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PASylated interferon α efficiently suppresses hepatitis B virus and induces anti-HBs seroconversion in HBV-transgenic mice

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ABSTRACT

Interferon α (IFNα) so far is the only therapeutic option for chronic hepatitis B virus (HBV) infection that can lead to virus clearance. Unfortunately, its application is limited by side effects and response rates are low. The aim of this study was to generate a novel long-acting IFNα with the help of PASylation technology that adds a polypeptide comprising Proline, Alanine and Serine (PAS) to increase plasma half-life. Following evaluation of four selected recombinant murine IFNα (mIFNα) subtypes in cell culture, the most active subtype, mIFNα11, was fused with a 600 amino acid PAS chain. The activity of PAS-mIFNα was assessed by interferon bioassay and further evaluated for induction of interferon-stimulated genes (ISG) and antiviral efficacy in cell culture as well as in HBV-transgenic mice. PAS-mIFNα induced expression of ISG comparable to unmodified mIFNα and, likewise, evoked dose-dependent reduction of HBV replication *in vitro*. *In vivo*, PAS-mIFNα led to pronounced suppression of HBV replication without detectable liver damage whereas conventional mIFNα treatment only had a modest antiviral effect. Importantly, all PAS-mIFNα treated mice showed an anti-HBs antibody response, lost HBsAg and achieved seroconversion after three weeks. PASylated IFNα showed a profoundly increased antiviral effect *in vivo* compared to the non-modified version without toxicity, providing proof-of-concept that an improved IFNα can achieve higher rates of HBV antiviral and immune control.

1. Introduction

Despite the availability of an effective vaccine, Hepatitis B virus (HBV) remains a major public health threat. Without a cure, around 260 million individuals with chronic HBV infection worldwide are at high risk of developing liver cirrhosis and hepatocellular carcinoma (Liang et al., 2015; Polaris Observatory, 2018). HBV is a small, enveloped DNA virus that specifically targets hepatocytes. The virus infects host cells through initial interaction with heparin sulfate proteoglycans, followed by binding to the sodium-taurocholate cotransporting polypeptide (NTCP) as specific receptor (Yan et al., 2012). After entry, the viral capsid is transported to the nucleus, where the relaxed circular

Abbreviations: ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA; EC_{50}, half-maximal effective concentration; HBc, hepatitis B virus core protein; HBeAg, hepatitis B e antigen; HBsAg, Hepatitis B surface antigen; HBV, hepatitis B virus; HBV-tg, HBV transgenic; H&E, hematoxylin and eosin staining; IFNα, interferon alpha; i.p., intraperitoneal; ISG, interferon-stimulated genes; MCMV, murine cytomegalovirus; mIFNα, murine IFNα; MX1, myxovirus resistance 1; NUC, nucleoside/nucleotide analog; NTCP, sodium-taurocholate cotransporting polypeptide; OAS1, 2'-5'-Oligoadenylate synthetase 1; PAS, Proline, Alanine and/or Serine; PAS-mIFNα, PASylated mIFNα; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; pgRNA, pregenomic RNA; qRT-PCR, quantitative reverse transcription PCR; qPCR, real-time PCR; rcDNA, relaxed circular DNA; SEAP, secreted alkaline phosphatase; SEC, size exclusion chromatography; VSV, vesicular stomatitis virus

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DNA (rcDNA) genome is released and converted by host enzymes into a covalently closed circular DNA (cccDNA) (Qi et al., 2016). HBV cccDNA persists as a minichromosome and serves as a template for transcription. The 3.5 kb pregenomic RNA (pgRNA) transcript is encapsidated and reverse-transcribed into new rcDNA in the viral capsid which finally becomes enveloped and released as progeny virus.

Currently, there are two types of approved treatments for chronic hepatitis B: (i) nucleoside/nucleotide analogs (NUC) and (ii) interferon (IFN) α. NUCs target the viral polymerase, hence inhibiting virus replication, however this therapy doesn't target the HBV persistence form, the so called cccDNA. Consequently, patients need long-term or lifelong treatment with the risk of developing side effects and severe hepatitis flares if treatment is interrupted. Since immune control rather than complete elimination of HBV cccDNA is observed when an acute or chronic infection is resolved, a functional cure rather than sterilizing immunity is the treatment goal (Lok et al., 2017). A functional cure is defined by sustained loss of hepatitis B surface antigen (HBsAg) with or without hepatitis B surface antibody (anti-HBs) seroconversion.

IFNs are a group of cytokines with pleiotropic biological activities. Among those, IFNα and also IFNβ, which belong to the type I IFN family, serve for various medical applications (Nagata et al., 1980). However, apart from receptor-mediated endocytosis, IFNα is quickly eliminated from the blood via renal filtration (Bino et al., 1982), which results in a very short plasma half-life ($t_{1/2}$) of merely ~44 min in the case of murine IFNα (mIFNα) (Bohoslawec et al., 1986) and of a couple of hours in humans (Tang et al., 2004). To allow longer dosing intervals in a clinical setting, the plasma half-life of human IFNα2b was extended by PEGylation, leading to the approved drug PEGIntron®, which shows retarded kidney clearance and $t_{1/2} \approx 40$ h (Wang et al., 2002). Similarly, Pegasys® was developed and approved for chronic hepatitis B treatment (Marcellin et al., 2004).

As the treatment of chronic diseases with PEGylated biologics bears potential disadvantages, such as vacuolization of the synthetic PEG polymer (Binder and Skerra, 2017), PASylation technology was developed as a biological alternative (Schlapschy et al., 2013). Indeed, a PASylated human IFN was successfully applied for the treatment in a mouse model of multiple sclerosis (Harari et al., 2014). PASylation involves the conjugation or genetic fusion of a biopharmaceutically active protein or peptide with a long hydrophilic polypeptide comprising Pro, Ala and/or Ser residues (PAS). Such PAS sequences adopt random coil conformation, which increases the hydrodynamic volume of the PASylated therapeutic protein above the pore size of the glomerular filter without being immunogenic (Schlapschy et al., 2013). Up to now, PASylation has been used to extend the plasma half-life of various biologics, including antibody fragments, a complement inhibitor, leptin, erythropoietin and human IFNβ1b (Binder and Skerra, 2017).

IFN-mediated antiviral effects on HBV are multifunctional and target several key steps of the viral life cycle (Xia et al., 2017a). IFNα treatment can also stimulate antiviral immunity and cause HBsAg loss and anti-HBs seroconversion, which is regarded a “functional cure” of hepatitis B (Lok et al., 2017; Zoulim et al., 2016). Response rates, however, remained low while the side effects are often limiting its application and dosing. The aim of the present study was to generate a novel long-acting IFNα based on PASylation technology and to evaluate its effect on HBV replication in a validated mouse model. Here we describe that PASylated mIFNα (PAS-mIFNα) effectively inhibits HBV replication *in vitro* and even more pronounced *in vivo*. Compared to unmodified mIFNα, PAS-mIFNα showed significantly increased antiviral effects and efficient anti-HBs seroconversion in HBV transgenic mice. PASylated interferon can thus be considered as a novel therapy to treat chronic hepatitis B for a functional cure.

