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Specific and non-hepatotoxic degradation of nuclear hepatitis B virus cccDNA

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Abstract

Current antivirals can control but not eliminate hepatitis-B-virus (HBV), because HBV establishes a stable nuclear cccDNA. Interferon- α treatment can clear HBV but is limited by systemic side effects. Here we describe how interferon- α can induce specific degradation of the nuclear viral DNA without hepatotoxicity and propose lymphotoxin- β -receptor activation as a therapeutic alternative. Interferon- α and lymphotoxin- β -receptor activation up-regulated APOBEC3A and 3B cytidine-deaminases, respectively, in HBV-infected cells, primary hepatocytes and human liver-needle biopsies. HBV-core protein mediated the interaction with nuclear cccDNA resulting in cytidine-deamination, apurinic/apyrimidinic site formation and finally cccDNA degradation that prevented HBV-reactivation. Genomic DNA was not affected. Thus, inducing nuclear deaminases - e.g. by lymphotoxin- β -receptor activation - allows development of new therapeutics that combined with existing antivirals may cure hepatitis B.

Main text:

Hepatitis B virus (HBV) infection remains a major public health threat with more than 350 million humans chronically infected worldwide at risk of developing end-stage liver disease and hepatocellular carcinoma. Each year, more than 600,000 humans die from consequences of chronic HBV infection. A prophylactic vaccine has been available for hepatitis B for almost thirty years, but the overall number of chronic infections remains high.

HBV is a small, enveloped DNA virus replicating via an RNA intermediate. The encapsidated viral genome consists of a 3.2 kb partially double-stranded relaxed circular DNA (rcDNA) molecule. The virus has optimized its life-cycle for long-term persistence in the liver (1). Upon translocation to the nucleus, the rcDNA genome is converted into a covalently closed circular DNA (cccDNA), which serves as the template for viral transcription and secures HBV persistence. Nucleos(t)ide analogues are efficient antivirals but only control and do not cure HBV infection owing to the persistence of HBV cccDNA. Therefore, long-term treatment is required, which is expensive and may lead to concomitant resistance (2). Interferon (IFN)- α is licensed for hepatitis B therapy and treatment with this cytokine can result in virus clearance in a proportion of patients; however, its efficacy is limited and high doses are not tolerated (3). Thus, efficient and non-toxic elimination of cccDNA in hepatocytes is a major goal of HBV research.

Using animal models, it has been shown that HBV replication, and in particular the cccDNA content of the liver, can be affected by non-cytopathic mechanisms involving cytokines such as interferons and tumor necrosis factor (TNF), which influence RNA and capsid stability (4-7). Here, we describe a novel antiviral mechanism that interferes with cccDNA stability and is distinct from influences of antiviral cytokines on cccDNA activity (8).

High-dose IFN-a leads to cccDNA degradation in HBV-infected hepatocytes

IFN- α is known to exert transcriptional, post-transcriptional and epigenetic antiviral effects on HBV (8-12). To study the effect of IFN- α on HBV cccDNA, we used HBV-infected, differentiated HepaRG (dHepaRG) cells and primary human hepatocytes (PHH). These are human cell types susceptible to HBV infection (13, 14) and responsive to IFN- α treatment *in*

vitro (Figure S1A). IFN- α treatment did not lead to detectable hepatotoxicity, even at very high doses (Figure S1B). Treating dHepaRG cells with 500 or 1000 IU/ml IFN- α controlled HBV-DNA synthesis as efficiently as 0.5 μ M (5-fold EC50) of the nucleoside analogue lamivudine (LAM). IFN- α , however, unlike LAM also significantly reduced expression of HBV-RNA and hepatitis B surface (HBsAg) and e (HBeAg) antigens (Figure 1A, S1C).

