The Journal of Infectious Diseases

MAJOR ARTICLE

Comparative Analysis of the Antiviral Effects Mediated by Type I and III Interferons in Hepatitis B Virus–Infected Hepatocytes

Jan-Hendrik Bockmann,[1,2,3] Daniela Stadler,[1] Yuchen Xia,[1,4] Chunkyu Ko,[1] Jochen M. Wettengel,[1] Julian Schulze zur Wiesch,[2,3] Maura Dandri,[2,3] and Ulrike Protzer[1,3]

[1]Institute of Virology, Technische Universität München/Helmholtz Zentrum München, Munich, [2]I. Department of Internal Medicine, Center for Internal Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, and [3]German Center for Infection Research, Munich and Hamburg partner sites, Germany; and [4]State Key Laboratory of Virology, School of Basic Medical Sciences, Wuhan University, China

Background. Type III interferons (IFNs) (λ1–3) activate similar signaling cascades as type I IFNs (α and β) via different receptors. Since 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 DNA and covalently closed circular DNA (cccDNA) levels in HBV-infected cells. IFN-β and -λ were at least as efficient as IFN-α. Differential DNA-denaturing polymerase chain reaction 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 apolipoprotein B messenger RNA–editing enzyme–catalytic polypeptide-like (APOBEC) deaminases 3A and 3G within 24 hours 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.

Keywords. HBV; interferon-lambda; interferon-beta; HepaRG cells; primary human hepatocytes.

Hepatitis B virus (HBV) is a major human pathogen with >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 hepatitis B surface antigen seroconversion, referred to as "functional cure" in patients with chronic hepatitis B [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, posttranscriptional, and epigenetic effects on HBV [8–11]. In addition, a potentially curative molecular mechanism of noncytolytic purging of HBV cccDNA induced by IFN-α and mediated by cytosine deamination has been reported [12]. Apolipoprotein B messenger RNA–editing enzyme–catalytic polypeptide-like 3 (APOBEC3) proteins have long been known as cytosine deaminases involved in immune responses against HIV, HBV and plasmid DNA [13, 14]. Now, APOBEC3A and APOBEC3B 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 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 the JAK/STAT pathway, finally inducing the expression of interferon-stimulated genes (ISGs) [17, 18]. In the present study, we used

Received 21 October 2018; editorial decision 23 March 2019; accepted 27 March 2019; published online March 29, 2019.

Correspondence: Jan-Hendrik Bockmann, MD, I. Department of Internal Medicine, Center for Internal Medicine, University Medical Center Hamburg-Eppendorf, Martinistr. 52, D-20246 Hamburg, Germany (j.bockmann@uke.de).

The Journal of Infectious Diseases® 2019;XX(XX):1–11

DOI: 10.1093/infdis/jiz143

primary human hepatocytes (PHHs) and differentiated HepaRG cells (dHepaRG) as in vitro infection models for studying innate immune responses induced by human IFN-β1a and IFN-λ1/2 on HBV replication and on HBV cccDNA in human hepatocytes. In particular, we addressed the question whether human IFNs 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]. PHHs were obtained within the ethical framework of the nonprofit foundation Human Tissue and Cell Research (Germany). After obtaining informed consent from patients, PHHs 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 viral particles (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 infection of HepaRG cells, respectively [25].

Analysis of HBV Infection

Hepatitis B e antigen (HBeAg) was determined by commercial immunoassay (Siemens Molecular Diagnostics). Total DNA and nuclear DNA were purified from infected cells. HBV DNA and cccDNA were detected using selective polymerase chain reaction (PCR) primers and normalized to single-copy gene Prnp [26, 27]. PCR products of cccDNA quantitative PCR (qPCR) assays 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]. The performance and quality of HBV DNA and cccDNA qPCRs were evaluated earlier [16].

Quantitative Reverse-Transcription PCR

RNA was extracted from cell layers using the peqGOLD Total RNA Kit (Peqlab) and transcribed into cDNA with the SuperScript III First-Strand Synthesis SuperMix kit for quantitative reverse-transcription PCR (Invitrogen). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. GAPDH, Mx1, and APOBEC 3A, B, and G, primers have been described previously [12]. Other primers were as follows: SOCS1-forward (fw): TTTTTCGCCCTTAGCGTGA; SOCS1-reverse (rev): AGCAGCTCGAAGAGGCAGTC; USP18-fw: CTCAGTCCCGACGTGGAACT; USP18-rev: ATCTCTCAAGCGCCATGCA; IL28R-fw: CCCAAGGGT AAGAGCTTCGAT; IL28R-rev: CCTTCATATTTTACTGAC ATGGACAAG; IL10R-for: TTGCTGTGGTGCGTTTACAAG; IL10R-rev: CTTTCAGGTGCTGTGGAAGAGA.

cccDNA Southern Blot

dHepaRG cells overexpressing a tetracycline-regulated Na+-taurocholate cotransporting polypeptide (NTCP) as HBV receptor (HepaRG-TR-NTCP cells) were infected with HBV at a multiplicity of infection of 1000 DNA-containing vp/cell. NTCP expression was induced either 2 days prior to or simultaneously with HBV infection. After 7 days, IFN 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 PHHs were lysed in Pierce radioimmunoprecipitation assay buffer (Thermo Scientific) and cooked at 95°C for 5 minutes after addition of loading dye (50 mM Tris-HCl pH6.8, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.01% bromophenol blue, 1% β-mercaptoethanol). Proteins were separated by 12% SDS–polyacrylamide gel electrophoresis, transferred onto a methanol-activated polyvinylidene difluoride (PVDF) membrane by wet blot procedure, and stained by primary (rabbit-anti-A3A 1:250 [Sigma; catalog number SAB4500753]; rabbit-anti-Mx1 1:1000 [Proteintech; catalog number 13750-1-AP]; mouse-anti-GAPDH 1:5000 [Acris Antibodies; catalog number ACR001PT]) and secondary antibodies (1:10000, Sigma-Aldrich). Membranes were blocked with Roti-Block (Roth).

