Comparative analysis of the antiviral effects mediated by type I and III interferons in hepatitis B virus infected hepatocytes

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Abbreviations: HBV, Hepatitis B virus; ISGs, interferon stimulated genes; IFN-λ, interferon-lambda; IFN-β, Interferon-beta

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Summary:

Simlar to interferon-α, interferon-β and –λ can purge the hepatitis B virus persistence form, cccDNA, from infected hepatocytes and control virus replication without killing the cells. All interferons induce cytosine deamination of cccDNA with interferon-α showing the least favourable kinetics.

Abstract

Background: Type III interferons (λ1-3) activate similar signaling cascades as type I interferons (α and β) via different receptors. Since interferon (IFN)-α and lymphotoxin-β activate cytosine deamination and subsequent purging of nuclear hepatitis B virus (HBV) DNA, we investigated whether IFN-β and -λ may also induce these antiviral effects in differentiated, HBV infected hepatocytes. **Methods**: After determining the biological activity of IFN-α2, -β1, -λ1 and –λ2 in differentiated hepatocytes, their antiviral effects were analyzed in HBV infected primary human hepatocytes and HepaRG cells. **Results**: Type I and III IFNs reduced nuclear open-circle and covalently closed circular (ccc)DNA levels in HBV infected cells. IFN-β and -λ were at least as efficient as IFN-α. Differential DNA-denaturing PCR and sequencing analysis revealed G-to-A sequence alterations of HBV cccDNA in IFN-α, -β and –λ treated liver cells indicating deamination. All IFNs induced APOBEC deaminases 3A and 3G within 24h of treatment, but IFN-β and -λ induced longer-lasting expression of APOBEC deaminases in comparison to IFN-α. **Conclusions**: IFN-β, IFN- λ1 and IFN-λ2 induce cccDNA deamination and degradation at least as efficiently as IFN-α, indicating that these antiviral cytokines are interesting candidates for the design of new therapeutic strategies aiming at cccDNA reduction and HBV cure.

Introduction

Hepatitis B virus (HBV) is a major human pathogen with more than 250 million chronically infected patients worldwide at high risk to develop liver cirrhosis and hepatocellular carcinoma [1, 2]. Chronic HBV infection is characterized by the inability of the host to raise an adequate immune response against the virus. To persist, the virus deposits a covalently closed circular DNA (cccDNA) form of its genome in the nucleus of infected hepatocytes [3]. This persistence form is not affected when patients receive antiviral treatment with nucleoside analogues which effectively control but cannot cure the infection. While HBV induces little interferon (IFN) response [4, 5], administration of pegylated IFN-α2 provides the option of HBsAg seroconversion referred to as functional cure in chronic hepatitis B patients [6]. Nevertheless, its clinical efficacy is limited and the treatment has strong side effects [7].

The antiviral mechanisms of IFN-α have been intensively studied and include transcriptional, post-transcriptional and epigenetic effects on HBV [8-11]. In addition, a potentially curative molecular mechanism of non-cytolytic purging of HBV cccDNA induced by IFN-α and mediated by cytosine deamination has been reported [12]. Apolipoprotein B mRNA-editing enzyme–catalytic polypeptide-like 3 (APOBEC3) have long been known as cytosine deaminases involved in immune responses against HIV, HBV and plasmid DNA [13, 14]. Now, APOBEC3A and B proteins were identified as the responsible cytosine deaminases locating and targeting the cccDNA in the nucleus. Deamination of cytosine to uracil in the target DNA results in DNA hypermutations. Deamination sites are recognized by DNA glycosylases that cut out

the base and leave apurinic/apyrimidinic (AP)-sites that can either be repaired or cleaved by endonucleases [12, 15].

In contrast to the well-studied antiviral effects of IFN-α and IFN-γ [16], little is known about the ability of other type I or type III interferons to induce APOBECs and purge HBV cccDNA. While type I and III IFNs are recognized by different receptors, both cytokine families signal through the activation of JAK/STAT pathway, finally inducing the expression of interferon-stimulated genes (ISGs) [17, 18]. In the present study we used primary human hepatocytes (PHH) and differentiated HepaRG cells (dHepaRG) as *in vitro* infection models for studying innate immune responses induced by human IFN-β1a and IFN-λ 1/2 on HBV replication and on HBV cccDNA in human hepatocytes. In particular, we addressed the question, whether human interferons other than IFN-α or IFN-γ can promote cccDNA degradation, thus being interesting candidates for antiviral therapy.

Materials and methods

HBV inocula, cell culture, HBV infection and treatments. HBV particles were concentrated from the supernatant of HepG2.2.15 cells as previously described. [16, 19, 20]. PHH were obtained within the ethical framework of the nonprofit foundation Human Tissue and Cell Research (Germany). After obtaining informed patient's consent, PHH were isolated from waste material during surgical liver resections, cultured and infected with HBV as previously described. [16, 21-23]. HepaRG cell culture, differentiation and infection at a multiplicity of 200 vp/cell were also performed as described [24]. Human IFN-α (Roferon-A, Roche) and recombinant human IFN-β/λ1/λ2 (Peprotech, Hamburg, Germany) were used at 100 U/ml unless

otherwise indicated. Each IFN activity was determined by a classical IFN bioassay based on its ability to prevent cytopathic effects induced by vesicular stomatitis virus (VSV) infection of HepaRG cells, respectively [25].

Analysis of HBV infection: HBeAg was determined by commercial immunoassay (Siemens Molecular Diagnostics, Marburg, Germany). Total DNA and nuclear DNA was purified from infected cells. HBV-DNA and cccDNA were detected using selective PCR primers and normalized to single-copy gene Prnp [26, 27]. PCR products of cccDNA qPCRs were used for differential DNA denaturation PCR (3D-PCR) using primers in the HBx gene (5'HBxin: ATGGCTGCTARGCTGTGCTGCCAA, 3'HBxin: AAGTGCACACGGTYYGGCAGAT) [12, 28]. Purified PCR products were cloned by TA cloning and sequenced [12]. Performance and quality of HBV DNA and cccDNA qPCRs was evaluated earlier [16].

qRT-PCR. RNA was extracted from cell layers using the peqGOLD Total RNA Kit (Peqlab, Erlangen, Germany) and transcribed into cDNA with the “SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR” kit (Invitrogen, Karlsruhe, Germany). GAPDH was used as housekeeping gene. GAPDH, Mx1 and APOBEC 3A, B and G primers have been described [12]. Other primers were: SOCS1-forward (fw): TTTTTCGCCCTTAGCGTGA, SOCS1-revers (rev): AGCAGCTCGAAGAGGCAGTC, USP18-fw: CTCAGTCCCGACGTGGAACT, USP18-rev: ATCTCTCAAGCGCCATGCA, IL28R-fw: CCCAAGGGTAAGAGCTTCGAT, IL28R-rev: CCTTCATATTTTAC-TGACATGGACAAG, IL10R-for: TTGCTGTGGTGCGTTTACAAG, IL10R-rev: CTTTCAGGTGCTGTGGAAGAGA.

cccDNA Southern Blot. dHepaRG cells overexpressing a tetracyclin regulated Na+-taurocholate cotransporting polypeptide (NTCP) as HBV receptor (HepaRG-TR-NTCP cells) were infected with HBV at a multiplicity of infection (MOI) of 1000 DNA-containing vp per cell. NTCP expression was either induced 2 days prior to or simultaneously with HBV infection. After 7 days, interferon treatment was started and maintained for 18 days with drug refreshment twice a week. For cccDNA Southern blot, a modified Hirt extraction protocol was performed to extract protein-free DNA as described [29].

