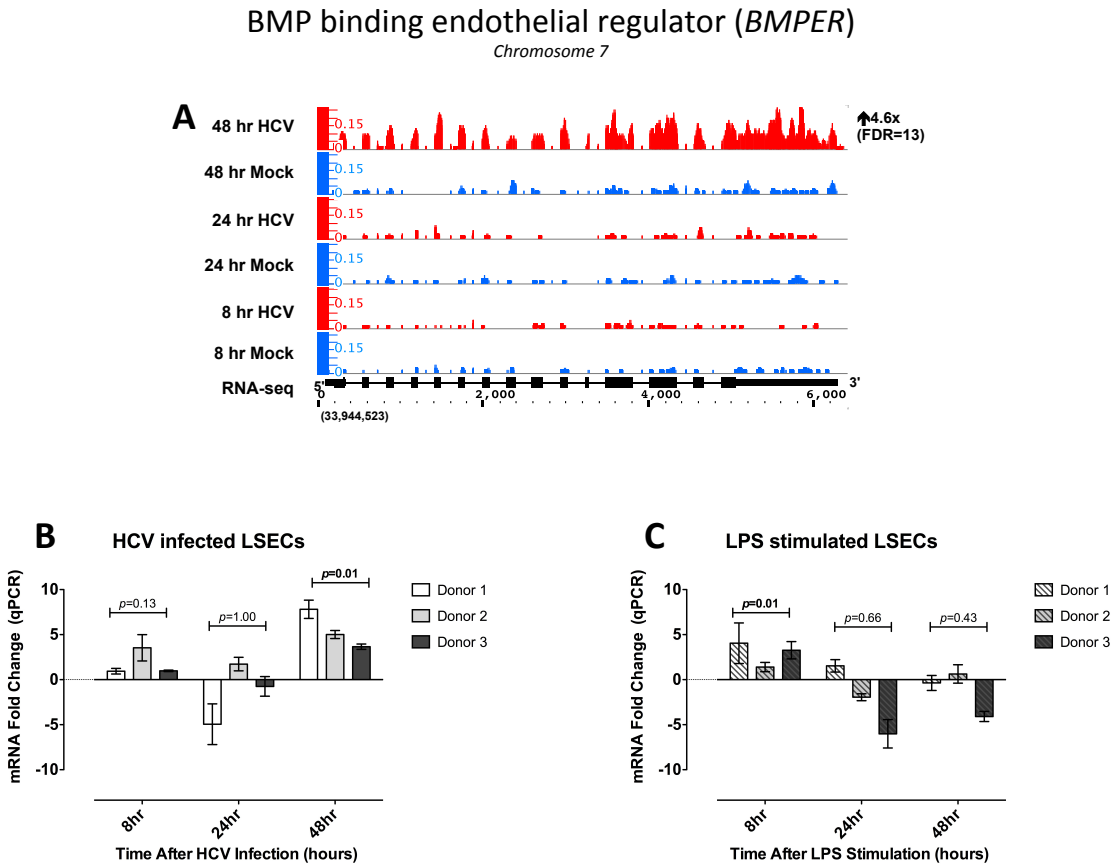


Figure 22. Increased expression of BMP binding endothelial regulator (*BMPER*) in HCV exposed LSEC. **Panel A.** Gene expression is displayed as the number of sequencing reads per kilobase of gene length per million reads (RPKM) using the Integrated Genome Browser (IGB). **Panel B.** and **Panel C.** show qPCR verification analysis of 9 additional HCV+/- and LPS +/- samples.

BMPER is a secreted glycoprotein that binds directly to BMPs and behaves as a BMP-antagonist.(94) RNA-seq revealed 6.3-inrease of *BMPER* mRNA, which was confirmed by qPCR analysis (Figure 19, Panel A and B). BMPER was shown to protect endothelial cells from TNFα-induced expression of proinflammatory

cytokines, and the loss of BMPER leads to inflammation primary due to enhanced BMP-induced NFKB activity.(95, 96) *BMPER* is regulated by transcription factor Krüppel-like factor 15 (*KLF15*), a gene commonly downregulated in endothelial dysfunction.(97) *KLF15* was 2.1-fold increased in HCV infected LSECs. Furthermore, among genes related to BMPER function that are inhibited in endothelial dysfunction, *NOS3* and *NOSTRIN* involved in NO synthesis, were upregulated in HCV infection, 1.5- and 2.0-fold increase, respectively.

Many other transcripts enrolled in negative regulation of inflammatory and defense responses were significantly upregulated in HCV infected cells, such as:

- *C1QTNF1,* C1q and tumor necrosis factor related protein 1 (3.7-fold increase) with anti-inflammatory and insulin sensing effects,(98)
- *NT5E,* 5'-nucleotidase, 1.5-fold increase, that participates in the extracellular pathway that converts ATP to adenosine on the surface of various types of cells, therefore by increasing extracellular levels of anti-inflammatory adenosine molecules;
- *APOE*, apolipoprotein E (2.1-fold increase) primary synthesized in the liver that mediates inflammation independently of its role in lipid metabolism by modulating type I inflammatory responses;
- *IGF2,* Insulin-like growth factor 2 (2.1-fold) with metabolic and growth-promoting effects;
- *SERPING1,* serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 (6.8-fold) a plasma protein that inhibits complement activation;
- *HPX,* hemopexin (2.5-fold) a protector of cells from oxidative stress previously shown to diminish increase of TNFα and IL6 in LPS stimulated macrophages.

Although partly contradictory, these results provide an interesting insight intro molecular mechanisms that might reduce inflammatory and promote anti-inflammatory response in HCV infected LSECs that may answer questions regarding mechanism of HCV infection chronicity, relatively slow process of fibrogenesis, which is primary related to low level of tissue inflammation.

5.6. CHANGES IN TRANSCRIPTOME RELATED TO THE REGULATION OF ADAPTIVE IMMUNE RESPONSE

Endothelial cells were recognized as critical in both limiting and enabling the trafficking of various immune cell populations out of the vasculature and into tissue. LSEC express and differentially regulate after HCV infection both class I (HLA-A 1.32x, HLA-B 1.75x, HLA-C 1.48x, HLA-E 1.70x, HLA-G 1.65x, HLA-H 1.64- fold increase after HCV exposure) and class II MHC molecules. The expression of MHC class II molecules was significantly lower than class I, what is similar as previously reported on HUVEC model. Therefore LSEC are able to provide signal 1 to resting T cells. In addition, LSEC provide a variety of co-stimulators.

Of note, cultured human LSEC lack both CD80 and CD86 ligands for T cell activation (signal 2). Since CD80 and CD86 are considered as the prototypic co-stimulators of T cell activation, this might suggest that LSEC are unable to provide second signal for T-cell activation in the setting of HCV infection. However, CD40, described as a possible co-stimulator of T cell activation, was 2.4-fold upregulated after HCV infection. *TNFSF4*, which produces a potent co-stimulatory signal for activated CD4+ T cells, was 1.9-fold decreased after HCV exposure.

A negative co-stimulator of T-cell, Programmed cell death ligand 1 (*PD-L1*), was 1.3-fold decreased after HCV infection. It was shown that PD-L1 and PD-L2 blocking results in increased endothelial transmigration by both $CD4^+$ and $CD8^+$ T-cells.(99) While normal endothelium expresses PD-L2 and reduces tissue inflammation, PD-L1 and PD-L2 expression by endothelial cells from multiple sclerosis lesions was significantly reduced, suggesting an important role of these molecules in chronic inflammation.(99) These findings are in contrast to plasmacytoid DCs and myeloid DCs (mDCs) isolated from HCV-infected patients, where the effect of inhibitory marker PD-L1 overwhelmed the effect of co-stimulatory markers and downregulated DC-T activation in HCV-infected patients.(100) Meanwhile, the increased interactions between PD-1(+) KCs and PD-L1(+) LSECs appear to lead to the decay of normal endothelial functions

critical to support vascular integrity and prevent acute liver failure as investigated at the model of murine sepsis, and this is probably independent of its normal, immune-suppressive activity.(101)

Interestingly, several other T cell co-inhibitory ligands were significantly altered after HCV exposure. These include:

- *TNFRSF14* (or HVEM, 1.8-fold increased), a TNF superfamily of co-stimulatory molecules, which bind to B- and T-lymphocyte associated (BTLA) co-inhibitory receptors. The interaction of TNFRSF14 with BTLA and multiple other ligands results in transmission of an inhibitory signal.(102)
- Galectin 9 (LGALS9, 2.3-fold increased), recognized by T cell immunoglobulin-3 (TIM3) glycoprotein receptor, induces intracellular calcium flux, cell aggregation and the death of Th1 but not Th2 cells which provides a negative feedback loop to prevent uncontrolled harmful Th1 responses.(102)

While this occur through direct cell-cell contact, LSEC are armed with a variety of soluble inhibitory mediators through secretion of which might provoke immune suppression. This might include:

- *NOS3* (nitric oxide synthase 3), 1.5-fold increase, reported to induce apoptotic cell death in a variety of cells, and has been proposed to alter the responsiveness of T-lymphocytes to antigen or mitogenic stimuli.(103)
- *HPX* (hemopexin), 2.5-fold increase, functions as an iron source for T-cells and is involved in the regulation of cell growth. It was suggested that hemopexin, by controlling heme-iron availability in lymphocytes, modulates responsiveness to IFN-gamma, which might have a negative regulatory role in Th17-mediated inflammation.(104)
- *IL33* (interleukin 33), 5.2-fold increased, which drives Th2-immune responses.(91)

The adhesion of leukocytes to vascular endothelium is a hallmark of the inflammatory process. These interactions are initiated by a variety of chemical mediators and the entire process of leukocyte–endothelial cell adhesion is regulated by the sequential activation of different families of adhesion molecules that are expressed on the surface of leukocytes and LSEC, as reviewed in (105, 106). Lectin-like adhesion glycoproteins, called the *selectins*, mediate leukocyte rolling, while the firm adhesion and subsequent transendothelial migration of leukocytes are mediated by the interaction of integrins on leukocytes with immunoglobulin-like adhesion molecules on endothelial cells (e.g., ICAM-1, VCAM-1).(106)

Inspection of the leukocyte transendothelial migration pathway revealed that 23 of 113 genes were core enrichment genes, which included genes encoding for the following proteins: chemokine receptor *CXCR4* and its ligand *CXCL12*, which activates through PI3K (encoded by *PIK3CB* and *PIK3CG*) and *RAC2*, eventually impacts cell motility; integrin *ITGB1* (-1.4x) involved in cellular adhesion; transmembrane protein *ICAM1* (+1.4x) that facilitates leukocyte endothelial transmigration; components of tight junction strands claudin -1 and -11 (*CLDN1* -3.7x, *CLDN11* -1.6x), upregulated claudin 5, -10 and -15 (*CLDN5* +1.6x, *CLDN10* +1.5x, *CLDN15* +1.4x), which also play critical roles in maintaining cell polarity and signal transductions; component of the hexameric ATPase cellular motor protein myosin 5 and 9 (*MYL5* +1.3x, *MYL9* +1.3x).

Along the passage of ICAM1-mediated signals was the coordinated down-regulation of PKC encoded by *PRKCA* (-2.9x) and upregulation of phospholipase C (*PLCG2* +1.6x) required for ICAM1 dependent leukocyte migration. Along the VCAM1-mediated signal passage were down-regulation of PI3K encoded by *PIK3CB* (-1.4x) and *PIK3CG* (-3.1), downregulation of protein tyrosine phosphatase (*PTPN11* -1.5x); upregulation of subunit of NADPH oxidase (encoded by *CYBA*, +1.5x) whose activation is required for the production of reactive oxygen species in a Rac-mediated manner (*RAC2* -1.3x) with subsequent activation of matrix metallopoteinases and loss of VE-cadherin-mediated adhesion. Along the Thy-1-signal passage, except Thy1 cell surface antigen (+2.7x), other

signal transducer was downregulated, ROCK2 (-1.3x), activation of which leads to myosin phosphorylation, resulting in retraction of the actin cytoskeleton.

After HCV infection selectin L, selectin P and selectin P ligand were 1.6-fold, 3.2- and 5.5-fold increased, respectively. Intercellular adhesion molecule 1 (ICAM-1) was 1.4-fold increased and vascular cell adhesion molecule 1 (VCAM-1) 1.6-fold decreased. Interestingly, several endogenous anti-adhesion molecules were also significantly altered. These include:

- *NOS3* (nitric oxide synthase 3), 1.5-fold increase, whose inhibitors were shown to stimulate the recruitment of adherent leukocytes.(107)
- Upregulation of the prostaglandin I2 (prostacyclin) synthase (2-fold increase), prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase, 1.5-fold increase) and phospholipase C (1.9-fold increase), all involved in production of prostacyclin (PGI2). Activation of the PGI_2 is a potent vasodilator and an inhibitor of platelet aggregation, leukocyte adhesion, and vascular cell proliferation
- Thrombomodulin (1.6-fold increase) shown to directly inhibit leukocyte adhesion to activated endothelium by sequestering proinflammatory proteins.(108) Other member of the C-type lectin transmembrane family, endosialin, with similar function was significantly increased (2.6-fold increase)
- Activated leukocyte cell adhesion molecule (*ALCAM* or CD166, 1.8-fold decreased), binds to T-cell differentiation antigene CD6, and is implicated in the processes of cell adhesion and migration

These results might suggest a different means of LSEC regulation of adaptive immune response than previously thought. However, the model of primary cell LSEC culture does not provide appropriate model for the research of adaptive HCV immune response.

5.7. DOWN-REGULATION OF CELL CYCLE AND APOPTOSIS PATHWAYS

The cell cycle pathway was significantly downregulated in HCV exposed LSEC and the coordinated down-regulation of these genes appeared in all phases of cell cycle including G1 to S, G2 to M, and metaphase to anaphase transition. Down-regulated genes encoding for proteins for G1 to S transition included: *CDNK2B*, cyclin-dependent kinase inhibitor; *CCNE1* encoding for cyclin E; *CDC7* cell division cycle 7. Downregulated were genes essential for genome replication and regulation of transcription *CDK6* and *CDK7*, encoding for cyclin-dependent kinase 6 and 7; mini-chromosome maintenance (MCM) complex (gene *MCM4*, 6) essential for the initiation of eukaryotic genome replication. These most likely represent the coordinated alteration as the down-regulation of the core enrichment genes directly involved in the transition promotion implicates G1/S arrest. In the G2 to M transition, the down-regulation of *CCNA1* and *CCNB1* (cyclin A and B) was observed. Genes encoding proteins that promote the activity of CDK1 complexes including *CDC25A*, *-B*, and *-C*, were not significantly altered, which may implicate G2/M arrest. Additionally, genes involved in DNA damage checkpoint were coordinately down-regulated in the such as *CHEK1*, *ATR* and *ATM*. Genes involved in activation of the activation of the anaphase promoting complex/cyclosome (*BUB1*, *BUB3*, *BUB1B*, *MAD2*), and components of anaphase-promoting complex (*ANAPC1* and *CDC27*), were also significantly down-regulated.

Closely associated with the cell cycle was the apoptosis pathway. While LPS induced major changes in both pro- and anti-apoptotic pathway, mainly suggesting induction of apoptosis, HCV induced minor changes that implicated inhibition of apoptosis after HCV infection. To examine the impact of HCV infection and LPS stimulation on the cells, we measured the cell death and apoptosis at 24 and 48 hours post-exposure. As shown in Figure 25., there was no difference in the percentage of dead or apoptotic cells between MOCK or HCV infected cells. However, the LPS exposed cells had significantly increased level of cell death and apoptosis. This supports transcriptome changes in key transcripts in apoptosis and cell death pathways.

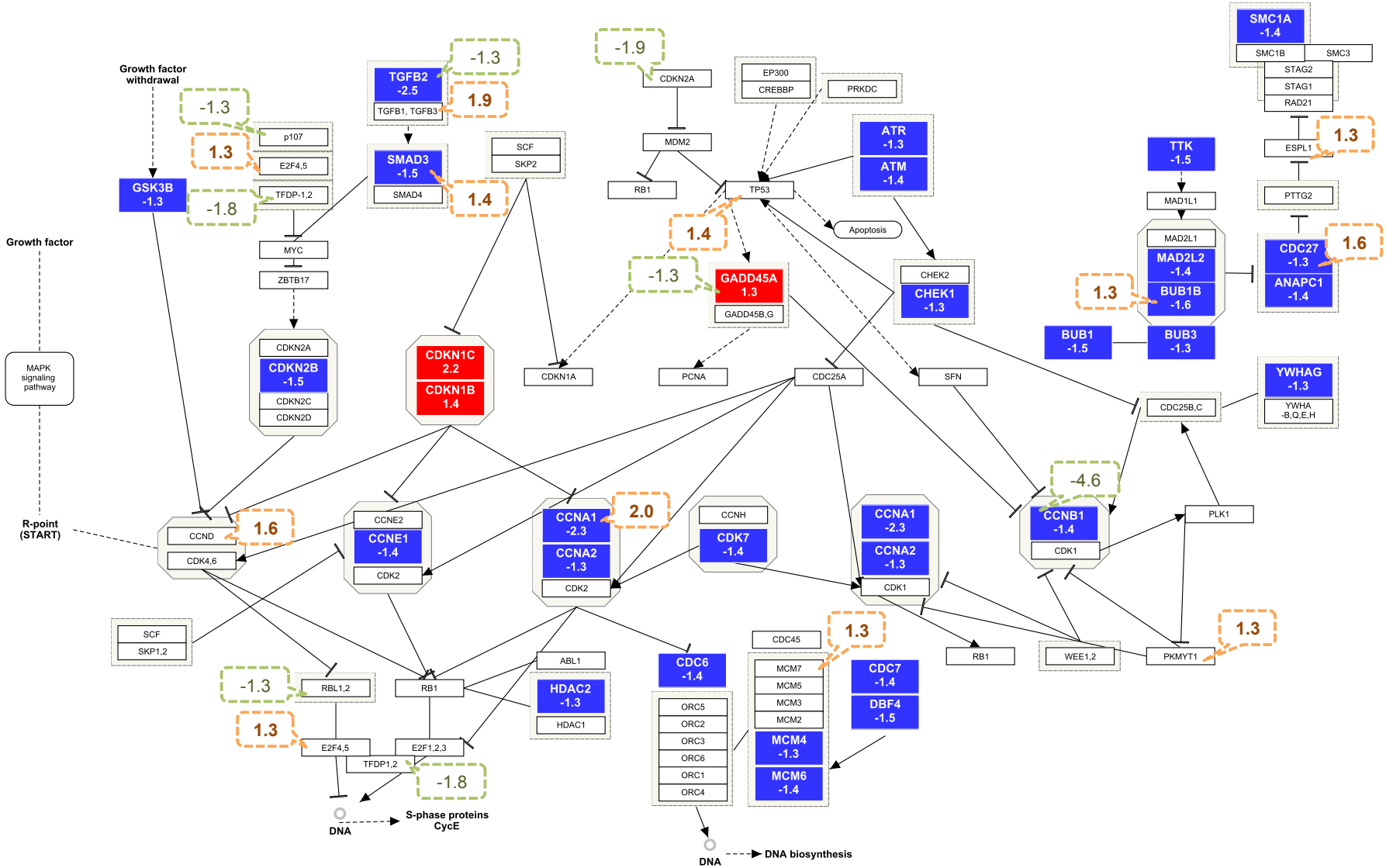


Figure 23. Downregulation of cell cycle pathway in HCV infected LSEC. Downregulated genes are shown in blue and upregulated in red. Fold changes of LPS stimulated LSECs are shown in orange (upregulated) and green (downregulated), dashed brackets.