2. Materials and methods

2.1. Analysis of HBV replication

Hepatitis B surface antigen (HBsAg) in cell culture samples was measured using the AXSYM system (Abbott Laboratories, Abbott Park, IL), and hepatitis B e antigen (HBeAg) was quantified by means of the BEP III system (Siemens, Munich, Germany). Total cellular DNA or DNA from cell culture supernatant was purified from infected cells using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). Total RNA was extracted from infected cells by means of Trizol reagent (Invitrogen, Thermo Fisher Scientific, St. Leon-Roth, Germany) and transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen). Quantitative reverse transcription PCR (qRT-PCR) or Real-time PCR (qPCR) was performed using the LightCycler system (Roche Diagnostics, Mannheim, Germany). HBV-DNA, pgRNA, and HBV-RNA were detected using specific PCR primers (listed in Supplementary Table 1). HBV-DNA from cell culture supernatant was quantified relative to an external plasmid standard as previously described (Xia et al., 2017a).

2.2. Animals

Seven-months old HBV-transgenic (HBV-tg) mice (kindly provided by H. Schaller, Heidelberg, Germany) were selected for comparable levels of HBeAg in serum. Legal standards for biological safety and animal care were met according to local requirements, and the experimental set-up was approved by the Regierung von Oberbayern. All animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the US Academy of Sciences and published by the National Institutes of Health. Intraperitoneal (i.p.) injection was performed and blood samples were collected from facial vein at indicated time points. Serum samples were prepared by resting whole blood at room temperature for 15 min followed by centrifuging at 2,000 × *g* for 10 min at 22 °C to remove the clot.

2.3. Serological analysis

Alanine aminotransferase (ALT) activity was measured murine serum using a Reflovet Plus reader (Roche Diagnostics). HBsAg, HBeAg and HBV small surface protein antibodies (anti-HBs) were quantified in 1:20 dilutions of murine serum using corresponding AXSYM assays (Abbott Laboratories). For quantification of serum HBV titers, DNA was extracted from 50 µL murine serum using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and subjected to qPCR as described (Protzer et al., 2007).

2.4. Statistical analysis

Data were subjected to an unpaired, 2-tailed Student's *t*-test and statistical significance was determined setting confidence intervals of 95% using Prism 7.0 software (Graph Pad Software, La Jolla, CA). Data are means ± standard deviation (s.d.). Two-sided P values $< .05$ were considered significant. *P $< .05$, **P $< .01$, ***P $< .001$.

3. Results

3.1. Preparation of recombinant PASylated and unmodified recombinant mIFNα subtypes

While essentially binding to the same receptors, different IFNα subtypes mediate diverse biological functions and display distinct antiviral activities (Gibbert et al., 2013). Clinically, human IFNα2 has been used to treat hepatitis B for more than 25 years (Hoofnagle et al., 1988). However, it may not constitute the most potent IFNα subtype in this regard (Schulte-Frohlinde et al., 2002; Song et al., 2017). To

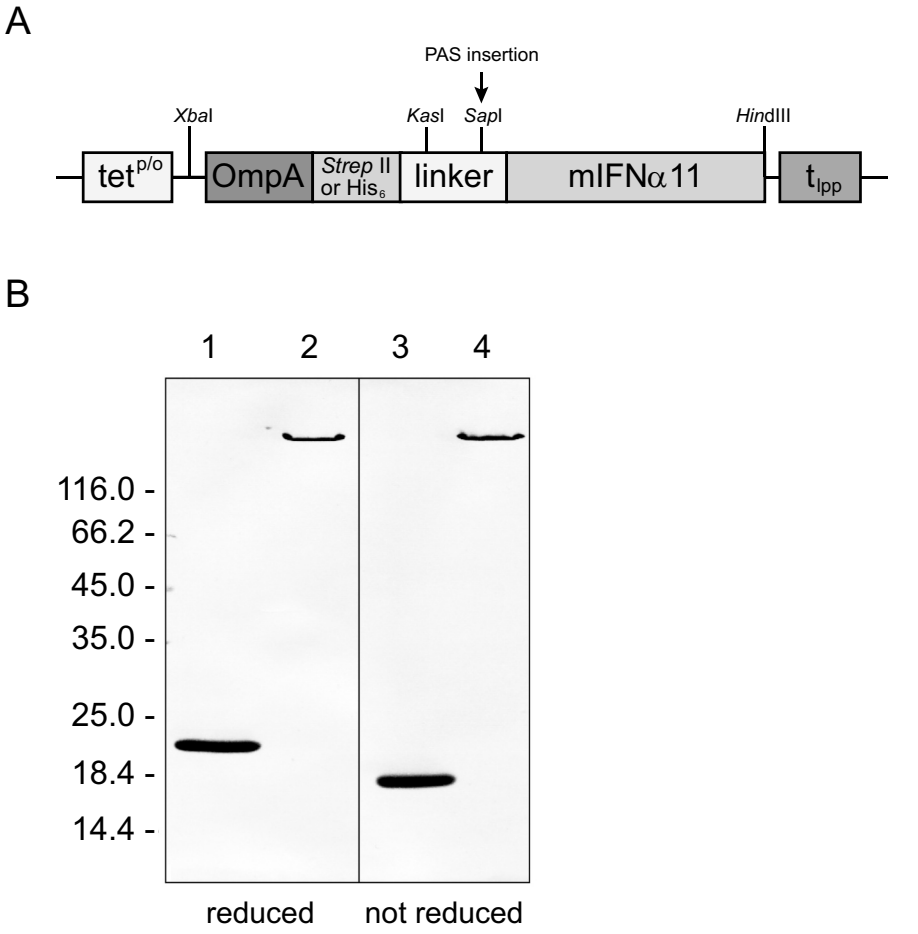


Fig. 1. Preparation of recombinant murine IFNα and its PASylated versions. (A) Expression plasmids are based on the generic pASK75 vector comprising the chemically inducible *tet*$^{p/o}$ promotor, an OmpA signal sequence for periplasmic secretion, a *Strep*-tag II or His_6-tag for affinity purification, a cloning site allowing insertion of the PAS gene cassette (here comprising 600 residues) and the coding region for the mature mIFNα subtype. (B) SDS-PAGE of purified recombinant mIFNα and PASylated mIFNα: lane 1, mIFNα11 reduced; lane 2, PAS-mIFNα11 reduced; lane 3, mIFNα11 not reduced; lane 4, PAS-mIFNα11 not reduced. (C) Analytical size exclusion chromatography of mIFNα11 and PAS-mIFNα11. Interpolation from a half-logarithmic calibration curve indicated apparent molecular masses of 19 kDa and 577 kDa for mIFNα11 and PAS-mIFNα11, respectively.

evaluate the antiviral effect of murine IFNα subtypes on HBV replication in mice, we investigated 4 different mIFNα subtypes, namely 1, 4, 9 and 11, which were chosen according to their known strong suppression of Friend virus replication (Gerlach et al., 2009; Gibbert et al., 2012).