Statistical Analysis

The Student unpaired 2-tailed *t* test was performed using Prism 6 software (GraphPad). Data are shown as mean ± standard deviation. *P* values < .05 were considered significant.

RESULTS

Type I and III IFNs Reduce HBV cccDNA in Primary Human Hepatocytes and dHepaRG Cells

Lymphotoxin (LT)–β receptor agonization 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 PHHs for 7 days with different doses of the type III IFNs, IFN-λ1 or IFN-λ2 (Figure 1A and 1B). 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/ml IFN-λ1 and 100 U/ml IFN-λ2, respectively.

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

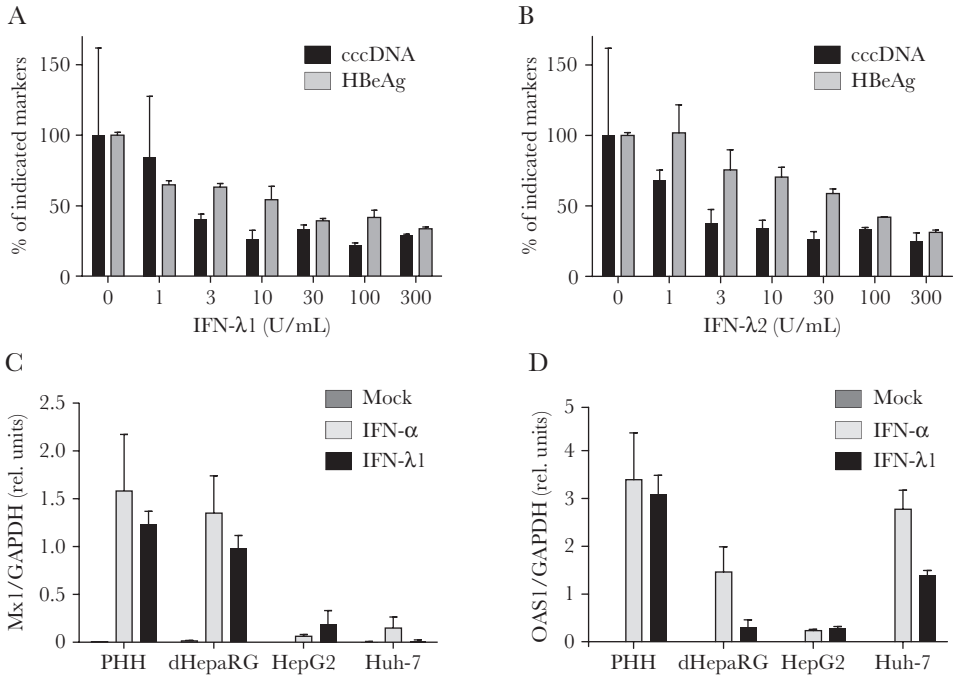

Figure 1. Comparison of type I and type III interferons (IFNs) in primary human hepatocytes (PHHs). *A* and *B*, PHHs were infected with hepatitis B virus (HBV) at a multiplicity of infection of 200 DNA-containing particles/cell for 10 days and treated for another 7 days with 1–300 U/mL of IFN-λ1 (*A*) or IFN-λ2 (*B*). HBV covalently closed circular DNA (cccDNA) amounts were determined by quantitative polymerase chain reaction (qPCR) and normalized to Prnp. Hepatitis B e antigen (HBeAg) in cell culture medium was analyzed by enzyme-linked immunosorbent assay. *C* and *D*, IFN-stimulated gene induction in PHHs was compared to different cell lines. Indicated cells were treated with 100 U/mL IFN-α or IFN-λ1 for 12 hours, and messenger RNA expression levels of Mx1/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (*C*) and 2'-5'-oligoadenylate synthetase 1/GAPDH (*D*) were assessed by reverse-transcription qPCR.

responsiveness to IFN. dHepaRG cells responded to both type I and III IFN treatment by up-regulating IFN-stimulated gene Mx1 with a strength comparable to PHHs (Figure 1C). Upregulation of 2'-5'-oligoadenylate synthetase 1 (OAS1) in IFN-α/-λ1–stimulated dHepaRG cells was lower than in PHHs or Huh-7 cells (Figure 1D). In contrast, HepG2 cells 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 activity, 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 (IC_{50}). 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 IC_{50} values of 0.3 pg/mL (IFN-β), 0.1 ng/mL (IFN-λ1), and 0.2 ng/mL (IFN-λ2), respectively, and these 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 PHHs (Figure 1A and 1B), 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). 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