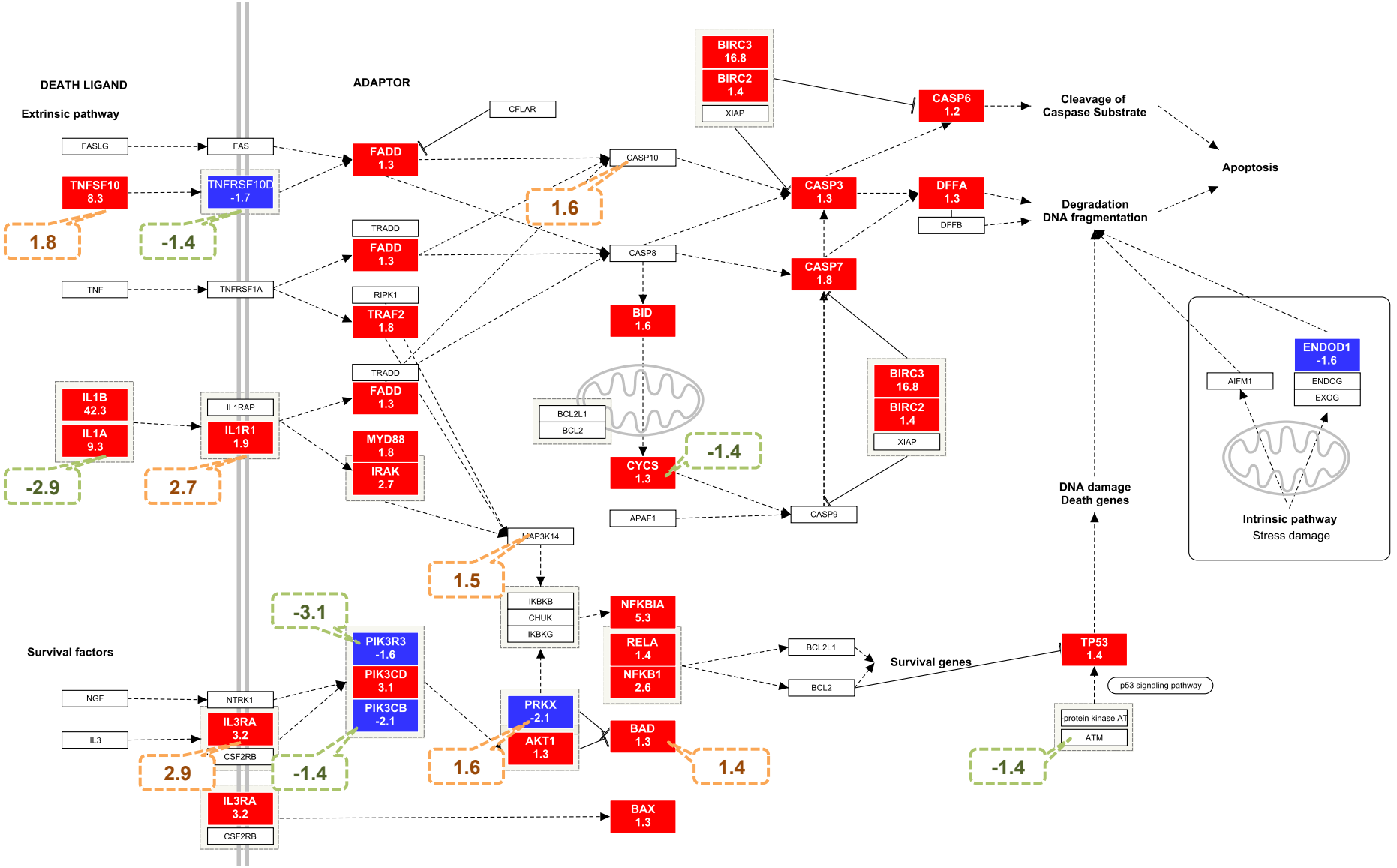


Figure 24. Activation of apoptosis pathway in LPS exposed LSEC cultures. Downregulated genes are shown in blue and upregulated in red. Fold changes of HCV exposed LSECs are shown in orange (upregulated) and green (downregulated), dashed brackets.

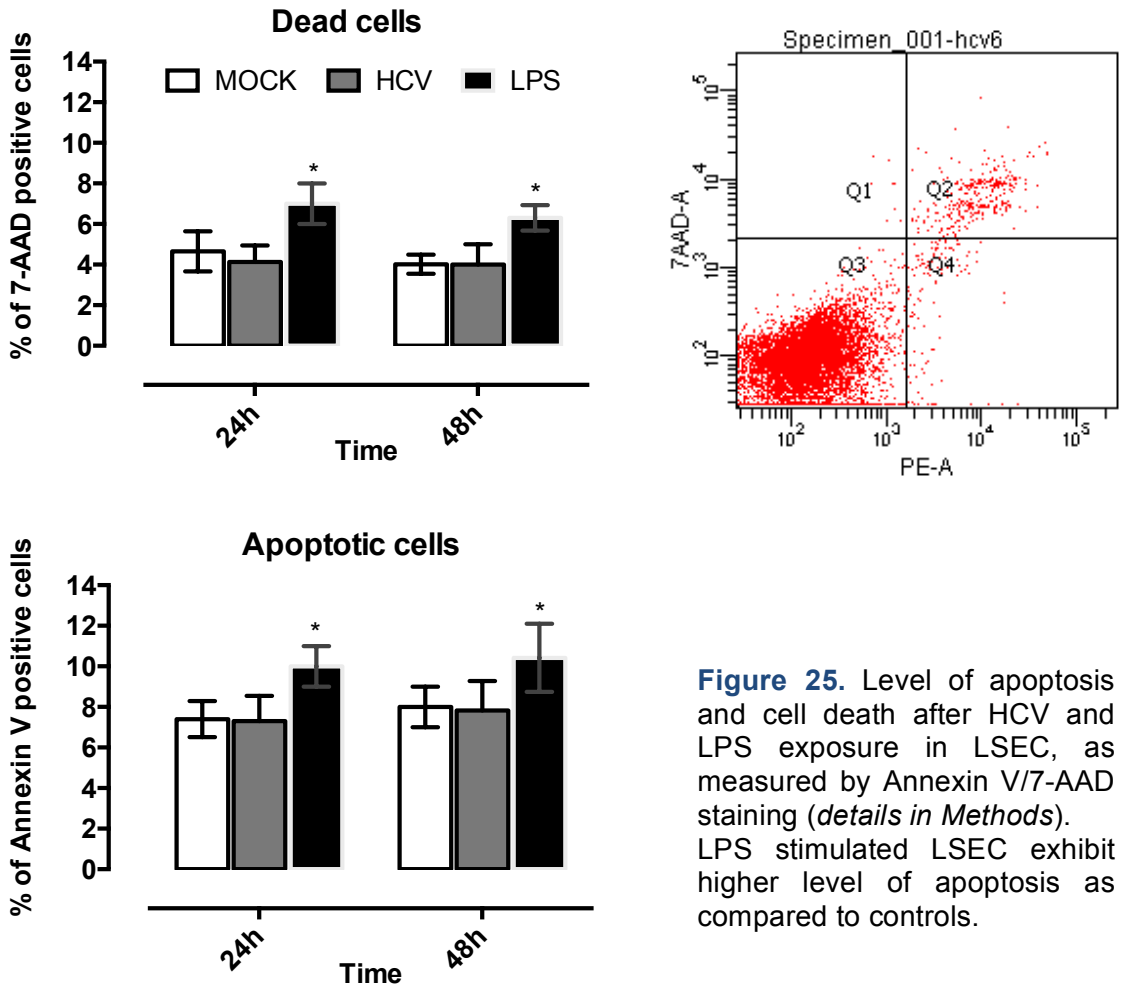


Figure 25. Level of apoptosis and cell death after HCV and LPS exposure in LSEC, as measured by Annexin V/7-AAD staining (*details in Methods*). LPS stimulated LSEC exhibit higher level of apoptosis as compared to controls.

5.8. LSEC RESPONSE TO HCV INFECTION MIGHT PLAY DUAL ROLE IN LIVER REMODELING AND FIBROGENESIS.

Significant number of DEGs (and Kegg pathways) was related to blood vessel development.(109) The formation of new vessels and the establishment of an abnormal angioarchitecture of the liver is a process strictly related to the progressive fibrogenesis. The process of liver remodeling typical for chronic viral hepatitis was illustrated by an overexpression of several growth factors, cytokines and metalloproteinases that have been shown to exert a potent profibrogenic role

and stimulate LSEC proliferation and migration.(109) However, RNA-seq analysis revealed downregulated vascular endothelial growth factor (*VEGFC,* 2.1-fold, Figure 20), angiopoietin 2 (*ANGPT2*, 1.5-fold), fibroblast grow factor 5 (*FGF5*, 1.6-fold), and 16 (*FGF16,* 2.1-fold), all playing crucial role in angiogenesis.

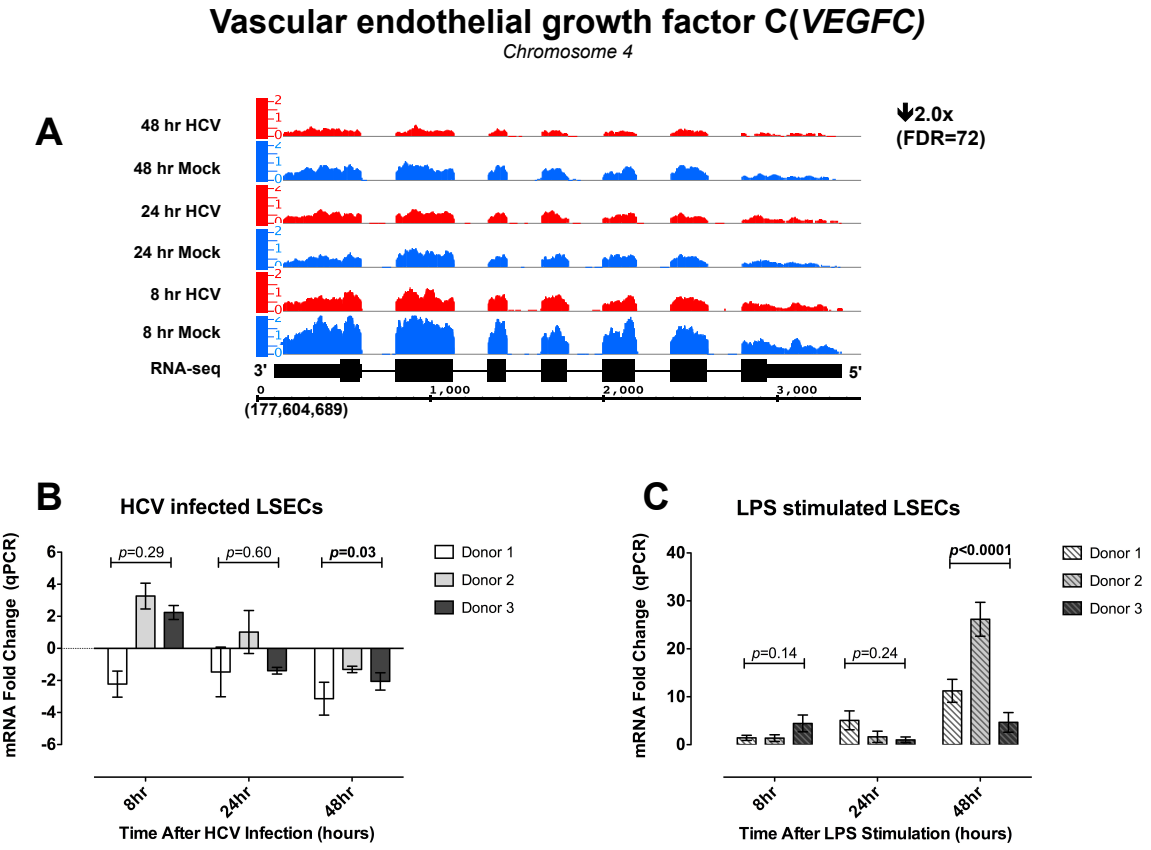


Figure 26. Decreased expression of *VEGFC* in HCV exposed LSEC. **Panel A.** Gene expression is displayed as the RPKM using the Integrated Genome Browser (IGB). **Panel B.** and **Panel C.** show qPCR verification analysis of 9 additional HCV+/- and LPS +/- samples

VEGF-driven responses in endothelial cells are considered to be a crucial in both physiologic and pathologic angiogenesis. Upregulation of transcripts involved in cell growth and survival (*PLCG2*, *SHC2*, *BAD*), actin organization and cell migration (*NOS3*, *MAPK11*, *MAPK12*, *HSPB1*) was observed after HCV infection.

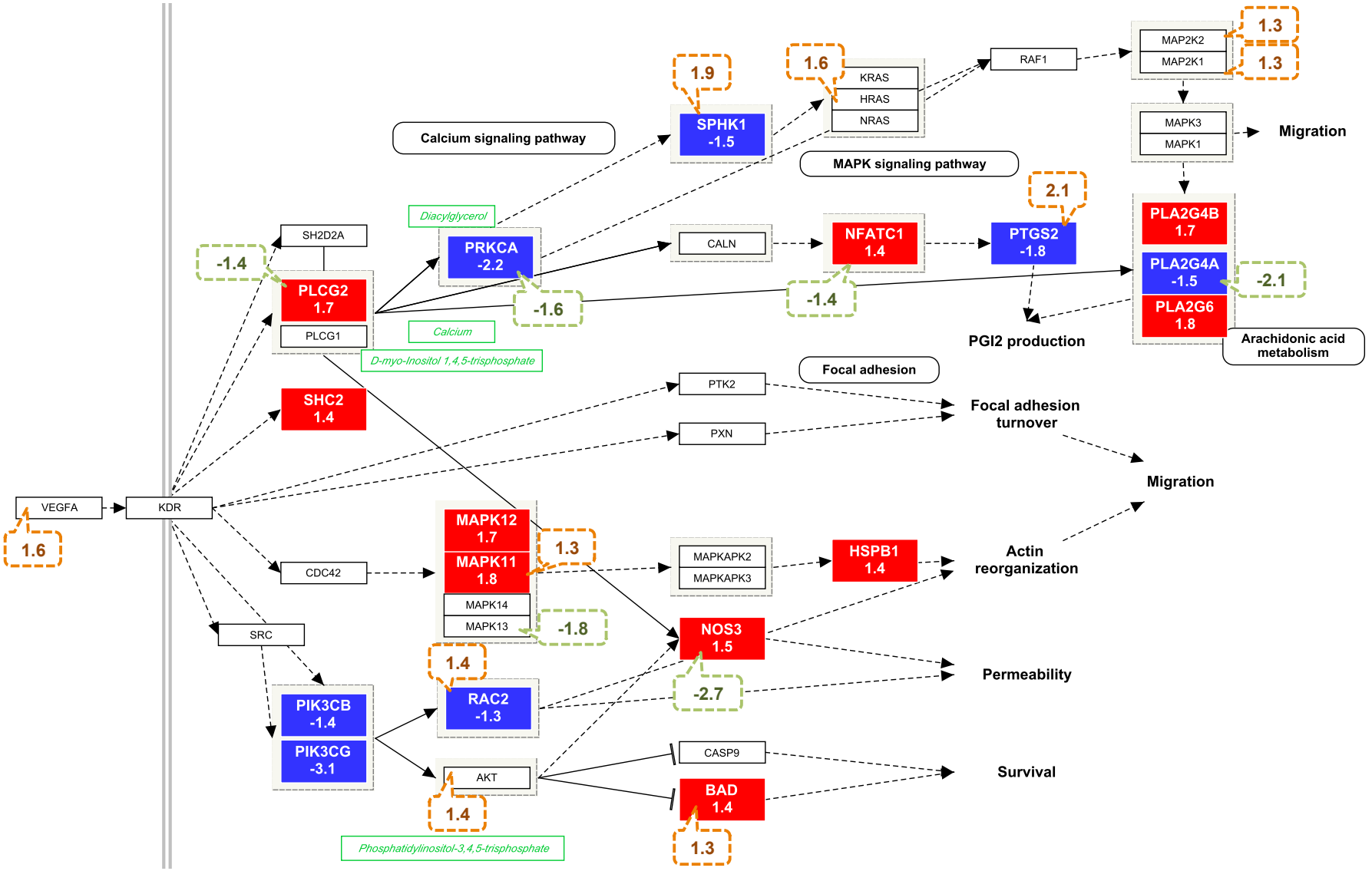


Figure 27. Enrichment of VEGF signaling pathway in HCV infected LSEC. Downregulated genes are shown in blue and upregulated in red. Fold changes of LPS exposed LSECs are shown in orange (upregulated) and green (downregulated), dashed brackets.

Several members of metalloproteinase family were also downregulated in addition to previously described absence of potent inflammatory response and changes in pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-6) that have been shown to stimulate angiogenesis. In contrast, several transcripts involved in endothelial invasion were significantly upregulated. Aquaporins are proteins embedded in the cell membrane that regulate the flow of water.(110, 111) However, they play an important role in cell migration and invasion that may contribute to angiogenesis and cirrhosis.(112, 113) Interestingly an AQP single-nucleotide polymorphism was recognized as part of a genetic signature that can identify patients at risk for HCV disease progression to cirrhosis.(114)

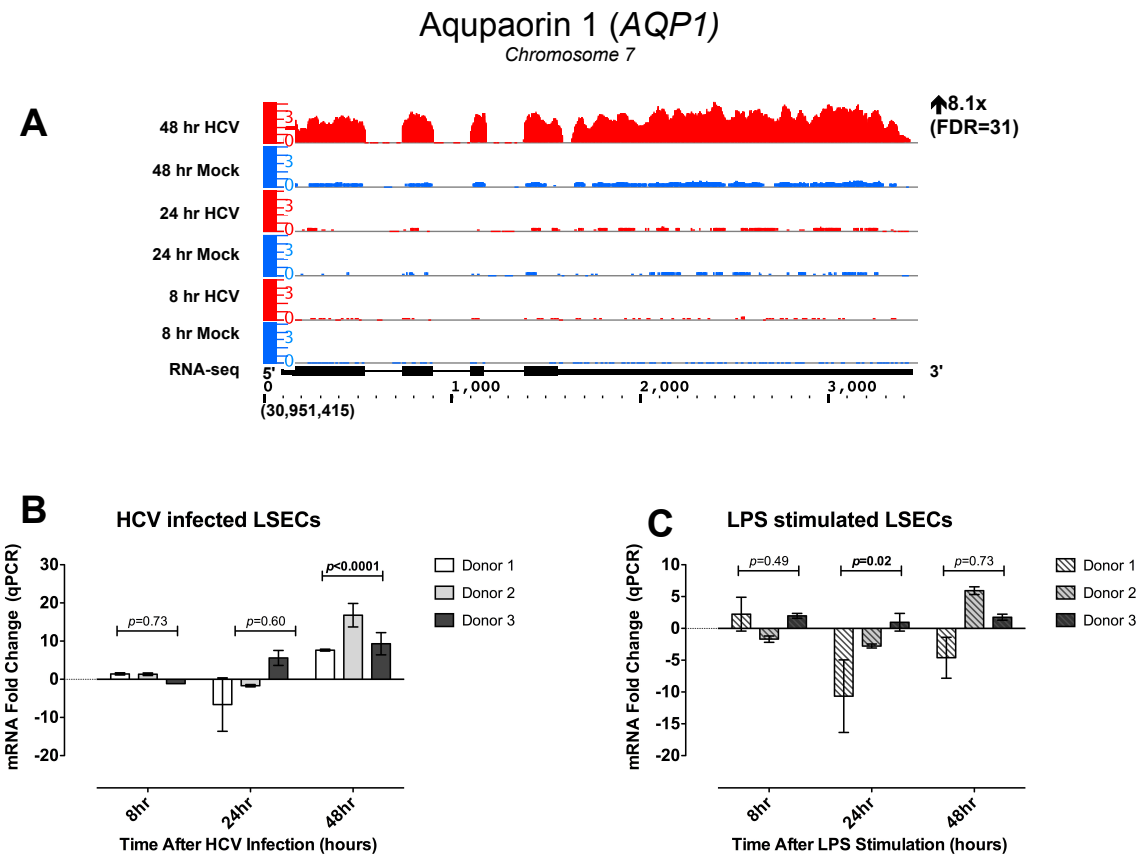


Figure 28. Increased expression of *AQP1* in HCV exposed LSEC. **Panel A.** Gene expression is displayed as the number of sequencing reads per kilobase of gene length per million reads (RPKM) using the Integrated Genome Browser (IGB). **Panel B.** and **Panel C.** show qPCR verification analysis of 9 additional HCV+/- and LPS +/- samples.

We found upregulated several members of aquaporin family, such as *AQP1*, *AQP3*, *AQP5* with 6.23-, 4.02-, 2.31-fold increase, respectively. We confirmed upregulation of *AQP1* with qPCR analysis on 27 additional samples. Interestingly, all *AQP1*, *AQP3*, *AQP5* were significantly downregulated in LPS stimulated LSECs (3.0-, 2.1-, 2.0-fold decrease, respectively) suggesting that upregulation of AQPs contributes to HCV specific liver pathology.