In patients, interruption of LAM treatment results in a rebound of HBV replication (2). Using IFN- α , we observed only a partial or no rebound in HBV-infected dHepaRG cells after treatment cessation (Figure 1A). Because dHepaRG don't allow virus spread, reduction of HBeAg and lacking rebound indicated an effect of IFN-α on the established HBV cccDNA transcription template besides the known antiviral effects on viral replication (14). By cccDNAspecific qPCR, we determined an 80% reduction of cccDNA after 10 days of treatment (Figure 1B). Reduction of cccDNA was confirmed by Southern blot analysis (Figure S1D) and was dose dependent (Figure S1E). cccDNA reduction could be induced at any time point (Figure 1C) and persisted over time (Figure 1A, 1C). The effect was corroborated in HBV-infected primary human hepatocytes (PHH) (Figure 1D). In contrast to IFN- α , LAM and even more potent nucleoside analogue entecavir (ETV) at very high doses (0.5 µM, 1000-fold IC-50) only inhibited reverse transcription and thus HBV replication, but not viral persistence (Figure 1E). Pretreatment with ETV did not enhance the effect of IFN- α (Figure 1F) indicating that IFN- α induces the decay of established HBV cccDNA. Since the doses of IFN-α used to achieve this effect were high, we screened for other cytokines showing similar antiviral effects at moderate doses.

LTβR-activation controls HBV and leads to cccDNA degradation in HBV-infected cells

IFN- γ and TNF- α are known to control HBV in a non-cytopathic fashion (4, 7), but cannot be used as therapeutics because they cause severe side effects. We tested the effect of lymphotoxin (LT) β receptor (LT β R) activation as an alternative therapeutic option. TNF superfamily members LT α , LT β and CD258 are the physiological ligands for LT β R and activate several inflammatory, anti-inflammatory, pro- and anti-survival pathways (15). Like hepatocytes (16), dHepaRG (14) and HepG2-H1.3 cells permit HBV replication (17) and express the LT β R

(Figure S2A, S2B). To activate LT β R, we used a super-agonistic tetravalent bispecific antibody (BS1) and a bivalent anti-LT β R monoclonal antibody (CBE11) (*18, 19*). As expected, LT β R agonists activated canonical (*20*) and non-canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathways to trigger p100 cleavage (Figure S2C), RelA phosphorylation (Figure S2D), nuclear RelB and RelA translocation (Figure S2E, S2F), and up-regulation of known target genes (Figure S2G) without causing any detectable hepatocytotoxicity (Figure S2H).

To test the effect of LT β R-activation on HBV infection, dHepaRG cells were treated with BS1 for 12 days starting 24 hours prior to HBV infection. LT β R-activation decreased levels of all HBV-markers, including cccDNA by approximately 90% without toxicity (**Figure 2A**). The antiviral effect was highly potent with an EC₅₀ of approximately 0.01 µg/mL (**Figure S3A**). Inhibition of apoptosis did not alter antiviral activity (**Figure S4**). Neither IFN- β nor classic IFNstimulated genes were up-regulated upon BS1-treatment (**Figure S2G**) and antiviral activity was independent of IFN-induction (**Figure S5**).

In vivo, activation of the murine LT β R by systemic application of an agonistic antibody (ACH6) induced RelA and RelB nuclear translocation in hepatocytes of HBV-transgenic mice (**Figure S6A**), reduced HBV viremia (**Figure S6B**), HBV RNA (**Figure S6C**) and HBV core (HBc) protein expression in the liver (**Figure S6D and S6E**). Neither signs of hepatocyte apoptosis (**Figure S6F**) or elevation of aminotransferases (ALT) (**Figure S6G, right panel**) were observed indicating good *in vivo* tolerability of LT β R-activation. Since HBV-transgenic mice do not establish HBV cccDNA, this indicated additional antiviral effects of LT β R-activation on HBV RNA transcription or stability. Accordingly, discontinuation of LT β R-activation induced an immediate, strong rebound of HBV replication (**Figure S6G**).

To investigate whether LTβR-activation would affect established HBV cccDNA in the context of a persistent infection and prevent HBV reactivation, dHepaRG cells were treated with LTβR agonists BS1 or CBE11 when a stable, nuclear cccDNA pool had established. All HBV markers, including HBV cccDNA, were reduced upon LTβR-activation in HBV-infected dHepaRG cells (**Figure 2B, 2C, S3**) as well as in stably transfected HepG2H1.3 cells containing high levels of cccDNA (**Figure 2C**). In HBV-infected primary human hepatocytes (PHH), LTβR agonisation reduced HBV cccDNA, HBeAg secretion and even more pronounced HBV-DNA

replication (Figure 2D). cccDNA degradation was more effective (up to 95%) when treatment was prolonged (Figure S3C and S3D). Treatment interruption for 10 days was almost as efficient as continuous treatment (Figure S3C) indicating that $LT\beta R$ agonists induce a persistent antiviral effect. In contrast to LAM treatment, no rebound of HBV-replication was observed when BS1 treatment stopped (Figure 2E). Hence, $LT\beta R$ activation not only suppressed HBV replication but also caused nuclear cccDNA degradation, needed to achieve virus elimination.

