Interferon signalling in the liver

Implications for the natural course and therapy of hepatitis C

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Contents

1 Introduction

1.1 Hepatitis C

1.1.1 Hepatitis C virus

Hepatitis C virus (HCV) infection is a considerable health burden worldwide, affecting an estimated 170 million individuals [1]. HCV infects only humans and chimpanzees. The main target of HCV are hepatocytes, although it has been reported that the virus can also infect immune cells [2]. Phylogenetic analysis of HCV isolates enabled viral classification into six major genotypes and more than 100 subtypes. This variability, which reflects the low-fidelity rate of the viral RNA-dependant RNA polymerase, is also evidenced by the existence of highly diverse quasispecies in the individual patient [3].

HCV is a positive-strand RNA virus whose life cycle is completed in the cytoplasm of the host cell. The viral lifecycle comprises viral entry, uncoating and release of the viral genome into the cytoplasm followed by the translation and replication of the RNA, assembly into new particles and egress (Figure 1.1). The uptake of the viral particles depends on the expression of four obligatory entry factors: CD81, claudin, occludin and scavenger receptor BI [4, 5, 6, 7]. Other factors implicated in HCV attachment and entry include glycosaminoglycans such as heparan sulfate [8, 9], the lectins DC-SIGN and liver-specific L-SIGN [10], low-density lipoprotein receptor [11], epidermal growth factor receptor, ephrin receptor A2 [12] and Niemann-Pick C1-like 1 cholesterol absorption receptor [13]. Following internalization via clathrin-dependent endocytosis, the viral genome is delivered from the early endosome to the cytoplasm by a pH-dependent fusion process. Upon virus uncoating, the internal ribosome entry site (IRES)-dependent translation of HCV proteins is initiated on the template of the viral genome. The HCV genome encompasses 9.6 kb and encodes a single open reading frame. Translation of the viral ORF produces a 3000 amino acids-long polyprotein which is cleaved by host and virus proteases into 3 structural and 7 non-structural HCV proteins (reviewed in [14]). HCV non-structural proteins assemble into replication complexes on the membranes of the endoplasmic reticulum, inducing formation of specific structures known as the membranous web [15, 16]. Newly synthesized positive-strand viral RNA translocates to the surface of the lipid droplets, where the virion assembly is thought to occur [17, 18, 19, 20]. The viral particles leave the cell in a complex with lipids making use of the the very-low-density lipoprotein secretion pathway [21, 22, 23].

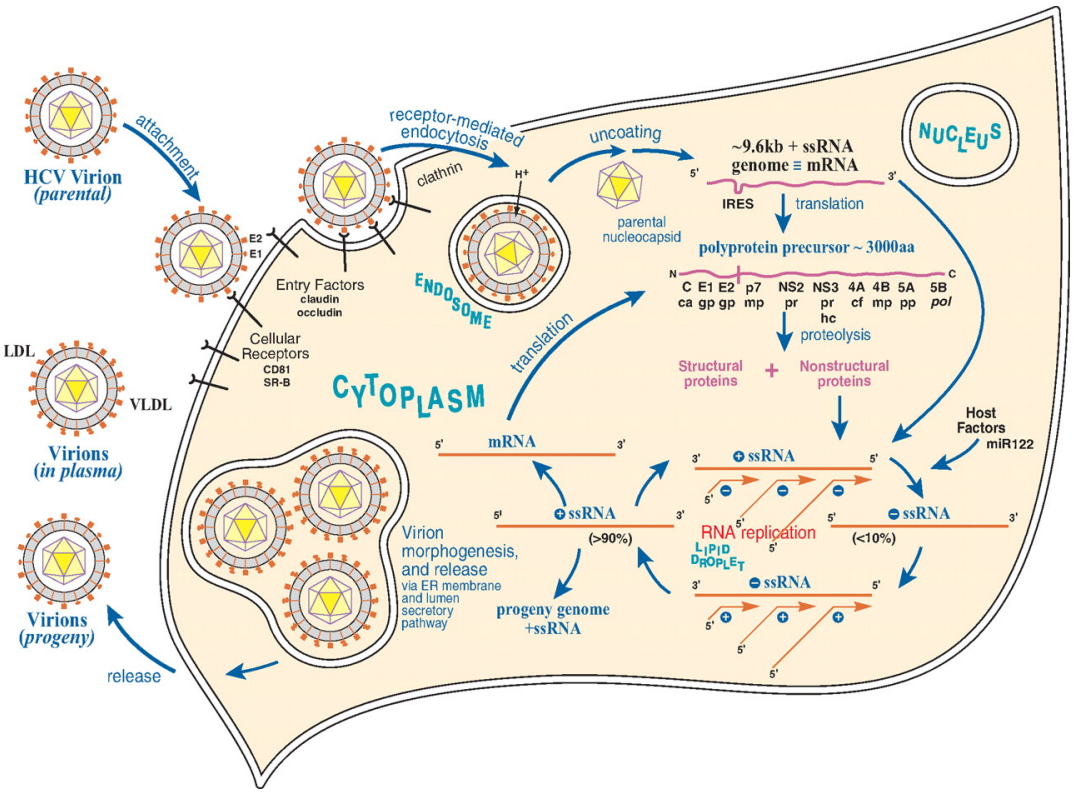

Figure 1.1: **HCV replication cycle** HCV particles in the plasma are associated with cellular lipoproteins (low density (LDL) and very low density (VLDL) lipoproteins). Following virus entry via receptor-mediated endocytosis, uncoating results in the release of the positive-sense, single-stranded RNA (ssRNA) genome. The 5' untranslated region contains an internal ribosome entry site (IRES) which drives the synthesis of a single viral polyprotein of about 3000 amino acids (aa). Subsequently, HCV polyprotein is processed by virus and host proteases to produce ten mature viral proteins. Three of these proteins have structural functions: core capsid protein (designated C ca) and envelope glycoproteins E1 (E1 gp) and E2 (E2 gp). There are seven non-structural HCV proteins: p7 membrane protein (p7 mp), NS2 protease (NS2 pr), NS3 protease and helicase (NS3 pr hc), NS4A cofactor for NS3 (4A cf), NS4B membrane protein (4B mp), NS5A phosphoprotein (5A pp), and NS5B RNA-dependent RNA polymerase (5B pol). In addition to its role as mRNA, the positive-sense RNA genome also serves as the template for RNA replication catalyzed by the viral RNA-dependent RNA polymerase (NS5B) that occurs in association with the ER membrane. Other components of the HCV replication complex include viral proteins and cellular factors, such as microRNA-122 (miR122). The complementary minus-sense RNA serves as the template for synthesis of positive-sense RNA that fulfills three functions: mRNA for translation, template for RNA replication, and progeny genome that undergoes encapsidation into new virions. Adapted from [24]

Several host cell factors are required for virus translation, replication and production. These include liver-specific microRNA-122 (miR-122) (Figure 1.1), which was shown to interact with the 5' untranslated region of the HCV genome and increase HCV abundance in replicon models [25]. miR-122 was also implicated in HCV translation, reportedly by enhancing the association of ribosomes with the viral RNA [26]. More recently, autophagy proteins such as BECN1, ATG4B, ATG5 and ATG12 have been suggested as host factors required for the initial translation of HCV RNA, enabling the subsequent step of HCV replication [27]. Another essential proviral host factor is cyclophilin A [28, 29]. Cyclophilin A was shown to interact with HCV NS5A protein and stimulate RNA binding in the domain II of NS5A [30]. Finally, lipid droplets in the vicinity of active replication structures are essential for viral assembly in cell culture [31]. Viral core and NS5A protein co-localize at the surface of the lipid droplets where they take part in the viral particle assembly process [32]. Also the subsequent step of the export of HCV particles through very-low-density lipoprotein secretion pathway necessitates further cofactors such as apolipoproteins B and E [33, 22].

1.1.2 Natural history of hepatitis C

HCV infection is transmitted by exposure to contaminated blood. HCV viremia can be first observed 1-2 weeks after transmission (Figure 1.2A) [34]. The virus replicates at high levels for several weeks before the adaptive immune response is activated. Elevations of serum levels of liver enzymes such as alanine aminotransferase (ALT) levels are usually observed 6-12 weeks after the onset of infection. ALT surge is attributed to the liver damage inflicted by effective immune response, as it coincides in most cases with a major decrease in the HCV viral load [34, 35].

Viral clearance is observed in approximately 30% of infected individuals [34, 36, 37]. In the remaining 70% of the infected population the immune system fails to clear the virus and a chronic persistent infection is established. This is associated with stabilizing of the serum viral load levels and ALT activity, indicating that a state of balance has settled between the host immune system and the virus. At this stage spontaneous clearance of infection is a rare event [38].

Chronic HCV infection, when untreated, persists and in the course of time the chronic inflammatory state of the liver may lead to pathological states including fibrosis, cirrhosis, hepatic decompensation and hepatocellular carcinoma (Figure 1.2A) [39, 40]. Although the rate of new HCV infections is decreasing in recent years, the number of infected people with complications of the advanced disease is

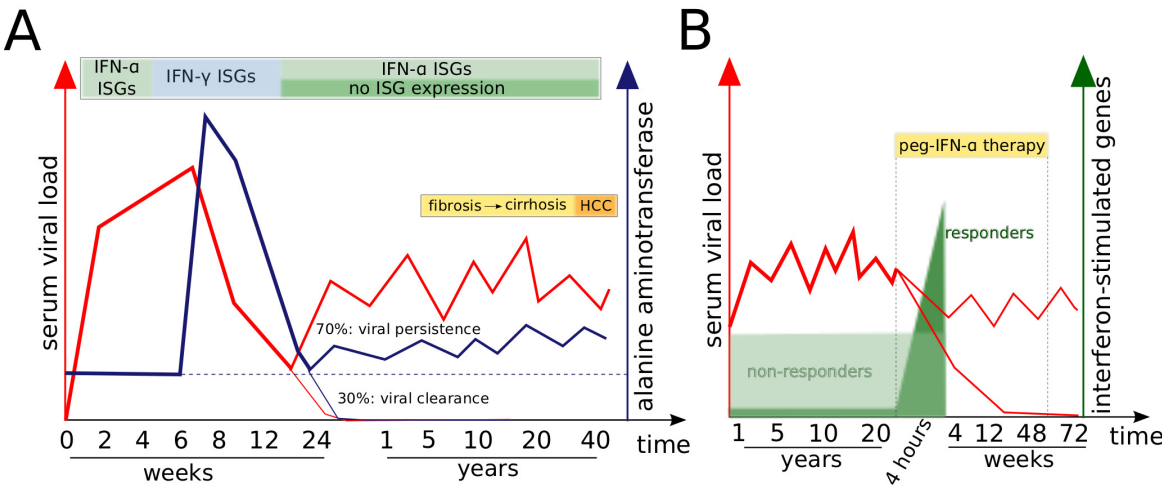

Figure 1.2: **(A) Natural course of hepatitis C virus infection.** Changes in serum HCV load, alanine aminotransferase levels (ALT), IFN-stimulated gene expression in the liver (ISGs) as well as the development of liver disease are shown as a function of time from transmission. The dashed line shows the upper limit of normal alanine aminotransferase levels. **(B) Treatment of chronic hepatitis C with peg-IFN-α and ribavirin.** Changes in serum HCV load and IFN-stimulated gene expression in the liver are shown as a function of time from transmission or initiation of peg-IFN-α and ribavirin therapy.

still on the rise (reviewed in [41]). Progressive hepatic fibrosis leading to cirrhosis is the major complication of chronic HCV infection. It is estimated that over the course of 20-40 years 20-30% of the patients with chronic hepatitis C progress to liver cirrhosis [42]. Patients with HCV-associated cirrhosis are at high risk of developing hepatic decompensation, manifesting as hepatic synthetic dysfunction or complications of portal hypertension. Among patients with cirrhosis and active hepatitis C 2-5% a year develop hepatocellular carcinoma. End-stage liver cirrhosis associated to chronic HCV infection is a leading cause of liver transplantation in developed countries.

1.1.3 Therapy of hepatitis C virus infection

The current standard of care for chronic hepatitis C consists of weekly injections of pegylated interferon (peg-IFN)-α2 combined with orally administered unspecific antiviral drug, ribavirin. Among patients with chronic hepatitis C response rates to the peg-IFN-α 2 combined with ribavirin vary from about 50% for HCV genotypes 1 and 4 to approximately 80% for viral genotypes 2 and 3 [43, 44]. For HCV genotype

1 the peg-IFN-α/ribavirin regimen is since recently complemented by HCV-specific antivirals (boceprevir or telaprevir [45, 46]) which act by inhibiting the viral NS3-4A protease, an enzyme essential for the HCV replication cycle [47](Figure 1.1). Introduction of these direct-acting antiviral drugs has been shown to raise the cure rates for the difficult-to-treat HCV genotype 1 to about 65% [45, 46].

A significant number of new anti-HCV drugs are currently in clinical development and even more in pre-clinical evaluation. Most of these compounds have been designed to specifically target HCV genotype 1 and show low efficacy against other viral genotypes. Moreover, high replicative potential of HCV combined with the lack of proofreading activity of its RNA polymerase often result in emergence of resistant HCV variants. This is especially frequent in patients with peg-IFN-α non-response in the setting of triple therapies employing peg-IFN-α, ribavirin and a direct-acting antiviral drug (reviewed in [48]). In addition to the problems related to non-response due to the refractoriness of endogenous IFN system, IFN-α-based therapies provoke systemic side effects owing to the ubiquitous expression of type I IFN receptor. The adverse effects of peg-IFN-α treatment include influenza-like symptoms, hematologic abnormalities and neuropsychiatric disorders [49]. Triple combination therapies add to this spectrum additional side effects of protease inhibitors with most common being rash, pruritus, dysgeusia, diarrhea and thrombocytopenia [45, 46].

The future direction is towards development of IFN-α-free, preferably all-oral combination therapies. Such therapies should combine marked antiviral efficacy with high barrier to viral resistance. This can be achieved by targeting elements of host cell indispensable to the HCV life cycle. Alisporivir is an example of such approach - it targets human cyclophilin A which is a cofactor essential for HCV replication. Resistance development to alisporivir in HCV cell culture system required on average 20 weeks, while upon treatment with polymerase or protease inhibitors resistant variants emerged in less than 2 weeks [50]. Moreover, peg-IFN-λ could replace peg-IFN-α in the future anti-HCV therapies. Treatment with peg-IFN-λ resulted in superior viral response in HCV genotypes 1 to 4 compared to peg-IFN-α [51]. Additionally, thanks to the restricted expression of IL28Rα chain of the IFN-λ receptor, the therapy was safer and better tolerated.

1.2 Interferon signal transduction pathway

1.2.1 Interferons and their receptors

IFNs are immune response mediators that constitute the first line of defence against viral infections. They are classified as type I, II or III IFNs based on their use of specific receptors (Figure 1.3) (reviewed in [52]). Human type I IFNs include 12 highly similar members of IFN-α family, a single IFN-β as well as IFNs-ϵ, -κ and -ω. The members of type I IFN family bind to a common, ubiquitously expressed IFN-α/IFN-β receptor (IFNAR) which consists of two major subunits, IFNAR1 and IFNAR2 (reviewed in [52]). Type I IFNs are produced primarily in response to the viral infection [53, 54]. The only type II IFN, IFN-γ, is produced mainly by NK and T cells in response to stimulation with antigens or mitogens [55, 56, 57]. IFN-γ binds to heterodimeric IFN-γ receptor (IFNGR), which, similarly to IFNAR, is expressed in a ubiquitous manner [58].

The recently discovered type III IFNs include IFN-λ1, -λ2 and -λ3 (also referred to as IL29, IL28A and IL28B, respectively)[59]. The receptor for the IFN-λ family consists of the IL10R2 chain, which is shared with the interleukin 10 receptor, and a unique IFN-λ chain, IL28Rα [59]. Contrary to IL10R2, IL28Rα is expressed in a tissue-specific manner, restricting the activity of IFN-λs to the cells of epithelial origin [60]. IFN-λs are produced in response to viral infection in both immune and nonimmune cells. It has also been postulated that IFN-λs might be induced by stimulation of cells with type I or III IFN, suggesting that this class of cytokines belongs at the same time to IFN-stimulated genes [61].

All IFNs signal through the Janus kinase-signal transducer and activator of transcription (Jak-STAT) pathway to regulate the expression of their target genes in the nucleus (Figure 1.3, 1.4). IFN receptor subunits are constitutively associated with tyrosine kinases from the Jak/Tyk family (Figure 1.3) [62]. The first step in the IFN signal transduction pathway is ligand-dependent rearrangement and dimerization of receptor subunits, which leads to autophosphorylation of the receptor and activation of the associated Jaks. Receptor phosphotyrosines act as docking sites for the STATs, which undergo tyrosine phosphorylation mediated by the activated Jaks. IFN-γ-induced signalling involves mainly phosphorylation of STAT1, which assembles into homodimeric complexes and translocates to the nucleus where it binds to promoter regions containing a specific gamma-activated sequence (GAS) to activate the transcription of downstream genes [63, 64]. Stimulation of cells with type I and III IFNs leads to STAT1 and STAT2 phosphorylation and assembly of

two types of transcriptional activators: homodimeric phospho-STAT1 complex and heterotrimeric complex composed of phospho-STAT1, phospho-STAT2 and IRF9 [65, 66]. This heterotrimeric complex drives the expression of genes whose promoters contain specific interferon-stimulated response elements (ISREs) [67]. The sets of genes induced by type I and III IFNs are almost identical, and partially overlap with the distinct set of IFN-γ-stimulated genes [68, 69].

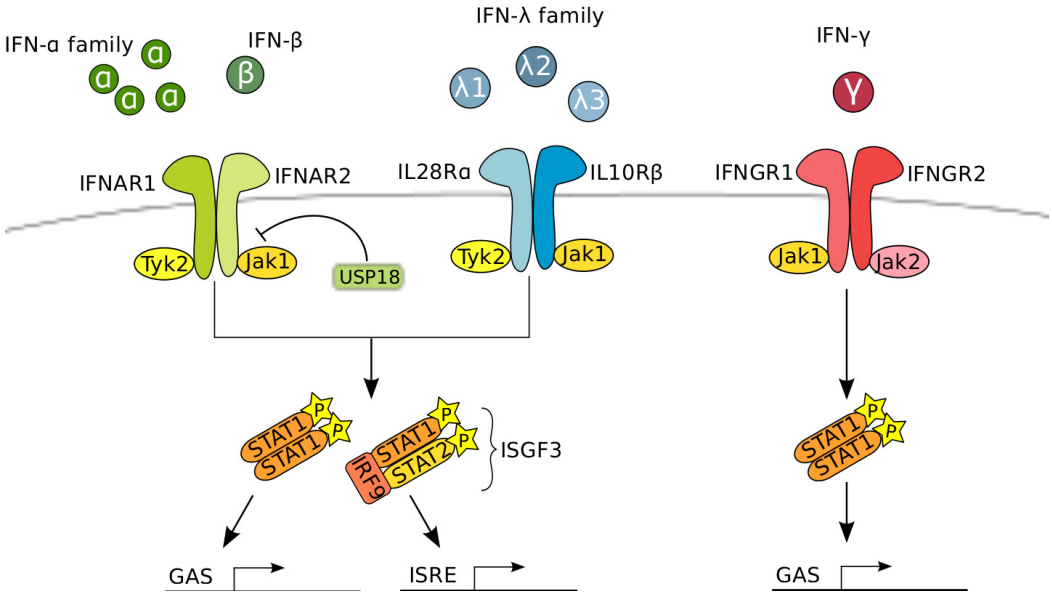

Figure 1.3: **Type I, II and III interferon signalling through the Jak-STAT pathway.** Type I and III IFNs bind to distinct receptors, but activate the same downstream signalling events, inducing almost identical sets of genes through the activation of ISGF3 and STAT1 homodimers. IFN-γ treatment leads to activation of STAT1 homodimers, but not ISGF3, inducing a distinct gene signature which partly overlaps with type I and III IFN target genes.

Apart from the antiviral functions of IFNs, signalling through the Jak-STAT pathway also produces growth-inhibitory and proapoptotic effects in a cell type-specific manner (reviewed in [70]). Among the intracellular Jak-STAT pathway factors, IRF9 appears to be the key component required for eliciting the antiproliferative activity of IFN-α, and it is possible that pro-apoptotic factor TRAIL is one of the important mediators [71]. The antiproliferative effects of IFNs are the rational basis for their use in the treatment of different malignancies (Figure 1.5).

The Jak-STAT pathway is the principal and the best studied signal transduction pathway involved in IFN signalling, but it is not the only one. IFNs can also medi-

ate biological effects by activation of the mitogen-activated protein kinase pathway (particularly of the p38 kinase family), the phosphatidylinositol 3-kinase pathway and the mammalian target of rapamycin pathway. These non-canonical IFN signal transduction pathways play a role in the efficient induction of the antiviral state, regulate IFN-induced mRNA translation and contribute to the growth-inhibitory effects of IFNs (reviewed in [62]).

1.2.2 Antiviral properties of the interferon-stimulated genes

Genes induced by IFN stimulation contribute to the establishment of the so-called antiviral state. IFN stimulation typically leads to up- and downregulation of several hundred genes, many of which are regulated in a cell-type specific manner. Only a small number of the IFN-induced antiviral effectors have been studied in detail and their mode of action in inhibiting the viral infections is known. To date, four main effector pathways of the IFN-mediated antiviral response have been described: the Mx GTPase pathway, the ISG15 ubiquitin-like pathway, the OAS-RNaseL pathway and the protein kinase R pathway (reviewed in [72]).

Mx GTPases are guanine-hydrolyzing proteins located in the smooth endoplasmic reticulum, where they regulate exocytosis and vesicle trafficking to trap essential viral components [73]. Additionally, Mx proteins are able to specifically interfere with influenza virus life cycle by binding and inhibiting the viral polymerase and therefore blocking transcription of viral mRNAs [74].

ISG15 is a ubiquitin-like small protein which can be conjugated to target proteins through a three-step cascade involving E1 activating enzymes, E2 conjugating enzymes and E3 ligases. The ISGylation (attachment of an ISG15 tag) is reversible and deconjugation is catalyzed by proteins from USP family such as USP18. ISGylation was shown to prolong target protein half-life [75], or modulate their function, such as increasing or decreasing the substrate affinity of enzymes [76, 77]. Additionally, free (unconjugated) ISG15 is known to be secreted from the cells and was proposed to act as a cytokine to regulate immune responses [78]. However, the mechanism of action of extracellular ISG15 is not well described to date.

Contrary to Mx and ISG15, which are virtually absent from unstimulated cells, the OAS and PKR proteins are constitutively expressed at low levels which enables them to function as intracellular pathogen recognition receptors. 2'-5' oligoadenylate synthetases (OAS) are a group of enzymes which catalyze the synthesis of 2'-5'-oligoadenylates from ATP in response to viral double-stranded RNA (reviewed in [79]). These adenosine polymers specifically activate the latent form of RNAse L

enzyme, leading to RNA degradation. Fragmented RNA can subsequently activate cytosolic receptors such as Mda5 and RIG-I, leading to production of IFNs [80].

PKR belongs to a family of protein kinases that regulate protein synthesis in response to environmental stress. Similar to OAS proteins it becomes activated upon double-stranded RNA binding [81]. Activated PKR phosphorylates the translation initiation factor eIF2α which results in general blockade of translation (reviewed in [72]).

Recently, a high-throughput study of IFN-inducible antiviral effectors reported a number of ISGs capable of potently inhibiting HCV replication in cell culture upon overexpression [82]. The most prominent HCV inhibitors included pattern-recognition receptors such as RIG-I and MDA5, signalling molecules such as MAP3K14 and transcription factors IRF1, IRF2 and IRF7. It is likely that overexpression of each of these genes led to induction of a number of other IFN-stimulated genes, demonstrating that the antiviral action of IFN is mediated by a joint action of many effector molecules. In line with this hypothesis, the strongest inhibition of HCV replication was achieved by overexpression of combination of different ISGs.

1.2.3 Negative regulation of the interferon signal transduction pathway

Administration of IFN-α results in the activation of the Jak-STAT pathway and induction of IFN-stimulated genes. Prolonged and intense IFN response can be detrimental and the Jak-STAT pathway is tightly controlled by several IFN-inducible negative feedback mechanisms in order to protect the organism from deleterious consequences of exaggerated immune activation. Jak-STAT pathway activation and gene induction were shown to strongly decrease after several hours of continuous IFN-α treatment in cultured cells as well as *in vivo* in mouse or chimpanzee liver [83, 84, 85, 86]. This downregulation of the IFN-α signalling is a result of the action of IFN-induced Jak-STAT pathway inhibition. Several IFN-inducible mechanisms exist to curtail the activation resulting from IFN stimulation, including *de novo* production of signalling inhibitors like SOCS1, SOCS3 or USP18 and activation of receptor-associated phosphatases such as SHP2 [87, 88, 89, 90, 91].