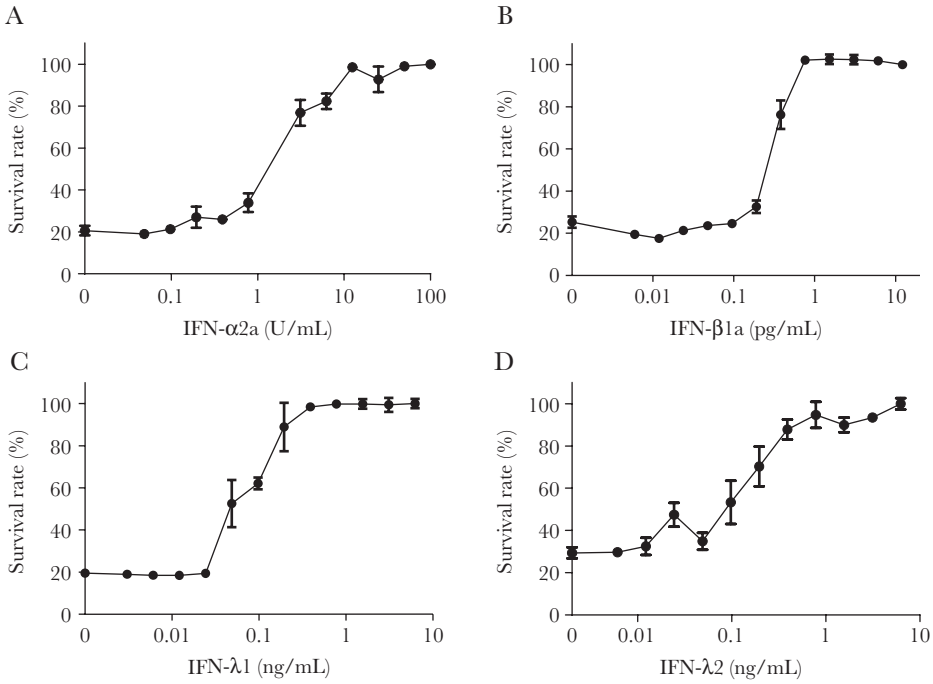

Figure 2. Interferon (IFN) activity in differentiated HepaRG (dHepaRG) cells determined by IFN bioassay. dHepaRG cells were treated with indicated doses of IFN-α (*A*), IFN-β (*B*), IFN-λ1 (*C*), or IFN-λ2 (*D*). Cells were then infected with vesicular stomatitis virus and virus-induced cytotoxicity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

reductions of cccDNA already after 7 days of treatment with type III IFN (IFN-λ1: 74.14%, $P = .0323$; IFN-λ2: 79.49%, $P = .0351$), whereas significant cccDNA reduction by type I IFNs was only determined at day 10 (IFN-α: 86.92%, $P = .0192$; IFN-β: 86.20%, $P = .0226$). Between days 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 noncytolytic 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, that is, 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-λ receptor subunits IL-10R or IL-28R 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 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 PHHs 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