All four mIFNα subtypes were produced in *E. coli* via periplasmic secretion according to a published procedure in a soluble and folded state (Schlapschy et al., 2013). In addition, these mIFNα subtypes were genetically fused at the N-terminus with a polypeptide comprising 600 PAS residues (Fig. 1A), which was previously observed to significantly

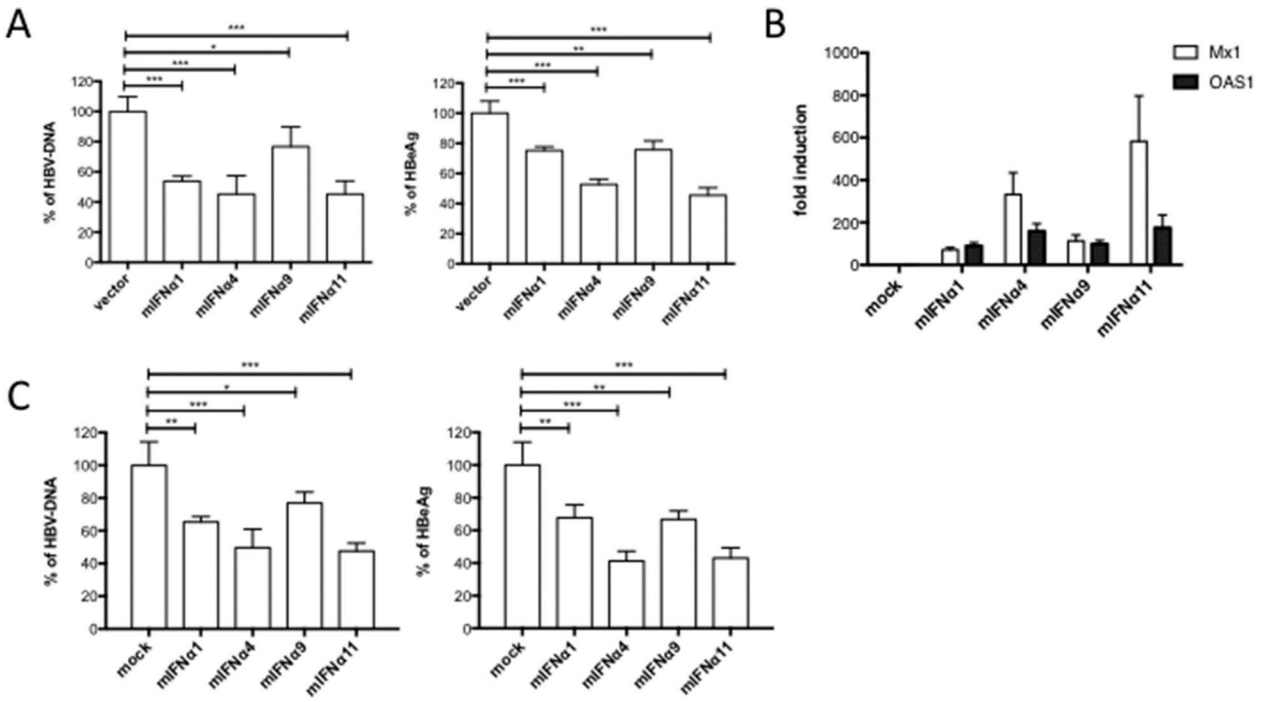


Fig. 2. Anti-viral effect of different mIFNα subtypes. (A) HBV-Met cells were co-transfected with plasmids encoding the indicated mIFNα subtype and SEAP. Seven days after transfection, HBV-DNA and HBeAg were determined in cell culture supernatants by qPCR and ELISA, respectively. Values were normalized by transfection efficiency as determined by SEAP activity assay. (B, C) HBV-Met cells were differentiated and treated with 1000 U/mL of the indicated mIFNα subtype (purified recombinant protein) for 4 days. (B) Expression of Mx1 and OAS1 was determined by qPCR. (C) HBV-DNA and HBeAg in cell culture supernatant were quantified by qPCR or ELISA, respectively.

reduce kidney filtration and to extend plasma half-life of the protein to 16 h while still allowing high production yields (Schlapschy et al., 2013). Both the plain and the PASylated IFNs were purified by means of the *Strep*-tag II or His_6-tag, followed by size exclusion chromatography (SEC). In this way, homogeneous functional protein preparations with yields up to 50 µg per liter bacterial culture were obtained. PAS-mIFNα11 was the candidate that showed the best expression level.

All unmodified recombinant mIFNα subtypes showed sizes of approximately 20 kDa in SDS-PAGE if treated with reducing sample buffer, as expected, while exhibiting slightly higher electrophoretic mobility in the oxidized state, thus indicating the presence of the pair of disulfide bonds. In contrast, the PASylated proteins exhibited strongly retarded electrophoretic mobility with an apparent size of ~300 kDa (for the PAS(600) fusion protein) as shown exemplarily for PAS-mIFNα11 (Fig. 1B). A similar effect, with an apparent mass much higher than predicted from the calculated mass (~70 kDa), was previously observed for other PASylated proteins (Morath et al., 2015; Schlapschy et al., 2013) and can be explained by the weak interaction between the hydrophilic PAS polymer and SDS, whose charged head groups provide the driving force during electrophoresis.

The effect of PASylation on the molecular size of the mIFNα versions in physiological buffer solution was studied by analytical SEC (Fig. 1C). Both the unmodified recombinant mIFNα and its PASylated versions exhibited a single peak, indicating monodisperse behavior. Interpolation of the SEC elution volume *versus* a set of globular standard proteins suggested a molecular size of 19 kDa (predicted: 20,803 Da) for the unmodified mIFNα11 but of 577 kDa (predicted: 70,141 Da) for its PAS(600) fusion. This phenomenon clearly revealed the drastically enlarged hydrodynamic molecular volume of the fusion protein caused by the expanded random coil conformation of the PAS tag.