SHP2 is a ubiquitously expressed SH2 domain-containing protein tyrosine phosphatase (Figure 1.4) (reviewed in [92]). SHP2 is not an IFN-inducible gene and it was shown to constitutively associate with IFNAR2 [91]. However, IFN stimulation leads to an increase in SHP2 enzymatic activity, creating a negative feedback loop [93]. In SHP2-deficient mouse fibroblasts, IFN-γ and IFN-α treatment resulted in elevated tyrosine phosphorylation levels of Jak1 (but not Jak2), STAT1 and STAT2,

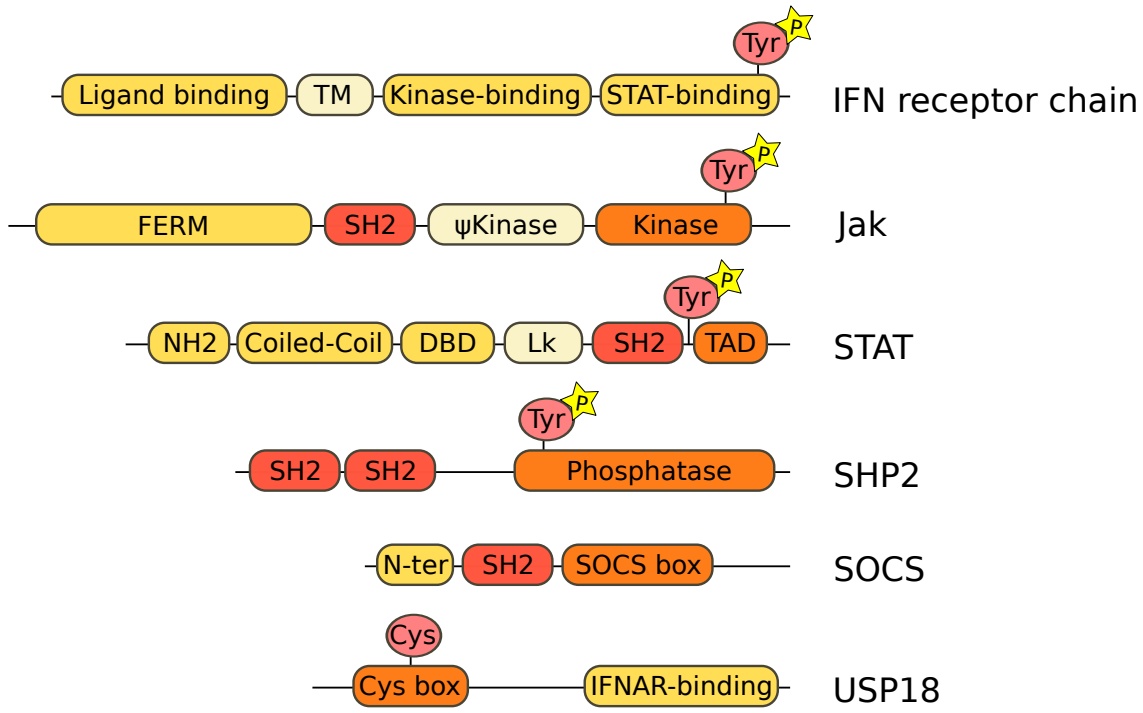

Figure 1.4: **Domain organization of Jak-STAT pathway components and signalling inhibitors** Jak-STAT pathway signalling components share general domain organization. **Receptor chains** for type I, II and III IFNs consist of the extracellular ligand recognition domain, the short transmembrane (TM) domain and intracellular regions responsible for interactions with Jak kinases and STATs. **Jak kinases** comprise the FERM domain responsible for the association with receptors, SH2-related domain of unknown function as well as pseudokinase and kinase domains in the carboxy-terminus. **STAT proteins** are composed of the amino-terminal (NH2) domain important for homodimerization of inactive STATs, the coiled-coil domain which can bind signalling regulators, the DNA-binding domain (DBD), conserved linker sequence (Lk), the SH2 domain which directs receptor binding and dimerization and the tyrosine activation domain (TAD). **SHP2 phosphatase** includes two SH2 domains which direct binding to tyrosine-phosphorylated substrates and a C-terminal catalytic domain. **SOCS family proteins** consist of the N-terminal domain (N-ter) which in case of SOCS1 and SOCS3 includes a kinase-inhibitory region, the central SH2 domain which binds phosphorylated tyrosine residues and the C-terminalSOCS box responsible for interaction with elongins B and C. **USP18 protein** is composed of the N-terminal peptidase domain with conserved cysteine-box containing the Cys61 active site and the C-terminal domain responsible for binding to the IFNAR2 chain of type I IFN receptor.

and augmented suppression of cell viability [94]. It is not clear whether SHP-2 directly acts on and inactivates the Jak1 kinase, although physical interaction between SHP-2 and Jak1 and 2 kinases has been observed [95]. Another mechanism by which SHP2 was proposed to exert its inhibitory action on Jak-STAT cascade is by removing the phosphate group from phospho-STAT1 in the nucleus. Direct interaction of SHP2 with phosphorylated STAT1 in the cell nucleus has been reported, leading to dephosphorylation of STAT1 on both tyrosine and serine residues [94].

SOCS1 and SOCS3 belong to a family of cytokine-inducible inhibitors of signalling. The SOCS proteins have a central SH2 domain that allows them to bind to phosphotyrosine residues in cytokine receptors or receptor-associated kinases [96, 97] and a C-terminal SOCS box domain that was reported to interact with elongins B and C and direct the SOCS-bound proteins for proteasomal degradation (Figure 1.4) [98, 99, 100]. SOCS1 and SOCS3 also contain a kinase inhibitory region that is dispensable for the target binding but necessary to inhibit the signalling [99, 96]. The expression of SOCS1 and SOCS3 has been shown to mediate potent inhibitory effects on type I and II IFN signal transduction and gene regulation in several experimental systems [87, 90]. SOCS1 has been found to co-immunoprecipitate with IFNAR1 and IFNGR1 as well as phosphorylated Jak2 and Tyk2 kinases [101, 102, 103, 104]. Moreover, SOCS1 interaction with the activated receptor-associated kinases was reported to regulate their ubiquitin-mediated degradation [102, 104]. SOCS3 was shown to associate with IFNGR1 at the phosphotyrosine residue [105]. SOCS3 was also shown to inhibit type I IFN-dependent signals, but the mode of inhibition has not been demonstrated so far. Contrary to SOCS1, SOCS3 was not able to inhibit Jak kinase activity in vitro, suggesting that SOCS3-receptor interactions are indispensable for the signalling inhibition [96].

USP18, also known as UBP43, was initially identified as an enzyme that catalyzes the removal of ISG15 conjugates from proteins. Genetic ablation of USP18 in mice leads to type I, but not type II IFN hypersensitivity and hyperactivation of ISGs in response to type I IFNs [88]. Recent work has demonstrated that the role of USP18 in the regulation of the signalling through the Jak-STAT pathway was independent of its ISG-deconjugating activity [89]. It was revealed that USP18 reduces signalling through the Jak-STAT pathway by specific binding of its C-terminal domain to the IFNAR2 subunit of the type I IFN receptor, while it is unable to bind to IFNAR1 or IFNGR1 (Figure 1.4) [89]. By specific binding to IFNAR2 USP18 was able to inhibit the Jak-STAT pathway by restricting the access of the Jak1 kinase to its docking site at the receptor (Figure 1.3) [89].

SOCS1 and SOCS3 are induced rapidly upon IFN treatment, remain detectable for a short time and contribute to reducing the strength and duration of the ongoing activation of the Jak-STAT pathway [87, 90]. On the other hand, USP18 upregulation mediates a long-lasting refractory state even after the initial IFN stimulus is not present anymore [84]. The cells which are refractory fail to respond to IFN-α stimulation with activation of the Jak-STAT pathway. The phenomenon of IFN-induced refractoriness has potential implications in the clinical practice, since IFNs-α, -β and -γ are currently used as treatment agents, and a pegylated form of IFN-λ is under evaluation in clinical trials (Figure 1.5).

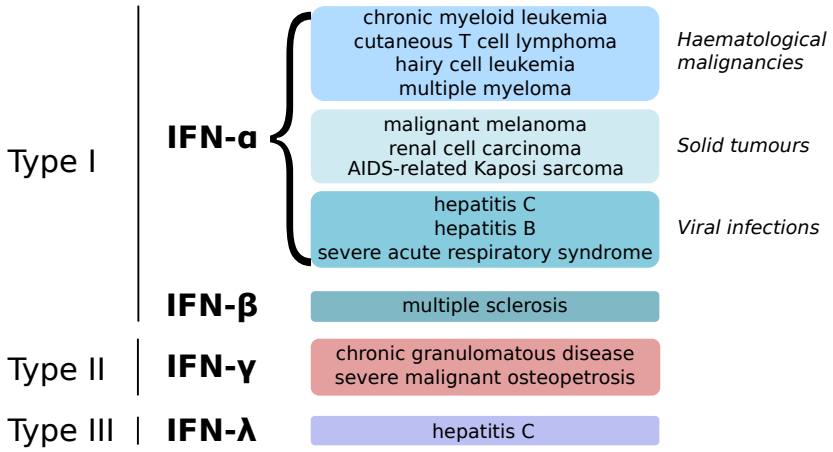

Figure 1.5: **Clinical applications of type I, II and III interferons.** Type I and II interferons are used in various clinical settings. Type III IFN - pegylated IFN-λ - is currently undergoing clinical trials for the treatment of chronic hepatitis C.

IFN signalling through the Jak-STAT pathway is also controlled through non-inducible mechanisms, such as constitutive expression of PIAS1 or 3 (protein inhibitors of activated STAT) in some cells types. Proteins from the PIAS family are SUMO E3 ligases (reviewed in [106]) and most of their biological functions are related to SUMOylation. However, PIAS1 and 3 proteins additionally exert repressive action on the Jak-STAT pathway by blocking the DNA binding of the dimers of phosphorylated STATs 1 and 3, respectively, without influencing their SUMOylation [107, 108].

1.3 Interferon signalling in chronic hepatitis C and treatment outcome

1.3.1 Preactivation of endogenous IFN system in chronic hepatitis C

Recent progress in research on IFN signalling in the liver has improved our understanding of the molecular mechanisms behind the non-response to IFN-based therapies observed in a significant proportion of chronic hepatitis C patients. Therapy of chronic hepatitis C with peg-IFN-α and ribavirin achieves viral clearance in approximately half of the patients [43, 45]. Non-response to therapeutically administered IFN-α is associated with constitutive upregulation of endogenous IFN system in the liver (Figure 1.2B) [109, 110, 111]. Patients with preactivated IFN system fail to respond to peg-IFN-α injection with further stimulation of STAT1 phosphorylation, nuclear translocation and induction of target genes in the liver [110], demonstrating an apparent refractory state.

Similarly to what is observed in mouse liver after repeated stimulation with IFN, the lack of sensitivity to IFN-α in chronic hepatitis C patients correlates with elevated levels of USP18 [84, 110]. This suggests that some patients react to HCV infection with production of endogenous IFNs which leads to upregulation of the IFN signalling inhibitors such as USP18 and therefore compromises the action of therapeutically administered peg-IFNα.

It is unclear why the activated endogenous IFN system of the peg-IFN-α non-responders is unable to inhibit viral replication and clear the infection. It has been proposed that HCV can block the effector function of IFN-stimulated genes [112]. According to this model, HCV infection induces phosphorylation of PKR and eIF2-α, leading to the global downregulation of cellular mRNA translation. As a result, the antiviral action of IFN-α is hampered, while at the same time the IRES-dependent translation of HCV RNA remains unaffected.

Another hypothesis which could explain why the preactivated state does not lead to viral clearance relies on spatial resolution of the cells with high ISG levels and the HCV-infected cells. It remains unclear what proportion of the hepatocytes contain HCV during the infection. Different studies report the percentage of HCV-infected hepatocytes in the human liver to vary from 4 to 25%[113], 7 to 20% [114] or 0 to 100% with an average of 40% [115]. It has been shown that HCV can inhibit IFN-α signalling through the Jak-STAT pathway by inducing upregulation of protein phosphatase PP2A [116, 117, 118]. With the majority of hepatocytes remaining free of the virus, it is possible that the strong ISG expression observed in

the preactivated livers is contributed by endogenous IFN stimulation of uninfected cells, whereas high levels viral replication preclude the Jak-STAT pathway activation and target gene expression in cells harbouring HCV.

1.3.2 Possible sources of preactivation

The cellular source and type of IFN which drives the preactivated state in the liver of non-responder patients remain a matter of speculation. It has been reported that HCV-infected cells induce IFN-α production in plasmacytoid dendritic cells (pDCs) through toll-like receptor 7 signalling in a manner that necessitates direct contact of pDCs with the virus-replicating cells [119]. It is however unclear if this mechanism is also active *in vivo* in the setting of human infection. The activation of the Jak-STAT pathway in liver biopsies of non-responder patients despite high levels of USP18 protein expression suggests that the cytokine which triggers the preactivated state is not sensitive to the USP18-mediated inhibition [110]. This would exclude IFN-α as the driver of pre-treatment IFN-stimulated gene induction in the liver of IFN non-responders.

3' untranslated region of HCV RNA contains a conserved poly-uridine motif. When full-length HCV RNA or the polyuridine motif alone are transfected into cells, they are recognized by RIG-I pattern recognition receptor and induce transcription of IFN-β gene [120]. In order to activate IFN-β production, RIG-I is required to interact with MAVS adaptor protein (also known as Cardif, IPS-1 or VISA) [121, 122, 123, 124]. HCV NS3-4A protease has been shown to cleave and inactivate MAVS, thereby preventing the induction of IFN-β in the infected cells [121]. MAVS cleavage has been documented in liver biopsies from patients chronically infected with HCV, and the patients with cleaved MAVS less frequently showed preactivation of endogenous IFN system, which correlated with improved response rates [125]. These data suggest that IFN induction through RIG-I-MAVS-dependent pathway could contribute to the preactivation in the liver of later peg-IFN-α non-responders.

Another cellular pattern recognition receptor which senses viral infection by recognizing double-stranded RNA is toll-like receptor 3 (TLR3) [126]. TLR3 signals through TRIF to activate IRF3, which drives IFN-β production [127]. HCV NS3-4A protease has been reported to be capable of cleaving TRIF, which could circumvent the induction of endogenous IFN-β through TLR3-mediated signalling [128]. TRIF cleavage has so far not been documented in liver tissue from hepatitis C patients. Moreover, despite the evidence of viral interference with the induction of IFN-β, RIG-I-dependent production of this cytokine was observed in primary human hep-

atocytes infected with cell culture-derived HCV [129].

Recent progress in high-throughput approaches made possible the discovery of single nucleotide polymorphisms (SNPs) which correlate with treatment outcome of chronic hepatitis C patients [130, 131, 132, 133]. Patients homozygous for major alleles of SNPs in proximity of IL28B (IFN-λ3) locus were about 2-fold more likely to respond to the standard therapy than carriers of the minor alleles [130, 131, 132, 133]. These findings have attracted attention to the role of IFN-λs in the chronic HCV infection and the possible contribution to the pre-treatment IFN-stimulated gene expression. Recent work in primary human fetal liver cell cultures infected with cell culture-derived HCV documented induction of IFN-λ upon HCV infection, accompanied by upregulation of IFN target genes [134].

Neither IFN-β, IFN-λ, nor any of the members of IFN-α family have been found upregulated in the liver of humans or chimpanzees chronically infected with HCV [135, 136]. Despite considerable research efforts, it is still unclear which IFN subtype is responsible for the Jak-STAT pathway activation in the chronic hepatitis C.

1.4 Interferon signalling in acute hepatitis C

Our understanding of the early hepatic events in the HCV infection derives from serial liver biopsy studies in experimentally infected chimpanzees [137, 138, 139, 140, 141]. In the first two weeks of infection HCV titers increase rapidly, followed by slowing down of the viral replication which occurs in concert with elevated mRNA levels of type I IFN target genes in the liver. The activation of the endogenous type I IFN system, believed to impede the viral replication at this stage, is observed in all animals irrespective of the outcome of the disease. It is not clear to date which subtype of IFN drives the hepatic activation at this early step of HCV infection.

Effective control of the acute infection in chimpanzees is observed 8-12 weeks post-inoculation and is attributed to IFN-γ induction and upregulation of IFN-γ-stimulated genes in the liver. Increases in the hepatic IFN-γ are in most cases followed by reductions in HCV viremia and ALT elevation which could result from killing of infected hepatocytes. Progression to chronicity was shown to be associated with reduced hepatic CD3e and CCL3, whereas spontaneous clearance was related to the induction of CD8+ T cell markers [139, 140]. Major decreases in viral titers in experimentally infected animals were accompanied by an early, multispecific, IFN-γ-producing intrahepatic CD4+and CD8+ response [142].

Acute infection with HCV in humans is mostly asymptomatic. As a result, few

patients are diagnosed during the first months after transmission. So far, the majority of studies of acute HCV infections in the human subjects focused on analyzing the circulating immune cells. In agreement with the observations from the chimpanzee models it has been shown that spontaneous clearance of infection is associated with vigorous, strong and multispecific T cell responses [34, 142, 143, 144]. Although CD4+ and CD8+ T cell responses are generated in the majority of acutely infected patients irrespective of outcome, the distinguishing feature of spontaneous recovery apperas to be the ability to maintain such responses over time [143, 145].

1.5 Toll-like receptor 9 agonists as therapeutic agents

1.5.1 Toll-like receptor 9 signalling in innate immunity

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs). There has been 10 functional TLRs identified in humans and 12 in mice, with TLRs 1-9 conserved in both species. TLRs comprise an extracellular leucine-rich repeat and a cytoplasmic TIR (Toll/interleukin-1 receptor) domain, connected through a transmembrane domain (reviewed in [146]). TLRs are sensors of microbial infection which recognize lipids, proteins and nucleic acids of bacteria, viruses, protozoan parasites and fungi (reviewed in [147]). Sensing these patterns by innate immune cells activates and directs the immune system response against pathogens.

TLR9 specifically recognizes unmethylated 2-deoxyribo - [cytidine-phosphate-guanosine] (CpG) DNA motifs that are frequently present in genomes of bacteria and viruses but rare in mammalian cells [148]. TLR9 is highly expressed on endosomal membranes of the cells of the immune compartment. Human TLR9 is expressed in memory B cells [149, 150] and pDCs [151, 152, 153]. Expression of TLR9 on human monocyte-derived DCs and monocytes has been reported, but is still a matter of debate [154, 155]. On the other hand, mouse TLR9 expression is not limited to B cells and pDCs, but is also detected in monocytes, macrophages and DCs [148, 156].

In non-activated immune cells TLR9 is expressed in the endoplasmic reticulum. Upon cellular activation, TLR9 traffics to endosomal and lysosomal compartments where the receptor can interact with endocytosed ligand at acidic pH, a condition that is probably necessary for DNA recognition [157, 158, 159]. The molecular basis of the TLR9 retention in the endoplastic reticulum membranes in unstimulated cells and the subsequent trafficking to the endosome upon cellular stimulation is unclear.

After the engagement of TLR9 by CpG DNA, the receptor recruits a TIR-domain containing adapter MyD88 (Figure 1.6, left). MyD88 forms a complex with members of IRAK (IL-1-receptor-associated kinase) family and TRAF6, which activates TAK1 kinase. TAK1 subsequently activates the IKK complex (IKKα, IKKβ, and IKKγ) to catalyze phosphorylation of IκB proteins. Phosphorylated IκB proteins are targeted for proteasomal degradation, allowing NFκB to translocate to the nucleus. At the same time, TAK1 activates the MAPK pathway by inducing the phosphorylation of MAPK kinases, which then activate transcription factors such as AP-1. NFκB and MAPK pathways control inflammatory responses by inducing production of cytokines and chemokines (reviewed in [160]).

In the plasmacytoid dendritic cells, TLR9 signalling through a Myd88-dependent

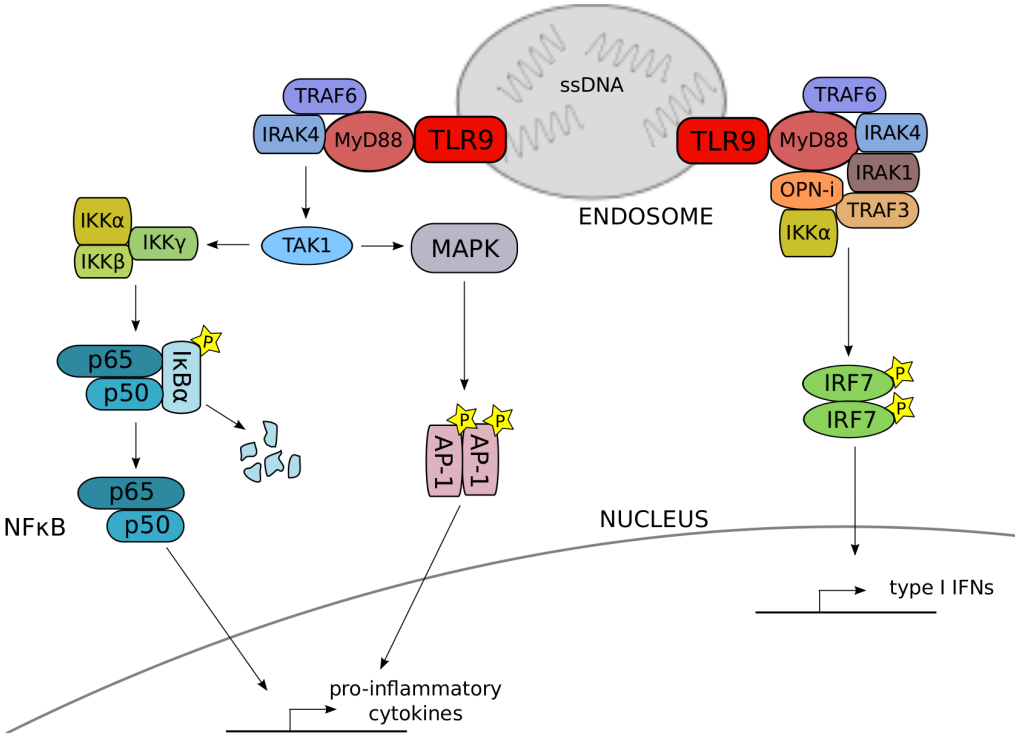

Figure 1.6: **Toll-like receptor 9 signalling pathway.** Toll-like receptor 9 recognizes endocytosed microbial DNA and activates signalling cascades that result in activation of transcription factors such as NFκB and AP1 and subsequent production of pro-inflammatory cytokines (illustrated on the left side of the graph). In plasmacytoid dendritic cells toll-like receptor 9 stimulation additionally leads to activation of IRF7, which results in the induction of type I interferons (right side of the graph).

pathway additionally leads to production of type I IFNs (Figure 1.6, right). IRF7, which is constitutively expressed by pDCs, binds MyD88 and forms a multiprotein signaling complex with IRAK4, TRAF6, TRAF3, IRAK1, OPN-i and IKKα. Within this complex, IRF7 becomes phosphorylated by IRAK1 and/or IKKα, dissociates from the complex and translocates into the nucleus. Phosphorylated IRF7 homodimers mediate production of type I IFNs by pDCs. Produced IFNs are secreted and activate the Jak-STAT pathway through IFN-α/β receptor which is ubiquitously expressed on cell membranes (see Section 1.2.1). Signal transduction through the Jak-STAT pathway leads to induction of IFN-stimulated genes, which have antiviral functions and contribute to the control of the infection.

In vivo TLR9 recognizes CpG motifs in genetic material of a variety of bacteria and some DNA viruses such as murine cytomegalovirus and herpes simplex

virus 1 and 2, resulting in production of inflammatory cytokines and type I IFNs [161, 162, 163, 164]. Overall, TLR9 stimulation induces cell maturation and production of proinflammatory cytokines including TNF-α, IL-1 and IL-6 as well as regulatory cytokines such as IL-12 and IL-18 that induce Th1-type cellular and humoral effector functions. These properties make TLR9 ligands interesting candidates for therapeutic intervention in infectious diseases, treatment of cancer and allergy [165].

1.5.2 Synthetic ligands of toll-like receptor 9 and their anti-HCV properties

The immunostimulatory effects of microbial DNA can be mimicked by synthetic oligodeoxynucleotides containing a CpG-motif (CpG-ODN). Stimulation of TLR9 through administration of synthetic oligonucleotide agonists has demonstrated potential in a variety of medical applications including use as vaccine adjuvants as well as mono- or combination therapies for the treatment of cancer and infectious diseases (reviewed in [165]).

Three major classes of structurally and phenotypically different CpG-ODNs have been described [166, 153, 167]. The A-class oligonucleotides are potent inducers of IFN-α secretion from pDCs, but poor inducers of B cell stimulation. B-class CpG-ODNs have a phosphorothioate backbone and mediate strong B cell stimulation but only weak IFN-α production (reviewed in [168]). The C-class TLR9 ligands show immunomodulatory properties which are intermediate between the A and B classes, inducing both B cell activation and IFN-α secretion. The unique structure of these ODNs with a 5' CpG-motif and a 3' palindrome enables duplex formation within the endosomal environment leading to specific profile of cytokine production [169, 170, 171].

Supernatants from human peripheral blood mononuclear cells (PBMCs) treated with class B CpG oligonucleotides showed potent antiviral activity in HCV replicon cells [172]. Similar results were obtained using mouse bone marrow-derived myeloid DCs and HCV replicon-bearing murine MH1 cells [173]. Class C CpG ODNs have also been shown to induce robust IFN-α production in pDCs from patients chronically infected with HCV [174].

Immunomodulatory oligonucleotides (IMOs) are a novel class of TLR9 agonists which incorporate synthetic cytosine or guanine analogues. These second generation oligonucleotides have the advantage of greater metabolic stability, species-independent activity and clear structure-activity relationship [175, 176, 177, 178].

IMOs containing a secondary structure-forming sequence and a CpR motif (where R is a synthetic analogue of deoxyguanosine) were shown to induce IFN-α production in human PBMC cultures as well as *in vivo* in nonhuman primates [176, 177]. In particular, IMO-2125 induced high and sustained levels of IFN-α and activated natural killer cells in non-human primates. Based on these properties, IMO-2125 has been selected as a candidate for the development of new therapies for chronic hepatitis C [179].

2 Aims of the PhD project

IFN-induced regulators of the Jak-STAT signalling are known to involve in negative feedback loops and affect the response to exogenously administered IFN-α. IFN-based therapies are in clinical use for treatment of diseases such as HCV infection or multiple sclerosis. In this context it is important to understand which IFN subtypes are potent inducers of the negative regulators and whether all IFNs are equally sensitive to the inhibitory mechanisms. To tackle this question we attempted to characterize and compare response patterns to IFN-α, -β and -λ in a setting of continuous and repeated stimulation (see Section 3.1).

The acute phase of HCV infection in humans (first 6 months after transmission) is characterized by high rates of spontaneous clearance and excellent treatment response (>90% cure rate). As the infection at that stage is mostly asymptomatic, it is rarely diagnosed and, in comparison to the chronic phase of HCV infection, little is known about the human liver response to acute HCV infection and the host-virus interactions during this time. In the second part of this PhD project we made use of the acute hepatitis C liver biopsies collected over the course of several years at the University Hospital of Basel to describe human hepatic response to acute HCV infection and gain an insight into the mechanism of improved cure rate compared to chronic hepatitis C (see Section 3.2).

Chronic hepatitis C is currently treated with combination therapies based on pegylated IFN-α. A significant proportion of patients fails to respond to the current treatment options, probably due to the refractory state of the preactivated endogenous IFN system in the liver. Several compounds are currently in clinical development with the aim to improve the treatment outcome of peg-IFN-α non-responders. In the last part of this work we investigated *in vivo* the mode of action of a novel synthetic TLR9 agonist which is a clinical candidate for anti-HCV therapy and characterized the hepatic response to this compound (see Section 3.3).