LTβR-activation and IFN-α treatment induce deamination and apurinic/apyrimidinic (AP) site formation in cccDNA

To investigate if cccDNA degradation upon LT β R-activation or IFN- α treatment was a result of DNA damage, we examined cccDNA deamination by differential DNA denaturation PCR (3D-PCR) (21). Low denaturing temperatures were sufficient for cccDNA amplification from HBV-infected dHepaRG cells and for PHH treated with IFN-a or BS1, compared with untreated, LAM- or ETV-treated cells (Figures 3A and S7C and S7D). Using a cocktail of recombinant proteins containing all enzymes necessary for DNA repair (preCR mix), we could reverse the denaturation of cccDNA (Figure 3A, lower panels). The fact, that denaturation temperatures of mock, LAM and ETV treated cells also shifted, indicated that this modification of HBV cccDNA existed even without exposure to exogenous drugs. Deamination of cccDNA (Figure 3A, right panel) and a drop in cccDNA levels after treatment with CBE11 (Table S1) was confirmed in vivo in human liver chimeric uPA-SCID mice infected with HBV. Sequencing analyses showed G/A transitions occurred under treatment (Figure S7A, S7B, 3B) indicating deamination of cytidines to uridine in the HBV cccDNA minus strand. At lower denaturation temperatures G/A transitions became more obvious (Figures S7A and 3C). These data showed that both LT β R-activation and IFN- α treatment led to cccDNA deamination *in vitro* and *in vivo*, and help to explain the G/A hypermutation observed in patient samples (21).

Importantly, neither deamination nor mutations of genomic DNA were observed by 3D-PCR (**Figure S8A**) or by deep sequencing of selected housekeeping or IFN- and LT β R-target genes (**Figure S8B**). This indicated that DNA modifications were specifically targeted to viral cccDNA. After cytidine deamination, DNA-glycosylases recognize the damaged DNA and cleave N-glycosidic bonds to release the base and create an accessible AP site that can then be cleaved by endonucleases (22). These AP sites can either be repaired, can lead to mutations upon DNA replication or can induce DNA degradation (23). We quantified AP sites created by LT β R-activation or IFN- α treatment. However, no increase of AP sites in total DNA extracts from dHepaRG cells or PHH treated with IFN- α or LT β R-agonists (**Figure S8C**) was found, reassuring that our treatments did not lead to detectable damage in genomic DNA. Because AP sites in the small (3.2 kb) cccDNA are very likely to be missed by this analysis, we digested total DNA extracts with an AP-endonuclease (APE1) and then amplified cccDNA by qPCR. APE digestion further decreased cccDNA extracted from dHepaRG cells and PHH treated with IFN- α or LT β R-agonists but not with LAM (**Figure 3D**). Taken together, our data indicate that both, LT β R-activation or IFN- α treatment induced deamination and AP-site formation in HBV cccDNA leading to its degradation, but did not affect genomic DNA.

LTβR-activation and IFN-α treatment up-regulate expression of nuclear APOBEC3 deaminases

IFN- α is known to induce several cytidine deaminases (23, 24). We performed genomewide expression profiling of HBV-infected dHepaRG cells after LT β R-activation (Figure S9A) and classified regulated genes according to their activity and properties (Figure S9B). Hereby, APOBEC3B (A3B) was identified to be the most up-regulated gene with nucleic acid binding properties (Figure S9C).

Analysis of all APOBEC3 family members showed that LT β R-activation leads to strong up-regulation of A3B and to minor extent A3G in HBV-infected dHepaRG and PHH, and after systemic application in human liver chimeric uPA-SCID mice (**Figure S10A**). A3B expression was induces by LT β R-activation in a dose-dependent manner and expression levels steadily increased during continuous treatment (**Figure S11**) correlating with a concomitant increase in treatment efficacy over time (**Figure S3C**). Treatment of PHH isolated from different donors with LT β R-agonist BS1 resulted in cccDNA degradation at different levels (**Figure S10B**, **Figure 3E**), which could neither be explained by the level of A3B upregulation (**Figure 3E**) nor by detection of a previously described (25) genomic deletion of the A3B allele, which seems to correlate with HBV persistence in infected patients (**Figure S10B, S10C**).