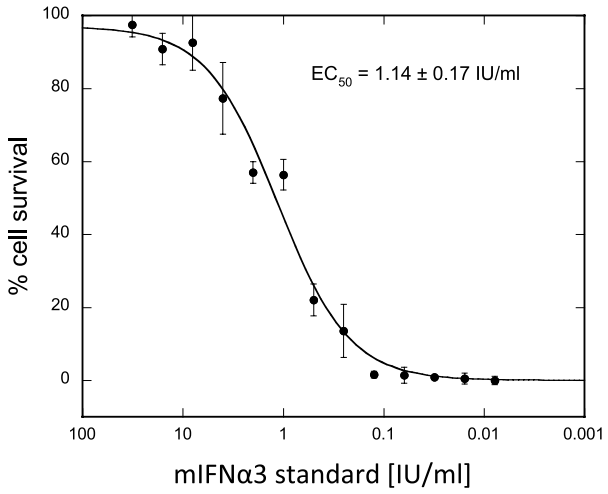
3.2. Anti-viral effect of different mIFNα subtypes

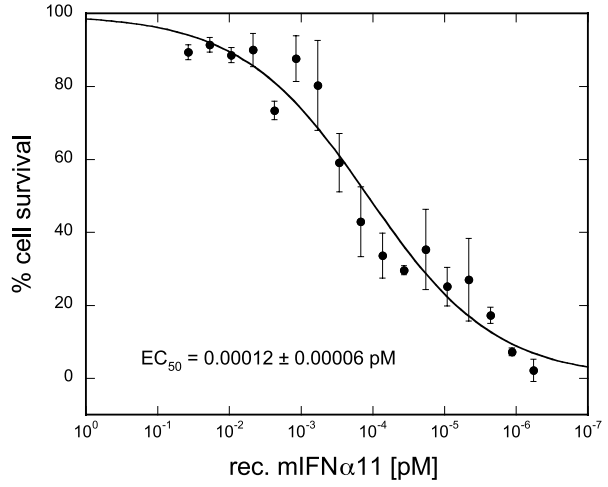
The HBV-Met cell line comprising immortalized HBV-expressing hepatocyte cells derived from HBV-tg mice (Robek et al., 2004), was used to investigate the antiviral effect of IFNα subtypes. First, HBV-Met cells were transfected with mIFNα expression plasmids, then HBV replication markers were measured (Fig. 2A). A plasmid encoding secreted alkaline phosphatase (SEAP) was co-transfected to normalize for the transfection efficiency. All four plasmids encoding the mIFNα subtypes 1, 4, 9 and 11 showed antiviral effects as indicated by the decrease of both HBV-DNA and HBeAg in the cell culture supernatant (Fig. 2A). Both mIFNα4 and mIFNα11 resulted in around 50% inhibition whereas mIFNα1 and mIFNα9 led to only 20–40% inhibition.

To further investigate the antiviral effect of the mIFNα subtypes, the same amount of each purified recombinant protein produced in *E. coli* was administered to cultured HBV-Met cells. The cells treated with mIFNα11 showed stronger expression of ISGs, including myxovirus resistance 1 (Mx1) and 2'-5'-oligoadenylate synthetase 1 (OAS1), compared to the other three subtypes (Fig. 2B). As observed after plasmid transfection, both recombinant mIFNα4 and mIFNα11 showed a better antiviral effect according to HBV-DNA and HBeAg analysis than mIFNα1 and mIFNα9 (Fig. 2C). In the light of its higher ISG induction and better antiviral effect as well as more efficient production in *E. coli*, we chose PASylated mIFNα11 for further study.

3.3. Comparison of unmodified recombinant mIFNα11 and its PASylated version in vitro

The biological activities of PAS-mIFNα11 and mIFNα11 were first compared in an IFN bioassay (Widman, 2013). The murine fibroblast cell line L929 was treated with serially diluted mIFNα11, PAS-mIFNα11, or a commercial mIFNα standard (mIFNα3, PBL Assay Science, Piscataway, NJ), and then challenged with vesicular stomatitis virus (VSV). The protective effect of mIFNα was evaluated by assaying cell viability, and the half-maximal effective concentration (EC_{50}) was determined for each mIFNα version (Fig. 3). As expected, the mIFNα standard showed an EC_{50} value around 1 U/mL (1.14 ± 0.17 U/mL,





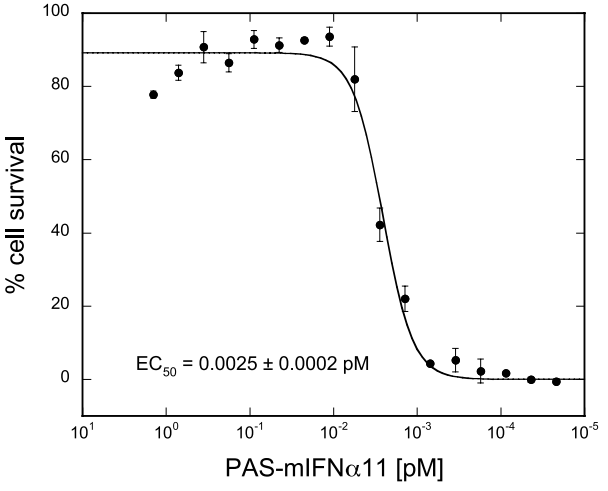


Fig. 3. Comparison of the antiviral activities of PAS-mIFNα11 and mIFNα11. The activities of PASylated and unmodified recombinant mIFNα11 in cell culture were determined by IFN bioassay. L929 cells were treated with a dilution series of a commercial mIFNα standard, recombinant mIFNα11 (from this study) or PAS-mIFNα11 for 24 h prior to infection with VSV. Cell viability was quantified using the XTT assay and plotted as percentage in comparison to uninfected cells. Data were fitted to a logistic function: $A = A_{max}/(1 + 10^{Slope \cdot log(EC50/[L])})$.

9.06×10^7 units/mg according to vendor). Our own IFN preparations showed EC_{50} values of 1.2×10^{-4} pM for mIFNα11 and 2.5×10^{-3} pM for PAS-mIFNα11. The calculated specific activity of mIFNα11 and PAS-mIFNα11 was 2.5×10^8 units/mg or 5×10^6 units/mg, respectively. Consequently, PASylation lead to a 20-fold reduction in potency, which can be explained by some sterical hindrance of the voluminous PAS moeity during ternary complex formation with the interferon-α/β receptor (IFNAR1/2) subunits (Schreiber and Piehler, 2015). Nevertheless, the effective concentration of PAS-mIFNα11 is extremely low compared with other typical biopharmaceuticals and its diminished receptor affinity is overcompensated by the 60-fold longer plasma half-life (Schlapschy et al., 2013).

To compare their specific antiviral activities on HBV replication, 100 or 1000 U/mL of mIFNα11 or PAS-mIFNα11 were used to treat HBV-Met cells (Fig. 4). First, induction of ISG mRNAs was measured by means of qRT-PCR (Fig. 4A). Both mIFNα11 and PAS-mIFNα11 showed comparable ability to induce Mx1 and OAS1 genes in a dose-dependent manner. Notably, no cytotoxic effect upon treatment of the cells was observed for either mIFN version (Fig. 4B). To check the inhibitory effect on HBV replication, HBeAg as well as HBV DNA were measured using ELISA and qPCR, respectively (Fig. 4C). Likewise, both versions of mIFNα showed a comparable antiviral effect. 100 U/mL of each mIFNα11 or PAS-mIFNα11 resulted in about 15–20% decrease in HBeAg and 30% decrease in HBV-DNA levels. 1000 U/mL of each mIFNα11 or PAS-mIFNα11 resulted in about 30% and 50% reduction of HBeAg and HBV DNA, respectively.