3 Materials, Methods and Results

3.1 Interferon-β and interferon-λ signaling is not affected by interferon-induced refractoriness to interferon-α *in vivo*

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Interferon-β and Interferon-λ Signaling Is Not Affected by Interferon-Induced Refractoriness to Interferon-α *In Vivo*

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Therapy of chronic hepatitis C with pegylated interferon α (pegIFN-α) and ribavirin achieves sustained virological responses in approximately half of the patients. Nonresponse to treatment is associated with constitutively increased expression of IFN-stimulated genes in the liver already before therapy. This activation of the endogenous IFN system could prevent cells from responding to therapeutically injected (peg)IFN-α, because prolonged stimulation of cells with IFN-α induces desensitization of the IFN signal transduction pathway. Whether all types of IFNs induce refractoriness in the liver is presently unknown. We therefore treated mice with multiple injections and different combinations of IFN-α, IFN-β, IFN-γ, and IFN-λ. Pretreatment of mice with IFN-α, IFN-β, and IFN-λ induced a strong expression of the negative regulator ubiquitin-specific peptidase 18 in the liver and gut. As a result, IFN-α signaling was significantly reduced when mice where reinjected 16 hours after the first injection. Surprisingly, both IFN-β and IFN-λ could activate the Janus kinase–signal transducer and activator of transcription (STAT) pathway and the expression of IFN-stimulated genes despite high levels of ubiquitin-specific peptidase 18. IFN-λ treatment of human liver biopsies *ex vivo* resulted in strong and maintained phosphorylation of STAT1, whereas IFN-α–induced STAT1 activation was transient. *Conclusion:* Contrary to the action of IFN-α, IFN-β, and IFN-λ signaling in the liver does not become refractory during repeated stimulation of the IFN signal transduction pathway. The sustained efficacy of IFN-β and IFN-λ could be an important advantage for the treatment patients who are nonresponders to pegIFN-α, through a preactivated endogenous IFN system. (HEPATOLOGY 2011;53:1154-1163)

Abbreviations: CHC, chronic hepatitis C; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; hu, human; IFN, interferon; IFNAR, interferon-α/β receptor; IFNGR, interferon-γ receptor; IL, interleukin; ISG, interferon-stimulated gene; ISGF3, interferon-stimulated gene factor 3; ISRE, interferon-stimulated response element; m, murine; PBS, phosphate-buffered saline; pegIFN, pegylated interferon; PKR, protein kinase R; RPL19, ribosomal protein L19; RT-PCR, real-time polymerase chain reaction; SEM, standard error of the mean; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; USP18, ubiquitin-specific peptidase 18.

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The interferons (IFNs) are a group of cytokines that induce an antiviral state. They are currently classified into three groups: type I, type II, and type III IFNs.[1,2] The largest group comprises the type I IFNs including all members of the IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, and IFN-ν families.[3] Humans have 12 different IFN-αs and a single IFN-β. Type I IFNs are induced in response to viral infections. All type I IFNs bind to the same IFN-α/IFN-β receptor (IFNAR) that consists of two major subunits: IFNAR1 (the a subunit in the older literature)[4] and IFNAR2c (the βL subunit in older literature).[5,6] The different IFN-α and IFN-β members have substantial differences in their specific antiviral activities. However, the molecular basis of these differences is not yet known.

There is only one class II IFN: IFN-γ, which is produced by T lymphocytes when they are stimulated with antigens or mitogens. IFN-γ binds to a distinct receptor, the IFN-γ receptor (IFNGR) that consists of the two subunits IFNGR1 (previously, the α chain)[7]

and IFNGR2 (previously, the β chain or accessory factor).[8,9]

The recently described type III IFNs IFN-λ2, IFN-λ3, and IFN-λ1 are also known as interleukin-28A (IL-28A), IL-28B, and IL-29, respectively. Similar to type I IFNs, they are also induced by viral infections.[10] They signal through the IFN-λ receptor consisting of the IL-10R2 chain that is shared with the IL-10 receptor, and a unique IFN-λ receptor chain.[11,12] Unlike IFNAR, the IFN-λ receptor is not expressed ubiquitously, but is mainly restricted to epithelial cells.[2] IFN-λ receptors are present in human hepatocytes.[13] In the mouse liver, the IFN-λ receptor is expressed at very low levels, and systemic application of IFN-λ had very little effects in the liver compared to other tissues such as intestine, heart, lung, and skin.[2,14]

All IFNs signal through the Janus kinase–signal transducer and activator of transcription (Jak-STAT) pathway to regulate the expression of their target genes in the nucleus. IFN-γ predominantly stimulates STAT1 and induces a homodimeric transcription factor complex, whereas members of the IFN-α, IFN-β, and IFN-λ families strongly activate STAT1 and STAT2 and induce the heterotrimeric transcription factor complex interferon-stimulated gene factor 3 (ISGF3). The different IFN subtypes induce overlapping but distinct sets of target genes.[15]

The activation of the Jak-STAT pathway is tightly controlled by several negative regulatory mechanisms. Suppressor of cytokine signaling 1 (SOCS1) and SOCS3 are rapidly induced by IFNs and prevent further STAT activation by inhibiting the Jak kinases.[16] Likewise, ubiquitin-specific peptidase 18 (USP18) is a classical ISG that provides a strong negative feedback loop at the level of the receptor-kinase complex.[17] As a result of the induction of these negative regulators, cultured cells become rapidly unresponsive (refractory) to continuous stimulation with IFNs, a phenomenon that has been known for more than 20 years.[18] We have recently shown that refractoriness also occurs in the liver of mice injected with IFN-α.[19] Repeated injection of mouse IFN-α (mIFN-α) at regular intervals resulted in constantly elevated serum concentrations, similar to what is observed in patients receiving pegylated IFN-α (pegIFN-α). Within hours after the first injection of mIFN-α, IFN-α signaling in the liver became refractory to further stimulation. Neither SOCS1 nor SOCS3 were instrumental for this long-lasting refractoriness. Instead, USP18 was identified as the key mediator.[19]

PegIFN-α2 together with ribavirin is the current standard of care for the treatment of chronic hepatitis C (CHC). The treatment achieves a sustained viral clearance in only 50%-60% of patients. The molecular mechanisms underlying treatment failure are still incompletely understood. In recent years, we and others have provided evidence that the endogenous IFN system is already activated in the liver of a substantial number of patients before the therapeutic application of pegIFN-α, and that such a preactivation prevents treatment responses.[20-22] It is not known why this preactivation of the endogenous IFN system inhibits the response to therapeutically injected pegIFN-α, but it is conceivable that a constant stimulation of liver cells by endogenous IFNs induces refractoriness to pegIFN-α stimulation.

Comparatively few clinical studies have been performed to assess the efficacy of IFN-β for the treatment of CHC. In treatment-naive Asian patients, 24 weeks of therapy with IFN-β and ribavirin achieved a sustained virological response in 57% of treated patients.[23] Interestingly, IFN-β is also effective in some patients who did not respond to previous therapies with IFN-α.[24] More recently, pegIFN-λ1 was found to be effective for the treatment of CHC in a phase 1b study with 49 IFN-α–treated patients with relapse and seven treatment-naive patients.[25]

In the present study, we analyzed the activation patterns of the Jak-STAT signal transduction pathway and the induction of ISGs in different organs after single and repeated subcutaneous injection of IFN-α, IFN-β, and IFN-λ in mice. Unexpectedly, marked refractoriness to repeated stimulation was observed only in case of repeated stimulation with IFN-α. The sustained sensitivity to IFN-β and IFN-λ despite preactivation of the signal transduction pathways with IFN-α provides support for the further clinical exploration of IFN-β and IFN-λ for the treatment of IFN-α nonresponders.

Materials and Methods

Cell Culture and Reagents. Huh7 cells were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. All cell culture reagents were from Gibco, Basel, Switzerland. Human IFNs used for Huh7 cells treatment were IFN-α-2b (Intron A; Essex Chemie AG, Luzern, Switzerland), IFN-β-1b (Betaferon; Bayer Schering Pharma, Zürich, Switzerland), or IFN-λ2 (Peprotech Inc., Rocky Hill, NJ).

Animals. Four- to 6-week-old male C57Bl/6 mice were used for all experiments. The animals were bred

in the animal facility of the Department of Biomedicine of the University Hospital Basel under specific pathogen-free conditions. All animal experiments were conducted with the approval of the Animal Care Committee of the Canton Basel-Stadt, Switzerland.

The animals were injected subcutaneously with murine IFNs alpha-4 (mIFN-α), beta (mIFN-β), lambda2 (mIFN-λ), and gamma (mIFN-γ) in sterile phosphate-buffered saline (PBS). Control animals were injected with PBS only. The mIFN-α4 was produced as described,[26] and the concentration was measured by mIFN-α enzyme-linked immunosorbent assay (PBL Interferon Source, Piscataway, NJ). Recombinant mIFN-β and mIFN-γ were purchased from Millipore (Axxora Europe, Lausen, Switzerland) and recombinant IFN-λ2 from Peprotech (Peprotech Inc., Rocky Hill, NJ). Specific activities of recombinant IFN-β and IFN-γ were 10^7 IU/mg and 1.15×10^7 IU/mg, respectively. The animals were euthanized by CO_2 narcosis. Samples from the liver, lung, kidney, and small intestine were collected and immediately frozen in liquid nitrogen and stored at $-80°C$ until further processing.

Western Blot Analysis. Tissue extracts and western blots (protein immunoblots) were done as described.[19] Proteins were detected with primary antibodies specific to phospho-STAT1 (Tyr701, catalog No 9171; Cell Signaling Technology, Bioconcept, Allschwil, Switzerland), STAT1 (catalog no. 610186; Transduction Laboratories, BD Biosciences Pharmingen, San Diego, CA), phospho-STAT2 (Tyr 689, catalog no. 07-224; Upstate Biotechnology, Lake Placid, NY), STAT2 (catalog no. sc950; Santa Cruz Biotechnology, LabForce AG, Nunningen, Switzerland), phospho-STAT3 (Tyr705, catalog no. 9131; Cell Signaling Technology), STAT3 (catalog no. sc482; Santa Cruz Biotechnology), and β-actin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Electrophoretic Mobility Shift Assay. Nuclear extracts from 150-200 mg of liver tissue were prepared as described.[27] Electrophoretic mobility shift assays were done as described.[28]

RNA Isolation and Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction. RNA was isolated from Huh7 cells or shock-frozen liver and gut samples using Trizol Reagent (Invitrogen AG, Basel, Switzerland). Isolated RNA was quantified and 1 μg was reverse-transcribed with random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega Biosciences Inc., Wallisellen, Switzerland). Prior to enzyme mix addition, the reaction mixture was incubated for 3 minutes at 70°C and then cooled on ice. Following the addition of the enzyme, reverse transcription was carried out for 1 hour at 37°C and stopped by incubation at 95°C for 5 minutes. Quantitative real-time polymerase chain reaction (RT-PCR) was performed based on SYBR green fluorescence (Applied Biosystems, Foster City, CA). The primers were: 5′-ATC CGC AAG CCT GTG ACT GT-3′ and 5′-TCG GGC CAG GGT GTT TTT-3′ for murine ribosomal protein L19 (mRPL19), 5′-CGG CGG AGA GAG CTT TGC-3′ and 5′-AGC TGA AAC GAC TGG CTC-3′ for mSTAT1, 5′-GTG GTT GTG GAG GGT GAG ATG-3′ and 5′-GGG ATG AGG TCT CCA GCC A-3′ for mSOCS1, 5′-AAG AGC CCG CCG AAA ACT-3′ and 5′-AGC CAC TGA ATG TAG ATG TGA CAA C-3′ for murine protein kinase R (mPKR), and 5′-CGT GCT TGA GAG GGT CAT TTG-3′ and 5′-GGT CGG GAG TCC ACA ACT TC-3′ for mUSP18. For Huh7 cell samples, the primers were: 5′-CTC AGT CCC GAC GTG GAA CT-3′ and 5′-ATC TCT CAA GCG CCA TGC A-3′ for huUSP18 and 5′-GCT CCT CCT GTT CGA CAG TCA-3′ and 5′-ACC TTC CCC ATG GTG TCT GA-3′ for human glyceraldehyde 3-phosphate dehydrogenase (huGAPDH). All reactions were run in duplicate using an ABI 7500 detection system (Applied Biosystems). The ΔCT value for mouse samples was derived by subtracting the threshold cycle (CT) value for mRPL19, which served as an internal control, from the CT values for mSTAT1, mSOCS1, mPKR, and mUSP18. The messenger RNA (mRNA) expression levels of the transcripts were calculated relative to mRPL19 using the formula $2^{-\mathrm{d}CT}$. For human cell samples, the internal control used was human GAPDH.

Ex Vivo *Liver Biopsy Treatment.* Freshly obtained liver biopsies were immersed in PBS-diluted human IFN-α-2b (Intron A; Essex Chemie AG, Luzern, Switzerland), IFN-β-1b (Betaferon; Bayer Schering Pharma, Zürich, Switzerland), or IFN-λ2 (Peprotech) and incubated for 10-60 minutes at 37°C. Longer treatment periods are not feasible *ex vivo* because of tissue degradation at 37°C. The liquid was then removed and the biopsy material immediately frozen in liquid nitrogen. The protocol was approved by the Ethics Committee of the University Hospital of Basel, Switzerland. Written informed consent was obtained from all patients. Biopsies were obtained from patients suffering from hepatitis C virus infection (biopsy 1), graft-versus-host disease after a liver transplant (biopsy 2), nodular regenerative hyperplasia (biopsy 3), and alcoholic steatohepatitis with liver cirrhosis (biopsy 4).

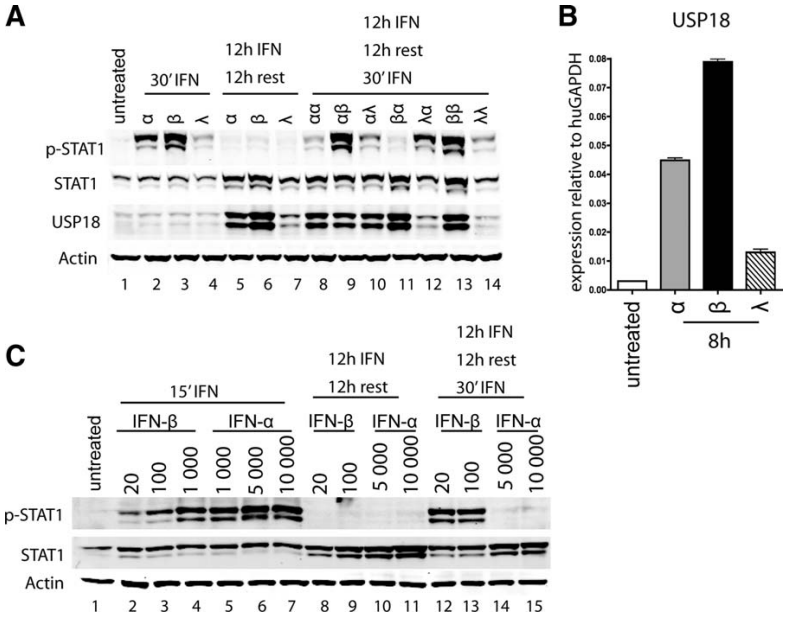

Fig. 1. Repeated stimulation with IFN-α, IFN-β, and IFN-λ results in different response patterns in Huh7 cells. (A) Huh7 cells were stimulated with 1000 IU/mL human IFN-α, 1000 IU/mL human IFN-β, or 500 ng/mL human IFN-λ using the following protocols: (1) single stimulation for 30 minutes (lanes 2-4), (2) single stimulation for 12 hours followed by a 12-hour resting period in complete growth medium (lanes 5-7), (3) initial stimulation for 12 hours followed by a 12-hour resting period and then restimulation for 30 minutes with the same or different IFN (lanes 8-14). IFN-induced tyrosine phosphorylation of STAT1 was assessed by immunoblotting of whole-cell extracts. Blots were then reprobed for total STAT1, USP18, and β-actin. (B) Quantitative RT-PCR analysis of USP18 mRNA expression in Huh7 cells treated for 8 hours with 1000 IU/mL human IFN-α, 1000 IU/mL human IFN-β or 500 ng/mL human IFN-λ. The data are plotted as the amount of USP18 mRNA relative to GAPDH mRNA (mean ± standard error of the mean [SEM]). (C) Huh7 cells were stimulated with 1000, 5000, or 10000 IU/mL human IFN-α or 20, 100, or 1000 IU/mL human IFN-β using the following protocols: (1) single stimulation for 15 minutes (lanes 2-7), (2) single stimulation for 12 hours followed by a 12-hour resting period in complete growth medium (lanes 8-11), (3) initial stimulation for 12 hours with 5000 or 10000 IU/mL human IFN-α or 20 or 100 IU/mL human IFN-β followed by a 12-hour resting period and then restimulation for 30 minutes with 1000 IU/mL of the same IFN as used for initial stimulation (lanes 12-15). IFN-induced tyrosine phosphorylation of STAT1 was assessed by immunoblotting of whole-cell extracts. Blots were then reprobed for total STAT1 and β-actin.

Results

Contrary to IFN-α, IFN-β Signaling Is Not Affected by Prior Stimulation with Type I IFNs. - Repeated or continuous stimulation with IFN-α rapidly induces a refractory state in cultured cells[18] and in mouse liver.[19] To test if prolonged stimulation with IFN-β and IFN-λ also desensitizes cells to further stimulation, and if pretreatment of cells with IFN-α induces refractoriness to IFN-β or IFN-λ and vice versa, we stimulated Huh7 cells with the different IFNs for 12 hours, and restimulated them after an additional 12-hour resting period (at the 24-hour time-point). As expected, pretreatment of cells with IFN-α induced a refractory state that prevented the activation of the Jak-STAT pathway by IFN-α at the 24-hour time-point (Fig. 1A, lane 8). IFN-α–induced STAT1 phosphorylation was also strongly reduced by pretreatment of cells with IFN-β (Fig. 1A, lane 11). Because IFN-β binds to and signals through the same receptor as IFN-α, we expected the same pattern of refractoriness. However, IFN-β signaling was not attenuated by IFN-α or IFN-β pretreatments. The phospho-STAT1 signals 30 minutes after the first and the second stimulation with IFN-β showed the same intensity (Fig. 1A, lanes 3, 9, and 13). We conclude that pretreatment of Huh7 cells with type I IFNs induces a refractory state that affects IFN-α–induced signaling, but not the response to IFN-β. Consistent with previous reports,[29] IFN-λ was less potent than IFN-α in regard to STAT1 activation. However, the signal intensity after restimulation was not decreased in cells pretreated with IFN-λ or IFN-α compared to treatment-naive cells (Fig. 1A, lane 4, 10, and 14).

We have previously shown that USP18 is a key mediator of refractoriness to IFN-α *in vivo*.[19] Pretreatment of cells with IFN-α and IFN-β strongly induced USP18 in Huh7 cells (Fig. 1A,B), but despite this, STAT1 phosphorylation induced by the second treatment with IFN-β and IFN-λ was not impaired.

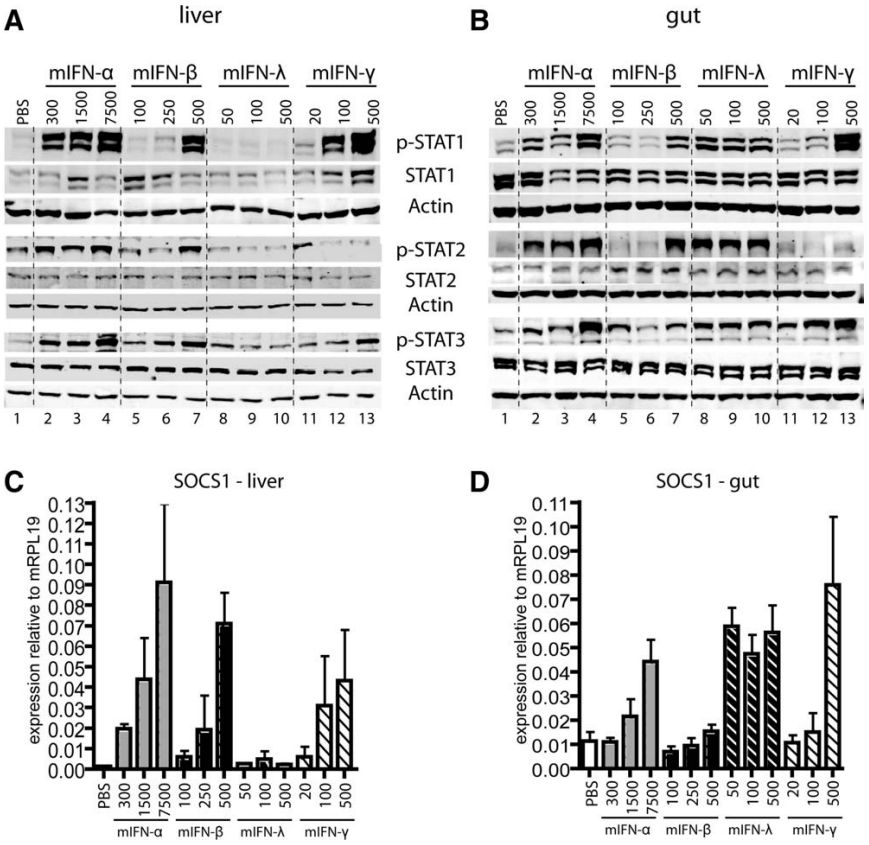

Fig. 2. Dose-response relationship of IFN-α, -β, -λ and -γ in liver and gut. C57Bl/6 mice were injected subcutaneously with increasing doses of murine IFN-α, -β, -λ and -γ and sacrificed 1 hour after injection. IFN doses were: 300, 1500 and 7500 pg/g body weight IFN-α; 100, 250, and 500 IU/g body weight IFN-β; 50, 100, and 500 ng/g body weight IFN-λ and 20, 100 and 500 IU/g body weight IFN-γ. IFN-induced phosphorylation of STAT1, STAT2 and STAT3 was assessed in the (A) liver and (B) small intestine. IFN-induced expression of SOCS1 was quantified by RT-PCR in liver (C) and small intestine (D). The data are plotted as the amount of SOCS1 mRNA relative to RPL19 mRNA (mean $\pm$ SEM).

A single stimulation of cells with 1000 IU/mL IFN-α resulted in a slightly weaker activation of STAT1 compared to 1000 IU/mL IFN-β (Fig. 1A, lanes 2 and 3). To exclude that this difference caused the differential response to repeated stimulation, we pretreated cells with higher concentrations of IFN-α and lower concentrations of IFN-β. In these conditions, IFN-α induced STAT1 activation was similar or stronger compared to IFN-β (Fig. 1C, lanes 2 to 7). However, restimulation with 1000 IU/mL IFN-α or IFN-β (Fig. 1C, lanes 12-15) revealed the same pattern of refractoriness as observed after pretreatment with 1000 IU/mL (Fig. 1A). We conclude that the different sensitivity of IFN-α and IFN-β to pretreatment-induced refractoriness is not influenced by the strength of the initial stimulation, but is an inherent characteristic of the IFN species.

IFN Subtypes Elicit Overlapping but Distinct Responses in the Liver and the Gut of Mice. In order to gain insight into the *in vivo* responses to type I, II, and III IFNs, we studied the dose-response curve to IFN-α, IFN-β, IFN-λ, or IFN-γ after subcutaneous administration. Mice were sacrificed 1 hour after the injection, and liver and small intestine samples were collected and analyzed for the activation of Jak-STAT pathway components and ISG induction (Fig. 2). IFN-α, IFN-β, and IFN-γ induced a dose-dependent phosphorylation of STAT1, STAT2, and STAT3 and a dose-dependent induction of the classical ISGs SOCS1, STAT1, and USP18 both in the liver and in the gut (Fig. 2 and Supporting Fig. 1). At the lowest dose, IFN-λ already strongly induced STAT phosphorylation and ISG expression in the gut, but had no effect in the liver even at the highest dose. This ineffectiveness in the liver is most likely due to the absence or very low expression of the IFN-λ receptor chain in the mouse liver.[2]

IFN-β–Induced Signaling in the Liver Is Not Refractory After Pretreatment. We then analyzed the *in vivo* patterns of refractoriness using mouse samples obtained after repeated administration of different combinations of type I, II, and III IFNs. Both for the first and the second stimulation we chose doses of the different IFNs that resulted in a similar STAT1 phosphorylation 1 hour after a single injection, as established in previous experiments (Fig. 2), i.e., 300 pg/g

body weight IFN-α, 500 IU/g body weight IFN-β, 50 ng/g body weight IFN-λ, and 100 IU/g body weight IFN-γ. At 16 hours after the first injection, the animals were sacrificed or injected again, either with the same IFN or with IFN-α. Mice receiving the second injection were sacrificed 1 hour later for the collection of liver, small intestine, kidney and lung samples. Signaling through the Jak-STAT pathway was analyzed at the level of STAT activation by tyrosine phosphorylation, at the level of binding of activated STAT1 to the IFN-stimulated response element (ISRE) found in promoters of ISGs, and at the level of transcriptional induction of ISGs.

Consistent with our previous findings,[19] mice pretreated with IFN-α showed little response to the second injection with IFN-α (Fig. 3 and Supporting Fig. 2). The same attenuation of signals and ISG induction was observed when IFN-β-pretreated mice were injected 16 hours later with IFN-α. IFN-λ pretreatment had no effect on later IFN-α responses, most likely because of the lack of IFN-λ receptors in mouse liver. Likewise, IFN-γ pretreatment did not induce refractoriness to subsequent stimulation with IFN-α (Fig. 3 and Supporting Fig. 2). This can be explained by the observation that IFN-γ treatment did not induce an up-regulation of USP18 (Fig. 3B and Supporting Fig. 1), the key mediator of refractoriness to IFN-α *in vivo*.[19]

Interestingly, IFN-β–pretreated mice showed a strong response to the second injection with IFN-β. Phosphorylation of STATs, DNA binding, and ISG induction were slightly decreased compared to the first injection of IFN-β. However, ISGs such as USP18 were again strongly induced relative to the expression level at time point 16 hours, demonstrating that the mouse liver remains responsive to repeated injections of IFN-β (Fig. 3A-D and Supporting Fig. 2). The same responses to IFN-α and IFN-β were found in kidney and lung (Supporting Fig. 3). We conclude that consistent with our findings in cultured cells (Fig. 1), cells in the liver, kidney, and lung remain responsive to IFN-β *in vivo*, whereas IFN-induced signaling becomes refractory to IFN-α.

To check if IFN-β could also overcome refractory state induced by a prolonged stimulation with IFN-α, we administered a single injection of IFN-β in mice that were continuously stimulated with IFN-α for an extended period of time. For this continuous stimulation mice were injected every 3 hours with 300 pg/g body weight mIFN-α to ensure constantly elevated IFN-α serum concentrations. In agreement with previously reported data,[19] IFN signaling was refractory to the second, third, and fourth injection of IFN-α (Fig. 3E, lanes 5 to 8). In contrast, IFN-β could still induce STAT1 activation in mice that have been previously injected three times with mIFN-α (Fig. 3E, lane 9).

Repeated Administration of IFN-λ Does Not Induce a Refractory State in the Gut. To investigate the effects of repeated administration of IFN-λ *in vivo* we analyzed STAT1 and 2 phosphorylation and ISG induction in small intestine samples from the same animals that were used to study the hepatic response (Fig. 4). Compared to IFN-α and -β, IFN-λ induced a stronger phosphorylation of STAT1 and STAT2 and stronger ISG up-regulation (Fig. 4A-D and Supporting Fig. 4). This finding is in line with recent studies demonstrating a primary role of IFN-λ in antiviral responses of tissues of epithelial origin.[30] Remarkably, repeated administration of IFN-λ did not lead to any detectable decrease in the levels of Jak-STAT pathway activation or transcriptional induction. This apparent lack of refractoriness related to signaling through interferon-λ receptor occurred despite an important up-regulation of USP18 mRNA 1 hour after injection, suggesting that the inhibitory effect of USP18 protein may be specific to stimulation of cells with IFN-α.[17]

IFN-λ signaling in the gut was also not affected after prolonged stimulation with IFN-α. Repeated injections of mice with mIFN-α resulted in a stepwise reduction of the phospho-STAT1 signals 1 hour after every injection (Fig. 4E, lanes 5 to 8), whereas IFN-λ still induced a strong STAT1 activation in animals injected repeatedly with IFN-α (Fig. 4E, lane 10).

