Interferon signaling in viral hepatitis

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Summary

Hepatitis C virus (HCV) is a single stranded positive RNA virus classified in 6 different genotypes. Hepatocytes are the main targets of HCV infection. It has been estimated that 60 to 70% of the infected patients develop chronic infection. If left untreated, chronic hepatitis C (CHC) results in cirrhosis in 10 to 20% of the cases. Once cirrhosis is established, the risk of hepatocellular carcinoma (HCC) development increases dramatically, with an estimated annual rate of 1% to 4%. The standard of care (SOC) for CHC treatment is based on pegylated IFNα (peg-IFNα) and Ribavirin administration. Peg-IFNα injection activates the Jak-STAT signaling pathway that leads to the phosphorylation of STAT1 and culminates in the up-regulation of hundred of genes in the liver, establishing an antiviral state. However, peg-IFNα-based therapy achieves the clearance of HCV only in half of the chronic infected individuals. In the recent past, the lack of response to peg-IFNα-based therapy in CHC have been associated to the broad up-regulation of interferon regulated genes (IRGs) in the liver of CHC patients, already before treatment. The reason why the *pre-activated* hepatic IFN system fails to clear HCV remains to be elucidated. Furthermore, the molecular mechanisms that define the level of activation of the hepatic IFN system in CHC are not clear. In the recent past, several genome-wide association studies have reported a strong association of treatment-failure with minor (less frequent in the population) alleles at single nucleotide polymorphisms (SNPs) located in the IL28B locus on chromosome 19. Minor alleles at SNPs in the IL28B locus have also been associated to the up-regulation of the hepatic IFN system pre-treatment in CHC patients. So far the molecular mechanisms that links allelic variants at IL28B locus, the pre-activation of the hepatic IFN system and treatment-response in CHC patients remain to be elucidated. The present work is aimed to investigate two of the possible molecular mechanisms that could mediate the *pre-activation* of the IFN system in the liver of CHC patients that do not respond to therapy.

In the first part of the thesis the role of unphosphorylated-STAT1 (U-STAT1) in mediating the up-regulation of hepatic IRGs in CHC patients was investigated. We have reported that STAT1 accumulates in the liver of CHC patients non-responders. Furthermore, experimental evidences suggest that STAT1 could play a role as

transcription factor independently by its phosphorylation on tyrosine 701 and its unphosphorylated form can drive the expression of a subset of IRGs. In the present study we took advantage of a cell line constitutively lacking STAT1 expression and we exogenously re-expressed a mutant form of STAT1 that can not be phosphorylated, mimicking U-STAT1. We proved that U-STAT1 *per se* is not able to induce the expression of IRGs and it is unlikely to be the cause of the *pre-activated* IFN system observed in the liver of non-responders CHC patients.

In the second part of the thesis, we investigated the role of IFNλs signaling pathway in the definition of the *pre-activated* hepatic IFN system in CHC. IFNλs are the most recently group of IFNs. IFNλs signal through the cells via a different receptor compared to the one of IFNα. However, the intracellular signaling pathway of the two class of cytokines is completely overlapping, leading to the up-regulation of the same IRGs. We demonstrated that in an hepatoma cell line Huh7 the over-expression of *IL28Rα,* one of the two chains of INFλ receptor complex, mediates the long lasting up-regulation of IRGs upon IFNλ stimulation. We confirmed our results in human liver biopsies, where we found a significant positive correlation between *IL28Rα* and IRGs expression. We observed that *IL28Rα* is an IRG itself but its level of expression is modulated by allelic variants at SNPs mapping in the IL28B locus, that have been associated to treatment response in CHC patients.

In conclusion we provide evidences of a molecular mechanism that links the pre-activation of the hepatic IFN system (and non-response) and allelic variants at IL28B locus.

Abbreviations

AHC	Acute hepatitis C
ALT	Alanine-aminotransferase
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
CHC	Chronic hepatitis C
CLDN	Claudin 1
Da	Dalton
DAAs	Direct-acting antivirals
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EGFR	Epithelial growth factor receptor
eiF2α	Eukaryotic translation initiation factor 2α
eiF3	Eukaryotic translation initiation factor 3
ER	Endothelial reticulum
EVR	Early virological response
GAGs	Glycosaminoglycans
GAS	Gamma-activation sequence
GTP	Guanosine triphosphate
GWAS	Genome wide association studies
HBV	Hepatitis B virus
HCCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Cell culture derived hepatitis C virus
HFLC	Human fetal liver cells
HIV	Human immunodeficiency virus
IFIT	Interferon-induced protein with tetratricopeptide repeats 1
IFN	Interferon
IRES	Internal ribosome entry site
IRF3	IFN regulatory factor 3
IRG	Inteferon regulated genes
ISRE	IFN-stimulated response element
IU	International units
Jak	Janus kinase
LD	Lipid dropplets
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
MAVS	Mitochondrial antiviral signaling protein
Mio	Million
miRNA	Micro RNA
NANBH	Non-A non-B hepatitis
NLR	Nucleotide oligomerization domain like receptor
NR	Non-responsders
OAS	2'-5'oligodanylate synthase
OCLN	Occludin
ORF	Open reading frame
PAMPs	Pattern associated molecular patterns
PBMCs	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
pDCs	Plasmacytoid dendritic cells
PEG	Polyethylenglycol
PHHs	Primary human hepatocytes

PIAS	Protein inhibitors of activated STAT
PKR	Protein kinase R
PP2A	Protein phosphatase 2A
PRMT1	Protein arginine N-methyltransferase 1
PRRs	Pattern recognition receptors
RdRp	RNA dependent RNA polymerase
RLRs	Retinoic acid inducible gene I like receptor
RNA	Ribonucleic acid
RNaseL	RNA nuclease L
RVR	Rapid virological response
SAMe	S-Adenosyl methionine
SH2	Scr homology domain 2
SHPs	SH2 domain containing peptidases
SOC	Standard of care
SR-B1	Scavenger receptor class B1
STAT	Signal Transducer and Activator of Transcription
SVR	Sustained virological response
Tc-PTP	T cell protein tyrosine phosphatase
TLRs	Toll like receptors
TRIF	TIR domain containing adapter inducting IFNβ
U-STAT1	unphosphorylated STAT1
USP18	Ubiquitin-specific peptidase 18
UTR	Untranslated region
VLDL	Very low density lipoprotein

1. Introduction

1.1 Hepatitis C virus

Hepatitis C virus (HCV) infection is cause of chronic liver disease worldwide[1]. It has been estimated that more than 130 million of people are chronically infected and many will develop chronic liver disease and hepatocellular carcinoma (HCC)[1].

1.1.1 HCV genome and classification.

HCV is a positive-strand RNA virus of the *Flaviviridae* family[2]. HCV genome is 9600 nucleotides in length and it encodes for a single open reading frame (ORF), flanked by 5'- and 3'- untranslated regions (UTRs) (Fig. 1.1) [2]. The 5'-UTR contains an internal ribosome entry site (IRES) and essential signals for the synthesis of the negative RNA strand which serves as replicative intermediate[2]. A liver specific microRNA, miR-122, have been reported to bind the 5'-UTR[3], resulting in the modulation of HCV RNA replication[3,4]. The ORF encodes for a polyprotein precursor that is post-translationally cleaved by viral and host proteases resulting in the production of structural and non-structural proteins[2].

HCV is classified in 6 major genotypes based on the genome sequences. Each genotype is divided in subtypes (designated with a small letter, e.g. 1a, 1b) that differ in their genomic sequences of 20-25%[5]. HCV has a high replicative rate and mutations are often found in the viral genome because of the lack of a proofreading activity of the HCV RNA dependent RNA polymerase (RdRp). Consequently, a heterogeneous population of HCV viruses (termed quasispecies) coexists within the same infected individual.

1.1.2 HCV lifecycle

HCV structure has not been completely clarified. It is believed that HCV virions comprise a nucleocapside core surrounded by a host derived membrane containing the glycoproteins E1 and E2[6]. Results of electron microscopy studies indicate that HCV particles are 40-70 nm in diameter[2]. The virus circulates free in the blood stream or bound to low density lipoproteins (LDL), very low density lipoproteins (VLDL) and immunoglobulins[2].

HCV entry.

Hepatocytes are the main targets of HCV infection [2]. Many host factors are involved in HCV entry: glycosaminoglycans (GAGs)[7], the low density lipoprotein receptor (LDLR)[8,9], the high density lipopoprotein receptor scavenger receptor class B 1 (SR-B1)[10], tetraspanin CD81[11], the tight junctions claudin 1 (CLDN1)[12] and occluding (OCLN)[13], and, most recently, the epithelial growth factor receptor (EGFR)[14]. The current model for HCV entry predicts a multistep process that includes attachment and receptor binding, post-binding association to tight junctions and then internalization via clathrin-mediated endocytosis[6]. Endocytosis is followed by the transit in an endosomal low-pH compartment that results in the fusion of the membranes and release of viral RNA in the cytoplasm[6].

Translation and polyprotein processing.

HCV RNA does not contain a 5'-cap and uses an IRES-based cap-independent approach for protein translation[15]. Translation initiation of HCV RNA occurs through the formation of a complex between IRES, the 40S ribosomal subunit and the eukaryotic initiation factor 3 (eIF3)[15]. Subsequently, the 80S complex is formed upon GTP hydrolysis and binding to the 60S ribosomal subunit[15]. It has been reported that conformational change in the 40S subunit induced by the binding of IRES is required for the assembly of an active 80S complex in the absence of a 5'-cap[16].

HCV RNA is translated in a precursor polyprotein of 3000 amino acids that is subsequently processed to generate the mature structural and non-structural proteins (Fig. 1.1)[2]. The structural proteins core, E1, and E2 (envelope glycoproteins) form the viral particle[2]. The non-structural proteins include the p7 ion channel, the NS2-3 protease, the NS3 serine protease/RNA helicase, the NS4A proteins, and the NS5B RNA dependent RNA polymerase (RdRp)[2].

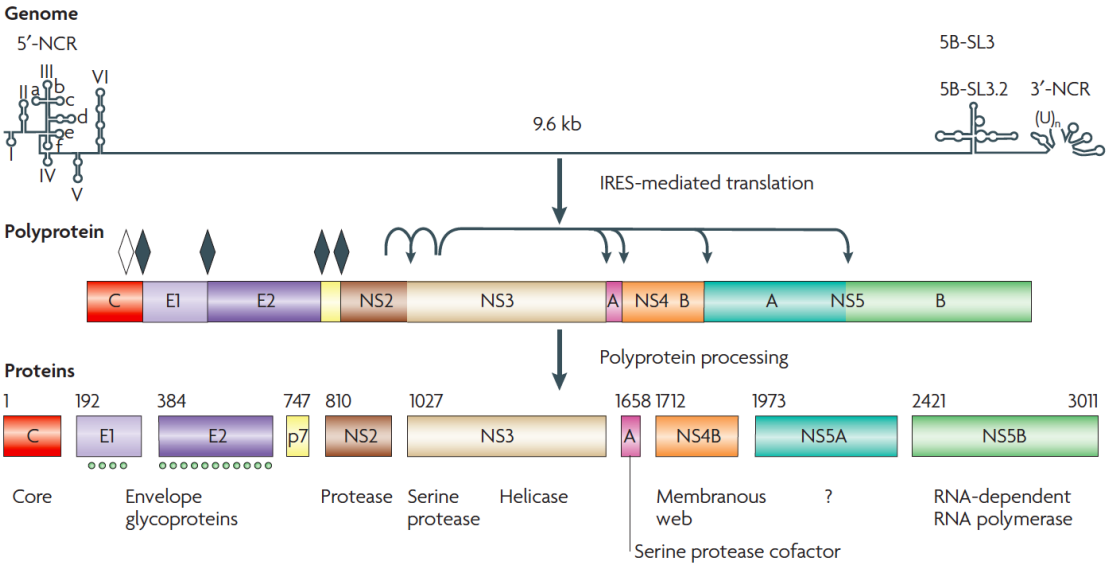


Figure 1.1. *HCV genome organization and viral protein functions*[2].

Replication.

HCV replication is achieved by generating a complementary negative-stranded RNA that serves as a template to synthesize a positive-stranded RNA[2]. NS5B RdRp is the key enzyme in this process[2]. The precise composition of HCV replication complex is not completely understood. However, as for all positive stranded RNA viruses, HCV replication requires intracellular membranes[2]. A specific membrane alteration (membranous webs) derived from the endothelial reticulum (ER) has been considered as putative site of HCV replication[2]. The membranous webs could have a role in supporting the organization of the replication complex concentrating the viral products, providing lipids, and protecting the viral RNA from host defense mechanisms.

Assembly, maturation, and release.

The late steps of the viral lifecycle are not completely understood. HCV assembly results in the formation of the nucleocapsid and loading of the HCV RNA[17]. It has been reported that p7, NS2, NS5A, NS4B, and NS3 are involved in HCV assembly (reviewed in [17]). This evidence suggests a close link between HCV replication and assembly.

HCV assembly is tightly linked to lipid metabolism[17]. Many independent evidence supports the concept that the core association with lipid droplets (LD) is essential for HCV assembly (reviewed in [17]).

Maturation and release of viral particles depends on the very low density lipoprotein (VLDL) pathway[17]. Several reports emphasize the role of apolipoprotien E (apoE) in the formation of infective viral particles[18,19,20]. Instead, Apolipoprotein B (apoB) contribution to HCV release remains controversial[18,20]. Mature HCV particles containing both apoB and apoE are finally released from the hepatocytes through the VLDL secretory pathway[17].

1.1.3 *In vitro* and *in vivo* models for HCV research.

HCV, first termed non-A, non-B hepatitis (NANBH), was initially described in 1975 in sera of post-transfusion hepatitis patients[21]. However, due to the lack of *in vitro* and *in vivo* models of infection, more than one decade had been required to clone the sequence of HCV genome[22]. In 1997, it was demonstrated that HCV cn infect chimpanzees.[23] In 1999, a major breakthrough in HCV research was achieved with the development of the sub-genomic replicon system[24] that allows the long-term HCV RNA replication in cell culture. However, this system does not permit the study of HCV life cycle since no viral particles are produced. Most recently, a complete HCV cell culture system has been developed (JFH1/HCVcc), opening the possibility to study HCV lifecycle *in vitro.* The HCVcc system is based on a unique isolate from a Japanese patient affected by fulminant hepatitis that was found to replicate in a hepatoma cell line (Huh7)[25]. Indeed, viral particles are produced in Huh7 cells transfected with the JFH1 RNA[25] and HCV viruses generated in culture are able to infect naïve Huh7 cells as well as chimpanzees[25,26].

No vaccine against HCV infection is available so far. The design of a successful vaccine requires the study of HCV in *in vivo* models in order to investigate the components of the adaptive immune response against the virus[27]. Chimpanzees are the only animals that can support the complete HCV life cycle[28]. Many efforts have been made to generate mouse models that could support HCV infection. So far, only immunodeficient mice engrafted with human hepatocytes support a complete viral life cycle[29,27]. However, these mice are not useful to study the adaptive immunoresponse against HCV and to develop vaccine[29,27]. To overcome the limitations, a new humanized mouse model has been recently proposed. A fusion protein of FK506 binding protein and caspase 8 under control of the albumin promoter (AFC8) was expressed in immune-deficient mice, resulting in the hepatocytes cell death upon

administration of a specific drug[30]. Subsequently, co-injection in the liver of new born mice of hepatocytes progenitor cells and $CD34^+$ human hematopoietic stem cells resulted in hepatocytes repopulation and detection of T cells, NK cells, plasmacytoid and myeloid dendritic cells[30]. The humanized mice were permissive for HCV infection, generate a specific immune response against the virus, and develop liver diseases (hepatitis and fibrosis)[31]. However, the use of such mouse model to develop vaccines is limited since the mice are unable to support the complete HCV lifecycle[31].

1.2 Innate immune response in HCV infection

HCV is sensed by the host innate immune system and then interferons (IFNs) are produced[32]. IFNs are the key mediators of antiviral response[32]. HCV interferes with the innate immune system at different levels in order to block IFN production and establish a persistent infection.

1.2.1. HCV sensing by the innate immune system.

The innate immune response is activated when pattern-associated molecular patterns (PAMPs) interact with pattern recognition receptors (PRRs)[32]. PRRs are divided in three major classes: Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide oligomerization domain (NOD)-like receptors (NLRs)[32]. The first two classes display a major role in HCV sensing and both mediate the production of type I and III IFNs (Fig 1.2).

Toll-like receptors.

TLRs family comprises at least 10 members that are expressed in various immune cells (macrophages, DCs, and B cells,) and in other cell types like, fibroblasts and epithelial cells[32]. Three members of the TLRs family are primarily involved in the sensing of viral infection: TLR3, TLR7, and TLR9[32]. Unlike other TLRs that display a cell membrane expression, TLR3, TLR7, and TLR9 are localized in the endosomes[32]. All these three TLRs detect viral nuclear acids: TLR9 senses unmethylated CpG motifs, TLR7 binds to uridine- and guanosine rich single stranded RNA (ssRNA), and TLR3 recognizes double stranded RNA (dsRNA)[32]. TLR3 and

TLR7 have been reported to play a role in HCV infection[33,34]. Upon activation, TLR7 binds the adaptor protein MyD88 inducing a kinase cascade that leads to the formation of the complex MyD88-IRAK1-IRAK4-TRAF6[35]. This complex ultimately activates NF-κB and IRF7[32]. MyD88 is a common adaptor protein for TLRs[32]. However TLR3 transduces the signal through the binding to TRIF[32]. TRIF, together with TBK1, activates ultimately IRF3[35]. IRF3, IRF7, and NF-κB translocate into the nucleus[32] and induce the expression of type I and type III IFNs that mediates the antiviral response[35].

RLR family.

RLR family comprises the cytoplasmic proteins RIG-I, MAD5, and LPG2[32]. RIG-I is primarily involved in HCV sensing[36]. HCV RNA contains two motifs that are targeted by RIG-I: the 5'-triphosphate and the stem loop structure in the 3'-UTR[36]. Of note, the 3'-UTR is highly conserved among the HCV genotypes and it is essential for HCV replication[32]. Binding of RNA to RIG-I induces a conformational change that results in the association of the mitochondrial antiviral signaling protein (MAVS), a key adaptor protein localized in the mitochondria cell membrane[32]. MAVS induces a signal cascade that involves TKB and IKKi resulting in the activation of IRF3 and NF-κB[32]. Ultimately, IRF3 and NF-κB induce the expression of type I and type III IFNs through the binding to response elements in their promoter region[32]. The key role of RIG-I in HCV RNA sensing has been demonstrated in hepatoma cells[36]. Indeed, in RIG-1 -/- Huh7.5 cells, HCV RNA fails to induce IFNβ[36]. Moreover HCV replication appears to be more efficient in cell lacking a functional RIG-I signal[37].

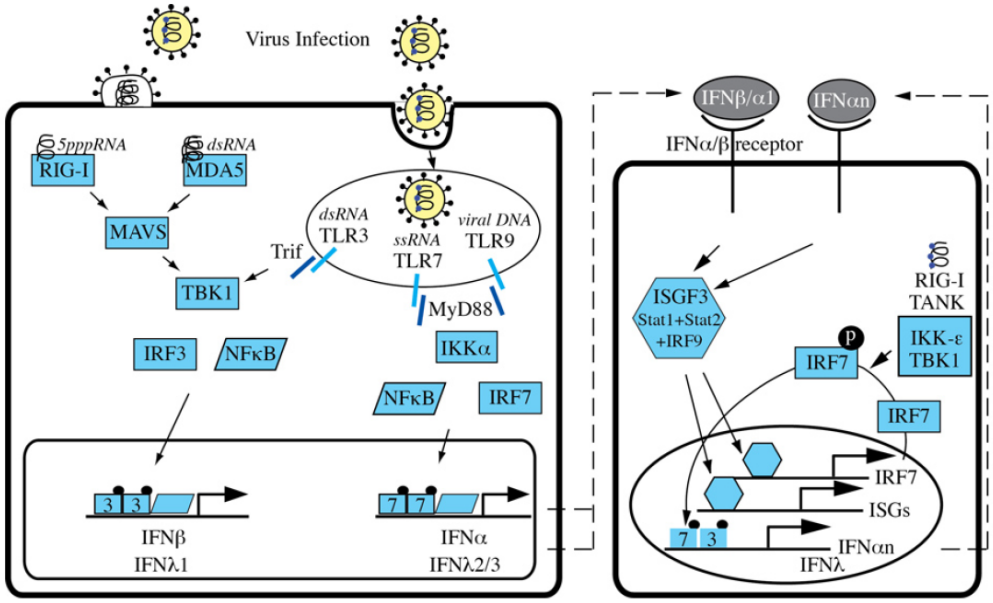


Figure 1.2. *Viral sensing and autocrine/paracrine IFNs production.*[38].

1.2.2 HCV interference with viral sensing.

In order to establish a persistent infection, HCV has developed different strategies to evade the host immune response[38]. HCV interference with the viral sensing pathways has been well characterized. Indeed, viral protease NS3 displays a major role in this context. NS3 binds to the co-factor NS4A allowing the complex to be anchored to the intracellular membranes and to facilitate the activation of the protease domain of NS3[39]. The membrane-bound localization of NS3/4A is essential to interfere with viral sensing[39]. Indeed, NS3/4A cleaves MAVS leading to the impairment of RIG-I signaling and IFNs production[40,41,42]. Importantly, MAVS cleavage by NS3/4A has been confirmed in HCV-infected liver biopsies[43].

The role of NS3/4A in TRIF cleavage remains controversial. TRIF cleavage has been reported[44] but this finding is not supported by others publications[39]. Moreover, TRIF cleavage has not been reported in HCV infected human liver biopsies[39].

1.2.3 Interferons and their receptors.

Since the first discovery in 1957 by Isaac and Lindenmann, many IFNs types and subtypes are now known[38]. IFNs are currently classified in three major classes: type I, type II and type III. Each class of IFNs signals into the cell by engaging different receptor complexes (Fig 1.3).

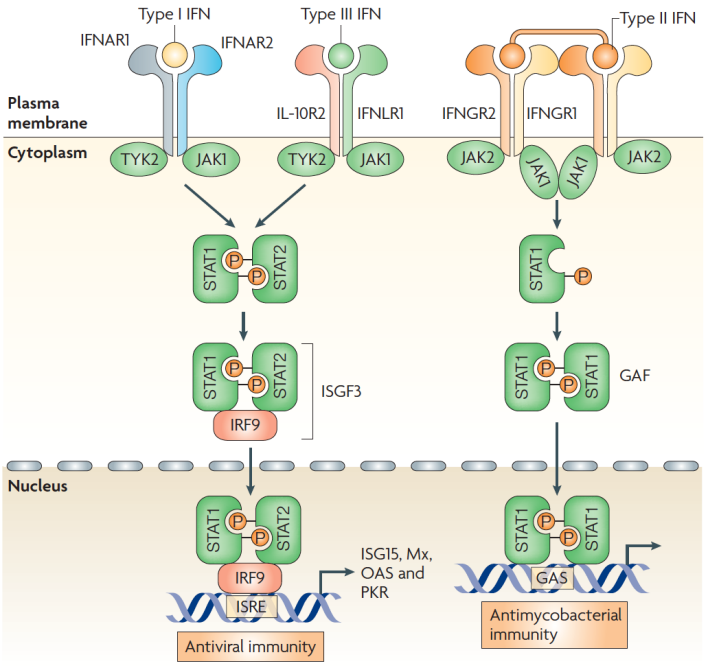


Figure 1.3. *Interferons, their receptors and the Jak-STAT pathway*[45].

Type I IFNs.

Type I IFNs comprise at least 13 IFNα subtypes and one single IFNβ[46]. Genes encoding for type I IFNs cluster are located on chromosome 9, lack introns, and are regulated by their own promoter[46]. The intracellular signaling pathway is mediated by the binding to a receptor comprising two chains ubiquitously expressed, IFNAR1[47] and IFNAR2c[48]. It has been reported that mice deficient for IFNAR chains are more susceptible to viral infection but maintain resistance to other pathogens[49,50].

IFNα has a key role in the antiviral activity against HCV infection. Indeed, since 20 years, IFNα has constituted the backbone of the standard of care (SOC) for the treatment of chronic HCV infection (see section 1.4.1)[51].

Type II IFNs.

IFNγ is the sole type II IFN and binds to a receptor composed by two subunits IFNGR1 and IFNGR2[52]. IFNγ is produced by immune cells like T-lymphocytes, B-cells, NK cells, and antigen presenting cells (monocyte/macrophage and dendritic cells)[52]. IFNγ -/- and IFNGR1 -/- show deficiencies to bacterial, parasitic, and viral infection[53,54]. Both type I and type II IFNs are required to efficiently clear some

viruses suggesting that the two class of IFNs complement each other providing protection against a broad spectrum of pathogens[50].

Type III IFNs.

Type III IFNs have been more recently described[55,56]. This IFN class includes: IFNλ1 (or IL29), IFNλ2 (or IL28A), and IFNLλ3 (or IL28B)[56] . Type III IFNs genes display high sequence similarities with each other[55,56]. In particular, IFNλ2 and IFNλ3 have almost identical sequence not only in the coding region but also in the upstream and downstream flanking regions[56]. Indeed, in the promoters of type III IFNs, computational analysis have predicted binding sites for transcription factors like AP1, NFκB, and IRF that have been described to mediate also the expression of type I IFNs[57]. During the last few years, the interest of the scientific community has been focused on the allelic variants at the IFNλ3 (IL28B) locus. Indeed, several genome wide association studies (GWAS) have reported an association between allelic variants at single nucleotide polymorphism (SNPs) mapping in the IL28B locus and response treatment in CHC patients[58,59,60]. This topic will be further discussed in section 1.4.2.