Western Blot. dHepaRG cells and PHH were lysed in Pierce RIPA buffer (Thermo Scientific) and cooked at 95°C for 5 min after addition of loading dye (50 mM Tris-HCl pH6.8, 2%SDS, 10% glycerol, 0.01% bromophenolblue, 1% beta-mercaptoethanol). Proteins were separated by 12 % SDS-PAGE, transferred onto a methanol-activated PVDF-membrane by wet blot procedure and stained by primary (Rabbit-anti-A3A 1:250 (Sigma; #SAB4500753); Rabbit-anti-Mx1 1:1000 (#13750-1-AP); Mouse-anti-GAPDH 1:5000 (#ACR001PT)) and secondary antibodies (1:10000, Sigma-Aldrich). Membranes were blocked with Roti-Block (Roth, Karlsruhe, Germany).

Statistical Analysis

The Student unpaired 2-tailed t test was performed using Prism6 (GraphPad, La Jolla, CA). Data are shown as means ± SD. P values less than .05 were considered significant.

Results

Type I and III interferons reduce HBV cccDNA in primary human hepatocytes and dHepaRG cells.

LT-β receptor agonisation and IFN-α have been reported to purge cccDNA from HBV-infected hepatocytes [12]. To assess if other interferons activating similar signaling pathways have the same effect on the HBV cccDNA transcription template, we treated PHH for 7 days with different doses of type III IFNs, IFN-λ1 or IFN-λ2 **(Figure 1A,B)**. Interestingly, a 74.21% and 65.98% reduction of HBV cccDNA was achieved already with relatively low doses of 10 U/ml IFN-λ1 and IFN-λ2. A maximum reduction of HBeAg by 60.64% and 58.02% was achieved at 30 U/L IFN-λ1 and 100 U/L IFN-λ2, respectively.

In order to further study and compare the effects of type I and III IFNs on the HBV cccDNA transcription template in a systematic fashion, we first compared different cell lines for their responsiveness to IFN. dHepaRG cells responded to both type I and III IFN treatment by upregulating IFN-stimulated gene Mx1 with a strength comparable to PHH (**Figure 1C**). Upregulation of OAS1 in IFN-α/λ1 stimulated dHepaRG cells was lower than in PHH or Huh-7 cells (**Figure 1D)**. HepG2 cells, in contrast, barely and Huh-7 cells only partially responded to the treatment with the different IFNs.

To be able to compare the effect of the different IFN batches by their biological ativity, we determined IFN activity in dHepaRG cells by classical IFN bioassay. To compare antiviral activity of the different IFNs, IFN Units were determined using the half maximal inhibitory concentration in this system (IC50). With an IC 50 value of 1 IU/ml, the units determined for IFN-α in the HepaRG cell culture system were congruent with the units given by the manufacturer (**Figure 2A**). The titration curve

analysis resulted in IC50 values of 0.3 pg/ml (IFN-β), 0.1 ng/ml (IFN-λ1) and 0.2 ng/ml (IFN-λ2), respectively, and were thus defined as 1 unit (**Figure 2B-D**). Importantly, treatment of HepaRG cells with the indicated type I or III IFNs did not result in any cytotoxic effects even when treated with higher doses.

Type I and type III IFNs show similar antiviral effects but no synergism

Next we determined whether treatment of dHepaRG cells with IFN-α, IFN-β, IFN-λ1 or IFN-λ2 resulted in a decrease of intracellular cccDNA or secreted HBeAg. In accordance to the effects observed in PHH (**Figure 1A,B**), all 4 cytokines significantly reduced intracellular cccDNA in a dose-dependent manner in dHepaRG cells **(Figure 3A-D)**. Maximum cccDNA reduction was achieved by 100 U/ml IFN-α (71.38%, **Figure 3A**), 100 U/ml IFN-β (50.29%, **Figure 3B**), 100 U/ml IFN-λ1 (76.11%, **Figure 3C**) and 10 U/ml IFN-λ2 (83%, **Figure 3D**), respectively. HBeAg levels further dropped when higher amounts of IFN were applied. Secreted HBeAg was reduced by 81.31% (IFN-α, ≥ 1000 U/ml), 85.33% (IFN-β, ≥ 1000 U/ml), 69.68% (IFN-λ1 ≥ 100 U/ml) and 65.29 % (IFN-λ2 ≥ 100 U/ml), respectively (**Figure 3A-D**). To confirm the IFN-induced cccDNA reduction detected by qPCR we treated HBV infected HepaRG-TR-NTCP cells with 100 U/ml IFN-α, IFN-β, IFN-λ1 or IFN-λ2 for 18 days and analyzed protein-free HBV DNA by Southern blot analysis. Southern blot analysis revealed a marked loss of protein-free open-circle DNA, which most likely represents the transcriptionally active form of cccDNA, and confirmed that all cytokine treatments resulted in a 30-60% reduction of closed-circular cccDNA **(Figure 4)**.

To investigate whether type I and type III IFNs differ in the kinetics of cccDNA reduction, we treated infected dHepaRG cells for 3, 7 and 10 days with 100 U/ml of IFN-α, IFN-β, IFN-λ1 or IFN-λ2, respectively, and analyzed intranuclear HBV cccDNA **(Figure 5A)**. Interestingly, we found significant reductions of cccDNA already after 7 day treatment with type III IFN (IFN-λ1: 74.14%, p=0.0323; IFN-λ2: 79.49%, p=0.0351), while significant cccDNA reduction by type I IFNs was only determined at day 10 (IFN-α: 86.92%, p=0.0192; IFN-β: 86.20%, p=0.0226). Between day 7 and 10, a further decline of cccDNA levels (75-87%) was observed in all conditions and cccDNA dropped to levels comparable between all 4 cytokines **(Figure 5A).** A certain proportion of cccDNA, however, remained unaffected in all conditions. Thus, although type I and type III IFNs had a similar antiviral effect and were able to significantly reduce HBV cccDNA in a non-cytolytic fashion, type III IFNs displayed a more rapid effect in the same cell culture system.