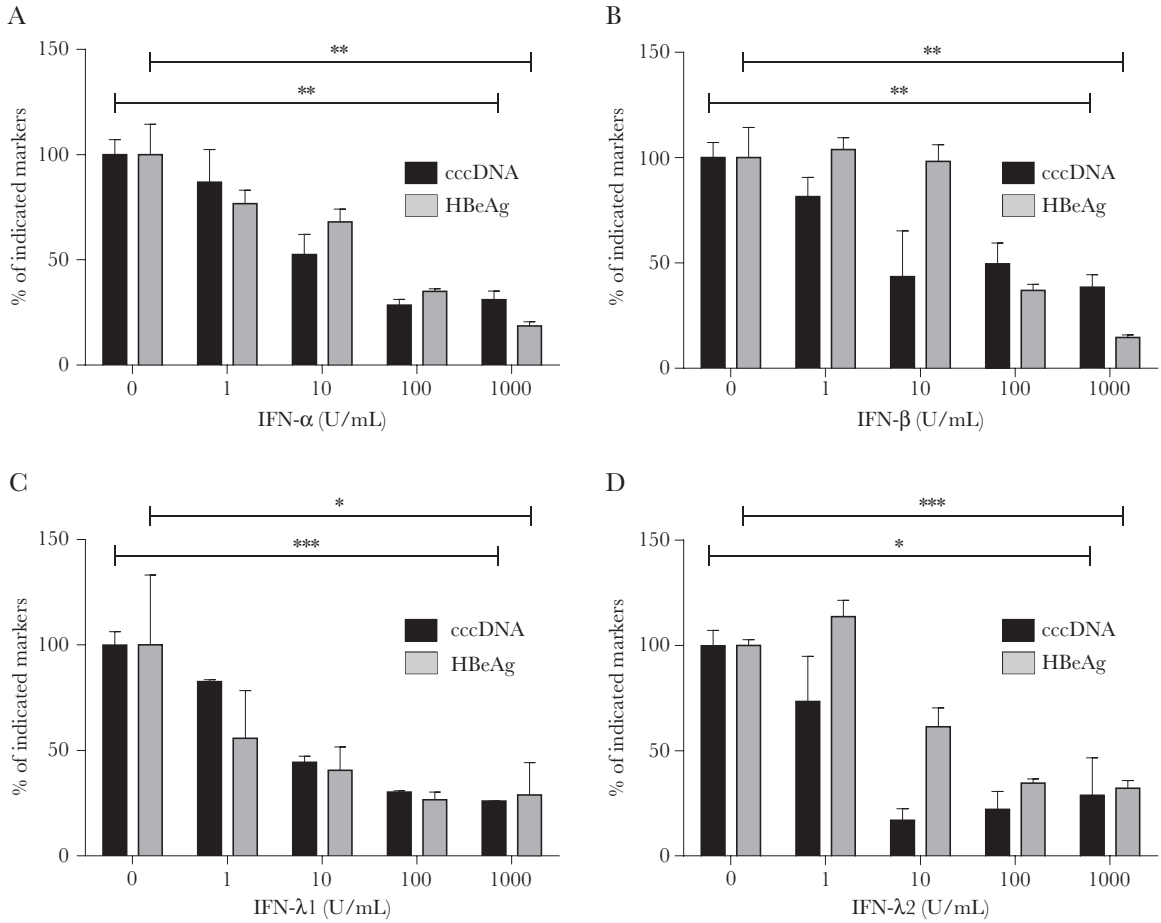

Figure 3. Influence of type I and III interferon (IFN) treatment on hepatitis B e antigen (HBeAg) secretion and intracellular hepatitis B virus (HBV) covalently closed circular DNA (cccDNA). Differentiated HepaRG cells were infected with HBV at a multiplicity of infection of 200 DNA-containing particles/cell for 10 days and treated for another 7 days with 1–1000 U/mL of IFN-α (*A*), IFN-β (*B*), IFN-λ1 (*C*), or IFN-λ2 (*D*). HBV cccDNA amounts were determined by quantitative polymerase chain reaction and normalized to the Prnp gene. HBeAg was measured by enzyme-linked immunosorbent assay in cell culture medium collected from day 7 to day 10. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ by Student unpaired *t* test.

in the PCR products obtained from IFN-α, IFN-β, IFN-λ1, or IFN-λ2–treated samples, but not in 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 4 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 LT-β receptor agonization, respectively [12]. In contrast to LT-β receptor agonization, which activates APOBEC3B via the NF-κB pathway, IFN-α via activation of STAT signaling only induces APOBEC3A. As shown in Figure 7A, IFN-β (5.73-fold, 24 hours), IFN-λ1 (8.83-fold, 2 hours), and IFN-λ2 (4.06-fold, 24 hours) can also induce APOBEC3A. The induction of APOBEC3A in dHepaRG cells and PHHs was confirmed on protein level by Western blot analysis (Figure 7B). In line with the messenger RNA kinetics, APOBEC3A protein was induced 24 hours after IFN-α stimulation and 48 hours after IFN-β, IFN-λ1, and IFN-λ2 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, which 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

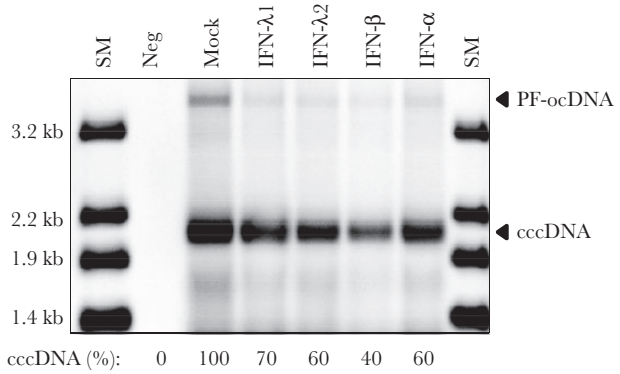

Figure 4. Southern blot analysis of hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) in type I and III interferon (IFN)–treated cells. Differentiated HepaRG-TR-NTCP cells were infected with HBV at a multiplicity of infection of 1000 viral particles per cell and treated with 100 U/mL of IFN-α, IFN-β, IFN-λ1, or IFN-λ2 for 18 days. Protein-free intracellular HBV DNA was analyzed by Southern blot using an HBV DNA probe. HBV protein-free open-circle DNA and cccDNA are indicated. Relative quantification of signal density is indicated below the blot. Abbreviations: cccDNA, covalently closed circular DNA; IFN, interferon; ocDNA, open-circle DNA; PF, protein-free; SM, size marker.

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 and Supplementary Figure 2*A* and 2*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 6 hours (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, suppressor of cytokine signaling 1 (SOCS1), was induced by all 4 IFNs very rapidly (2 hours), but already down-regulated again after 12 hours (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 up-regulated cytosine deaminase APOBEC3A and induced cccDNA deamination as well as cccDNA loss in treated cells. All 4 IFNs were titrated for their antiviral effect in an IFN bioassay in dHepaRG cells, that is, 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 4 cytokines and nonsynergistic in combined treatment. This was somewhat unexpected and indicated that all 4 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 PHHs, 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 noncytolytic fashion by activation of APOBEC3 deaminases [33]. The differences may relate to differences in