IFN-λ Induces Long-Lasting STAT1 Activation in Human Liver. To study the response patterns to different IFNs in human liver, we treated human liver biopsies *ex vivo* with IFN-α, -β, and -λ. At a dose of 500 ng/mL, IFN-λ elicited comparable STAT1 phosphorylation as stimulation with 1000 IU/mL IFN-α or -β (Fig. 5A). Using these equipotent doses, we next studied the time-course of STAT1 activation. Consistent with our previous results,[31] IFN-α induced a transient phosphorylation of STAT1 that returned to baseline within 60 minutes (Fig. 5B). Interestingly, IFN-λ induced STAT1 was still maximal after 60 minutes.

Discussion

Patients with CHC and an induction of the endogenous IFN system in the liver do not respond to therapeutically injected pegIFN-α with further stimulation of STAT1 phosphorylation, STAT1 nuclear translocation, or induction of IFN target genes.[20] This apparent refractoriness of IFN signaling could explain why

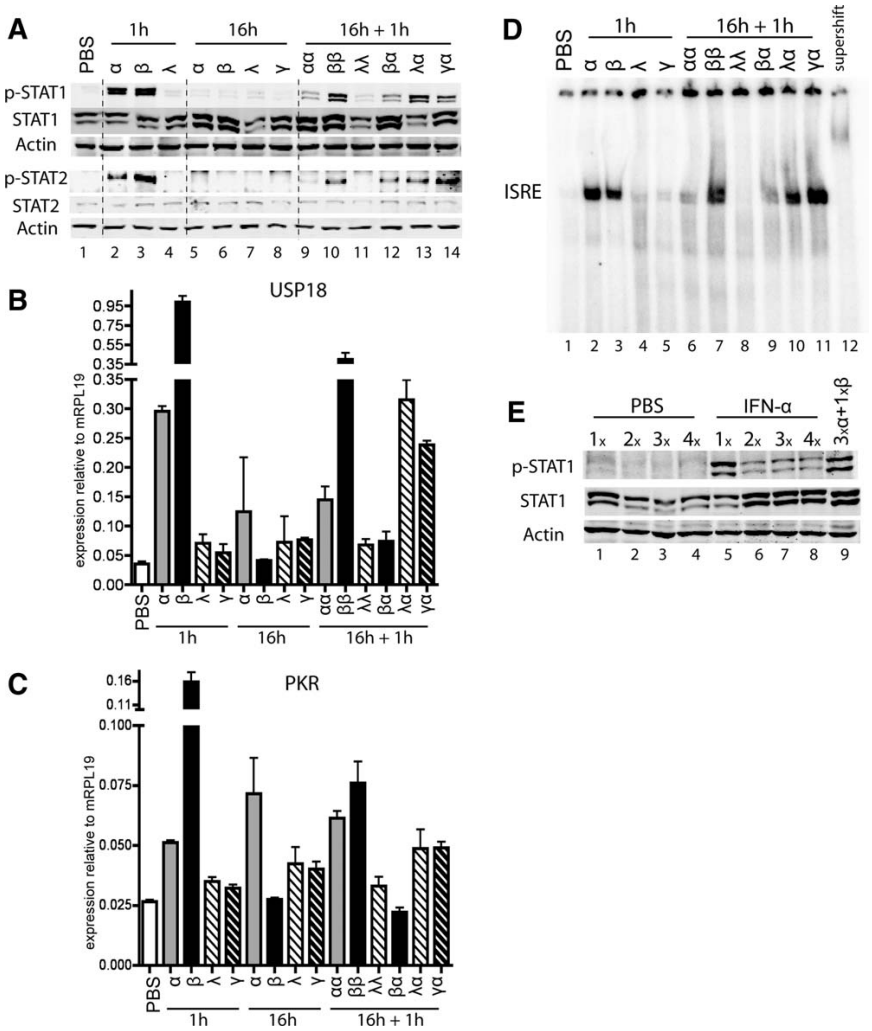

Fig. 3. IFN-β-induced signaling in the mouse liver is not abrogated after pretreatment. (A, B, C, D) C57Bl/6 mice were injected subcutaneously with different murine IFNs and sacrificed 1 hour or 16 hours later, or injected again 16 hours after the first administration and then sacrificed 1 hour after the second injection. IFN doses were 300 pg/g body weight IFN-α, 500 IU/g body weight IFN-β, 50 ng/g body weight IFN-λ, or 100 IU/g body weight IFN-γ. (A) Liver samples were subjected to immunoblotting for tyrosine phosphorylation of STAT1 and STAT2 as well as STAT1 and STAT2 total protein and β-actin as a loading control. (B, C) Quantitative RT-PCR analysis of USP18 (B) and PKR (C) mRNA expression in the liver. The data are plotted as the amount of USP18 or PKR mRNA relative to RPL19 mRNA (mean $\pm$ SEM). (D) Liver nuclear extracts were analyzed in EMSAs using ISRE oligonucleotide probe. Supershift of the ISGF3 band was performed using antibody specific for STAT1 (lane 12). (E) C57Bl/6 mice were injected every 3 hours with 300 pg/g body weight mIFN-α or PBS (control). Liver samples were collected 1 hour after the first, second, third, and fourth injections, as well as after three injections of mIFN-α and subsequent (3 hours later) single injection of mIFN-β (500 IU/g body weight). Whole-cell extracts were subjected to immunoblotting for tyrosine phosphorylation of STAT1 as well as STAT1 total protein and β-actin as a loading control.

most patients with a preactivated IFN system are nonresponders to the current standard of care with pegIFN-α and ribavirin.[20-22] The molecular mechanisms of nonresponse to therapeutically applied pegIFN-α in such preactivated patients have not been conclusively identified. One of the key components responsible for nonresponse could be USP18. USP18 (also known as UBP43) binds to IFNAR2 and inhibits the interaction of Jak1 with the receptor, thereby preventing the activation of STAT1 and STAT2.[17] USP18 was found to be up-regulated in pretreatment liver biopsies of nonresponders[20,22] and it was identified as an important regulator of the antiviral activity of interferon against hepatitis C virus infection *in vitro*.[32]

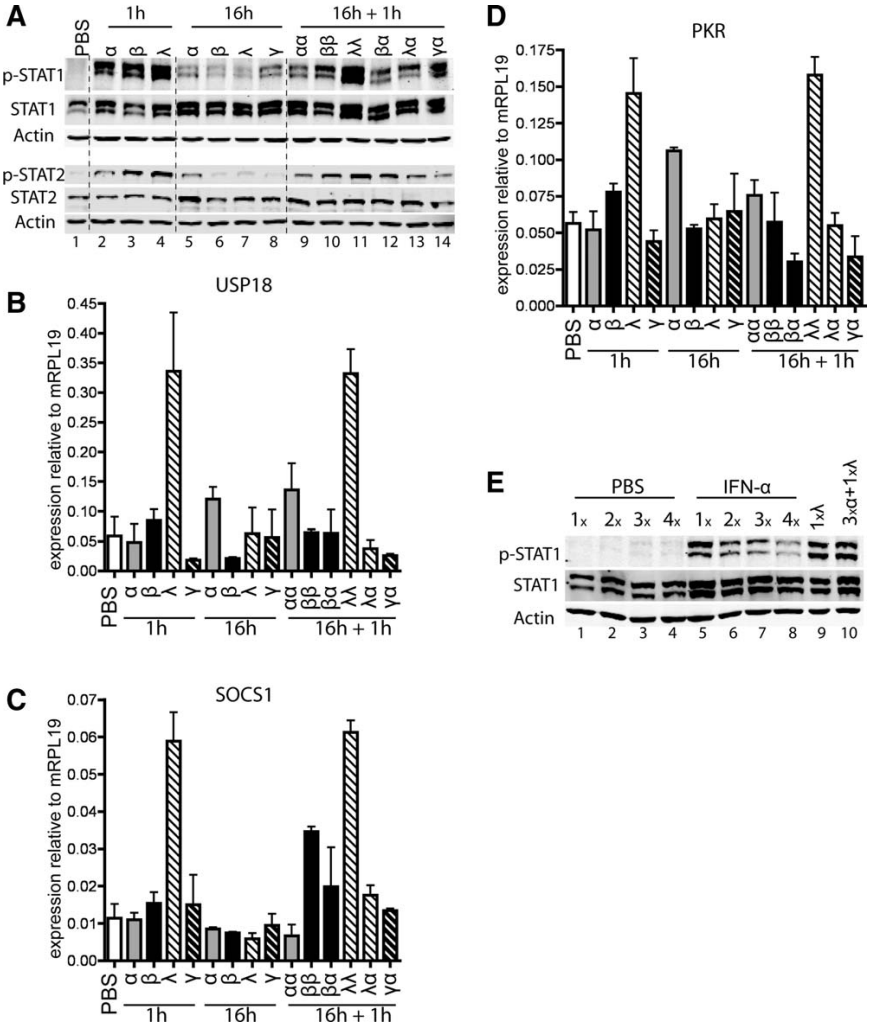

Fig. 4. Repeated administration of IFN-λ does not lead to the reduction of the signaling in the gut. (A, B, C, D) C57Bl/6 mice were injected subcutaneously with different murine IFNs and sacrificed 1 hour or 16 hours later, or injected again 16 hours after the first administration and then sacrificed 1 hour after the second injection. IFN doses were 300 pg/g body weight IFN-α, 500 IU/g body weight IFN-β, 50 ng/g body weight IFN-λ or 100 IU/g body weight IFN-γ. (A) Small intestine samples were subjected to immunoblotting for tyrosine phosphorylation of STAT1 and STAT2 as well as STAT1 and STAT2 total protein and β-actin as a loading control. (B, C, D) Quantitative RT-PCR analysis of USP18 (B), SOCS1 (C) and PKR (D) mRNA expression in the small intestine samples. The data are plotted as the amount of USP18, SOCS1, or PKR mRNA relative to RPL19 mRNA (mean $\pm$ SEM). (E) C57Bl/6 mice were injected every 3 hours with 300 pg/g body weight mIFN-α or PBS (control). Small intestine samples were collected 1 hour after the first, second, third, and fourth injections, as well as after a single injection of mIFN-λ (50 ng/g body weight) or three injections of mIFN-α and subsequent (3 hours later) single injection of mIFN-λ (50 ng/g body weight). Whole-cell extracts were subjected to immunoblotting for tyrosine phosphorylation of STAT1 as well as STAT1 total protein and β-actin as a loading control.

Furthermore, USP18 has been identified as a key mediator of refractoriness to repeated or prolonged stimulation with IFN-α in the mouse liver.[19]

IFN-β and IFN-αs bind to the same receptor, IFNAR. It has also been shown that IFN-β induced signaling is enhanced in USP18 deficient mouse embryonic fibroblasts.[17] However, IFN-β signaling in the mouse liver was largely unaffected in mice pretreated with IFN-α or IFN-β despite high expression levels of USP18 (Fig. 4). There is experimental evidence that IFN-β has a higher affinity to IFNAR2 compared to IFN-αs.[33] It is also conceivable that such different affinity bindings might induce different conformational changes to the receptor molecules and thereby differential sensitivities to inhibition by USP18. However, solid experimental evidence supporting such a model is still lacking.

The absence or low-level expression of the IFN-λ receptor in the mouse liver prevented us from studying

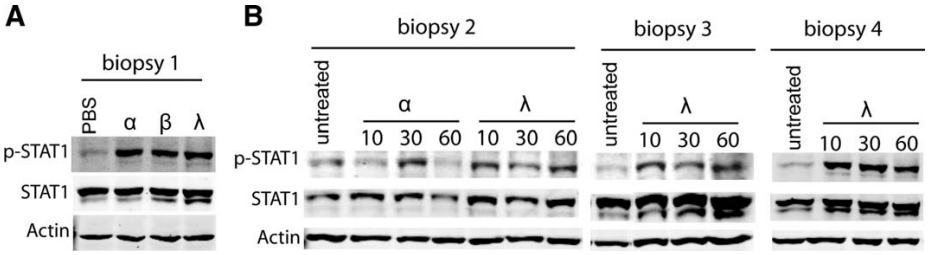

Fig. 5. IFN-λ induces STAT1 activation in the human liver biopsies. (A) Freshly obtained human liver biopsy sample was incubated for 20 minutes at 37°C with human IFN-α (1000 IU/mL), -β (1000 IU/mL), -λ (500 ng/mL) or PBS as a control. Whole-cell extracts were subjected to immunoblotting for tyrosine phosphorylation of STAT1, STAT1 total protein, and β-actin. (B) Freshly obtained human liver biopsy samples were incubated for 10, 30, or 60 minutes at 37°C with human IFN-α (1000 IU/mL) or IFN-λ (500 ng/mL). Whole-cell extracts were subjected to immunoblotting for tyrosine phosphorylation of STAT1, STAT1 total protein, and β-actin.

the induction of refractoriness of the type III IFN signaling system in the liver. We therefore used the gut as a model tissue *in vivo* (Fig. 4). Sensitivity to IFN-λ remained unaffected by repeated injections of IFN-λ or IFN-α, again despite high expression levels of USP18. In the case of IFN-λ, the use of a different and structurally unrelated receptor that is not bound by USP18 is a likely explanation.

IFN-λ has been shown to activate the Jak-STAT pathway in human hepatoma cells and to inhibit hepatitis C virus replication.[13,29] Using liver biopsies treated *ex vivo*, we provide evidence that IFN-λ is also active in the human liver. Moreover, contrary to the transient STAT1 phosphorylation signal detected in IFN-α–stimulated samples, IFN-λ induced STAT1 phosphorylation was maintained (Fig. 5).

It is widely assumed that the constant high serum concentrations achieved with pegIFN-α provide the decisive advantage over nonpegylated forms of recombinant IFN-α, because the permanent stimulation of the IFN signal transduction pathway will induce an uninterrupted antiviral activity in the infected hepatocytes. However, there is no experimental evidence to support this hypothesis. On the contrary, in previous work we observed a long-lasting refractoriness to IFN-α in mice that were repeatedly injected in short intervals with mIFN-α in order to maintain high serum concentrations over a prolonged period of time. The present observation that IFN-λ signaling is unaffected by IFN induced up-regulation of USP18 could provide an alternative explanation for the increased efficacy of pegIFN-α. Similar to IFN-αs, IFN-λs can be induced by type I IFNs.[34] If refractoriness to IFN-α would be restricted to hepatocytes, pegIFN-α could still stimulate dendritic cells or macrophages to secrete IFN-λ and thereby indirectly sustain the expression of antiviral genes in hepatocytes. Indeed, we have previously found that pegIFN-α injections could still activate STAT1 in nonparenchymal, sinusoidal cells in patients with a preactivated hepatic IFN system whereas no further increase in phospho-STAT1 signals were found in hepatocytes.[20]

In conclusion, contrary to IFN-α, both IFN-β and IFN-λ continue to induce signaling through the Jak-STAT pathway in the setting of repeated or prolonged stimulation with type I or type III IFNs *in vivo*. We propose that pegylated IFN-β and pegylated IFN-λ could be promising treatment options specifically for patients with a preactivated hepatic IFN system who have little chances to be cured by the current standard of care with pegIFN-α and ribavirin, and for patients who are known nonresponders to previous therapies with (peg)IFN-α–based regimens.

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3.2 IFN-γ-stimulated genes, but not USP18, are expressed in livers of patients with acute hepatitis C

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Interferon-γ–Stimulated Genes, but Not USP18, Are Expressed in Livers of Patients With Acute Hepatitis C

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BACKGROUND & AIMS: Approximately 50% of patients with chronic hepatitis C (CHC) have a sustained virologic response to treatment with pegylated interferon (pegIFN)-α and ribavirin. Nonresponse to treatment is associated with constitutively increased expression of IFN-stimulated genes (ISGs) in the liver. Treatment of patients with acute hepatitis C (AHC) is more effective, with sustained virologic response rates greater than 90%. We investigated mechanisms of the different responses of patients with CHC and AHC to pegIFN-α therapy. **METHODS:** We analyzed IFN signaling and ISG expression in liver samples from patients with AHC, patients with CHC, and individuals without hepatitis C (controls) using microarray, immunohistochemical, and protein analyses. Findings were compared with those from primary human hepatocytes stimulated with IFN-α or IFN-γ, as reference sets. **RESULTS:** Expression levels of hundreds of genes, primarily those regulated by IFN-γ, were altered in liver samples from patients with AHC compared with controls. Expression of IFN-γ–stimulated genes was induced in liver samples from patients with AHC, whereas expression of IFN-α–stimulated genes was induced in samples from patients with CHC. In an expression analysis of negative regulators of IFN-α signaling, we did not observe differences in expression of suppresor of cytokine signaling 1 or SOCS3 between liver samples from patients with AHC and those with CHC. However, USP18 (another negative regulator of IFN-α signaling), was up-regulated in liver samples of patients with CHC that did not respond to therapy, but not in AHC. **CONCLUSIONS: Differences in expression of ISGs might account for the greater response of patients with AHC, compared with those with CHC, to treatment with pegIFN-α and ribavirin. Specifically, USP18 is up-regulated in liver samples of patients with CHC that did not respond to therapy, but not in patients with AHC.**

Keywords: HCV; Jak–STAT Signaling; Host–Virus Interaction; Immune Response.

Chronic infection with hepatitis C virus (HCV) is a major cause of liver disease worldwide.[1] For the past decade, a combination of pegylated interferon-α (pegIFN-α) with ribavirin was the standard therapy for chronic hepatitis C (CHC). This treatment achieves an overall sustained virologic response (SVR) in approximately 55% of patients.[2] Recently, 2 HCV protease inhibitors used in conjunction with pegIFN-α and ribavirin were approved for the treatment of CHC, and triple combination therapies most likely will be the standard of care for a majority of patients in developed countries.[3] However, nonresponse to pegIFN-α remains an important problem in the setting of triple therapy because it significantly increases the rate of viral breakthrough during therapy caused by the emergence of HCV variants resistant to protease inhibitors.[4,5] It has been well documented that nonresponse to pegIFN-α is associated with persistent induction of IFN-stimulated genes (ISGs) in the liver.[6–8] The very same set of hundreds of ISGs is induced by therapeutically applied pegIFN-α in patients without pretreatment activation of ISGs who have a good response to treatment.[7] Paradoxically, activation of the endogenous IFN system not only is ineffective in clearing the infection, but also impedes response to pegIFN-α therapy, possibly because of refractoriness of the IFN-α signal transduction pathway. We previously showed that IFN-α signaling in the mouse liver becomes unresponsive within hours after the injection of IFN-α and have identified USP18 as a key mediator of refractoriness.[9]

Contrary to patients with CHC, most patients with acute hepatitis C (AHC) respond very well to monotherapy with (peg)IFN-α.[10,11] The reasons for the discrepant response to pegIFN-α are unknown. Given the association of intrahepatic ISG expression and nonresponse to pegIFN-α in CHC, an obvious explanation for the good response to therapy in AHC could be a lack of ISG induction in AHC. The intrahepatic immune response has not been studied in patients with AHC, but serial liver biopsy specimens in chimpanzees obtained during the first 6–8 months after infection with HCV have revealed a strong induction of ISGs.[12–14] In the present study, we analyzed inflammatory infiltrates, the activation of IFN

Abbreviations used in this paper: AHC, acute hepatitis C; CHC, chronic hepatitis C; CHC-NR, CHC nonresponder; CHC-R, CHC responder; HCV, hepatitis C virus; HPF, high-power field; ISG, interferon-stimulated gene; JAK, Janus kinase; MAVS, mitochondrial antiviral signaling protein; mRNA, messenger RNA; PCR, polymerase chain reaction; pegIFN-α, pegylated interferon alfa; PHH, primary human hepatocytes; pSTAT1, phosphorylated STAT1; RT, reverse-transcription; SVR, sustained virologic response.

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Table 1. Patient Characteristics

Patient number	Age, *y*	Sex	HCV genotype	Viral load at Bx, *log IU/mL*	Alanine aminotransferase level at Bx, *U/L*	Interleukin-28B rs12979860	Week 4 response	Response	HIV	ΔT Inf-Bx, *mo*
1	31	M	3	<12	60	CC	—	SC	—	3
2	17	F	1	<12	421	CT	—	SC	—	3
3	16	F	1	3.53	86	CT	RVR	SVR[a]	—	4
4	30	M	4	2.49	125	CT	RVR	SVR	—	2
5	44	M	3	5.98	571	CC	—	Interrupted	—	2–5
6	56	M	3	4.15	155	TT	RVR	EoTR[b]	—	3–4

Bx, biopsy; EoTR, end of treatment response; HIV, human immunodeficiency virus; Inf, infection; RVR, rapid virologic response (below limit of detection at week 4); SC, spontaneous clearance; ΔT, time interval.
[a]Treatment with pegIFN-α-2a for 12 weeks.
[b]Treatment with pegIFN-α-2b and ribavirin.

signal transduction pathways, and gene expression profiles in liver biopsy specimens of 6 patients with AHC.

Materials and Methods

Patients

All patients were recruited in the Hepatology Outpatient Clinic of the University Hospital Basel (Basel, Switzerland). From October 2007 to December 2010, 6 patients with AHC (between 0 and 6 months after the HCV transmission) gave written informed consent to participate in this study and donated a liver biopsy specimen for research purposes. The study was approved by the Ethics Committee of Basel.

Liver biopsy specimens from 16 patients with CHC and 4 normal liver tissue samples were used for comparative analysis. These samples were obtained during a previous study that has been published previously.[7] The data reported in that article have been deposited in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo, accession no. GSE11190). An additional 17 liver biopsy specimens of patients with CHC were used for immunostaining analyses. All patients with CHC were treated with pegIFN-α and ribavirin, the standard of care during the study period. Patients with a sustained virologic response defined as undetectable HCV-RNA level 6 months after the end of treatment were classified as responders (R); all others were classified as nonresponders (NR). Serum HCV RNA was quantified using the Cobas Amplicor Monitor (Roche, Basel, Switzerland). AHC patients were monitored closely for transaminases and HCV polymerase chain reaction (PCR), and if there was no decline of the viral load below the limit of detection within a month after the first visit, they were treated with 1.5 μg/kg body weight pegIFN-α-2b (PegIntron; Essex Chemie, Luzern, Switzerland) monotherapy for 24 weeks, unless indicated otherwise (Table 1).

Cell Culture

Primary human hepatocytes (PHH) were isolated from liver resections obtained from noninfected patients as described previously.[15] Freshly isolated PHH were seeded on 6-well plates precoated with collagen (BD Biosciences, Allschwil, Switzerland) and maintained in culture in William's E medium (Sigma, Buchs, Switzerland), supplemented with 1% Glutamax (Gibco, Zug, Switzerland), 1% insulin transferrin selenium (Gibco, Aidenbach, Germany), 10^{-7} mol/L dexamethasone (Sigma), 0.15% bovine serum albumin (Sigma), and 10% fetal bovine serum (PAN Biotec, Aidenbach, Germany). Huh-7 cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum. PHH and Huh-7 cells were treated with 1000 U/mL of human IFN-α (Roferon; Roche), human IFN-α (BioLegend, Luzern, Switzerland), or 70 ng/mL of human pegIFN-α (Pegasys; Roche).

RNA Extraction and Microarray Hybridization

Gene expression was assessed by microarray analysis using Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) for human liver specimens and Affymetrix Human Gene 1.0 ST arrays for PHH. Detailed information is included in the Supplementary Materials and Methods section. All original array data are deposited at the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE38598.

Statistical Analysis

Microarray analysis was performed with Bioconductor packages of R statistical environment (R foundation for Statistical Computing, Vienna, Austria).[16] Detailed information on the statistical procedures is included in the Supplementary Materials and Methods section. Statistical analyses of real-time reverse-transcription (RT)-PCR and immunohistochemical data were performed using GraphPad Prism (La Jolla, CA) software version 4.0.

Interleukin-28B Genotyping

Extraction of DNA and genotyping for the single-nucleotide polymorphism rs12979860 near the *interleukin-28B* gene was performed as described previously.[17]

Real-Time RT-PCR

Reverse transcription was performed as described.[7] For the measurement of IFN-α and IFN-β genes containing only one exon, the same amount of RNA also was mock reverse-transcribed to control for genomic DNA contamination. SYBR real-time PCR was performed using SYBR green (Applied Biosystems, Foster City, CA). The intron-spanning primers are listed in Supplementary Table 1. IFN-α primers were designed to detect all 13 IFN-α genes. All reactions were run in duplicate with an ABI 7500 Real-Time PCR System (Applied Biosystems). Messenger RNA (mRNA) expression levels of the transcripts were normalized to *glyceraldehyde-3-phosphate dehydrogenase* using the delta Ct method.

Western Blot

Whole-cell extracts and blotting of human liver samples were performed as described.[7] The membranes were incubated

with primary antibodies (listed in Supplementary Table 1) in Tris-buffered saline Tween-20 overnight at 4°C. After 3 washes with Tris-buffered saline Tween-20, membranes were incubated with fluorescent secondary goat anti-mouse (IRDye 680) or anti-rabbit (IRDye 800) antibodies (both LI-COR Biosciences, Bad Homburg, Germany) for 1 hour at room temperature. Blots were scanned by the Odyssey Infrared Imaging System (LI-COR). For mitochondrial antiviral signaling protein (MAVS), the membrane was incubated with HRP-conjugated goat anti-mouse antibody (Pierce, Lausanne, Switzerland), and developed on Biomax MR films (Kodak, Chalon-sur-Saone, France).

Immunohistochemistry

Serial sections (4-μm thick) were cut from formalin-fixed, paraffin-embedded, liver biopsy specimens, rehydrated, pretreated for 20 minutes in epitope retrieval solution 2, incubated with the respective primary antibody, and counterstained with hematoxylin. Standard indirect immunoperoxidase procedures were used for immunohistochemistry (ABC-Elite; Vectra Laboratories, Peterborough, UK). The staining procedure was performed with an automated stainer (Bond; Vision BioSystems, Newton Aycliffe, UK). The primary antibodies are listed in Supplementary Table 2.

For the co-localization analysis, each section was photographed at 50× magnification with an 11.7-megapixel Axio Zeiss (Jena, Germany) camera (picture size, 3900 × 3000 pixels), choosing the same area of the biopsy. Five random high-power fields (HPF, 279 × 252 pixels) were chosen within the parenchyma of the biopsy of the first section (Supplementary Figure 1). Then, identical HPFs of the other sections were defined, all HPFs were enlarged digitally, and the amount of positive hepatocytes or immune cells were counted by 2 independent observers (M.T.D., F.M.) (Supplementary Table 6). To ensure the quality of the count on the digitally enlarged HPFs, the corresponding HPFs also were counted by microscopic assessment. For the digital processing, Adobe Photoshop and Illustrator version 5 were used.