In contrast to LT β R-activation, IFN- α treatment induced mainly A3A, but also A3F and A3G expression in HBV-infected dHepaRG cells and PHH (**Figure S12A**), and A3D expression in isolated PHH. By systemic IFN treatment of chimpanzees (*26*), A3A was strongly upregulated in liver needle biopsies (**Figure S12B**). Activation of A3A, A3F and A3G after IFN- α treatment was dose- and time-dependent, and decreased after an initial peak despite continuous treatment indicating that cells become refractory to IFN- α (**Figure S13**). In patients treated with subcutaneous pegylated IFN- α , needle biopsies obtained at different time points confirmed a rapid, strong upregulation of A3A and to a lower extend of A3G in the liver peaking at 16 hrs post treatment (**Figure S12C**). Expression levels declined after this time point and remained low until day 6 post treatment confirming a fast but only transient induction of A3A by IFN- α treatment. Interestingly, the level of A3B or A3A induction in BS-1 and IFN- α treated PHH, respectively, did not directly correlate with the level of cccDNA degradation (**Figure 3E**). The fact that IFN- α only induces a transient A3A induction and cells rapidly become refractory to IFN- α treatment in HBV-infected patients (*3*).

APOBEC3A or APOBEC3B activity is essential to induce cccDNA degradation

Among the APOBEC3 family members up-regulated in our experiments, only A3A and A3B located to the nucleus (**Figure S14**) where they can gain access to cccDNA. To verify that they are indeed responsible for the induction of cccDNA degradation, we overexpressed the HIV-Vif protein (known to promote the degradation of all APOBEC3 proteins except A3B (*27, 28*)) in dHepaRG cells in a tetracycline-regulated fashion. Expression of HIV-Vif reduced A3A, A3F and A3G expression (**Figure S15A**), reverted IFN- α -induced cccDNA deamination and prevented cccDNA degradation induced by IFN- α treatment (**Figure 4A**). However, expression of HIV-Vif did not alter A3B levels (**Figure S15B**) and had no impact on cccDNA degradation by LT β R-activation (**Figure S15C**). To specifically address the role of A3A or A3B in cccDNA degradation we further knocked down A3A and A3B in dHepaRG cells under IFN- α or LT β R-agonist treatment, respectively, and observed reduced cccDNA deamination (**Figure 4B and 4C**, **left panels**). A3A as well as A3B knock-down completely reverted cccDNA degradation, but

could not rescue the additional effect of IFN- α or LT β R-activation on HBV replication (Figure 4B and 4C, right panels).

To confirm the impact of A3A and A3B on cccDNA deamination, we over-expressed A3A and A3B, respectively, in HBV-replicating HepG2-H1.3 (Figure 4D, 4E). Cytidinedeamination of nuclear cccDNA by A3A and A3B is in accordance with other studies showing that both localize to the nucleus (29) and may be involved in the elimination of foreign DNA (23).

APOBEC3A interacts with the HBV core protein and binds to cccDNA

APOBECs have evolved to restrict retroviral replication (30) as well as DNA transfer into cells. They are able to clear foreign nuclear DNA (23, 31), but it remains unclear how HBV cccDNA DNA was recognized and whether it was specifically targeted in our experiments. To assess specificity, we generated cell lines replicating a mammalian replicon plasmid pEpi containing a linear HBV 1.3-fold overlength sequence. From the linear HBV-genome, HBV replication was initiated and in addition to the pEpi-H1.3 replicon HBV cccDNA was established in the nucleus. Treatment with either IFN- α or LT β R-agonist BS1 inhibited HBV replication and resulted in deamination and degradation of HBV cccDNA, but not of the HBV-sequence containing replicon (**Figure S16**). This indicated that deamination and subsequent degradation induced by both treatments is HBV cccDNA specific.