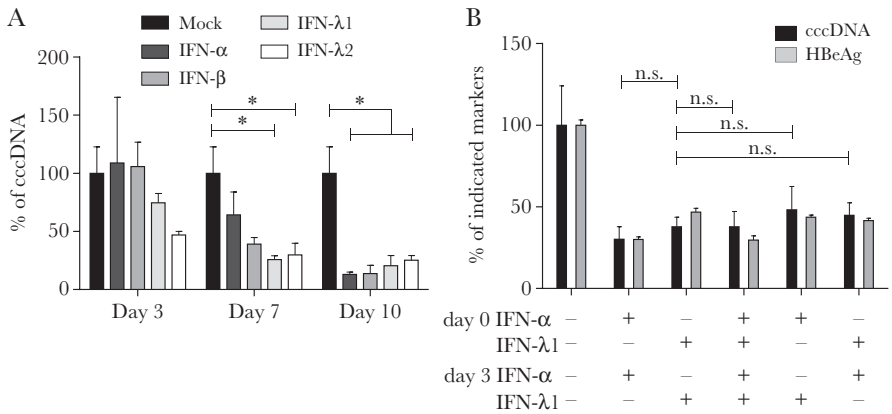

Figure 5. Kinetics and synergistic effects of type I and III interferons (IFNs). *A*, Differentiated HepaRG (dHepaRG) cells were treated with 100 U/mL of IFN-α, IFN-β, IFN-λ1, or IFN-λ2. Cells were lysed after 3, 7, or 10 days, and the intranuclear hepatitis B virus covalently closed circular DNA (cccDNA) amount was evaluated by quantitative polymerase chain reaction (qPCR) and normalized to Prnp. *B*, Infected dHepaRG cells were treated with 100 U/mL of IFN-α or 5 U/mL of IFN-λ1 on day 0 and day 3 to achieve submaximum reduction of cccDNA/hepatitis B e antigen (HBeAg) amounts after 7 days of treatment. 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 enzyme-linked immunosorbent assay. **P* < .05; n.s., not significant by Student unpaired *t* test.