Role of the LSECs in liver fibrosis has been controversial. Since changes in LSEC can be detected significantly before fibrosis is microscopically visible it was suggested that LSEC might drive or even initiate fibrosis, particularly in the early stages before hepatic stellate cells undergo myofibroblastic differentiation. RNA-seq results suggest that LSEC may be involved in liver fibrosis on several different levels.(115)

First, we found upregulated several transcripts coding for basement membrane glycoproteins, such as collagen IV (*COL4A1* 2.1- and *COL4A2* 1.25-fold increase), laminin (*LAMB2* 1.3-fold increase), enactin (*NID1* 1.27- and *NID2* 2.15-fold increase), lectins (*LGALS3BP* 1.96-, *LGALS9* 2.32-fold increase), aggrecan (*ACAN* 2.68-fold increase), thrombospondins (*THBS2* 1.95- and *THBS3* 1.51-fold increase). Integrins (*ITGB2* 2.7, *ITGA2* 2.3, *ITGB8* 2.2, *ITGAV* 1.9-fold decreased), which play major role LSEC activation, were significantly decreased. In addition, gap junction molecules were upregulated after HCV infection (such as *GJA4* 2.56- and *GJA5* 2.1-fold increase). Upregulation of these transcripts might suggest capillarization, loss of fenestrations and formation of an organized basement membrane in the Space of Disse that has been recognized as one of the hallmarks of liver fibrosis. Moreover, it appears that HCV by itself is sufficient to induce these changes in the absence of signals from surrounding cells in liver microenvironment.

Second, many transcripts coding the fibrillar collagens were upregulated (*COL1A* 2.56-, *COL1A2* 1.48-, *COL3A1* 1.77-, *COL5A3* 2.15-, *COL6A1* 2.55-, *COL6A2* 3.02-. *COL6A3* 2.13-, *COL7A1* 2.4- fold increase). Although stellate cells are main source of extracellular matrix (ECM) synthesis and deposition,(116) our

study showed that LSECs can synthesize these molecules and participate in ECM deposition.

Third, we found more than 130 DEGs related to cell adhesion and ECM-cell interaction (such as *SELPLG* 5.44- and *SELP* 3.26-, *ITGB4* 3.26-, *ITGA9* 2.57-, *PCDHB7* 2.56- *SPON2* 2.55-, *NLGN3* 2.51-, *ADAMTS13* 2.43- fold increase). Many of these DEGs are coding for soluble, secreted molecules that are involved in regulation of inflammatory, angiogenic and fibrogenic processes and has not been previously related with HCV infection (such as *CLEC3B* 4.1-, *MATN2* 3.2-, *SMOC1* 2.1-, *TNXB* 2.6- fold increase).

Forth, some of the enriched Kegg pathways (e.g. Peroxisome proliferator-activated receptors (PPAR) signaling pathway, ECM-receptor interaction, Renin-angiotensin system) are involved in fibrosis progression. Angiotensin I converting enzyme (*ACE*) was upregulated 3.4-fold in HCV infected LSECs, but not in LPS stimulated LSECs (1.6-fold decrease). Recent studies have shown that the renin-angiotensin system (RAS) plays a pivotal role in liver fibrosis and the clinically used angiotensin-converting enzyme (ACE) inhibitor (ACE-I), and AT1-R blockers (ARB) significantly attenuated experimental liver fibrosis.(117, 118)

However, the efficiency of these changes and contribution to the fibrosis development is unclear. At the same time pathways like TGFβ and inflammatory molecules are downregulated, suggesting that fibrotic role of LSECs could be more complicated in *in vivo* environment.

5.9. CHANGES IN KEGG PATHWAYS IN HCV INFECTED LSEC

In order to further investigate and visualize genes interaction, the RNA sequencing database of differentially expressed genes was analyzed to determine which of the 228 KEGG pathways were significantly enriched (altered) during HCV infection in LSECs. We identified a total of 31 significantly enriched pathways (Table 7.) The majority of enriched KEGG pathways were observed at 48 hours after infection.

KEGG PATHWAY	8h DEGs	*p* value	24h DEGs	*p* value	48h DEGs	*p* value
RIG-I-like receptor signaling pathway					16	0.04
Cytokine-cytokine receptor interaction			8	0.04	50	0.02
Complement and coagulation cascades			4	0.02		
Leukocyte transendothelial migration					24	0.05
Fc gamma R-mediated phagocytosis					21	0.03
Lysosome					28	<0.001
Cell adhesion molecules (CAMs)			5	0.05	34	<0.001
Focal adhesion			7	<0.001	47	<0.001
Axon guidance	13	<0.001	5	0.04	35	<0.001
TGF-beta signaling pathway					19	0.04
MAPK signaling pathway					49	0.05
Cell Cycle					30	<0.001
VEGF signaling pathway					17	0.04
Hematopoietic cell lineage					19	0.04
Renin-angiotensin system					6	0.03
PPAR signaling pathway			4	0.02	18	<0.001
ECM-receptor interaction			4	0.03	29	<0.001
Ubiquitin mediated proteolysis	10	0.03				
ABC transporters			5	<0.001		
Apoptosis					19	0.04
mTOR signaling pathway	5	0.05				
p53 signaling pathway	6	0.04			16	0.03
Wnt signaling pathway	10	0.05				
Pathways in cancer	29	<0.001			61	0.02
Basal cell carcinoma	6	<0.001				
Pancreatic cancer	7	0.01				
Bladder cancer	5	0.02				
Small cell lung cancer	7	0.03			20	<0.01
Amino sugar and nucleotide sugar metabolism					13	<0.001
Other glycan degradation					6	<0.01
Purine metabolism					31	0.03

Table 7. List of enriched KEGG pathways (Kyoto Encyclopedia of Genes and Genomes).

Enriched were KEGG pathways related to cell scavenger functions (e.g. Fc gamma R-mediated phagocytosis, Cell adhesion molecules, Focal adhesion, Leukocyte transendothelial migration, Axon guidance), innate immune responses (RIG-I-like receptor signaling, cytokine-cytokine receptor interaction, complement and coagulation cascades) and cancer development (Pathways in cancer, Apoptosis, p53 signaling, Wnt signaling).

The TGFβ signaling pathway was previously reported deregulated during acute HCV infection and regulates cell cycle arrest, apoptosis, and fibrosis under experimental conditions.(10, 62) Interestingly, pathways that might be involved in fibrosis progression (e.g. PPAR-signaling pathway, ECM-receptor interaction, Renin-angiotensin system) were also significantly altered.

.

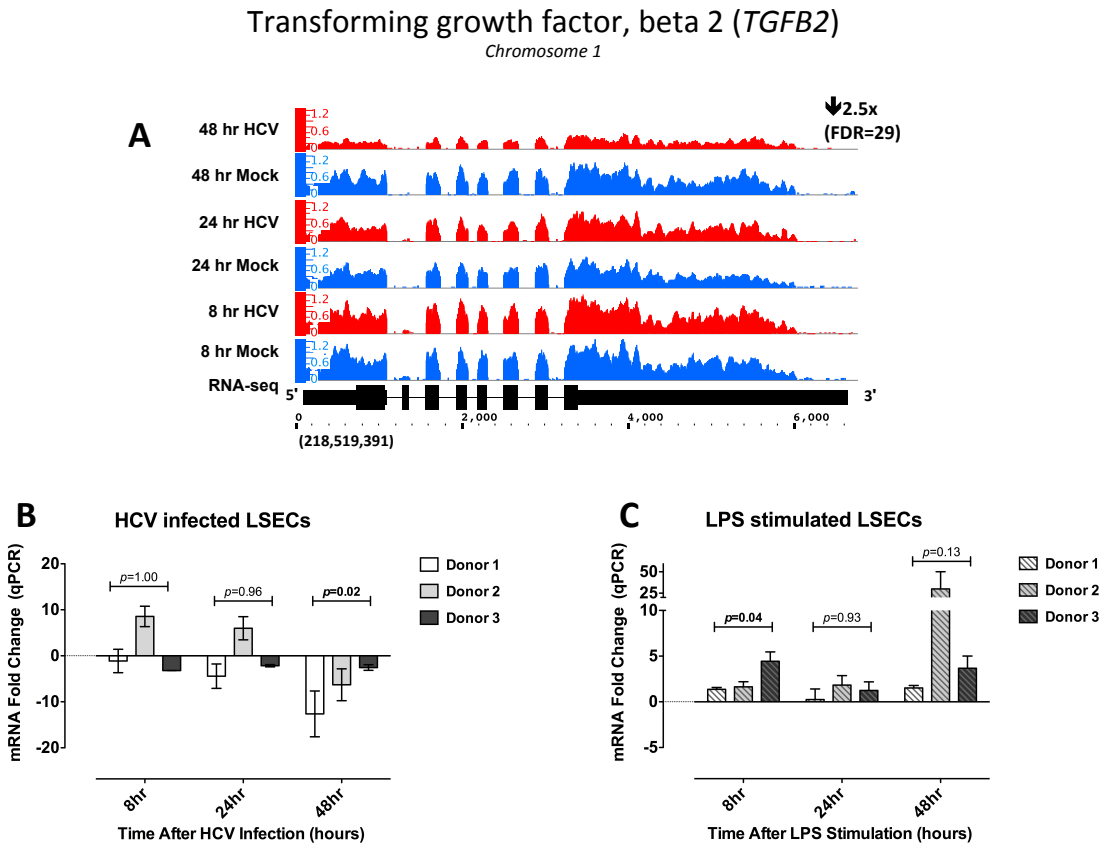


Figure 29. Panel A. Gene expression is displayed as the RPKM using the Integrated Genome Browser (IGB). **Panel B.** and **Panel C.** show qPCR verification analysis of 9 additional HCV+/- and LPS +/- samples.

Furthermore, in closer look to the gene changes within the specific pathways, some of them are differently regulated than in other cell lines, such as TGFβ/BMP that is upregulated in Huh 7.5 cells. In addition to downregulated *TGFB2* (2.5- fold decrease, Figure 29.), we found significantly downregulated SMAD family members, *SMAD3* and *SMAD5* (1.5- and 1.4-fold decrease, respectively) important for signal transduction. Furthermore, inhibitory *SMAD6* and *SMAD7* were upregulated 1.4- and 1.25-fold, and transcriptional target of TGF-β/BMP signaling, *RUNX1* transcriptional factor, was downregulated 2.1-fold. Genes involved in TGF-β trafficking and activation *LTBP1* and *LTBP2* were significantly downregulated, both 1.6-fold. These data suggest that TGF-β pathway is inhibited n HCV infected LSECs on several different levels (Figure 30.).

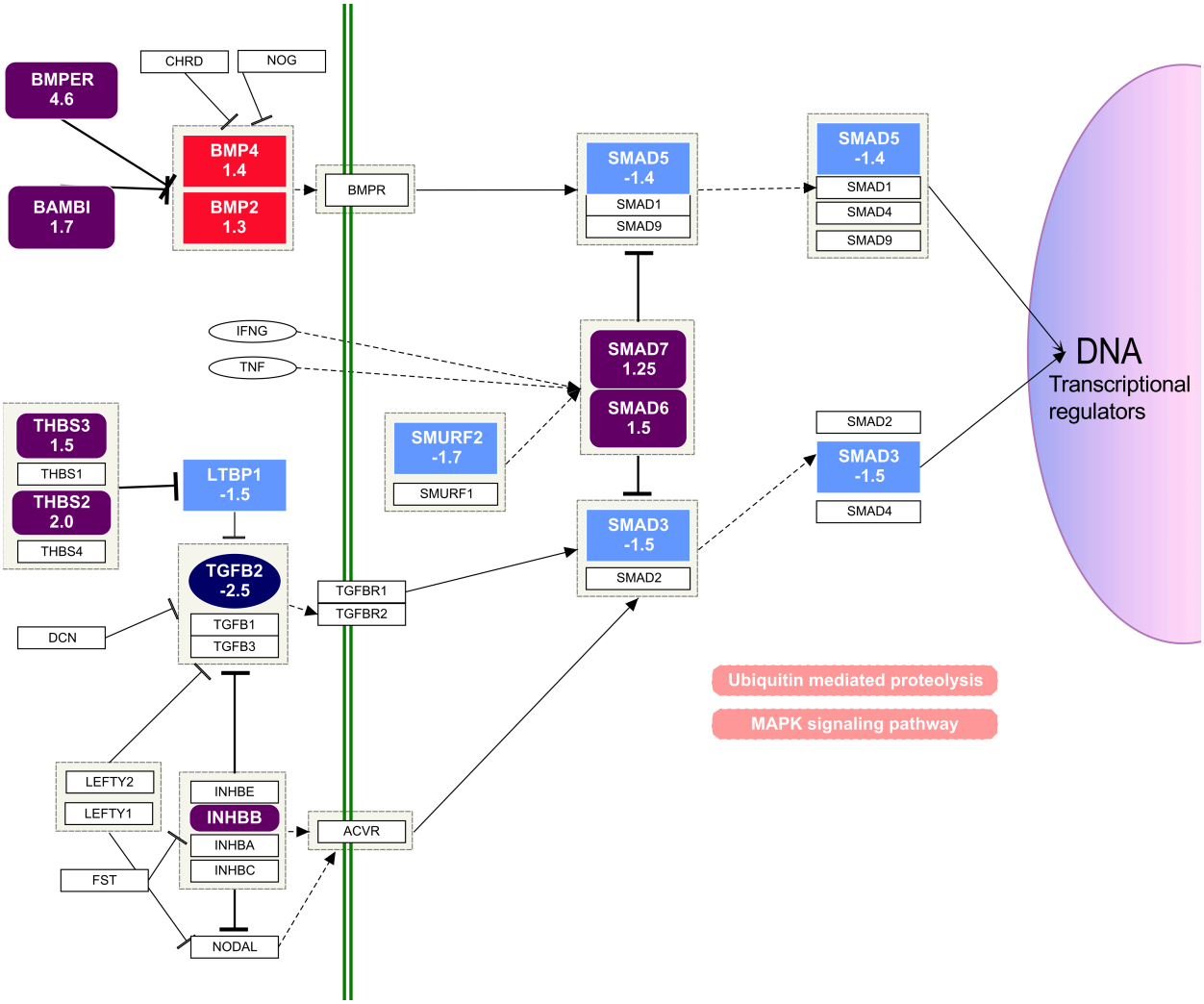


Figure 30. Enrichment of TGF-β signaling pathway 48 hours after HCV exposure

Interestingly, several pathways and more than 100 DEGs were directly related to cytoskeleton reorganization or vesicle mediated transport. Fc gamma R-mediated phagocytosis plays an essential role in host defense response through the uptake and destruction of infectious pathogens. In addition, Cell adhesion molecules, Focal adhesion, Leukocyte transendothelial migration and Axon guidance pathways play important roles in biological processes including cell motility, proliferation, differentiation, regulation of gene expression and cell survival. Signaling events of these pathways culminate in reorganization of actin cytoskeleton and membrane remodeling. These processes may be hijacked by HCV to facilitate its infectivity and entry into relatively safe intracellular environment. LSECs may use them as a detour for activating gene expression in the presence of inefficient PRR-signaling that does not result in the activation of the inflammatory responses.

Since it was reported that HCV utilizes endocytosis pathway to escape lysosomal degradation,(57) we examined related pathways. In Fc gamma R-mediated phagocytosis pathway, transcripts involved through the actin cytoskeleton rearrangements and membrane remodeling to the formation of phagosomes were significantly upregulated. These include: Dynamin 1 (*DNM1*, +1.9x), MARCKS-like 1 (+1.5x), phospholipase A2 (encoderd by *PLA2G6*, +1.8x), regulator of actin polymerization encoded by *TTLL3* (+1.7x) and gelosolin (+2.1x). Furthermore, several lysosomal acid proteases were significantly upregulated: cathepsins (*CTSA* +1.4x, *CTSC* -1.4x, *CTSD* +1.6x, *CTSF* +2.0x, *CTSH* +1.5x, *CTSL2* +2.1x), glycosidases (*GAA* +2.0, *IDUA* +1.8x, *HEXA* +1.4x, *NEU1* +1.3x), sulfatases (*ARSA* +1.5x, *ACP2* +1.4, *ACP5* 9.1x). Meanwhile clathrin (*CLTC* -1.34x), lysosomal structural proteins (*NPC1* -1.4x, *LAMP3* -1.5x) and *AP4E1* (-1.5x) were downregulated. Upregulated were also genes coding for early and late endosome (*FAM125A* 1.4x, *VPS28* 1.4x, *VPS37D* 2.0x, *EHD3* 1.7x).

Surprisingly the only pathway enriched at all time points was axon guidance. Axon guidance represents a key stage in the formation of neuronal network and involves a variety of guidance factors, such as semaphorins. However, accumulating evidence indicates that several semaphorins play additional diverse roles in unrelated processes to axon guidance, including organogenesis,

vascularization, angiogenesis, neuronal apoptosis, and neoplastic transformation.(119) Moreover, newly recognized “immune semaphorins”, are crucial to various phases of the immune response, from initiation to terminal inflammatory processes.(119, 120) The Figure 31 shows changes in mRNA levels of molecules within sempahorins signaling pathway (as a part of the axon guidance pathway) in HCV infected LSECs. It seems that final outcome of semaphorins signaling could be inhibition of pathway on receptor or downstream levels. Interestingly, semaphorins can exist in membrane-bound and soluble form, which can target immune cells.(119, 120)

Examples include:

- SEMA3A (2.1-fold decrease) known to inhibit spontaneous monocytic cell migration.
- SEMA4A (2.4-fold increase) that can provide T cell costimulation and the induction of either Th1-cell-mediated IFN-γ production or Th2-cell-mediated IL-4 production, depending on the respective culture conditions.
- *SEMA6B* (1.4-fold increase) that might bind to and activate dendritic cells and increase type I interferon production.
- *SEMA7A* (2.6-fold increase) stimulates monocytes/macrophages to provoke proinflammatory cytokine production.

So far semaphorins have not been related with HCV infection.

In our previous study on HCV infected Huh 7.5 cells we found upregulated several semaphorins (such as *SEMA3B* 2.4, *SEMA4C* 1.5, *SEMA6A* 1.7, *SEMA7A* 3.2) and their receptors (*PLXNA1* 1.9-, *PLXNA3* 2.0-, *PLXNB1* 1.3-, *PLXNAB2* 1.6-fold). Similarly, *SEMA3C*, *SEMA4A* and *SEMA6B* were upregulated in HCV infected TPH-1 cells (16.5-, 2.2- and 18.5- fold). This suggests an important, previously unexplored role of these molecules in HCV pathogenesis

.

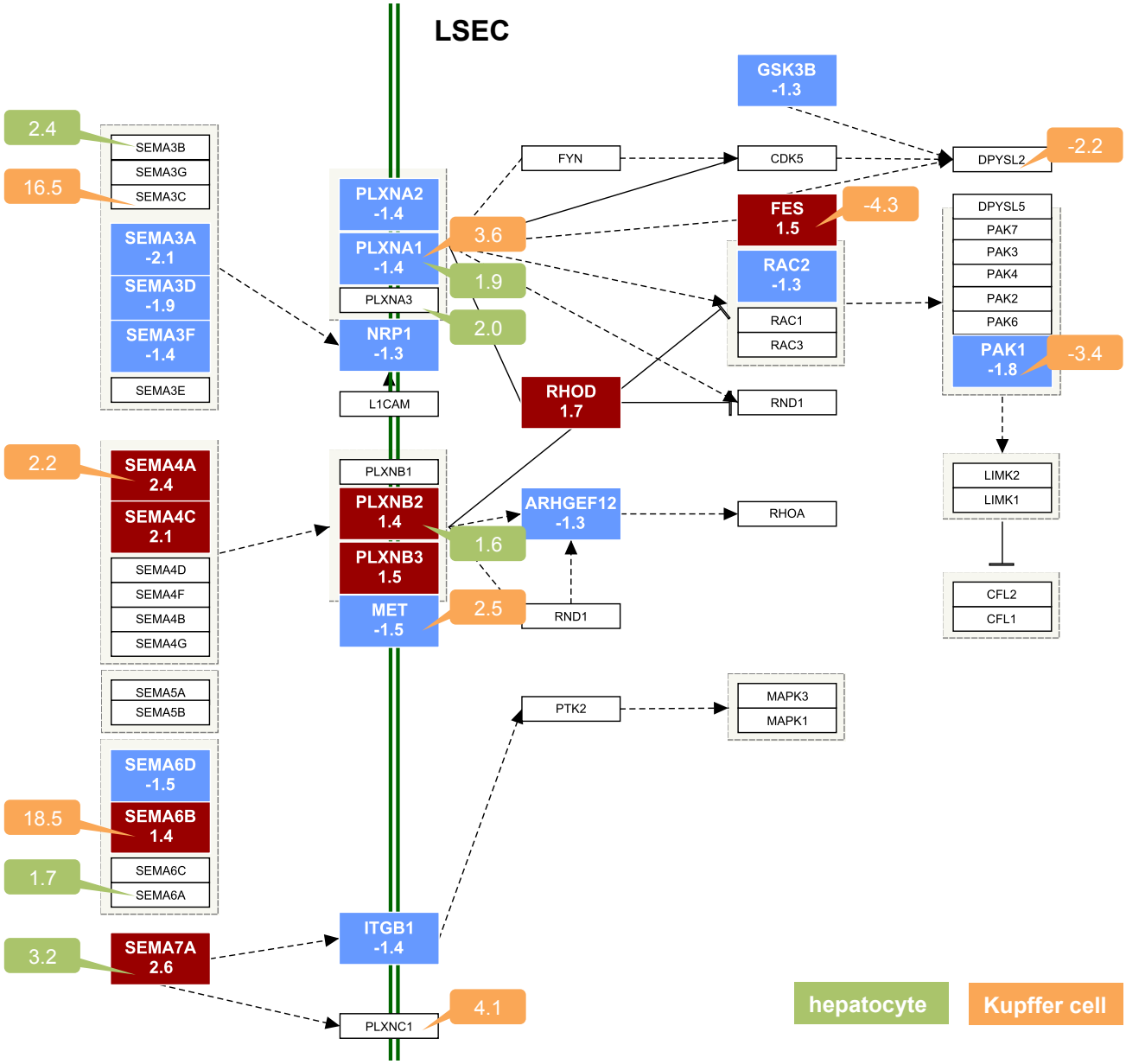


Figure 31. Enrichment of “semaphorins” pathway signaling 48 hours after HCV exposure. Downregulated genes are shown in blue and upregulated in red boxes. Green boxes show fold changes 72h after HCV infection of Huh7.5 cells, and orange 16 hours after HCV infection of THP-1 cells.