3.4. Comparison of PASylated mIFNα11 with the unmodified recombinant mIFNα11 in vivo

PASylation of therapeutic proteins was previously shown to strongly extend plasma half-life and, consequently, enhance *in vivo* efficacy (Binder and Skerra, 2017). To examine the advantage of PASylation on the antiviral activity of mIFNα11 *in vivo*, the HBV-tg mouse model was used (Guidotti et al., 1995). First, we evaluated the dose range using unmodified recombinant mIFNα (Fig. S1). HBV-tg mice (N = 3 per group) were injected i.p. four times with 10 pmol, 30 pmol or 100 pmol mIFNα11, or with PBS as vehicle control (Fig. S1A). Serum levels of the liver enzyme ALT were normal, demonstrating that application of up to 4 × 100 pmol of mIFNα11 did not induce liver damage (Fig. S1B). Notably, the HBV replication markers HBV-DNA, HBsAg and HBeAg were reduced in all mice treated with the 30 pmol and 100 pmol mIFNα doses, while one mouse from the 10 pmol mIFNα11 treatment group showed a less pronounced response (Figs. S1C–D). Evaluation of HBV core protein expression in the liver revealed a similar pattern for all doses (Fig. S1E).

Chronic hepatitis B patients under IFNα treatment receive three injections of IFNα2b or one injection of PEG-IFNα2a/b per week. Considering the generally shorter plasma half-life of therapeutic proteins in mice (Schlapschy et al., 2013), a regimen of two injections per week and in total three weeks treatment of HBV-tg mice was chosen for our comparative animal study of the PASylated mIFNα (Fig. 5 and Fig. S2). 20 HBV-tg mice were separated into five groups (N = 4) and each

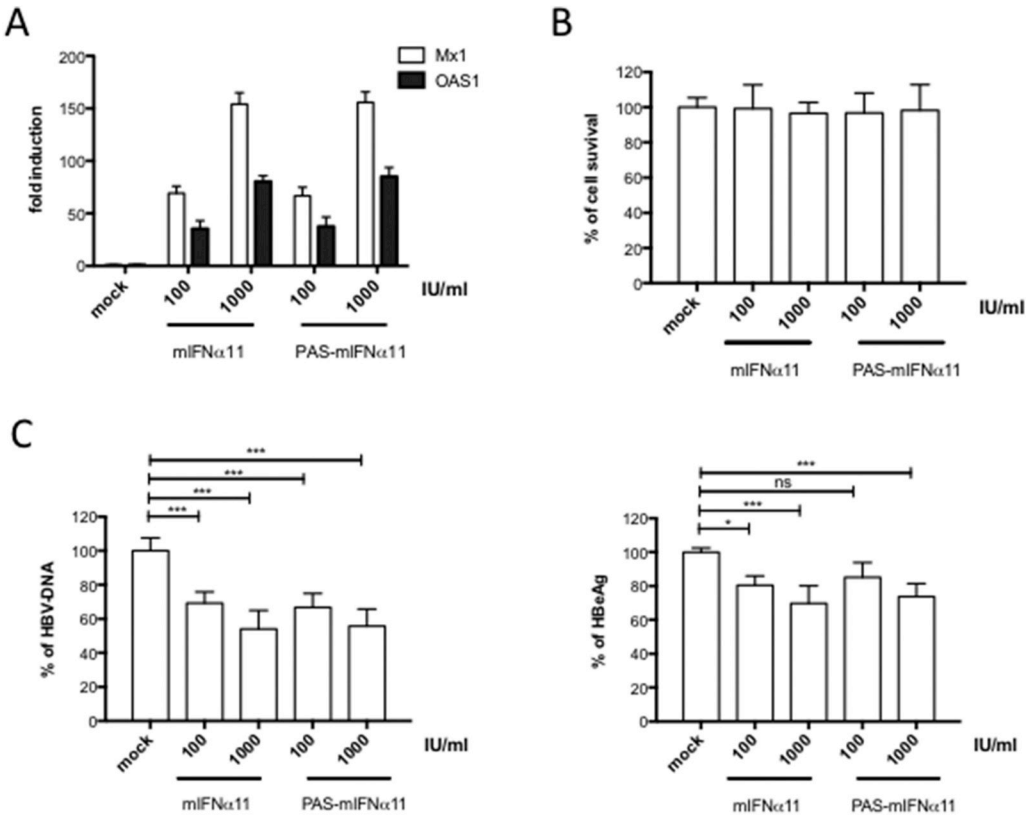


Fig. 4. Comparison of the gene induction activities of PAS-mIFNα11 and mIFNα11. HBV-Met cells were treated with the indicated dose of unmodified or PASylated mIFNα11 for four days. (A) Expression levels of Mx1 and OAS1 mRNA were determined by qPCR. (B) Cytotoxic effects of mIFNα11 and PAS-mIFNα11 were determined using an XTT assay. (C) HBV-DNA and HBeAg in the cell culture supernatant were measured by qPCR and ELISA, respectively.

treated with 10 pmol or 100 pmol of PAS-mIFNα11 or mIFNα11 per injection, or the same volume of PBS. None of the treatments resulted in a body weight change (Fig. S2B) or elevation of ALT activity (Fig. 5A) in any of the treated animals. Liver histology confirmed that neither mIFNα11 nor PAS-mIFNα11 caused tissue damage at the doses applied (Fig. 5B, Fig. S3). Taken together, PAS-mIFNα11 was well tolerated and showed no detectable side effects in the HBV-tg mice, like the unmodified recombinant mIFNα.

To assess the persistence of PAS-mIFNα11 in the blood, we measured the presence of PAS-mIFNα11 or mIFNα11 in serum samples by ELISA at four time points. With a dose of 100 pmol sustained presence of PAS-mIFNα11 in the serum was measurable whereas PAS-mIFNα11 at the lower dose of 10 pmol and mIFNα11 at both doses remained below the detection level (Fig. 5C, upper panel). To assess ISG induction *in vivo*, the kinetics of OAS1 gene expression in blood cells was evaluated by qRT-PCR. PAS-mIFNα11 clearly induced a stronger OAS1 response than the unmodified mIFNα11 at both doses (Fig. 5C, lower panel). Furthermore, in the liver the higher dose of 100 pmol PAS-mIFNα11 induced significant ISG expression (Fig. 5D).