Immunofluorescence

Sections (6-μm thick) were cut from fresh-frozen liver biopsy specimens embedded in optimal cutting temperature medium, fixed in periodate-lysine-paraformaldehyde for 10 minutes, and then incubated with primary antibodies (Supplementary Table 2) for 1 hour. CD8 was detected with goat anti-mouse-Cy3 antibody (1:600, 115-166-068; Jackson ImmunoResearch, Newmarket, England) and IFN-γ with goat anti-rabbit-Alexa488 (1:100, A11070; Invitrogen, Lucerne, Switzerland). Pictures were recorded with a 40× objective using an LSM 710 confocal microscope (Carl Zeiss, Feldbach, Switzerland).

Results

Host–Virus Interactions During Acute Hepatitis C Induce a Distinct Pattern of Gene Expression in the Liver

Six patients with HCV monoinfection underwent a liver biopsy 2–5 months after HCV transmission (ie, during the acute phase of HCV infection) (Table 1). Gene expression in these liver biopsy specimens was analyzed with Affymetrix U133 Plus 2.0 arrays and compared with 4 samples from patients without liver disease (controls) and 16 samples from patients with CHC recruited in a previous study.[7] We found between 203 and 492 genes (average, 312) up-regulated and 239 to 374 genes (average, 294) down-regulated more than 2-fold in the liver of patients with AHC compared with the healthy controls (Figure 1*A*). The extent of up-regulation or down-regulation was not associated with response to treatment, spontaneous clearance, estimated time from infection to biopsy, serum viral load, or interleukin-28B genotype (data not shown). Transcriptome profiles of AHC liver samples were highly homogenous: between 50% and 80% of genes altered in a particular patient also were changed in at least 2 other AHC patients (Figure 1*A*). Genes up-regulated in AHC patients compared with healthy liver included chemokines and their receptors, ISGs, and genes involved in cellular immune responses (Figure 1*B* and Supplementary Table 3). Many of the down-regulated genes are involved in intermediate metabolism and lipid homeostasis (Supplementary Table 4).

A comparable number of genes were dysregulated in the group of CHC patients who were nonresponders to pegIFN-α (CHC-NR), but intersecting the sets of differentially regulated genes showed only a limited overlap between CHC and AHC patients, with 147 genes up-regulated and 138 genes down-regulated specifically in AHC (Figure 1*C*). Genome-wide unsupervised clustering of the healthy liver, CHC, and AHC samples (Supplementary Figure 2) showed that AHC samples form a well-defined, separate cluster, further showing the specific molecular signature of this group of patients.

Activation of Janus kinase-Signal Transducer and Activator of Transcription Signaling and ISG Induction in AHC

As outlined earlier, functional annotation of the genes dysregulated in AHC identified several classic ISGs (Supplementary Table 3). To investigate more rigorously to what extent ISGs were induced in AHC, we made use of a list of bona fide hepatic ISGs. This list was compiled in a previous study in which we obtained paired biopsy specimens before and 4 hours after the first injection of pegIFN-α in 10 selected patients with CHC who did not show induction of ISGs before treatment and responded well to pegIFN-α.[7] The list contains 167 genes (242 probe sets) significantly (paired t test, $P < .05$) changed more than 2-fold by pegIFN-α (157 up-regulated, 10 down-regulated). Of the 167 genes, there were 125 detected above the minimal expression cut-off level in our dataset. Unexpectedly, only 30 of these 125 IFN-α–regulated genes were regulated more than 2-fold in AHC. This low number of induced ISGs could be explained by a relatively weak activation of IFN signaling pathways in AHC. However, when we analyzed phosphorylation and nuclear translocation of STAT1 in the AHC samples, we found a strong activation of this central mediator of the IFN signaling pathway (Figure 2*A–C*). Alternatively, the absence of a broad induction of pegIFN-α–induced genes in AHC could be explained by the activation of STAT1 by IFN-γ, another strong inducer of STAT1 phosphoryla-

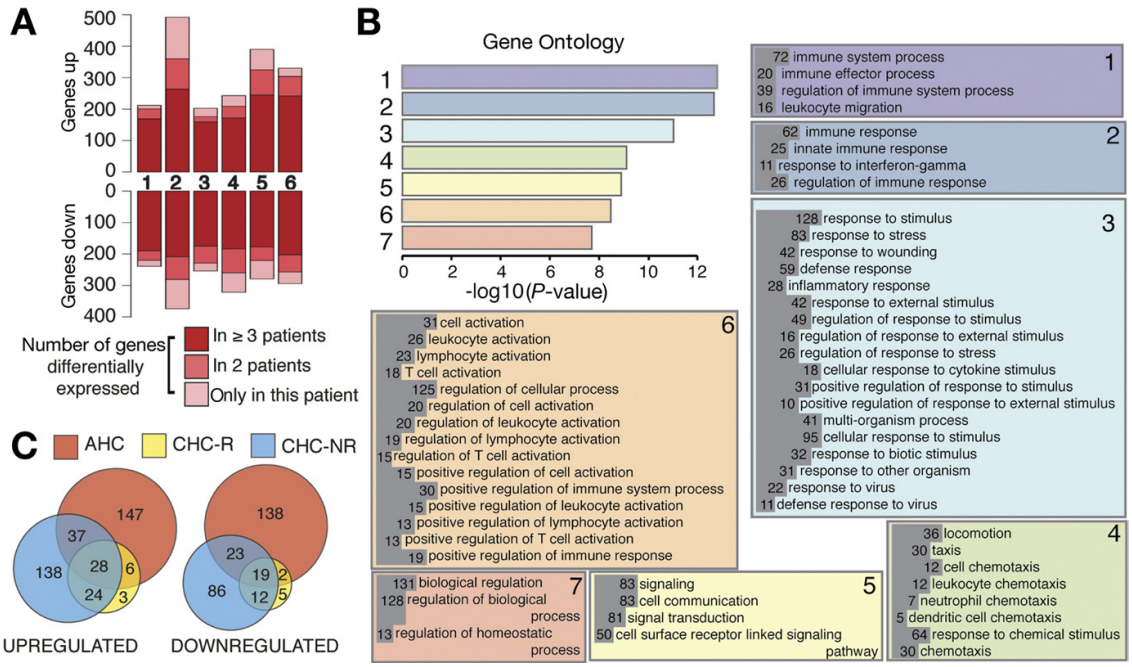

Figure 1. Acute hepatitis C patients show a distinct pattern of gene expression in the liver. (*A*) Number of genes 2-fold up-regulated or down-regulated in each AHC patient compared with the mean gene expression in healthy liver samples (n = 4). *Different shades* show the extent of overlap between patients as indicated. (*B*) Enrichment of gene ontology biological process terms among genes up-regulated in AHC patients with respect to control patients. Terms with *P* values less than 10^{-6} were clustered based on the gene ontology hierarchy. The *bar plot* shows the enrichment score for each cluster. The *numbers to the left* of the term name show the number of genes that represent a given gene ontology term in AHC patients. (*C*) Venn diagram of genes identified as up-regulated or down-regulated in AHC (n = 6), CHC-NR (n = 6), or CHC-R patients (n = 10) compared with healthy liver.

tion[18] that has been implicated in the immune response during AHC in chimpanzees and human beings.[19,20] We therefore measured the mRNA expression levels of IFN-γ, and found them indeed significantly up-regulated in AHC biopsy specimens compared with CHC (Figure 2*D*). Of note, we were not able to detect any up-regulation of IFN-α and IFN-β levels in all biopsy specimens (data not shown). Also, we could not detect phosphorylation of type I IFN-specific STAT2 in AHC biopsy specimens (Figure 2*E*). We conclude that in AHC, STAT1 activation is caused by IFN-γ and not by IFN-α/β.

IFN-γ–Specific Gene Signature Is Enriched in the AHC Gene Expression Profiles, Whereas IFN-α–Induced Transcription Patterns Characterize CHC-NR Patients

To further study the pattern of ISG induction in AHC and CHC-NR, we generated IFN-α– and IFN-γ–induced gene lists and compared them with the ISG expression in the biopsy specimens. Because ISG expression differs considerably between different cells and tissues, we did not use published ISG lists obtained in nonhepatic cells, but stimulated primary human hepatocytes (PHH) from 2 donors with 1000 IU/mL of human IFN-α and IFN-γ for 6 and 24 hours and performed microarray analysis (Figure 3*A*). There were 256 genes up-regulated more than 2-fold in the PHH from both donors after IFN-α stimulation, with the majority of the genes induced already after 6 hours of treatment. IFN-γ induced a comparable number of genes (288), but with different kinetics. The majority of the IFN-γ–induced genes were detected after 24 hours of treatment, which was in accordance with a previous study.[21] Treatment of PHH with IFN-α led to a very broad gene down-regulation: transcript levels of 850 genes were reduced more than 2-fold in PHH from both donors. Interestingly, the observed suppression was very transient and only 15 genes were found down-regulated after 24 hours of IFN-α exposure. Gene down-regulation after IFN-γ treatment involved 123 genes, with a slightly larger number of genes found suppressed after 24 hours of treatment (77) compared with 6 hours (60), and a limited overlap between the 2 time points (14).

Comparison of the gene sets induced by IFN-α or IFN-γ identified 149 common genes, but also a similar number of genes specifically induced by either IFN-α or IFN-γ (Figure 3*A* and Supplementary Tables 5 and 6). This allowed us to generate 2 gene lists representative of IFN-α– or IFN-γ–stimulated genes (Supplementary Figure 3*A*). These gene sets then were used to assess the enrichment of specific IFN-α and IFN-γ signatures in liver

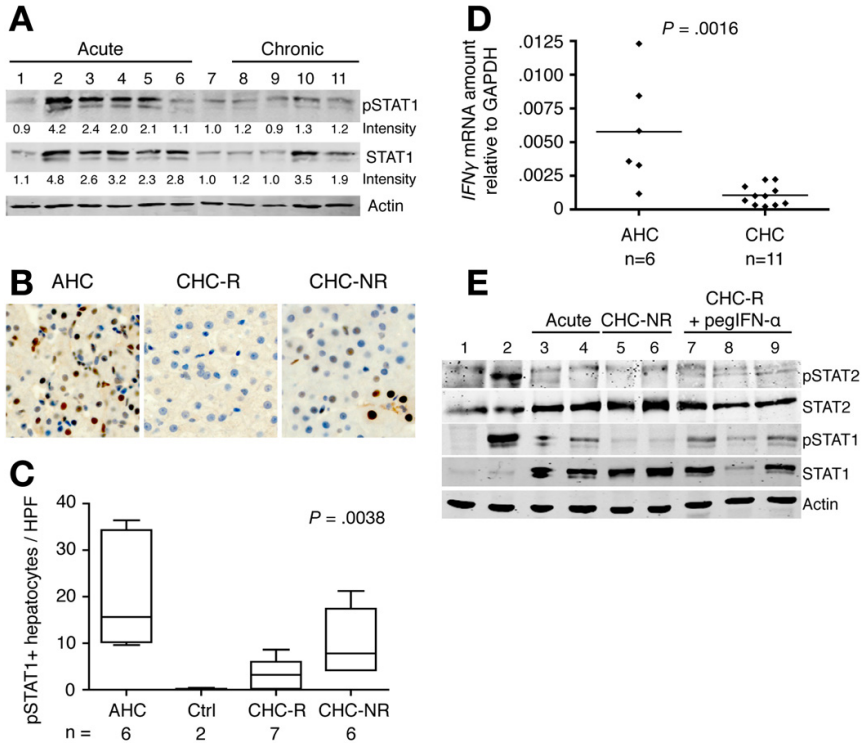

Figure 2. Jak-STAT pathway activation in acute hepatitis C. (*A*) STAT1 phosphorylation and STAT1 protein expression by Western blot analysis using whole-cell extracts of liver samples from AHC (*lanes 1–6*, according to Table 1), healthy liver (*lane 7*), CHC-R (*lanes 8* and *9*), and CHC-NR (*lanes 10* and *11*). (*B*) Representative pictures of immunohistochemistry for phosphorylated STAT1 (pSTAT1) showing strong nuclear staining in AHC and moderate staining in CHC-NR, although CHC-R is not positively stained. (*C*) Quantification of phospho-STAT1 nuclear staining in hepatocytes per HPF in AHC, healthy liver (Ctrl), and CHC-R and CHC-NR. There were significant differences of the mean amount of positive hepatocytes between the 4 groups (*P* value obtained by 1-way analysis of variance). (*D*) Measurement of hepatic expression of *IFN-γ* mRNA in AHC and CHC by quantitative PCR normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Each *dot* represents one sample. *P* value was obtained with the Student *t* test. (*E*) STAT1 and STAT2 phosphorylation and whole protein expression by Western blot analysis using whole-cell extracts of Huh7 cells untreated (*lane 1*) or treated for 30 minutes with 1000 U/mL IFN-α (*lane 2*) or liver samples of 2 AHC patients (*lanes 3* and *4*), 2 CHC-NR patients (*lanes 5* and *6*), and 3 CHC-R patients undergoing a biopsy 4 hours after subcutaneous pegIFN-α injection (*lanes 7–9*). Phospho-STAT2 signals are detected in IFN-α–treated Huh7 cells (*lane 2*) and weakly in biopsy specimens from pegIFN-α–treated patients (*lanes 7–9*). Phospho-STAT1 signals are strong in biopsy specimens from patients with AHC (*lanes 3* and *4*) and after pegIFN-α treatment (*lanes 7–9*), and weak in CHC-NR samples (*lanes 5* and *6*).

biopsy specimens of AHC and CHC-NR patients using the gene set enrichment analysis algorithm[22] (Figure 3*B* and Supplementary Table 7). We observed a significant enrichment of IFN-γ–regulated genes in AHC compared with CHC-NR (enrichment score, 0.52; $P = .04$). On the other hand, the genes up-regulated in PHH by IFN-α were enriched in CHC-NR samples (enrichment score, −0.83; $P < .001$). By selecting interferon, alpha-inducible protein 27 and interferon-induced protein with tetratricopeptide repeats 1 as IFN-α–specific ISGs as well as guanylate binding protein 5 and major histocompatibility complex, class II, DM beta for IFN-γ specificity we were able to confirm the data obtained from the microarrays in the PHH and the liver biopsy specimens by quantitative RT-PCR (Supplementary Figure 4). These results disclose a predominant role of IFN-γ in driving the ISG transcription in the acute phase of HCV infection, whereas ISG expression in pre-activated patients in the chronic phase shows a type I IFN-specific pattern.

CD8+ T Cells Co-localize With Phosphorylated STAT1-Positive Hepatocytes in AHC

To investigate the source of IFN production in the infected liver, serial sections from AHC and CHC liver biopsy specimens were stained for phospho-STAT1 and markers for T cells (CD3, CD8), for B cells (CD20), for natural killer cells (CD56), and for plasmacytoid dendritic cells (CD123) (Figure 4*A*, Supplementary Figure 1 and Supplementary Table 8). In general, the liver parenchyma of AHC showed more inflammatory infiltrates than CHC and most of these cells were positive for CD3 (Figure 4*B*). Co-localization analysis revealed that AHC areas with

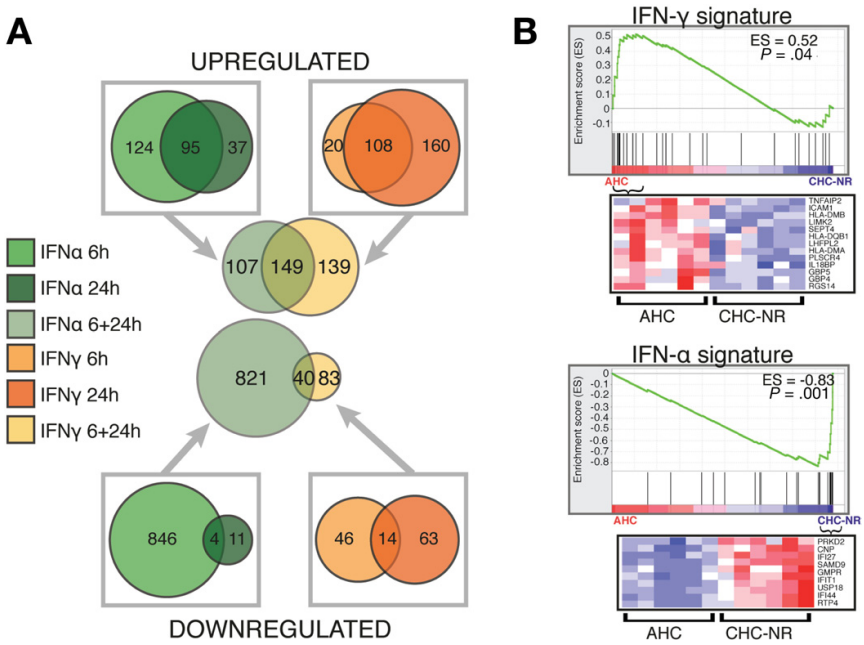

Figure 3. IFN-γ–specific gene signature is enriched in the AHC gene expression profiles, whereas IFN-α–induced transcription patterns characterize CHC-NR patients. (*A*) Venn diagrams of genes differentially expressed in PHH upon IFN-α or IFN-γ treatment. Diagrams in *gray boxes* show temporal patterns of IFN-induced gene expression in PHH, with genes differentially regulated at 6 and at 24 hours. In the *middle*, the overlap between the sets of genes differentially regulated by IFN-α (*green*) and IFN-γ (*orange*) at any of the 2 time points is shown. (*B*) Genes were rank-ordered based on differential expression between the AHC and CHC-NR patients and the overrepresentation of the experimentally defined IFN-α– and IFN-γ–specific gene sets at the top and bottom of the list was assessed by the gene set enrichment analysis (GSEA) algorithm. *Below* the GSEA plots are heatmaps of the genes that contribute to the enrichment score of the gene set tested.

high amounts of phospho-STAT1–positive hepatic nuclei were associated with high numbers of CD3+ and CD8+ cells, but not with CD20+, CD56+, or CD123+ cells (Figure 4*A*). We did not observe a co-localization of any of these cell types with phospho-STAT1–positive hepatocytes in CHC-NR samples (Figure 4*A*). There was a statistically significant correlation of STAT1 phosphorylation with the amount of CD3+ cells (Spearman $r = 0.70$; $P < .0001$) and CD8+ cells in AHC (Spearman $r = 0.69$; $P < .0001$; Figure 4*C*). We detected a positive correlation of CD8+ cells and IFN-γ mRNA levels in AHC and a co-localization of CD8 and IFN-γ (Figure 4*D* and *E*). In addition, enrichment analysis of gene ontology terms and *Kyoto Encyclopedia of Genes and Genomes* pathways revealed a significant overrepresentation of categories related to T-cell activation in AHC compared with CHC-NR patients (Supplementary Figure 3*B*). Taken together, these data provide strong evidence that infiltrates of CD8+ T cells in the liver of patients with AHC are responsible for IFN-γ production and induction of the Janus kinase-Signal Transducer and Activator of Transcription (Jak-STAT) signaling pathway.

USP18 *Expression Correlates With Treatment Response to PegIFN-α*

Nonresponse to treatment with pegIFN-α and ribavirin in CHC is associated with a general up-regulation of ISGs in the liver, but the molecular mechanism linking ISG induction to IFN nonresponse remains unknown.[6–8] In the present study, we found a similar extent of ISG up-regulation in AHC samples, but most of the patients either cleared HCV spontaneously or responded to therapy (Figure 1 and Table 1). We therefore hypothesized that the IFN-α–driven ISG set in CHC included specific genes that are not up-regulated by IFN-γ in AHC. Because negative feedback inhibition of Jak-STAT signaling pathways could underlie treatment nonresponse,[9,23] we analyzed the expression of pathway inhibitors in AHC and CHC liver biopsy samples. Suppresor of cytokine signaling 1 (*SOCS1*) and *SOCS3*, 2 IFN-induced negative regulators of IFN signaling, showed no difference between AHC and CHC (data not shown). However, ubiqitin-specific peptidase 18 (*USP18*), a more recently discovered negative regulator that is instrumental for the refractory state of IFN signaling in the mouse liver,[9,24] was up-regulated significantly in CHC-NR patients compared with CHC-R and AHC (Figure 5*A*). The induction of USP18 in CHC-NR also was apparent on the protein level (Figure 5*B* and *C*). In our microarray analysis of IFN-treated PHH, *USP18* was induced preferentially by IFN-α (Supplementary Table 5). This finding also was confirmed on the protein level in Huh-7 cells stimulated by either IFN subtype (Figure 5*D*). Indeed, *USP18* was induced

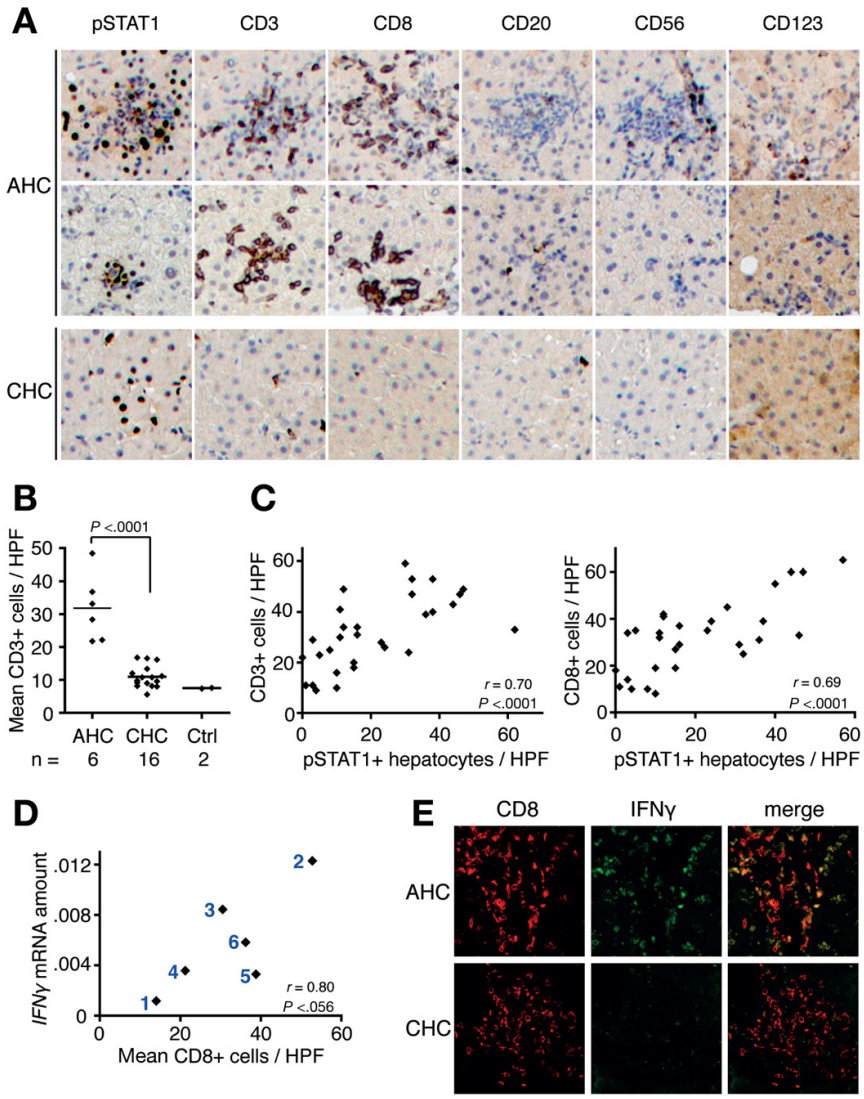

Figure 4. In AHC, phospho-STAT1–positive hepatocytes co-localize with CD8+ T cells. (*A*) Representative pictures of serial sections from liver biopsy specimens analyzed immunohistochemically for phospho-STAT1 and markers for T cells (CD3), cytotoxic T cells (CD8), B cells (CD20), natural killer cells (CD56), and plasmacytoid dendritic cells (CD123). For each section within the sample, the same detail is shown. In AHC pSTAT1+ hepatocytes co-localized with immune cells positive for CD3 and CD8. (*B*) Number of mean CD3+ cells per HPF of the liver parenchyma. Each *dot* represents the mean number per patient. *P* value was obtained by the Student *t* test. (*C*) Correlation analysis of the number of CD3+ cells and CD8+ cells/HPF with the number of nuclear phospho-STAT1 signals in hepatocytes/HPF (n = 30). The values represent the number of positive cells counted in 5 random HPF in the parenchyma of each biopsy specimen in AHC patients, which are shown in Supplementary Figure 4 and listed in Supplementary Table 8. Each *dot* represents 1 HPF. Association was assessed by Spearman correlation analysis. (*D*) Correlation analysis of the mean number of CD8+ cells with the *IFN-γ* mRNA amount in AHC (Pearson correlation). *Numbers* denote AHC patients listed in Table 1. (*E*) Co-localization analysis of IFN-γ and CD8 in AHC and CHC-NR biopsy specimens by immunofluorescence. For CHC-NR, a portal tract is depicted owing to lack of parenchymal T-cell infiltration.

almost exclusively by IFN-α. The low-level induction of USP18 by IFN-γ was not sufficient to attenuate (peg)IFN-α–mediated signaling (Figure 5*E*).