Plasmacytoid dendritic cells (pDCs) are currently considered as the "professional" producers of IFNλs upon viral infection[61]. However, many other cell types have been reported to produce type III IFNs upon viral infection[62]. Indeed, early phase of HCV infection has been associated with type III IFNs production[63,64] and induction of type III IFNs has been reported in HCV infected primary human hepatocytes (PHH) [63,64] as well as in primary human fetal liver cells (HFLC)[65].

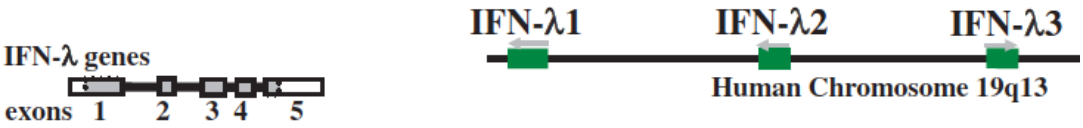


Figure 1.4 *Organization of human IFNλs gene cluster on chromosome 19*[57].

Type III IFNs bind to a receptor complex that comprises the unique IL28Rα and the IL-10R2 chain that is shared with the receptors of IL-10, IL-22, and IL-26[56]. The gene encoding for IL28Rα is located on chromosome 1[55,56], whereas the one encoding for of IL-10R2 maps on chromosome 21. Despite the distant chromosomal localization, IL28Rα and IL10-R2 genes share a similar genetic structure. The coding region

comprises 7 exons: exon 1 encodes for the 5'-UTR and for the plasma membrane signal peptide; exons 2, 3, 4, 5, and part of exon 6 encode for the extracellular domain; the transmembrane domain is encoded by exon 6; the intracellular domain is encoded by part of exon 6 and exon 7; exon 7 encodes also for the 3'-UTR[55,56]. Three different splice variants have been described for the human IL28Rα. IL28Rα-variant 1 comprises all the previously described exons and encodes for the functional chain of the receptor[56]. IL28Rα-variant 2 is generated by a partial splicing of exon 7 resulting in a signaling incompetent protein that lacks most of the intracellular domain[56]. IL28Rα-variant 3, originating from the splicing of exon 6, encodes for a soluble form of the receptor and lacks both the transmembrane and intracellular domain[55,56]. The biologic function of the signal-incompetent variants 2 and 3 has not been completely elucidated. It has been suggested that splice variant 3 could act as a decoy receptor, partially subtracting type III IFNs from the binding to the functional receptor[66].

A computational analysis suggests the presence of binding sites for the transcription factors AP-2, c-Jun, p53, and STAT1 in the promoter of human IL28RA gene[67].

IL28Rα, unlike IL10-R2, display cell and tissue specific expression. Indeed, lungs and many organs of the immune system (spleen, thymus, PBMCs) express high level of IL28Rα[66]. The brain displays a low IL28Rα expression level[66]. Keratinocytes and melanocytes, unlike fibroblast, endothelial cells and adipocytes, express high level of IL28Rα[66].

1.2.4. Interferons and the Jak-STAT signaling pathway.

Interferons mediate antiviral, antiangiogenic immunoregulatory, and antiproliferative effects[68]. They exert their activity mainly through the activation of the Jak-STAT signaling pathway and induction of interferon regulatory genes (IRGs) [68]. However, the effects mediated by IFNs result also through the activation of other signaling pathways, such as the p38-Map kinase cascade[69] and the phosphatidylinositol-3-kinase – Akt pathway[70].

The Janus kinase (Jak) - signal transducer and activator of transcription (STAT) signaling pathway is induced by IFNs and many other cytokines. In mammalian, 5 Jaks (Jak 1-3 and Tyk2) and 7 STATs (STAT1-4, STAT5A, STAT5B and STAT6) genes have been mapped[71,72,73]. Of note, different splicing and post-translational cleavages can form multiple STATs variants, that can act as dominant[71]. Indeed,

STAT1 displays two splicing variants: STAT1α (full length) and STAT1β (lacking the carboxy-terminal part of the protein)[68].

Jaks are tyrosine kinases, whereas STATs are latent transcription factors, found inactive in the cytoplasm. Specific Jaks associate to the intracellular domain of each IFN receptor chains: Jak1 binds to IFNAR2, IFNGR1, and IL10-R2; Jak2 associates to IFNAR1; Tyk2 binds to IL28Rα, IFNAR1 and IL28Rα (Fig. 1.3)[68]. Upon binding of IFN, the receptor chains are brought in close proximity and the Jaks trans-activate each other and then phosphorylate specific tyrosine residues in the intracellular domain of the receptors. The phospho-tyrosine residues become docking sites for the STATs that bind to the receptors through the Src homology domain 2 (SH2)[68]. The Jaks then activate STATs by phosphorylation of specific tyrosine residues[68]. Activated STATs form homo or heterodimers that move into the nucleus and bind to the promoter of interferon regulated genes (IRGs) through the DNA binding domain, inducing gene transcription[72].

Type II IFNs mediate the activation of STAT1 (phosphorylation on tyrosine 701), resulting in the formation of STAT1 homodimers[73] (fig 1.3). Despite the engagement of different receptor complexes, type I and type III IFNs intracellular signaling pathways are overlapping and result in the phosphorylation of STAT1, STAT2 (phosphorylation on tyrosine 689), and STAT3 (phosphorylation on tyrosine 705)[74]. STAT1 homodimers and STAT1-STAT2 and STAT1-STAT3 heterodimers are then generated[74]. STAT1 homodimers and STAT1-STAT3 heterodimers associate to gamma activates sequences (GAS) in the promoter region of IRGs[71]. STAT1-STAT2 heterodimers bind to IRF9 and generate the complex IFN-stimulated gene factor 3 (ISGF3) that associates to IFN-stimulated responsive elements (ISRE) in the promoter of the IRGs[71].

IFN stimulation induces the expression of hundred of IRGs, however, the function of some of them has so far been investigated[75].

ISG15.

ISG15 is an ubiquitin-like protein. The enzymatic cascade that mediates ISGylation comprises the activation of an E1 activating enzyme (UBE1L), an E2 conjugating enzyme (UbcH8), and an E3 ligase (HERC5 and TRIM25)[75]. ISGylation is reversible and USP18/UBP43 is one of the key enzymes that catalyzes the hydrolysis of ISG15

from the targets[76]. Interestingly, most of the enzymes involved in ISGylation are induced upon IFN stimulation[75]. More than 150 putative targets of ISGylation have been identified, and many are involved in interferon signaling and viral sensing[77]. ISGylation does not mediate the degradation of the target protein[75]. Accordingly, it has been reported that ISGylation prevent virus-mediated degradation of IFN regulatory factor 3 (IRF3), increasing the production of IFNβ[78]. The prominent role of ISG15 in the antiviral activity has been confirmed in knock-out mice, that display an increased susceptibility to the infection by a number of viruses[79,80].

Mx family proteins.

MX1 and MX2 display a GTPase activity[75]. Point mutations in the genes encoding the two MX proteins confer to mice a high susceptibility to viral infection[81]. The MX proteins seem to target viral components that are subsequently trapped and targeted for degradation[82].

OAS and the RNAseL pathway.

The 2'-5' oligoadenylate synthase (OAS) family comprises four genes (OAS1, OAS2, OAS3 and OASL)[75]. Several splice isoforms are generates[75]. The 2'-5' oligoadnylate activates the latent RNA nuclease (RNAseL) that degrades single stranded RNA[75].

PKR.

PKR is a protein kinase that is ubiquitously expressed and upregulated upon type I and type III IFN stimulation[75]. PKR is maintained inactive in the cytoplasm and it is activated through binding of viral RNA[75]. Activated PKR mediates the phosphorylation of eukaryotic initiation factor 2 (eIF2α), leading to the block of translation[75].

IFIT family.

The interferon-induced protein with tetratricopeptide repeats (IFIT1 and IFIT6) have also been shown to display anti viral activity[38]. They bind to eIF3 and block translation[38].

1.2.5. Negative regulators of the Jak-STAT pathway.

The Jak-STAT signaling pathway is modulated by a number of negative regulators (reviewed in [71,38,83], Fig 1.5).

SHPs.

SH2 domain containing phosphatases, SH1 and SH2 are ubiquitously expressed and reside in the cytoplasm[83]. SHPs impair the Jak-STAT signaling pathway by de-phosphorylating phospho-tyrosine residues in the intracellular domain of the receptors or on the Jaks, resulting in a reduced STAT phosphorylation[83].

Suppressor of cytokine signaling proteins (SOCSs).

SOCS family comprises 8 members (CIS and SOCS1 to SOCS7) that are rapidly induced by IFNs, resulting in an early negative feedback loop of the Jak-STAT pathway[38]. SOCSs are cytoplasmic proteins that contain an SH2 domain[38]. They can exert the inhibitory activity on the Jak-STAT pathway in various ways: by binging and inhibiting Jaks, by competing with STATs for the binding to the receptors or by mediating the protein turnover of the receptors through ubiquitine-proteasome degradation[83].

Ubiquitin specific peptidase (*USP18/UBP43).*

USP18/UBP43 has been first described as a protease mediating the cleavage of ubiquitine-like ISG15 conjugated to target proteins[76]. However, it has been recently shown that USP18/UBP43 exerts an inhibitory effect on the Jak-STAT signaling pathway independently from the peptidase activity[84]. Indeed, USP18/UBP43 has been reported to inhibit Jak1 activation via impairment of the Jak1 binding to IFNAR2c[84]. USP18/UBP43 is induced upon IFN treatment[85,86]. Accordingly, in Huh7 cells, type I and III IFNs[85], and at less extent type II IFN[86], up-regulate USP18/UBP43 at mRNA and protein levels.

PIAS.

Protein inhibitor of the activated signal transducer and activator of transcription STATs (PIAS) exert an inhibitory effect on the Jak-STAT pathway in the nucleus. PIAS1 and PIAS3 bind to activated STAT1 and STAT3, respectively, and prevent STAT dimers to associate to the DNA[87].

TcPTP.

STAT1 activation is abrogated in the nucleus via specific de-phosphorylation of tyrosine 701[88]. This process is mediated by the T-cell protein tyrosine phosphatase, TcPTP[88].

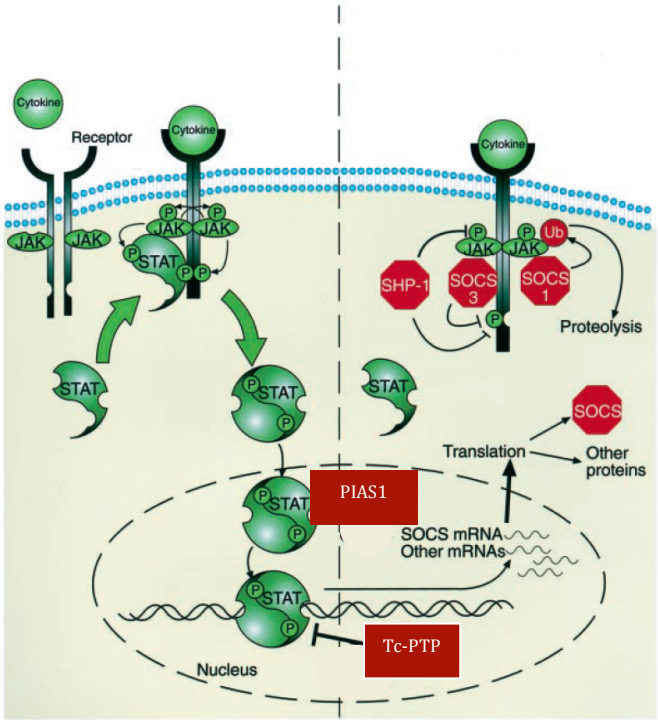


Figure 1.5. *Negative regulators of the Jak-STAT signaling pathway (modified from*[83]*).*

1.2.6. Refractoriness of IFN signaling pathway.

Upon treatment with saturating doses of IFNα, py-STAT1 is induced at maximum level in hepatoma cells and in mouse liver after 30 minutes or one hour, respectively[89,90]. pY-STAT1 activation results in IRGs induction in hepatoma cella and in mouse liver[89,90]. However, the constant exposure of cells to IFNα or repeated injections of IFNα in mice resulted in the "desensitization" (*refractoriness*) of the Jak-STAT signaling pathway, leading to the impairment of STAT1 phosphorylation and IRGs expression[89,90]. The role of negative regulators in mediating the refractoriness of the Jak-STAT signaling pathway has been investigated in the mouse liver[90]. It has been shown that the refractoriness phenomenon does not depend on SOCS1 and SOCS3[90]. Instead, it has been proven that USP18/UBP43 mediates the refractory state of the IFNα signaling pathway in mouse liver[90]. Indeed, mice knock-out for USP18/UBP43 and repeatedly injected with IFNα display a long-lasting STAT1 tyrosine phosphorylation in the liver[90]. Interestingly, we have recently described that *in vivo* INFλ and IFNβ signaling pathways are not refractory[85]. The lack of desensitization of the Jak-STAT pathway was confirmed also via the prolonged *ex-vivo* treatment of human liver biopsies with INFλ[85]. Of note, since USP18/UBP43 is

induced by both IFNα and IFNβ, which share the same receptor complex, the mechanism that allows IFNβ to escape from the refractoriness state remains to be elucidated.

1.2.7. Interference of HCV with the Jak-STAT signaling pathway.

In vitro evidences suggests that HCV has developed different strategies to interfere with the Jak-STAT signaling pathway and to block the host antiviral response. Indeed, transient transfection of HCV RNA in hepatoma cells has revealed that HCV core inhibits STAT1 activation via SOCS3 up-regulation[91,92]. It has also been reported that HCV core over-expression is associated with proteasome-dependent degradation of STAT1[93]. Another group, however, observed that HCV core and NS5A over-expression do not affect STAT1 degradation but alter the nuclear transport of activated STATs[94]. Our group described a reduction of STAT1-DNA binding in hepatoma cells expressing HCV open reading frame, in transgenic mice expressing HCV proteins, and in liver biopsies from patients chronically infected with HCV[95,96]. We reported that protein phosphatase 2A (PP2A) is the key mediator of the impairment of STAT1-DNA binding[95]. Furthermore, we observed that HCV-induced an ER stress response that mediates PP2A catalytic subunit over-expression[97]. PP2A induction was confirmed in hepatoma cells over-expressing HCV protein, in HCV transgenic mice, and in human liver biopsies from HCV chronically infected patients[98]. We have shown that PP2A binds to protein arginine methyl transferase 1 (PRMT1) impairing its enzymatic activity resulting in a reduced methylation of STAT1[99]. Despite the still controversial finding that STAT1 methylation modulates its association to PIAS1[100,101], we have provided evidence that the inhibition of PRMT1 activity mediated by PP2A resulted in an increased STAT1-PIAS1 association impairing the binding of pY-STAT1 to the DNA[98,99] (schematic summary in Fig. 1.6). Finally, we have proven that the treatment with the methyl donor S-adenosyl-methionine (SAMe) restores the normal IFN signaling in HCV replicon cells[102].

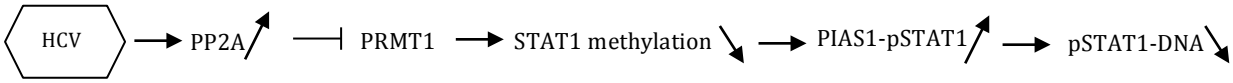


Figure 1.6. *PP2A-mediated inhibition of STAT1-DNA binding.*

Another model of HCV interference with the host antiviral response has been recently proposed. It has been reported that HCV impairs of eukaryotic translation[103]. HCV activates PKR, leading to the phosphorylation of eIF2α and the inhibition of eukaryotic cap-dependent mRNA translation[103]. On the contrary, phosphorylation of eIF2α does not modulate the IRES-dependent HCV RNA translation[103]. However, the impact of HCV-mediated mRNA translation impairment remains to be assessed in human liver biopsies.

1.3 Host-virus interaction during acute and chronic HCV infection.

1.3.1 Natural history of HCV infection

Transmission.

HCV infection occurs via exposure to infected blood and sexual transmission. Blood transfusions have been tested since 1992, leading to the virtually complete elimination of HCV transmission through donated blood[104]. In developed countries, intravenous drug injection is the major source of HCV infection[104]. In developing countries, instead, the use of contaminated equipment in the medical practice is one of the most prominent way of HCV transmission[105].

Acute hepatitis C (AHC).

AHC is difficult to diagnose since the patients are asymptomatic or (in 30% of the cases) develop non-specific symptoms like fatigue, myalgia, vomiting, and jaundice[106]. The symptoms may develop between 2 and 12 weeks after infection[107].

Within days after exposure HCV RNA is detectable in the blood[107]. Four to twelve weeks after viral exposure, an increase of alanine aminotransferase (ALT) levels may occur[108]. Fulminant liver injury is rare and occurs in less than 1% of the infected

individuals[108]. Seroconversion may occur between 4 and 12 weeks after exposure to the virus[109,108]. Early studies on infected patients due to blood transfusions have demonstrated that 15-30% of the patents clear the virus spontaneously[110,111].

Chronic hepatitis C (CHC), cirrhosis and hepatocellular carcinoma HCC.

HCV infection is defined as chronic when HCV RNA is detectable in the blood after 6 months of viral exposure[110]. It has been estimated that 75-80% of the infected patients progress to chronicity[110,111]. Interestingly, the rate of CHC appears to be lower in young individuals[112], in women[113], and in patients who develop jaundice or other clinical manifestations during the acute phase[107,114]. Persistent HCV infection can lead to liver disease (fibrosis, cirrhosis and HCC)[115]. Heretofore, liver biopsy is considered as the gold standard for the assessment of the liver disease status[116]. A systematic analysis of published epidemiological studies indicates that, after 20 years of infection, the rate of progression to cirrhosis varies between 10 to 20% in CHC patients[115]. Once cirrhosis is established, the risk of HCC development increases dramatically, with an estimated annual rate of 1% to 4%[117,118]. Progression of CHC to liver disease varies between subjects, since it is influenced by many risk factors like sex, race, alcohol consumption, and co-infection with HBV and HIV (reviewed in [119]).

1.3.2 Host response during acute HCV infection.

Acute HCV infection can be divided in an early-acute phase (1 to 6 weeks post infection) and a late acute phase (6 to 24 weeks post infection) (Fig. 1.7). The early acute phase of HCV infection has been studied exclusively in chimpanzees[120,121,122]. In these animals, HCV titer increases few days after infection and concomitantly IRGs are up-regulated in the liver[120,121]. However, the type of IFNs responsible for this effect remains to be elucidated. Of note, the IRGs up-regulation fails to clear HCV[120,121]. In the late acute phase, a cellular immune response occurs and HCV viral titer decreases[120,121,122]. Indeed, in the liver of chimpanzees, CD8+ T cells can be detected concomitantly with an increase of IFNγ mRNA and alanine aminotransferase, indicating damage of the liver , and IFNγ mRNA[120,121]. In vitro data using the HCV replicon system support the role of CD8+ T cells in HCV clearance. CD8+ T cells inhibits HCV replication by inducing hepatocytes lysis and through a non-cytolytic way, mediated by IFNγ production[123]. We have recently

assessed that a strong induction of IFNγ-stimulated genes can be detected in liver biopsies of AHC patients[124].

After the late acute phase, chimpanzees that do not clear HCV are characterized by the rise of viral titer after its transient diminution[122]. HCV titer is 10 times lower in chronic infection than in AHC, and HCV infection becomes chronic[122]. In the chronic infection both type I or type III IRGs are detectable in the liver of chimpanzees[125].

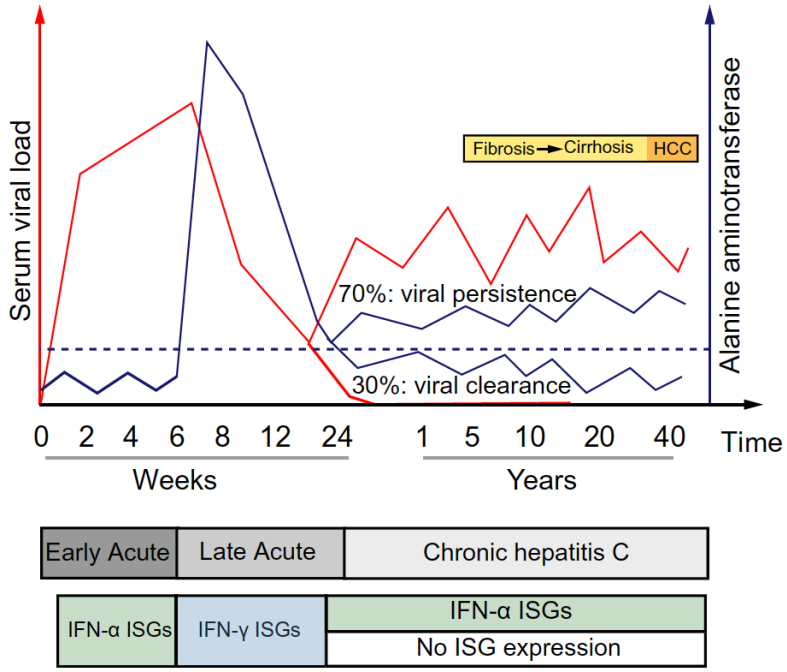


Figure 1.7. *Natural history of HCV infection*[68].

1.3.3 Host response during chronic HCV infection.

In individuals chronically infected with HCV, the hepatic up-regulation of type I and type III IRGs varies greatly[68]. Interestingly, in the Caucasian population, it has been observed that almost half of the CHC patients display a broad induction of IRGs in the liver (*pre-activation of IFN system*) despite the persistence of HCV infection, whereas the rest of the patients have no detectable induction of the innate immune system in the liver[68]. The molecular mechanism that mediates the differential induction of IRGs in the liver remains to be elucidated. The pre-activation of the IFN system could be driven by a specific IFN subtype. Expression of IFNα, IFNβ, and

IFNγ has not been consistently observed in liver biopsies of CHC patients[68]. On the contrary, IFNλs can be detected in the liver biopsies of CHC patients[126]. IFNγ can be further excluded as driving force of the pre-activated IFN system since we have recently assessed the presence of type I/III IRGs, and not type II IRGs, in the liver of CHC patients[122,124,127]. IFNα signaling pathway, as described in session 1.2.6, undergoes a refractory state after the first stimulation, which would prevent the long lasting induction of ISGs in the liver of CHC patients. IFNλs and IFNβ signaling pathways, on the contrary, are not refractory[85]. Indeed, the involvement of those two cytokines in the activation of the endogenous IFN system in the liver of CHC patients is tempting.

CHC patients that display an up-regulation of the hepatic endogenous IFN system are characterized by an up-regulation of STAT1 at mRNA and protein levels in the liver[127]. As assessed by Western blotting, STAT1 it is mot likely to be in an un-phosphorylated state (U-STAT1) in the liver of pre-activated patients[127]. It has been reported that U-STAT1 can shuttle in the nucleus independently of tyrosine 701 phosphorylation[128] suggesting a role of U-STAT1 as active transcription factors. The possibility that U-STAT1 could maintain the up-regulation of ISGs in the liver of CHC pre-activated patients is tempting. Indeed, a gene expression profile of cells over-expressing a STAT1 mutant that can not be phosphorylated suggests that U-STAT1 maintain high expression of a subset of IRGs[129]. However several criticisms to the experimental settings make questionable the results of this study.

Several reports indicate that the up-regulation of the endogenous IFN system in the liver of CHC patients is associated to the minor allelic variant at single nucleotide polymorphisms (SNPs) mapping at the *IL28B* locus[126,130,131] (discussed also in 1.4.2, see fig. 1.8). In particular, the minor (less frequent in the population) allele at rs12979860[130,126] and rs8099917[130,131] were associated with an increased baseline expression of IRGs in the liver of CHC patients. However, the molecular mechanism that links the polymorphisms at *IL28B* locus to the IRGs up-regulation in a subset of CHC patients needs to be clarified.

1.4 Treatment of chronic hepatitis C.

1.4.1 Interferon α-based therapy.

The aim of CHC treatment is the achievement of a sustained virological response (SVR), defined as undetectable HCV RNA with PCR assay (<50 International Units [IU]/mL) 24 weeks after the end of the antiviral therapy[51].

In the last 20 years, IFNα has been the key component for CHC treatment[51]. Recombinant IFNα was first introduced in 1986 for the treatment of non-A, non-B hepatitis, even before HCV was first described[132]. The therapy consisted of IFNα2 (3 Mio IU) subcutaneous injections 3 times a week for 24-48 weeks, resulting in the eradication of HCV in 15-25% of the patients[132]. In the late 1990s, the introduction of the orally administrated ribavirin, a broad spectrum antiviral agent, improved viral clearance up to 30-40% of the cases[133]. More recently, unmodified IFNα2 has been replaced with pegylated IFNα2 (pegIFNα2)[134,135]. There are two licensed pegIFNα2, that show overlapping clinical response: pegIFNα2b, with a 12KDa linear polyethylene glycol (PEG) moiety covalently linked to the standard IFNα2, and pegIFNα2a with a covalently linked 40KDa branched PEG moiety. PegIFNα2 has a longer serum half-life than unmodified IFNα2[134,135]. Consequently, the dosing interval has been adjusted to one weekly subcutaneous injection. Any differences in the clinical response have been observed for the two different pegIFNα2 available[134,135]. The administration of pegIFNα2 and ribavirin for 24-48 weeks has improved HCV clearance in up to 55%[134,135].