5.10. LSEC, HEPATOCYTES AND KUPFFER CELLS MIGHT PLAY DISTINCT ROLES DURING HCV INFECTION

Applying the system biology approach to determine the drivers of inflammation in HCV infected liver, a comprehensive RNA sequencing gene expression analysis of models of hepatocytes (Huh 7.5 cells), substitute model of Kupffer cells (THP-1), primary human LSECs, plus mild (inflammation but no fibrosis) and severe (cirrhosis) chronic hepatitis C liver specimens were performed.(10, 26, 27) This helped to identify distinct gene expression profiles in HCV infected LSECs, KCs and hepatocytes, suggesting their different roles during HCV infection.

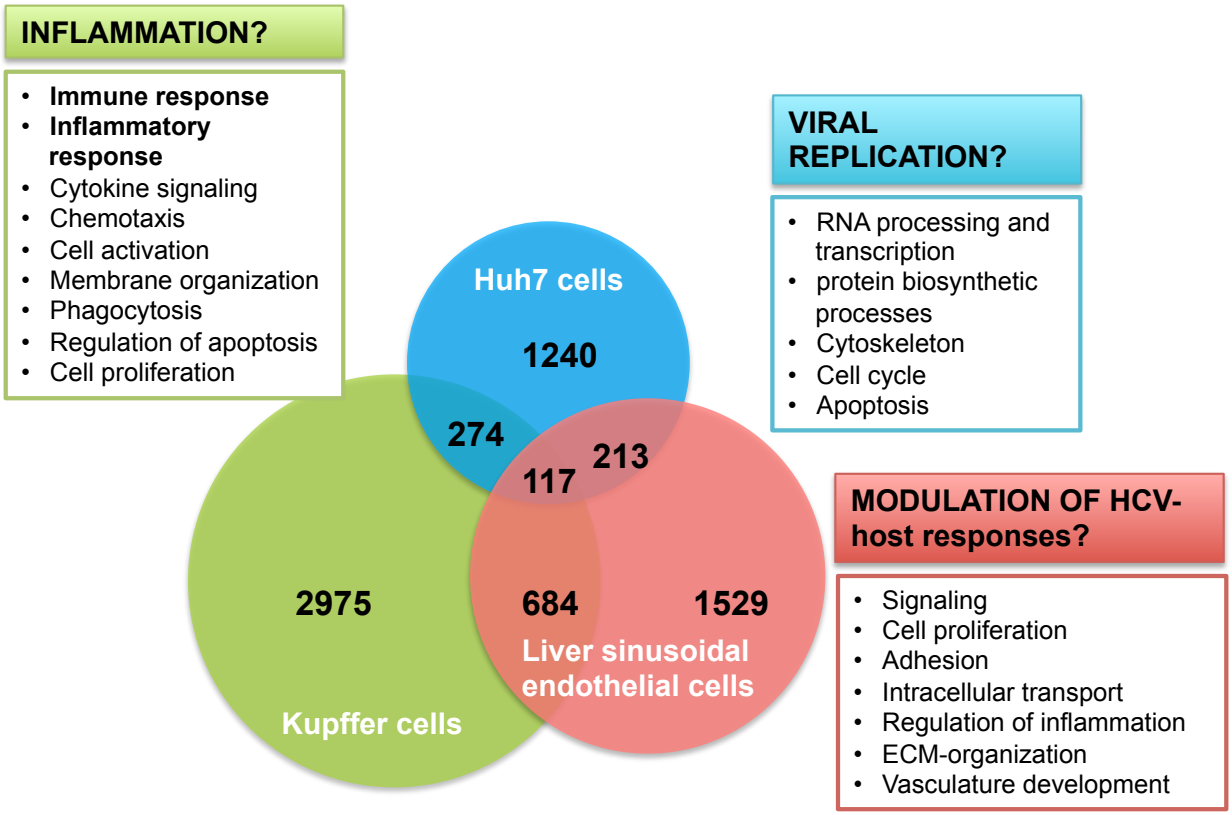


Figure 32. Differentially expressed genes after HCV infection of Huh 7.5, THP-1 and LSEC cell cultures including the most enriched GO-pathways representative to each cell type.

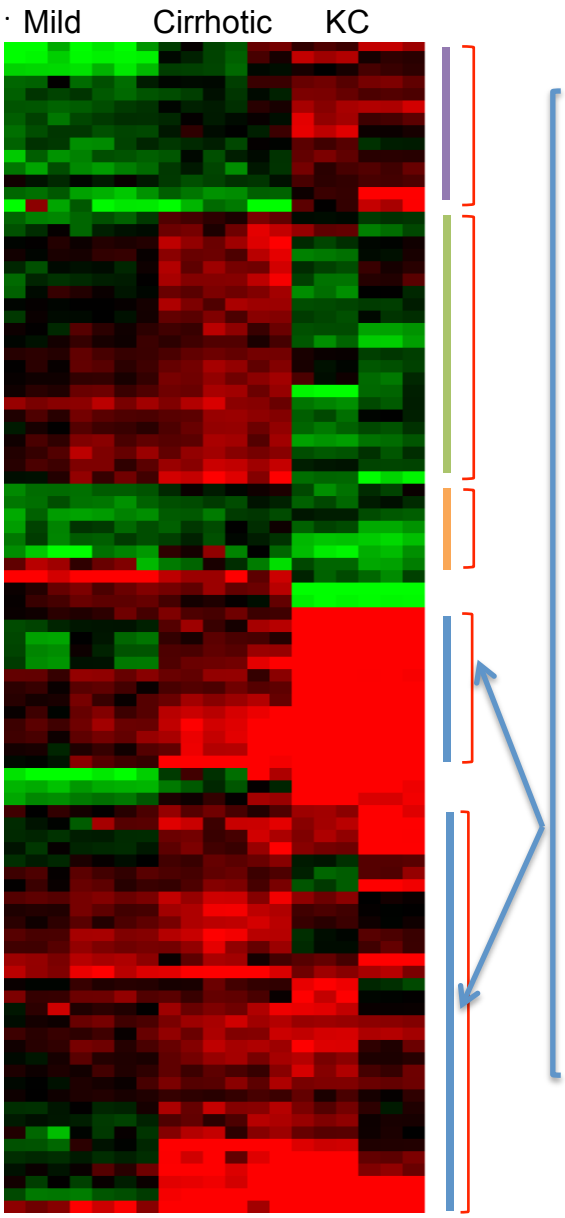
5.10.1. KUPFFER CELLS DRIVE INFLAMMATORY RESPONSES IN CHRONIC HEPATITIS C

RNA-seq analysis of HCV infected THP-1 cells revealed a broad and complex cellular transcriptional reprograming in wide range of gene functional categories. Analysis of differentially expressed genes (DEGs) identified 4050 and 3079 significantly altered genes in THP-1 cells at 6 and 16 hours post HCV-exposure, respectively. In contrast to similar studies on hepatoma cell lines and LSEC, where the overall effect of HCV infection on cellular gene expression was subtle in the early phases of infection, in macrophages fold change ranges from 324-fold increase of *CTGF* (connective tissue growth factor) to 60-fold decrease of *DHRS9* (dehydrogenase/reductase (SDR family, member 9).

Similarly as in LSEC, THP-1 cells do not support viral replication, so these changes are not related with HCV replication cycle, viral titer or presence of dsRNA, as in Huh 7.5 cells where the host transcriptional response corresponds closely to the levels of HCV replication. Interestingly, in comparison to acutely infected Huh 7.5 cells, approximately 80% of changed transcripts were unique for KCs. In addition, HCV-activation of KC results in the release of an array of inflammatory and immune mediators.

Several hundreds of genes participate in the inflammatory response and their coordinated expression is tightly regulated. Most of these genes share some remarkable features regarding their role in biosynthesis and degradation of inflammatory mediators. This includes a dramatic and broad increase in *IL-1β* (81-fold increase 16h after HCV exposure in contrast to 27-fold increase after LPS stimulation) and NFκB responsive proinflammatory cytokine and chemokines expression (such as *IL12*, 132-increase; *IL8*, 38x; MIP1b or *CCL4*, 34x; MIP1a or *CCL3*, 24x; *CCL22* 176x; *CXCL1* 100x, *CXCL2* 68x, *LIF* 48x; *CSF3* 189x). Meanwhile, modest increases in anti-inflammatory cytokines occurred (*IL10* 16x; *IL1RN* 7x; *IL11* 15x; *TNFRp75* 3x). Interestingly, this significantly correlated with LPS stimulated cells, therefore suggesting a similar pattern of inflammatory response, in contrast to LSEC. Importantly, in comparison with chronic hepatitis C

liver specimens, the expression of this gene correlated with disease severity. These observations imply that in patients with chronic hepatitis C, Kupffer cells and/or infiltrating liver macrophages produce IL-1β, driving a hepatic response that includes the expression of a wide range of proinflammatory mediators of liver inflammation, fibrogenesis and disease.



IL8	38x	**CCL17**	22x
CCL22	176x	**CD70**	9x
IL1B	22x	**CCL24**	5x
CXCL2	68x	**OSM**	4x
CXCL3	40x	**CXCL5**	5x
CXCL1	100x	**RELT**	3x
CD40	30x	**CCL5**	2x
TNFRSF9	20x	**NCF1**	8x
TNFRSF18	40x	**CXCL12**	6x
CCL2	35x	**ITK**	32x
CCL3	25x	**CCR4**	7x
CCL4L2	32x	**IL2RG**	7x
CCL3L1	28x	**IL7R**	39x
TIAM2	9x	**INHBB**	9x
IL10RA	4x	**CXCR4**	7x
IL3RA	7x	**IL6**	64x
CSF2	161x	**LIF**	48x
VEGFC	3x	**CXCL6**	60x
CXCL14	3x	**PDGFB**	43x

Figure 33. Cluster map showing the expression of genes linked with inflammation in THP-1 cells and in chronic HCV livers. Selected genes that were commonly expressed in both THP-1 cells and chronic HCV livers are shown in table.

Unlike RNA-seq analysis of macrophages, HCV did not induce a marked increase in such inflammatory signals in both LSECs and Huh7.5 cells. In contrast, HCV in general induced a downregulation of inflammatory signals in LSEC.

IL8	-3x	**ITLN1**	7x	**CEBPD**	3x
IL10	2x	**C1QTNF**	4x	**CSF3**	-4x
A2M	6x	**SERPING1**	7x	**HIF3A**	3x
MAFB	4x	**APOE**	2x	**IRF6**	-2x

Modulation of Immune response

IL-1β inflammatory networks

IL8	38x	**CCL17**	22x	**TIAM2**	9x
IL1B	22x	**CCL24**	5x	**INHBB**	9x
CXCL2	68x	**OSM**	4x	**PDGFB**	43x
CXCL1	100x	**CXCL6**	60x	**IL7R**	39x
CCL3	25x	**CCR4**	7x	**CSF2**	161x

Figure 34. Differentially expressed genes (Gene Ontology: Immune response) in THP-1 and LSECs cultures. Venn diagram shows a different pattern of gene expression in THP-1 and LSECs. Examples of significantly changed genes are shown in tables.

Thus, selective dysfunction of KC and LSEC might be relevant by profoundly affecting the immune responses against HCV at the level of the infection site.

5.10.2. TRANSCRIPTIONAL RESPONSE IN HCV INFECTED HEPATOCYTES

RNA-seq analysis identified 100, 684, and 1,844 significantly differentially expressed annotated genes in acutely infected Huh 7.5 cells at 6, 48, and 72 hours, respectively. The number of differentially expressed genes correlated closely with HCV replication, which was not the case in both THP-1 and LSEC cultures.

Gene ontology analysis of those, identified major changes within regulation of transcription, RNA processing, metabolism, signaling, cell cycle and apoptosis pathways (Figure 35.). Significantly, a subset of genes within these pathways was similarly regulated in HCV-infected livers and their expression correlated with fibrosis level. Meanwhile, only small changes within inflammatory cytokine-chemokine networks were observed.

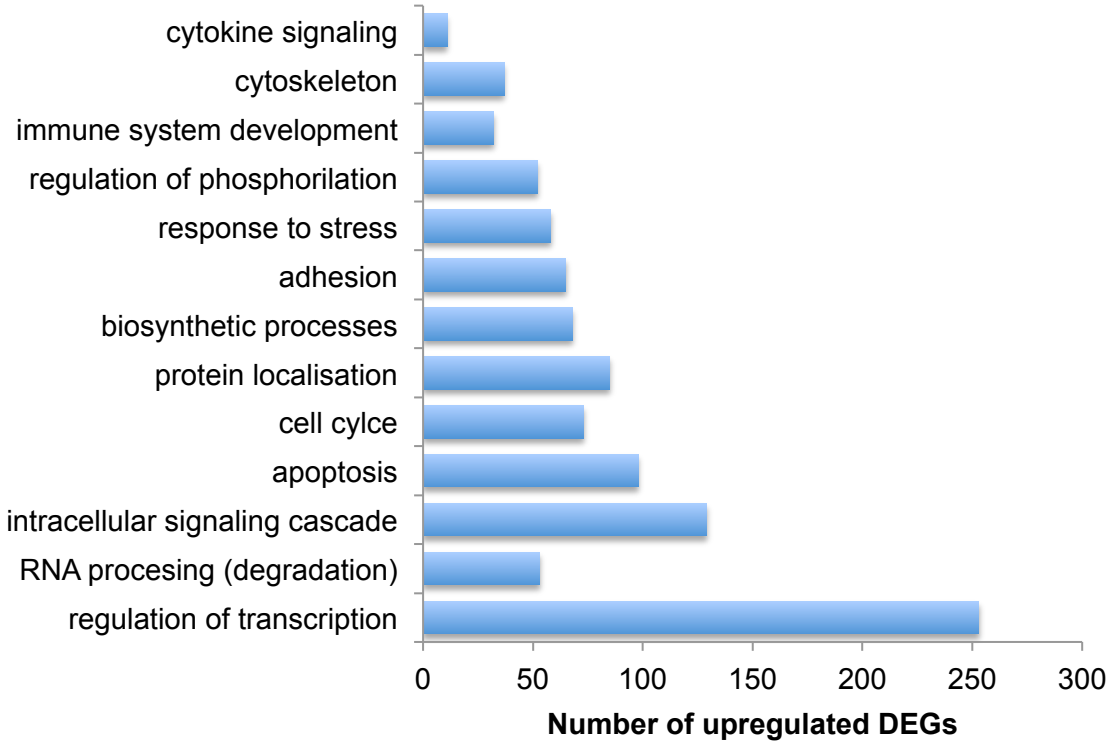


Figure 35. Gene ontology analysis of HCV infected Huh7.5 cells.

Furthermore, a significant number of altered genes is coding for a building blocks of the cell membrane, cytoskeleton, endoplasmic reticulum and nucleus.

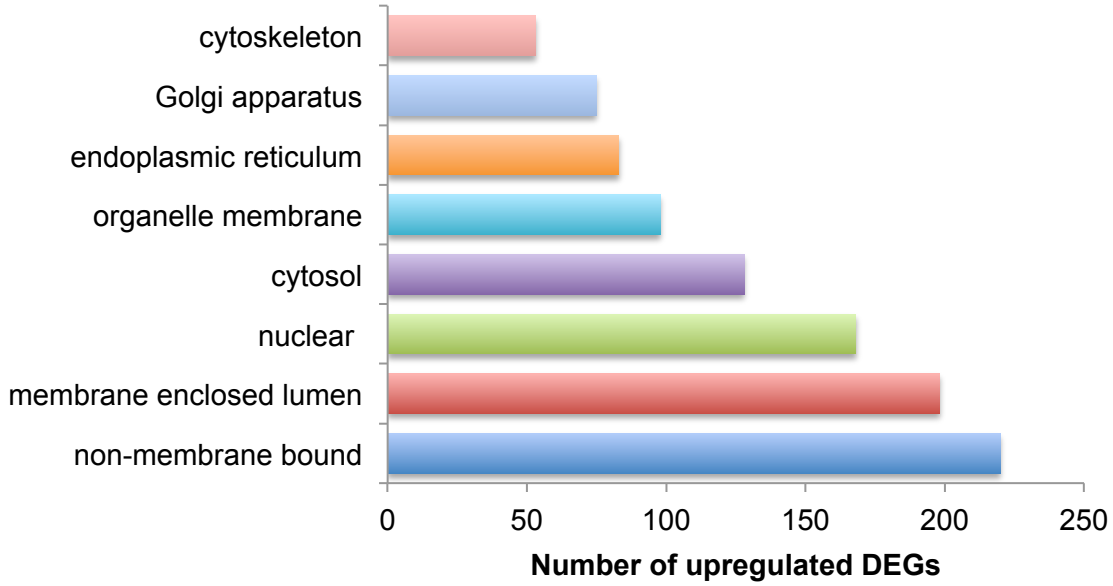


Figure 36. Functional analysis of DEGs in HCV infected Huh7.5 cells.

This is suggesting that in order to establish efficient and persistent infection, HCV rearrange cytoskeleton and membranes that are required for formation of replication complex. Follow up studies on some of these genes supports this concept. Silencing of fucosyltransferase 1 (*FUT1*, involved in protein glycosylation) in Huh 7.5 cells inhibited HCV RNA replication by up to 80% of controls and infectious virion production was inhibited by 50%.(10) Similarly, silencing of kelch-domain containing 7B (KLHDC7B, regulates protein-protein interactions), inhibited HCV RNA replication and virion production by 70%.(10)

Pathways regulating cell-survival, proliferation and growth responses (insulin signaling, Notch signaling and *c-myc* pathways) were significantly enriched as well as did the pathways stimulating apoptosis and cell death. Since all these changes are growing demand for energy, alternation of metabolic pathways (such as insulin signaling, amino acid and lipid metabolism) was not surprise.

These results suggest, that HCV has developed mechanisms to subvert this aspect of the host antiviral response to establish a safe harbor for replication and viral persistence.

5.10.3. LIVER SINUSIDAL ENDOTHELIAL CELL SPECIFIC PATHWAYS IN CHRONIC HEPATITIS C LIVERS

A global transcriptome analysis of RNA recovered from liver biopsies of normal control (donor) or chronic HCV-infected livers of patients staged according to mild (mild inflammation and no fibrosis) or severe (cirrhosis undergoing liver transplantation) disease was performed, and the list of differentially expressed genes was compared with LSEC-RNAseq data. Total of 633 DEGs were commonly expressed in LSEC and mild, and 671 in LSEC and cirrhotic livers.