To address the question if a combination of type I and III IFNs would enhance antiviral effects, we treated dHepaRG cells simultaneously with 100 U/ml IFN-α and 5 U/ml IFN-λ1, i.e. at doses that showed submaximal effects in individual treatment. In addition, we added the second cytokine after 3 days of treatment and compared cccDNA and HBeAg amounts of these samples on day 7 to those treated with either IFN-α or IFN-λ1 alone. Neither the combination of both cytokines, nor the alternating therapy for 7 days resulted in a stronger effect on cccDNA or HBeAg compared to the single treatments (50-60% reduction of cccDNA and HBeAg) **(Figure 5B).** Of note, a longer combination treatment with IFN-α and IFN-λ1 for 10 days could also not achieve significantly stronger cccDNA reductions than treatment with IFN-α or IFN-λ1 alone for 10 days **(Supplementary Figure 1)**. In addition, we did not observe an

induction of the IFN-lambda receptor subunits IL10R or IL28R by any of the treatments (data not shown), further supporting the notion that type I and III IFNs do not act synergistically.

Type I and III IFNs induce cytosine deamination of HBV cccDNA in human hepatocytes.

The decline of HBV cccDNA upon treatment with IFN-α, IFN-β, IFN-λ1 or IFN-λ2 indicated cccDNA modifications as described before [12]. To investigate if the cccDNA loss detected was the result of DNA damage, we analyzed cccDNA deamination by differential DNA denaturation PCR (3D-PCR) as a method to discover GC to AT transitions [14]. Because AT-rich regions within the virus genome melt at lower temperatures than GC-rich regions, lower denaturation temperatures can be used to amplify AT-rich virus DNA compared to GC-rich DNA. As shown in **Figure 6A**, cccDNA isolated from dHepaRG cells treated for 3 days with IFN-α, IFN-β, IFN-λ1 or IFN-λ2 could be amplified at lower denaturing temperatures compared to cccDNA isolated from mock treated samples. This was confirmed by type III IFN treatment of PHH for 7 days with IFN-λ1 or IFN-λ2, where HBV cccDNA could also be amplified at lower denaturing temperatures compared to mock treated samples **(Figure 6B)**. To detect maximal deamination, PCR products resulting from reactions with the lowest denaturation temperature possible (84°C and 86°C, respectively) were cloned and subjected to Sanger sequencing. The resulting sequencing data **(Figure 6C)** show G to A transitions in the PCR products obtained from IFN-α, IFN-β, IFN-λ1 or IFN-λ2 treated samples, but not in from mock treated samples that matched the HBV input sequence. Thus, not only IFN-α, but also IFN-β, IFN-λ1 and IFN-λ2 induced cytosine deamination of HBV cccDNA. The quantification of base

frequencies of the PCR products revealed a higher frequency of G to A conversion in PCR products of IFN-α, -β, -λ1 and -λ2 treated cells as compared to the mock control, whereas C and T counts were unaffected **(Figure 6D)**. This showed that all four IFNs induced the deamination of cytosine to uracil in the HBV cccDNA minus strand.

Type I and III IFNs induce APOBEC 3A but not 3B deaminases.

To analyze the enzymes involved in type I and III IFN-induced deamination of HBV cccDNA, we examined expression levels of different deaminases upon IFN stimulation. APOBEC3A and APOBEC3B have recently been shown to be crucial for cccDNA deamination and degradation by IFN-α or LTβ receptor agonisation, respectively [12]. In contrast to LT-β receptor agonisation, that activates APOBEC3B via the NFκB pathway, IFN-α via activation of STAT-signaling only induces APOBEC3A. As shown in **Figure 7A**, IFN-β (5.73-fold, 24h), IFN-λ1 (8.83-fold, 2h) and IFNλ2 (4.06-fold, 24h) can also induce APOBEC3A. The induction of APOBEC3A in dHepaRG cells and PHH was confirmed on protein level by Western blot analysis (**Figure 7B**). In line with the mRNA kinetics, APOBEC3A protein was induced 24 hours after IFN-α stimulation and 48 hours after IFN-β, IFN-λ1 and IFNλ2 treatment. Like IFN-α, the other IFNs did not induce APOBEC3B, another family member with nuclear localization [12], but induced APOBEC3G (**Figure 7C**) that, however, acts in the cytoplasm and therefore cannot deaminate HBV cccDNA. Activation Induced Cytidine Deaminase (AICDA) that was previously shown to be capable of inducing G to A transitions in HBV nucleocapsid DNA [30] was not induced by any of the 4 IFNs in our analyses (data not shown).

Despite ongoing stimulation for up to 7 days, expression of deaminases was transient upon IFN treatment. While APOBEC3A expression induced by IFN-α, IFN-β and IFN-λ2 rapidly declined again after 24 hours, IFN-λ1 had a more sustained effect for up to 7 days (**Figures 7A**). Induction of APOBEC3G and Mx1, a dynamin-like GTPase with antiviral activity, was also somewhat more sustained after treatment with type III than with type I IFNs **(Figure 7C, Supplementary Figure 2A,B)**. These transient expression levels of deaminases and ISGs indicate a refractory state upon type I and III IFN stimulation. We therefore also analyzed negative regulators of IFN response. Ubiquitin Specific Peptidase 18 (USP18) has been reported to regulate long-term refractory states [31] in IFN-stimulated cells and was strongly induced by all IFNs within the first 6h **(Figure 7D)**. Although slowly fading out, elevated levels of USP18 were detected for 7 days. Interestingly, the mediator of short-term refractoriness upon IFN stimulation (SOCS1) was induced by all 4 IFNs very rapidly (2h), but already downregulated again after 12h (**Figure 7D**). These analyses revealed that type I as well as type III IFNs induce APOBEC3A deaminase rapidly but transiently probably due to the parallel induction of negative regulators.

Discussion

In this study we analyzed the antiviral effects of type I and III IFNs on HBV cccDNA in infected dHepaRG cells and primary human hepatocytes. IFN-α2a, IFN-β1a, IFN-λ1 and IFN-λ2 upregulated cytosine deaminase APOBEC3A and induced cccDNA deamination as well as cccDNA loss in treated cells. All four IFNs were titrated for their antiviral effect in an IFN bioassay in dHepaRG cell, i.e. in differentiated

hepatocytes. Based on this titration, IFN-λ1 showed the most favorable antiviral effect on HBV with a high activity already at low doses. The magnitude of cccDNA reduction, however, was comparable between all four cytokines and non-synergistic in combined treatment. This was somewhat unexpected and indicated that all four cytokines act via the same mechanism of cccDNA deamination but also that part of the cccDNA is refractory to the IFN-mediated effects.