In case of non-response (NR, failure to clear HCV RNA from serum after 24 weeks of therapy), treatment options are limited. Due to the very low probability of achieving an SVR (less than 5%), retreatment is not recommended[136].

Side effects are very common in patients treated with IFNα-based therapy[134,135]. In the two clinical trials for the registration of pegIFNα2a and pegIFNα2b, more than half of patients experienced influenza-like symptoms such as fatigue, headache, fever and, rigor [134,135]. Twenty two to thirty one percent of patients experienced psychiatric effects like depression, irritability, and insomnia[134,135]. Neutropenia has been observed in 18% of patients, but it was not associated to an increased susceptibility to infections[134,135]. Approximately one third of patients experienced anemia[134,135].

1.4.2 Prediction of response to IFNα-based therapy.

Since IFNα-based therapy is not effective in a significant percentage of CHC patients, predictors of response to treatment are useful to advise the patients for their likelihood to achieve the eradication of the viral infection.

Baseline predictors.

HCV genotype.

HCV genotypes display different sensitivity to pegIFNα-based therapy[137]. Indeed, patients infected with genotypes non-1 (mostly 2 and 3) can be cured in over than 75% of the cases by a combined therapy[137]. Patients infected with genotype 1, though, achieve eradication of the virus in less than 50% of the cases[137]. This phenomenon is puzzling since different HCV genotypes induce similar type of disease[137].

Ethnicity, gender, age and diseases.

A *low viral load* (600.000-800.000IU/mL or less) before treatment is a predictor of SVR independently from the HCV genotype[137]. *Non-African-American race*[138,139], *female gender*[140], *age less than 40 years*[51] and *absence of insulin resistance*[51] have been associated to a higher rate of SVR. A lower rate of response to IFNα-based therapy has been associated to advanced *liver fibrosis* and *cirrhosis*[140].

Pre-activation of the endogenous IFN system in the liver.

Non-response to IFN-based therapy has been repeatedly associated to the activated endogenous IFN system in the liver of CHC patients (described also in section 1.3.3.)[127,130,141,142]. Indeed, gene expression profile on paired liver biopsies before and after peg-IFNα2 injection revealed that non-responder patients display an a pre up-regulation of the endogenous IFN system in the liver, preventing the further induction of IRGs by exogenous peg-IFNα2[127]. Responder CHC patients, though, have low levels of IRGs in the liver before treatment and exogenous peg-IFNα2 induces hepatic IRGs at high levels[127]. Expression of hepatic IRGs is a potent predictor of response to treatment in CHC patients. Indeed, we have recently developed an algorithm based on the hepatic expression level of 4 classifier genes (ISG15, RSAD2, IFI27 and HTAITP2) that allows the prediction with high accuracy of treatment-outcome in CHC patients[126].

Allelic variants at IL28B genotype.

In the last few years, several genome wide associations studies have reported a strong association between treatment-response in CHC patients and allelic variants at single nucleotide polymorphisms (SNPs) located at the *IL28B* locus on chromosome 19[143, 144,145,146]. The SNPs found mostly associated are the following: rs12979860 (C major allele, T minor allele) mapping 3Kb upstream the *IL28B* gene[143], rs8099917 (T major allele, G minor allele) located 7.5Kb upstream the *IL28B* gene[144,145,146], rs12980275 (A major allele, G minor allele) located 2.5Kb downstream the *IL28B* gene[146] (Fig. 1.8). For all these SNPs, the minor allele is associated to treatment failure in both heterozygosity and homozygosity, pointing out its dominant effect[143,144,145,146]. Indeed, in the first published genome wide association study on CHC Caucasian patients, C/C, C/T and TT genotypes were associated with ≈ 80%, 40% and 35% SVR rate, respectively[143]. In African-Americans, the rate of SVR was reduced, maintaining, though, the dominant effect of the minor allele on treatment outcome[143]. Interestingly, the frequency distribution of the major allele at rs12979860 varies among East Asians (90%), European Americans and Hispanics (70%), and African-Americans (40%)[143]. This finding correlates with the observation that treatment outcomes in CHC differ among ethic groups: 70% in East-Asian, 50% in Hispanics and European-Americans, and around 25% in African-Americans[143].

So far, the molecular mechanism that links polymorphisms at *IL28B* locus with treatment outcome has not yet been elucidated.

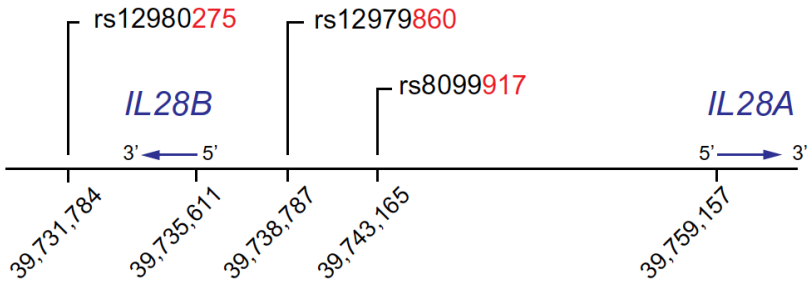


Figure 1.8 *Localization of SNPs at IL28B locus (chromosome 19) that are mostly associated to treatment response in CHC*[68].

Predictors during therapy.

HCV kinetic during treatment is a useful tool to predict response to therapy in CHC patients (refer to Table 1.1 for definitions) [51]. HCV RNA is currently measured during

therapy at weeks 4, 12, 24 or 48[51]. Patients are then tested 24 weeks after the end of the treatment for SVR assessment[51]. In the early phase of the therapy, achieving a rapid virological response (RVR, HCV RNA negative at treatment week 4) is highly predictive of obtaining an SVR, regardless the HCV genotype[51]. However, only 15 to 20% of the patients with genotype 1 and 65% with genotype 2 or 3 achieve an RVR[147,148]. Monitoring the early virological response (EVR, HCV RNA decrease ≤ 2 logs at treatment week 12) is useful for treatment-prediction, especially in patients with HCV genotype 1[51]. Indeed, data of two retrospective studies revealed that 97% to 100% of genotype 1 patients that do not show an EVR fail to achieve an SVR[134,149]. EVR is less helpful to predict the treatment-response in genotype 2 and 3 infection since most of the patients clear the virus at week 12 and respond to therapy[51].

Virological response	**Definition**
Rapid virological response (RVR)	HCV RNA negative at treatment week 4
Early virological response (EVR)	≥ 2 log reduction in HCV RNA level compared to baseline
End of treatment response (ETR)	HCV RNA negative at the end of 24 or 48 weeks of treatment
Sustained virologic response (SVR)	HCV RNA negative 24 weeks after cessation of treatment
Breakthrough	Reappearance of HCV RNA while still on therapy
Relapse	Reappearance of HCV RNA after therapy is discontinued
Non responders (NR)	Failure to clear HCV RNA after 24 weeks of therapy
Null responder	Failure to decrease HCV RNA by ≤ 2 log after 24 weeks of therapy
Partial responder	Two log decrease in HCV RNA but still HCV RNA positive at week 24

Table 1.1*. Virological responses during therapy (modified from [51]).*

1.4.3 IFNλ-based therapy.

Since IFNλs display an antiviral effect against HCV *in vitro* and activate an intracellular signaling pathway that overlaps the one of IFNα, clinical trials are currently ongoing to assess the safety and efficacy of peg-IFNλ in the treatment of CHC patients[150,151]. The tissue-restricted localization of IFNλ receptor makes this cytokine very appealing for clinical use since the therapy should be associated with fewer side effects compared to IFNα. A study conducted on healthy volunteers assessed the safety of peg-IFNλ1[150]. Administration of a single dose up to 7.5μg/Kg

pegIFNλ1 revealed minor side effects up to 50 days of follow up[150]. On the basis of this results, a phase 1b clinical trial with peg-IFNλ1 in combination of not with ribavirin has been conduced on genotype 1 treatment-naïve or treatment-relapse CHC patients[151]. Different doses of peg-IFNλ1 were tested (between 1.5 and 35μg/Kg) and patients were treated with a weekly single subcutaneous injection up to 4 weeks[151]. The results of the clinical trial show an antiviral activity of peg-IFNλ1 in all the doses selected[151]. Twenty nine percent of the naïve patients achieved a RVR and in general the treatment was associated with reduced side effects[151]. However, this study lacks comparison with peg-IFNα treatment[151]. At the last EASL meeting in Barcelona, results from a phase 2b clinical trial were presented. In this study, SVR was assessed in CHC naïve patients with HCV genotype 2 and 3 treated with peg-IFNλ1-ribavirin or peg-IFNα2-ribavirin. SVR rate was similar in the two arms of the clinical trial[152]. However, treatment with peg-IFNλ1 was associated with reduced side effects compared to peg-IFNα2 treatment[152].

These results point out a possible role of peg-IFNλ1 as substitute of peg-IFNα2 in the treatment of CHC. Further investigation is required to assess the rate of SVR achieved in case of treatment-relapse in CHC patients.

1.4.4. Direct-acting antiviral agents (DAA) for CHC treatment

Since INFα-base therapy is only partially effective in the treatment of CHC patients and it is associated with side effects, new antiviral strategies are required. Intense studies in the past years resulted in the elucidation of the crystal structure of several HCV viral proteins, allowing the design of direct-acting antiviral agents (DAA). In 2011, for the first time, two DAA (Telaprevir and Boceprevir) have been approved for the treatment of genotype 1 infected CHC patients in combination with INFα-based therapy[153,154,155,156]. Telaprevir and Boceprevir are both inhibitors of NS3/4A proteases and they differ for the nature of the covalent complex generated with the target (irreversible for Telaprevir, reversible for Boceprevir). Phase III randomized clinical trials on treatment-naïve genotype 1 infected patients revealed that the triple therapy, compared to peg-IFNα and ribavirin, led to a higher SVR rate: 75% for Teleprevir, 66% for Boceprevir[156,154]. Previously treated patients have been also assessed for their response to the triple therapy. In this case SVR rates varies from the 30% of prior null response to the 85% in prior reponders/relapsers[156,154].

Of note, patients treated with the triple therapy experienced additional side effects like anemia, pruritus, and rash[156,154].

Teleprevir and Boceprevir can not be administrated as a monotherapy because resistant viral variants are selected within 1-2 weeks, leading to virological breakthrough[157,158]. Therefore, the association of the two DAA with IFNα-based therapy is mandatory to achieve SVR.

Other DAA targeting HCV non-structural proteins, like NS2/3, NS4B, NS5A and NS5B, are currently in pre-clinical or clinical evaluation (reviewed in [159]). However, IFNα-based therapy remains the backbone of HCV treatment.

In the future, emerging of DAA that do not require IFNα-based therapy is expected.

1.4.5 Host-targeting antiviral agents (HTA)

An additional category of potential drug target consists in the host cell factors required for HCV replication. Cyclophillin A, a cellular peptidil-prolylisomerase that acts on NS5A[160] and has a key role in HCV replication[161] is a promising target for HCV antiviral therapy. The cyclophillin A-binding molecule Alisporivir has been reported to induce genotype 1 resistant variant at lower time rate compared to Teleprevir/Boceprevir (20 weeks and less than 2 weeks, respectively)[162]. Recent results from a phase II clinical trial suggests that triple therapy with Alisporivir results in a higher SVR rate compared to pegIFNα/ribavirin regimen in genotype I infected patients[163]. Phase III clinical trials are ongoing.

Another promising target for HCV antiviral therapy is the liver-specific miRNA, miR-122. Mir-122 is required for HCV replication[4]. Miravirsen has been designed to target and sequester miR-122[164]. Pre-clinical studies on chimpanzees indicate that Miravirsen successfully long-lasting prevents HCV viremia without the emerging of resistant viral variants and side effects[164]. Clinical trials to assess safety and efficacy for Miraviresn are currently ongoing.

2. Aim of the thesis

In chronically infected HCV patients, the response to IFNα-based therapy depends on the induction of the IFN system in the liver before the therapy. Indeed, non-responder patients are characterized by a broad pre-up-regulation (*pre-activation*) of IRGs in the liver[127]. It has also been reported that in CHC patients, the expression level of hepatic IRGs[130,131,126] and the clinical outcome[143, 144,145,146] are associated to allelic variants at *IL28B* locus.

So far, the molecular mechanisms that link the hepatic expression level of IRGs, the allelic variants at *IL28B* locus, and the treatment outcome in CHC patients have not yet been clarified. The aim of the thesis is to investigate molecular mechanisms that could explain these observations.

a) Unphosphorylated STAT1 (U-STAT1) induces a long-lasting up-regulation of IRGs.

STAT1 is over-expressed at protein level in liver biopsies of non-responder CHC patients compared to responder patients[127]. In non-responder patients, the IFNα signaling is refractory since an exogenous administration of peg-IFNα does not lead to a significant increase of pY-STAT1 in the liver[127]. It has been reported that STAT1 can be detected in the nucleus independently from its tyrosine phosphorylation, indicating a possible role of U-STAT1 as a transcription factor[128]. The role of U-STAT1 in prolonging IRGs expression is tempting and it has been already investigated in a report published in 2009 by the Stark group[129]. However, criticisms to the experimental settings in this paper make the reported results not conclusive. Therefore, we decided to investigate the role of U-STAT1 in the maintenance of IRGs expression using a different experimental set up.

b) IFNλ signaling mediates the pre-activation of the IFN system in the liver of CHC non-responder patients.

The *pre-activation* of the IFN system in the liver of CHC non- responder patients could be due to a constant activation of the Jak-STAT signaling pathway. We have recently assessed that IFNλ signaling pathway is not desensitized in case of repeated injection of IFNλ in the mice leading to a prolonged pY-STAT1 signal in the mouse

guts[85]. IFNλ activates a type-I like intracellular signaling pathway[57] and it can be detected in liver biopsies of CHC patients[126]. Therefore, we wanted to assess if the absence of refractory state in the IFNλ signaling pathway could mediate the long-lasting up-regulation of ISGs in the liver of non-responder CHC patients.

3. Materials and methods

3.1. Role of unphosphorylated STAT1 (U-STAT1) in the *pre-activation* of the hepatic IFN system.

The following material and methods refer to the results described in the chapter 4.1.

3.1.1 Cells and reagents.

2fTGH and U3A cell lines were described in[165,166]. Briefly, human sarcoma cell line HT 1080 has been transfected with a selectable marker (guanine phosphoribosyltransferase) regulated by an IFNα-dependent promoter, allowing the generation of 2fTGH cell line[165]. 2fTGH cells were chemically mutagenized with ICR-191 (Polysciences) and selected as described in[165]. Selected cells were characterized for the response to IFNα and the expression of Jak-STAT signal components. The U3A clone lacks STAT1 expression at protein level and, consequently is not responsive to IFNα stimulation[166]. 2fTGH and U3A cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 250μg/ml of hygromycin B (cat n. 10843555001, Roche Pharma). Stable transfected cells were selected with 800μg/ml of G418 (Calbiochem, cat n. 345810). Experiments were performed in serum-starved culture medium. IFNα-2a (Roferon-A) was purchased by (Roche Pharma).

3.1.2 Site-directed mutagenesis and cloning.

STAT1α-FLAG-pcDNA3 was provided by J.E. Darnell. STAT1α(Y701F)-FLAG pcDNA3 was generated from STAT1α-FLAG-pcDNA3 according to the method described in[167] with the following primers: 5'-CTGGCACCAGAACGAATGA-3', 5'-ATTTAGGTGACACTATAG-3', 5'-GGAACTGGATTCATCAAGACTGAG-3', 5'GGAACTGGATTCATCAAGACTGAG-3', 5'-CTCAGTCTTGATGAATCCAGTTC-3', the restriction sites BlpI and ApaI and *Pfu* DNA polymerase (Promega Biosciences Inc.). Mutation of tyrosine 701 to phenylalanine was confirmed by sequencing.

STAT1α-FLAG-pcDNA3 and STAT1α-FLAG-pcDNA3 were transfected in U3A STAT1 knock out (STAT1 -/-) cells using Fugene HD (Roche Pharma, Basel,

Switzerland), selection with hygromycin B was carried out for 15 days and single clones were picked. Over-expression of STAT1α WT of STAT1α-(Y701F) was assessed by Western blotting.

3.1.3 Cell lysis and Western blotting.

Cells were lysed for 20 minutes on ice with a buffer containing 100mM NaCl, 50mM Tris pH 7.5, 1mM EDTA, 0.1% Triton X-100, 10mM NaF, 1mM phenylmethyl sulfonyl fluoride, and 1M sodium orthovanadate and protease inhibitors. Lysates were centrifuged at 14,000 rpm for 15 minutes, and protein concentration was determined by Bio-Rad protein assay. 10μg of whole protein cell lysate was loaded on 8% SDS/PAGE and transferred onto nitrocellulose membrane (Schleicher & Schuell, Switzerland). After blocking for 1 hour with 5% BSA in TBS-Tween (20nM Tris pH 7.4, 0.15 M NaCl, 0.1%), the membranes were incubated overnight with the following antibodies diluted in TBS-Tween: STAT1 N-terminus (cat. n. 610119, BD Transduction Laboratories), phosphpo-Y701-STAT1 (cat. n. 9167, Cell Signaling), β-actin (cat. n. A2228, Sigma-Aldrich). After washing with TBS-Tween membranes were incubated at room temperature for 1h with secondary infrared antibodies goat anti-mouse (IRDye 680) or goat anti-rabbit (IRDye 800) form LI-COR Bioscience. Membranes were scanned with Odyssey Infrared Imaging System (LI-COR).

3.1.4 RNA extraction, reverse transcription and quantitative real-time polymerase analysis.

RNA extraction was performed with NucleoSpin RNA II (Macherey-Nagel, GmbH & Co. KG) according to the manufacturer's instructions. 1μg of RNA was reverse-transcribed with random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega Biosciences Inc.). Quantitative real-time polymerase chain reaction was performed with SYBR green fluorescence (Applied Biosystems) and an ABI 7500 detection system (Applied Biosystems). Gene expression was normalized to human GAPDH using the ΔCt method. Primers used for quantitative real-time polymerase chain reaction are listed in the following table:

Target	Forward primer	Reverse primer
BST2	5'-TCTCCTGCAACAAGAGCTGA-3'	5'-TCTTCTCAGTCGCTCCACCT-3'
HERC6	5'-CACTACCACTCCCTGGCATT-3'	5'-TGTTACTTCCCCAGCCAAAV-3'
IFI27	5'-GGCAGCCTTGTGGCTACTCT-3'	5'-CCCAGGATGAACTTGGTCAATC-3'
IFI44L	5'-GCTGCGGGCTGCAGAT-3'	5'-CTCTCTCAATTGCACCAGTTTCC-3'
ISG15	5'-TCCTGCTGGTGGTGGACAA-3'	5'TTGTTATTCCTCACCAGGATGCT-3'
MX1	5'-GTGCATTGCAGAAGGTCAGA-3'	5'-TCAGGAGCCAGCTTAGGTGT-3'
OAS1	5'-TGATGCCCTGGGTCAGTTG-3'	5'-TCGGTGCACTCCTCGATGA-3'
OAS2	5'ACAGCTGAAAGCCTTTTGGA-3'	5'-AAGTTTCGCTGCAGGACTGT-3'
RSAD2	5'-CTTTGTGCTGCCCCTTGAG-3'	5'-TCCATACCAGCTTCCTTAAGCAA-3'

Table 3.1. *Real-time PCR primers list.*

3.1.5 Statistical Analysis

Statistical analysis was performed using Prism4 (GraphPad software Inc).

3.2. The interferon λ receptor chain α (IL28Rα) triggers high expression levels of interferon stimulated genes in non responsive chronic hepatitis C patients.

The following material and methods refer to the results described in the chapter 4.2.

3.2.1 Reagents and antibodies.

The following human IFNs were used: IFNα-2a (Roferon-A, Roche Pharma, Basel, Switzerland); IFNβ-1β Betaferon (Bayer Schering Pharma, Zürich, Switzerland); IFNλ2 (Peprotech Inc., Rocky Hill, NJ). LPS was purchased from Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany L5293). The following antibodies were used for Western blotting and immunofluorescence staining: phospho-STAT1, phospho-STAT1 Alexa Fluor® 555 conjugated, p53, and $pSer_{18}$-p53 (Cell Signaling Technology, Bioconcept, Allschwil, Switzeland), STAT1 (Transduction Laboratories, BD Biosciences Pharmingen, San Diego CA), β-actin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and IL28RA (ProSci Inc. cat. n. 26-279, Poway, CA).

3.2.2 Cell culture.

Huh7 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. The coding sequence for IL28Rα was amplified from a commercially available IMAGE human cDNA clone (cat.n. EHA10001-99865534 Open Biosystems, Thermo Fisher Scientific Inc.) with the primers 5'-TTTTCTAGACGGCAGGAAGGCCATGGC-3' and 5'TCTAGACCTGGCCATGTAATGCCCCAAT-3', and subsequently cloned into pcDNA3.V5-His vector with XbaI restriction enzyme. The expression plasmid was transfected into Huh7 cells using Fugene HD (Roche Pharma, Basel, Switzerland). Cells were selected with DMEM culture medium supplemented with 10% FBS, penicillin/streptomycin and 1mg/ml G418 (cat.n. 345810; Calbiochem EMD Chemicals, Inc. San Diego) to generate stable over-expressing clones.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Plaque (Life Technologies, Cergy Pontoise, France) density gradient centrifugation. Monocytes were purified from PBMCs by positive selection using a magnetic cell separator and CD14 microbeads (cat. n. 130-050-201;MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Dendritic cells (DCs) were differentiated into culturing monocytes for six days in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin/streptomycin, 20ng/ml IL4, and 50ng/ml GM/CFS.

Primary human hepatocytes (PHHs) were isolated from liver resections from patients at the Strasbourg University Hospitals with approval from the Institutional Review Board. Briefly, liver specimens were first perfused with calcium-free 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid buffer containing 0.5 mM ethylene glycol tetra-acetic acid followed by a second perfusion with 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid containing 0.05% collagenase at 37°C. After several washes with PBS, non-viable cells were removed by Percoll gradient centrifugation. Freshly isolated hepatocytes (3×10^5 cells/well) were then plated in 24-well plates pre-coated with collagen (Biocoat, BD Biosciences). Cells were then cultured in William's E medium (Sigma-Aldrich) supplemented with 1% Glutamax (Gibco), 1% insulin transferrin selenium (Gibco), 10^{-7} M dexamethasone (Sigma), 0.15% bovine serum albumine (Sigma), and 10% fetal bovine serum (PAN Biotec).

3.2.3 Patients.

Liver biopsies from CHC and non-HCV infected patients were obtained during diagnosis workup. A liver biopsy for research purposes was obtained upon informed consent, in accordance with the Ethics Committee of Basel. Grading and staging of CHC was defined according to METAVIR classification. Serum HCV RNA was quantified using the Cobas AmpliPrep/COBAS TaqMan HCV Test and the Cobas Amplicor Monitor from Roche Molecular Systems (Basel, Switzerland). Response to treatment in CHC patients was defined as described previously[126]. Diagnosis of non-HCV infected patients was obtained by histopatologic assessment. CHC and non-HCV infected patients characteristics are summarized in Supplementary Tables 1 and 3, respectively. Liver specimens for PHH isolation were obtained from donors listed in Supplementary table 4.

3.2.4 Western blotting.

Protein extracts from adherent cells and immunoblotting were performed according to the protocol described in 3.1.3.

3.2.5 RNA extraction, reverse transcription and quantitative real-time polymerase analysis.

RNA isolation from Huh7 cells was performed with Trizol reagent (Invitrogen, Basel, Switzeland) according to the manufacturer's instruction. DNA digestion was performed with RQ1 RNase-Free DNase (Promega Biosciences Inc., Wallisellen, Switzerland). RNA isolation from PHH was performed with NucleoSpin RNA II (Macherey-Nagel, GmbH & Co. KG) according to the manufacturer's instructions. 1μg of RNA was reverse-transcribed with random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega Biosciences Inc., Wallisellen, Switzerland). Quantitative real-time polymerase chain reaction was performed with SYBR green fluorescence (Applied Biosystems, Foster City, CA) and an ABI 7500 detection system (Applied Biosystems). Primers used for quantitative real-time polymerase chain reaction are listed in Table 3.2. For IL28Rα variant 1, IFNλ1, and IFNλ2 the specificity of the polymerase chain reaction was assessed on 3% agarose gel and by sequencing the PCR product. Gene expression was normalized to human

GAPDH using the ΔCt method. Complete gene expression data from CHC patients and PHHs are listed in Supplementary Tables 2 and 4.