To compare the antiviral effects of mIFNα11 and PAS-mIFNα11, HBV replication markers were determined in the animals. Treatment with 100 pmol PAS-mIFNα11 resulted in more than 95% suppression of both HBsAg and HBeAg levels, while the other treatment regimens reduced HBsAg and HBeAg by max. 50% (Fig. 6A and B). Viremia was only reduced when dosing 100 pmol PAS-mIFNα11, with a fast decline already one week after start of treatment and reaching around 80% suppression by the end of week 3 (Fig. 6C). Accordingly, intrahepatic total HBV RNA as well as pgRNA were significantly reduced exclusively in the group dosed with 100 pmol PAS-mIFNα11 (Fig. 6D). Staining for HBcAg in liver sections (Fig. 7A, Fig. S4) and quantification of HBc-positive nuclei (Fig. 7B) showed that a dose of 100 pmol PAS-mIFNα11 cleared more than 80% of HBc from the hepatocytes in all mice treated thus indicating activation of antiviral immunity. Most interestingly, quantification of anti-HBs titers in serum revealed that all four HBV-tg mice treated with 100 pmol PAS-mIFNα11 for 3 weeks started to generate anti-HBs antibodies at titers between 320 and > 1000 IU/mL (Fig. 7C). This kind of HBsAg loss and anti-HBs seroconversion is considered the hallmark of successful induction of an immune response, indicating functional cure of HBV, and is a prerequisite for complete and durable control of HBV infection.

4. Discussion

Based on the long-term follow-up of patients, current IFNα and/or NUC treatment achieve HBsAg clearance in less than 10% of the patient population (Zoulim and Durantel, 2015). Here, we describe that different murine IFNα subtypes show distinct activities on inducing downstream gene expression as well as inhibiting HBV replication in HBVtg mouse cells. The most effective subtype, mIFNα11, was improved with regard to its pharmacokinetic properties via PASylation. PAS-mIFNα11 exhibited slightly lower activity than unmodified mIFNα11 *in vitro*, but has much longer plasma half-life (Schlapschy et al., 2013), leading to drastically enhanced antiviral activity *in vivo* and effecting anti-HBs seroconversion of the animals treated – a hallmark of HBV cure. These results suggest that a novel long-acting IFNα of appropriate subtype generated by PASylation could be a promising therapy to achieve functional cure from HBV infection in humans.

With 13 subtypes for IFNα, murine IFNs show a pronounced redundancy. The reasons for this remain unclear but include differences in tissue-specific expression, temporal variations in expression and distinct activities (van Boxel-Dezaire et al., 2006). In our experiments with individual subtypes, we observed mIFNα 4 and 11 to be the most active versions (Fig. 2B and C). By comparison of their amino acid sequences, we found several unique residues within the IFNAR1 binding region shared by mIFNα4 and mIFNα11 (Fig. S5). Of those, especially Arg^{58} and Asp^{59} in the mature mIFN are known to play an important role in the interaction with the IFNAR1 receptor chain (Thomas et al., 2011). While at these positions His and Glu are conserved among all human IFNα subtypes (Lavoie et al., 2011), the murine IFNs show variations (Fig. S5). Since the antiviral activity of IFNα mainly depends

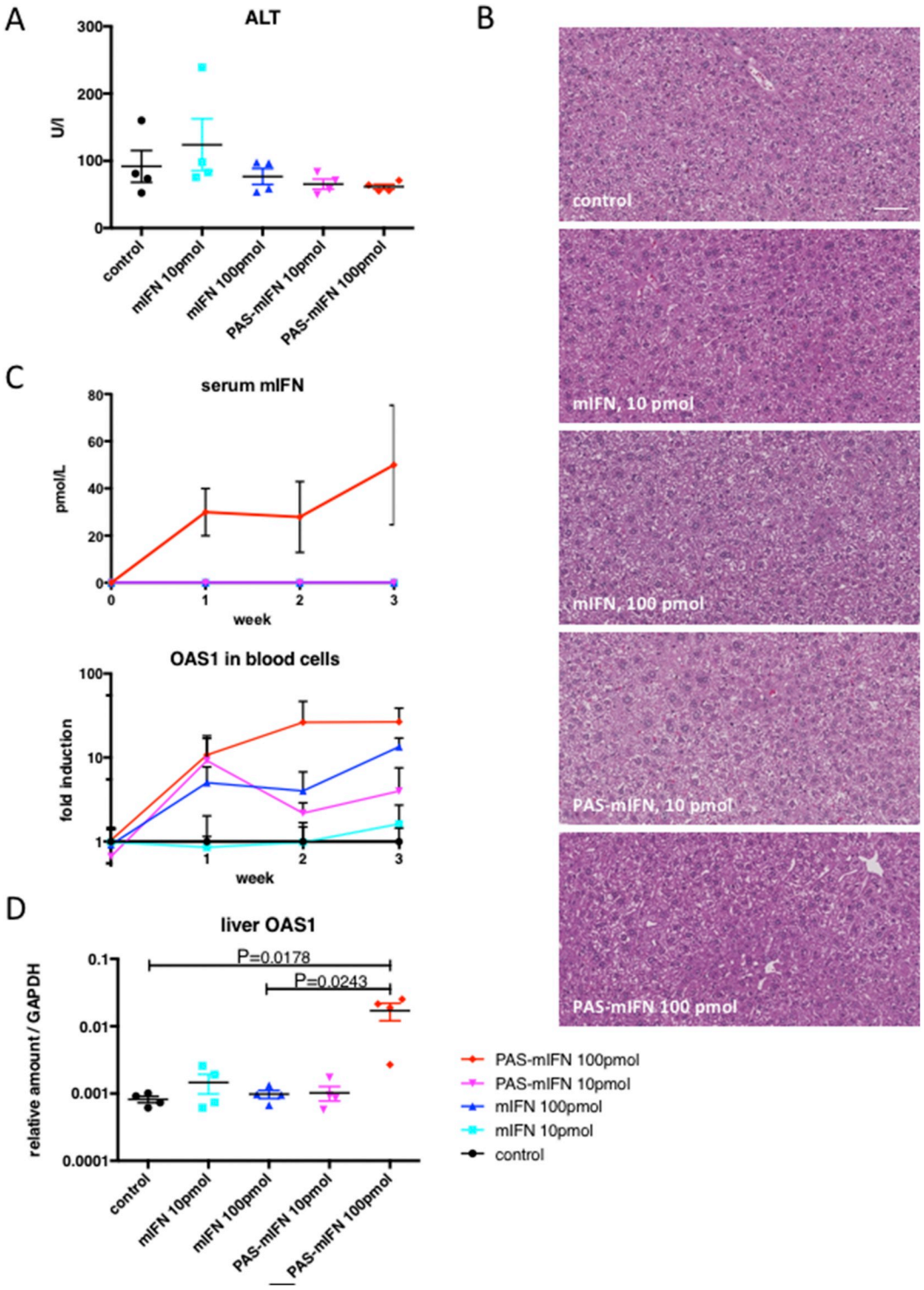


Fig. 5. PAS-mIFNα11 induces strong IFN signaling *in vivo*. HBVtg mice were injected i.p. with unmodified or PASylated mIFNα11 at a dose of 10 or 100 pmol per mouse twice weekly as indicated in Supplementary Fig. 1. (A) Serum ALT activities were measured on day 21. (B) H&E staining of liver sections was performed at the end of the experiment (scale bar = 100 µm). (C) Serum titers of mIFNα at indicated time points were quantified by ELISA (upper panel). Expression kinetics of OAS1 in blood cells was determined by qPCR (lower panel). (D) Expression of OAS1 was determined in total liver RNA at the end of the experiment using qRT-PCR.