Our data are contrary to those reported by *Isorce et al* [32], who could not observe any effect of human type I and III IFNs on HBV cccDNA levels in PHH, but are in accordance with a recent report in HBV infected, humanized mice in which T-cell derived cytokines were able to limit HBV infection in a non-cytolytic fashion by activation of APOBEC3 deaminases [33]. The differences may relate to differences in the experimental settings, as well as methodologies of cccDNA quantification. We confirmed our qPCR results by Southern blot analysis which showed that open-circle HBV-DNA was affected stronger than cccDNA. Sequencing analysis revealed preferred deamination of the DNA minus-strand. As active transcription requires unwinding of the DNA strand so that the minus strand becomes accessible for RNA polymerase II, we hypothesize that the same applies for accessability for cytidine deaminases. The fact that the cytokine-induced effects observed were not curative and we always detected remaining cccDNA even after long-term treatment indicates that a proportion of the cccDNA minichromosomes may be epigenetically modified [8] and hence not accessible to cytosine deamination and/or endonucleases [12].

Previous studies have shown that type I and type III IFNs induce the same pattern of ISGs but with different kinetics and magnitude [34, 35]. Comparative analyses should not only be based on molarity but should take functional effects of the respective

cytokine in account. A unique characteristics of this study was the establishment of the IC 50 of each IFN in the same cell culture system. Using this approach, IFN-α2a, IFN-β1a, IFN-λ1 and IFN-λ2 induced very similar patterns of ISGs, but with different kinetics and at different doses. In the in vitro systems here analyzed, the dose of IFN-λ1 and IFN-λ2 needed to reach maximum cccDNA destabilization was lower than of IFN-α2a.

Previous reports indicated that IFN-α-induced antiviral states become refractory upon 3 days of treatment, while IFN-β and IFN-λ have been shown to induce less refractory states compared to IFN-α [31, 36]. This renders IFN-β and IFN-λ cytokines of interest for antiviral therapeutic approaches. In line with previous data, we observed that IFN-β and -λ induced a more prolonged expression of ISGs compared to IFN-α if treated with identical amount of IFN units determined by IFN bioassay in the same cell culture system. In particular the induction of APOBEC3A, which was shown to be crucial for IFN-α mediated cccDNA deamination and degradation [12], varied between the different IFNs analyzed. APOBEC3A induction was more sustained after treatment with IFN-β and IFN-λ compared to IFN-α with IFN-λ1 showing the longest and most favorable activity of all four cytokines analyzed. IFN-β seemed to display antiviral properties that are more similar to IFN-λ than to IFN-α. It is worth mentioning that the life time of IFN-β in complex with the IFN receptor subunit IFNAR1 is substantially longer than that of IFNα2 and different receptor affinities of these type I IFNs correlated with different antiproliferative and antiviral activities [37]. These results indicate that IFN-β and IFN-λ1 may be preferable over IFNα2 as therapeutic agents aiming at promoting cccDNA reduction.

The early decline of IFN-induced APOBEC3A was closely associated with a strong up-regulation of USP18 and SOCS1. USP18 is known to interfere with IFN effects by cleaving interferon-stimulated gene 15 (ISG15) from its ISGylated substrates. Even more important for its potency as an IFN inhibitor, USP18 is recruited to the IFNAR2 chain of the IFN type I receptor via STAT2 [38], but is also recruited to the IFNLR1 chain of the IFN type III receptor where it blocks further signaling via JAK-STAT [39]. SOCS1 is an important regulator for both cytokine and TLR-induced responses in the liver has been implicated in preventing liver pathophysiology. It abrogates IFN's antiviral effect on hepatitis C virus replication [40].

Despite a favorable profile of IFN-λ in our analysis, recent clinical studies were disappointing and found pegylated IFN-λ to be inferior compared to pegylated IFN-α [41]. While this study shows similar effects of type I and III IFNs in cell culture systems and in the absence of adaptive immunity, the effects of both IFN types might be more complex in patients, e.g. by exerting different effects on T cells or NK cells, human hepatocytes or intrahepatic non-parenchymal cells. It is worth noting that recent *in vivo* data showed similar direct antiviral effects of type III and I IFNs on hepatitis Delta virus in the setting of HBV/HDV coinfection in humanized mice [42]. Thus, it may be worthwhile to further elucidate the antiviral and immunomodulatory effect of the different IFNs in different cell types and in the setting of chronic HBV infection, including co-infection with hepatitis Delta virus.

In summary, our results show that IFN-β and type III IFNs can reduce cccDNA in infected hepatocytes in a non-cytolytic fashion. Both IFN families activate the same cytosine deaminase, APOBEC3A. Kinetics and in particular duration of activation by IFN-β or IFN-λ seemed to be favourable compared to IFN- α. In particular IFN-β,

which is licensed for the treatment of multiple sclerosis in a pegylated form, might represent an interesting candidate for a potentially curative treatment of chronic hepatitis B.

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Figure legends

Figure 1. Comparison of type I and type III IFN in primary human hepatocytes. **(A,B)** PHH were infected with HBV at an MOI of 200 DNA-containing particles per cell for 10 days and treated for another 7 days with 1-300 U/ml of **(A)** IFN-λ1 or **(B)** IFN-λ2. HBV cccDNA amounts were determined by qPCR and normalized to Prnp. HBeAg in cell culture medium was analyzed by ELISA. **(C,D)** ISG induction in PHH was compared to different cell lines**.** Indicated cells were treated with 100 U/ml IFN-α or IFN-λ1 for 12 hours and mRNA expression levels of **(C)** Mx1 and **(D)** OAS1 were assessed by qRT-PCR.

Figure 2. IFN activity in dHepaRG cells determined by IFN bioassay. dHepaRG cells were treated with indicated doses of IFN-α **(A)**, IFN-β **(B)**, IFN-λ1 **(C)** or IFN-λ2 **(D)**. Cells were then infected with vesicular stomatitis virus (VSV) and virus-induced cytotoxicity was assessed by measuring cell viability 48 hours after infection by cell titer blue assay.

Figure 3. Influence of type I and III IFN treatment on HBeAg secretion and intracellular HBV cccDNA. dHepaRG cells were infected with HBV at an MOI of 200 DNA-containing particles per cell for 10 days and treated for another 7 days with 1-1000 U/ml of **(A)** IFN-α, **(B)** IFN-β, **(C)** IFN-λ1 or **(D)** IFN-λ2. HBV cccDNA amounts were determined by qPCR and normalized to Prnp gene. HBeAg was measured by ELISA in cell culture medium collected from day 7 to 10. *p < 0.05, **p < 0.01, ***p < 0.001 by Student's unpaired t test.