Target	Forward primer	Reverse primer
ATF3	5'-CTCCTGGGTCACTGGTGTTT-3'	5'-AGGCACTCCGTCTTCTCCTT-3'
GAPDH	5'-GCTCCTCCTGTTCGACAGTCA-3'	5'-ACCTTCCCCATGGTGTCTGA-3'
HTATIP2	5'-GGGCGGAGGGATTTGTTC-3'	5'-TGCCAGCTCTGCAGACTTCA-3'
IFI27	5'-GGCAGCCTTGTGGCTACTCT-3'	5'-CCCAGGATGAACTTGGTCAATC-3'
IFI44L	5'-GCTGCGGGCTGCAGAT-3'	5'- CTCTCTCAATTGCACCAGTTTCC -3'
IFNl1	5'-CACAGGAGCTAGCGAGCTTCA3'	5'- TTTTCAGCTTGAGTGACTCTTCCA -3'
IFNl2	5'-TTTCTTCTGCTGACAAAGACC3'	5'- AGCGACTCTTCTAAGGCATCTTT -3'
IL28Rav1	5' CAGTGTCCCGAAATACAGCA -3'	5'- TGTGTCCAGAAAAGTCCAGGGC -3'
ISG15	5'- TCCTGCTGGTGGTGGACAA -3'	5'- TTGTTATTCCTCACCAGGATGCT -3'
NOXA	5'-AGAGCTGGAAGTCGAGTGT-3'	5'-GCACCTTCACATTCCTCTC-3'
RSAD2	5'-CTTTGTGCTGCCCCTTGAG-3'	5'-CTTTGTGCTGCCCCTTGAG-3'
STAT1	5'-TCCCCAGGCCCTTGTTG-3'	5'-CAAGCTGCTGAAGTTCGTACC-3'
USP18	5'-CTCAGTCCCGACGTGGAACT-3'	5'-ATCTCTCAAGCGCCATGCA-3'

Table 3.2. *Real-time PCR primers list.*

3.2.6 *Ex vivo* treatment of human liver biopsies

Freshly obtained liver biopsies from CHC patients were incubated with PBS, IFNα-2α 1000U/ml, or IFNλ2 100ng/ml for 15min at 37°C. Specimens were then embedded in Tissue-Tek OCT (Sakura Finetek USA. Inc). Sections of 8μm were prepared.

3.2.7 Immunofluorescence

Liver biopsies sections were fixed in freshly prepared 4% formaldehyde for 15 minutes. After a wash with PBS, they were permeabilized in cold methanol. Background was then removed with Background Buster (Innovex Bioscience). Phosphorylated Y701-STAT1 was then detected using phospho-STAT1-Alexa Fluor® 555 conjugated antibody. Sections were mounted with Mount FluorCare DAPI (Roth, Germany).

Adherent cells were incubated with cold methanol for 10 minutes, washed with TBS-Tween, and blocked with blocking solution (5.5% FBS in TBS-Tween) for 60min at room temperature. Cells were then washed and incubated with antibodies against phosphorylated Y701-STAT1 or IL28Rα.

3.2.8 DNA isolation and single nucleotide polymorphisms (SNPs) genotyping.

Genomic DNA was isolated from liver biopsies using Trizol reagent (Invitrogen, Basel, Switzeland) and DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. Genomic DNA from donors listed in Supplementary Table 4 was obtained from non-parenchymal liver cells using DNeasy Blood & Tissue Kit (Qiagen).

rs12979860 and rs8099917 genotyping was performed with TaqMan SNP genotyping assays (Applied Biosystems Inc, Foster City, CA). TaqMan probes and primers were designed and synthesized by Applied Biosystems: rs12979860, forward 5′-TGTACTGAACCAGGGAGCTC-3′, reverse 5′-GCGCGGAGTGCAATTCAAC-3′; Vic probe 5′-TGGTTCGCGCCTTC-3′, Fam probe 5′-CTGGTTCACGCCTTC-3′; rs8099917, ABI reference C_11710096_10.

3.2.9 Statistical Analysis

Statistical analysis was performed using Prism4 (GraphPad software Inc).

4. Results

4.1. Role of unphosphorylated STAT1 (U-STAT1) in the *pre-activation* of the hepatic IFN system.

We have observed that CHC patients who do not respond to IFN-based therapy display a *pre-activation* of the hepatic IFN system, resulting in an up-regulation of a subset of IRGs, including STAT1. The *pre-activation* of the IFN signaling cascade is believed to impair the antiviral effect of exogenous administration of pegIFNα by preventing further stimulation of the Jak-STAT pathway (Fig. 4.1.1)[127]. Heretofore, the molecular mechanism that leads to the *pre-activation* of the IFN system remains undetermined.

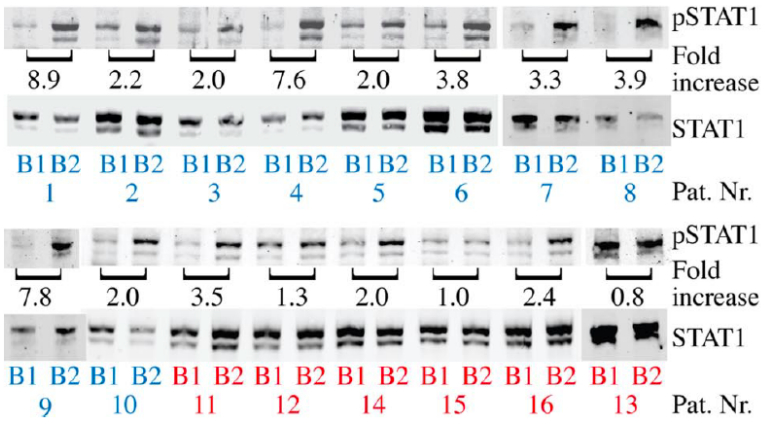


Figure. 4.1.1. *Analysis of the Jak-STAT signaling in liver biopsies from CHC patients before (B1) and after (B2) 4 hours of peg-IFNα administration. In blue CHC responder patients, in red CHC non-responder patients (from*[127]*, Fig.4A).*

Because of the high expression level of STAT1 observed in CHC non-responsive patients (Fig. 4.1.1), we hypothesized that this transcription factor could induce and maintain the elevated expression level of IRGs in the liver. The transcriptional activity of STAT1 requires its activation through phosphorylation on tyrosine 701 residue. Activated STAT1 are then translocated from the cytoplasm to the nucleus where they trigger gene transcription. Nevertheless, it has been reported that unphosphorylated STAT1 (U-STAT1) shuffles between cytoplasm and nuclei

independently from its tyrosine phosphorylation suggesting its potential transcriptional activity [128]. U-STAT1 enters the nucleus via carrier-free mechanism that requires the interaction with nucleoporins whereas pY701-STAT1 dimers are transported in the nucleus in an energy-dependent manner that relies on importins[128]. Cheon H. and Stark G.R. recently investigated U-STAT1-mediated gene expression in fibroblasts (BJ cells) and in mammary epithelial cells (hTERT-HME1 cells)[129]. They over-expressed STAT1 wild type (STAT1-WT) or an unphosphorylable STAT1 mutant containing a substitution of tyrosine 701 to phenylalanine (STAT1Y701F) (Figure 4.1.2) and analyzed gene expression profile[129].

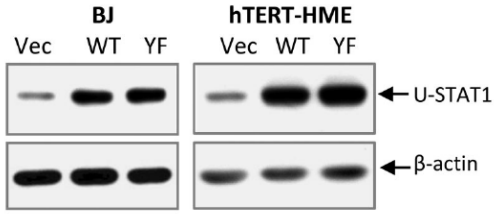


Figure 4.1.2. *Infection of BJ and hTERT-HME with lentivirus expressing STAT1-WT (WT), STAT1Y701F (YF) and empty vector (Vec) (From[129], Fig 2A).*

The authors concluded that the high expression level of U-STAT1 is sufficient to mediate the transcription of a subset of IRGs (Table 4.1.1)[129].

Cheon and Stark performed the experiments in cell lines that retain the endogenous STAT1 expression. Since the experiments were not preformed in serum-starved condition, the presence of a basal level of STAT1 phosphorylation can not be excluded. Furthermore, the authors claim the use of a monoclonal antibody specific for U-STAT1 (cat. n. 610185, BD Bioscience). However, there is no experimental evidence that this monoclonal antibody is sufficiently specific to discriminate between STAT1 and pY701-STAT1. Therefore, the conclusion of major nuclear localization of U-STAT1 is questionable. Based on these critical points, we decided to reassess the transcriptional activity of U-STAT1 suing a different experimental setting.

		BJ	
Gene symbol	PROBE_ID	WT	YF
IFI27*	ILMN_1661581	4.3	8.1
BST2	ILMN_1723480	3.7	8.1
OAS1*	ILMN_1658247	3.1	6.3
	ILMN_1675640	5.1	6.9
OAS2*	ILMN_1674063	4.1	6.2
OAS3	ILMN_1745397	2.5	2.6
STAT1	ILMN_1690105	2.2	2.8
	ILMN_1777325	1.7	2.0
IFI44	ILMN_1760062	2.6	3.3
IFI44L	ILMN_1723912	5.2	7.7
IFIH1	ILMN_1781373	2.7	4.0
IFITM1	ILMN_1801246	2.0	2.5
IFI35	ILMN_1745374	2.1	2.2
IFIT3*	ILMN_1701789	2.9	2.4
MX1*	ILMN_1662358	3.6	5.3
IRF7	ILMN_1798181	3.0	4.3
G1P2	ILMN_1813289	2.7	3.0
IFIT1*	ILMN_1707695	2.3	2.9
PLSCR1	ILMN_1752889	2.4	3.2
HERC6	ILMN_1654639	3.4	3.7
FLJ20035	ILMN_1795181	2.1	2.9
EPSTI1	ILMN_1688566	2.5	3.4

Table 4.1.1. *Genes induced by U-STAT1 in a gene expression profile analysis. WT, BJ cells over-expressing STAT1-WT; YF, BJ cells over-expressing STAT1-Y701F. Results expressed as fold change induction relative to the empty-vector transduced BJ cells. (From*[129]*, part of Table 1).*

4.1.1. Generation and characterization of U3A clones expressing STAT1-WT or STAT1Y701F.

We generated STAT1-WT and STAT1Y701F expressing clones by transfecting U3A cells that lack STAT1 expression[165,166], with mammalian expression vectors containing either STAT1-WT or STAT1Y701F coding sequences. The clones were selected according to the expression level of STAT1-WT or STAT1Y701F compared to the maximal expression level of endogenous STAT1 induced by IFNα in 2fTGH cells, from which U3A cells derive. For instance, we show that the level of STAT1 expression is equal in WT_clone1, Y701F_clone1, and IFNα-stimulated 2fTGH cells (Fig. 4.1.3A and 4.1.3B, lanes 5 *versus* 6). Next, we analyzed and confirmed the absence of STAT1 activation in Y701F clones upon IFNα stimulation. Indeed, a pY-STAT1 signal was detected in STAT1-WT clones but absent in Y701F clones (Fig.4.1.3A & B). We confirmed the transcriptional activity of these phosphorylated STAT1 by measuring the expression of *OAS1* upon IFNα stimulation by quantitative

real time PCR. As expected, *OAS1* was detected only in U3A cells containing STAT1-WT but not in STAT1Y701F expressing cells (Fig.4.1.4.).

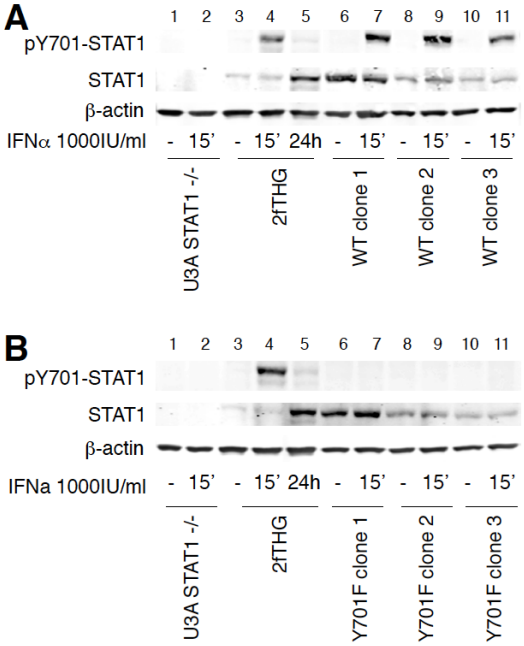


Figure 4.1.3. Expression of STAT1WT and STAT1Y701F in U3A STAT1-/- cells. *A. U3A STAT1 -/-, 2fTGH cells and U3A clones 1 to 3 expressing STAT1WT were stimulated with 1000 IU/mL of IFNα for the indicated time. Whole cell protein extracts were subjected to Western blotting for the detection of STAT1, pY701STAT1 and β-actin. B. U3A STAT1 -/-, 2fTGH cells and U3A clones 1 to 3 over-expressing STAT1Y701F were stimulated with 1000IU/mL for the indicated time. Whole cell protein extracts were subjected to Western blotting for the detection of STAT1, pY701STAT1 and β-actin.*

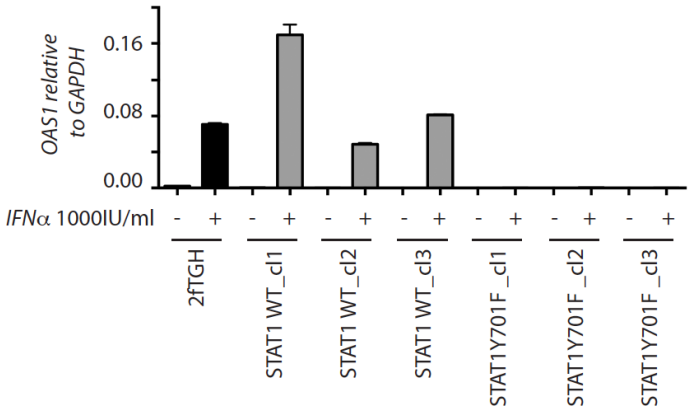


Figure 4.1.4 OAS1 expression upon IFNα stimulation in STAT1-WT and STAT1Y701F clones. *Cells were stimulated for 8 hours with 1000IU/ml of IFNα. Total RNA was extracted and OAS1 expression level was assessed by quantitative real time PCR. Relative expression to GPADH is reported.*

4.1.2. STAT1-WT and STAT1Y701F expression does not induce IRGs expression.

Because Choen and Stark proposed that U-STAT1 is transcriptionally active and maintains a high expression level of a subset of IRGs, we therefore analyzed the ability of STAT1Y701F to trigger IRGs. The expression of selected IRGs such as *OAS1*, *HERC6*, *BST2*, *MX1*, *IFI44L*, *IFI27*, *ISG15*, and *RSAD2,* was analyzed by qPCR in overnight serum starved STAT1-WT and STAT1Y701F clones. We included STAT1-WT clone 3 stimulated with 1000IU/ml for 4h as a positive control for the induction of gene transcription. As shown on Fig.4.5.1, none of the STAT1Y701F clones, neither the STAT1-WT clones were able to induce IRGs expression despite the elevated expression level of STAT1.

Our data clearly demonstrate that unphosphorylated STAT1 is transcriptionally inactive and, thus, it can not mediate the *pre-activation* of the hepatic IFN system observed in CHC non-responsive patients.

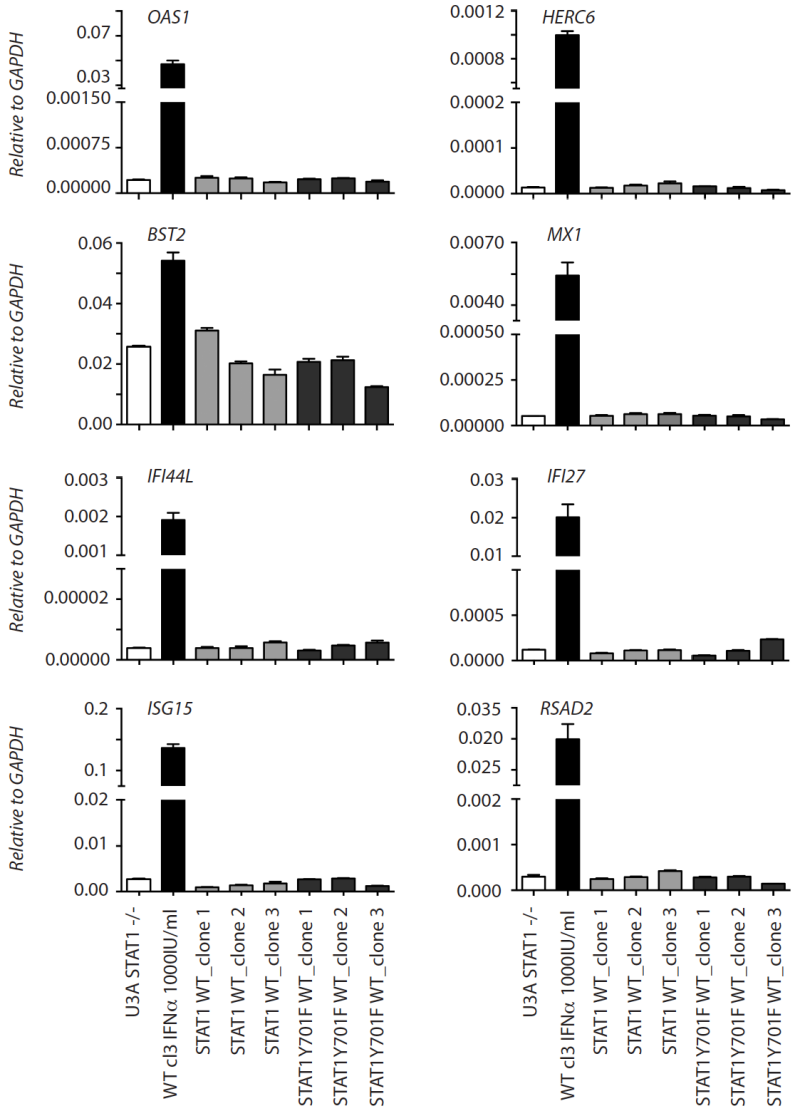


Fig. 4.1.5. IRGs expression is U-STAT1 independent. *RNA was extracted from STAT1-WT and STAT1Y701F cells serum-starved over night. Expression of indicated IRGs was assessed by real time PCR and values expressed as relative to GAPDH. STAT1-WT_clone 3 treated with 1000 IU/ml of IFNα for 8 hours and U3A STAT1 -/- cells were included as positive and negative control of the experiment.*

4.2. The interferon λ receptor chain α (IL28Rα) triggers high expression levels of interferon stimulated genes in non-responsive chronic hepatitis C patients.

From our previously shown data, we exclude unphosphorylated STAT1 as a key player in the pre-activation of the IFN system in the liver of CHC patients non-responder. The transcriptional activity of STAT1 requires its activation through tyrosine phosphorylation[71]. Therefore, we reasoned that the up-regulation of hepatic IRGs could result from a prolong activation of STAT1 mediated by a specific type of cytokine. Indeed, we have recently reported that the Jak-STAT signaling pathway is not desensitized by repeated exposure to IFNβ or IFNλ resulting in a prolonged STAT1 phosphorylation[85]. On the contrary, we observed that the Jak-STAT pathway is refractory to repeated exposure to IFNα leading to the impairment of STAT1 phosphorylation[90]. These data strongly suggest that IFNβ or IFNλ-mediated STAT1 activation may contribute to elevated hepatic IRGs expression in the liver of CHC patients non-responders.

4.2.1. IFNα up-regulates *IL28Rα* and enhances IFNλ2-mediated STAT1 phosphorylation in primary human hepatocytes (PHHs).

We analyzed the response of non-infected primary human hepatocytes (PHHs) to IFNα, IFNβ, or IFNλ2 stimulation. PHHs were exposed to saturating doses of the three cytokines and pY701-STAT1 signal was assessed by Western blot. We observed that IFNλ2 is a weak inducer of pY701-STAT1 presumably due to the low abundance of the IFNλ receptor[168] (Fig 4.2.2A). Nevertheless, an *in silico* promoter analysis of IL28RA gene, encoding for the *IL28Rα,* revealed the presence of a putative STAT1 binding site[67]. This finding suggests that IL28Rα is an IRG and that the response to IFNλ in PHHs could be amplified by pYSTAT1-induced *IL28Rα* up-regulation. Therefore, we stimulated PHHs with IFNα, IFNβ, or IFNλ2 and assessed *IL28Rα* expression by quantitative PCR. PCR primers specific for *IL28Rα* full length (variant 1) were designed as indicated in Figure 4.2.1A. The specificity of the PCR product was confirmed on agarose gel, according to the product size (Figure 4.2.1B), and by sequencing.

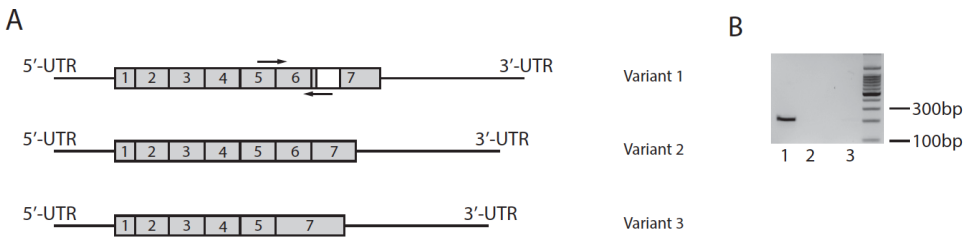


Figure 4.2.1. Primers validation for IL28Rα transcript variant 1 qPCR. *(A) Schematic representation of three IL28Rα transcription variants. Arrows indicates the position of specific forward and reverse primers used for the analysis of variant 1. The white box indicates the missing sequence in variant 2. (B) Validation of IL28RA primers by end-point PCR in Huh7 cells. cDNA (lane 1), RNA (lane 2), and H_2O (lane 3).*

We show that IFNα up-regulates *IL28Rα* in PHHs with a maximal level reached at 4 hours (Fig 4.2.2B; left panel), demonstrating that *IL28Rα* is an IRG. Surprisingly, we observed that the IFNα-mediated up-regulation of *IL28Rα* was impaired in Huh7 cells, although STAT1 expression was induced (Fig. 4.2.2B; right panel). Analysis of the response to INFλ2 stimulation after pre-exposure to IFNα shows that the pre-incubation of PHHs with IFNα for 4 hours improved the IFNλ2-induced pY701 STAT1 signal (Fig 4.2.2C: left panel, lane 4 *versus* lane 6). In line with the absence of IFNα-mediated *IL28Rα* expression in Huh7 cells, we show that a pre-incubation with IFNα did not lead to the amplification of the IFNλ signaling (Fig. 4.2.1C, right panel). Using immunofluorescent staining for IL28Rα and pY701-STAT1 we confirmed that IFNα induces IL28Rα expression and therefore amplifies the IFNλ-mediated pY701-STAT1 signal in PHHs from two different donors (Fig. 4.2.2D and 4.2.2E).

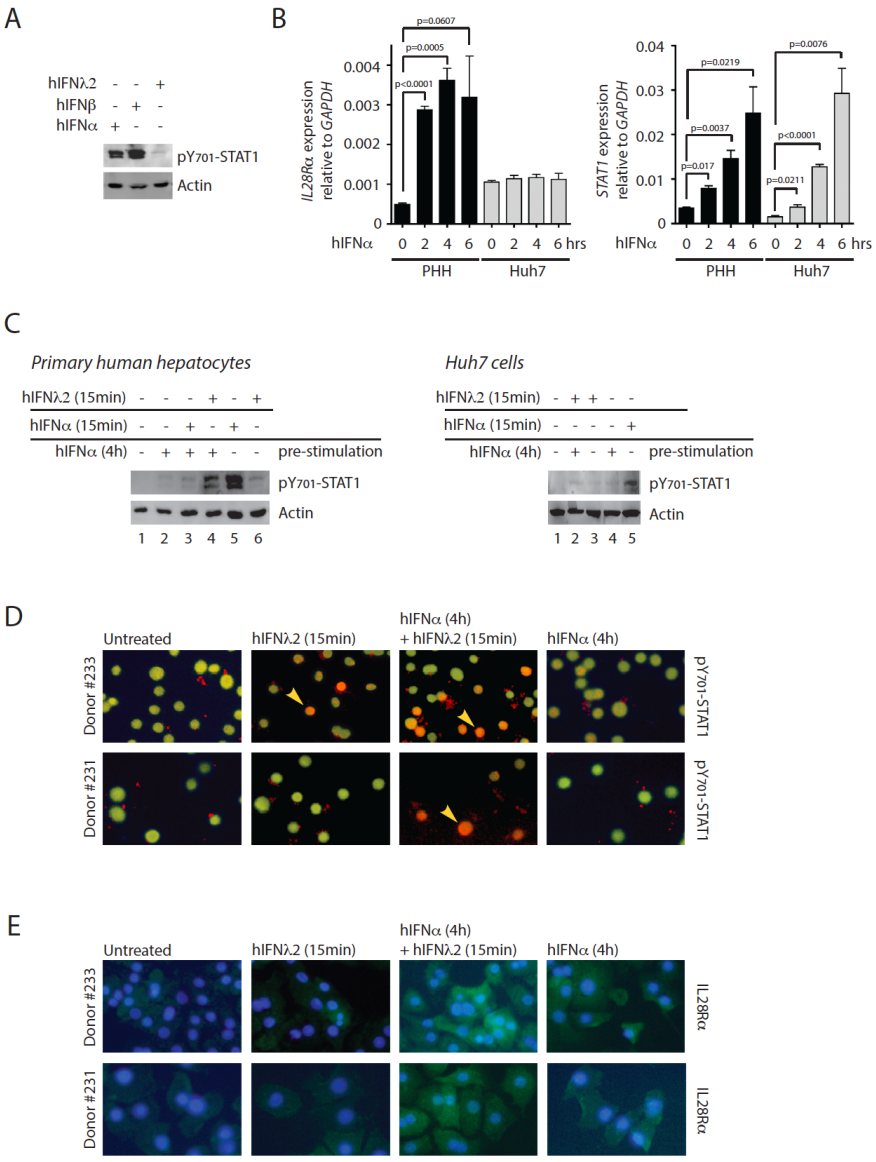


Figure 4.2.2. IFNα induces IL28Rα expression and amplifies pY-STAT1 signal upon IFNλ2 stimulation in human primary hepatocytes. *(A) Primary human hepatocytes were stimulated with 1000IU/ml IFNα, 1000IU/ml IFNβ, or 100ng/ml IFNλ2 for 15 minutes and pY-STAT1 signal was analyzed by Western blotting. Shown is a representative result from 2 independent experiments. (B) IFNα induces IL28Rα transcription in PHHs but not in human hepatoma cells. PHHs (black bars) and Huh7 cells (grey bars) were stimulated with 1000IU/ml IFNα for 0, 2, 4, and 6h. IL28Ra and STAT1 expression were analyzed by qPCR. Statistical analysis was performed using a Fisher's exact t-test. Shown are the results from 3 independent experiments. (C) IFNα pre-stimulation enhances the IFNλ2-induced STAT1 phosphorylation in PHHs but not in human hepatoma cells. PHHs and Huh7 cells were incubated with 1000IU/ml IFNα for 4h prior being stimulated with 1000IU/ml IFNα or100ng/ml IFNλ for another 15min. (C) Whole cell lysates were then prepared and STAT1 activation was measured by immunoblotting. Shown is a representative blot from at least 3 independent experiments. (D) & (E) PHHs isolated from two non-HCV donors were pre-stimulated with 1000IU/ml IFNα for 4h prior being exposed to IFNλ2 for another 15min. STAT1 activation and IL28Rα were monitored by*

immunofluorescence. Phosphorylated STAT1 signal is shown in red, IL28Rα in green, and nuclear staining (DAPI) in yellow or blue (panels D and E, respectively). Yellow arrow indicates nuclear localization of pY-STAT1.