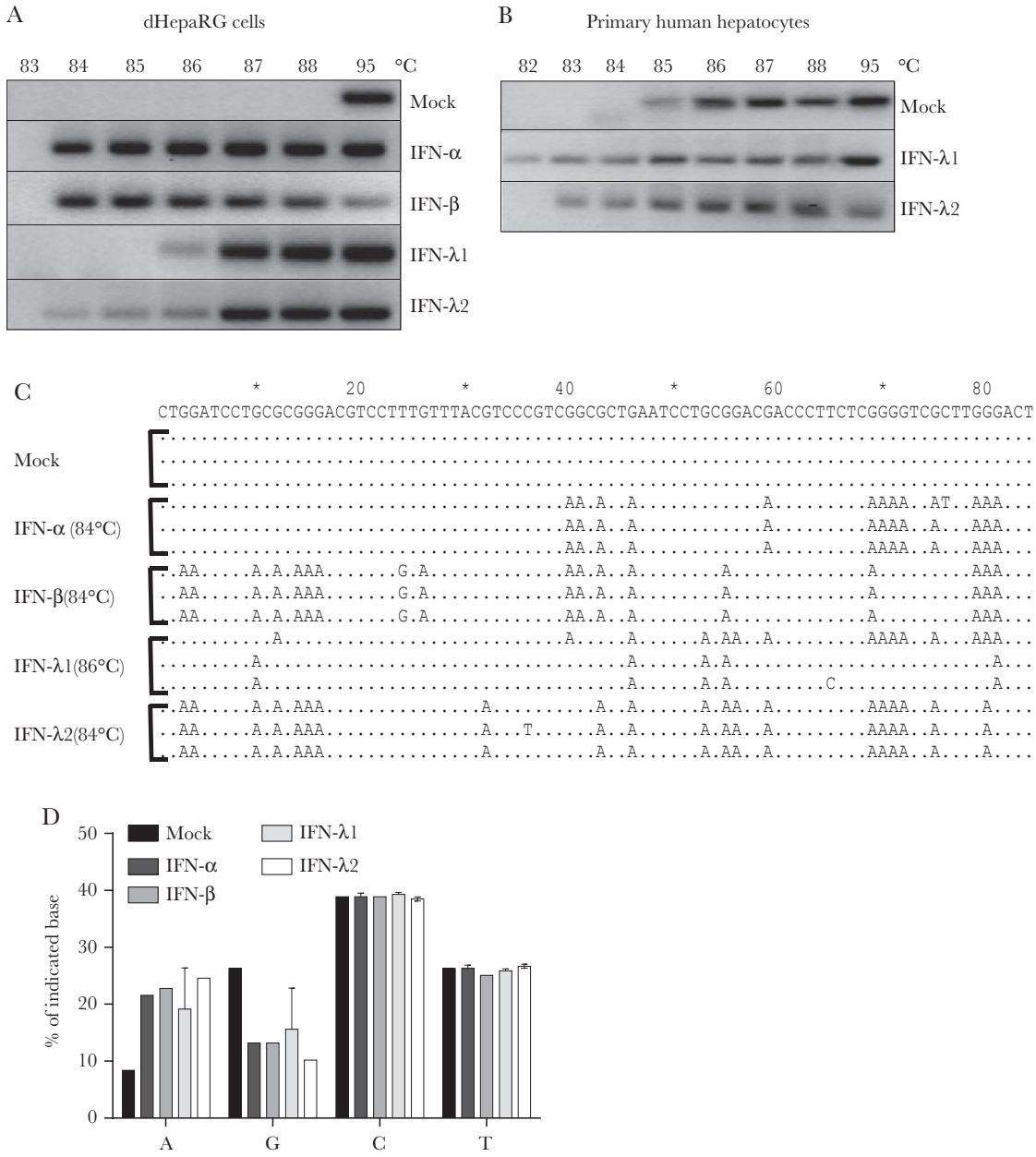

Figure 6. Deamination of hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) after treatment with type I and III interferons (IFNs). *A*, Differentiated HepaRG (dHepaRG) cells were treated with 100 U/mL of IFN-α, IFN-β, IFN-λ1, or IFN-λ2. Cells were lysed after 3 days and intranuclear HBV cccDNA was analyzed by differential DNA denaturation polymerase chain reaction (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.

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 more strongly affected 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

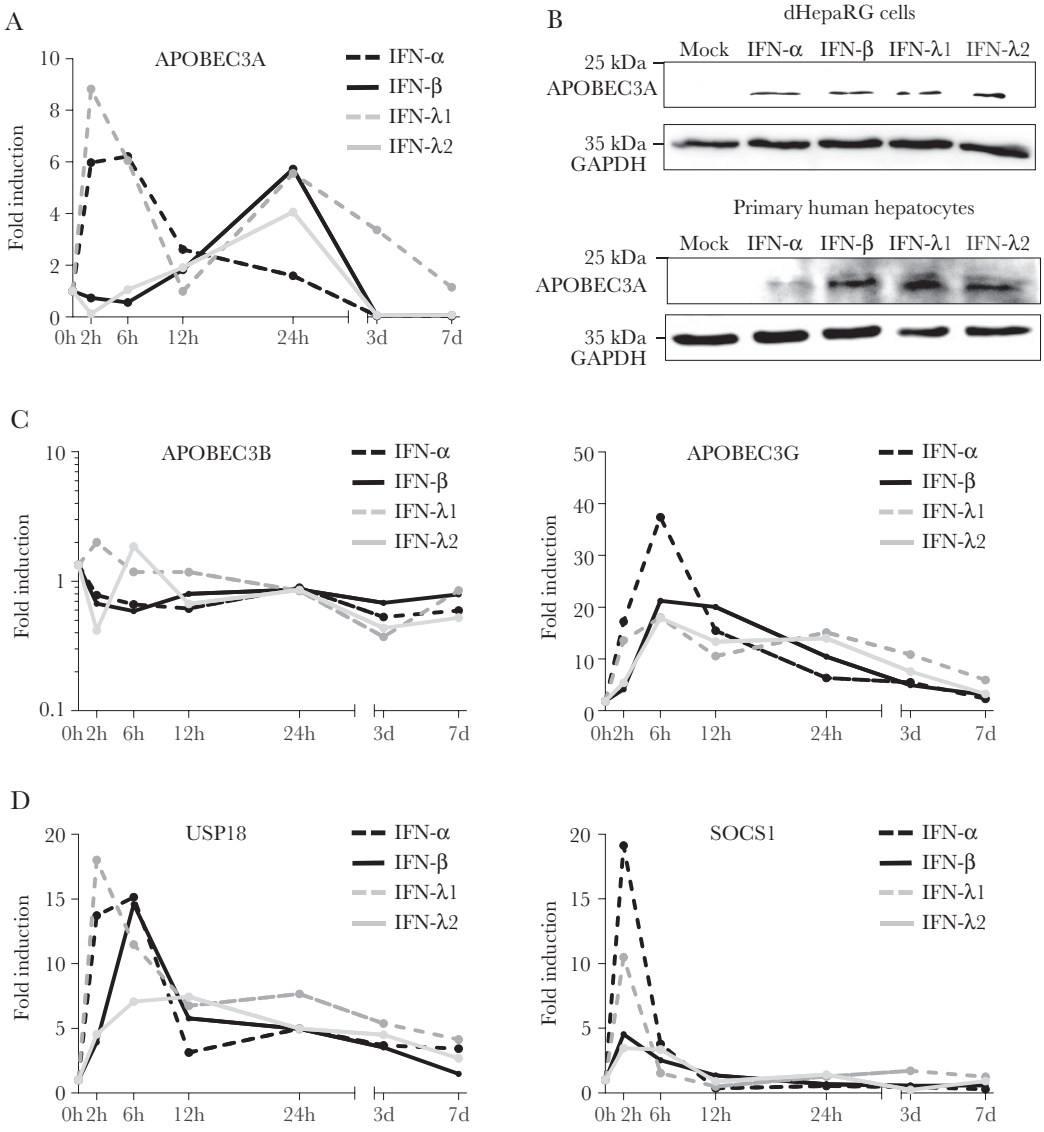

Figure 7. Induction of apolipoprotein B messenger RNA–editing enzyme–catalytic polypeptide-like (APOBEC) deaminases and regulators of interferon (IFN) signaling. Differentiated HepaRG (dHepaRG) cells were stimulated with 100 U/mL of IFN-α, IFN-β, IFN-λ1, or IFN-λ2. *A*, Messenger RNA expression levels of cytosine deaminase APOBEC3A were analyzed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) after 2 hours, 6 hours, 12 hours, 24 hours, 3 days, and 7 days of treatment and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). *B*, APOBEC3A protein induction was analyzed by Western blot analysis after 24 hours of IFN-α as well as 48 hours of IFN-β, IFN-λ1, or IFN-λ2 stimulation of dHepaRGs and primary human hepatocytes. *C* and *D*, APOBEC3 deaminases B and G (*C*) and negative regulators of IFN signaling ubiquitin-specific peptidase 18 (USP18) and suppressor of cytokine signaling 1 (SOCS1) (*D*) were analyzed by qRT-PCR and normalized to GAPDH expression after 2 hours, 6 hours, 12 hours, 24 hours, 3 days, and 7 days of stimulation with the indicated IFNs.