Figure 4. Southern blot analysis of HBV cccDNA in type I and III IFN treated cells. Differentiated HepaRG-TR-NTCP cells were infected with HBV at an MOI of 1000 vp per cell and treated with 100 U/ml of IFN-α, IFN-β, IFN-λ1 or IFN-λ2 for 18 days. Protein-free (PF) intracellular HBV DNA was analyzed by Southern blot using an HBV-DNA probe. HBV PF open-circle (oc)DNA and cccDNA are indicated. Relative quantification of signal density is indicated below the blot.

Figure 5. Kinetics and synergistic effects of type I and III IFN. (A) dHepaRG cells were treated with 100 U/ml of IFN-α, IFN-β, IFN-λ1 or IFN-λ2. Cells were lysed after 3, 7 or 10 days, intranuclear HBV cccDNA amount was evaluated by qPCR and normalized to Prnp. **(B)** Infected dHepaRG cells were treated with 100 U/ml of IFN-α or 5 U/ml of IFNλ-1 on day 0 and day 3 to achieve sub-maximum reduction of cccDNA/HBeAg amounts after 7 days of treatment. In order to analyze potential synergistic effects, infected cells were treated with both IFNs on day 0 and day 3 or with one IFN type on day 0 and with the other on day 3, respectively. After 7 days cccDNA amounts were measured by qPCR and normalized to Prnp. Secreted HBeAg was analyzed by ELISA. *$p < 0.05$, n.s.: not significant by Student's unpaired t test.

Figure 6. Deamination of HBV cccDNA after treatment with type I and III IFN. (A) dHepaRG cells were treated with 100 U/ml of IFN-α, IFN-β, IFN-λ1 or IFN-λ2. Cells were lysed after 3 days and intranuclear HBV cccDNA was analyzed by differential DNA denaturation (3D)-PCR to detect deaminated cccDNA. **(B)** Primary human

hepatocytes were treated with 100 U/ml of IFN-λ1 or IFN-λ2 for 7 days and PCR products of HBV cccDNA were analyzed by 3D-PCR. **(C)** 3D-PCR products of treated HepaRG cells obtained at indicated temperatures were cloned and sequenced. (D) Base frequencies in 3D-PCR products of mock, IFN-α, IFN-β, IFN-λ1 and IFN-λ2 treated cells were quantified.

Figure 7. Induction of APOBEC deaminases and regulators of IFN signaling. Differentiated HepaRG cells were stimulated with 100 U/ml of IFN-α, IFN-β, IFN-λ1 or IFNλ2. **(A)** mRNA expression levels of cytosine deaminase APOBEC3A were analyzed by qRT-PCR after 2h, 6h, 12h, 24h, 3d and 7d of treatment and normalized to GAPDH. **(B)** APOBEC3A protein induction was analyzed by Western blot analysis after 24h of IFN-α as well as 48h of IFN-β, IFN-λ1 or IFNλ2 stimulation of dHepaRGs and PHH. **(C,D)** APOBEC deaminases B and G **(C)** and negative regulators of IFN signaling USP18 and SOCS1 **(D)** were analyzed by qRT-PCR and normalized to GAPDH expression after 2h, 6h, 12h, 24h, 3d and 7d stimulation with the indicated IFNs.