Similarly to IFNα, we show that IFNβ and IFNλ are capable to upregulate *IL28Rα* expression. IFNβ induces a high expression level of *IL28Rα* whereas IFNλ2 only slightly increases *IL28Rα* expression *(*Fig. 4.2.3).

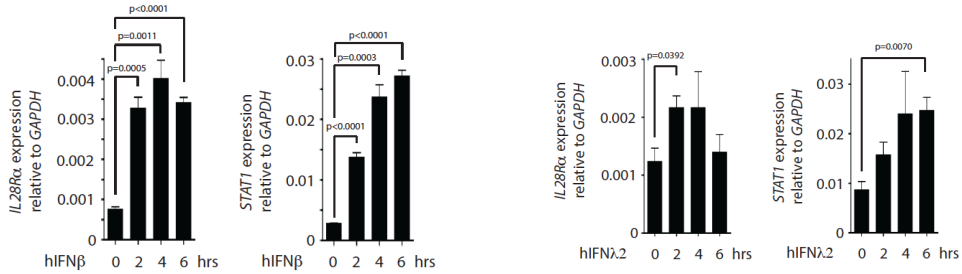


Figure 4.2.3. IL28Rα is induced by IFNβ and IFNλ. *(A) PHHs from non-HCV donors were stimulated with 100ng/ml IFNλ2 or with 1000IU/ml of IFNβ for 0, 2, 4, and 6h. IL28Rα and STAT1 expression were analyzed by qPCR. Results are expressed as mean ± s.e.m. and are representative of 2 independent experiments. Statistical analysis was performed using a Fisher's exact t-test.*

Interestingly, while analysing the IFNα-mediated *IL28Rα* expression, we observed that the induction of *IL28Rα* varied among PHHs donors, suggesting a differential response to IFNλ stimulation. We therefore selected 4 donors who differentially express *IL28Rα* in response to IFNα stimulation and assessed pY-STAT1 signal upon IFNλ treatment. As expected, we show that the response to IFNλ-mediated STAT1 activation is dependent on the expression level of *IL28Rα* (Fig 4.1.4A and B).

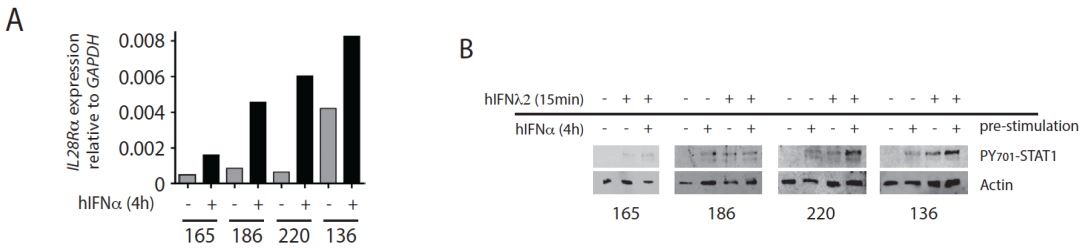


Figure 4.1.4. IFNα-induced IL28Rα up-regulation varies among PHHs donors. *(A) PHHs from four non-HCV donors were stimulated with 1000IU/ml IFNα for 4h and the expression level of IL28Rα was measured by qPCR. (B) The response to IFNλ2 stimulation depends on the expression level of IL28Rα. PHHs from four non-HCV donors previously analyzed for the*

expression level of IL28Rα were pre-stimulated with 1000IU/ml IFNα prior being exposed to 100ng/ml IFNλ2 for additional 15min. The phosphorylation of STAT1 was then monitored by immunoblotting.

4.2.2 The Jak-STAT signaling pathway is not refractory to continuous stimulation of IFNλ and leads to high expression levels of *pre-activated* IRGs.

In order to further investigate the IFNλ signaling pathway, we stably over-express *IL28Rα* in Huh7 cells. Three clones (LR-clone 1, 2 and 3) with increasing *IL28Rα* expression were selected. The over-expression of IL28Rα was assessed by quantitative PCR and by immunofluorescence staining (Fig. 4.2.5A; Fig. 4.2.4B). *IL28Rα* over-expression resulted in an increased IFNλ2-induced pY701-STAT1 signal without affecting the response to IFNα stimulation (Fig. 4.2.5C). We then analyzed the desensitization of the Jak-STAT signaling pathway upon IFNα and IFNλ2 stimulation in *IL28Rα* over-expressing cells. We observed that the Jak-STAT pathway became refractory to further stimulation by IFNα (Fig 4.2.5D; lanes 11 *versus* 13 and 2 *versus* 4) in Huh7 and in *IL28Rα* over-expressing cells. Interestingly, the prolonged exposure to IFNλ2 in *IL28Rα* over-expressing cells resulted in a robust maintenance of pY-701STAT1 signal (Fig. 4.2.5D; lane 2 *versus* 5). Furthermore, the repeated stimulation of *IL28Rα* over-expressing cells with IFNλ2 increased pY701-STAT1 signal (Fig. 4.2.5D; lane 7 *versus* lane 6). These data suggest that IFNλ2 stimulation does not lead to the desensitization of the Jak-STAT signaling pathway and, therefore, propose the lack of refractoriness of the IFNλ signaling pathway as a potential event that contribute to the maintenance of high expression levels of IRGs.

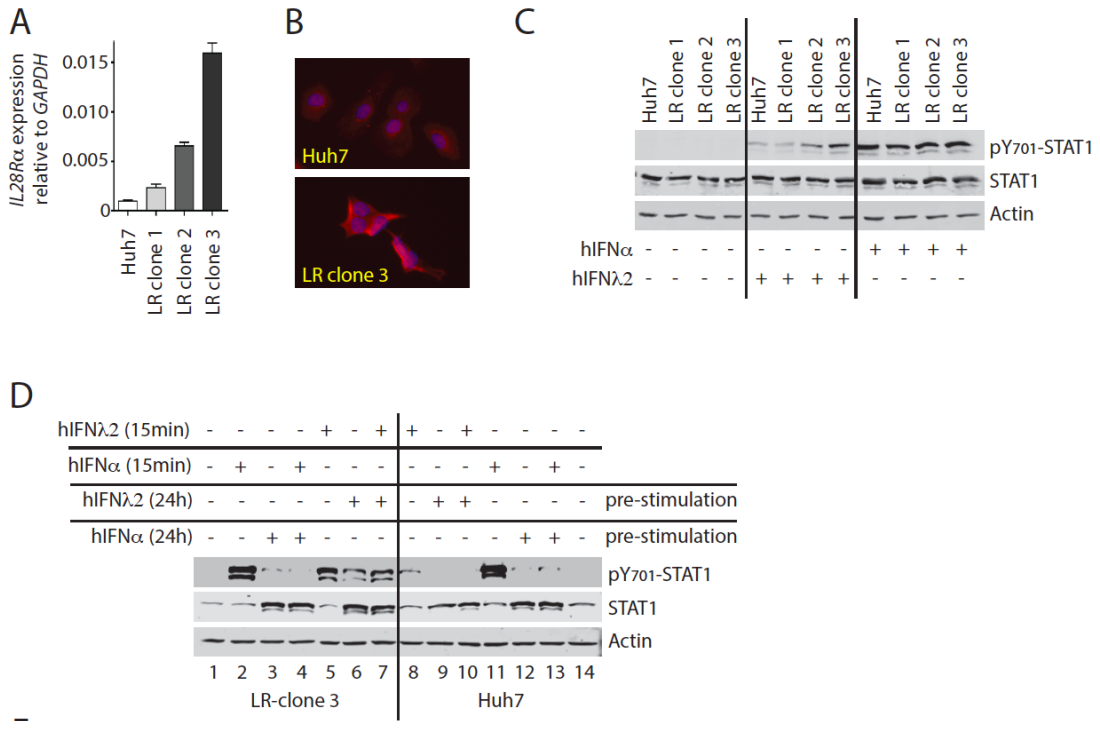


Figure 4.2.5. Absence of desensitization of the Jak-STAT signaling pathway in response to IFNλ2 stimulation. *(A) & (B) Huh7 cells were stably transfected with a plasmid containing IL28Rα coding sequence. The over-expression of IL28Rα was confirmed by qPCR and by immunofluorescence. (C) The over-expression of IL28Rα does not alter IFNα-induced STAT1 activation but enhances STAT1 phosphorylation upon IFNλ2 stimulation. Huh7 and Huh7LR cells were stimulated with 1000IU/ml IFNα or 100ng/ml IFNλ2 for 15 minutes and pY-STAT1 signal was analyzed by immunoblotting from whole cell extract. Shown is a representative blot from 2 independent experiments. (D) The IFNλ signaling is not refractory to further stimulation by IFNλ2 in human hepatoma cells that over-express IL28Rα. Huh7 and LR cells were pre-stimulated for 24h with 1000IU/ml IFNα or 100ng/ml IFNλ2 prior being incubated with IFNα or IFNλ2 for additional 15 minutes. Phosphorylated STAT1, STAT1, and β-actin signals were analyzed by Western-blotting from whole cell extracts. Shown is a representative blot of at least 3 independent experiments.*

Next, we investigated whether IFNλ-mediated Jak-STAT signaling is able to maintain elevated the expression of IRGs in cells that over-express the IFNλ receptor. For that purpose, Huh7 cells and LR clones were stimulated with IFNλ2 up to 96 hours and the expression level of *RSAD2*, *USP18*, *ISG15* and *IFI44L* (four described pre-activated genes in the liver of CHC nonresponsive patients[127]) was measured by

quantitative real time PCR. Our results demonstrate that IFNλ2 up-regulates IRGs in an *IL28Rα*-dependent manner (Fig. 4.2.6). Furthermore, we observed that IFNλ2 was capable to prolong the expression of IRGs up to 96 hours whereas IFNα failed (Fig. 4.2.6).

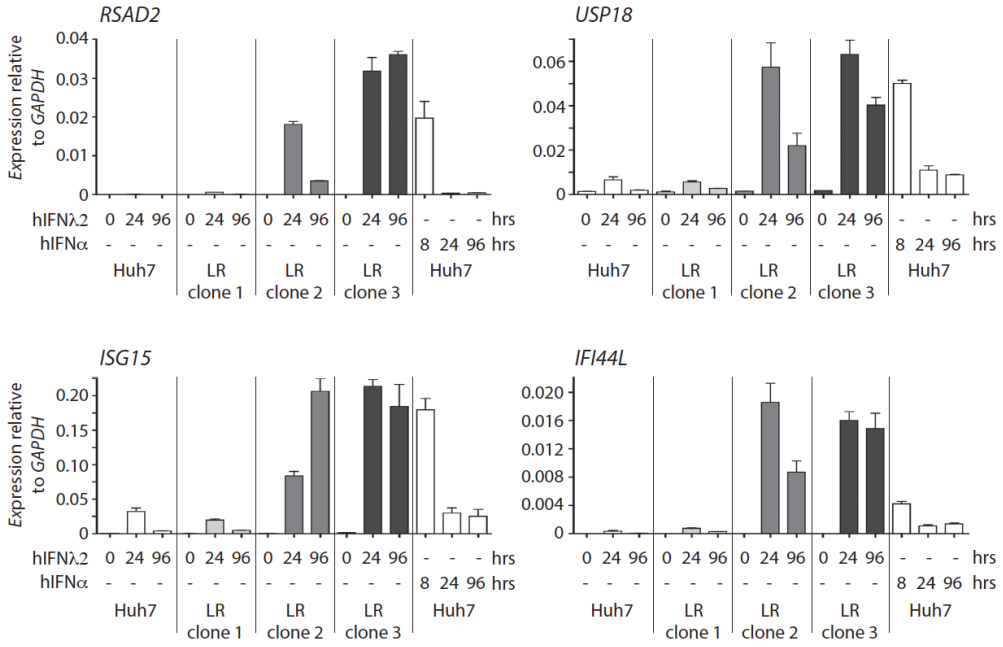


Figure 4.2.6. IFNλ2, but not IFNα, maintains high expression level of a set of IRG identified as pre-activated genes in non-responsive CHC patients. *IFNλ2 induces a long-lasting expression of pre-activated genes in IL28Rα over-expressing cells. Huh7 (n=3) and LR clones (n=3) were stimulated with 1000IU/ml IFNα or 100ng/ml IFNλ2 for 0, 8, 24, and 96h. The expression of RSAD2, ISG15, USP18, and IFI44L was assessed by qPCR. Results are expressed as mean ± s.e.m and are representative from 3 independent experiments.*

Additionally, analysis of pY-STAT1 signal showed that STAT1 remained phosphorylated up to 96 hours in *IL28Rα* over-expressing cells upon IFNλ2 stimulation (Fig. 4.2.7A; lanes 4, 5, 6 versus 3 and lanes 8, 9, 10 versus 7) whereas we observed a complete disappearance of STAT1 phosphorylation at this time point when cells were stimulated with INFα (Fig. 4.2.7; lane 1 versus 2).

In order to confirm that the IFNλ-mediated specifically an upregulation of pre-activated genes, we analyzed the expression level of *ATF3* and *NOXA*, two IRGs that are not highly induced in the liver of CHC non-responder patients[127]. We show that both IFNα and IFNλ2 failed to maintain a high expression level of *ATF3* and *NOXA* (Fig. 4.2.7B). All together these findings support our conclusion that the elevated

expression of *pre-activated* ISGs in CHC non-responder patients is triggered by the IFNλ2 signaling pathway.

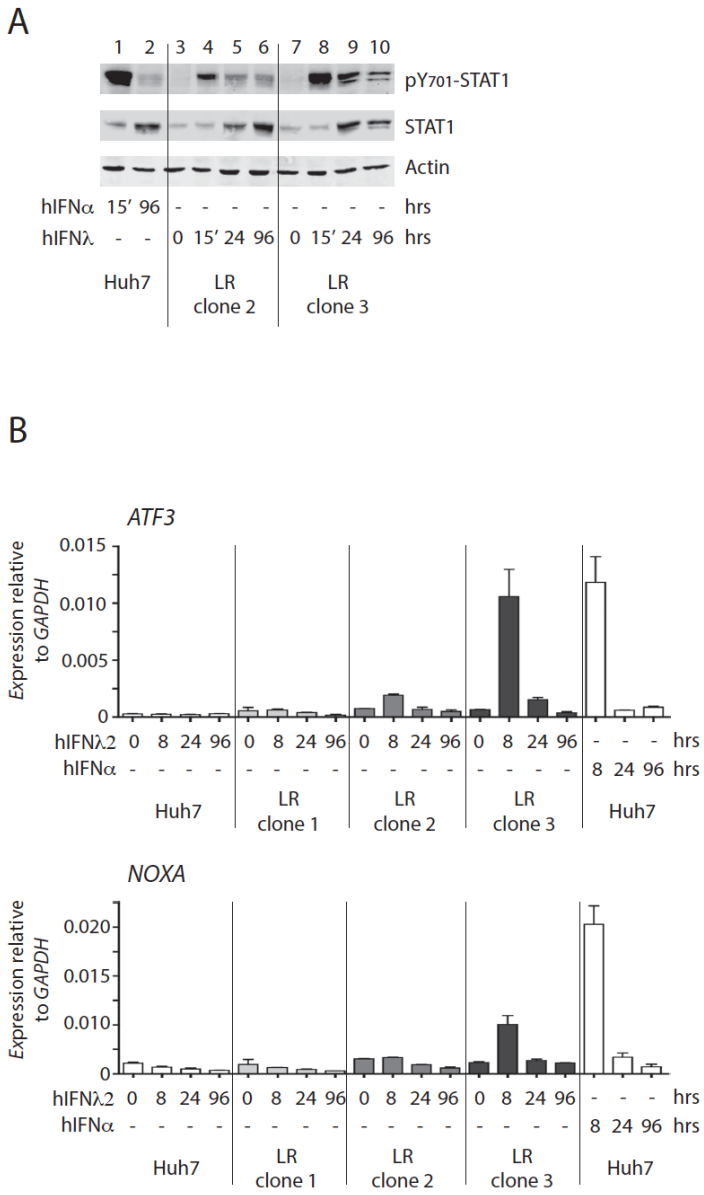
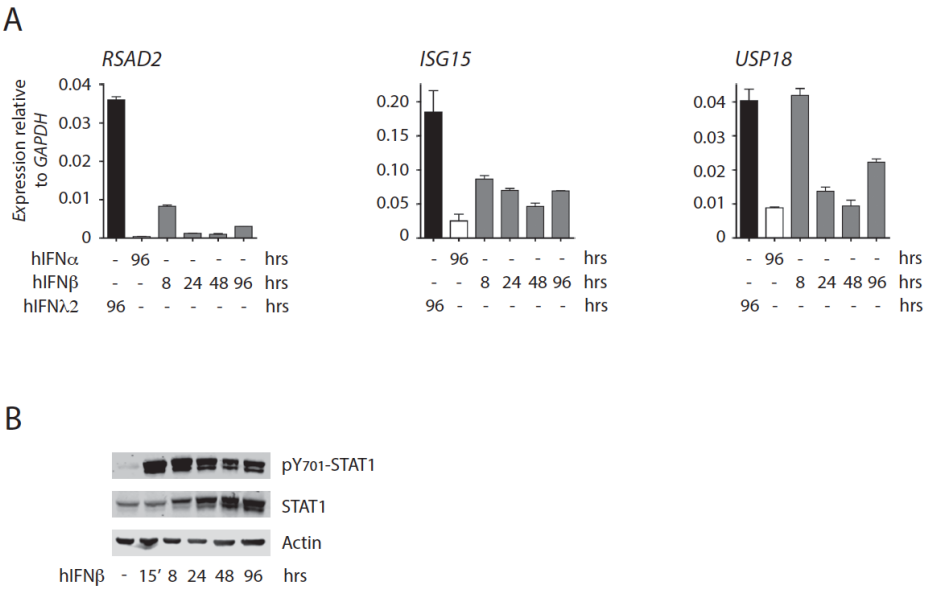


Figure 4.2.7. IFNλ2 mediates a prolonged STAT1 activation but fails to induce a long lasting expression of non pre-activated IRGs. *(A) Huh7, LR clone 2, and LR clone 3 were stimulated with IFNα 1000IU/ml or with IFNλ2 100ng/ml up to 96h. pY-STAT1, STAT1, and actin signals were visualized by immunoblotting. Shown is a representative blot from at least 2 independent experiments. (B) Huh7 (n=3) and LR clones (n=3) were stimulated with 1000IU/ml IFNα or 100ng/ml IFNλ2 for 0, 8, 24, and 96h. The expression of ATF3 and NOXA were assessed by qPCR. Results are expressed as mean ± s.e.m. and are representative from 3 independent experiments.*

4.2.3 The Jak-STAT signaling pathway is not refractory to continuous stimulation of IFNβ but lead to intermediate expression levels of *pre-activated* IRGs.

Since we previously reported that the IFNβ signaling pathway is not refractory in mouse liver[85], we analyzed the ability of IFNβ to induce a prolonged expression of *RSAD2*, *ISG15*, and *USP18* in Huh7 cells. Our data show that IFNβ up-regulated the IRGs at intermediate extent (Fig. 4.2.8A), despite a strong pY-STAT1 signal detected up to 96h of continuous stimulation by IFNβ (Fig. 4.2.8.B). However, we did not observe any significant correlation between *IFNβ* and IRGs (*IFI27*, *ISG15*, and *RSAD2)* expression in liver biopsies from CHC patients (Fig. 4.2.8.C). Additionally, no correlation was observed between *IFNAR1* and *IFI27*, *ISG15*, and *RSAD2* (Fig. 4.2.8.D). Our data exclude the involvement of IFNβ in the prolonged expression of IRGs in non-responder CHC patients.



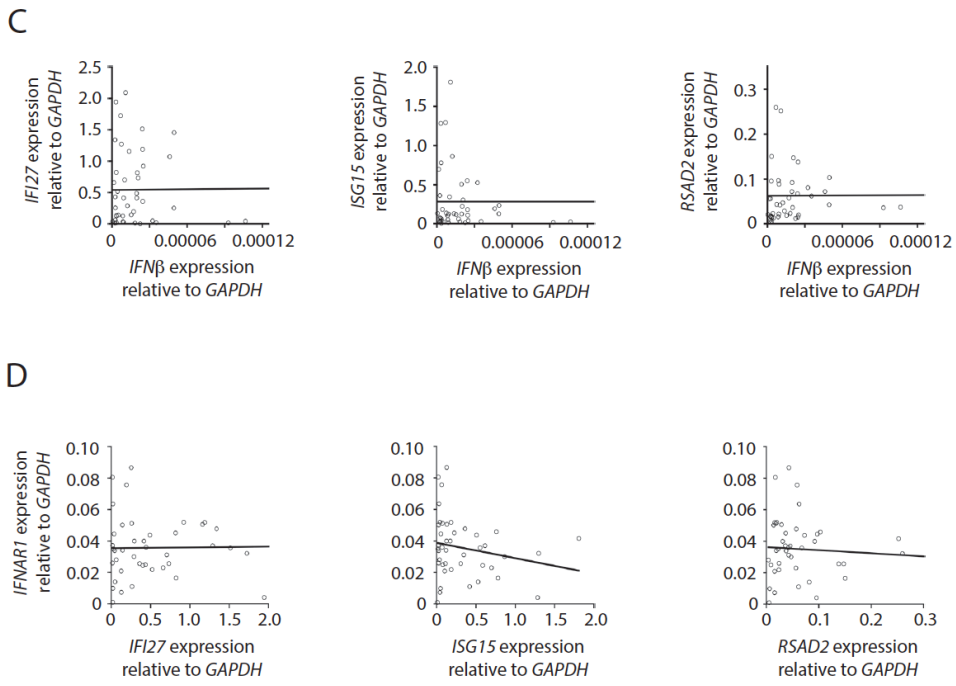


Figure 4.2.8. IFNβ induces intermediate expression of pre-activated in vitro *(A) IFNβ failed to induce a long-lasting expression of IRGs in Huh7 cells. Huh7 cells were stimulated with 1000IU/ml IFNα, 1000IU/ml IFNβ, or 100ng/ml IFNλ2 for indicated time points. IRGs expression of RSAD2, ISG15, and USP18 was assessed by qPCR. (B) IFNβ treatment prolongs STAT1 phosphorylation in Huh7 cells. Huh7 cells where treated for 15', 8, 24, 48 and 96h with 1000IU/ml IFNβ. Phosphorylated STAT1, STAT1, and β-actin measurement was assessed by immunoblotting from whole cell lysate. (C) IFNβ expression does not correlate with IRGs expression in liver biopsies of CHC patients (n=46). IFNβ, IFI27, ISG15, and RSAD2 expression was assessed by qPCR. Association was assessed by Spearman correlation analysis. (D) IFNAR1 expression does not correlate with ISGs expression in liver biopsies of CHC patients (n=46). IFNAR1, IFI27, ISG15, and RSAD2 expression was analyzed by qPCR and the association was assessed by Spearman correlation analysis.*

4.2.4. IL28Rα is highly expressed and strongly correlated with IRGs in liver biopsies from CHC patients.

In order to validate our *in vitro* findings, we analyzed the expression level of *IL28Rα* in liver biopsies from non-HCV and CHC patients. We assessed a significant over-expression of *IL28Rα* in CHC compared to non-HCV patients (Fig. 4.2.9A). Furthermore, we observed a significant correlation between the expression of *IL28Rα* and *pre-activated* IRGs, such as *IFI27*, *ISG15*, and *RSAD2* in CHC liver biopsies (Fig. 4.2.9B). Using a previously published algorithm for the prediction of the probability of SVR (pSVR) in CHC patients treated with IFNα-based therapy[126], we showed that patients with pSVR value below 0.5 (meaning less than 50% chance to clear HCV) displayed a significant higher expression of *IL28Rα* compared to the ones with a pSVR above 0.5 (meaning more than 50% chance to clear HCV) (Fig. 4.2.9C). In line with this observation, analysis of CHC patient clinical outcome upon IFNα-based therapy indicated a significant higher expression of *IL28Rα* in non-responder (NR) than in SVR (Fig. 4.2.9D).