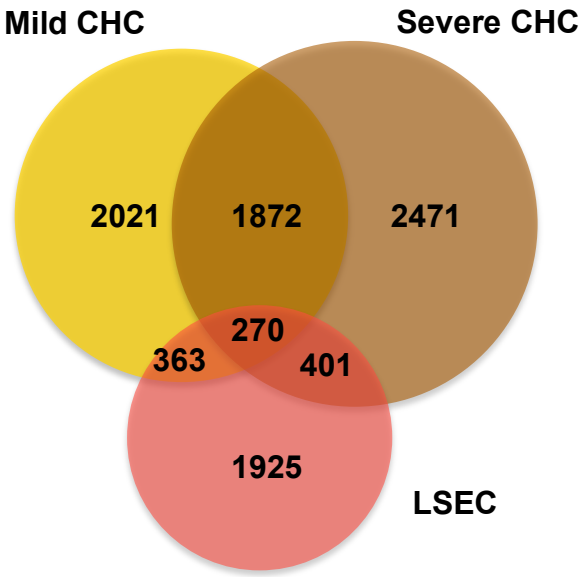


Figure 37. Differently expressed genes (DEGs; FDR.13 and fold change ≥1.5) HCV+/LSECs, mild chronic hepatitis C (CHC) (HCV+, no fibrosis) and severe CHC (HCV+, cirrhotic liver biospecimens.

Majority of these genes were involved in regulatory pathways including: regulation of cell proliferation and migration (84 DEGs), adhesion (70 DEGs), response to wounding (50 DEGs), defense response (46 DEGs). The list of commonly expressed Gene Ontology terms is shown in Figure 38.

Significantly, the integration of the *in vitro* and patient liver gene expression data also suggests that these processes contribute to liver disease progression. Interestingly, the increased expression of a subset of these genes appeared to be associated with liver disease progression.

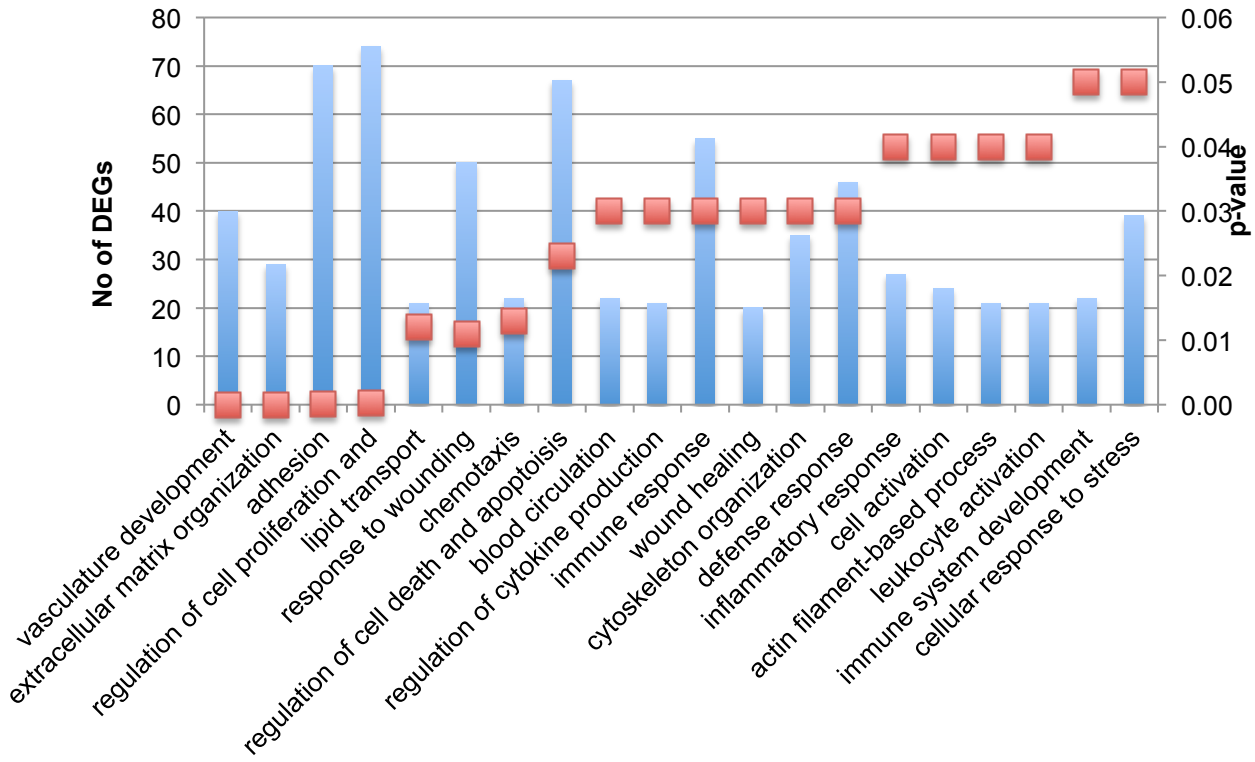


Figure 38. Gene Ontology analysis shows significant overlap of pathways between chronic livers and LSECs.

Collectively, these data demonstrate that, despite JFH-1 being somewhat of an atypical HCV, transcriptional changes which occur in HCV-infected LSEC parallel those which occur specifically during fibrosis development in HCV-infected patients.

Since previous reports suggested that LSEC acquire enhanced immunogenicity in the setting of liver fibrosis related to the establishment of an abnormal angioarchitecture,(41) we examined the expression of key transcripts involved in angiogenesis and vasculature remodeling in liver biospecimens.

The VEGF/VEGFR pathway is a key mediator of angiogenesis.(109) We found increasing expression of VGEFA and VGEFC with level of fibrosis (*VGEFA* 1.2-fold decrease vs. 2.6-fold increase in cirrhotic livers, and *VGEFC* 1.4-fold decrease vs. 1.6-fold increase). Similarly, TGFβ was significantly upregulated in cirrhotic livers (TGFB2 13.5-fold, TGFB1 2.6-fold), while not significantly altered in mild fibrosis.

Several angiopoietins were significantly changed. In mild fibrosis, Angiopoietin-like 3 (*ANGPTL3*,), which induces endothelial cell migration and cell adhesion, was 2.5-fold increased. Meanwhile, in cirrhotic liver Angiopoietin 2 (*ANG2*) was 3.6-fold increase. ANG2 functions as an autocrine-acting, antagonistic ligand of the vessel maturation and remodeling by controlling ANG1 signaling axis. As such, ANG2, being almost exclusively produced by endothelial cells and function as a vessel-destabilizing molecule that facilitates the activities of other cytokines involved that promotes or constrains angiogenesis. Interestingly, ANG2 was identified as an autocrine regulator of endothelial cell inflammatory responses, by permitting the activation of endothelial cells by proinflammatory cytokines.(121)

Thrombomodulin (*THBD*), involved in angiogenesis by regulating the ability of endothelial cells to proliferate, invade, and adhere to each other, was downregulated in mild (4.2-fold), and not changed in cirrhotic livers.(122) Meanwhile, Thrombospondin (*THBS1*) was downregulated in mild (5.3-fold), but upregulated in cirrhotic livers (9.53-fold), as well as other *THBS2* (4.4-fold). They serve as inhibitors of angiogenesis through direct effects on endothelial cell migration and survival.(123)

One of the highly upregulated transcripts in cirrhotic livers was *RGC32* (Response gene to complement 32), 20.8-fold increased. RGC32 is induced by activation of complement and regulates cell proliferation and is recognized as an antiangiogenic factor in endothelial cells.(124)

CMA1 (chymase 1) was upregulated 9.7-fold. CMA1 is in blood vessels, rather than angiotensin converting enzyme, largely responsible for converting angiotensin I to the vasoactive peptide angiotensin II that induces angiogenesis.(125)

The abundance of these transcripts confirms the important role of LSEC in disease progression and pathogenesis.

6.

DISCUSSION

This study represents the first comprehensive snapshot into the liver sinusoidal endothelial cell transcriptome by investigating the global dysregulation of the biological pathways in HCV and LPS exposed primary human LSEC. The main objective of the study was to identify genomic signatures associated with immune response in LSEC triggered by HCV, in order to increase our understanding of the HCV disease development.

Changes in gene expression revealed a broad and complex cellular transcriptional reprograming in wide range of gene functional categories and many of these DEGs haven't been previously linked with HCV infection. Importantly, as LSEC cells do not support viral replication, these changes should not be related with HCV replication cycle, viral titer or presence of dsRNA, as in hepatocytes where the host transcriptional response corresponds closely to the levels of HCV replication. Interestingly, other characteristic of HCV induced LSEC transcriptome changes was a significant downregulation of gene expression, which was not the case in LPS stimulated cells, especially since downregulation of genes is relatively uncommon event in gene expression analysis. This might suggest a different pattern of gene reprograming as a special characteristic of LSECs, where cells shut down their normal functions that can benefit or limit virus infectivity. Indeed, in the HCV exposed cells, the key innate immune response pathways were significantly downregulated (such as RIG-I and TLR-signaling pathways), which correlated with the expression of inflammatory mediators. Notably, HCV infection induced completely diverse gene expression profiles in LSEC, hepatoma and monocyte cell cultures, suggesting LSEC's unique role in disease pathogenesis

Our data indicated that several hundreds of genes participate in the immune response and their coordinated expression is tightly regulated. These genes can be grouped into subsets as coding for various transcription factors (*NUPR1*, *IL33*, *HEYL*, *MAF*), cytokines and chemokines (*CXCL1*, *CXCL2*, *IL8*, *CCL2*, *LIF*), cellular

growth factors (*VGEFC*, *FGF*, *TGFB2*) and corresponding receptors (*CD40*, *IL6R*, *IL3RA*, *FLT4*, *EGF4*), as well as those coding for adhesion molecules (*SELP*, *SELE*, *ICAMs*). Expression patterns of the most of those genes share some remarkable features regarding their role in the modulation of inflammatory responses. In addition, we found 31 significantly enriched pathways. Since the immune-related pathways have the most direct relevance to the immune dysfunction characterizing HCV disease and this aspect has been under constant investigations, my discussion centered on the immune-related pathways top-ranked by our analysis.

The primary function of innate immunity is to recognize pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs).(11) Genome profiling of LSEC revealed expression of a large repertoire of PRRs, indicating a broad sentinel function, as previously reported in microarray analysis of murine LSEC cultures. Of those, TLR- and RIG-I system represent an important category of pathogen recognition receptors that are crucial in the first-line defense and are also important for modulating the adaptive immune response.(11-13) At the transcriptome level, the overall down-regulation of the PRR- signaling pathways manifested by the decreased TLR4 and intracellular sensors of double-stranded RNA, RIG-I and PK3 expression with subsequent tuning down of the expression of the genes encoding for adaptor proteins, JAK-STAT, NF-κB and IRF signaling cascades, which resulted in the reduced expression of cytokine genes and reflected the deficiencies of innate immune response in HCV+ LSEC cultures. In contrast, LPS (TLR4 agonist) stimulation shows the ability of LSEC to initiate inflammatory response.

The possible link between HCV infection and PRRs expression was suggested by the finding that specific agonists stimulated myeloid and plasmocytic dendritic cells from HCV-infected patients induced a significantly lesser amounts of IFNβ, TNFα and IL-12 than those from the healthy counterparts.(126, 127) This implies that alteration of the TLR/RIG-I system is instrumental in impairment of innate immunity in HCV infection.

Furthermore, the importance of TLR-signaling pathway was suggested in previously published study that showed TLR3 agonists could induce antiviral mediators by LSEC and inhibit murine encephalo-myocarditis virus replication (EMCV, a positive sense, single-stranded RNA virus).(128) This raises the question if PRRs' ignorance of HCV in LSEC, enables undisturbed HCV replication in underlying hepatocytes. Recently, it was suggested that paracrine signals from LSEC could stimulate HCV replication.(129) JFH-1 replication in Huh7.5 cells in the presence of conditioned media from HCV non-infected LSEC cultures was significantly enhanced.(129) Since LSEC are the first cells exposed to the HCV entering the liver, it remains unanswered if the same effect would be observed with the media of HCV exposed LSEC.

The down-regulation of the genes encoding for IRF3/7 arm of the TLR/RIG-I pathways is of particular interest as it plays crucial role in activation of IFNs transcription. Although the capacity of cultured endothelial cells to produce interferons (IFNs) was previously described,(130, 131) in LSEC expression of types I interferon and interferon-stimulated genes was unaffected after HCV exposure, at least for the time-points used in this experiment. The ability of HCV to dampen IFN-responses is well described. While transcriptome analysis of liver homogenates from chimpanzees with chronic HCV infection revealed a strong induction of hundreds of ISGs, the situation is more complex in patients with CHC.(132) 50%-70% of Caucasian patients show very little or no induction of ISGs, whereas the remaining 30%–50% have a permanent high-level expression of hundreds of ISGs.(133, 134) Paradoxically, activation of the endogenous IFN system in the liver seems to be ineffective against HCV and is related with negative response to IFN-based treatment.(135) In addition to inhibited TLR/RIG-I pathways, the possible explanation of IFN-system inhibition might include changes in transcriptional factors, expression of inflammation and immune system inhibitors.

The changes in gene expression are usually driven by transcription factors, which are master-control proteins regulation activation and/or suppression of gene expression through binding to specific regulatory sequences of target genes. Our

transcriptomic analysis identified upregulated transcription factors essential for induction of anti-inflammatory state, such as *MAFB*, *NUPR1*, *IL33*, *KLF15*.

The member of Maf family, v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (*MAFB*), was shown to be a constitutive inhibitor of the type I interferon pathway.(77) Interestingly, it was shown that during the acute phase of viral infection, MAFB expression decreases, which allows activation of IFNβ transcription and subsequent antiviral activity.(78) The prolonged poorer expression of MAFB leads to the hyperactivation of IFNβ that might induce the development of autoreactive immune cells, therefore playing a possible role in mechanism of autoimmunity.(77) In contrast, if high MAFB expression is sustained, IFNβ expression is insufficient, which results in vulnerability to viral infection.(77, 78) In addition to disrupting IFNβ-enhanceosome, MAFB might suppress transcription of RANTES.(77, 78) MAFB was 2-fold upregulated in mild, but not significantly changed in cirrhotic livers, which corresponded with the observed level of ISG expression in liver samples.

NUPR1, nuclear protein, transcriptional regulator 1, is stress-induced protein, implicated in diverse functions, including transcription regulation, modulation of apoptosis and autophagy, and cell cycle regulation.(79, 80) NUPR1 overexpression is additionally associated with tumor progression.(79) The upregulation of NUPR1 in acute pancreatitis was correlated with the ability of the defense mechanism of the pancreatic cells by inducing the expression of the anti-inflammatory genes. In addition, activation of NURP1 was found essential for the ability of tissues to tolerate lipopolysaccharide (LPS) treatment.(81) Interestingly, in THP-1 HCV infected cells, which showed a broad activation of inflammatory responses, NUPR1 was significantly downregulated.

Interleukin 33, IL-33, an unconventional member of IL1 cytokine family, is mainly produced by structural and lining cells, so its expression was not changed in hepatocytes or Kupffer cells. Interestingly, IL-33 interacts with the transcription factor NF-kB and dampens its activity.(93) It is suggested that IL-33 prevents undesirable activation of inflammatory processes in endothelia despite constant exposure to NF-kB stimulators in the blood stream.(91-94) In addition, IL-33

serves as alarmin and has an important role in sensing damage in various infectious and inflammatory diseases.(91-94) IL-33 affects the various cell types, enhances the production of Th2 cytokines and Th2 cell mobilization.(91) IL-33 can amplify polarization of alternatively activated macrophages to produce increased levels of CCL17 and CCL24 and induce proliferation and collagen production. Serum IL-33 levels were significantly upregulated in acute liver failure, suggesting it role as a potential marker of inflammation.(94)

Collectively this data suggest an activation of transcriptional regulators that prohibit potentially dangerous and undesirable endothelial inflammation and provide anti-inflammatory state.

The decreased expression of genes observed in the PRR-signaling pathways and activation of negative transcriptional regulators of the inflammatory response was further manifested by the downregulation of the cytokine–cytokine receptor interaction pathway. One of the most pronounced changes in this pathway was actually the significant absence of the expression of genes encoding proinflammatory cytokines including IL-1β, IL-6 or TNF-α. In contrast, chronic alcohol consumption was related with increase in RANTES production from normal endothelium, and ethanol induced a significant inflammatory response in rats LSEC.(136) Furthermore, attenuated inflammatory response in HCV exposed LSEC was in clear contrast to hepatoma and monocyte cell lines that expressed an increased expression of these genes. On the other hand, while conventional anti-inflammatory cytokine genes did not show major changes, a plethora of less known immunomodulatory genes were significantly upregulated. These include:

ACP5 (acid phosphatase 5, tartrate resistant) gene encodes an iron containing glycoprotein (TRAP), which catalyzes the conversion of orthophosphoric monoester to alcohol and orthophosphate. Although originally discovered as a regulator of bone morphogenesis, TRAP role has been expanded to the regulation of innate immune responses and deficient ACP5 and TRAP function has been related with autoimmune disorders.(90, 137) Knock-out studies of ACP5 in mice model resulted in abnormal immunomodulatory cytokine responses; after LPS or *S.aureus* infection, TNF-α and IL-1β secretion was markedly increased in TRAP

deficient mice, which correlated with impaired microbicidal activity.(89) Interestingly, mutations in ACP5 genes were discovered in patients with immuno-osseus dysplasia spondyloenchondrodysplasia (SPENCD) syndrome.(90) In these patients loss of TRAP protein results in a dramatic up-regulation of IFNα and type I interferon-stimulated genes.(90) More recently, TRAP was suggested as a macrophage-derived inflammation marker associated with cerebrovascular disease risk and with coronary vessel disease.(138, 139) Similarly as in LSEC, ACP5 was increased in THP-1 cells (4-fold). Thus, the marked upregulation of ACP5 expression in HCV infected LSEC might suggest a way of switching off inflammatory response as a way to prohibit a pathological inflammation that might damage the host.

Furthermore, the abundance of transcripts coding for soluble inhibitors of inflammation suggest the more expanded role of LSEC in controlling inflammation within the liver. The most noticeable examples include: *A2M* inhibits biological effects of inflammatory cytokines and chemokines; *C1QTNF1* has anti-inflammatory and insulin sensing effects, *NT5E* increases extracellular levels of anti-inflammatory adenosine molecules; *APOE* modulates type I inflammatory responses; *SERPING1* inhibits complement activation; *HPX* diminishes increase of TNF-α and IL6 in LPS stimulated macrophages.

One of the significantly downregulated cellular pathways was TGF-β signaling. TGF-β is a central regulator in chronic liver disease contributing to all stages of disease progression from initial liver injury through inflammation and fibrosis to cirrhosis and hepatocellular carcinoma.(140, 141) The intracellular mechanism of TGFβ- signaling via kinase receptors and SMAD effectors is firmly established.(142, 143) TGF-β pathway was upregulated in previously published expression studies in hepatocytes, macrophages and chronic liver samples.(10, 26, 61, 62) Furthermore, patients with chronic HCV infection have elevated plasma levels of TGF-β1 and increased expression of TGF-β1 in the liver, while the clearance of HCV infection with anti-viral treatment is associated with normalization of plasma TGF-β1 levels.(144) In contrast, we found significantly downregulated TGF-β2 with subsequent suppressed expression of the genes encoding for effector SMAD3, SMAD5 and SMURF and increased inhibitory

SMAD6 and SMAD7 transcripts. SMAD7 is a general antagonist of TGF-β family, while SMAD6 is specific for BMP signaling.(142, 143) Since, TGF-β is considered indispensable for angiogenesis, its activation in endothelial cells was extensively studied. It is proposed that the activation state of endothelial cells is regulated by TGF-β in two distinct ways. Whereas activation of the ALK1-Smad1/5 pathway induces the expression of proangiogenic genes leading to endothelial cell proliferation, migration and organization, the activation of the ALK5-Smad2/3 pathway results in different signaling events associated with the expression of maturation-specific genes and inhibition of angiogenesis.(145) To our knowledge, this study is the first to report the simultaneous tuning down of the genes encoding for both signaling cascades integrated within the TGF-β pathway during HCV infection.

Importantly, soluble inhibitors of TGF-β signaling pathways were significantly changed, suggesting that this might *in vivo* extent to surrounding cell types. These includes upregulation of thrombospondin- 2 and 3 (THBS2 and THBS3), both with a shown function as potent inhibitors of tumor growth and angiogenesis; upregulation of BMPER (BMP endothelial cell precursor-derived regulator), a secreted glycoprotein that binds directly and inhibits BMPs function; downregulation of latent transforming growth factor beta binding protein 1 and 2 (LTBP1 and LTBP2) involved in immature TGF-β trafficking and activation in extracellular space. BMP signaling (part of the TGF-β signaling pathway) is essential for the inflammatory response of vascular endothelial cells. The BMPs (BMP2 and BMP4) might directly influence lesion progression through endothelial inflammation and cell differentiation.(95, 96) BMP4 was upregulated after HCV infection in LSEC, and its expression was significantly increased in mild and cirrhotic HCV+ livers, but not expressed in both hepatoma and THP-1 cells. This is supported by recent publication that has shown that LSEC are the major source of BMP4 in the liver and that BMP4 promotes HCV replication in hepatocytes.(129) In addition, in LSEC BMPER was significantly increased. BMPER is a key modulator in BMP signaling particularly in blood vessel formation.(96) It was shown that, lack of BMPER confers a proinflammatory endothelial phenotype with reduced eNOS levels and enhanced expression of adhesion molecules leading to increased leukocyte adhesion and extravasation in *ex vivo* and *in vivo* experiments.(96) Vice

versa, addition of BMPER exerts endothelium protective functions and antagonizes TNFα induced inflammation.