HBV core protein associates with A3G (32) and HBV cccDNA (33) and thus was a candidate to mediate the targeting of A3 deaminases to HBV cccDNA. Confocal microscopy indicated a colocalization of A3A and A3B with HBV core in different cell lines and PHH (**Figure 5, S17**). Chromatin immunoprecipitation (ChIP) experiments using stably (**Figure S18A**) or transiently transfected HepG2H1.3 cells or HBV-infected and IFN- α treated dHepaRG cells, showed that HBV core protein and A3A both bind to the cccDNA minichromosome (**Figure 6A**). Supporting the possibility that a guardian protein prevents A3A direct binding to DNA (34), we could not detect A3A binding to genomic DNA (**Figure S18B**) even in the presence of HBV core, which has been reported to also bind to cellular DNA (35). HBV core protein co-immunoprecipitated A3A in HepG2H1.3 cells and transfected HuH7 cells indicating physical interaction with A3A (Figure S19). Direct interaction of HBV core expressed after HBV infection and A3A induced by IFN- α was confirmed by proximity ligation assay (PLA) (Figure 6B, S20) and fluorescence resonance energy transfer (FRET) analysis (Figure 6C). By deletion analysis, we determined that the central region of HBc (aa 77 to 149) is involved in the interaction with A3A (Figure 6C and S21).

These data suggest that A3A is targeted to cccDNA by interaction with HBV core. No such targeting to genomic DNA has been described so far. Since APOBEC3 deaminases are thought to act on single stranded DNA (*36*), one possibility is that A3A and A3B act on cccDNA when it is transiently rendered single-stranded by RNA polymerase II before transcription initiation.

We suggest, therefore, the following mechanism of APOBEC-dependent degradation of HBV cccDNA (Figure 6D). High dose IFN- α treatment or LT β R-activation up-regulate the expression of A3A and A3B, respectively, which subsequently co-localize or directly interact with HBV core in infected hepatocytes, translocate to the nucleus, where they are brought into close contact with cccDNA by HBV core. Now, APOBECs can deaminate cccDNA that is transiently rendered single-stranded during transcription. Uracils in HBV cccDNA are recognized and excised by cellular DNA glycosylases leading to formation of AP sites, which are then recognized by cellular AP endonculeases (23) leading to cccDNA digestion. Why cccDNA is degraded instead of being repaired by the cellular DNA repair machinery remains elusive so far. Using a mixture of various enzymes, we were able to repair deaminated cccDNA *in tubo* (Figure 3A) suggesting induction of an additional factor promoting DNA degradation or an impaired function of the repair machinery rather than a lack of recognition by the repair machinery. Thus, we can only speculate that either the number of AP sites introduced after treatment is too high and exceeds the capacity of the cellular repair machinery or that IFN-a treatment or LT β R-activation or even HBV itself (37) modulate the repair machinery. This may shift the equilibrium from cccDNA repair (38) to degradation.

Ideally, a cure for HBV infection needs to eliminate cccDNA. Therefore, cytokines or cytokine-receptor agonists that can trigger HBV cccDNA deamination and its degradation are

interesting antiviral candidates. Antivirals that induce A3A/B activity should be combined with nucleos(t)ide analogues to avoid the replenishment of nuclear cccDNA after degradation. LT β R-agonists were active at low doses and we did not observe any toxicity *in vitro* or *in vivo* nor did we detect any modification of genomic DNA. Constitutive overexpression of LT α/β for more than one year has been associated with inflammatory liver disease and hepatocellular carcinoma (*16*). As antivirals, however, LT β R-agonists would only be used for a limited period of time minimizing the risk of side effects. Moreover, LT β R-activation was already explored as a cancer treatment (*18*).

A recent study has shown a significantly higher frequency of an A3B deletion allele in persistent HBV carriers and hepatocellular carcinoma patients compared with healthy controls (25). This finding was further supported by the moderate deamination of cccDNA even in absence of treatment, and by the observation that knockdown of A3B in the absence of any treatment increased cccDNA levels. Although deregulated expression of A3A and A3B has been shown to correlate with genomic DNA mutations (39, 40), we did not detect any alterations of genomic DNA using analyses of AP sites, 3D-PCR analysis and deep sequencing of a set of human genes.

Our data indicate that cccDNA degradation is possible and can be induced without sideeffects on the infected host cell. An important task will be testing of combinations of nucleos(t)ide analogues with novel anti-viral strategies (e.g. LT β R agonists or adoptive T-cell therapy (41)) to activate A3A or A3B to cure hepatitis B.