We assessed the predictive power of *IL28Rα* for treatment-response in CHC patients by calculating the receiver operating characteristic (ROC) curve for *IL28Rα*. The area under the curve (AUC) for *IL28Rα* is 0.744 (Std Err=0.071; p=0.002) indicating that the expression level of *IL28Rα* could be used to discriminate NR from SVR (Fig. 4.2.9E). Furthermore, we observed that *IL28Rα* has similar performance as the one of two SNPs mapping at IL28B locus (rs12979860 and rs8099917) in predicting treatment-response in CHC patients (AUC=0.73; Std Err=0.16)[126].

In order to be activated, the IFNλ signaling pathway requires the presence of both IFNλ and receptor. Therefore, we measured by quantitative real-time PCR the expression of *IFNλ1* and *IFNλ2* in liver biopsies from non-HCV and CHC patients. The specificity of the primers for *IFNλ1* and *IFNλ2* was validated by assessing the PCR product size on agarose gel and by sequencing (Fig. 4.2.10A). Further analysis showed that quantitative real time PCR signals were detected only in samples from monocyte-derived dendritic cells (MDDCs) stimulated with LPS confirming the specificity of our *IFNλ1* and *IFNλ2* detection method (Fig. 4.2.10B & C). *IFNλ1* and *IFNλ2* were not detectable in liver biopsies from non-HCV patients whereas we could measure *IFNλ1* in 63% and *IFNλ2* in 46% of CHC liver biopsies samples (Fig. 4.2.10C). Further analysis showed no correlation between *IFNλ2* and *IFI27*, *ISG15*, or

RSAD2 in liver biopsies form CHC patients (Fig. 4.2.9D). These results suggest, that the *pre-activation* of the hepatic IFN system in CHC non-responder patients is not triggered by the elevated expression of IFNλs.

Taken together, our results provide evidences that the continuous activation of the IFNλ signaling pathway leads to a prolonged expression of pre-activated IRGs in CHC patients and point out a key role for IL28Rα in such mechanism.

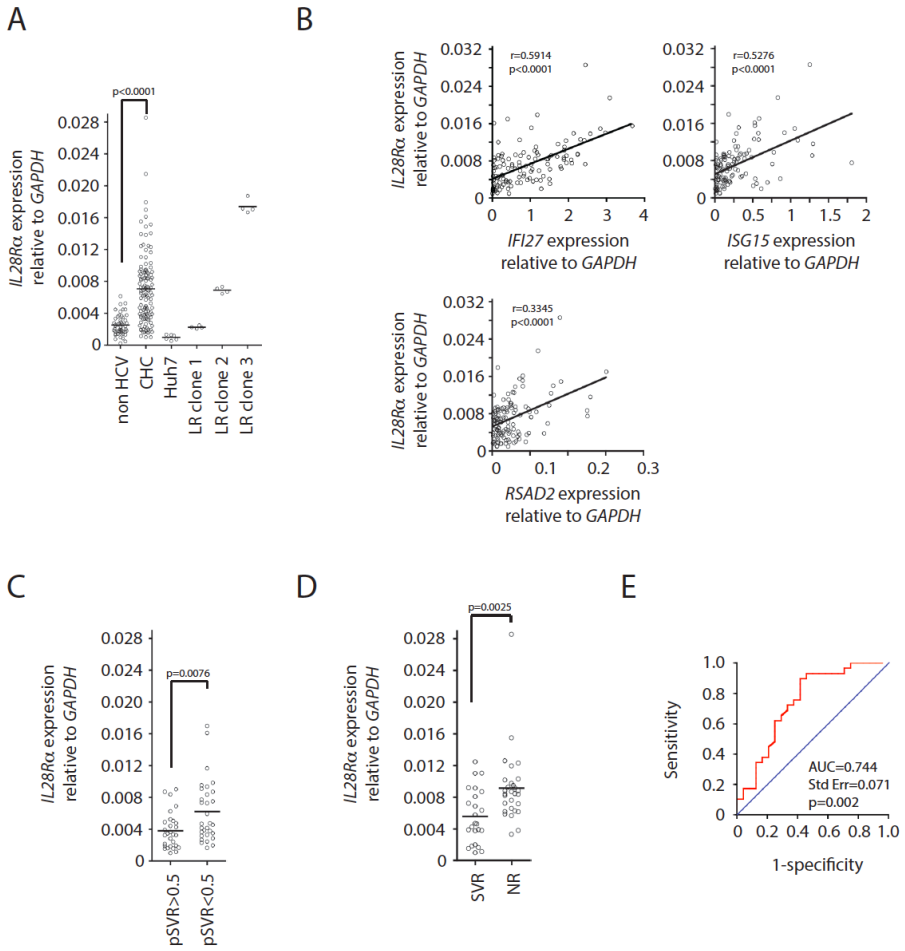


Figure 4.2.9. IL28Rα is over-expressed in nonresponsive CHC patients and correlates with the expression of pre-activated IRGs. *(A) IL28Rα is upregulated in the liver of CHC patients. The hepatic expression of IL28Rα was quantified in CHC patients (n=122) and in non-HCV patients (n=53) by qPCR. Statistical analysis was performed using a Mann-Whitney's test. (B) Positive correlation between IL28Rα and the pre-activated IRGs the liver biopsy from CHC patients (n=122). IL28Rα, IFI27, ISG15 and RSAD2 were analyzed by qPCR. The relationship between IL28Rα and the pre-activated ISGs was estimated by Spearman's correlation analysis. (C) IL28Rα is highly expressed in the liver biopsy from CHC patients with a low pSVR. The hepatic expression level of IL28Rα was measured by qPCR and then*

sorted according to the predicted SVR values (pSVR≤0.5; n=29 and pSVR≥0.5; n=31). Statistical analysis was performed using a Mann-Whitney's test. (D) IL28Rα is over-expressed in nonresponsive CHC patients to peg-IFNα/ribavirin therapy. The hepatic expression level of IL28Rα was quantified by qPCR in SVR (n=24) and in NR (n=29) patients. Statistical analysis was performed using a Mann-Whitney's test. (E) ROC curve for the clinical treatment response with the expression level of IL28Ra (n=53).

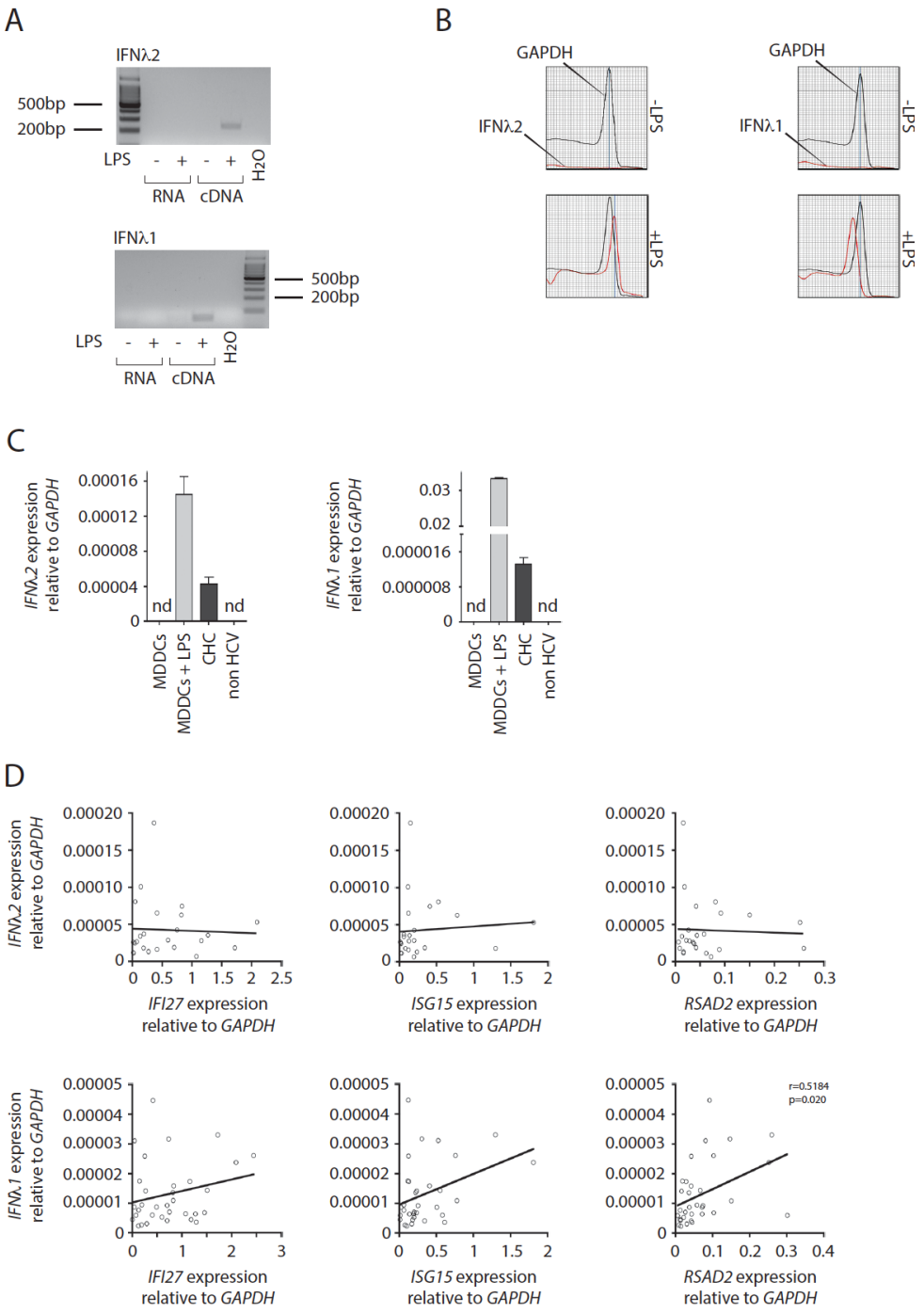


Figure 4.2.10. IFNλ1 and IFNλ2 are detectable at mRNA level but did not correlate with the pre-activated IRGs expression in the liver biopsies from CHC patients. *(A) Validation of specific primers for IFNλ1 and IFNλ2. MDDCs were stimulated or not with 1μg/ml LPS for 2h and the expression of IFNλ1 and IFNλ2 were analyzed by end-point PCR. (B) Melting curves for IFNλ1 and IFNλ2 (in red) and GAPDH (in black) were reported for MDDCs stimulated or not with LPS for 2h. (C) IFNλ1 and IFNλ2 were measured in CHC patients by qPCR (n=53; positive for IFNλ2 n=24, positive for IFNλ1n=33). MDDCs stimulated with LPS were used as a positive control. (D) IFNλ1, IFNλ2, IFI27, ISG15, and RSAD2 were quantified by qPCR in liver biopsy from CHC patients (IFNλ1 n=33, IFNλ2 n=24). The relationship between the genes was assessed by Spearman's correlation analysis.*

4.2.5 The response to IFNλ2 stimulation in liver biopsies from CHC patients is dependent on *IL28Rα* expression level.

In order to prove that the response to IFNλ *in vivo* is regulated by the expression level of *IL28Rα*, we performed a functional assay by stimulating *ex-vivo* liver biopsies from CHC patients with IFNλ2. Activated STAT1 was monitored by immunofluorescence and the results were classified on the basis of the hepatic expression level of *IL28Rα.* We sorted patients with low (Fig. 4.2.11A) and high (Fig. 4.2.11B) expression level of *IL28Rα.* Our results show an absence of pY-STAT1 signal in PBS-treated biopsies from patients with low hepatic expression of *IL28Rα* (Fig. 4.2.11A; left column) whereas cells are positively stained for pY-STAT1 in the PBS-treated biopsies from the patients with high hepatic expression of *IL28Rα* (4.2.11B; left column). This finding suggests that patients expressing a high amount of the IFNλ receptor have a pre-activated hepatic IFN system. Upon IFNα stimulation, all samples with a low *IL28Rα* expression showed a strong positive pY-STAT1 staining (Fig. 4.2.11A; middle column) whereas in only 2 samples on 9 (B761 and B757) from the high *IL28Rα* expressing group were positive for pY- STAT1 (Fig. 4.2.11B; middle column). Finally, the stimulation with IFNλ2 resulted in an enhanced pY-STAT1 staining in all the samples with high expression of *IL28Rα* (Fig. 4.2.11B; right column) whereas pY-STAT1 signal was much less pronounced in the samples with low expression of *IL28Rα* (Fig. 4.2.11A; right column).

These data clearly demonstrate that the hepatic response to IFNλ stimulation is dependent on the expression level of *IL28Rα*.

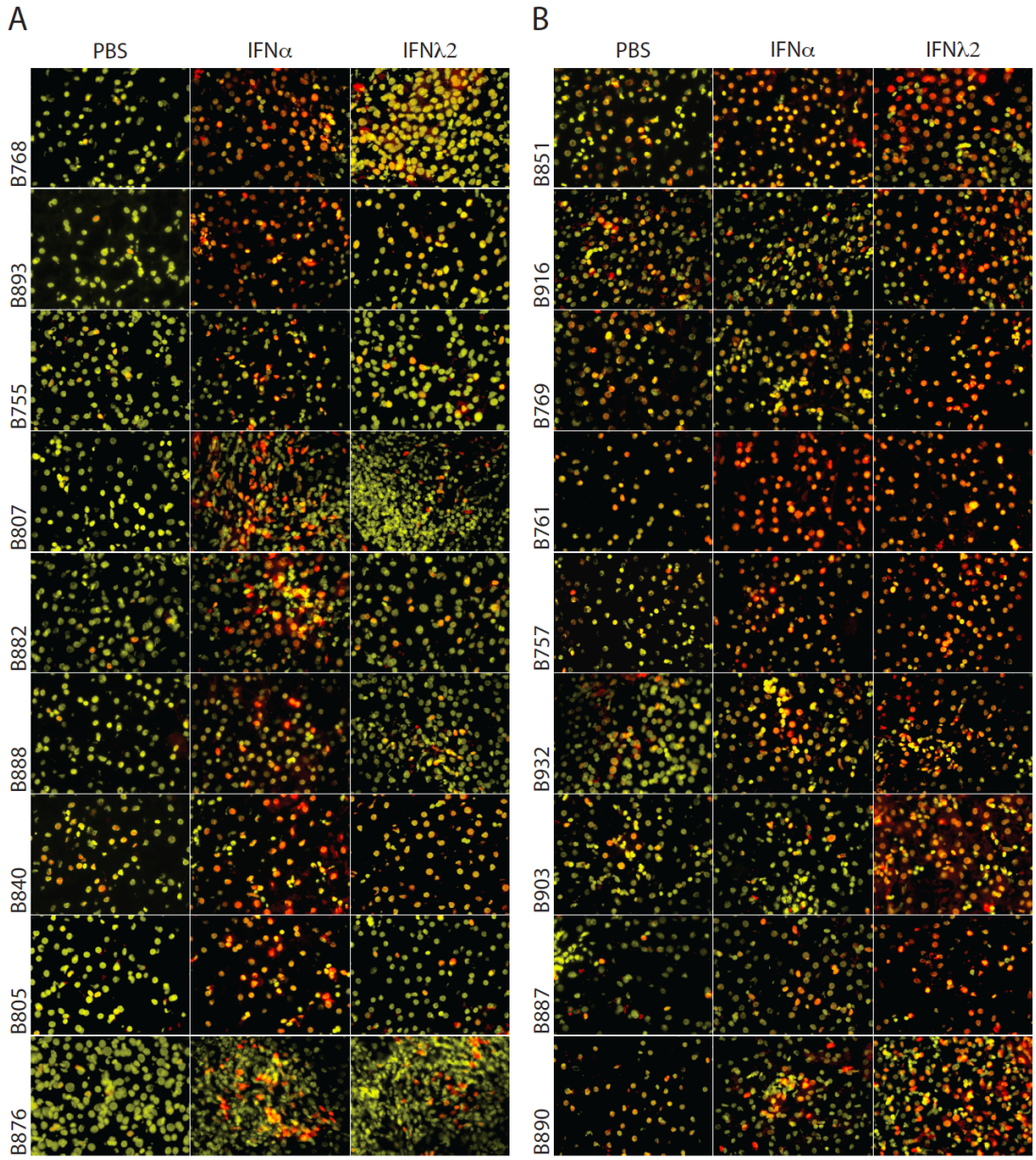


Figure 4.2.11. The ability of IFNλ2 to induce STAT1 phosphorylation in human liver biopsies depends on the hepatic expression of IL28Rα. *(A) & (B) Needle liver biopsies freshly obtained from CHC patients were incubated in 1000IU/ml IFNα, in 100ng/ml IFNλ2, or in PBS for 15min at 37℃. pY-STAT1 signal (in red) was monitored by immunofluorescence. Nuclei were stained with DAPI (in yellow). Panel A shows the results obtained from CHC patients (n=9) with a hepatic expression of IL28Rα <0.004. Panel B shows the data from CHC patients (n=9) with a hepatic expression of IL28Rα > 0.005.*

4.2.6 IFNα-mediated *IL28Rα* up-regulation is associated to allelic variants at IL28B locus in PHHs.

Several genome wide association studies reported that minor alleles at two SNPs (rs12979860 and rs8099917) mapping at the IL28B locus are associated to high hepatic expression level of IRGs[130,131,126] and with poor treatment-outcome[143,144,145,146] in CHC patients. We have observed that the IFNα-mediated *IL28Rα* up-regulation varies among different PHH donors (Fig. 4.1.4A). Therefore, we wanted to investigate whether the differential *IL28Rα* induction in PHHs upon IFNα stimulation correlates with allelic variants at the *IL28B* locus. Genotyping of rs12979860 and rs8099917 was performed on all the donors from whom PHHs were isolated. Our data show that donors harboring the homozygous major allele at the two selected SNPs (CC for rs12979860; TT for rs8099917) display a low expression level of *IL28Rα* (median<0.004) upon IFNα stimulation, while donors harboring the minor allele at the two SNPs (CT/TT for rs12979860; TG/GG for rs8099917) show a significantly more pronounced induction of *IL28Rα* expression (median>0.006) in response to IFNα treatment (Fig. 4.2.12A & B). Further analysis of the IFNα-mediated *STAT1* and *RSAD2* up-regulation in PHHs revealed no significant difference among the genotypes demonstrating a specific effect of *IL28B* polymorphisms on the IFNα-induced *IL28Rα* expression.

We then calculated ROC curves for the SNPs (rs12979860 and rs8099917) using the expression levels of *IL28Rα* and *STAT1* upon IFNα stimulation. The AUC for *IL28Rα* and *STAT1* were 0.779/0.854 and 0.625/0.593 for rs12979860/rs8099917 respectively (Fig. 4.2.13A&B). These data indicate that the expression level of IFNα-induced IL28Rα is a better parameter than STAT1 to discriminate the genotypes at *IL28B* locus.

Finally, we show a higher expression of *IL28Rα* in liver biopsies of CHC patients with CT/TT (rs12979860) or TG/GG (rs8099917) genotypes compared to the samples with a CC or a TT genotype (rs12979860 and rs8099917 respectively) (Fig. 4.2.12C).

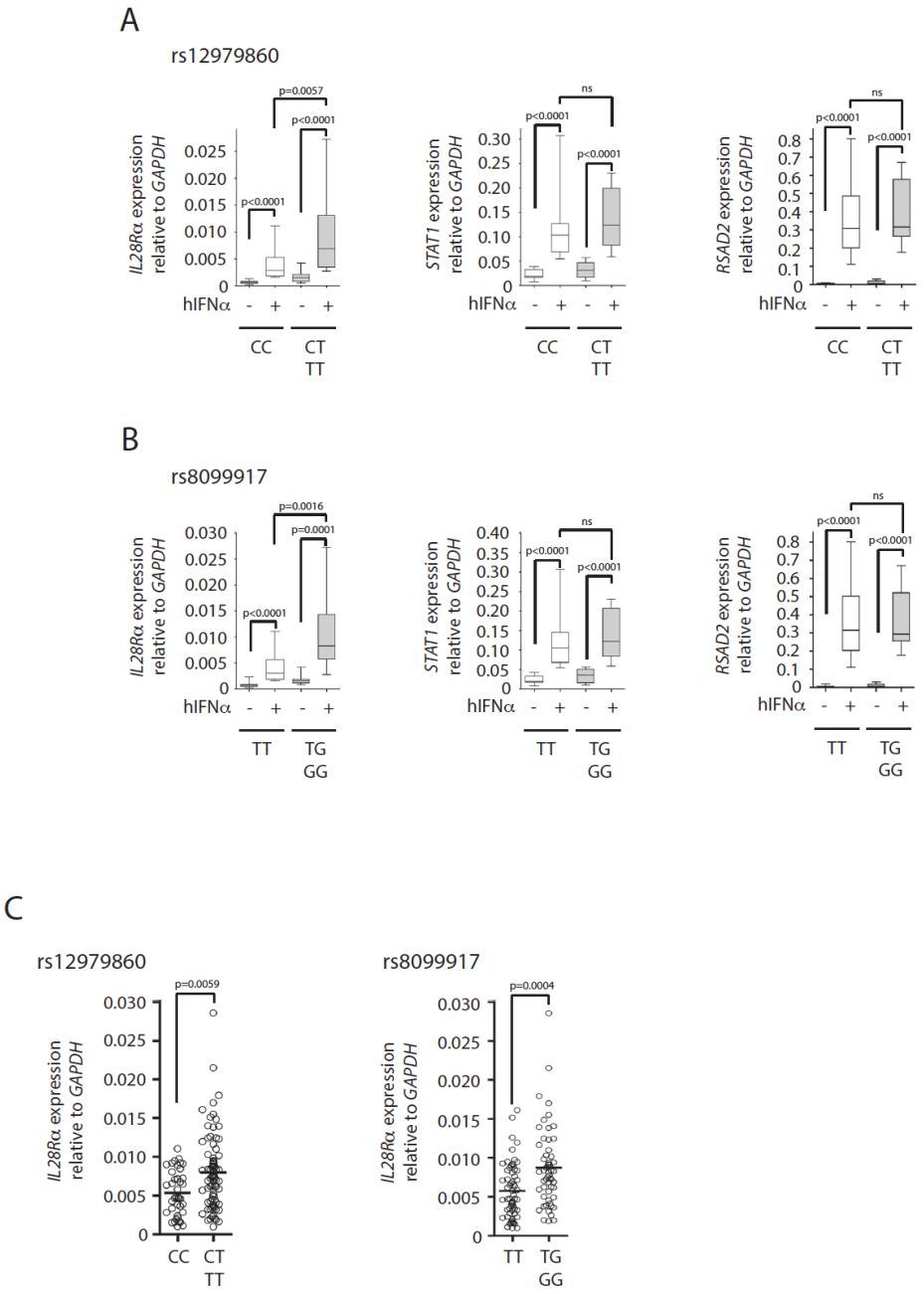


Figure 4.2.12. IFNα induces a high expression level of IL28Rα in PHHs carrying the minor allele at rs12979860 and rs8099917. *PHHs from 30 non-HCV donors were stimulated for 4h with 1000IU/ml IFNα. IL28Rα, STAT1 and RSAD2 expression were measured by qPCR. Statistical analysis was performed using a Mann-Whitney's test. (A) IL28Rα, unlike STAT1 and RSAD2, is strongly induced by IFNα in PHHs carrying the minor allele (CT; n=13) and (TT; n =1) compared to the ones carrying the major allele (CC; n=16) at rs12979860. (B) IL28Rα, unlike STAT1, is strongly induced by IFNα in PHHs carrying the minor allele (GT; n=17) and (GG; n =2) compared to the ones carrying the major allele (TT; n=19) at rs8099917. (C) Liver biopsies from CHC patients carrying the minor allele at*

rs12979860 and rs8099917 have a higher hepatic expression of IL28Rα than the ones that carry the major allele. The IL28Rα expression levels were quantified by qPCR in liver biopsies from CHC patients and the results are presented according to the genotypes rs12979860 (CC; n=39), (CT; n=68), and (TT; n=13). rs8099917 (TT; n=64), (GT; n=47), and (GG; n=9).

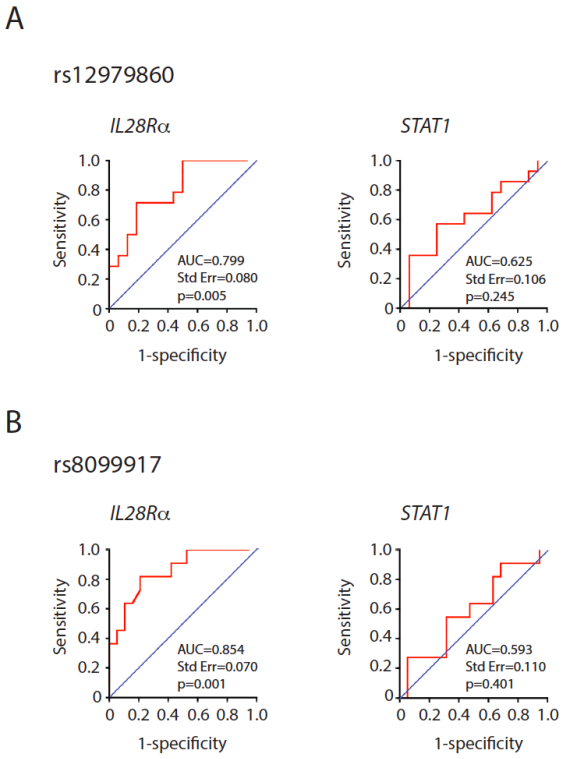


Figure 4.2.12. ROC curves for rs12979860 and rs8099917 with the expression levels of IL28Rα and STAT1. *PHHs from non-HCV donors (n=30) were stimulated with 1000IU/ml IFNa for 4h and ROC curves were calculated for rs12979860 (A) or for rs8099917 (B).*

In conclusion, the data presented support the hypotehsis that the *pre-activation* of the hepatic IFN system observed in the liver of CHC non-responder patients is mediated by IFNλ signaling pathway and requires a minimal expression level of *IL28Rα*. We also provide evidence that the IFNα-mediated *IL28Rα* expression is linked to allelic variants at SNPs at IL28B locus.