There is a wealth of evidence that definitively establish TGF-β as a primary mediator in pathological fibrosis; increased expression of TGF-β1 correlates with ECM deposition, and delivery of exogenous TGF-β1 by various means to liver, lung or kidney tissues results in severe fibrosis in experimental animals.(145-148) There are now multiple reports that the therapeutic administration of TGF-β inhibitors ameliorates experimental fibrosis.(149-151) There are several reports that provide evidence that TGF-β1 positively regulates HCV RNA replication, and overexpression of TGF-β1 is an independent predictor for poor outcome of interferon and ribavirin therapy.(152-154) Our results might suggest that LSEC modulate TGF-β signaling pathway to control HCV replication in underlying hepatocytes and slow-down fibrogenesis. Functional studies are required to elucidate the mechanisms of these processes.

Remarkably, semaphorin pathway emerged as a consistent finding in transcriptome analyses. The importance of plexins and semaphorins has been emphasized by their discovery in many organ systems including the nervous, epithelial, and immune systems as well as diverse cell processes including angiogenesis, embryogenesis and cancer.(119, 120) Studies of plexins and semaphorins have revealed that several members of these families are involved in a series of immune cell interactions, which ultimately influence the outcome of the immune response and substantially influence the level of inflammation by inducing proinflammatory cytokine production.(119, 120) Interestingly, semaphorins play the opposite roles in innate versus adaptive immune response, amplifying inflammation while dampening T-cell proliferation and activation.(119, 155, 156) Although “immune semaphorins” are crucial to various phases of the immune response, so far semaphorins have not been linked with HCV infection. While upregulation of downstream genes in both Kupffer cells and hepatocytes seems to activate the pathway, in LSEC seems that final outcome of semaphorins signaling could be inhibition of pathway on receptor or downstream levels (as presented in Figure 31). This might be explained by distinct roles of each cell type in HCV pathogenesis model: to amplify inflammatory response, KC activate semaphorin

pathways, while LSEC attenuate semaphorin signaling to prevent vascular inflammation. This data strongly indicate a pivotal role for these molecules in HCV pathogenesis. This is supported with finding of their increasing expression with the progression of the disease.

Since LSEC can express MHC I and II class molecules and process antigens, have the potential of acting as antigen-presenting cells. This action has been well documented *in vitro*, but whether it occurs *in vivo* is still unclear. Although this experiment was not designed to answer the questions of T- and B-cell activation and antigen-presentation, it revealed changes in expression of genes that might be biologically relevant in regulating these processes. In LSEC, T-cell co-inhibitory ligands (HVEM, galectin) and soluble mediators (eNOS, HPX, IL33) were significantly upregulated. Meanwhile, prototypic co-stimulators of T-cell activation, CD80 and CD86, were not expressed, and negative co-stimulator of T-cell, PD-L1 was downregulated. Recently it was reported that TLR-1, -2 and -6 stimulated LSEC induced T-cell proliferation as assessed by IFNγ production and proliferative activity, in the absence of significant upregulation of MHC class II, CD40, CD80 and CD86.(128) It was shown that the expression of PD-L1 in LSEC and KC could inhibit the function of activated T cells via a PD-1 dependent and independent mechanism.(157, 158) *Von Oppen et al* described the mechanism of peripheral CD8+ tolerance in which antigen-specific retention of naive CD8+ T-cells in the liver represents the first step in the induction of CD8 T-cell tolerance toward circulating antigens that is executed by liver-resident scavenger LSECs.(152) Most of the studies examining LSEC anti-viral responses were performed using the murine LSEC *in vitro* models. Viral infection with murine cytomegalovirus caused functional maturation of antigen-presenting LSECs and was sufficient to promote antigen-specific differentiation into effector CD8+ T cells in the absence of dendritic cells and independent of CD80/86.(49) It is frequently suggested that uptake of viral particles by LSEC primes regulatory CD4+ T cells and impairs CD8+ T cells that finally fails to eradicate the virus from the liver.(51) This is generally supported of findings that HCV-specific CD8+ T cells in the liver frequently become dysfunctional and unable to secrete IFNγ or IL-2.(159) Notably, the studies describing potency of LSEC to induce HCV-specific T-cell tolerance or activation are required.

In general, hepatocytes are not easily accessible to immunocompetent leukocytes because LSECs may form an effective barrier between hepatocytes and the sinusoidal lumen. The trafficking of immune cells through the vascular endothelium is crucial in limiting tissue inflammation and unwanted damage.(160) Contradictory, we found upregulated both adhesion molecules permitting leukocyte extravasation, as well as endogenous anti-adhesion molecules. Liver biopsy staining analyses support this dual role of LSEC that simultaneously enable and limit cell-mediated inflammation. While neutrophil, macrophages and lymphocyte infiltration of liver is a hallmark of chronic hepatitis, the degree of which is usually mild and reflects in the progression that usually takes decades until cirrhosis and end stage disease is developed.

Control of the cell trafficking is closely related with sinusoids structural integrity. While apoptotic death of HCV infected hepatocytes in clinically “silent” process, apoptosis of endothelial cells would cause disruption of their barrier function. As described in acute liver ischemia/reperfusion injury and acetaminophen induced liver necrosis, LSEC cell death is manifested with organ failure and fulminant course of the disease. In addition, the damage of LSEC is the first sign of graft rejection in liver transplant. In contrast to previous reports of HCV induced apoptosis in HUVEC, RNA-seq analysis revealed significant changes in anti-apoptotic genes.(42) In addition, follow up apoptosis and cell death assays revealed no changes in the degree of apoptosis between HCV+ and control LSEC. Meanwhile, LPS significantly induced apoptosis. Similar observations were noticed in murine sepsis models, where LSEC apoptosis led to the organ dysfunction.(101)

However, this is in contrast to *in vivo* observations of no local inflammation in the liver sinusoids resulting from portal blood endotoxaemia.(46) It was show that the proinflammatory cytokine IL-6, which is central to the development of inflammatory reactions in the liver, is produced by LSEC in response to low concentrations of endotoxin (100 pg/ml to 1 ng/ml) that was attenuated by KC, which locally release anti-inflammatory cytokines (such as IL-10) and down-regulated liver inflammatory reactions to endotoxaemia.(161) Indeed, we have found a significant increase in anti-inflammatory cytokines in LPS exposed THP-1 cells (*IL10*, *IL4*, *IL11 IL13*, *ILRA*) all able to provide inhibitory stimulus to LSEC. It

is important to note that our intention was to simulate overwhelming endotoxin exposure, so we used high LPS levels of 10µg/mL that would presumably overcome LSEC “intrinsic” insensitivity to low-level LPS exposure. Notably, we found a dramatic increase in inflammatory transcripts expression at early time point after LPS exposure, however this effect was attenuated by 48 hours time point. This correlated with significant upregulation of negative regulators of inflammatory response.

The integrity of the liver sinusoidal endothelium is of highest relevance for the maintenance of liver physiology, and disruption of sinusoidal endothelial function have a prominent role in liver pathophysiology. LSEC dysfunction, with decreased intrahepatic NO production, has been considered as a relevant pathogenic factor in the progression of liver cirrhosis, while healthy sinusoidal endothelium is essential for liver regeneration.(162, 163) Early reports suggested that capillarization, loss of fenestration and formation of a basement membrane, precedes liver fibrosis that finally supports the activation of hepatic stellate cells.(164) Here we suggested new possible molecular mechanisms of these processes.

However, a limitation to this study is that modeling LSEC function *in vitro* may not reflect their function in the liver milieu. LSEC immune phenotype may also be unavoidably modified upon isolation. In addition, this is a transcriptomic study, which provides changes only at *m*RNA level and functional studies at the protein level in future would help to confirm the changes reported here and transform them into a mechanistic overview. Time consuming and technologically challenging studies of LSEC isolated form HCV infected patients at different stages of disease are required. Notably, our study has already provided a number of possible new biomarkers and therapeutic targets for HCV infection. As Butler et al. showed, LSEC have multiple roles in maintaining tissue architecture through expression of soluble mediators.(165) This characteristic has already been exploited in the development of anti-tumor therapies targeting angiogenic factors.(166, 167) The data presented here suggests that LSEC dictate the immune/inflammatory response to HCV infection. Given this influential role, it seems reasonable to look at LSEC as therapeutic targets to reinstitute normal immunity and resolve

inflammation. Due to the LSEC diverse functions, there are plentiful therapeutic strategies, ranging from exploitation of already present anti-inflammatory molecules to the blockade of leukocyte adhesion and antiangiogenic approaches, inhibition of LSEC proliferation and activation. Some of these have already been shown effective in human and experimental systems.(166, 167)

7.

CONCLUSION

To the best of my knowledge, this is the first comprehensive gene expression study on human LSEC in the course of HCV infection; therefore it has a very high significance for HCV research. Through the development of this project a series of novel results was obtained:

1) Whole genome sequencing analysis revealed that LSEC expressed a wide repertoire of transcripts required for efficient immune response activation (including pathogen-recognition receptors, adaptor proteins, pro-/anti-inflammatory cytokines, MHC molecules).
2) Although HCV does not replicate in LSEC, a non-replicative HCV infection is sufficient to cause a broad and complex cellular transcriptional reprograming in wide range of gene functional categories. However, while HCV induced a significant upregulation of gene expression in hepatoma and macrophages *in vitro* cell culture systems, in LSEC a significant downregulation of gene expression was observed.
3) Kinetics of gene expression might suggest early *vs.* late response to HCV infection, since the majority of gene changes were restricted to 8, 24 and 48 hours time point rather than persisting across time points. This might be reflection of HCV potential for chronicity, where by shutting down LSEC «defense» properties, HCV delays cellular changes in order to prevent the activation of immune response.
4) In HCV infected LSEC, the key innate immune response pathways were significantly downregulated (RIG-I and TLR-signaling pathways). This manifested by the decreased PRR transcripts with subsequent tuning down of the expression of the genes encoding for adaptor proteins, JAK-STAT, NF-κB and IRF signaling cascades, which resulted in the reduced expression of cytokine genes and reflected the deficiencies of innate immune response. The ability of LSEC to activate innate immune response was confirmed with LPS stimulation.

5) In control LPS stimulated cells, a dramatic increase in inflammatory transcripts expression at early time point after LPS exposure was observed. This effect was attenuated by 48 hours time point, which correlated with significant upregulation of negative regulators of inflammatory response.

6) HCV induced activation and upregulation of transcriptional factors essential for the induction of anti-inflammatory state (such as MAFB, NUPR1, IL33, KLF15, CEBPD). Meanwhile, HCV downregulated transcriptional factors shown to promote inflammatory responses (ETV1, MITH, HDAC9, EGR3, IRF6). This data suggest an activation of transcriptional regulators that prohibit potentially dangerous and undesirable endothelial inflammation and provide anti-inflammatory state.

7) In contrast to LPS, HCV did not induce expression of proinflammatory cytokines. Meanwhile, a plethora of immunomodulatory genes were significantly upregulated (such as ACP5, A2M, C1QTNF1, NT5E, SERPING1, BMPER) that might extend to surrounding cells and attenuate liver inflammation.

8) While cultured human LSEC lack the prototypic co-stimulators of T cell activation CD80 and CD86 ligands for T cell activation, several T-cell co-inhibitory ligands (TNFRSF14 and LGALS9) and soluble inhibitory mediators (NOS3, HPX, IL33) were significantly increased.

9) The cell cycle pathway was significantly down-regulated in HCV exposed LSEC and the coordinated down-regulation of these genes appeared in all phases of cell cycle including G1 to S, G2 to M, and metaphase to anaphase transition.

10) While LPS induced major changes in pro-apoptotic pathway, HCV induced only minor changes that implicated inhibition of apoptosis. Follow up apoptosis and cell death assays revealed no changes in the degree of apoptosis between HCV+ and control LSEC, while LPS significantly induced apoptosis.

11) Total of 31 KEGG pathways was significantly enriched in HCV infected LSEC. Enriched were KEGG pathways related to cell scavenger functions (e.g. Fc gamma R-mediated phagocytosis, Cell adhesion molecules, Focal adhesion, Leukocyte transendothelial migration, Axon guidance), innate immune responses (RIG-I-like receptor signaling, cytokine-cytokine receptor

interaction, complement and coagulation cascades) and cancer development (Pathways in cancer, Apoptosis, p53 signaling, Wnt signaling).

12) TGF-β signaling pathway was significantly downregulated in HCV infected LSEC. This reflected in the simultaneous tuning down of the TGF-β2 expression with subsequent suppressed expression of the genes encoding for effector SMAD3, SMAD5 and SMURF and increased inhibitory SMAD6 and SMAD7 transcripts. Meanwhile soluble inhibitors of TGF-β signaling pathways were significantly changed (BMPER, THBS2, THBS3), suggesting that this might *in vivo* extent to surrounding cell types.
13) The only pathway enriched at all time points in LSEC (as well as in KC and Huh7.5 cells) was axon guidance, more specifically «immune semaphorins pathway». While upregulation of downstream genes in both Kupffer cells and hepatocytes seems to activate the pathway, in LSEC seems that final outcome of semaphorins signaling could be inhibition of pathway on receptor or downstream levels.
14) The system biology approach helped to identify distinct gene expression profiles in HCV infected LSECs, KCs and hepatocytes, suggesting their different roles during HCV infection. Unlike RNA-seq analysis of macrophages that demonstrated a broad increase in IL1β and NFκB-responsive proinflammatory cytokines and chemokines, HCV did not induce a marked increase in such inflammatory signals in both LSECs and Huh7.5 cells. Analysis of genes within these pathways showed increased IL1β levels with increasing severity of liver disease and marked overlap in upregulated expression in macrophages exposed to HCV and liver specimens.
15) In contrast, analysis of genes commonly expressed in LSECs exposed to HCV with HCV infected liver showed significant overlap of pathways associated with angiogenesis, adhesion, ECM-organization, and regulation of defense/immune responses among other.
16) Selective dysfunction of KC and LSEC might be relevant by profoundly affecting the immune responses against HCV at the level of the infection site.
17) RNA-sequencing approach proposed new potential biomarkers that need to further be investigated in clinical samples (such as BMPER, IL33, semaphorins).

Taken all together these results suggest that HCV triggers downregulation of cellular regulators of inflammation in LSEC, which is manifesting in disabled host-cell innate immune response possibly spreading to surrounding cells by secretion of soluble immune mediators. Meanwhile, HCV activates a very potent inflammatory response in Kupffer cells that might be harmful and lead to tissue destruction without LSEC's attenuating effects. Gene changes in hepatocytes imply a serving role for HCV production where virus can undisturbedly replicate in high titers, successfully inhibiting host-cell defense mechanism.

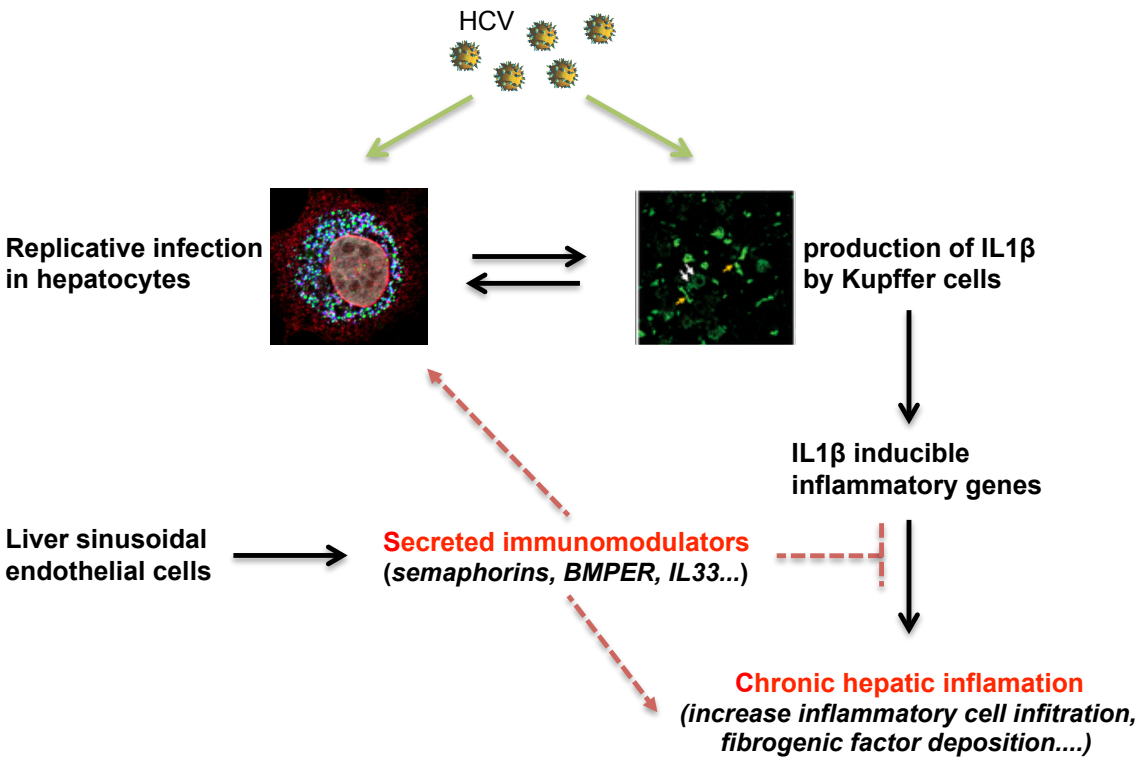


Figure 39. Proposed model of HCV disease pathogenesis

Finally, this research identified specific host genes and molecules that are part of the human antiviral response to HCV and possibly other viral infections. This might facilitate the development of more effective biomarkers, interventions against HCV infection and might aid in the development of novel strategies for the design of vaccines against HCV infection.

8.

SAŽETAK / ABSTRACT in CROATIAN

UVOD: Jetrene sinusoidalne endotelne stanice (LSEC) igraju ključnu ulogu u eliminaciji krvlju prenosivih virusa. LSEC čine 20% stanica jetre i predstavljaju jedinstvenu staničnu populaciju važnu za degradaciju bakterijskih produkata, prezentaciju antigena i indukciju «imunološke tolerancije». Iako su ti procesi od posebne važnosti u patogenezi HCV infekcije, uloga LSEC u kroničnom C hepatitisu je nepoznata.

MATERIJALI I METODE: Cilj ove studije je primjenom metodologije sistemske biologije istražiti ulogu LSEC u HCV infekciji. Pol II selektirane RNK iz HCV, MOCK ili LPS stimuliranih LSEC bile su analizirane upotrebom RNK-sekvenciranja cijelog staničnog transkriptoma, s ciljem identificikacije diferencijalno eksprimiranih gena (DEG) i staničnih putova. Ekspresija gena je uspoređena s uzorcima jetre blage i uznapredove fibroze, hepatocitima i Kupfferovim stanicama.