Discussion

The study of the acute phase of HCV infection in human beings is hampered by the fact that most cases are asymptomatic. Spontaneous clearance occurs in about 20%–30% of patients.[25] Studies of subjects after needle-stick injuries revealed a very rapid increase of HCV viral load to maximal levels within the first 2–4 weeks.[20] Viral replication then is slowed down, most likely by an innate immune response involving the induction of ISGs in the liver.[14,19] HCV-specific T cells are detectable 5–9 weeks

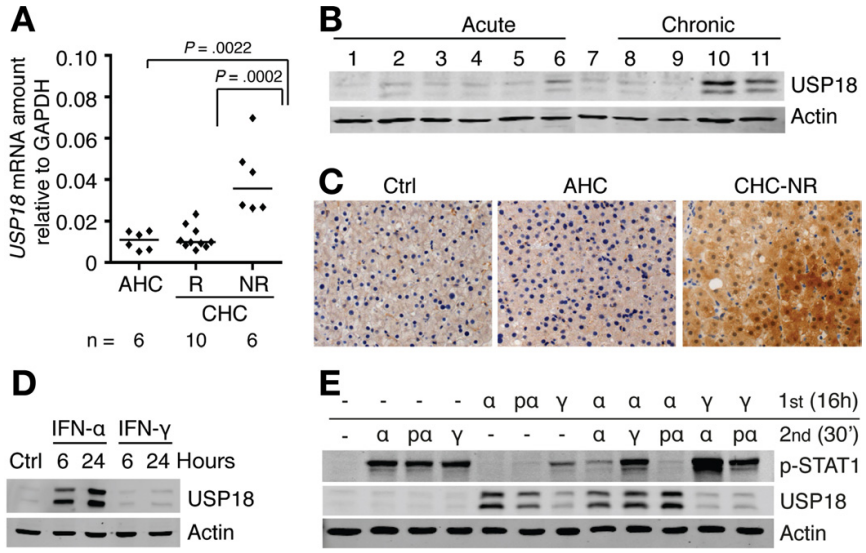

Figure 5. *USP18* expression in AHC and CHC liver biopsy specimens. (*A*) Hepatic expression of *USP18* mRNA measured by quantitative PCR and normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Each *dot* represents one sample. The *line* indicates the median. *P* values were obtained by Mann–Whitney tests. (*B*) USP18 protein expression by Western blot analysis using whole-cell extracts of liver samples from AHC (*lanes 1–6*, number according to Table 1), healthy liver (*lane 7*), CHC-R (*lanes 8* and *9*), and CHC-NR (*lanes 10* and *11*). (*C*) Representative pictures of immunohistochemistry for USP18 in healthy liver (Ctrl), AHC, and CHC-NR, showing a strong cytoplasmic and patchy staining in CHC-NR (magnification, 400×). (*D*) USP18 protein expression by Western blot analysis using whole-cell extracts of Huh-7 cells treated with IFN-α and IFN-γ for 6 or 24 hours, and untreated cells (Ctrl). (*E*) Huh-7 cells were treated for 16 hours with IFN-alfa (α), pegIFN-alfa (pα), or IFN-γ (γ), rested for 8 hours in IFN-free medium, and restimulated for 30 minutes. Expression of USP18 and STAT1 phosphorylation were assessed by Western blot.

after infection, accompanied by an increase in alanine aminotransferase levels and a decline of the serum viral load.[20] Liver biopsy studies in chimpanzees documented the presence of HCV-specific CD8+ T cells and an increase in intrahepatic IFN-γ mRNA during this period of viral decline.[14,19] In the present study, we analyzed human liver biopsy specimens obtained 2–5 months after HCV infection (ie, during the early phase of the adaptive immune response). In accordance with the chimpanzee studies, we found CD8+ T-cell infiltrates, increased intrahepatic IFN-γ mRNA expression, and an increase in alanine aminotransferase level. Importantly, T-cell infiltrates positive for IFN-γ were found in direct proximity of hepatocytes positive for nuclear phospho-STAT1 immunostaining, providing evidence that the predominant mediator of STAT1 activation in the hepatocytes is IFN-γ secreted by infiltrating T cells (Figure 4). The microarray analysis of ISG expression revealed a strong enrichment of IFN-γ–specific ISGs in AHC liver biopsy samples, further confirming that the predominant IFN in this phase of HCV infection is IFN-γ and not IFN-α. These results do not support the hypothesis that liver-infiltrating HCV-specific T cells are stunned, with impaired IFN-γ production, and are therefore not capable to clear the infection.[20,26,27] Our results are more consistent with a model in which recruitment of T cells, IFN-γ secretion by T cells, and IFN signaling in hepatocytes is intact, but the induction of hundreds of ISGs is barely effective, either because of a block of translation of ISG mRNAs[28] or because of interference of viral proteins with antiviral effector systems.

Up-regulation of ISGs during the chronic phase of HCV infection also is ineffective in clearing the virus, and even strongly associated with nonresponse to therapy with pegIFN-α and ribavirin.[6–8] It is presently still not clear which IFN is the driver of this induction of ISGs because despite an IFN-α–like signature, we and others have failed to detect up-regulation of type I IFNs in human or chimpanzee CHC liver biopsy specimens.[29,30] We have shown previously that in liver biopsy specimens of CHC patients with persistently induced ISG expression, nuclear phospho-STAT1 staining is detectable in 40%–80% of hepatocytes already in pretreatment samples, and that this number does not increase in biopsy specimens obtained 4 hours after the injection of pegIFN-α.[7] In such pre-activated livers, STAT1 phosphorylation seems to be refractory to further IFN-α stimulation. These findings can explain why about half of the patients with CHC do not respond to treatment with pegIFN-α and ribavirin. On the other side, patients with AHC have an excellent, more than 90%, response rate to treatment with pegIFN-α, even when given as monotherapy. Before our present study, an attractive hypothesis to explain the efficacy of pegIFN-α in AHC postulated the lack of ISG induction in AHC. The seminal findings that the HCV protease NS3/4A can cleave and inactivate TRIF (also known as TICAM1, toll-like receptor adaptor molecule 1) and MAVS, two impor-

tant components of cellular pathways involved in viral sensing and IFN-β induction, provided a molecular mechanism to explain the lack of induction of the endogenous hepatic IFN system.[31,32] However, we could not detect cleaved MAVS in any of the 6 AHC biopsy samples (Supplementary Figure 5). Furthermore, microarray analysis studies of liver biopsy specimens from chimpanzees during the acute phase of HCV infection revealed a strong induction of ISGs.[12,13,33] These findings do not support the hypothesis that efficient MAVS cleavage is a central viral escape mechanism by preventing the induction of the IFN system. Our present study in human liver biopsy specimens confirms these findings by showing a strong activation of STAT1 and ISG induction during AHC. However, although biopsy specimens were obtained during the entire course of AHC in the chimpanzee studies, we obtained the biopsy specimens in the late phase of AHC. Therefore, we cannot exclude that TRIF and/or MAVS cleavage are important viral escape mechanisms in the very first weeks after infection in human beings.

In a previous study in mice, we identified USP18 as a key mediator of IFN-α refractoriness.[9] Here, we show that USP18 is up-regulated in CHC-NR but not in AHC patients. Comparison of responders vs nonresponders to pegIFN-α in a combined analysis including AHC, CHC-R, and CHC-NR showed that USP18 induction is associated with nonresponse to pegIFN-α. Its preferential induction by IFN-α can explain the low expression levels in patients with AHC, in which ISG induction is predominantly IFN-γ driven. Because USP18 is an important mediator of refractoriness to IFN-α signaling, the apparent lack of its induction in AHC might explain the markedly improved response rate to pegIFN-α treatments in these patients compared with patients with CHC.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2012.05.044.

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M.T.D. and Z.M. contributed equally to this work.

Conflicts of interest
The authors disclose no conflicts.

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Supplementary Materials and Methods

RNA Extraction and Microarray Hybridization

Total RNA was extracted from human liver tissue and PHH using Qiazol reagent and the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instruction. For the 3' in vitro transcription (IVT) arrays, 1 μg of total RNA from each sample was reverse transcribed using Genechip 3' IVT Express Kit (Affymetrix) according to the manufacturer's instructions. For the whole-transcript arrays, 500 ng of total RNA was reverse transcribed and biotinylated with the whole-transcript Expression Kit (Ambion, Zug, Switzerland) and whole-transcript Terminal Labeling Kit (Affymetrix) according to the manufacturer's instructions. The Hybridization and Wash Kit (Affymetrix) was used to hybridize all samples.

Biostatistical Analysis

Microarray data were preprocessed using standard robust multiarray averaging (RMA) algorithm. Batch effects observed between the human liver samples processed and hybridized at different times were corrected using the ComBat algorithm.[1] Probe sets with very low expression intensities (<80 in the highest-expressing sample) as well as the control probe sets were excluded from the subsequent analyses. Genome-wide hierarchical clustering of the human liver samples was performed using Ward's linkage method, with 1 – Pearson correlation as a distance metric. Differential gene expression for AHC, CHC-R, and CHC-NR vs control samples was assessed using the *limma* package,[2] with fold-change cut-off value of 2 and a false discovery rate cut-off value of 0.05. To calculate the false discovery rate, moderated t-statistics were first generated using the empiric Bayes method, as implemented in the *limma* package, and the obtained *P* values were corrected for multiple testing using Benjamini and Hochberg[3] adjustment. Enrichment of gene ontology biological process terms was performed using the list of genes significantly up-regulated in AHC patients with respect to CTRL patients. Significance estimation of the enrichment analysis was performed using a hypergeometric test as implemented in g:Profiler software.[4] Terms with *P* values less than 10^{-6} were clustered into distinct groups based on the gene ontology hierarchy. The enrichment scores were calculated for each cluster ($-\log_{10}$ of the geometric mean of the *P* values for all categories in a cluster).

Area-proportional Venn diagrams were created with the help of BioVenn software.[5]

Two gene sets for the gene set enrichment analysis[6] were obtained as follows (Supplementary Figure 3*A*): (1) 2 initial probe set lists were derived from the PHH expression data set based on up-regulation in IFN-α– or IFN-γ–treated samples compared with untreated samples (fold-change between the means of treated and untreated samples above 2 at least at one time point); (2) within the 2 lists we selected probe sets for which at least at one time point there was more than a 2-fold difference between the means of IFN-α– and IFN-γ–treated samples, with the *P* value from a Welch *t* test between the corresponding samples less than .05. These 2 lists then were annotated with gene symbols and their enrichment was assessed in AHC vs CHC-NR samples with java gene set enrichment analysis software version 2.07 (Broad Institute, Cambridge, MA), using the signal-to-noise ratio as a ranking metric. On the gene set enrichment analysis plot (Figure 3*B*), the x-axis represents a list of all genes on the array rank-ordered according to their decreasing correlation with AHC phenotype (red, genes overexpressed in AHC; blue, genes overexpressed in CHC-NR). Black ticks along the x-axis show positions of genes that are part of the tested gene set. The y-axis unit is the enrichment score defined as a running-sum statistic calculated walking down the ranked gene list. The running-sum increases when a gene in the ordered list is present in the gene set in question and decreases when it is absent. The increment of the enrichment score depends on the value of the ranking metric.

Enrichment of *Kyoto Encyclopedia of Genes and Genomes* pathways and gene ontology biological process terms in lists of genes significantly altered between AHC and CHC-NR was assessed using DAVID software version 6.7.[7] To facilitate the interpretation, terms with *P* values (modified Fisher exact test) less than .05 were grouped based on the overlapping gene membership. The enrichment score is equal to $-\log_{10}$ of the geometric mean of the *P* values for all categories in a cluster.

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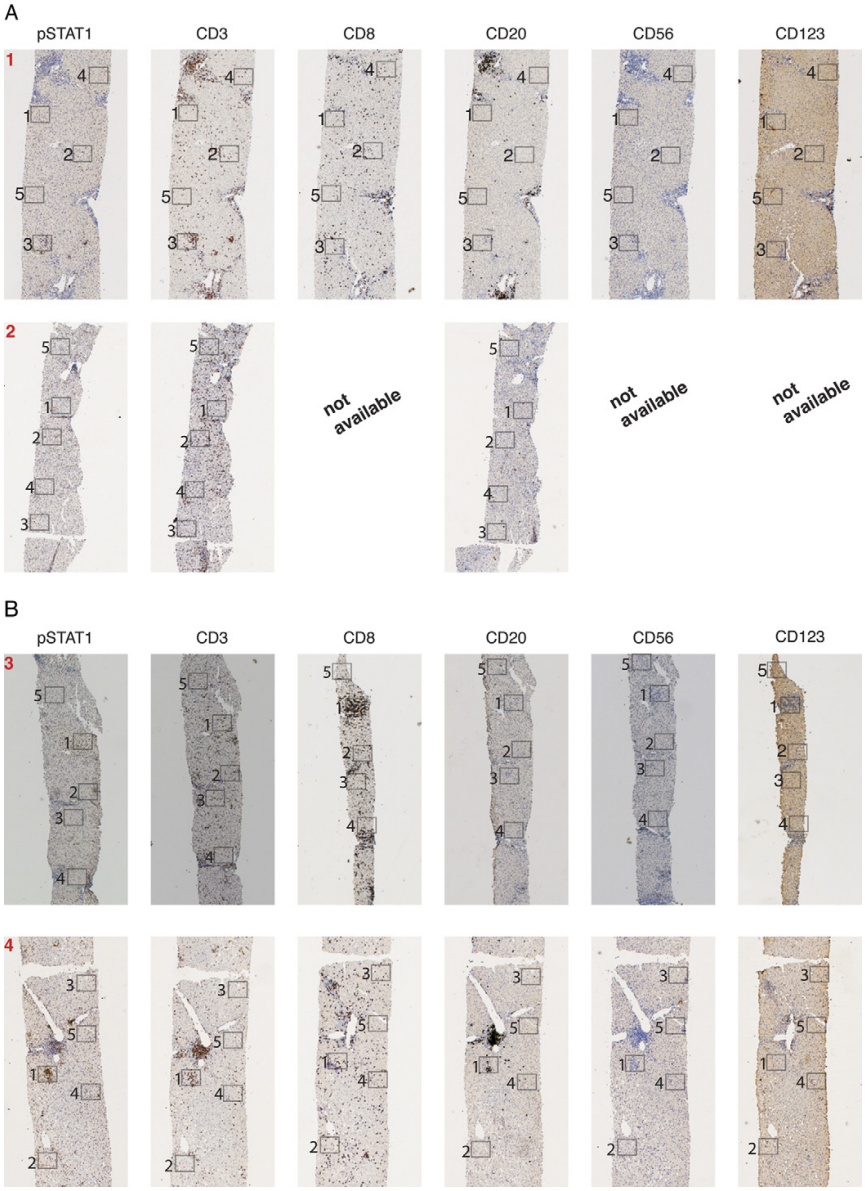
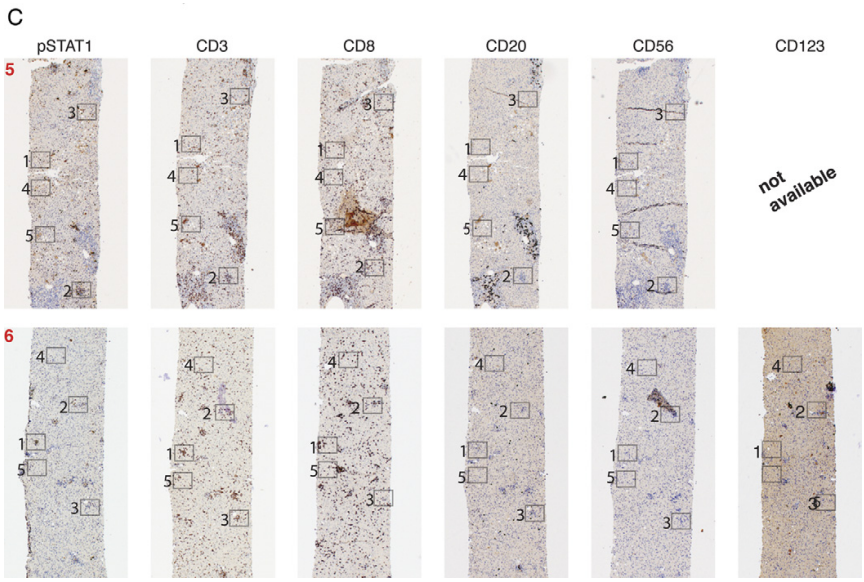
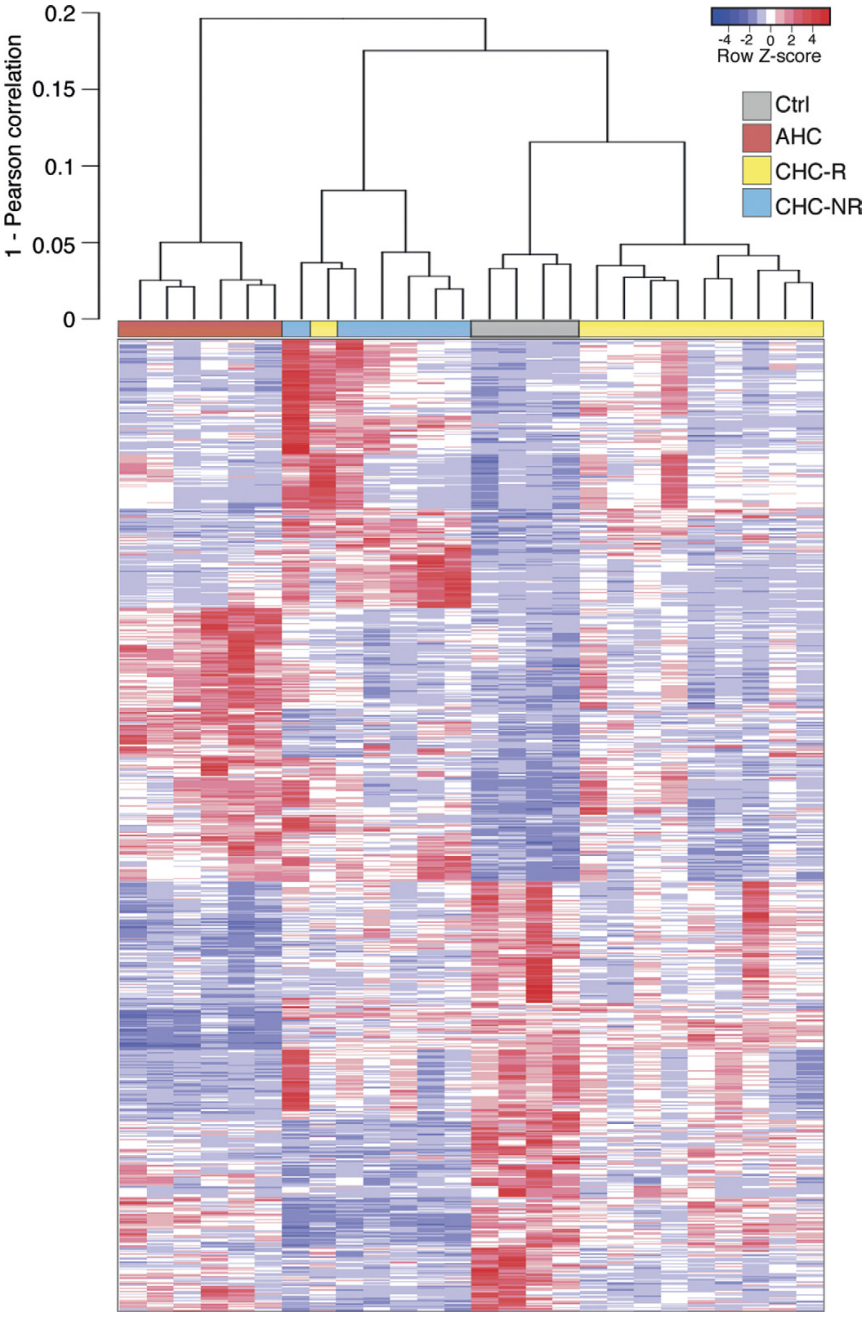


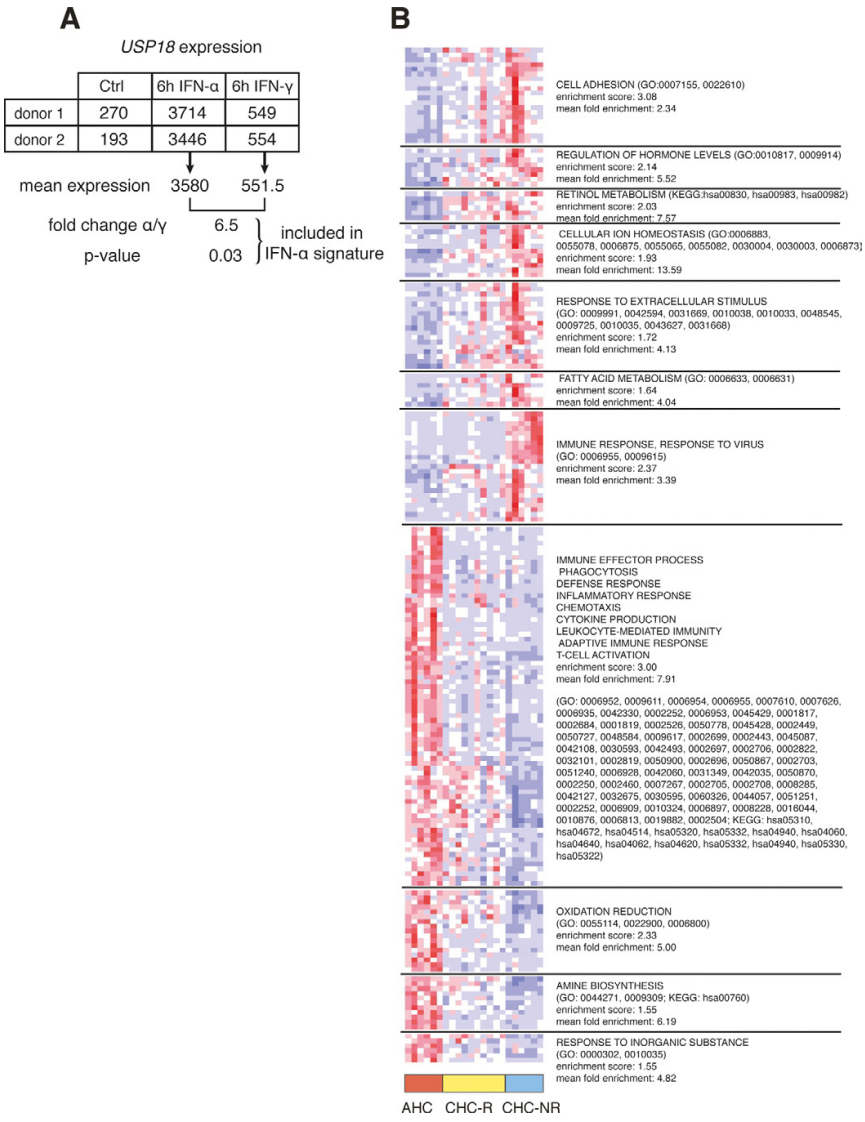
Supplementary Figure 1. Collection of all liver biopsy serial sections from AHC patients numbers 1–6 (*red number*), immunohistochemically stained for pSTAT1 and markers for T cells (CD3), cytotoxic T cells (CD8), B cells (CD20), natural killer (NK) cells (CD56), and plasmacytoid dendritic cells (CD123). The 5 boxes per slide indicate the high-power fields in the liver parenchyma that were chosen randomly for the co-localization analysis.



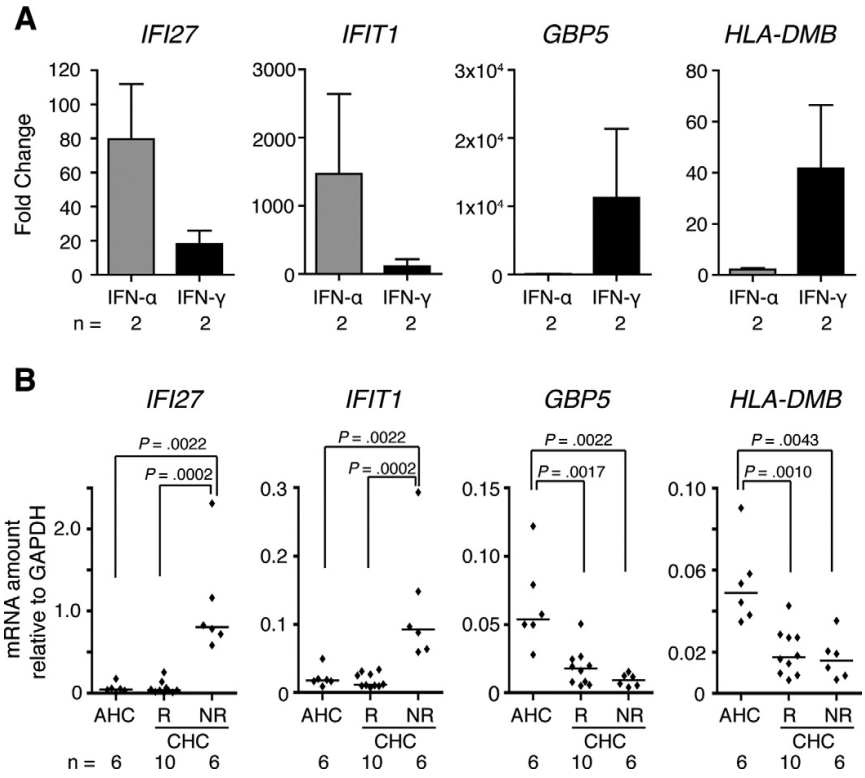
Supplementary Figure 1. (Cont'd).



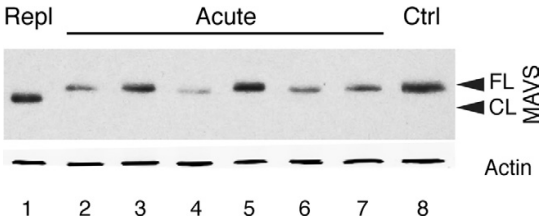
Supplementary Figure 2. Genome-wide unsupervised hierarchical clustering groups all AHC patients in a distinct cluster, separate from the CHC or control samples. The heatmap shows the expression patterns of 1003 probe sets identified as up-regulated or down-regulated in at least one of the HCV-infected groups (AHC, CHC-NR, CHC-R) compared to the healthy liver control group.



Supplementary Figure 3. (*A*) Scheme representation of the generation of gene sets for gene set enrichment analysis (GSEA). A gene was included if the difference between the means of IFN-α– and IFN-γ–treated PHH was larger than 2-fold and the *P* value from a Welch *t* test of the corresponding samples was less than .05. The example in the scheme shows expression values for *USP18* after 6 hours of IFN-α or IFN-γ treatment. (*B*) Gene ontology biological process terms and *Kyoto Encyclopedia of Genes and Genomes* collection pathways were tested for overrepresentation in lists of genes significantly altered between AHC and CHC-NR patients. Enriched categories then were clustered to bring together closely related terms. The heatmap shows expression patterns of all differentially expressed genes that belong to one of the enriched categories.



Supplementary Figure 4. Confirmation of microarray data by quantitative RT-PCR. (*A*) Quantification of IFN-α–specific ISGs (*IFI27* and *IFIT1*) and IFN-γ–specific ISGs (*GBP5* and *HLA-DMB*) by quantitative RT-PCR in IFN-α– or IFN-γ–treated PHH confirmed specific induction as previously assessed by the microarray analysis (time point, 24 h). (*B*) Quantitative RT-PCR confirmed up-regulation of IFN-α ISGs (*IFI27* and *IFIT1*) in CHC-NR and up-regulation of IFN-γ ISGs (*GBP5* and *HLA-DMB*) in AHC. (*P* values were by Mann–Whitney test).



Supplementary Figure 5. Analysis of MAVS cleavage by Western blot. *Arrowheads* indicate full-length (FL) and cleaved (CL) MAVS. Lysates from Huh-7.5 cells harboring a subgenomic HCV replicon (Repl, *lane 1*) and healthy liver (Ctrl, *lane 8*) served as controls for cleaved and full-length MAVS. Lysates from AHC patients 1–6 (according to Table 1) are displayed in *lanes 2–7*.

3.3 Induction of type I and II interferons drives the biomodal kinetics of response to a novel toll-like receptor 9 agonist in mouse liver

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Sequential induction of type I and II interferons mediates a long-lasting gene induction in the liver in response to a novel synthetic toll-like receptor 9 agonist

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Abbreviations: HCV, hepatitis C virus; IFN, interferon; TLR, Toll-like receptor; Jak-STAT, Janus kinase-signal transducer and activator of transcription; ISG, interferon-

stimulated gene; PBMCs, peripheral blood mononuclear cells; IMO, immunomodulatory oligonucleotide; KO, knockout; PBS, phosphate-buffered saline; RT-PCR, real-time quantitative polymerase chain reaction; CT, threshold cycle; FDR, false discovery rate; GO, gene ontology; NPLC, non-parenchymal liver cell

ABSTRACT

Background & Aims: The toll-like receptor 9 (TLR9) agonist IMO-2125 is currently evaluated in clinical trials for chronic hepatitis C therapy. The aim of this study was to investigate the *in vivo* mode of action of a closely related compound, referred to as immunomodulatory oligonucleotide (IMO).