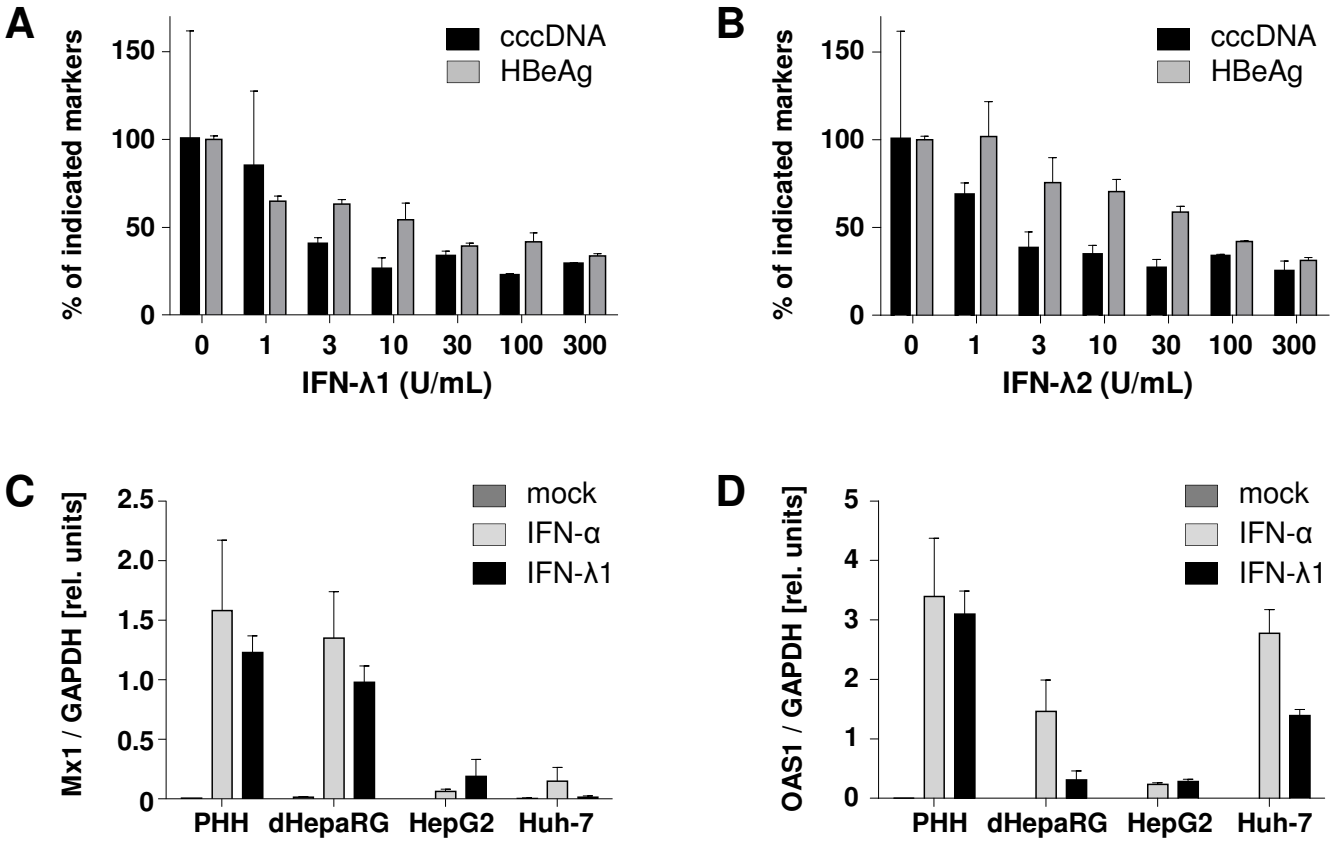

Figure 1

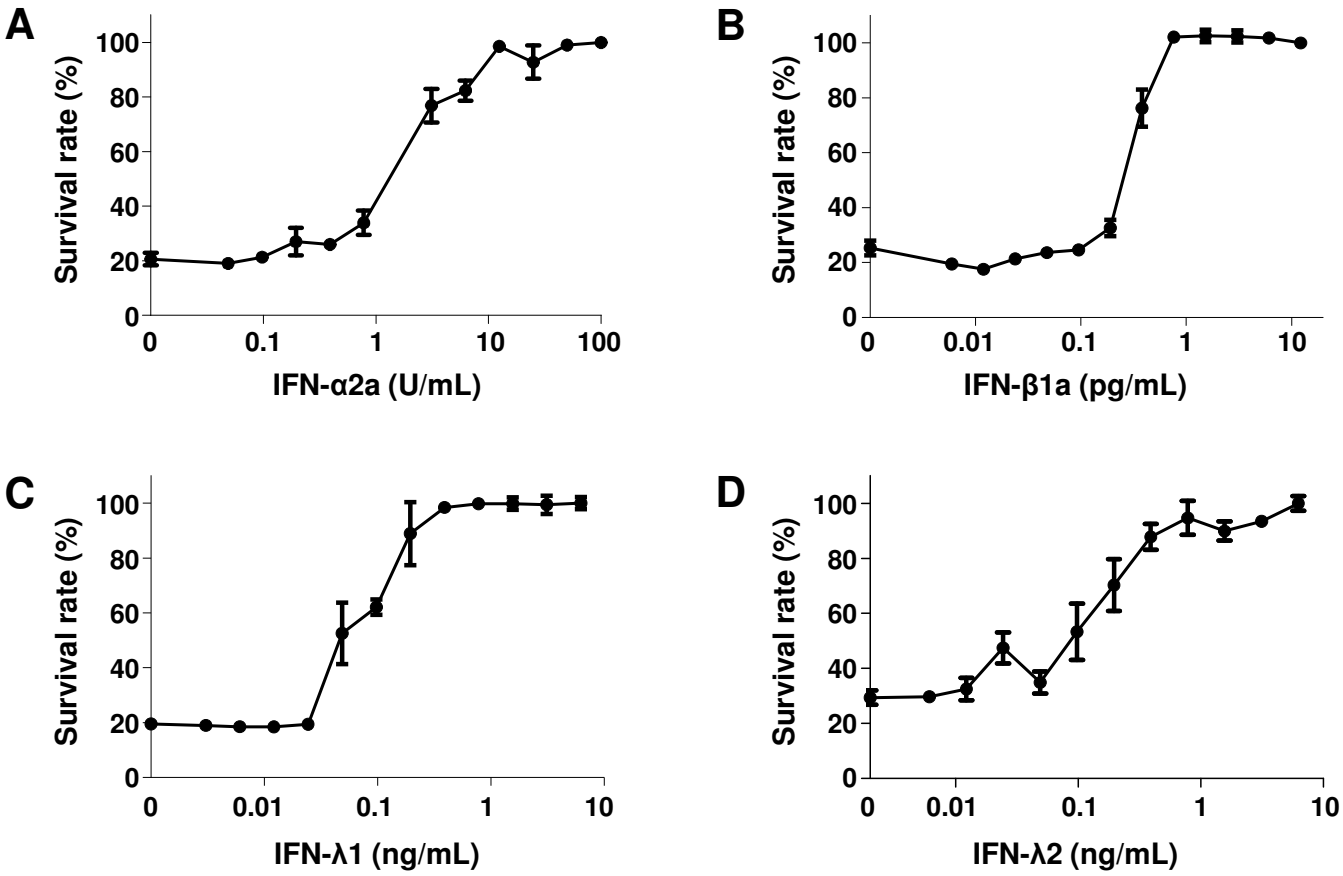

Figure 2