References

- 1. U. Protzer, M. K. Maini, P. A. Knolle, Living in the liver: hepatic infections. *Nat Rev Immunol* **12**, 201 (Mar, 2012).
- 2. F. Zoulim, Hepatitis B virus resistance to antiviral drugs: where are we going? *Liver Int* **31 Suppl 1**, 111 (Jan, 2011).
- 3. K. Wursthorn *et al.*, Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. *Hepatology* **44**, 675 (Sep, 2006).
- 4. L. G. Guidotti *et al.*, Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a noncytolytic mechanism in transgenic mice. *Proc Natl Acad Sci U S A* **91**, 3764 (Apr 26, 1994).
- 5. L. G. Guidotti *et al.*, Viral clearance without destruction of infected cells during acute HBV infection. *Science* **284**, 825 (Apr 30, 1999).
- S. F. Wieland, H. C. Spangenberg, R. Thimme, R. H. Purcell, F. V. Chisari, Expansion and contraction of the hepatitis B virus transcriptional template in infected chimpanzees. *Proc Natl Acad Sci U S A* 101, 2129 (Feb 17, 2004).
- 7. H. McClary, R. Koch, F. V. Chisari, L. G. Guidotti, Relative sensitivity of hepatitis B virus and other hepatotropic viruses to the antiviral effects of cytokines. *J Virol* 74, 2255 (Mar, 2000).
- 8. L. Belloni *et al.*, IFN-alpha 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* **122**, 529 (Feb 1, 2012).
- 9. A. Rang, S. Gunther, H. Will, Effect of interferon alpha on hepatitis B virus replication and gene expression in transiently transfected human hepatoma cells. *J Hepatol* **31**, 791 (Nov, 1999).
- 10. V. Pasquetto, S. F. Wieland, S. L. Uprichard, M. Tripodi, F. V. Chisari, Cytokine-sensitive replication of hepatitis B virus in immortalized mouse hepatocyte cultures. *J Virol* 76, 5646 (Jun, 2002).
- 11. S. F. Wieland, L. G. Guidotti, F. V. Chisari, Intrahepatic induction of alpha/beta interferon eliminates viral RNA-containing capsids in hepatitis B virus transgenic mice. *J Virol* 74, 4165 (May, 2000).
- S. L. Uprichard, S. F. Wieland, A. Althage, F. V. Chisari, Transcriptional and posttranscriptional control of hepatitis B virus gene expression. *Proc Natl Acad Sci U S A* 100, 1310 (Feb 4, 2003).
- 13. P. Gripon *et al.*, Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. *J Virol* **62**, 4136 (Nov, 1988).
- 14. P. Gripon *et al.*, Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci U S A* **99**, 15655 (Nov 26, 2002).
- M. J. Wolf, G. M. Seleznik, N. Zeller, M. Heikenwalder, The unexpected role of lymphotoxin beta receptor signaling in carcinogenesis: from lymphoid tissue formation to liver and prostate cancer development. *Oncogene* 29, 5006 (Sep 9, 2010).
- 16. J. Haybaeck *et al.*, A lymphotoxin-driven pathway to hepatocellular carcinoma. *Cancer Cell* 16, 295 (Oct 6, 2009).
- 17. S. Jost, P. Turelli, B. Mangeat, U. Protzer, D. Trono, Induction of antiviral cytidine deaminases does not explain the inhibition of hepatitis B virus replication by interferons. *J Virol* **81**, 10588 (Oct, 2007).
- 18. M. Lukashev *et al.*, Targeting the lymphotoxin-beta receptor with agonist antibodies as a potential cancer therapy. *Cancer Res* **66**, 9617 (Oct 1, 2006).
- 19. X. Hu *et al.*, Lymphotoxin beta receptor mediates caspase-dependent tumor cell apoptosis in vitro and tumor suppression in vivo despite induction of NF-kappaB activation. *Carcinogenesis*, (Feb 13, 2013).
- 20. E. Dejardin *et al.*, The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways. *Immunity* **17**, 525 (Oct, 2002).