REZULTATI: LSEC ne podupiru HCV replikaciju, ali virus efikasno ulazi u stanicu. U LSEC identificirano je 754, 245 i 2543 DEG, 8, 24 i 48 sati nakon HCV ekspozicije. Dok je LPS stimulacija potaknula izuzetno snažnu aktivaciju upalnog odgovora, HCV je općenito doveo do utišavanja istog. Ključni putevi prirođene stanične imunosti bili su značajno utišani (RIG-I i TLR-signalni putovi), što se manifestiralo sniženom ekspresijom PRR gena, adaptorskih proteina, JAK-STAT, NF-κB i IRF signalnih kaskada, rezultirajući smanjenom ekspresijom citokina te odražavajući neučinkovitost imunološkog odgovora. Molekularni mehanizmi ovih procesa uključuju: aktivaciju transkripcijskih regulatora ključnih za indukciju protuupalnog odgovora (*MAFB*, *NUPR1*, *IL33*, *KLF15*, *CEBPD*) i inhibiciju onih koji promoviraju upalni odgovor (*ETV1*, *MITH*, *HDAC9*, *EGR3*, *IRF6*). Istovremeno solubilni imunomodulatori (*ACP5*, *A2M*, *C1QTNF1*, *NT5E*, *SERPING1*, *BMPER*) bili su pojačano eksprimirani, a potencijalno utišivaju upalni proces u jetri. Daljnja analiza je identificirala obogaćenim KEGG stanične putove povezane sa staničnom eliminacijskom funkcijom (Fc gamma R-fagocitozu, stanične adhezijske molekule, tkivna adhezija, transendotelna migracija leukocita, imuni semaforini), prirođenom i stečenom imunošću, karcinogenezom (putovi u razvoju karcinoma,

apoptoza, p53- i Wnt- stanični putovi). Za razliku od KC i hepatocita, TGF-β signalni put je bio značajno utišan u LSEC. Različiti profili ekspresije gena su identificirani u LSEC, KC i hepatocitima, sugerirajući njihovu različitu ulogu u patogenezi HCV infekcije. U makrofazima je identificirana pojačana ekspresija proupalnih citokina i kemokina, koja korelira sa stadijem jetrene bolesti. Istovremeno, usporedba LSEC i uzoraka HCV-om inficirane jetre pokazala je značajnu podudarnost u staničnim putovima povezanim s angiogenezom, adhezijom, organizacijom ekstracelularnog matriksa i regulacijom imunološkog odgovora.

ZAKLJUČAK: Ovo je prva analiza ekspresije gena u LSEC koja je identificirala promjene u transkriptomu povezane s HCV infekcijom. Identificirani geni čine ključne kompontente staničnog imunološkog i upalnog odgovora i sugeriraju da LSEC utišavaju upalu u tijeku HCV infekcije.

9.

ABSTRACT in ENGLISH

PhD thesis: The role of liver sinusoidal endothelial cells in HCV infection

PhD candidate: Neven Papic, MD

Mentors: Assistant Professor Adriana Vince, MD, PhD, Professor Curt Hagedorn, MD, PhD

Affiliations: University Hospital for infectious diseases Zagreb, Croatia. University of Utah, School of Medicine, Salt Lake City, Utah, USA.

INTRODUCTION: Liver sinusoidal endothelial cells (LSEC) due to their extraordinary scavenger activity are playing a pivotal role in blood-borne virus clearance. LSEC account for the 20% of hepatic cells and are unique organ-resident cell population with diverse functions, including degradation of bacterial by-products, antigen presentation and induction of tolerance. While these processes are particularly relevant to HCV infection, the role of LSEC in chronic hepatitis C is not defined.

MATERIALS AND METHODS: Aim of this study was to apply systems biology approaches to evaluate the role of LSEC in HCV infection. Poly(A) RNAs from HCV, MOCK or LPS treated primary LSEC cultures were analyzed by RNA-sequencing (Illumina) to identify differentially expressed genes (DEG) and biological pathways. Cell transcriptomes were compared to similar analysis with mild (no fibrosis) and severe (cirrhosis) hepatitis C livers, hepatoma and Kupffer cells.

RESULTS: Following exposure LSEC internalized HCV, but failed to support HCV replication. LSEC overall displayed 754, 245 and 2543 DEG at 8, 24 and 48h after HCV exposure, respectively. While LPS stimulation triggered exceptionally potent activation, HCV in general induced a downregulation of inflammatory signals. The key innate immune response pathways were significantly downregulated (RIG-I and TLR-signaling pathways), which manifested by the diminished PRR transcripts expression with subsequent tuning down of the expression of the genes encoding for adaptor proteins, JAK-STAT, NF-κB and IRF signaling cascades resulting in the

reduced expression of cytokine genes and reflecting in the deficiencies of innate immune response. Molecular mechanisms of these processes involve: upregulation of transcriptional factors essential for the induction of anti-inflammatory state (such as MAFB, NUPR1, IL33, KLF15, CEBPD) and downregulation of those shown to promote inflammatory responses (ETV1, MITH, HDAC9, EGR3, IRF6). Meanwhile, a plethora of immunomodulatory genes were significantly upregulated (ACP5, A2M, C1QTNF1, NT5E, SERPING1, BMPER) that might extend to surrounding cells and attenuate liver inflammation. Gene pathway analysis further highlighted enriched KEGG pathways related to cell scavenger functions (e.g. Fc gamma R-mediated phagocytosis, Cell adhesion molecules, Focal adhesion, Leukocyte transendothelial migration, Axon guidance), innate immune responses and cancer development (Pathways in cancer, Apoptosis, p53 signaling, Wnt signaling). In contrast to KC and hepatocytes, TGF-β signaling pathway was significantly downregulated in HCV infected LSEC. The system biology approach helped to identify distinct gene expression profiles in HCV infected LSECs, KCs and hepatocytes. Macrophages demonstrated a broad increase in IL-1β and NFκB-responsive proinflammatory cytokines and chemokines, which correlated with increasing severity of liver disease. In contrast, analysis of genes commonly expressed in LSEC with HCV infected liver showed significant overlap of pathways associated with angiogenesis, adhesion, ECM-organization, and regulation of defense/immune responses.

CONCLUSION: This is the first comprehensive gene expression analysis of LSEC that provided insight into the broad portrait of genomic changes associated with HCV infection. These genes are critical components of host immune and inflammatory pathways and provide new evidence that LSEC downregulate inflammation during HCV infection.

10.

LIST OF REFERENCES

1. Bialek SR, Terrault NA. The changing epidemiology and natural history of hepatitis C virus infection. Clinics in liver disease. 2006;10(4):697-715.
2. Di Bisceglie AM. Natural history of hepatitis C: its impact on clinical management. Hepatology. 2000;31(4):1014-8.
3. Dibonaventura MD, Yuan Y, Lescrauwaet B, L'Italien G, Liu GG, Kamae I, et al. Multicountry burden of chronic hepatitis C viral infection among those aware of their diagnosis: a patient survey. PloS one. 2014;9(1):e86070.
4. Ly KN, Xing J, Klevens RM, Jiles RB, Ward JW, Holmberg SD. The increasing burden of mortality from viral hepatitis in the United States between 1999 and 2007. Annals of internal medicine. 2012;156(4):271-8.
5. Kato T, Date T, Murayama A, Morikawa K, Akazawa D, Wakita T. Cell culture and infection system for hepatitis C virus. Nature protocols. 2006;1(5):2334-9.
6. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nature medicine. 2005;11(7):791-6.
7. Pareek CS, Smoczynski R, Tretyn A. Sequencing technologies and genome sequencing. Journal of applied genetics. 2011;52(4):413-35.
8. Moradpour D, Penin F, Rice CM. Replication of hepatitis C virus. Nature reviews Microbiology. 2007;5(6):453-63.
9. Sklan EH, Charuworn P, Pang PS, Glenn JS. Mechanisms of HCV survival in the host. Nature reviews Gastroenterology & hepatology. 2009;6(4):217-27.
10. Papic N, Maxwell CI, Delker DA, Liu S, Heale BS, Hagedorn CH. RNA-sequencing analysis of 5' capped RNAs identifies many new differentially expressed genes in acute hepatitis C virus infection. Viruses. 2012;4(4):581-612.
11. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783-801.
12. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nature immunology. 2004;5(10):987-95.

13. Yoneyama M, Fujita T. Function of RIG-I-like receptors in antiviral innate immunity. The Journal of biological chemistry. 2007;282(21):15315-8.
14. Foy E, Li K, Sumpter R, Jr., Loo YM, Johnson CL, Wang C, et al. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(8):2986-91.
15. Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, et al. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(8):2992-7.
16. van Boxel-Dezaire AH, Rani MR, Stark GR. Complex modulation of cell type-specific signaling in response to type I interferons. Immunity. 2006;25(3):361-72.
17. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, et al. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature. 2005;437(7062):1167-72.
18. Foy E, Li K, Wang C, Sumpter R, Jr., Ikeda M, Lemon SM, et al. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science. 2003;300(5622):1145-8.
19. Bode JG, Ludwig S, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, et al. IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2003;17(3):488-90.
20. Tang H, Grise H. Cellular and molecular biology of HCV infection and hepatitis. Clinical science. 2009;117(2):49-65.
21. Thimme R, Bukh J, Spangenberg HC, Wieland S, Pemberton J, Steiger C, et al. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(24):15661-8.
22. Bain C, Fatmi A, Zoulim F, Zarski JP, Trepo C, Inchauspe G. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. Gastroenterology. 2001;120(2):512-24.
23. Dolganiuc A, Kodys K, Kopasz A, Marshall C, Do T, Romics L, Jr., et al. Hepatitis C virus core and nonstructural protein 3 proteins induce pro- and anti-

inflammatory cytokines and inhibit dendritic cell differentiation. Journal of immunology. 2003;170(11):5615-24.
24. Francavilla V, Accapezzato D, De Salvo M, Rawson P, Cosimi O, Lipp M, et al. Subversion of effector CD8+ T cell differentiation in acute hepatitis C virus infection: exploring the immunological mechanisms. European journal of immunology. 2004;34(2):427-37.
25. Rehermann B. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. The Journal of clinical investigation. 2009;119(7):1745-54.
26. Negash AA, Ramos HJ, Crochet N, Lau DT, Doehle B, Papic N, et al. IL-1beta production through the NLRP3 inflammasome by hepatic macrophages links hepatitis C virus infection with liver inflammation and disease. PLoS pathogens. 2013;9(4):e1003330.
27. Papic N, Delker D, Rong X, Liu S, Negash AA, Gale M, et al. Kupffer Cells are a major source of IL-1 and other drivers of inflammation in Chronic Hepatitis C. Hepatology. 2012;56:704A-A.
28. Dinarello CA. Interleukin-1 and the pathogenesis of the acute-phase response. The New England journal of medicine. 1984;311(22):1413-8.
29. Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. Nature immunology. 2009;10(3):241-7.
30. Revie D, Salahuddin SZ. Human cell types important for hepatitis C virus replication in vivo and in vitro: old assertions and current evidence. Virology journal. 2011;8:346.
31. Yamada E, Montoya M, Schuettler CG, Hickling TP, Tarr AW, Vitelli A, et al. Analysis of the binding of hepatitis C virus genotype 1a and 1b E2 glycoproteins to peripheral blood mononuclear cell subsets. The Journal of general virology. 2005;86(Pt 9):2507-12.
32. el-Awady MK, Tabll AA, Redwan el RM, Youssef S, Omran MH, Thakeb F, et al. Flow cytometric detection of hepatitis C virus antigens in infected peripheral blood leukocytes: binding and entry. World journal of gastroenterology : WJG. 2005;11(33):5203-8.
33. Willems M, Peerlinck K, Moshage H, Deleu I, Van den Eynde C, Vermylen J, et al. Hepatitis C virus-RNAs in plasma and in peripheral blood mononuclear

cells of hemophiliacs with chronic hepatitis C: evidence for viral replication in peripheral blood mononuclear cells. Journal of medical virology. 1994;42(3):272-8.
34. Henin C, Makris M, Brown J, Peake IR, Preston EF. Peripheral mononuclear cells of haemophiliacs with chronic liver disease are infected with replicating hepatitis C virus. British journal of haematology. 1994;87(1):215-7.
35. Bouffard P, Hayashi PH, Acevedo R, Levy N, Zeldis JB. Hepatitis C virus is detected in a monocyte/macrophage subpopulation of peripheral blood mononuclear cells of infected patients. The Journal of infectious diseases. 1992;166(6):1276-80.
36. Lohr HF, Goergen B, Meyer zum Buschenfelde KH, Gerken G. HCV replication in mononuclear cells stimulates anti-HCV-secreting B cells and reflects nonresponsiveness to interferon-alpha. Journal of medical virology. 1995;46(4):314-20.
37. Muller HM, Pfaff E, Goeser T, Kallinowski B, Solbach C, Theilmann L. Peripheral blood leukocytes serve as a possible extrahepatic site for hepatitis C virus replication. The Journal of general virology. 1993;74 (Pt 4):669-76.
38. Wilkinson J, Radkowski M, Eschbacher JM, Laskus T. Activation of brain macrophages/microglia cells in hepatitis C infection. Gut. 2010;59(10):1394-400.
39. Arribas JR, Barbado FJ, Zapico R, Sendino A, Gonzalez I, Vazquez JJ. Association between hepatitis C virus and mixed cryoglobulinemia. Reviews of infectious diseases. 1991;13(4):770-1.
40. Monti G, Pioltelli P, Saccardo F, Campanini M, Candela M, Cavallero G, et al. Incidence and characteristics of non-Hodgkin lymphomas in a multicenter case file of patients with hepatitis C virus-related symptomatic mixed cryoglobulinemias. Archives of internal medicine. 2005;165(1):101-5.
41. Connolly MK, Bedrosian AS, Malhotra A, Henning JR, Ibrahim J, Vera V, et al. In hepatic fibrosis, liver sinusoidal endothelial cells acquire enhanced immunogenicity. Journal of immunology. 2010;185(4):2200-8.
42. Balasubramanian A, Munshi N, Koziel MJ, Hu Z, Liang TJ, Groopman JE, et al. Structural proteins of Hepatitis C virus induce interleukin 8 production and apoptosis in human endothelial cells. The Journal of general virology. 2005;86(Pt 12):3291-301.
43. Lau DT, Negash A, Chen J, Crochet N, Sinha M, Zhang Y, et al. Innate immune tolerance and the role of kupffer cells in differential responses to

interferon therapy among patients with HCV genotype 1 infection. Gastroenterology. 2013;144(2):402-13 e12.
44. Gershwin ME, Vierling JM, Manns MP. Liver immunology : principles and practice. Totowa, N.J.: Humana Press; 2007. xv, 486 p. p.
45. Braet F, Wisse E. Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. Comparative hepatology. 2002;1(1):1.
46. Knolle PA, Limmer A. Neighborhood politics: the immunoregulatory function of organ-resident liver endothelial cells. Trends in immunology. 2001;22(8):432-7.
47. Bottcher JP, Knolle PA, Stabenow D. Mechanisms balancing tolerance and immunity in the liver. Digestive diseases. 2011;29(4):384-90.
48. Smedsrod B. Clearance function of scavenger endothelial cells. Comparative hepatology. 2004;3 Suppl 1:S22.
49. Kern M, Popov A, Scholz K, Schumak B, Djandji D, Limmer A, et al. Virally infected mouse liver endothelial cells trigger CD8+ T-cell immunity. Gastroenterology. 2010;138(1):336-46.
50. Billiar TR, Curran RD, Williams DL, Kispert PH. Liver nonparenchymal cells are stimulated to provide interleukin 6 for induction of the hepatic acute-phase response in endotoxemia but not in remote localized inflammation. Archives of surgery. 1992;127(1):31-6; discussion 6-7.
51. Limmer A, Ohl J, Kurts C, Ljunggren HG, Reiss Y, Groettrup M, et al. Efficient presentation of exogenous antigen by liver endothelial cells to CD8+ T cells results in antigen-specific T-cell tolerance. Nature medicine. 2000;6(12):1348-54.
52. Breiner KM, Schaller H, Knolle PA. Endothelial cell-mediated uptake of a hepatitis B virus: a new concept of liver targeting of hepatotropic microorganisms. Hepatology. 2001;34(4 Pt 1):803-8.
53. Ganesan LP, Mohanty S, Kim J, Clark KR, Robinson JM, Anderson CL. Rapid and efficient clearance of blood-borne virus by liver sinusoidal endothelium. PLoS pathogens. 2011;7(9):e1002281.
54. Gardner JP, Durso RJ, Arrigale RR, Donovan GP, Maddon PJ, Dragic T, et al. L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(8):4498-503.

55. Pohlmann S, Zhang J, Baribaud F, Chen Z, Leslie GJ, Lin G, et al. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. Journal of virology. 2003;77(7):4070-80.
56. Cormier EG, Durso RJ, Tsamis F, Boussemart L, Manix C, Olson WC, et al. L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(39):14067-72.
57. Ludwig IS, Lekkerkerker AN, Depla E, Bosman F, Musters RJ, Depraetere S, et al. Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. Journal of virology. 2004;78(15):8322-32.
58. Berg M, Wingender G, Djandji D, Hegenbarth S, Momburg F, Hammerling G, et al. Cross-presentation of antigens from apoptotic tumor cells by liver sinusoidal endothelial cells leads to tumor-specific CD8+ T cell tolerance. European journal of immunology. 2006;36(11):2960-70.
59. von Oppen N, Schurich A, Hegenbarth S, Stabenow D, Tolba R, Weiskirchen R, et al. Systemic antigen cross-presented by liver sinusoidal endothelial cells induces liver-specific CD8 T-cell retention and tolerization. Hepatology. 2009;49(5):1664-72.
60. Lambotin M, Raghuraman S, Stoll-Keller F, Baumert TF, Barth H. A look behind closed doors: interaction of persistent viruses with dendritic cells. Nature reviews Microbiology. 2010;8(5):350-60.
61. Blackham S, Baillie A, Al-Hababi F, Remlinger K, You S, Hamatake R, et al. Gene expression profiling indicates the roles of host oxidative stress, apoptosis, lipid metabolism, and intracellular transport genes in the replication of hepatitis C virus. Journal of virology. 2010;84(10):5404-14.
62. Walters KA, Syder AJ, Lederer SL, Diamond DL, Paeper B, Rice CM, et al. Genomic analysis reveals a potential role for cell cycle perturbation in HCV-mediated apoptosis of cultured hepatocytes. PLoS pathogens. 2009;5(1):e1000269.
63. Woodhouse SD, Narayan R, Latham S, Lee S, Antrobus R, Gangadharan B, et al. Transcriptome sequencing, microarray, and proteomic analyses reveal cellular and metabolic impact of hepatitis C virus infection in vitro. Hepatology. 2010;52(2):443-53.

64. Folkers ME, Delker DA, Maxwell CI, Nelson CA, Schwartz JJ, Nix DA, et al. ENCODE tiling array analysis identifies differentially expressed annotated and novel 5' capped RNAs in hepatitis C infected liver. PloS one. 2011;6(2):e14697.
65. Morozova O, Marra MA. Applications of next-generation sequencing technologies in functional genomics. Genomics. 2008;92(5):255-64.
66. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome biology. 2009;10(3):R25.
67. Nicol JW, Helt GA, Blanchard SG, Jr., Raja A, Loraine AE. The Integrated Genome Browser: free software for distribution and exploration of genome-scale datasets. Bioinformatics. 2009;25(20):2730-1.
68. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nature methods. 2008;5(7):621-8.
69. Nix DA, Courdy SJ, Boucher KM. Empirical methods for controlling false positives and estimating confidence in ChIP-Seq peaks. BMC bioinformatics. 2008;9:523.
70. Carty M, Goodbody R, Schroder M, Stack J, Moynagh PN, Bowie AG. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. Nature immunology. 2006;7(10):1074-81.
71. Gong J, Wei T, Stark RW, Jamitzky F, Heckl WM, Anders HJ, et al. Inhibition of Toll-like receptors TLR4 and 7 signaling pathways by SIGIRR: a computational approach. Journal of structural biology. 2010;169(3):323-30.
72. Arimoto K, Takahashi H, Hishiki T, Konishi H, Fujita T, Shimotohno K. Negative regulation of the RIG-I signaling by the ubiquitin ligase RNF125. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(18):7500-5.
73. Stumpo DJ, Lai WS, Blackshear PJ. Inflammation: cytokines and RNA-based regulation. Wiley interdisciplinary reviews RNA. 2010;1(1):60-80.
74. Suehiro J, Hamakubo T, Kodama T, Aird WC, Minami T. Vascular endothelial growth factor activation of endothelial cells is mediated by early growth response-3. Blood. 2010;115(12):2520-32.
75. Bailey CM, Abbott DE, Margaryan NV, Khalkhali-Ellis Z, Hendrix MJ. Interferon regulatory factor 6 promotes cell cycle arrest and is regulated by the

proteasome in a cell cycle-dependent manner. Molecular and cellular biology. 2008;28(7):2235-43.

76. Kelly KF, Daniel JM. POZ for effect--POZ-ZF transcription factors in cancer and development. Trends in cell biology. 2006;16(11):578-87.

77. Motohashi H, Igarashi K. MafB as a type I interferon rheostat. Nature immunology. 2010;11(8):695-6.

78. Kim H, Seed B. The transcription factor MafB antagonizes antiviral responses by blocking recruitment of coactivators to the transcription factor IRF3. Nature immunology. 2010;11(8):743-50.

79. Cano CE, Hamidi T, Sandi MJ, Iovanna JL. Nupr1: the Swiss-knife of cancer. Journal of cellular physiology. 2011;226(6):1439-43.