respective cytokine in account. A unique characteristic 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, whereas 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 4 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 lifetime of IFN-β in complex with the IFN receptor subunit IFNAR1 is substantially longer than that of IFN-α2 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 IFN-α2 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 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 Toll-like receptor–induced responses in the liver and 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 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, for example, by exerting different effects on T cells or NK cells, human hepatocytes, or intrahepatic nonparenchymal 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 (HDV) 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 coinfection with HDV.

In summary, our results show that IFN-β and type III IFNs can reduce cccDNA in infected hepatocytes in a noncytolytic 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 favorable 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.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We are grateful to Kathrin Kappes, Theresa Asen, Nathalie Röder, and Romina Bester for their technical support.

Author contributions. J. B., D. S., Y. X., C. K., and J. W. performed experiments. U. P., J. S. W., and M. D. initiated the study. J. B., D. S., Y. X., and U. P. designed the experiments. J. B. and D. S. analyzed data. J. B. and U. P. wrote the manuscript.

Financial support. The study was funded by the Deutsche Forschungsgemein schaft (German Research Foundation; project number 272983813–TRR 179). J. B. received a clinical leave stipend from the German Center for Infection Research.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. MacLachlan JH, Cowie BC. Hepatitis B virus epidemiology. Cold Spring Harb Perspect Med **2015**; 5:a021410.
2. Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet **2015**; 386:1546–55.
3. Protzer U, Maini MK, Knolle PA. Living in the liver: hepatic infections. Nat Rev Immunol **2012**; 12:201–13.
4. Hösel M, Quasdorff M, Wiegmann K, et al. Not interferon, but interleukin-6 controls early gene expression in hepatitis B virus infection. Hepatology **2009**; 50:1773–82.
5. Cheng X, Xia Y, Serti E, et al. Hepatitis B virus evades innate immunity of hepatocytes but activates cytokine production by macrophages. Hepatology **2017**; 66:1779–93.
6. Wursthorn K, Lutgehetmann M, Dandri M, et al. Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. Hepatology **2006**; 44:675–84.
7. Lutgehetmann M, Bornscheuer T, Volz T, et al. Hepatitis B virus limits response of human hepatocytes to interferon-alpha in chimeric mice. Gastroenterology **2011**; 140:2074–83, 83.e1–2.
8. Belloni L, Allweiss L, Guerrieri F, et al. IFN-α inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. J Clin Invest **2012**; 122:529–37.
9. Rang A, Günther S, Will H. Effect of interferon alpha on hepatitis B virus replication and gene expression in transiently transfected human hepatoma cells. J Hepatol **1999**; 31:791–9.
10. Pasquetto V, Wieland SF, Uprichard SL, Tripodi M, Chisari FV. Cytokine-sensitive replication of hepatitis B virus in immortalized mouse hepatocyte cultures. J Virol **2002**; 76:5646–53.