on the affinity towards IFNAR (Lavoie et al., 2011), we assume that these residues play a role for the distinct antiviral potencies of these mIFNα subtypes. However, previous studies demonstrated that mIFNα4 and mIFNα9 mediate stronger antiviral activity against herpes simplex virus (Harle et al., 2002), whereas mIFNα4 and mIFNα11 are superior in blocking the replication of Mengo virus (van Pesch et al., 2004). In case of murine cytomegalovirus infection, mIFNα6 and mIFNα11 are effective in reducing viral replication, whereas treatment with mIFNα2 or mIFNα5 even enhanced infection (Gibbert et al., 2012). Human IFNα2b is well established for the treatment of hepatitis B (Hoofnagle et al., 1988). However, taking into account the differences observed among the mIFN subtypes it may not be the most potent IFN for this purpose in man (Schulte-Frohlinde et al., 2002).

The low response rates and significant side effects during IFNα

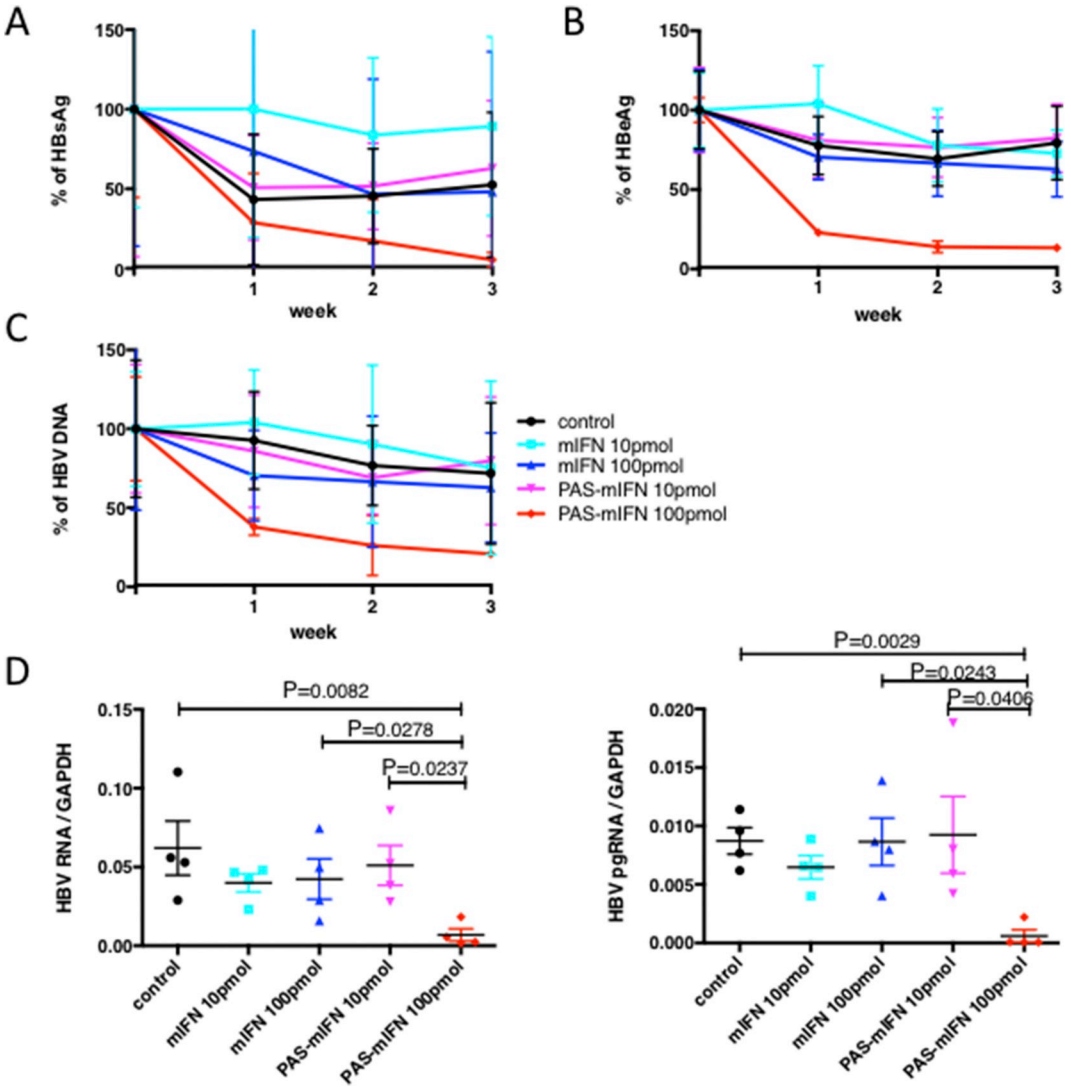
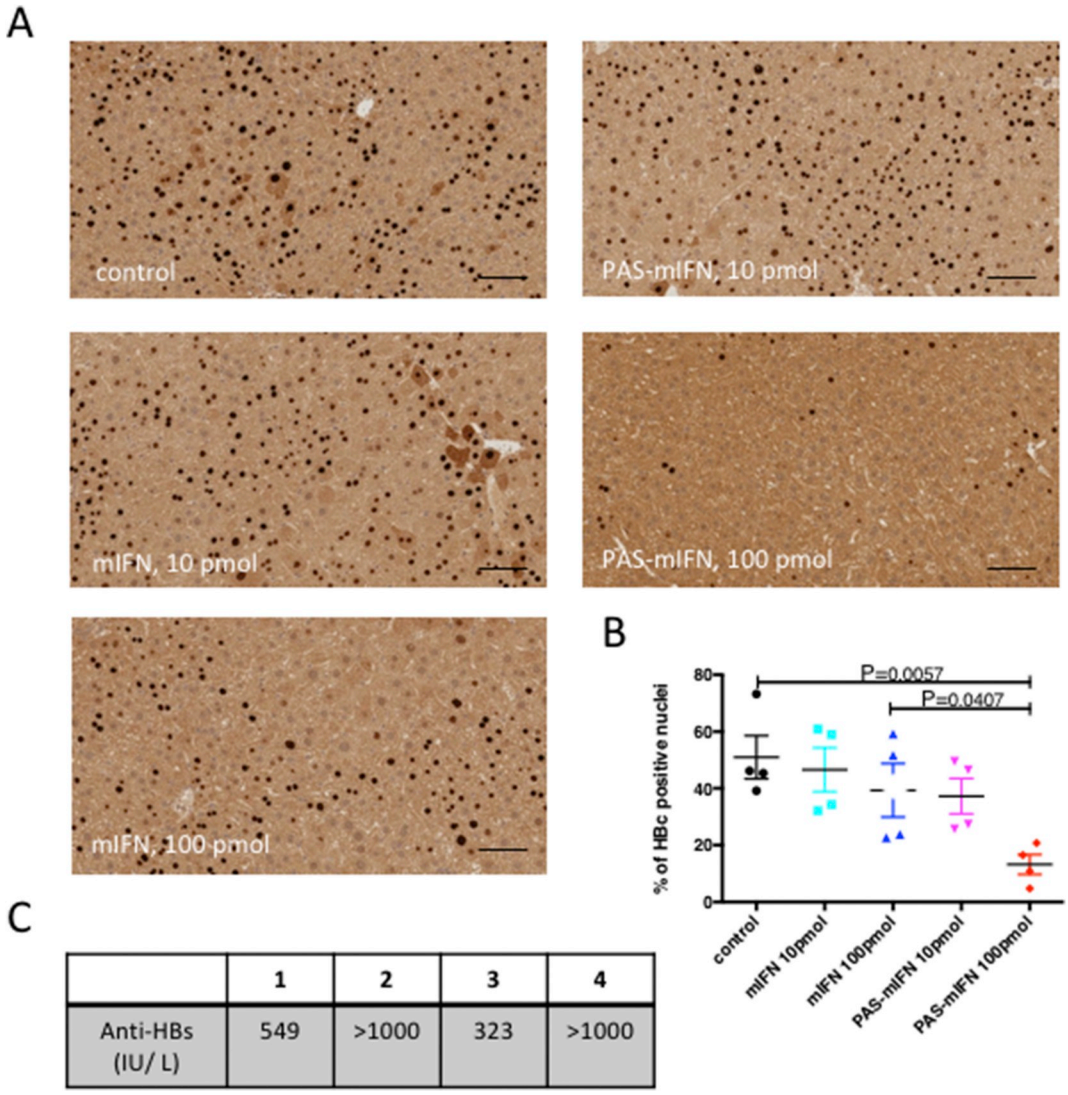