- R. Suspene *et al.*, 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* 102, 8321 (Jun 7, 2005).
- 22. J. I. Friedman, J. T. Stivers, Detection of damaged DNA bases by DNA glycosylase enzymes. *Biochemistry* **49**, 4957 (Jun 22, 2010).
- M. D. Stenglein, M. B. Burns, M. Li, J. Lengyel, R. S. Harris, APOBEC3 proteins mediate the clearance of foreign DNA from human cells. *Nature structural & molecular biology* 17, 222 (Feb, 2010).
- 24. M. Bonvin *et al.*, Interferon-inducible expression of APOBEC3 editing enzymes in human hepatocytes and inhibition of hepatitis B virus replication. *Hepatology* **43**, 1364 (Jun, 2006).
- 25. T. Zhang *et al.*, Evidence of associations of APOBEC3B gene deletion with susceptibility to persistent HBV infection and hepatocellular carcinoma. *Human molecular genetics* **22**, 1262 (Mar 15, 2013).
- 26. Y. Huang *et al.*, Defective hepatic response to interferon and activation of suppressor of cytokine signaling 3 in chronic hepatitis C. *Gastroenterology* **132**, 733 (Feb, 2007).
- 27. B. P. Doehle, A. Schafer, B. R. Cullen, Human APOBEC3B is a potent inhibitor of HIV-1 infectivity and is resistant to HIV-1 Vif. *Virology* **339**, 281 (Sep 1, 2005).
- G. Berger *et al.*, Functional analysis of the relationship between Vpx and the restriction factor SAMHD1. *J Biol Chem* 287, 41210 (Nov 30, 2012).
- 29. H. Muckenfuss *et al.*, APOBEC3 proteins inhibit human LINE-1 retrotransposition. *J Biol Chem* **281**, 22161 (Aug 4, 2006).
- 30. C. Munk, A. Willemsen, I. G. Bravo, An ancient history of gene duplications, fusions and losses in the evolution of APOBEC3 mutators in mammals. *BMC evolutionary biology* **12**, 71 (2012).
- 31. M. A. Carpenter *et al.*, Methylcytosine and normal cytosine deamination by the foreign DNA restriction enzyme APOBEC3A. *J Biol Chem* **287**, 34801 (Oct 5, 2012).
- 32. P. Turelli, B. Mangeat, S. Jost, S. Vianin, D. Trono, Inhibition of hepatitis B virus replication by APOBEC3G. *Science* **303**, 1829 (Mar 19, 2004).
- C. T. Bock *et al.*, Structural organization of the hepatitis B virus minichromosome. *Journal of molecular biology* 307, 183 (Mar 16, 2001).
- 34. M. M. Aynaud *et al.*, Human Tribbles 3 protects nuclear DNA from cytidine deamination by APOBEC3A. J Biol Chem 287, 39182 (Nov 9, 2012).
- 35. Y. Guo *et al.*, Hepatitis B viral core protein disrupts human host gene expression by binding to promoter regions. *BMC genomics* **13**, 563 (2012).
- 36. H. C. Smith, R. P. Bennett, A. Kizilyer, W. M. McDougall, K. M. Prohaska, Functions and regulation of the APOBEC family of proteins. *Seminars in cell & developmental biology* 23, 258 (May, 2012).
- T. H. Lee, S. J. Elledge, J. S. Butel, Hepatitis B virus X protein interacts with a probable cellular DNA repair protein. J Virol 69, 1107 (Feb, 1995).
- 38. K. Kitamura *et al.*, Uracil DNA Glycosylase Counteracts APOBEC3G-Induced Hypermutation of Hepatitis B Viral Genomes: Excision Repair of Covalently Closed Circular DNA. *PLoS Pathog* 9, e1003361 (May, 2013).
- S. Landry, I. Narvaiza, D. C. Linfesty, M. D. Weitzman, APOBEC3A can activate the DNA damage response and cause cell-cycle arrest. *EMBO reports* 12, 444 (May, 2011).
- 40. M. B. Burns *et al.*, APOBEC3B is an enzymatic source of mutation in breast cancer. *Nature* **494**, 366 (Feb 21, 2013).
- K. Krebs *et al.*, T Cells Expressing a Chimeric Antigen Receptor That Binds Hepatitis B Virus Envelop Proteins Control Virus Replication in Mice. *Gastroenterology*, (Apr 29, 2013).

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Figures legends

Fig. 1. Degradation of cccDNA in IFN- α treated HepaRG cells and primary human hepatocytes. (A, B, C, E, F) HBV-infected dHepaRG were treated with IFN- α at day 10 post-infection (dpi). Different regimens of treatment were applied as indicated. (D) HBV-infected primary human hepatocyte (PHH) were treated with IFN- α at dpi 3 for 13 days. Levels of HBeAg, total intracellular DNA and cccDNA are given relative to mock treated cells. LAM: lamivudine; ETV: entecavir. Mean values +/- standard deviation of replicates from independent experiments are given; data were analyzed by t-test. * p<0.05, ** p<0.01 and *** p<0.001.