Methods: We analyzed the Jak-STAT pathway activation and induction of interferon-stimulated genes in the liver of wild-type, interferon-α/β receptor-deficient and interferon-γ-deficient mice after administration of IMO.

Results: IMO induced a prolonged activation of the Jak-STAT pathway and upregulation of interferon-stimulated genes in mouse liver. Contrary to the response observed after interferon-α injection, the signalling induced by IMO was not abrogated following repeated administration.

At early time points after IMO injection STAT1 phosphorylation and interferon-stimulated gene induction required a functional interferon-α/-β receptor, whereas at the later time points the activation was type I interferon-independent. Microarray analysis revealed that IMO induced broad transcriptional response in the mouse liver. This included upregulation of cytokine and chemokine genes responsible for recruitment of IFN-γ producers such as T cells and natural killer cells. Interferon-γ-deficient mice showed a transient response to IMO, demonstrating the central role of interferon-γ in sustained activation of Jak-STAT pathway by IMO.

Conclusions: The bimodal kinetics of response to IMO in the mouse liver are driven by the sequential endogenous production of type I and II interferons. The lack of refractoriness to IMO combined with the long-lasting induction of interferon-stimulated genes reveal a favourable pharmacodynamics profile of this novel TLR9 agonist for the treatment of chronic viral hepatitis.

Keywords: Toll-like receptor 9**,** Interferon**,** Hepatitis C virus**,** Liver**,** Oligonucleotide

INTRODUCTION

Chronic infection with hepatitis C virus (HCV) is a major cause of liver disease worldwide (1). Current standard of care for chronic hepatitis C consists of pegylated interferon (IFN)-α and ribavirin, complemented with direct-acting antiviral drugs for HCV genotype 1 (2). A substantial proportion of patients has an induction of the endogenous IFN system in the liver already before therapy, and consequently respond poorly to IFN-α treatments (3-5), probably because of refractoriness of the IFN-α signal transduction pathway (6). A number of therapeutics are presently in clinical development with the aim to improve the treatment outcome of IFN-α non-responders. One of the compounds evaluated in current clinical trials for chronic hepatitis C therapy is IMO-2125, a novel synthetic Toll-like receptor (TLR) 9 agonist (7).

TLR9 ligands, such as CpG-motif containing oligodeoxynucleotides (ODNs), act by stimulating TLR9 present on the endoplasmic vesicles of the cells of the immune system (8). Ligand binding to TLR9 induces signalling cascade involving MyD88, IRAK4 and TRAF6 which culminates in nuclear translocation of transcription factors such as IRF7 and NFκB, resulting in the production of endogenous IFNs and other cytokines (reviewed in (9)). The secreted IFNs bind to their specific receptors on the cell surface and signal through the Janus kinase-signal transducer and activator of transcription (Jak-STAT) pathway to regulate the expression of the target genes. IFN-γ predominantly stimulates STAT1 and induces formation of homodimeric transcription factor complexes, whereas IFN-β and the members of IFN-α family activate both STAT1 and STAT2, resulting in the assembly of heterotrimeric transcription factor complex interferon-stimulated gene factor 3. The different IFNs induce overlapping but distinct sets of target genes (10). The products of interferon-stimulated genes (ISGs) function as the effectors of the antiviral state (11).

Oligonucleotide agonists of TLR9 have demonstrated potential in a variety of medical applications including use as vaccine adjuvants as well as mono- or combination therapies for the treatment of cancer and infectious diseases (reviewed in (12)). Supernatants from human peripheral blood mononuclear cells (PBMCs) treated with class B CpG oligonucleotides showed potent antiviral activity in HCV replicon cells (13). Similar results were obtained using mouse bone marrow-derived myeloid dendritic cells

and HCV replicon-bearing murine MH1 cells (14). Class C CpG TLR9 agonists have also been shown to induce robust IFN-α production in plasmacytoid dendritic cells from patients chronically infected with HCV (15). The activity of the CpG oligomers has been shown to depend on the sequence and secondary structures of the DNA flanking the CpG motif (16, 17). Immunomodulatory oligonucleotides (IMOs) are a novel class of TLR9 agonists which incorporate synthetic cytosine or guanine analogues. These second-generation oligonucleotides have the advantage of greater metabolic stability, species-independent activity depending on the synthetic dinucleotide motif incorporated and a clear structure-activity relationship (18-21). IMOs containing a secondary structure-forming sequence and a CpR motif (where R is a synthetic analogue of deoxyguanosine) were shown to induce IFN-α production in human PBMC cultures as well as *in vivo* in nonhuman primates (19, 20).

In the present study we investigated pharmacodynamics of an IMO, a novel TLR9 agonist related to IMO-2125, in mouse liver. The novel TLR9 agonist contains a 3'-3'-attached structure and CpR dinucleotide motifs (wherein R is a 7-deaza-deoxy-guanosine) within a sequence that allows duplex formation, but not a hairpin. It belongs to a class of compounds such as IMO-2125 that produce elevated levels of IFN-α in human plasmacytoid dendritic cells and stimulates B cell proliferation through TLR9 activation (19, 22). We found that shortly after administration IMO induced the production of type I IFNs and a variety of chemokines, leading to marked immune cell recruitment to the liver. This was followed by secretion of IFN-γ resulting in long-lasting stimulation of Jak-STAT pathway and induction of ISGs. There was no refractoriness to repeated injections of IMO. These pharmacodynamic properties support the development of TLR9 agonists with similar structure and activity as IMO-2125 for treatment of viral hepatitis.

MATERIALS AND METHODS

Compounds

TLR9 agonist used in this study, 5'-TCG1AACG1TTCG1-X1-G1CTTG1CAAG1CT-5' (wherein G1 is 7-deaza-dG and X1 is 1,2,4-butane triol linker) was synthesized on a MerMade 6 DNA/RNA synthesizer with phosphorothioate (PS) backbone using β-

cyanoethylphosphoramidite chemistry at Idera Pharmaceuticals as described previously (23). The oligonucleotide was purified on an anion-exchange HPLC, desalted, dialyzed and lyophilized. The purity of lyophilized compound was 95% with the rest being shorter by one or two nucleotides (n - 1 and n - 2) as determined by analytical anion-exchange HPLC and capillary gel electrophoresis. The sequence integrity was determined by MALDI-ToF mass spectrometry. Endotoxin was less than 0.1 EU/mL as determined by the Limulus assay.

Animals

Four- to 8-week-old male mice were used for all experiments. The animals were bred in the animal facility of the Department of Biomedicine of the University Hospital of Basel under specific pathogen-free conditions. All animal experiments were conducted with the approval of the Animal Care Committee of the Canton Basel-Stadt, Switzerland.

C57Bl/6 and IFNAR knockout (KO) mice (24) were obtained from BRL (Füllinsdorf, Switzerland) and IFN-γ KO mice (25) were from Doug Hilton. Genotyping primers for IFN-γ KO mice: Ivg33: 5'-TTC AAT GAC GCT TAT GTT GTT GCT G-3', Ivg31: 5'-CCT CAG AAC TCA AGT GGC ATA GAT-3', Ivg35: 5'-CAT TCG ACC ACC AAG CGA AAC ATC-3'. Genotyping primers for IFNAR KO mice: UM5: 5'-ATT ATT AAA AGA AAA GAC GAG GCG AAG TGG-3', UM4: 5'-AAG ATG TGC TGT TCC CTT CCT CTG CTC TGA-3', Neo: 5'-CCT GCG TGC AAT CCA TCT TG-3'. The animals were injected subcutaneously with IMO or murine IFN-α (CalBioChem) in sterile phosphate-buffered saline (PBS). Control animals were injected with PBS only. Mice were euthanized by CO_2 narcosis. Samples from the liver were collected, immediately frozen in liquid nitrogen and stored at -80°C until further processing.

RNA isolation, reverse transcription and quantitative RT-PCR

RNA was isolated from shock-frozen liver samples using Trizol Reagent (Invitrogen). Isolated RNA was quantified and 1 μg was reverse-transcribed with random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega). Prior to enzyme mix addition, the reaction mixture was incubated for 3 minutes at 70°C and then cooled on ice. Following the addition of the enzyme, reverse transcription was carried out for 1 hour at 37°C and stopped by incubation at 95°C for 5 minutes. Quantitative real-time polymerase chain reaction (RT-PCR) was performed based on SYBR green fluorescence

(Applied Biosystems). The primers were: 5'-CGG CGG AGA GAG CTT TGC-3' and 5'-AGC TGA AAC GAC TGG CTC-3' for STAT1, 5'-GTG GTT GTG GAG GGT GAG ATG-3' and 5'-GGG ATG AGG TCT CCA GCC A-3' for SOCS1, 5'-CGT GCT TGA GAG GGT CAT TTG-3' and 5'-GGT CGG GAG TCC ACA ACT TC-3' for USP18, 5'-ATC CGC AAG CCT GTG ACT GT-3' and 5'-TCG GGC CAG GGT GTT TTT-3' for RPL19. All reactions were run in duplicate using an ABI 7500 detection system (Applied Biosystems). The ΔCT value was derived by subtracting the threshold cycle (CT) value for RPL19, which served as an internal control, from the CT values for STAT1, SOCS1 and USP18. The RNA expression levels of the transcripts were calculated relative to RPL19 using the formula $2^{-\Delta CT}$.

Microarray analysis

RNA from shock-frozen liver samples was extracted with Qiazol (Qiagen) and purified on a RNeasy Mini column kit (Qiagen). Reverse transcription, second strand synthesis and *in vitro* transcription were performed according to manufacturer's instructions (Ambion). The samples were hybridized overnight to Affymetrix Mouse Gene ST 1.0 arrays (Affymetrix). Microarray analysis was carried out with Bioconductor packages of R statistical environment (26). Normalization, background correction and summarization on the level of transcript clusters was performed using RMA algorithm implementation of *oligo* package (27, 28). Genes were identified as differentially expressed if they showed a mean fold change above 2 between the treated and control samples with corresponding false discovery rate (FDR) below 0.1 (moderated t-test as implemented in the *limma* package followed by Benjamini-Hochberg *p*-value adjustment for multiple testing) (29). Significance estimation of the Gene Ontology (GO) Biological Process enrichment analysis was carried out using a hypergeometric test as implemented in g:Profiler software (30). Terms with p-values below 10^{-5} were clustered into distinct groups based on the GO hierarchy. The enrichment scores were calculated for each cluster (–log10 of the geometric mean of the p-values for all categories in a cluster).

Western blot

Tissue extracts and western blots (protein immunoblots) were done as described (6). Proteins were detected with primary antibodies specific to phospho-STAT1 (Tyr701, catalog No 9171; Cell Signaling Technology), STAT1 (catalog no. 610186; Transduction

Laboratories), phospho-STAT2 (Tyr 689, catalog no. 07-224; Upstate Biotechnology), phospho-STAT3 (Tyr 705, catalog no. 9131; Cell Signaling), STAT2 (catalog no. sc950, Santa Cruz Biotechnology), STAT3 (catalog no. sc482, Santa Cruz Biotechnology) and β-actin (Sigma-Aldrich Chemie GmbH).

Histochemical staining

Mouse liver samples were fixed overnight in 4% buffered formalin and embedded in paraffin blocks. Hematoxylin-eosin staining was performed according to the standard procedure.

Isolation of mouse hepatocytes and non-parenchymal liver cells

Buffer I containing 136mM NaCl, 2.7mM KCl, 0.8mM Na_2HPO4, 25mM Hepes, 0.5mM EGTA and buffer II containing 136mM NaCl, 2.7mM KCl, 0.8mM Na_2HPO4, 25mM Hepes, 5mM $CaCl_2$, 25μg/ml Liberase TM (Roche) were prepared and kept at 37°C prior to the isolation procedure. C57BL/6 mice were perfused through the portal vein with buffer I for 5 minutes at 37°C (flow rate 160ml/h) and then with buffer II for another 5 minutes at 37°C (flow rate 200ml/h). The liver was removed and cells were dispersed in cold Hank's balanced salt solution (HBSS, Gibco). Cell dispersion was filtered through a 70μm nylon cell strainer and then washed with 30ml of cold HBSS. Non-parenchymal cells were collected from the supernatant after the first wash with HBSS. Primary mouse hepatocytes were collected from the pellet.

Mouse IFN-γ ELISA

Mouse serum samples were diluted 1:10 in Assay Diluent and the concentration of murine IFN-γ was tested by High Sensitivity Femto-HS ELISA (eBioscience) according to manufacturer's instructions.

RESULTS

Administration of IMO leads to a sustained activation of Jak-STAT pathway

Injection of murine IFN-α leads to a transient activation of Jak-STAT pathway in mouse liver (6). To gain insight into the signalling patterns induced in the mouse liver by the novel TLR9 agonist, we injected the animals subcutaneously with 3 μg/g bodyweight (bw) IMO and analyzed liver samples collected at different time points after administration. We found that a single injection of IMO resulted in a bimodal pattern of

STAT1 activation (Figure 1A). Phospho-STAT1 signal first appeared 2 hours after IMO administration and decreased to almost undetectable levels 4 hours later. The second peak of phosphorylation was observed 12 hours post-injection, and the activation of STAT1 continued until as late as 96 hours after administration. On the other hand, STAT2 phosphorylation was only detectable 2 and 4 hours after administration. The signalling induced by IFN-α leads to activation of both STAT1 and STAT2 ((31), Figure 1A). The absence of STAT2 phosphorylation at the late time points after IMO injection suggested that the second peak of STAT1 activation was not mediated by IFN-α. STAT3 phosphorylation was first observed 1 hour after injection, peaked 1 hour later and remained detectable at low levels until 96 hours post-administration. The sustained signalling through the Jak-STAT pathway led to a long-lasting upregulation of ISGs such as STAT1, SOCS1 or USP18 (Figure 1B). This is in contrast to the activation pattern elicited by murine IFN-α, which was characterized by a potent, but short-lived Jak-STAT pathway stimulation.

Signalling induced by IMO does not become refractory

Administration of IFN-α is known to elicit a refractory state in mouse liver (6). Refractory cells are not responsive to further stimulation with IFN-α and this phenomenon was shown to depend on the presence of USP18 (6). We investigated whether repeated stimulation with IMO also results in desensitization of the mouse liver. Mice were injected with 1 μg/g bw IMO. At this dose of IMO induced STAT1 activation that began to recede 48 hours post-injection and returned to baseline 24 hours later. We administered a second dose of TLR9 agonist 48 hours after the first injection and analyzed Jak-STAT signalling in the liver. The second injection of IMO resulted in efficient re-induction of STAT1 and STAT3, but not STAT2 phosphorylation (Figure 2A). In contrast, the second dose of IFN-α administered 8 hours after the first injection did not lead to the Jak-STAT pathway activation. In consequence, transcription of ISGs could be re-induced in IMO-, but not IFN-α-treated mice (Figure 2B).

IMO upregulates cytokine and chemoattractant genes

To characterize the transcriptional response to IMO we performed microarray analysis of the mouse liver samples 4 hours after a single injection of 1000 IU/g bw IFN-α, 3 μg/g

bw IMO or PBS (control). We found a broad transcriptional induction following IMO injection, with over 450 genes upregulated more than 2-fold with false discovery rate below 0.1 compared to PBS-injected mice (Figure 3A). At the same time IFN-α administration led to upregulation of 296 genes in the mouse liver, and almost 80% of those were also significantly induced by IMO. Gene ontology enrichment analysis of the genes regulated by IMO revealed terms such as cytokine biosynthesis, activation of immune response, cellular response to interferon and regulation of cell death and proliferation (Supplementary Table 1). Several chemokine genes were significantly more induced by IMO than by IFN-α, including chemoattractants of T cells and NK cells such as *Cxcl11*, *Cxcl10*, *Cxcl9* and *Ccl5* (Figure 3B). Immune cell recruitment to the liver was readily observed by hematoxylin-eosin staining, with focal areas of lymphocyte concentration first appearing 48 hours after IMO injection and increasing with time in size and frequency (Figure 3C, 48 and 96 hours time points shown).

Signalling induced by IMO relies on endogenous production of type I and II interferons

We observed a sustained STAT1 activation in IMO-injected animals, whereas STAT2 phosphorylation was transient (Figure 1A). To further explore the molecular underpinnings of this phenotype we examined the hepatic response to IMO in IFN-γ KO and IFNAR1 KO mice. Animals lacking IFN-γ showed intact signalling and ISG induction at early time points after IMO administration, but 12 hours post-injection or later the response was completely abrogated (Figure 4A, C, D). On the other hand, animals deficient in a functional type I IFN receptor failed to respond to IMO treatment shortly after injection, but STAT1 phosphorylation and ISG induction 24 or 48 hours post-injection were identical to the wild-type levels (Figure 4B, C, D). In agreement with these data, we were able detect elevated levels of IFN-γ in the serum of WT mice 12, 24 and 48 hours after IMO injection (Figure 4E). These findings demonstrate the central role of IFN-γ in the sustained activation mediated by IMO as well as the importance of type I IFNs in early response to this agonist in mouse liver. It is also evident that the early events involving secretion of type I IFNs are not required for later IFN-γ production.

DISCUSSION

In this study we investigated the pharmacodynamics and the mode of action of a novel synthetic TLR9 agonist,. Administration of IMO resulted in two-phase pattern of Jak-STAT pathway stimulation in the mouse liver, with initial transient activation shortly followed by a long-lasting activated state. We demonstrated that these kinetics resulted from two independent events: initial production of type I IFNs and a subsequent production of IFN-γ. In contrast to the response elicited by exogenous IFN-α injections, repeated treatment with IMO did not result in the refractoriness of the Jak-STAT pathway. These findings improve our understanding of the *in vivo* action of the IMO class of TLR9 agonists, compounds currently evaluated in clinical trials for many diseases including chronic hepatitis C infection.

Chronic hepatitis C infection is presently treated with pegylated IFN-α combined with ribavirin and for some virus genotypes also with direct-acting antiviral drugs. Previous work of our group has demonstrated that mouse liver becomes unresponsive to IFN-α stimulation within hours after the first application of IFN-α and identified the interferon-stimulated gene USP18 as the key regulator of IFN-induced refractoriness (6). These findings suggest that desensitization may also occur upon clinical use of IFN-α and negatively influence the therapeutic outcome. Recent studies of our group and others showed that different IFN types are unequally affected by the USP18-mediated negative feedback loop (32, 33). Particularly, IFN-α-mediated signalling was demonstrated to be almost completely abrogated by previous exposure to IFN, whereas IFN-β, -λ or -γ-mediated signals were only marginally reduced. The long-lasting activation of the Jak-STAT pathway after IMO injection is induced by IFN-γ, which is not sensitive to the refractory state of the cells induced by USP18. The continuing strong activation of the signalling in the context of repeated administration could provide an advantage for TLR9 agonists such as IMO-2125 in the clinical setting.

We have not identified the cells that secrete type I and II IFNs in response to IMO in mice. We found very low levels of TLR9 expression in isolated mouse hepatocytes, suggesting that these cells are not the direct targets of the IMO (Supplementary Figure 1B). On the other hand, we observed high expression of TLR9 in isolated mouse non-parenchymal liver cells (NPLCs), raising the possibility that the NPLCs contribute to the

cytokine and chemokine production after IMO injection. In line with this hypothesis it has previously been shown that mouse non-parenchymal liver cells can produce interleukin 6, tumour necrosis factor α and IFN-β in response to TLR stimulation (14). Repeated stimulation of TLR9 in mice has been associated with adverse effects such as alterations in the architecture of lymphoid organs, liver damage and macrophage activation syndrome-like disease, raising concerns regarding the use of TLR9 agonists as treatment agents (34, 35). However, in these reports mice have been exposed to very high doses of CpG DNA: 2-3 mg/kg bw administered daily or every other day, whereas the minimal therapeutically effective dose of IMO-2125 in human subjects with chronic HCV infection has been established at 0.16 mg/kg bw twice or once weekly (7). In agreement with previous reports we found an increase in spleen size in mice 3 and 4 days after an injection of 3 μg/g bw IMO (data not shown), but no splenomegaly was found in the mice which received ten times lower dose. The dose of 0.3 μg/g bw IMO could still achieve Jak-STAT pathway activation in the mouse liver, however with altered kinetics (Supplementary Figure 1). Moreover, no severe treatment-related adverse effects have been noted in phase I clinical trials of IMO-2125 or CPG 10101, another TLR9 agonist clinical candidate (36). These findings suggest that side effects of TLR9 agonists observed in mice may be dose-dependent. The doses used in patients are likely too low to trigger the same serious adverse events described in the mouse model. Indeed, evidence of antiviral activity of IMO-2125, an analogue of the TLR9 agonist that was used in the present study, has been obtained in Phase I clinical trials in HCV patients (7). Moreover, it should be noted that TLR9 expression is wider across the cell types in mice than in humans, resulting in overestimation of TLR9 agonist-mediated toxicity in mice (rev in (37)).

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Figure 1

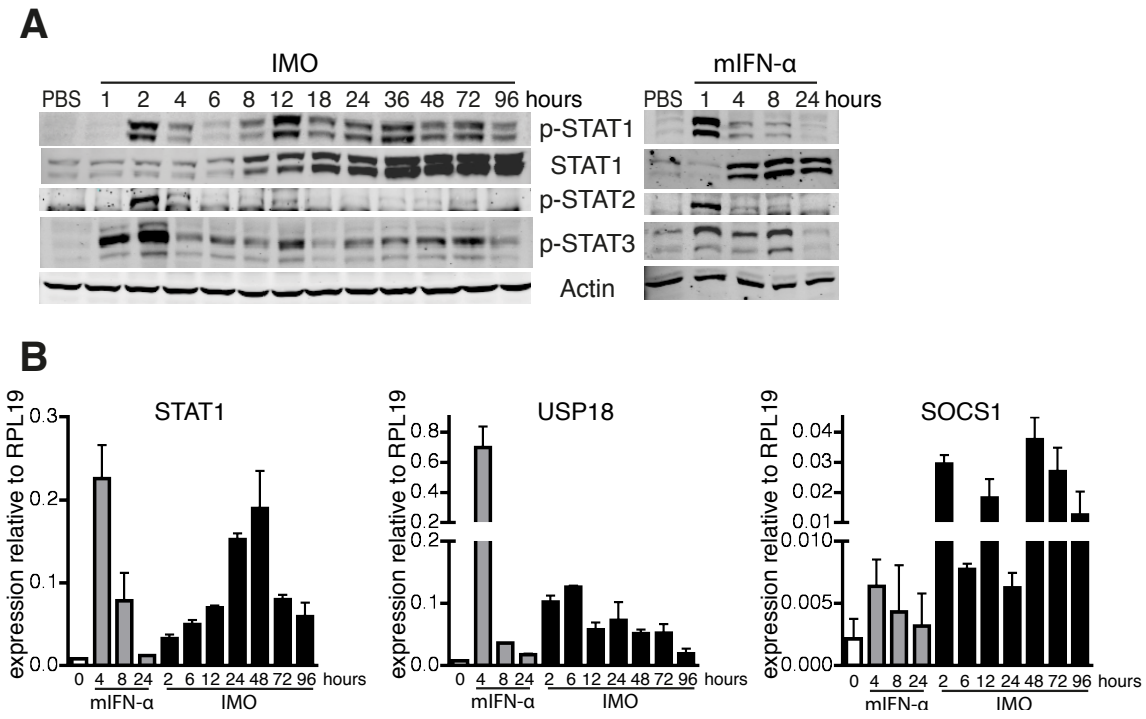

Figure 3.1: **Jak-STAT pathway activation in mouse liver after IMO and IFN-α injection** C57Bl/6 mice were injected subcutaneously with 3 μg/g bw IMO or 1000 IU/g bw mIFN-α and the liver samples were collected at indicated time points post-administration. (A) STAT1, STAT2 and STAT3 phosphorylation as well as total STAT1 protein levels induced by IMO and IFN-α treatment were assessed by immunoblot with specific antibodies. (B) IMO and IFN-α-induced expression of STAT1, SOCS1 and USP18 was quantified by RT-qPCR. The data are plotted as the amount of STAT1 or SOCS1 mRNA relative to RPL19 mRNA (mean and SEM).

Figure 2

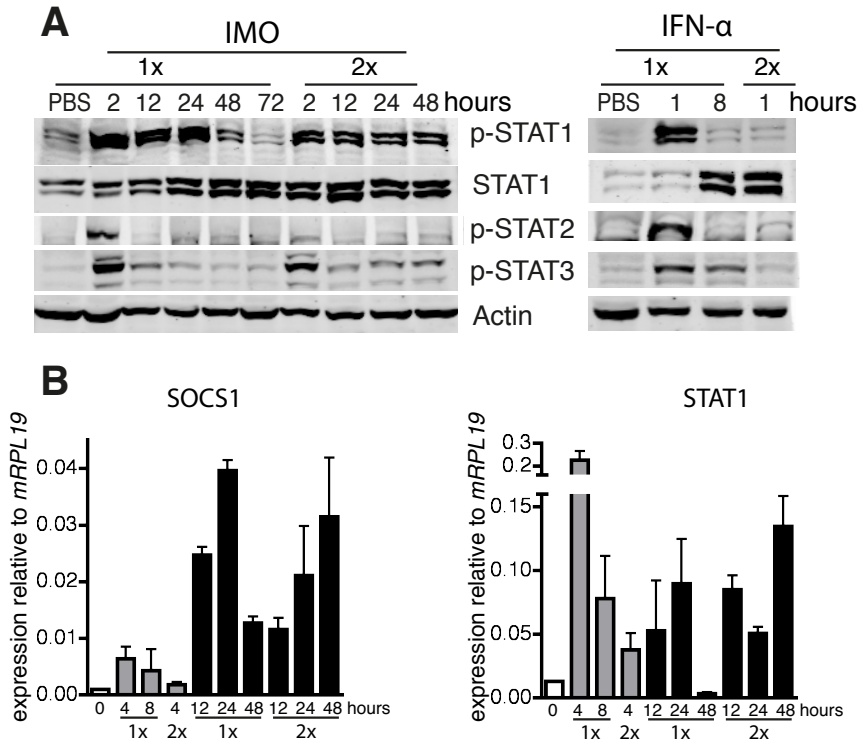

Figure 3.2: **Lack of refractoriness to IMO treatment in mouse liver** C57Bl/6 mice were injected subcutaneously with 1 μg/g bw IMO or 1000 IU/g bw mIFN-α and subsequently re-injected with the same compound 8 hours post-injection (mIFN-α) or 48 hours post-injection (IMO). Liver samples were collected at indicated time points post-administration. (A) STAT1, STAT2 and STAT3 phosphorylation as well as total STAT1 protein levels induced by single and repeated IMO and IFN-α treatments were assessed by immunoblot with specific antibodies. (B) IMO and mIFN-α-induced expression of STAT1 and SOCS1 was quantified by RT-qPCR. The data are plotted as the amount of STAT1 or SOCS1 mRNA relative to RPL19 mRNA (mean and SEM).