Supplementary table 1: CHC patients characteristics
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Patient #	Biopsy coding #	Gender	Age	HCV GT	Viral load	Clinical response	rs12979886	rs8099917
1	A478/B161	m	45	1	5632925	PNR	0	0
2	A502b	m	67	1b	2137002	No EoTR	1	1
3	A515	f	54	3a	75644	SVR	0	0
4	A518	m	48	4	45523	interrupted	2	2
5	A531	m	39	1a	3427262	no treatment	0	0
6	A532	m	55	1a	788024	no treatment	0	0
7	A544b	m	46	1a	1218968	no treatment	2	2
8	A551	m	46	1b	69000000	interrupted	0	0
9	A558c	f	47	1a	1444062	PNR	1	1
10	A563/A938	m	48	1b	713046	PNR	1	1
11	A566	m	42	1a	3556921	no treatment	1	1
12	A567	m	29	1b	384525	SVR	1	1
13	A574	m	43	1a	792776	no treatment	1	1
14	A582	m	35	1b	17594	SVR	0	0
15	A590	f	48	1a	3107291	No EoTR	0	0
16	A603	f	38	2	5780000	No EoTR	2	0
17	A622	f	48	4	88216	no treatment	2	2
18	A626	m	63	1	1752158	No EoTR	1	1
19	A643	f	52	2a/c	72157	Relapse	0	0
20	A650	f	44	3a	319000	SVR	1	1
21	A656	m	51	4e	864372	no treatment	1	1
22	A658	m	57	1	10327448	no treatment	1	1
23	A675	f	53	1	n.d.	no treatment	1	1
24	A687	f	69	1	1686605	no treatment	1	1
25	A688	f	48	3a	13538256	Relapse	1	0
26	A689	f	67	3a	265382	no treatment	1	1
27	A704	m	41	3a	15538256	SVR	0	0
28	A707	f	24	1b	440	SVR	0	0
29	A725	m	48	1a	36108528	PNR	1	0
30	A764	m	38	4	12000	SVR	0	0
31	A768	m	36	3a	1140000	EoTR	1	1
32	A770	m	49	4	165330	SVR	1	0
33	A795	m	54	1	222053	Breakthrough	0	0
34	A809	f	41	1a	4652760	PNR	1	1
35	A821	m	40	1a	15900000	Relapse	0	0
36	A847	m	49	4c	371535	Breakthrough	1	1
37	A864	f	50	3	1123718	Relapse	1	1
38	A865	m	35	1a	1578381	EoTR	1	0
39	A867	f	48	3a	28368	SVR	0	0
40	A897	f	34	1b	360000	Relapse	1	1

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41	A931	f	49	4	4100000	no treatment	1	1
42	A940	m	50	3	2230542	SVR	0	0
43	A942	m	42	1a	4291956	No EoTR	1	1
44	A954	m	44	1a	3884688	Relapse	1	1
45	A960	m	54	1a	4625025	no treatment	0	0
46	A962	m	53	1b	770000	Relapse	1	0
47	A998	m	51	1b	241223	Relapse	1	1
48	B007	m	37	3a	11426813	Relapse	0	0
49	B011	m	27	1b	3102	SVR	0	0
50	B020	m	43	1b	1089515	SVR	1	1
51	B022	m	35	4	4491065	SVR	0	0
52	B029	f	31	3a	249240	SVR	1	1
53	B061	f	44	1	90124	no treatment	1	1
54	B065	m	49	1a	1484271	Relapse	0	0
55	B078	m	55	3	578305	Relapse	1	0
56	B146	m	48	4b	3201553	PNR	2	0
57	B181	m	58	1b	57791	No EoTR	2	2
58	B261	f	40	1b	1145339	SVR	0	0
59	B271	f	52	1a	5800000	SVR	0	0
60	B292	m	60	1b	4326723	PNR	1	1
61	B293	m	55	1a	1710592	PNR	1	0
62	B357	f	51	1a	6487	SVR	1	1
63	B392	m	30	1a	1389686	no treatment	0	0
64	B411	f	53	2	7079458	SVR	1	0
65	B517	m	21	1	5011872	SVR	0	0
66	B523	f	55	1a	720851	SVR	1	0
67	B526	m	40	4	173269	interrupted	2	1
68	B528	m	44	1a	n.d.	no treatment	1	1
69	B585	f	51	3a	2398833	no treatment	0	0
70	B609	m	45	1a	7585775	SVR	1	1
71	B611	f	44	3a	11988	EoTR	1	0
72	B631	m	48	3a	2818383	no treatment	0	0
73	B636	m	51	1a	1202264	EoTR	0	0
74	B657	m	41	1a	270092	no treatment	1	2
75	B658	m	52	3a	6025596	no treatment	1	0
76	B659	m	49	4	3235937	no treatment	1	0
77	B669	m	47	4	139013	no treatment	1	1
78	B675	m	49	3a	672811	no treatment	1	0
79	B679	m	42	1a	n.d.	no treatment	0	0
80	B683	m	43	1a	n.d.	no treatment	1	1
81	B696	f	46	3	n.d.	no treatment	1	0
82	B723	m	59	1a	n.d.	no treatment	2	0
83	B728	m	37	1a	455164	ongoing	n.d.	n.d.

84	B736	m	43	1 a/b	941000	no treatment	0	0
85	B754	m	61	1a	n.d.	no treatment	0	0
86	B755	m	16	3a	3030490	no treatment	1	2
87	B757	m	43	3	1445439	no treatment	1	1
88	B761	m	44	3a	1148154	EoTR	1	0
89	B768	m	51	1	5754399	no treatment	0	0
90	B769	m	59	4	26700	no treatment	1	0
91	B780	f	53	3a	n.d.	no treatment	1	1
92	B784	f	48	1a	n.d.	no treatment	1	1
93	B785	m	46	3a	1572883	no treatment	0	0
94	B786	m	47	3	474333	ongoing	2	1
95	B790	m	52	3a	2884032	no treatment	1	0
96	B798	f	45	3a	115079	no treatment	n.d.	n.d.
97	B805	m	41	1a	385577	no treatment	1	0
98	B806	f	47	1a	5815142	no treatment	1	1
99	B807	f	72	1b	530221	ongoing	2	2
100	B819	f	45	1b	2398832	no treatment	0	0
101	B822	m	59	1a	836996	no treatment	0	0
102	B823	m	49	4	3162278	no treatment	2	1
103	B825	m	45	1a	445108	ongoing	1	1
104	B827	m	15	1a	274768	no treatment	1	1
105	B834	f	47	1a	5998161	no treatment	1	0
106	B840	f	60	1b	1479108	no treatment	1	1
107	B851	m	61	1b	6309573444	ongoing	2	1
108	B855	m	44	1b	373837	n.a.	0	0
109	B856	m	24	1	64646	no treatment	2	2
110	B860	m	49	3a	806067	no treatment	1	1
111	B875	m	53	1b	2940128	no treatment	0	0
112	B876	f	61	1a	1348962	ongoing	1	0
113	B882	f	52	1a	1230268	no treatment	0	0
114	B887	m	58	1a	n.d.	n.a.	1	1
115	B888	m	49	1a	n.d.	no treatment	1	1
116	B890	m	51	1a	224979	no treatment	1	1
117	B893	m	52	3a	n.d.	n.a.	1	2
118	B903	m	19	3a	8509	no treatment	1	1
119	B916	m	36	3a	802225	no treatment	1	0
120	B918	m	56	1a	32800	eatment planne	1	0
121	B929	f	38	1a	112857	no treatment	0	0
122	B932	m	37	2	2818383	ongoing	0	0

n.d: not determined
n.a.= not available
rs12979886 (0=CC; 1=CT; 2=TT)
rs8099917 (0=TT; 1=TG; 2=GG)

SVR= Sustained virological response
EoTR= End of treatment response
PNR= primary non responder

Supplementary table 2: Gene expression levels in CHC patients
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Patient #	Biopsy coding #	*IFI27*	*RSAD2*	*ISG15*	*HTATIP2*	*IL28Rα*	*IFNλ1*	*IFNλ2*	pSVR
1	A478/B161	0.3004	0.0354	0.0751	0.0439	0.0072	n.d	n.d	n.d
2	A502b	1.0175	0.0557	0.1593	0.0443	0.0057	n.d	n.d	n.d
3	A515	1.1487	0.0434	0.2698	0.0538	0.0110	n.d	n.d	n.d
4	A518	1.7963	0.0130	0.1649	n.d	0.0068	n.d	n.d	n.d
5	A531	0.0529	0.0054	0.0217	n.d	0.0065	n.d	n.d	n.d
6	A532	1.7053	0.0381	0.1326	n.d	0.0089	n.d	n.d	n.d
7	A544b	2.5758	0.0606	0.2842	n.d	0.0138	n.d	n.d	n.d
8	A551	0.0289	0.0028	0.0122	n.d	0.0071	n.d	n.d	n.d
9	A558c	2.4453	0.1792	1.2527	0.0606	0.0286	n.d	n.d	n.d
10	A563/A938	1.0497	0.0417	0.2689	0.0404	0.0062	n.d	n.d	n.d
11	A566	2.2423	0.0138	0.1127	n.d	0.0094	n.d	n.d	n.d
12	A567	1.8987	0.0298	0.3078	0.0343	0.0110	n.d	n.d	n.d
13	A574	2.8382	0.1817	1.0570	n.d	0.0149	n.d	n.d	n.d
14	A582	0.0495	0.0022	0.0124	0.0401	0.0081	n.d	n.d	n.d
15	A590	0.1528	0.0069	0.0201	0.0462	0.0085	n.d	n.d	n.d
16	A603	1.9453	0.0163	0.2842	0.0540	0.0076	n.d	n.d	n.d
17	A622	2.4200	0.1560	1.0943	n.d	0.0124	n.d	n.d	n.d
18	A626	1.4897	0.0619	0.2300	0.0499	0.0088	n.d	n.d	n.d
19	A643	1.2058	0.0396	0.1246	0.0686	0.0093	n.d	n.d	0.232
20	A650	0.7195	0.0581	0.5783	0.0313	0.0125	n.d	n.d	n.d
21	A656	2.9690	0.1610	0.9170	n.d	0.0140	n.d	n.d	n.d
22	A658	1.8921	0.0489	0.2862	n.d	0.0103	n.d	n.d	n.d
23	A675	1.8790	0.0324	0.2544	n.d	0.0083	n.d	n.d	n.d
24	A687	2.1361	0.0813	0.5724	n.d	0.0139	n.d	n.d	n.d
25	A688	0.1550	0.0046	0.0186	0.0364	0.0120	n.d	n.d	n.d
26	A689	0.2736	0.0084	0.0830	n.d	0.0081	n.d	n.d	n.d
27	A704	0.0887	0.0116	0.0251	0.0394	0.0047	n.d	n.d	n.d
28	A707	0.1539	0.0082	0.0197	0.0373	0.0092	n.d	n.d	n.d
29	A725	2.3214	0.0542	0.5176	0.0557	0.0126	n.d	n.d	n.d
30	A764	0.0349	0.0025	0.0075	0.0271	0.0038	n.d	n.d	n.d
31	A768	0.7145	0.0167	0.0794	0.0527	0.0070	n.d	n.d	n.d
32	A770	0.9862	0.0308	0.1416	0.0367	0.0043	n.d	n.d	n.d
33	A795	1.1728	0.0492	0.1908	0.0356	0.0098	n.d	n.d	n.d
34	A809	1.4641	0.0394	0.3099	0.0689	0.0084	n.d	n.d	n.d
35	A821	1.0140	0.0295	0.1768	0.0595	0.0072	n.d	n.d	n.d
36	A847	1.9319	0.0418	0.2500	0.0398	0.0123	n.d	n.d	n.d
37	A864	1.5529	0.0513	0.3287	0.0738	0.0092	n.d	n.d	n.d
38	A865	1.0246	0.0811	0.3153	0.0293	0.0151	n.d	n.d	n.d
39	A867	0.7448	0.0258	0.1216	0.0343	0.0091	n.d	n.d	n.d
40	A897	0.9593	0.0160	0.1119	0.0262	0.0088	n.d	n.d	n.d
41	A931	0.2719	0.0167	0.2361	0.0762	0.0049	1.4E-05	1.3E-05	0.79
42	A940	0.4161	0.0081	0.0353	n.d	0.0064	n.d	n.d	n.d
43	A942	1.8150	0.0201	0.1655	0.0515	0.0063	n.d	n.d	n.d

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44	A954	1.1134	0.0504	0.4522	0.0696	0.0102	n.d	n.d	n.d
45	A960	0.1934	0.0044	0.0183	n.d	0.0068	n.d	n.d	n.d
46	A962	2.2423	0.0890	0.3415	0.0522	0.0095	n.d	n.d	n.d
47	A998	3.6808	0.0725	0.5267	0.0587	0.0155	n.d	n.d	n.d
48	B007	0.0274	0.0024	0.0202	0.0269	0.0033	n.d	n.d	0.994
49	B011	0.1632	0.0135	0.0589	0.0504	0.0046	n.d	n.d	n.d
50	B020	0.8827	0.0138	0.0767	0.0565	0.0069	n.d	n.d	n.d
51	B022	0.0313	0.0071	0.0874	0.0440	0.0058	n.d	n.d	0.804
52	B029	0.5194	0.0118	0.0396	0.0520	0.0072	n.d	n.d	n.d
53	B061	3.0844	0.1212	0.8322	n.d	0.0215	n.d	n.d	n.d
54	B065	0.1001	0.0044	0.0221	0.0434	0.0066	n.d	n.d	n.d
55	B078	1.4340	0.1166	0.3909	0.0447	0.0084	n.d	n.d	n.d
56	B146	1.2269	0.0135	0.0991	0.0370	0.0059	n.d	n.d	n.d
57	B181	2.0720	0.0577	0.1327	0.0437	0.0082	n.d	n.d	n.d
58	B261	0.0725	0.0064	0.0133	0.0365	0.0015	5.9E-06	2.7E-05	0.646
59	B271	0.1321	0.0134	0.0621	0.0386	0.0011	7.7E-06	3.4E-05	0.982
60	B292	1.2720	0.0437	0.1376	0.0447	0.0038	6.4E-06	3.6E-05	0.126
61	B293	1.5138	0.0241	0.1038	0.0715	0.0062	n.d	n.d	0.454
62	B357	1.1607	0.0288	0.1299	0.0645	0.0039	1.7E-05	2.8E-05	0.154
63	B392	0.0199	0.0624	0.0291	0.0326	0.0021	und	1.1E-05	0.554
64	B411	0.0157	0.0361	0.0184	0.0389	0.0016	und	2.6E-05	0.356
65	B517	0.0361	0.0967	0.0506	0.0337	0.0039	und	und	0.768
66	B523	0.1937	0.0582	0.0616	0.0383	0.0018	9.4E-06	3.7E-05	0.818
67	B526	1.0736	0.0721	0.1938	0.0322	0.0036	6.4E-06	6.7E-06	0.154
68	B528	1.4554	0.1033	0.2342	0.0412	0.0073	6.8E-06	und	0.062
69	B585	0.0211	0.0059	0.0467	0.0703	0.0047	n.d	n.d	0.972
70	B609	0.3615	0.1377	0.1085	0.0411	0.0038	und	und	0.448
71	B611	0.0426	0.0374	0.0250	0.0357	0.0023	8.7E-06	2.4E-05	0.356
72	B631	0.2627	0.0615	0.4167	0.0700	0.0095	n.d	n.d	0.418
73	B636	0.0272	0.0221	0.0142	0.0298	0.0016	und	und	0.928
74	B657	0.7325	0.1472	0.3053	0.0371	0.0059	3.2E-05	und	0.278
75	B658	0.0478	0.0813	0.5245	0.0569	0.0161	3.1E-05	8.1E-05	0.384
76	B659	0.8349	0.0421	0.4101	0.0470	0.0052	1.6E-05	7.5E-05	0.554
77	B669	1.2880	0.0452	0.6129	0.0399	0.0020	3.6E-06	und	0.026
78	B675	0.2533	0.0428	0.1266	0.0563	0.0024	2.6E-05	und	0.378
79	B679	0.1404	0.0136	0.0248	0.0340	0.0028	und	und	0.944
80	B683	0.4019	0.3016	0.5822	0.0791	0.0170	6E-06	und	0.222
81	B696	0.0017	0.0123	0.0122	0.0192	0.0010	4.5E-06	und	0.928
82	B723	0.4166	0.0921	0.1243	n.d	0.0035	4.5E-05	6.6E-05	n.d
83	B728	0.4166	0.0888	0.1243	0.0336	0.0020	und	1.6E-05	0.496
84	B736	0.0146	0.0166	0.0194	0.0339	0.0019	und	1.2E-05	0.928
85	B754	0.0146	0.0238	0.0236	0.0333	0.0016	und	und	0.928
86	B755	0.0185	0.0049	0.0060	0.0295	0.0020	n.d	n.d	0.99
87	B757	1.8793	0.2513	0.4832	0.0967	0.0087	n.d	n.d	0
88	B761	0.0414	0.0207	0.1320	0.0265	0.0087	n.d	n.d	0.958
89	B768	0.4424	0.0420	0.0679	0.0583	0.0010	n.d	n.d	0.81

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90	B769	1.1237	0.0983	0.2962	0.0518	0.0084	n.d	n.d	0.004
91	B780	0.2572	0.0178	0.0713	0.0612	0.0050	n.d	n.d	0.996
92	B784	0.8230	0.1505	0.7798	0.0793	0.0098	1.1E-05	6.3E-05	0.418
93	B785	0.0202	0.0020	0.0141	0.0245	0.0017	n.d	n.d	0.994
94	B786	0.4332	0.0082	0.0780	0.0487	0.0084	n.d	n.d	0.992
95	B790	0.1273	0.0155	0.0394	0.0356	0.0044	n.d	n.d	0.986
96	B798	0.9200	0.0197	0.0397	0.0652	0.0039	n.d	n.d	0.214
97	B805	0.6583	0.0559	0.6949	0.0853	0.0033	und	und	0.382
98	B806	0.2894	0.0469	0.8630	0.0582	0.0041	und	und	0.262
99	B807	0.4892	0.0722	0.5070	0.0603	0.0026	8.6E-06	und	0.382
100	B819	0.1441	0.0187	0.1171	0.0305	0.0043	1.8E-05	0.0001	0.88
101	B822	0.8169	0.0366	0.2225	0.0629	0.0047	1.4E-05	und	0.458
102	B823	2.0930	0.2519	1.8082	0.0491	0.0075	2.4E-05	5.3E-05	0.004
103	B825	0.7043	0.0423	0.3440	0.0381	0.0045	4.1E-06	1.9E-05	0.456
104	B827	2.4412	0.1030	0.7577	0.0758	0.0073	2.6E-05	und	0
105	B834	0.1887	0.0091	0.0803	0.0457	0.0033	2.7E-06	1.8E-05	0.998
106	B840	0.7500	0.0267	0.2002	0.0325	0.0033	7E-06	4.2E-05	0.934
107	B851	0.5985	0.0223	0.1965	0.0546	0.0063	5.3E-06	2.9E-05	0.692
108	B855	0.1270	0.0154	0.1007	0.0502	0.0024	2.3E-06	und	0.984
109	B856	0.6080	0.0301	0.3918	0.0395	0.0093	und	und	0.452
110	B860	0.7025	0.0738	0.2513	0.0579	0.0077	9.2E-06	und	0.404
111	B875	0.4680	0.0232	0.1853	0.0437	0.0036	n.d	n.d	0.866
112	B876	0.3665	0.0162	0.1479	0.0525	0.0035	und	0.00019	0.782
113	B882	0.2927	0.0306	0.1708	0.0443	0.0029	3.1E-06	n.d	0.442
114	B887	1.7243	0.2595	1.2951	0.0659	0.0116	3.3E-05	1.8E-05	0
115	B888	1.5134	0.0672	0.5509	0.0430	0.0031	1.4E-05	und	0.008
116	B890	1.1876	0.0153	0.1824	0.0445	0.0179	4.4E-06	und	0.534
117	B893	0.0617	0.0036	0.0366	0.0348	0.0019	und	und	0.994
118	B903	1.9445	0.0954	1.2829	0.1105	0.0091	und	und	0
119	B916	0.5208	0.0232	0.1836	0.0604	0.0069	und	und	0.846
120	B918	1.4967	0.0691	0.5314	0.0566	0.0041	und	und	0
121	B929	1.3397	0.0566	0.3609	0.0803	0.0047	und	und	0
122	B932	0.2436	0.0154	0.1857	0.0925	0.0090	und	und	0.846

Supplementary table 3: Gene expression levels in non-HCV patients

Patient #	Biopsy coding #	Gender	Age	Diagnosis	*IL28Rα*	*IFNλ1*	*IFNλ2*
123	B447	m	31	Steatohepatitis	0.002689	n.d	n.d
124	B448	m	56	Steatosis	0.001848	n.d	n.d
125	B453	m	54	ALD	0.002212	n.d	n.d
126	B612	m	46	unclear hepatopathy	0.004467	und	und
127	B614	m	51	unclear hepatopathy	0.002765	und	und
128	B621	m	54	DILI	0.001397	und	und
129	B622	m	50	ASH/ NASH	0.001796	und	und
130	B627	m	60	ALD	0.001274	und	und
131	B632	m	73	NASH	0.002541	n.d	n.d
132	B633	m	61	ASH/ NASH	0.001660	und	und
133	B641	w	73	PBC	0.002373	n.d	n.d
134	B643	m	44	NAFLD	0.001547	und	und
135	B646	m	69	ALD with cirrhosis	0.000847	und	und
136	B652	w	39	normal liver	0.001393	und	und
137	B656	m	53	sclerotic hepatitis with cirrhosis	0.003741	und	und
138	B661	m	59	NAFLD	0.002330	n.d	n.d
139	B662	w	62	DILI	0.001800	und	und
140	B663	w	62	AIH	0.002538	und	und
141	B666	m	27	NAFLD	0.001699	und	und
142	B676	w	55	NASH	0.003754	n.d	n.d
143	B680	m	44	Steatosis, undetermined	0.005239	n.d	n.d
144	B686	m	78	DILI	0.001696	n.d	n.d
145	B700	m	39	NAFLD	0.003091	und	und
146	B705	m	61	NAFLD	0.003091	und	und
147	B707	w	75	ALD	0.000455	n.d	n.d
148	B717	w	39	DILI	0.002380	n.d	n.d
149	B718	m	42	unclear hepatopathy	0.003329	n.d	n.d
150	B720	m	34	NAFLD	0.004203	und	und
151	B725	w	49	NASH	0.002766	n.d	n.d
152	B731	m	67	unclear hepatopathy	0.004464	n.d	n.d
153	B734	m	40	NASH	0.002572	n.d	n.d
154	B735	w	73	NAFLD	0.003626	und	und
155	B745	w	59	unclear hepatopathy	0.005129	n.d	n.d
156	B746	w	53	unclear hepatopathy	0.006117	n.d	n.d
157	B747	m	65	NAFLD	0.002037	n.d	n.d
158	B762	m	54	Steatosis, undetermined	0.001320	n.d	n.d
159	B764	m	48	ALD	0.000757	n.d	n.d
160	B770	w	55	DILI	0.003574	n.d	n.d
161	B771	w	76	PBC	0.004478	n.d	n.d
162	B774	w	64	DILI	0.001913	n.d	n.d
163	B777	m	27	toxic hepatitis	0.001594	n.d	n.d
164	B779	w	56	NASH	0.001803	n.d	n.d
165	B782	w	84	unclear hepatopathy	0.001976	n.d	n.d
166	B787	m	33	ALD	0.002876	n.d	n.d
167	B791	w	67	PBC	0.004456	n.d	n.d
168	B795	m	71	NASH	0.001680	und	und
169	B797	m	65	ALD with cirrhosis	0.000978	und	und
170	B810	w	75	DILI	0.001749	und	und
171	B812	m	49	unclear hepatopathy	0.001950	n.d	n.d
172	B813	w	36	unclear hepatopathy	0.003455	und	und
173	B815	m	70	ALD	0.002705	und	und
174	B818	w	68	ALD with cirrhosis	0.000226	n.d	n.d
175	B821	m	64	ALD	0.001383	n.d	n.d

und: undetectable; n.d: not determined; DILI: drug induced liver injury; NASH: nonalcoholic steatohepatitis
AIH: autoimmune hepatitis; PBC: primary biliary cirrhosis; ASH: alcoholic steatohepatitis; ALD: alcoholic liver disease