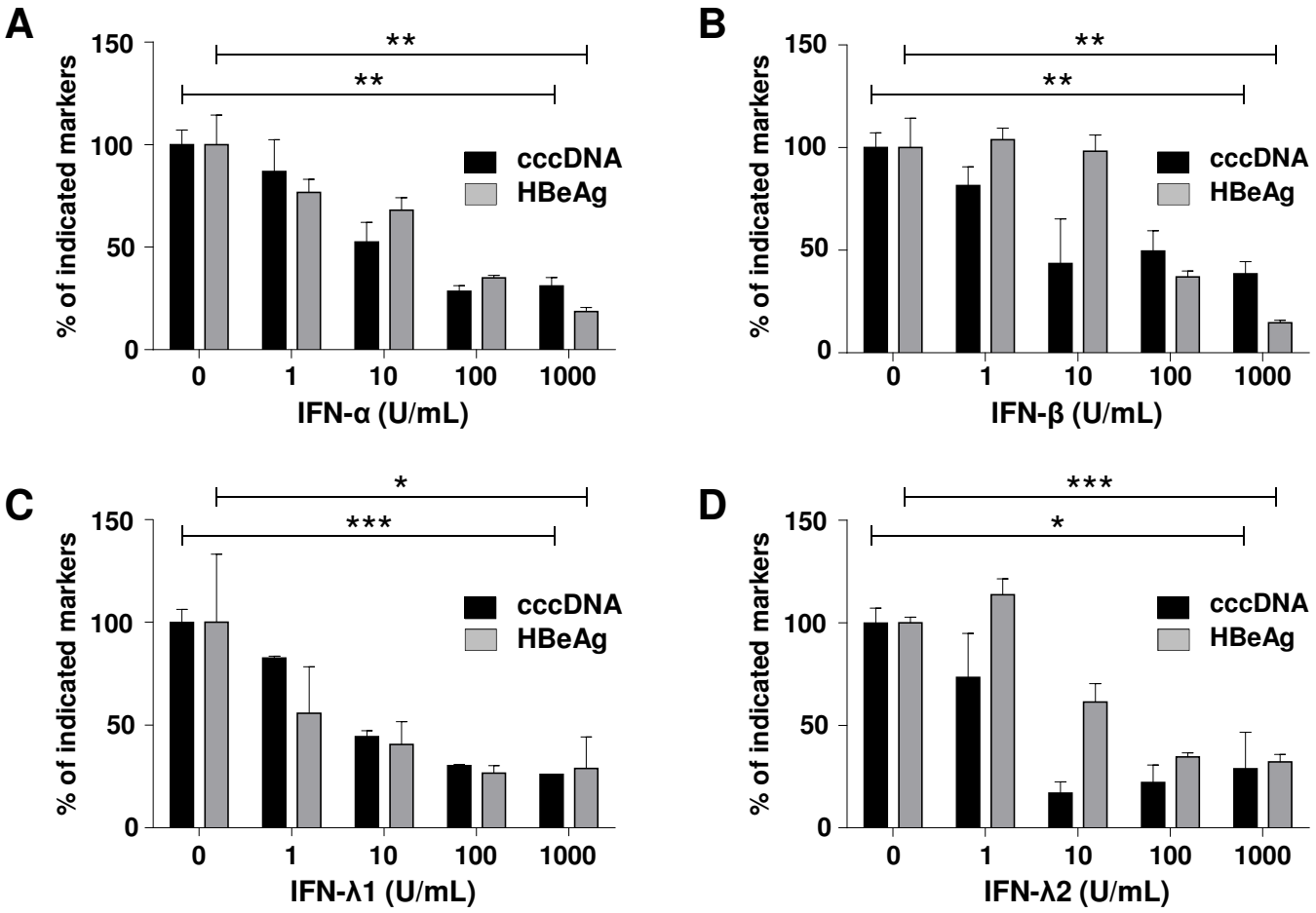

Figure 3

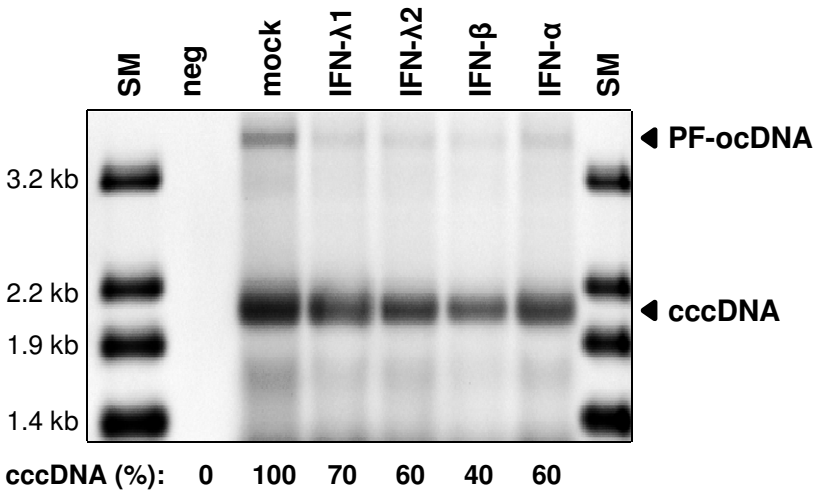

Figure 4

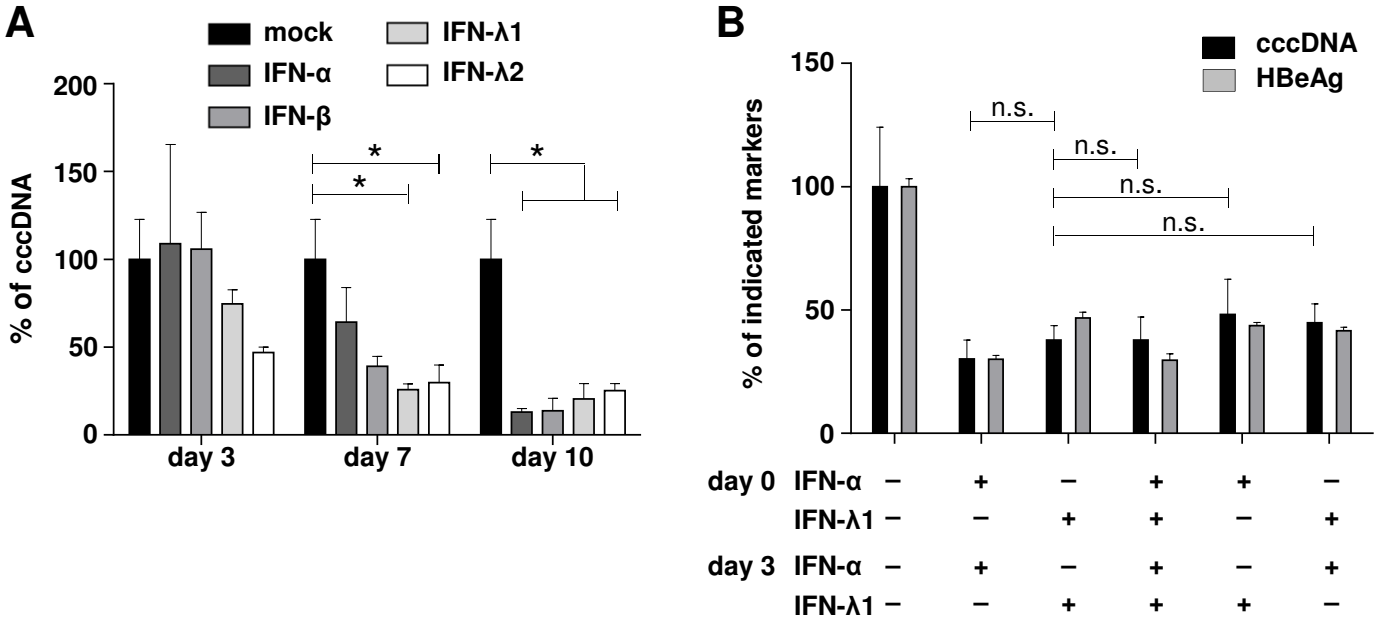