11. Wieland SF, Guidotti LG, Chisari FV. Intrahepatic induction of alpha/beta interferon eliminates viral RNA-containing capsids in hepatitis B virus transgenic mice. J Virol **2000**; 74:4165–73.
12. Lucifora J, Xia Y, Reisinger F, et al. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. Science **2014**; 343:1221–8.
13. Stenglein MD, Burns MB, Li M, Lengyel J, Harris RS. APOBEC3 proteins mediate the clearance of foreign DNA from human cells. Nat Struct Mol Biol **2010**; 17:222–9.
14. Suspene R, Guetard D, Henry M, Sommer P, Wain-Hobson S, Vartanian JP. Extensive editing of both hepatitis B virus DNA strands by APOBEC3 cytidine deaminases in vitro and in vivo. Proc Natl Acad Sci U S A **2005**; 102:8321–6.
15. Bonvin M, Achermann F, Greeve I, et al. Interferon-inducible expression of APOBEC3 editing enzymes in human hepatocytes and inhibition of hepatitis B virus replication. Hepatology **2006**; 43:1364–74.
16. Xia Y, Stadler D, Lucifora J, et al. Interferon-γ and tumor necrosis factor-α produced by T cells reduce the HBV persistence form, cccDNA, without cytolysis. Gastroenterology **2016**; 150:194–205.
17. Odendall C, Kagan JC. The unique regulation and functions of type III interferons in antiviral immunity. Curr Opin Virol **2015**; 12:47–52.
18. Donnelly RP, Kotenko SV. Interferon-lambda: a new addition to an old family. J Interferon Cytokine Res **2010**; 30:555–64.
19. Seitz S, Iancu C, Volz T, et al. A slow maturation process renders hepatitis B virus infectious. Cell Host Microbe **2016**; 20:25–35.
20. Ko C, Chakraborty A, Chou WM, et al. Hepatitis B virus genome recycling and de novo secondary infection events maintain stable cccDNA levels. J Hepatol **2018**; 69:1231–41.
21. Schulze-Bergkamen H, Untergasser A, Dax A, et al. Primary human hepatocytes–a valuable tool for investigation of apoptosis and hepatitis B virus infection. J Hepatol **2003**; 38:736–44.
22. Lee SM, Schelcher C, Demmel M, Hauner M, Thasler WE. Isolation of human hepatocytes by a two-step collagenase perfusion procedure. J Vis Exp **2013**. doi:10.3791/50615.
23. Thasler WE, Weiss TS, Schillhorn K, Stoll PT, Irrgang B, Jauch KW. Charitable state-controlled foundation human tissue and cell research: ethic and legal aspects in the supply of surgically removed human tissue for research in the academic and commercial sector in Germany. Cell Tissue Bank **2003**; 4:49–56.
24. Hantz O, Parent R, Durantel D, Gripon P, Guguen-Guillouzo C, Zoulim F. Persistence of the hepatitis B virus covalently closed circular DNA in HepaRG human hepatocyte-like cells. J Gen Virol **2009**; 90:127–35.
25. Bis RL, Stauffer TM, Singh SM, Lavoie TB, Mallela KM. High yield soluble bacterial expression and streamlined purification of recombinant human interferon α-2a. Protein Expr Purif **2014**; 99:138–46.
26. Protzer U, Seyfried S, Quasdorff M, et al. Antiviral activity and hepatoprotection by heme oxygenase-1 in hepatitis B virus infection. Gastroenterology **2007**; 133:1156–65.
27. Xia Y, Stadler D, Ko C, Protzer U. Analyses of HBV cccDNA quantification and modification. Methods Mol Biol **2017**; 1540:59–72.
28. Vartanian JP, Henry M, Marchio A, et al. Massive APOBEC3 editing of hepatitis B viral DNA in cirrhosis. PLoS Pathog **2010**; 6:e1000928.
29. Ko C, Lee S, Windisch MP, Ryu WS. DDX3 DEAD-box RNA helicase is a host factor that restricts hepatitis B virus replication at the transcriptional level. J Virol **2014**; 88:13689–98.
30. Liang G, Kitamura K, Wang Z, et al. RNA editing of hepatitis B virus transcripts by activation-induced cytidine deaminase. Proc Natl Acad Sci U S A **2013**; 110:2246–51.
31. Sarasin-Filipowicz M, Wang X, Yan M, et al. Alpha interferon induces long-lasting refractoriness of JAK-STAT signaling in the mouse liver through induction of USP18/UBP43. Mol Cell Biol **2009**; 29:4841–51.
32. Isorce N, Testoni B, Locatelli M, et al. Antiviral activity of various interferons and pro-inflammatory cytokines in non-transformed cultured hepatocytes infected with hepatitis B virus. Antiviral Res **2016**; 130:36–45.
33. Koh S, Kah J, Tham CYL, et al. Nonlytic lymphocytes engineered to express virus-specific T-cell receptors limit HBV infection by activating APOBEC3. Gastroenterology **2018**; 155:180–93.e6.
34. Bolen CR, Ding S, Robek MD, Kleinstein SH. Dynamic expression profiling of type I and type III interferon-stimulated hepatocytes reveals a stable hierarchy of gene expression. Hepatology **2014**; 59:1262–72.
35. Jilg N, Lin W, Hong J, et al. Kinetic differences in the induction of interferon stimulated genes by interferon-α and interleukin 28B are altered by infection with hepatitis C virus. Hepatology **2014**; 59:1250–61.
36. Makowska Z, Duong FH, Trincucci G, Tough DF, Heim MH. Interferon-β and interferon-λ signaling is not affected by interferon-induced refractoriness to interferon-α in vivo. Hepatology **2011**; 53:1154–63.
37. Piehler J, Thomas C, Garcia KC, Schreiber G. Structural and dynamic determinants of type I interferon receptor assembly and their functional interpretation. Immunol Rev **2012**; 250:317–34.
38. Arimoto KI, Löchte S, Stoner SA, et al. STAT2 is an essential adaptor in USP18-mediated suppression of type I interferon signaling. Nat Struct Mol Biol **2017**; 24:279–89.
39. Honke N, Shaabani N, Zhang DE, Hardt C, Lang KS. Multiple functions of USP18. Cell Death Dis **2016**; 7:e2444.

40. Shao RX, Zhang L, Hong Z, et al. SOCS1 abrogates IFN's antiviral effect on hepatitis C virus replication. Antiviral Res **2013**; 97:101–7.
41. Chan HLY, Ahn SH, Chang TT, et al; LIRA-B Study Team. Peginterferon lambda for the treatment of HBeAg-positive chronic hepatitis B: a randomized phase 2b study (LIRA-B). J Hepatol **2016**; 64:1011–9.
42. Giersch K, Homs M, Volz T, et al. Both interferon alpha and lambda can reduce all intrahepatic HDV infection markers in HBV/HDV infected humanized mice. Sci Rep **2017**; 7:3757.