Supplementary table 4: PHHs donors characteristics
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Donor #	Gender	Age	Ethnicity	rs12979886	rs8099917	IFNα 4h	*IL28Rα*	*STAT1*
93	m	78	Caucasian	CT	TT	-	0.0023	0.0179
						+	0.0030	0.0628
117	f	38	Caucasian	CC	TT	-	0.0011	0.0088
						+	0.0017	0.0546
124	f	66	Caucasian	CT	TG	-	0.0012	0.0152
						+	0.0060	0.1343
125	f	48	Caucasian	CT	TG	-	0.0016	0.0398
						+	0.0119	0.2302
127	f	68	Caucasian	CT	TT	-	0.0005	0.0254
						+	0.0060	0.1916
130	m	72	Caucasian	CC	TT	-	0.0013	0.0160
						+	0.0046	0.0682
134	f	44	Caucasian	CC	TT	-	0.0008	0.0324
						+	0.0111	0.3072
136	f	54	Caucasian	CT	TG	-	0.0042	0.0506
						+	0.0083	0.1063
139	f	44	African	CC	TT	-	0.0008	0.0312
						+	0.0018	0.1139
146	m	73	Caucasian	CC	TT	-	0.0004	0.0181
						+	0.0018	0.1010
156	m	78	Caucasian	CT	TT	-	0.0007	0.0425
						+	0.0027	0.1667
160	f	?	Caucasian	CT	GG	-	0.0017	0.0097
						+	0.0039	0.0588
163	m	58	Caucasian	CC	TT	-	0.0004	0.0172
						+	0.0020	0.0818
165	m	72	Caucasian	CC	TT	-	0.0005	0.0346
						+	0.0016	0.1529
167	f	55	Latin-American	CC	TT	-	0.0010	0.0179
						+	0.0021	0.1167
184	m	76	Caucasian	CC	TT	-	0.0008	0.0327
						+	0.0057	0.0706
186	m	52	Caucasian	CC	TT	-	0.0009	0.0383
						+	0.0046	0.1450
188	f	48	Caucasian	CT	TG	-	0.0008	0.0208
						+	0.0058	0.0805
192	m	57	Caucasian	CT	TG	-	0.0008	0.0186
						+	0.0028	0.0841
193	m	56	Caucasian	CC	TT	-	0.0008	0.0367
						+	0.0032	0.1112
213	m	56	Caucasian	CT	TG	-	0.0019	0.0536
						+	0.0095	0.0905

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216	m	68	Caucasian	TT	GG	-	0.0015	0.0567
						+	0.0078	0.1215
217	f	47	Caucasian	CC	TT	-	0.0002	0.0217
						+	0.0024	0.0594
220	m	70	Caucasian	CC	TT	-	0.0006	0.0087
						+	0.0060	0.0640
224	f	61	Caucasian	CC	TT	-	0.0006	0.0077
						+	0.0048	0.1367
228	m	62	Caucasian	CC	TT	-	0.0005	0.0178
						+	0.0019	0.0965
229	m	74	Caucasian	CT	TG	-	0.0013	0.0163
						+	0.0144	0.1267
230	f	61	Caucasian	CC	TT	-	0.0006	0.0195
						+	0.0090	0.1054
231	m	79	Caucasian	CT	TG	-	0.0016	0.0416
						+	0.0199	0.2186
233	f	56	Caucasian	CT	GG	-	0.0027	0.0362
						+	0.0272	0.2070

5. Discussion

The response to IFNα-based therapy in patients with chronic hepatitis C infection is closely associated to the hepatic expression level of IRGs prior peg-IFNα treatment[127,130,141,142]. Indeed, we have reported that in CHC patients, the basal hepatic expression level of four classifier genes (*ISG15, RSAD2, IFI27* and *HTAITP2*) can be used to predict treatment-response with good accuracy[126]. Despite the clinical relevance of the *pre-activated* hepatic IFN system, the molecular mechanisms that modulate the gene expression in the liver of CHC patients remain unclear. In the present study two putative molecular pathways mediating this phenomenon were investigated.

The hypothesis of U-STAT1 contribution to the *pre-activated* IFN system was quite appealing. Cheon H. and Stark G.R. recently investigated the role of U-STAT1 in triggering gene expression[129]. They concluded that the exogenous over-expression of STAT1 wild type or its unphosphorylable mutant form STAT1Y01F is sufficient *per se* to induce the expression o a set of IRGs in a fibroblast cell line (BJ)[129]. However, we believe that the some experimental setting and methods selected by Cheon H. and Stark G.R. make the results of the paper questionable:

1. The authors assess STAT1 up-regulation upon IFNβ and IFNγ stimulation of BJ and hTERT-HME cell lines by Western Blotting (Fig 1 and Fig. 2 of [129]). They claim that the monoclonal antibody used (cat. n. 610185, BD Biosciences) is U-STAT1 specific. However, experimental evidences indicate that this antibody is binding STAT1 independently from its phosphorylated status. Indeed, we have repeatedly observed[85,98] that in case of short stimulation of hepatoma cells with IFNα of IFNβ, in which STAT1 is mostly in its phosphorylated form, no difference in term of intensity can be detected between the band of STAT1 of untreated and IFN-stimulated cells. If the indicated STAT1 antibody would have been specific for the unphosphorylated form of the protein, we would have observed a decrease in the intensity of the STAT1 band in the IFN-treated samples compared to the un-stimulated one.

2. The authors assessed the role of U-STAT1 on gene transcription by performing a gene expression profile on BJ and hTERT-HME cells transfected with STAT1 wild type and STAT1Y701F. However, as indicated in Fig. 4.1.2 both cell lines retain the endogenous STAT1. Cells were not serum starved during the experiments, implying that a basal level of pY701-phosphprylation could be present in the experimental setting.
3. The author performed a gene expression profile on BJ and hTERT-HME transfected with STAT1-WT and STAT1-Y701F. However, results form the two cell lines are discrepant. Indeed, the authors state that only in BJ cells and not in hTERT-HME STAT1-transfected cells an up-regulation of a set of IRGs (like IFI27, BST2, OAS1, OAS2, OAS3, IFI44L and STAT1) was detected. The author do not discuss this difference in the paper.
4. In Table 4.1.1., the authors indicate a set of genes that are up-regulated upon over-expression of STAT1-WT or mutant STAT1-Y701F in BJ cells. The values are expressed as fold change compared to non-transfected BJ cells. BJ cells over-expressing STAT1-Y701F display a much higher fold change induction of the same set of genes compared to BJ cells over-expressing STAT1-WT. This discrepancy can not be justified by the extent of STAT1 over-expression because STAT1 up-regulation is comparable between the two in systems (Fig 4.1.2). The authors do not discuss this discrepancy in the paper.

On the basis of those observations, we decide to newly assess the role of U-STAT1 in gene transcription. In the present study, a more rigorous experimental setting was used. U3A STAT1 -/- is a well established cell line used in the IFN research field since many years[166]. Several STAT1 mutants are now available and the one harboring the single amino acid substitution Y701F has been well characterized[169]. This mutant constitutes the best option to mimic U-STAT1 since the amino acid change affects the ability of Jak1 to mediate activation of STAT1[169]. We have generated multiple cell clones expressing different levels of either STAT1WT or STAT1Y701F. Thus, we were able to assess the dose-dependent effect of U-STAT1 on gene expression, avoiding clonal effects. We demonstrate that U-STAT1 is unable to maintain a high expression of IRGs in hepatoma cells, regardless its level of expression. In particular, the expression of *IFI27, BST2, OAS1,* and *IFI44L*, the most up-regulated genes upon

STAT1Y701F over-expression in Cheon & Stark paper, was not influenced by U-STAT1 expression in our system. Taken together, our results do not support the hypothesis that U-STAT1 mediates the up-regulation of the IFN system in the liver of CHC patients who do not respond to IFNα-based therapy.

Subsequently, we reasoned that the broad up-regulation of IRGs detected in the liver of non-responder CHC patients could result from the prolonged activation of the hepatic IFN system by a specific cytokine. Indeed, our group previously reported that non-responder CHC patients display an appreciable nuclear pY-STAT1 staining in most of the hepatocytes, suggesting that the IFN system is activated[126]. According to our hypothesis, one or more cytokines may have been involved in the process.

In the present study, we demonstrated that IFNα fails to long-lasting up-regulate IRGs in a hepatoma cell line, making IFNα quite unlikely to be the driving force of the *pre-activated* hepatic IFN system in non-responder CHC patients. The drop of IRGs expression level observed after 8 hours of continuous stimulation with IFNα results from the desensitization of the IFN system as reported previously *in vivo* and *in vitro*[85,90]. Subsequently, we investigated the role of IFNβ and IFNλ in mediating the up-regulation of IRGs in the liver of CHC patients. Interestingly, we have reported that IFNβ and IFNλ signaling pathway is not subjected to desensitization *in vivo* upon repeated sub-cutaneous injections[85]. However, we observed that a continuous stimulation of hepatoma cells with IFNβ fails to long-lasting up-regulate the IRGs. Interestingly, the IRGs were not maintained up-regulated, despite the prolonged STAT1 activation upon continuous stimulation with IFNβ. We confirmed in liver biopsies from CHC patients that the IRGs expression does not correlate with *IFNβ* expression level. Finally, we reported that *IFNAR1* and IRGs expression levels were not correlated in liver biopsies from CHC patients, excluding the hypothesis that the distinct activation of the hepatic IFN system results from inter-individual difference in type I IFN receptor expression.

Heretofore, the difference of STAT1 phosphorylation intensity upon continuous stimulation with IFNα and IFNβ remains unclear, considering that these two cytokines engage the same receptor complex[85,170]. The negative regulator USP18 has been regarded as the major determinant of the refractoriness of IFNα signaling pathway[90]. Experimental evidences indicates that USP18 impairs *Ifnar2*-*Jak1* binding, leading to the impairment of STAT1 activation[90]. *USP18* is up-regulated by IFNβ but,

surprisingly, does not impair the signaling pathway[85,170]. It has been reported that IFNα and IFNβ show different affinities for their binding to the receptor[171]. This phenomenon may induce a different conformational change of *Ifnar2* upon binding of the cytokines that could influence the negative activity of USP18 on the IFN signaling pathway. Furthermore, one could hypothesize that the IFNβ signaling may lead to the expression of additional negative regulators that could specifically act on the transcription level, limiting the signaling pathway despite the long-lasting activation of STAT1.

The results of our study do not support the role of type I IFNs in mediating the *pre-activation* of the hepatic IFN system in CHC patients. Instead, our findings provide evidences for a key role of the IFNλ signaling pathway in defining the basal hepatic expression level of IRGs in CHC patients.

First, we show that the expression level of *IL28Rα* defines the response rate to IFNλ, assessed by STAT1 phosphorylation, in primary human hepatocytes, in Huh7 cells over-expressing *IL28Rα,* and in human liver biopsies from CHC patients. Accordingly, we observe that the extent of IRGs up-regulation correlates to the *IL28Rα* expression in Huh7 cells that over-express *IL28Rα* and in human liver biopsies from CHC patients. Interestingly, not only the maximal expression level but also the kinetic of IRGs up-regulation depends on the *IL28Rα* expression. Recently, it has been reported that infection of Huh7 cells with HCV viral particles leads to an up-regulation of *IL28Rα,* and thus a prolonged IRGs expression upon IFNλ stimulation[172]. Although the authors show a positive correlation between IRGs and *IL28Rα,* they failed to provide direct and convincing evidence that the prolonged IRGs expression is triggered by *IL28Rα* up-regulation[172]. Moreover, we could not reproduce these data showing an up-regulation of *IL28Rα* in Huh7 cells infected with HCVcc.

Second, we show that the long lasting up-regulation of IRGs *in vitro* is due to the lack of refractoriness of the IFNλ signaling pathway. These findings support data previously published[85]. The Jak-STAT signaling pathway is not desensitized by repeated stimulation with IFNλ, despite the induction of the negative regulator USP18. Since it has been reported that USP18 exert its inhibitory activity via the binding to *Ifnar2,* we could hypnotized that USP18 does not exerts its inhibitory effect because the IFNλ receptor engages IL28Rα.

Third, we were able to detect *IFNλ1* and *IFNλ2* in liver biopsies of CHC patients. This finding strongly suggests that, in the liver of CHC patients, the Jak-STAT signaling pathway could be activated by those cytokines. This finding support also previously published results[126]. Interestingly, we assessed that the level of induction of hepatic IRGs does not correlate with the amount of *IFNλ1* and *IFNλ2*. This result points out that the hepatic up-regulation of IRGs in the liver of CHC patients depend mainly on the IFNλ receptor abundance and not on the expression level of the cytokine.

Several reports show that allelic variants at SNPs mapping close to the *IL28B* are associated to the hepatic expression level of IRGs[130,131,126]. In particular, minor alleles (less frequent in the general population) at rs12979860 and rs8099917 have been associated to high expression level of IRGs in the liver[130,131,126] of patients chronically infected with HCV. We propose here a molecular mechanism that may explain this phenomenon. Despite the weak STAT1 activation upon IFNλ treatment, we showed that the response to IFNλ stimulation is highly dynamic in PHHs. Indeed, we demonstrated that *IL28Rα* is an IFN stimulated gene and that pre-treatment of PHH with IFNα, IFNβ, or IFNλ improves the response to IFNλ, due to the up-regulation of *IL28Rα*. Type III IFNs and, to lesser extent type I IFNs, have been detected in the liver of chimpanzees acutely infected with HCV[63,64], suggesting that those cytokines could modulate *IL28Rα* expression level in hepatocytes *in vivo* . Therefore, we postulate that an initial up-regulation of *IL28Rα* mediated by type I/III IFNs production upon HCV infection would be sufficient to improve the response to IFNλ stimulation (Fig. 5.1). Interestingly, we assessed that the extent of *IL28Rα* up-regulation upon IFNα stimulation varies among PHH donors. Particularly, the *IL28Rα* expression is highly induced in donors harboring the minor allele at rs12979860 or rs8099917. These data strongly indicate that genetic elements in the *IL28B* locus could modulate *in trans* the expression of *IL28Rα* that positively correlates with the IRGs expression in liver biopsies of CHC patients. However, the precise mechanism that links allelic variants at *IL28B* locus to the IFNα-mediated *IL28Rα* expression remains to be elucidated. We excluded the involvement of micro RNAs (miRNAs, short non-coding RNA of ≈ 20 nucleotides in length that modulate gene expression post-transcriptionally[173]), since we have performed a genomic analysis on *IL28B*

locus and failed to map miRNAs in this region (data not shown). Increasing evidence have pointed out the role of long non-coding RNAs (lncRNAs) in the regulation of gene transcription[174]. This class of RNAs comprehends trancripts that are more than 200 nucleotides in length and do not encode for proteins[174]. Despite the limited number of lncRNAs characterized so far, several mechanisms have been proposed to explain lncRNAs mode of action (reviewed in[174]). Some lncRNAs have been reported to bind to regulatory proteins impairing their association to the DNA, others act as scaffolds bringing together two or more proteins in discrete complexes[174]. The role of lncRNA has been related mainly to gene silencing and repressing chromatin modifier complexes[174,175]. However, it has been recently reported that lncRNAs are associated also to active transcribed chromatin and can act as enhancers[176]. It has been reported that lncRNA expression is mediated by the binding of known transcription factors to their promoters[175,177]. For instance, lncRNA-p21 is induced by p53 and acts by repressing gene transcription[177]. Thus, the identification of lncRNA mapping in the *IL28B* locus that are induced by IFNs concomitantly with the IRGs would help to clarify the mechanism that links these allelic variants at the *IL28B* locus to the *IL28Rα* expression level.

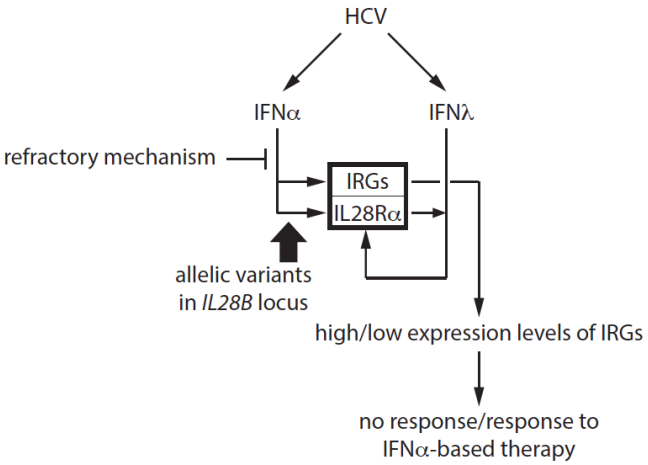

Fig. 5.1. Proposed mechanism for the pre-activated IFN system in the liver of CHC patients non-responders. *HCV infection stimulates the production of IFNα and IFNλ by dendritic cells. IFNα rapidly induces the IRGs expression including IL28Rα The strength of induction of IL28Rα upon IFNα stimulation is associated to allelic variants at SNPs mapping in the IL28B locus. Because of the refractory state of the IFNα signaling pathway, IFNα fails to maintain a long lasting up-regulation of the IRGs. However, in case the subject harbors the minor allele at SNPs mapping at IL28B locus, the increased IFNα-induced IL28Rα expression triggers a response to IFNλ stimulation that results in the maintenance of the elevated the expression of IL28Rα and IRGs.*

The IFNα-base therapy allows the clearance of HCV only in 50 to 60% of chronically infected patients[51]. The introduction of direct-acting antiviral agents (Telaprevir and Boceprevir) for the treatment of HCV genotype 1 chronic infection has improved the viral clearance in this difficult to treat HCV genotype[154,156]. However, Telaprevir and Boceprevir need to be administrated together with the IFNα-based therapy in order to avoid the appearance of viral resistant species[157,158]. Despite the use of those new drugs, a high rate of treatment failure is still registered[154,156].

Peg-IFNλ has been currently evaluated for its efficacy in HCV clearance *versus* peg-IFNα-based regimen. Recent clinical trials indicate that peg-IFNλ is at least as efficient as peg-IFNα for the treatment of chronic HCV infection and it is associated with less adverse effects[151]. A clinical trial conducted on treatment-naïve CHC patients showed a similar SVR rate for IFNλ and IFNα-treated patients [152]. Due to the promising performances of peg-IFNλ, it is quite likely that in the next future peg-IFNλ will substitute IFNα in the standard of care for CHC. Keeping this idea in mind, it would be useful to predict response to peg-IFNλ treatment in CHC patients. In the present study we propose *IL28Rα* as a suitable marker for the prediction of IFNλ based treatment-response. Indeed, we showed in liver biopsies that a minimal intrahepatic expression level of *IL28Rα* is required to achieve a significant activation of the IFN system in the liver of CHC patients. Furthermore, it would be interesting to evaluate the rate of SVR in previously non-responder CHC patients in case of re-treatment with peg-IFNλ. Indeed, the results presented in this study indicate that CHC patients non-responsive to IFNα display a higher hepatic expression of IFNλ receptor and would respond well to peg-IFNλ.

It remains unclear the reason why the *pre-activated* IFN system in CHC non-responder patients is unable to eradicate the virus. Indeed, one would expect that the strong activation of the IFN system is associated to a higher antiviral activity and therefore led to viral clearance. It has been proposed that HCV can block ISGs translation via PKR phosphorylation and inhibition of the eukaryotic translation initiation factor, eIF2α [103]. Though this mechanism, cap-dependent RNA translation is impaired, leading to a reduced production of antiviral proteins despite mRNA synthesis[103]. On the contrary, IRES-dependent RNA translation remains unaffected, allowing the synthesis of HCV proteins[103]. Furthermore, HCV interferes with viral sensing and IFN production through MAVS cleavage[41,43]. We have shown that HCV impairs the Jak-STAT pathway through an ER-stress mediated up-regulation of PP2Ac[97,98]. These findings suggest that cells infected with HCV fail to up-regulate ISGs. Thus, the high expression of IRGs may derive from non-infected hepatocytes. So far the percentage of hepatocytes that are positive for HCV infection in CHC patients is imprecise, with an average number of 40%. New techniques are required to achieve reliable HCV staining assessment *in vivo*. Moreover, it would be interesting to assess the spatial localization of HCV and IRG mRNAs in order to elucidate if the IRGs expression is limited to non-infected hepatocytes.

In conclusion, we exclude that the *pre-activated* hepatic IFN system before treatment in CHC patients is mediated by U-STAT1 or by type I IFNs. We propose instead, that the broad up-regulation of IRGs in the liver of CHC patients is mediated by IFNλ signaling pathway. In this context we assessed the prominent role of *IL28Rα* expression level in defining the response to IFNλ *in vivo*. We propose *IL28Rα* expression as a suitable marker for peg-IFNλ based therapy. Furthermore, we provide evidence that the amplitude of *IL28Rα* expression in hepatocytes is linked to genetic elements located at the IL28B locus. Our data provide the missing mechanism that explains the association of SNPs mapping at the *IL28B* and the up-regulation of the hepatic IRGs observed in CHC patients non-responders.

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Curriculum Vitae

Personal information	
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Nationality	Italian
Date of birth	28.01.1982
Work experience	
Date	Since March 2009
Position held	PhD student
Name and address of employer	Hepatology Research Group, Department of Biomedicine. Hebelstrasse 20, 4031 Basel, Switzerland.
Date	October 2006-February 2009
Position held	Fellowship
Name and address of employer	Research Unit "Polygenic Inheritance", Department of Experimental Oncology and Laboratories, Fondazione IRCCS Istituto Nazionale per lo Studio e la Cura e Tumori, via G. Venezian 1, 20133 Milan-Italy
Date	September 2005-October 2006
Position held	Master student
Name and address of employer	Research Unit "Polygenic Inheritance", Department of Experimental Oncology and Laboratories, Fondazione IRCCS Istituto Nazionale per lo studio e la Cura dei Tumori, via G. Venezian 1, 20133 Milan-Italy
Date	April 2004 - September 2004
Position held	Student
Name and address of employer	Human Genetics Unit, Department of Biotechnology and Molecular Sciences, Università degli Studi dell'Insubria, Via H. Dounant 3, 21100 Varese-Italy
Education and training	
Date	2004 – 2006
Title awarded	Master degree in Medical Molecular and Cellular Biotechnology (Score: 110/110)

Subjects covered	Thesis Title: "Hereditary susceptibility to lung cancer modulated by polyallelism at mouse Pas1 locus". Molecular biology, cancer research, life science
Name and type of organization	Università Vita-Salute San Raffaele via Olgettina 58, 20132 Milan-Italy.
Date	October 2001-September 2004
Title awarded	Bachelor in Biotechnology (Score: 110/110 with honours)
Subjects covered	Thesis Title "Expression of human RNASET2 protein lacking signal peptide for secretion: effect of cytoplasmic accumulation in human cell lines " Cellular and molecular biology, cancer research
Name and type of organization	Università degli Studi dell'Insubria Via H. Dounant 3 21100 Varese-Italy
Dates	September 1996- July 2001
Title of qualification awarded	Scientific High School degree (score: 100/100)
Name and type of organization	Liceo Scientifico Statale "Paolo Giovio", via Pasquale Paoli 28, 22100 – Como.

Personal skills and competences

Mother thongue	**Italian**
Other language(s)	**English:** independent user in listening, reading, writing and speaking **French:** basic user
Social skills and competences	I have good skills in team work and I've been working in in three different research groups. I also worked as secretary during elections in 2003, 2004 and 2005.
Organizational skills and competences	I have good ability in working in an autonomous way and in planning my work

Technical skills and competences	During the different experiences of lab work, I acquired competences in molecular biology such as: DNA and RNA purification from cultured cells and animal tissues, electrophoresis of nucleic acids on agarose gel, DNA cloning with restriction enzymes digestion in bacterial and mammalian cells, reverse-transcriptase PCR, PCR and quantitative PCR with Syber green method, analysis of Single Nucleotide Polymorphisms (SNPs) by Pyrosequencing (PSQ96MA system - Biotage AB, Uppsala, Sweden); analysis of Short Sequence Length Polymorphisms (SSLP) by PCR and polyacrylamide/agarose gel, Western blotting. I acquired competences in gene silencing using lentiviral system. I acquired basic competences in the use of HPLC and immunoflueorescence. I acquired competences in cell biology such as: maintain and culture human cancer cell lines, culture primary mouse hepatocytes, transfect cancer cells to obtain transient or stable overexpressing cell lines, set up clonogenicity tests. I acquired competences in working with mice: IP and subcute injection, organ removal, liver perfusion, isolation of primary mouse hepatocytes. In 2009 I attended and successfully completed the Introductory Course in Laboratory Animal Science (FELASA category B accredited) at the University of Zurich.
Computer skills	I have good knowledge of the Office Suite (Word Excel, Power Point); I have basic knowledge of Photoshop and ImageJ. I have good knowledge of bioinformatics programs and Databases
Artistic skills and competences	For seven years I had studied armonium and organ.
Driving licence	I obtained European driver's license B

Publications

Shnaker V. Trincucci G. Heim MH and Duong HTF
"Protein phosphatase 2A impairs IFNα-induced antiviral activity against the hepatitis C virus through the inhibition of STAT1 tyrosine phosphorylation". Journal of Virla Hepatatitis (in press).

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Mannati G., Trincucci G., Pettinicchio A., Amendola E., Scrfo' M., Dragani TA.
"Cis-acting genomic elements of the Pas1 locus control Kras mutability in lung tumors." Oncogene (2008=

Personal interests

I like reading romances and essays; I like visiting art exhibitions and going to the theatre. I prectice salsa dancing.