Fig. 6. PAS-mIFNα11 shows strong antiviral activity *in vivo*. HBsAg (A) and HBeAg (B) or HBV DNA (C) in the sera of HBVtg mice (from the experiment shown in Fig. 5) were quantified using ELISA and qPCR, respectively. (D) Intrahepatic HBV total RNA and pgRNA were measured by qPCR in total nucleic acid extracted from mouse livers.

treatment of chronic hepatitis B patients may be overcome by a higher local concentration in the liver and by a generally longer plasma half-life, thus avoiding exaggerated peak concentrations. PEGylation has been developed to counter this problem and widely used in several clinical applications (Jevsevar et al., 2010). However, the high cost of *in vitro* chemical coupling, accumulating evidence on the immunogenicity of PEG and the missing biodegradability of the unnatural PEG polymer hamper its future application (Binder and Skerra, 2017; Garay et al., 2012). Similar to PEGylation, N- or C-terminal genetic fusion with an unstructured hydrophilic polypeptide made of Pro, Ala and/or Ser, dubbed PASylation, increases the hydrodynamic volume of the conjugated protein, thus extending plasma half-life due to retarded renal filtration (Binder and Skerra, 2017).

In fact, PASylation offers several advantages and provides a superior alternative to PEG or related polymers: (i) PAS biopolymers adopt a stable random coil conformation under physiological conditions and, hence, occupy a large hydrodynamic volume; (ii) PAS biopolymers are resistant against serum proteases but are rapidly degraded by intracellular/lysosomal enzymes, thus avoiding organ accumulation; (iii) PAS biopolymers can be easily produced in *E. coli* as recombinant polypeptides with defined lengths, thus yielding a monodisperse drug substance; (iv) PAS biopolymers exhibit high solubility in water in the absence of any charged side chains and, hence, they do not alter the isoelectric point of the conjugated pharmacologically active molecule; (v) PAS biopolymers are non-toxic, lack T-cell epitopes and show no signs of immunogenicity in laboratory animals; (vi) PAS biopolymers can be adjusted to match desired pharmacokinetic parameters by varying the length of the coding DNA region, e.g. from 100 to 1000 residues. Altogether, PASylation offers unique advantages while sharing surprisingly similar biophysical properties with PEG (Binder and Skerra, 2017), hence rendering it a promising technology to improve current IFNα treatment.

As a multifunctional antiviral cytokine, IFNα has been shown to activate antiviral immunity and to restrict HBV infection at different steps. Transcriptional and post-transcriptional control of HBV replication by IFNα was demonstrated both *in vitro* and *in vivo* (Uprichard et al., 2003; Wieland et al., 2000). In addition, suppression of viral protein translation and virion secretion by IFNα were observed in cell cultures (Park et al., 2011; Yan et al., 2015). IFNα also inhibits HBV entry via secretion of soluble factors that bind to heparin sulfate proteoglycans and block viral attachment (Xia et al., 2017b), and it affects HBV cccDNA directly either through epigenetic transcriptional silencing or by reducing its stability (Belloni et al., 2012; Lucifora et al., 2014; Xia et al., 2016) – both effects that could not be addressed in the



	1	2	3	4
Anti-HBs (IU/ L)	549	>1000	323	>1000

Fig. 7. PAS-mIFNα11 induces anti-HBs seroconversion *in vivo*. (A) HBV core protein (HBc) was stained in liver sections and (B) the percentage of positive nuclei was calculated (scale bar = 100 μm). (C) Anti-HBs antibody titers in sera from four individual mice treated with 100 pmol PAS-mIFNα were quantified by ELISA.

HBV-transgenic mouse model in our study. To study both, immune stimulation and direct antiviral effects, an immune competent HBV infection model would be needed that is currently not available. It can however be anticipated that in a natural HBV infection, PAS-IFNα may even result in a more prominent antiviral effect since a larger number of virologically relevant targets are involved.

In theory, a complete cure of hepatitis B can be achieved via inhibition of HBV replication, degradation of its cccDNA and blockade of reinfection or viral spreading. The current therapeutic goal of chronic hepatitis B therapy is a functional cure of HBV infection comprising HBsAg loss with or without anti-HBs seroconversion. Unfortunately, this is rarely achieved with currently available treatments. Cytokines such as IFNα are believed to be involved in non-cytolytic clearance of HBV, yet their effects are multifunctional and target several key steps of viral infection. In this respect, a novel therapy based on a long-acting cytokine like PASylated IFNα offers prospects to achieve functional cure or even eradication of virus infection.

In summary, PAS-mIFNα11 showed no toxicity and was well tolerated in all animals tested in this study. PASylation significantly enhanced bioavailability and antiviral effects of mIFNα *in vivo*, providing proof-of-concept for the development of a more effective IFNα treatment with potential to achieve a “functional cure” from HBV in a larger proportion of patients.

Conflicts of interest

A.S. and M.S. are cofounders and shareholders of XL-protein GmbH.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.11.003.

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