Figure 5

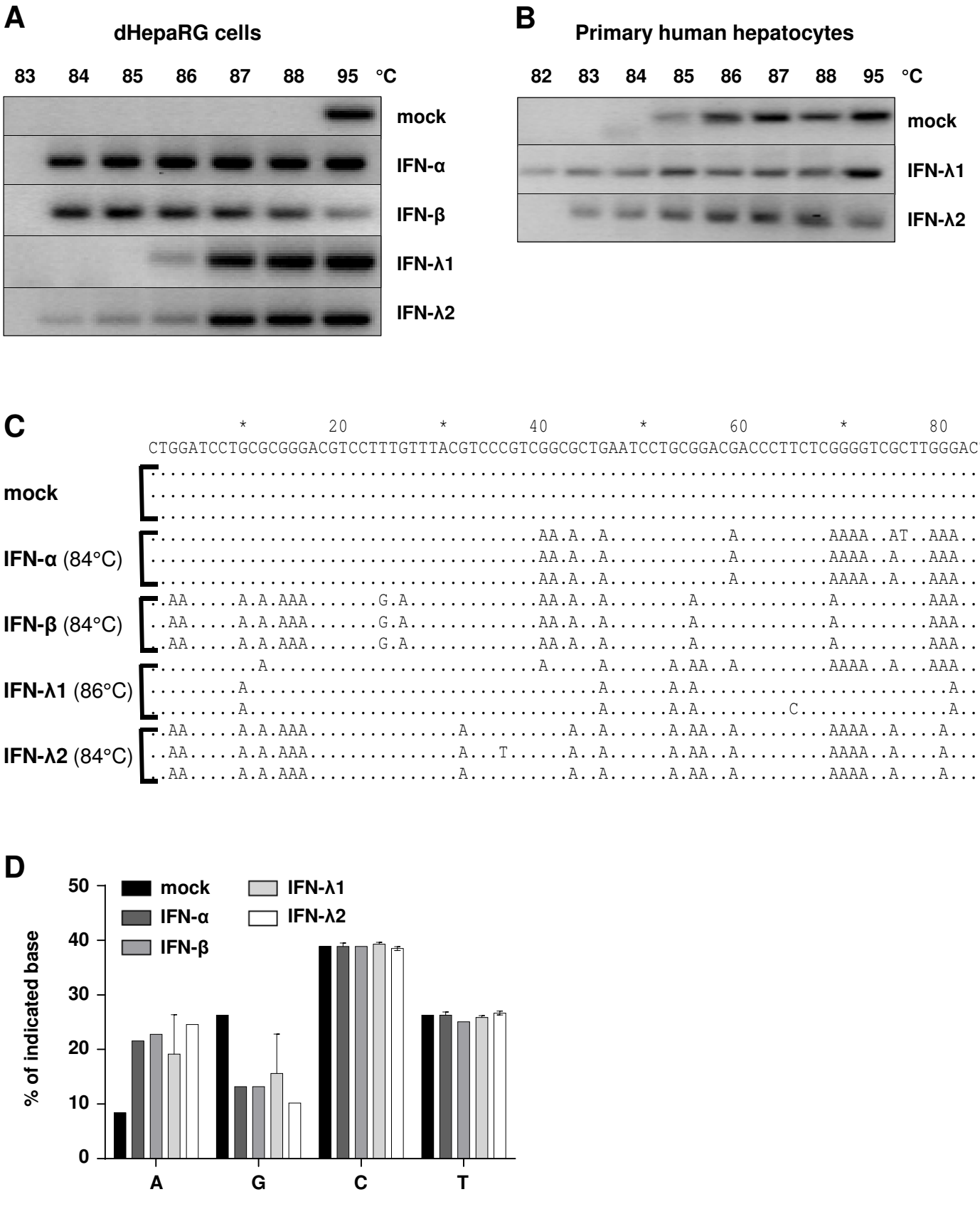

Figure 6

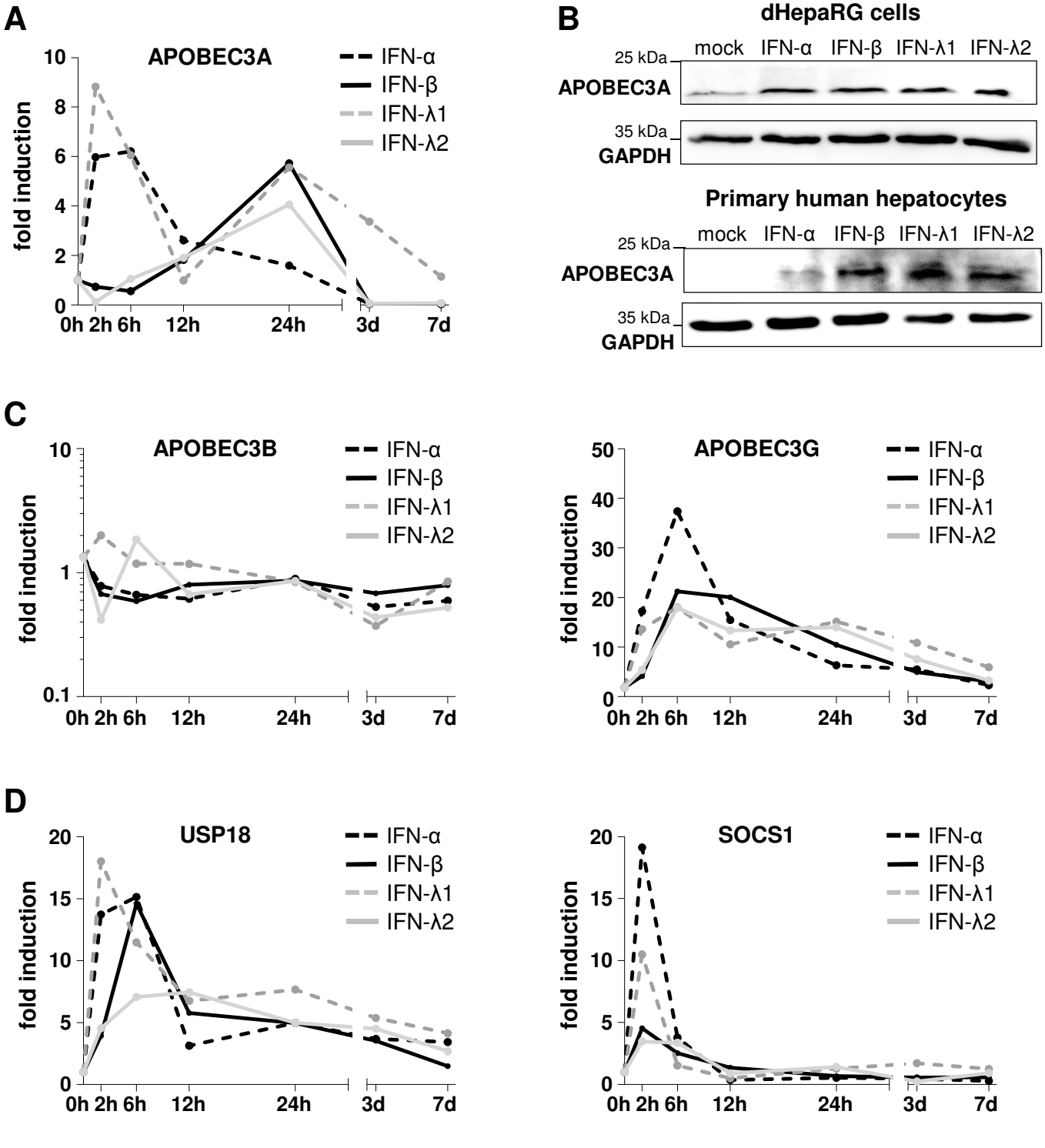

Figure 7