80. Kong DK, Georgescu SP, Cano C, Aronovitz MJ, Iovanna JL, Patten RD, et al. Deficiency of the transcriptional regulator p8 results in increased autophagy and apoptosis, and causes impaired heart function. Molecular biology of the cell. 2010;21(8):1335-49.

81. Vasseur S, Folch-Puy E, Hlouschek V, Garcia S, Fiedler F, Lerch MM, et al. p8 improves pancreatic response to acute pancreatitis by enhancing the expression of the anti-inflammatory protein pancreatitis-associated protein I. The Journal of biological chemistry. 2004;279(8):7199-207.

82. Heikkila M, Pasanen A, Kivirikko KI, Myllyharju J. Roles of the human hypoxia-inducible factor (HIF)-3alpha variants in the hypoxia response. Cellular and molecular life sciences : CMLS. 2011;68(23):3885-901.

83. Hsiao YW, Li CF, Chi JY, Tseng JT, Chang Y, Hsu LJ, et al. CCAAT/enhancer binding protein delta in macrophages contributes to immunosuppression and inhibits phagocytosis in nasopharyngeal carcinoma. Science signaling. 2013;6(284):ra59.

84. Genolet R, Wahli W, Michalik L. PPARs as drug targets to modulate inflammatory responses? Current drug targets Inflammation and allergy. 2004;3(4):361-75.

85. Heo SH, Choi YJ, Ryoo HM, Cho JY. Expression profiling of ETS and MMP factors in VEGF-activated endothelial cells: role of MMP-10 in VEGF-induced angiogenesis. Journal of cellular physiology. 2010;224(3):734-42.

86. Clark RB. The role of PPARs in inflammation and immunity. Journal of leukocyte biology. 2002;71(3):388-400.

87. Dalli J, Norling LV, Montero-Melendez T, Federici Canova D, Lashin H, Pavlov AM, et al. Microparticle alpha-2-macroglobulin enhances pro-resolving responses and promotes survival in sepsis. EMBO molecular medicine. 2014;6(1):27-42.
88. Rehman AA, Ahsan H, Khan FH. alpha-2-Macroglobulin: a physiological guardian. Journal of cellular physiology. 2013;228(8):1665-75.
89. Bune AJ, Hayman AR, Evans MJ, Cox TM. Mice lacking tartrate-resistant acid phosphatase (Acp 5) have disordered macrophage inflammatory responses and reduced clearance of the pathogen, Staphylococcus aureus. Immunology. 2001;102(1):103-13.
90. Briggs TA, Rice GI, Daly S, Urquhart J, Gornall H, Bader-Meunier B, et al. Tartrate-resistant acid phosphatase deficiency causes a bone dysplasia with autoimmunity and a type I interferon expression signature. Nature genetics. 2011;43(2):127-31.
91. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity. 2005;23(5):479-90.
92. Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: the new kid in the IL-1 family. Nature reviews Immunology. 2010;10(2):103-10.
93. Ali S, Mohs A, Thomas M, Klare J, Ross R, Schmitz ML, et al. The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription. Journal of immunology. 2011;187(4):1609-16.
94. Wang J, Zhao P, Guo H, Sun X, Jiang Z, Xu L, et al. Serum IL-33 levels are associated with liver damage in patients with chronic hepatitis C. Mediators of inflammation. 2012;2012:819636.
95. Yao Y, Bennett BJ, Wang X, Rosenfeld ME, Giachelli C, Lusis AJ, et al. Inhibition of bone morphogenetic proteins protects against atherosclerosis and vascular calcification. Circulation research. 2010;107(4):485-94.
96. Helbing T, Rothweiler R, Ketterer E, Goetz L, Heinke J, Grundmann S, et al. BMP activity controlled by BMPER regulates the proinflammatory phenotype of endothelium. Blood. 2011;118(18):5040-9.

97. Helbing T, Volkmar F, Goebel U, Heinke J, Diehl P, Pahl HL, et al. Kruppel-like factor 15 regulates BMPER in endothelial cells. Cardiovascular research. 2010;85(3):551-9.
98. Wong GW, Krawczyk SA, Kitidis-Mitrokostas C, Revett T, Gimeno R, Lodish HF. Molecular, biochemical and functional characterizations of C1q/TNF family members: adipose-tissue-selective expression patterns, regulation by PPAR-gamma agonist, cysteine-mediated oligomerizations, combinatorial associations and metabolic functions. The Biochemical journal. 2008;416(2):161-77.
99. Pittet CL, Newcombe J, Prat A, Arbour N. Human brain endothelial cells endeavor to immunoregulate CD8 T cells via PD-1 ligand expression in multiple sclerosis. Journal of neuroinflammation. 2011;8:155.
100. Shen T, Chen X, Chen Y, Xu Q, Lu F, Liu S. Increased PD-L1 expression and PD-L1/CD86 ratio on dendritic cells were associated with impaired dendritic cells function in HCV infection. Journal of medical virology. 2010;82(7):1152-9.
101. Hutchins NA, Wang F, Wang Y, Chung CS, Ayala A. Kupffer cells potentiate liver sinusoidal endothelial cell injury in sepsis by ligating programmed cell death ligand-1. Journal of leukocyte biology. 2013;94(5):963-70.
102. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nature reviews Immunology. 2013;13(4):227-42.
103. Wink DA, Hines HB, Cheng RY, Switzer CH, Flores-Santana W, Vitek MP, et al. Nitric oxide and redox mechanisms in the immune response. Journal of leukocyte biology. 2011;89(6):873-91.
104. Rolla S, Ingoglia G, Bardina V, Silengo L, Altruda F, Novelli F, et al. Acute-phase protein hemopexin is a negative regulator of Th17 response and experimental autoimmune encephalomyelitis development. Journal of immunology. 2013;191(11):5451-9.
105. Biedermann BC. Vascular endothelium: checkpoint for inflammation and immunity. News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society. 2001;16:84-8.
106. Radi ZA, Kehrli ME, Jr., Ackermann MR. Cell adhesion molecules, leukocyte trafficking, and strategies to reduce leukocyte infiltration. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine. 2001;15(6):516-29.

107. Sanz MJ, Hickey MJ, Johnston B, McCafferty DM, Raharjo E, Huang PL, et al. Neuronal nitric oxide synthase (NOS) regulates leukocyte-endothelial cell interactions in endothelial NOS deficient mice. British journal of pharmacology. 2001;134(2):305-12.
108. Lin WL, Chang CF, Shi CS, Shi GY, Wu HL. Recombinant lectin-like domain of thrombomodulin suppresses vascular inflammation by reducing leukocyte recruitment via interacting with Lewis Y on endothelial cells. Arteriosclerosis, thrombosis, and vascular biology. 2013;33(10):2366-73.
109. Fernandez M, Semela D, Bruix J, Colle I, Pinzani M, Bosch J. Angiogenesis in liver disease. Journal of hepatology. 2009;50(3):604-20.
110. Verkman AS, Hara-Chikuma M, Papadopoulos MC. Aquaporins--new players in cancer biology. Journal of molecular medicine. 2008;86(5):523-9.
111. Papadopoulos MC, Saadoun S, Verkman AS. Aquaporins and cell migration. Pflugers Archiv : European journal of physiology. 2008;456(4):693-700.
112. Huebert RC, Vasdev MM, Shergill U, Das A, Huang BQ, Charlton MR, et al. Aquaporin-1 facilitates angiogenic invasion in the pathological neovasculature that accompanies cirrhosis. Hepatology. 2010;52(1):238-48.
113. Huebert RC, Jagavelu K, Hendrickson HI, Vasdev MM, Arab JP, Splinter PL, et al. Aquaporin-1 promotes angiogenesis, fibrosis, and portal hypertension through mechanisms dependent on osmotically sensitive microRNAs. The American journal of pathology. 2011;179(4):1851-60.
114. Fabrega E, Berja A, Garcia-Unzueta MT, Guerra-Ruiz A, Cobo M, Lopez M, et al. Influence of aquaporin-1 gene polymorphism on water retention in liver cirrhosis. Scandinavian journal of gastroenterology. 2011;46(10):1267-74.
115. Iwakiri Y, Groszmann RJ. Vascular endothelial dysfunction in cirrhosis. Journal of hepatology. 2007;46(5):927-34.
116. Wells RG. Cellular sources of extracellular matrix in hepatic fibrosis. Clinics in liver disease. 2008;12(4):759-68, viii.
117. Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Nakatani T, et al. Angiotensin-II type 1 receptor interaction is a major regulator for liver fibrosis development in rats. Hepatology. 2001;34(4 Pt 1):745-50.
118. Yoshiji H, Noguchi R, Ikenaka Y, Kitade M, Kaji K, Tsujimoto T, et al. Renin-angiotensin system inhibitors as therapeutic alternatives in the treatment of chronic liver diseases. Current medicinal chemistry. 2007;14(26):2749-54.

119. Roney K, Holl E, Ting J. Immune plexins and semaphorins: old proteins, new immune functions. Protein & cell. 2013;4(1):17-26.
120. Kumanogoh A, Kikutani H. Immunological functions of the neuropilins and plexins as receptors for semaphorins. Nature reviews Immunology. 2013;13(11):802-14.
121. Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, et al. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nature medicine. 2006;12(2):235-9.
122. Martin FA, Murphy RP, Cummins PM. Thrombomodulin and the vascular endothelium: insights into functional, regulatory, and therapeutic aspects. American journal of physiology Heart and circulatory physiology. 2013;304(12):H1585-97.
123. Stenina-Adognravi O. Thrombospondins: old players, new games. Current opinion in lipidology. 2013;24(5):401-9.
124. An X, Jin Y, Guo H, Foo SY, Cully BL, Wu J, et al. Response gene to complement 32, a novel hypoxia-regulated angiogenic inhibitor. Circulation. 2009;120(7):617-27.
125. Uehara Y, Miura S, Yahiro E, Saku K. Non-ACE pathway-induced angiotensin II production. Current pharmaceutical design. 2013;19(17):3054-9.
126. Miyazaki M, Kanto T, Inoue M, Itose I, Miyatake H, Sakakibara M, et al. Impaired cytokine response in myeloid dendritic cells in chronic hepatitis C virus infection regardless of enhanced expression of Toll-like receptors and retinoic acid inducible gene-I. Journal of medical virology. 2008;80(6):980-8.
127. Gondois-Rey F, Dental C, Halfon P, Baumert TF, Olive D, Hirsch I. Hepatitis C virus is a weak inducer of interferon alpha in plasmacytoid dendritic cells in comparison with influenza and human herpesvirus type-1. PloS one. 2009;4(2):e4319.
128. Wu J, Meng Z, Jiang M, Zhang E, Trippler M, Broering R, et al. Toll-like receptor-induced innate immune responses in non-parenchymal liver cells are cell type-specific. Immunology. 2010;129(3):363-74.
129. Rowe IA, Galsinh SK, Wilson GK, Parker R, Durant S, Lazar C, et al. Paracrine signals from liver sinusoidal endothelium regulate hepatitis C virus replication. Hepatology. 2014;59(2):375-84.

130. Einhorn S, Eldor A, Vlodavsky I, Fuks Z, Panet A. Production and characterization of interferon from endothelial cells. Journal of cellular physiology. 1985;122(2):200-4.
131. Hagele H, Allam R, Pawar RD, Anders HJ. Double-stranded RNA activates type I interferon secretion in glomerular endothelial cells via retinoic acid-inducible gene (RIG)-1. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2009;24(11):3312-8.
132. Bigger CB, Guerra B, Brasky KM, Hubbard G, Beard MR, Luxon BA, et al. Intrahepatic gene expression during chronic hepatitis C virus infection in chimpanzees. Journal of virology. 2004;78(24):13779-92.
133. Asselah T, Bieche I, Narguet S, Sabbagh A, Laurendeau I, Ripault MP, et al. Liver gene expression signature to predict response to pegylated interferon plus ribavirin combination therapy in patients with chronic hepatitis C. Gut. 2008;57(4):516-24.
134. Chen L, Borozan I, Feld J, Sun J, Tannis LL, Coltescu C, et al. Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. Gastroenterology. 2005;128(5):1437-44.
135. Dill MT, Duong FH, Vogt JE, Bibert S, Bochud PY, Terracciano L, et al. Interferon-induced gene expression is a stronger predictor of treatment response than IL28B genotype in patients with hepatitis C. Gastroenterology. 2011;140(3):1021-31.
136. Wang BY, Ju XH, Fu BY, Zhang J, Cao YX. Effects of ethanol on liver sinusoidal endothelial cells-fenestrae of rats. Hepatobiliary & pancreatic diseases international : HBPD INT. 2005;4(3):422-6.
137. Hayman AR, Macary P, Lehner PJ, Cox TM. Tartrate-resistant acid phosphatase (Acp 5): identification in diverse human tissues and dendritic cells. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 2001;49(6):675-84.
138. Lang P, Zakaroff-Girard A, Wahlen K, Andersson J, Olsson T, Bambace C, et al. Expression and secretion of the novel adipokine tartrate-resistant acid phosphatase from adipose tissues of obese and lean women. International journal of obesity. 2011;35(12):1502-10.

139. Janckila AJ, Lin HF, Wu YY, Ku CH, Yang SP, Lin WS, et al. Serum tartrate-resistant acid phosphatase isoform 5a (TRACP5a) as a potential risk marker in cardiovascular disease. Clinica chimica acta; international journal of clinical chemistry. 2011;412(11-12):963-9.
140. Meindl-Beinker NM, Matsuzaki K, Dooley S. TGF-beta signaling in onset and progression of hepatocellular carcinoma. Digestive diseases. 2012;30(5):514-23.
141. Dooley S, ten Dijke P. TGF-beta in progression of liver disease. Cell and tissue research. 2012;347(1):245-56.
142. Guo X, Wang XF. Signaling cross-talk between TGF-beta/BMP and other pathways. Cell research. 2009;19(1):71-88.
143. Yan X, Liu Z, Chen Y. Regulation of TGF-beta signaling by Smad7. Acta biochimica et biophysica Sinica. 2009;41(4):263-72.
144. Flisiak R, Jaroszewicz J, Lapinski TW, Flisiak I, Prokopowiczi D. Effect of pegylated interferon alpha 2b plus ribavirin treatment on plasma transforming growth factor-beta1, metalloproteinase-1, and tissue metalloproteinase inhibitor-1 in patients with chronic hepatitis C. World journal of gastroenterology : WJG. 2005;11(43):6833-8.
145. Jakobsson L, van Meeteren LA. Transforming growth factor beta family members in regulation of vascular function: in the light of vascular conditional knockouts. Experimental cell research. 2013;319(9):1264-70.
146. Branton MH, Kopp JB. TGF-beta and fibrosis. Microbes and infection / Institut Pasteur. 1999;1(15):1349-65.
147. Roulot D, Sevcsik AM, Coste T, Strosberg AD, Marullo S. Role of transforming growth factor beta type II receptor in hepatic fibrosis: studies of human chronic hepatitis C and experimental fibrosis in rats. Hepatology. 1999;29(6):1730-8.
148. Sanderson N, Factor V, Nagy P, Kopp J, Kondaiah P, Wakefield L, et al. Hepatic expression of mature transforming growth factor beta 1 in transgenic mice results in multiple tissue lesions. Proceedings of the National Academy of Sciences of the United States of America. 1995;92(7):2572-6.
149. George J, Roulot D, Koteliansky VE, Bissell DM. In vivo inhibition of rat stellate cell activation by soluble transforming growth factor beta type II receptor: a

potential new therapy for hepatic fibrosis. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(22):12719-24.
150. Ueno H, Sakamoto T, Nakamura T, Qi Z, Astuchi N, Takeshita A, et al. A soluble transforming growth factor beta receptor expressed in muscle prevents liver fibrogenesis and dysfunction in rats. Human gene therapy. 2000;11(1):33-42.
151. Breitkopf K, Haas S, Wiercinska E, Singer MV, Dooley S. Anti-TGF-beta strategies for the treatment of chronic liver disease. Alcoholism, clinical and experimental research. 2005;29(11 Suppl):121S-31S.
152. Presser LD, Haskett A, Waris G. Hepatitis C virus-induced furin and thrombospondin-1 activate TGF-beta1: role of TGF-beta1 in HCV replication. Virology. 2011;412(2):284-96.
153. Lin W, Weinberg EM, Tai AW, Peng LF, Brockman MA, Kim KA, et al. HIV increases HCV replication in a TGF-beta1-dependent manner. Gastroenterology. 2008;134(3):803-11.
154. Gomaa WM, Ibrahim MA, Shatat ME. Overexpression of cyclooxygenase-2 and transforming growth factor-beta 1 is an independent predictor of poor virological response to interferon therapy in chronic HCV genotype 4 patients. Saudi journal of gastroenterology : official journal of the Saudi Gastroenterology Association. 2014;20(1):59-65.
155. Wen H, Lei Y, Eun SY, Ting JP. Plexin-A4-semaphorin 3A signaling is required for Toll-like receptor- and sepsis-induced cytokine storm. The Journal of experimental medicine. 2010;207(13):2943-57.
156. Yamamoto M, Suzuki K, Okuno T, Ogata T, Takegahara N, Takamatsu H, et al. Plexin-A4 negatively regulates T lymphocyte responses. International immunology. 2008;20(3):413-20.
157. Iwai Y, Terawaki S, Ikegawa M, Okazaki T, Honjo T. PD-1 inhibits antiviral immunity at the effector phase in the liver. The Journal of experimental medicine. 2003;198(1):39-50.
158. Dong H, Zhu G, Tamada K, Flies DB, van Deursen JM, Chen L. B7-H1 determines accumulation and deletion of intrahepatic CD8(+) T lymphocytes. Immunity. 2004;20(3):327-36.
159. Klenerman P, Thimme R. T cell responses in hepatitis C: the good, the bad and the unconventional. Gut. 2012;61(8):1226-34.

160. Danese S, Dejana E, Fiocchi C. Immune regulation by microvascular endothelial cells: directing innate and adaptive immunity, coagulation, and inflammation. Journal of immunology. 2007;178(10):6017-22.
161. Knolle PA, Loser E, Protzer U, Duchmann R, Schmitt E, zum Buschenfelde KH, et al. Regulation of endotoxin-induced IL-6 production in liver sinusoidal endothelial cells and Kupffer cells by IL-10. Clinical and experimental immunology. 1997;107(3):555-61.
162. Wiest R, Groszmann RJ. The paradox of nitric oxide in cirrhosis and portal hypertension: too much, not enough. Hepatology. 2002;35(2):478-91.
163. Ding BS, Nolan DJ, Butler JM, James D, Babazadeh AO, Rosenwaks Z, et al. Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. Nature. 2010;468(7321):310-5.
164. Gupta TK, Toruner M, Chung MK, Groszmann RJ. Endothelial dysfunction and decreased production of nitric oxide in the intrahepatic microcirculation of cirrhotic rats. Hepatology. 1998;28(4):926-31.
165. Butler JM, Kobayashi H, Rafii S. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. Nature reviews Cancer. 2010;10(2):138-46.
166. Schraa AJ, Everts M, Kok RJ, Asgeirsdottir SA, Meijer DK, de Leij LF, et al. Development of vasculature targeting strategies for the treatment of cancer and chronic inflammatory diseases. Biotechnology annual review. 2002;8:133-65.
167. Gacche RN, Meshram RJ. Angiogenic factors as potential drug target: Efficacy and limitations of anti-angiogenic therapy. Biochimica et biophysica acta. 2014;1846(1):161-79.

11.

LIST OF FIGURES AND TABLES

FIGURES

TABLES

12.

CURRICULUM VITAE

Neven Papic, born on January 5th 1984 in Zagreb, following graduation from School of Medicine at the University of Zagreb, worked as a Research Fellow at the University Hospital for Infectious Diseases in Zagreb where he gained interest in the field of viral hepatitis and has started infectious disease fellowship in 2010. In 2011 and 2012 he gained additional education in the field of genome analysis at the University of Utah, USA. These studies have provided insight into the broad portrait of genomic changes associated with inflammatory response to HCV infection. He received several prestige awards in 2011 ESCMID (European Society of Clinical Microbiology and Infectious Diseases) Training and Education Award and Croatian Science Foundation Scholarship for doctoral students and in 2014 ESCMID TAE Award for Training achievements. Neven is co-author on 10 science publications published in international peer-reviewed journals, and has presented his research in several international meetings. In his research he advocates the interdisciplinary approach that integrates the basic and clinical science to decode dynamic interactions between microbial pathogens and their hosts. Currently, he is completing his final year of ID training.