Figure 3

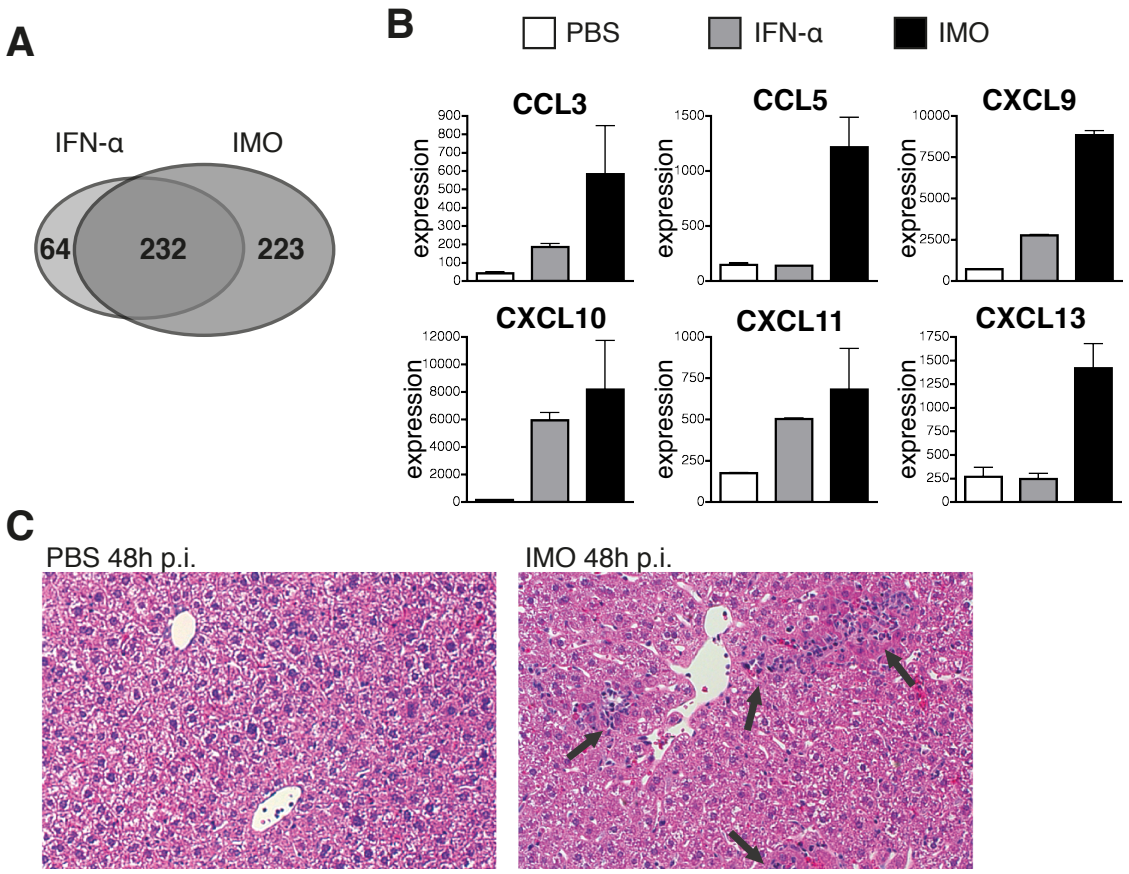

Figure 3.3: **Gene expression and immune cell recruitment induced by IMO application** C57Bl/6 mice were injected subcutaneously with 3 μg/g bw IMO and liver samples were collected at indicated time points post-administration. (A) Venn diagram showing the number of genes induced in mouse liver 4 hours after IMO (3 μg/g bw) or mIFN-α (1000 IU/g bw) treatment (fold-change above 2 and false discovery rate below 0.1). (B) Expression levels of chemoattractant genes CCL3, CCL5, CXCL9, CXCL10, CXCL11, CXCL13 in mouse liver 4 hours after PBS, IMO (3 μg/g bw) or mIFN-α (1000 IU/g bw) treatment quantified by microarray analysis (mean and SEM). (C) Hematoxylin-eosin staining of formalin-fixed, paraffin-embedded mouse liver sections 48 and 96 hours after injection of 3 μg/g bw IMO or 96 hours after PBS injection. Arrows indicate areas of lymphocyte concentration.

Figure 4

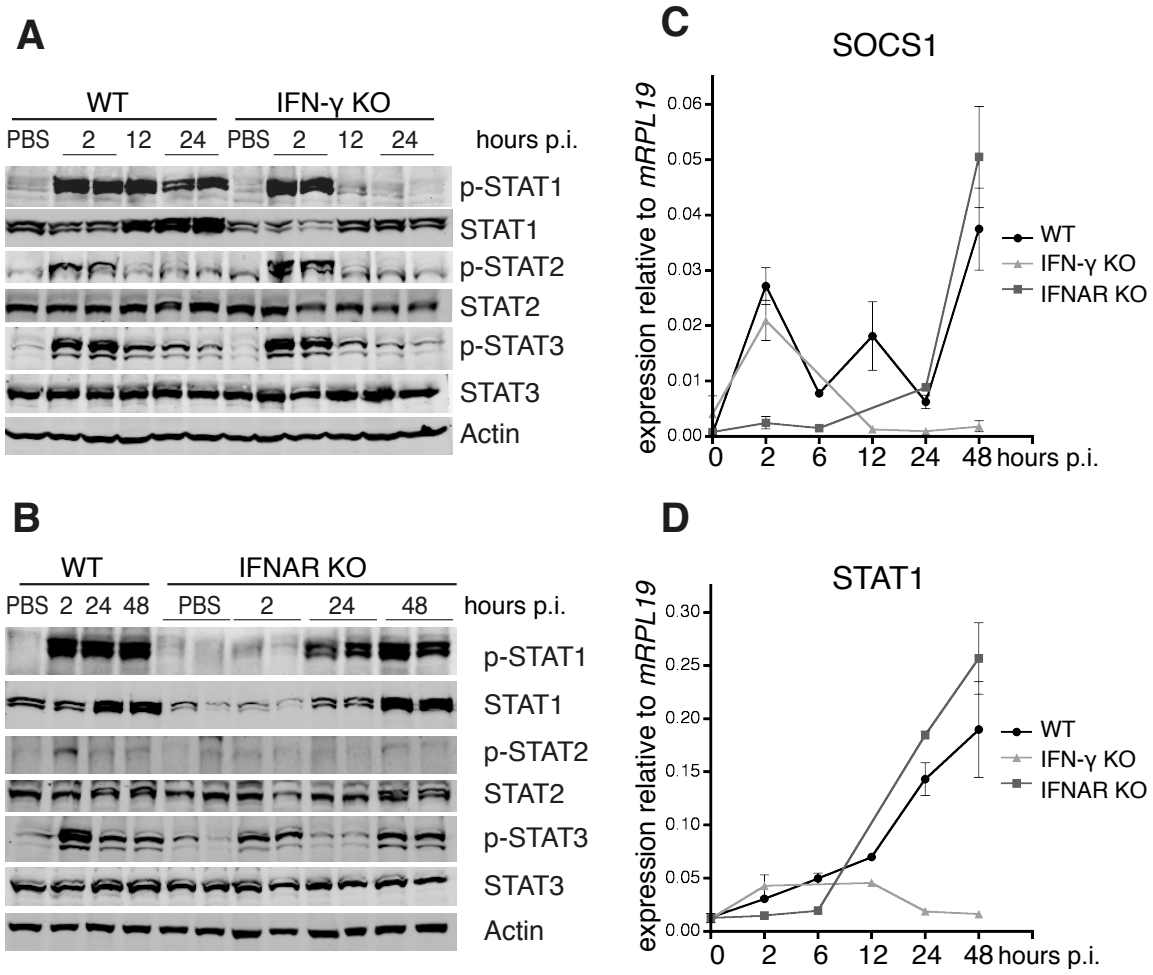

Figure 3.4: **Type I and II interferons mediate the response to IMO in mouse liver** C57Bl/6, IFNAR KO and IFN-γ KO mice were injected subcutaneously with 3 μg/g bw IMO and liver samples were collected at indicated time points post-administration. (A, B) STAT1, STAT2 and STAT3 phosphorylation as well as total STAT1 protein levels induced by IMO treatment were assessed by immunoblot with specific antibodies (C, D) IMO-induced expression of SOCS1 and STAT1 was quantified by RT-qPCR. The data are plotted as the amount of SOCS1 or STAT1 mRNA relative to RPL19 mRNA (mean and SEM). (E) mIFN-γ protein concentration was measured in the serum of WT mice in response to an injection of 3 μg/g bw IMO. The data are plotted as mean and SEM.

Supplementary Figure 1

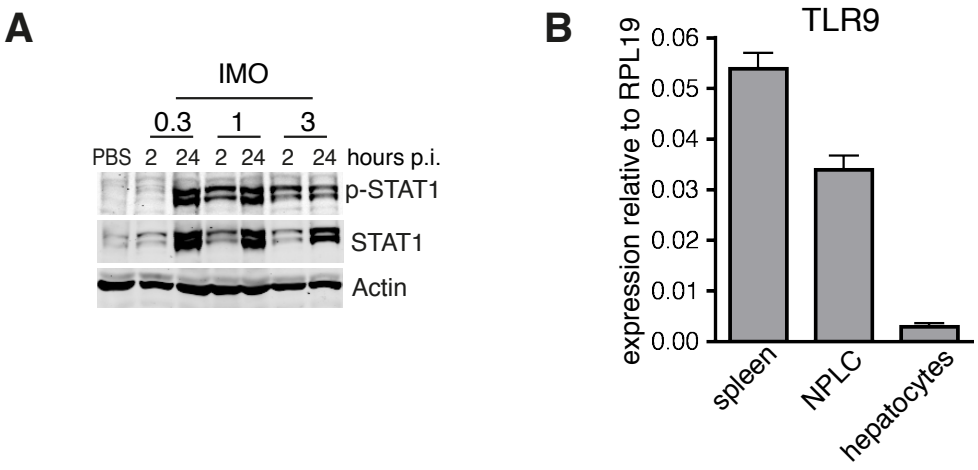

Figure 3.5: A) C57Bl/6 mice were injected subcutaneously with IMO (0.3, 1 or 3 μg/g bw) and the liver samples were collected at indicated time points post-administration. STAT1 phosphorylation as well as total STAT1 protein levels induced by the treatment were assessed by immunoblot with specific antibodies. B) Expression of TLR9 was quantified by RT-qPCR in mouse spleen, isolated non-parenchymal liver cells (NPLC) and hepatocytes (C57/Bl6 mice). The data are plotted as the amount of TLR9 mRNA relative to RPL19 mRNA (mean and SEM).

4 Discussion

4.1 Differential sensitivites of IFNs-α, -β and -λ to the negative feedback mechanisms of the Jak-STAT pathway

Over 20 years ago it has been demonstrated that cultured cells continuously exposed to IFN-α mount an initial transcriptional response which is followed by a long-lasting (up to 72 hours) refractory period. During this time, expression of IFN-stimulated genes was attenuated and re-stimulation with IFN was uneffective in re-inducing the signalling [85]. More recently it has been demonstrated that the same phenomenon occurs *in vivo* in mouse liver [84]. Experiments with knock-out mice showed that this refractory period following IFN-α exposure was specifically mediated by USP18 protein. Whereas the phenomenon of IFN-induced desensitization was described for IFN-α, it has not been studied whether it also affects other IFN subtypes.

In our present work we have investigated the Jak-STAT pathway activation and induction of IFN-stimulated genes in the setting of continuous and repeated administration with IFN-α, -β and -λ. In agreement with previous studies, we found that IFN-α-mediated signalling was almost completely abrogated in the cells or *in vivo* in the mouse liver when the cytokine was administered repeatedly. In contrast, the Jak-STAT pathway activation achieved by treatment with other type I IFN, IFN-β, was only marginally reduced through pretreatment of cultured cells or mice with IFN-α or -β. Similarly, the signalling induced by type III IFN was not attenuated by previous exposure to type I or III IFNs.

USP18 reduces signalling through the Jak-STAT pathway by specific binding of its C-terminal domain to the IFNAR2 subunit of the type I IFN receptor and restricting the access of the Jak1 kinase to its docking site at the receptor (Figure 1.3) [89]. Given this mechanism of action, it is not unexpected that the signalling which proceeds through IFN-λ receptor is intact despite high levels of USP18 resulting from previous IFN exposure. On the other hand, IFNs-α and IFN-β bind to the same receptor, but still exhibit differential sensitivity to USP18-mediated refractory state. Experimental evidence suggests that IFN-β has higher affinity to IFNAR2 compared to IFN-αs [180]. It is possible that such different affinity bindings may result in different conformational changes to the receptor molecules, affecting protein-protein interactions of the intracellular part of IFNAR2 and therefore leading to distinct sensitivities to inhibition by USP18. A different mechanism has been postulated by Francois-Newton and colleagues, whereby USP18 binding to the intracellular portion

of IFNAR2 would specifically inhibit the binding of IFN-α to the receptor on the cell surface [181].

Pegylated IFN-α is the backbone of chronic hepatitis C therapies, with cure rates ranging from 50 to 80% [43, 44]. It is conceivable that the USP18-mediated attenuation of IFN-α signalling underlies the mechanism of treatment non-response. In particular, it has been shown that patients who do not respond to the peg-IFN-α and ribavirin treatment have elevated levels of IFN-stimulated genes in the liver already before the commencement of the therapy [110]. One of the highly upregulated genes is USP18. Following the first therapeutic injection of peg-IFN-α these patients do not show efficient STAT1 phosphorylation in the hepatocytes or further IFN-stimulated gene induction in the liver. In contrast, the patients whose endogenous IFN system is not preactivated before the antiviral treatment respond fully to the therapeutically administered IFN-α and eventually succeed in clearing the HCV infection completely.

4.2 IFN-γ response in the liver during acute phase of hepatitis C virus infection

The first 6 months after HCV transmission are classified as the acute phase of the infection. During this time the patients have a considerable chance (about 30%) of clearing the infection spontaneously, without the need for antiviral therapy. With the time, as the infection progresses to the chronic phase, the odds for spontaneous cure drastically decrease. When treated with peg-IFN-α-based therapy, almost all patients with acute HCV monoinfection respond by eradicating the virus, a stark contrast to the patients in the chronic phase of the disease, when only about a half of the patient population can be cured with this regimen. These discrepancies suggest that the host-virus interactions during HCV infection change with progression to chronicity. To gain an insight into the nature of this change we investigated the host hepatic response during the late phase of acute HCV infection in humans and compared it to the chronic hepatitis C situation. We found evidence of a strong activation of the IFN signalling in liver biopsy samples obtained 2-5 months after the transmission of HCV infection. This activation of the Jak-STAT pathway was accompanied by a significant upregulation of IFN-γ, but not IFN-α target genes, marking an important difference to the preactivated state observed in the liver of the chronic hepatitis C non-responder patients. The source of the IFN-γ in the liver were the infiltrating CD8-positive T cells found in the direct proximity of the hepatocytes

showing nuclear phospho-STAT1 signals. These data are in line with the previous findings from the chimpanzee model of acute HCV infection, where elevated levels of IFN-γ and CD8-positive T cell infiltrates in the liver are noted several weeks after HCV transmission. These observations usually coincide with elevated levels of liver enzymes and transient or permanent suppression of HCV viral load.

The lack of induction of type I IFN-specific target genes in the liver of the acute hepatitis C patients despite ongoing viral replication could be explained by HCV interference with the immune system. However, contrary to the chronic hepatitis C situation, we could not detect MAVS cleavage in any of the acute hepatitis C patients. Moreover, strong phospho-STAT1 signals found in the liver of acute hepatits C patients suggest that Jak-STAT signalling is not globally impaired. It remains unclear what mechanisms circumvent type I IFN induction and/or signalling in the liver of the patients in the acute phase of disease.

Contrary to chronically infected individuals, patients with acute hepatitis C have an excellent, over 90% response rate to treatment with peg-IFN-α. Refractoriness to IFN-α-based therapies in the chronic hepatitis C patients correlates with a strong overexpression of an IFN-α, but not IFN-β or -λ inhibitor, USP18 [110]. USP18 is a target gene of type I and III IFNs, but it is only weakly induced by IFN-γ. IFN-γ stimulation is the main source of IFN target genes expression during the acute phase of HCV infection. Accordingly, no elevations in USP18 transcript or protein levels were found in the liver of acute hepatitis C patients. Moreover, it has been demonstrated that silencing of USP18 potentiates the ability of IFN-α to inhibit the replication and virion production of HCV in the cell culture model system which reproduces the full cycle of viral replication. Consequently, the lack of induction of USP18 in the liver of acute hepatitis C patient might explain the excellent treatment response rates in this group. USP18 could represent a potential therapeutic target for improving IFN-α responsiveness of the patients with preactivated endogenous IFN system.

4.3 Sequential induction of type I and II IFNs in response to a novel TLR9 agonist

Synthetic TLR agonists induce innate immune responses and regulate adaptive immunity by stimulating the signalling through the toll-like receptor pathways which evolved to detect and respond to microbial infections. This therapeutic strategy is currently intensively investigated in a number of clinical trials. TLR agonists

are tested in a variety of applications including cancer, viral infections, allergy and asthma as well as use in vaccine adjuvants. Three TLR agonists are currently approved for use in humans: bacillus Calmette-Guerin (TLR4 agonist, an attenuated strain of *Mycobacterium bovis*) is mainly used as a vaccine against tuberculosis, but also for the immunotherapy of *in situ* bladder carcinoma. Monophosphoryl lipid A (TLR4 agonist derived from the LPS of *Salmonella minnesota*) is included in the formulation of a vaccine against human papillomavirus-16 and -18. Imiquimod (TLR7 agonist, a synthetic imidazoquinoline) is employed for treatment of actinic keratosis, superficial basal cell carcinoma and external genital warts (reviewed in [182].

In this work we investigated the mouse liver response to IMO, a synthetic agonist of TLR9. This compound is closely related to a TLR9 agonist IMO-2125 which is currently developed for treatment of chronic hepatitis C. We found that a single injection of IMO induced a prolonged activation of the Jak-STAT pathway and IFN-stimulated gene induction in mouse liver. Repeated treatment with IMO did not lead to decrease in STAT1 activation or IFN-stimulated gene expression, demonstrating that contrary to IFN-α-induced signalling, there is no refractory period following IMO administration. Microarray analysis revealed a broad transcriptional response to IMO in the mouse liver, including production of a panel of chemoattractant molecules. Indeed, lymphocyte infiltration was readily observed in the liver of mice receiving IMO. Moreover, we were able to demonstrate that *in vivo* response to IMO was biphasic, with initial activation relying on signal transduction through type I IFN receptor and later an independent sustained phase of IFN-γ-mediated signalling.

Mouse liver becomes unresponsive to IFN-α within hours after administration and this refractory state depends on upregulation of USP18. The present work of our group and an independent recent study showed that different IFN types are unequally affected by the USP18-mediated negative feedback loop [181, 183]. Particularly, IFN-α-mediated signalling was demonstrated to be almost completely abrogated by previous exposure to type I IFN, whereas IFN-β, -γ or -λ-mediated signals were only marginally reduced. The long-lasting activation of the Jak-STAT pathway after IMO injection is induced by IFN-γ, which is not sensitive to the refractory state of the cells induced by USP18. The continuing strong activation of the signalling in the context of repeated administration could provide an advantage for TLR9 agonists such as IMO-2125 or similar compounds in the clinical setting.

5 Outlook

Considerable efforts have been made to understand the interaction of hepatitis C virus with the IFN system in the liver, but a number of key questions remain unanswered. One of the central questions is why the preactivated state of IFN-α non-responders is completely ineffective in clearing the virus. It has been proposed that the inability of the endogenous IFN system to combat the infection is due to the spatial resolution of the sites of HCV replication in the liver and the hepatocytes producing the antiviral IFN-stimulated genes. It is an attractive hypothesis, but the advances have so far been hampered by the lack of reliable protocol for *in situ* HCV detection in liver biopsies from infected patients.

Moreover, it is still not clear what subtype of IFN is responsible for the preactivated state of the liver of the patients who do not respond to therapy. No upregulation of known cytokines that signal through the Jak-STAT pathway and could explain the type I IFN-like transcriptional signature of the liver in chronic hepatitis C non-responders has been detected. It is conceivable that an as-yet undescribed cytokine or IFN subtype could be responsible for the observed activation.

Another central question in the field is that of the functional link between the IL28B genotype, preactivated state of the liver and treatment non-response. SNPs in proximity of the IL28B (IFN-λ2) gene and preactivated state of the liver are both highly related to treatment outcomes in chronic hepatitis C. It is a matter of debate whether the upregulation of IFN-stimulated genes in the liver is causally linked to the IL28B polymorphisms [184, 185, 186, 187]. IL28B genotype does not appear to impact the treatment response rates through variations in coding sequence of IFN-λ2 or changes in its expression levels. A more complex mechanism seems to be in play, warranting further research efforts to clarify the mechanisms of treatment failure in IL28B minor allele carriers.

6 Abbreviations

ALT	alanine aminotransferase
CpG	2-deoxyribo-[cytidine-phosphate-guanosine]
GAS	gamma activated sequence
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
IFN	interferon
IFNAR	interferon alpha/beta receptor
IFNGR	interferon gamma receptor
IL	interleukin
IMO	immunomodulatory oligonucleotide
IRAK	interleukin 1 receptor-associated kinase
IRES	internal ribosome entry site
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
ISGF3	interferon-stimulated gene factor 3
ISRE	interferon-stimulated response element
Jak	janus kinase
miR-122	microRNA-122
NF-κB	nuclear factor kappa B
NK	natural killer cell
NS	non-structural (protein)
OAS	2'-5' oligoadenylate synthetase
ODN	oligonucleotide
PBMCs	peripheral blood mononuclear cells
pDCs	plasmocytoid dendritic cells
peg	pegylated
PIAS	protein inhibitor of activated STAT
SNP	single nucleotide polymorphism
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
TIR	toll-interleukin 1 receptor
TLR	toll-like receptor
USP18	ubiquitin-specific peptidase 18

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7 *Curriculum vitae*

CURRICULUM VITAE

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EDUCATION:

2012	Postdoctoral research in Hepatology group, Biomedicine Department of the University of Basel, Switzerland
2009- 2012	PhD student in Hepatology group, Biomedicine Department of the University of Basel, Switzerland Research focus: Interferon signalling in the liver Thesis director: Prof. Markus Heim
2010	CSAMA course: Computational Statistics for Genome Biology; Brixen, Italy
2010	Introductory Course in Laboratory Animal Science (FELASA Category B); Zürich, Switzerland
2008-2009	MSc in Biological Science at Ecole Normale Superieure in Lyon, France
2009	Course in transmission electron microscopy; Lyon, France
2009	Internship in the Institute of Protein Biology and Chemistry in Lyon, France Research project: Visualizing morphology of mutant and wild-type hepatitis C virus particles under transmission electron microscope Internship director: Prof. François Penin
2008-2007	Erasmus fellowship at Ecole Normale Superieure in Lyon, France Internship in Human Virology Department of Ecole Normale Superieure in Lyon Research project: Role of hepatitis C virus core protein basic domains in viral replication cycle Internship director: Prof. Jean-Luc Darlix
2004-2007	BSc in Biotechnology at Adam Mickiewicz University in Poznań, Poland

LANGUAGES:

Polish – Native Speaker
English, French – Fluent (stays in the USA and France; IELTS score for 2008: 8)
German – Intermediate

COMPUTATIONAL SKILLS:

GNU-R – Basic programming knowledge and use of Bioconductor software libraries.

AWARDS:

2011 Research Prize of the Swiss Association for the Study of the Liver (SASL)/SGG/SGVC
2012 Junior Hepatology Prize of the Swiss Association for the Study of the Liver (SASL)/SGG/SGVC

CONFERENCE CONTRIBUTIONS:

ORAL PRESENTATIONS

2012 **Interferon-γ-stimulated gene expression and lack of USP18 induction in the liver of acute hepatitis C patients**
Annual meeting of the Swiss Association for the Study of the Liver (SASL)/SGG/SGVC; Interlaken, Switzerland

2011 **Characterization of response to toll-like receptor 9 agonist CpR-2252 in the mouse liver**
Annual meeting of the Swiss Association for the Study of the Liver (SASL)/SGG/SGVC; Lausanne, Switzerland

2011 **Interferon-β and -λ signalling is not affected by interferon-induced refractoriness to interferon-α**
The International Liver Congress of the European Association for the Study of the Liver; Berlin, Germany

INVITED TALKS

2011 **Negative regulation of type I and III interferon signalling**
Joint Meeting of the International Cytokine Society (ICS) and the International Society for Interferon and Cytokine Research (ISICR); Florence, Italy

POSTERS

2012 **Continuous exposure to peg-IFN-α only transiently activates Jak-STAT signalling in human liver**
Joint Meeting of the International Cytokine Society (ICS) and the International Society for Interferon and Cytokine Research (ISICR); Geneva, Switzerland

2011 **Endogenous production of type I and II interferons drives the bimodal kinetics of response to CpR-2252, a synthetic agonist of TLR9, in mouse liver**
Joint Meeting of the International Cytokine Society (ICS) and the International Society for Interferon and Cytokine Research (ISICR); Florence, Italy

2010 **Differential patterns of response to toll-like receptor 9 agonist and exogenous interferon-α in the mouse liver**
Joint Meeting of the International Cytokine Society (ICS) and the International Society for Interferon and Cytokine Research (ISICR); Chicago, USA

PUBLICATIONS:

2012 **Interferon signaling in the liver during hepatitis C virus infection**
Makowska Z, Heim MH, **Cytokine**, 2012, 59(3):460-466

2012 **Interferon-γ Stimulated Genes, but not USP18, are Expressed in Livers of Patients with Acute Hepatitis C**
Makowska Z, Dill MT, Duong FH, Merkofer F, Filipowicz M, Baumert TF, Tornillo L, Terraciano L, Heim MH, **Gastroenterology**, 2012, 143(3):777-786

2012 **Intrinsic flexibility of nucleic acid chaperone proteins from pathogenic RNA viruses**
Ivanyi-Nagy R, Makowska Z, Darlix JLD, in: Peptide Folding, Misfolding and Nonfolding; Schweitzer-Stenner R, Uversky V Eds., Wiley, ISBN: 978-0-470-59169-7

2011 **Interferon-β and interferon-λ signaling is not affected by interferon-induced refractoriness to interferon-α *in vivo***
Makowska Z, Duong FH, Trincucci G, Tough DF, Heim MH, **Hepatology**, 2011, 53(4):1154-63