Fig. 2. LTβR-activation inhibits HBV infection and leads to cccDNA degradation in HepaRG cells and PHH. (A, B) HBV-infected dHepaRG were treated with BS1, CBE11, hu-IgG control or lamivudine (LAM). (A) Treatment started 24h before infection for 12 days or (B) at 18 dpi for 10 days. Levels of the indicated HBV markers as well as cell viability are given relative to untreated controls (mock). (C) cccDNA levels were analyzed after 14 days of BS1 treatment by Southern blot in HBV-infected dHepaRG and HBV-replicating HepG2H1.3 cells. Supercoiled cccDNA bands were identified by their expected size and linearization upon *EcoRI* digestion (3,2 kb). (D) PHH were infected with HBV and treated with BS1 at 7 dpi for 10 days. Levels of the indicated HBV markers were compared to untreated PHH of the same donor (donor 3) (mock). (E) HBV-infected dHepaRG were treated with BS1, hu-IgG control or LAM. Intracellular HBV-DNA was analyzed 8 and 14 days after treatment cessation. Mean values +/standard deviation of replicates from independent experiments are given; data were analyzed by t-test. * p<0.05, *** p<0.001.

Fig. 3. Deamination and AP-site formation in cccDNA upon IFN- α treatment and LT β R-activation. (A) dHepaRG (left) and PHH (middle panel) were infected with HBV and treated with IFN- α , BS1 or LAM. Human chimeric uPA/SCID mice were treated with CBE11 or hu-IgG control (right panel). 3D-PCR analyses were performed on cccDNA left either untreated (upper panels) or treated with a PreCR mix (lower panels). (B, C) 3D-PCR products from HBV-infected dHepaRG cells treated as indicated (IFN- α , BS1 or mock) were cloned and sequenced and

mutations were analyzed. (D) Total DNA extracts from HBV-infected cells treated as indicated were digested with APE1, and cccDNA content was compared to mock-treated cells. (B,C,D) Mean values +/- standard deviation of biological triplicates from two independent experiments are given; data were analyzed by t-test. * p<0.05, ** p<0.01. (E) PHH were infected with HBV and treated with BS1 or IFN- α at 7 dpi for 10 days. Levels of the indicated cccDNA as well as A3A and A3B mRNA expression were compared to untreated PHH (mock) of the same donor.

Fig. 4. Analysis of cccDNA deamination and degradation. (A-C) cccDNA denaturation was analyzed by 3D-PCR (left panels); levels of HBeAg, total intracellular DNA and cccDNA are given relative to mock treated cells (right panels). (A) dHepaRG-tA-Vif cells treated with IFN- α for 10 days with and without doxycycline (dox)-induced HIV-Vif expression. HBV-infected dHepaRG cells treated with (B) IFN- α or (C) BS1 transfected with siRNA against A3A or A3B, respectively, or sequence non-specific siRNA (sicontrol). Mean values +/- standard deviation of independent replicates and experiments are given; data were analyzed by t-test. * p<0.05, ** p<0.01 and *** p<0.001. (D) cccDNA denaturation analysis by 3D-PCR in HepG2-H1.3 cells overexpressing A3A or (E) A3B from lentiviral vector plasmid pLenti6.3 or pTR600, respectively, for 5 days.

Fig. 5. Co-localization of A3A and A3B with HBV core protein (HBc). (A) HuH7 cells were co-transfected with an HBV1.1-fold genome and A3A-Flag or A3B-Flag expressing plasmids and stained using DAPI, anti-HBc and anti-FLAG antibodies. (B) HBV-infected dHepaRG and PHH were treated with IFN- α at day 7 post infection for 3 days. A3A and HBc were analyzed by immunofluorescence staining. Right panels indicate z stacks taken at the dotted lines.

Fig. 6. Interaction of A3A, HBV core protein (HBc) and cccDNA (A) Chromatin immunoprecipitation (ChIP) was performed using lysates of HepG2H1.3 cells transfected with A3A-expressing plasmid, or HBV-infected dHepaRG cells treated with IFN- α for 3 days. IPs using antibodies against histone H3, A3A, HBc and control rabbit IgG (RIgG) were analyzed by qPCR for cccDNA. (B) Interaction between HBc and A3A was assessed by proximity ligation assay (PLA) in HBV-infected, IFN- α treated dHepaRG. PLA-spots were quantified in single

cells by software-based spot-counting. Data were analyzed by one-way ANOVA. ** p<0.01 and *** p<0.001. (C) Serial HBV core-deletion mutants (left panel) were fused to CFP and interaction with A3A-YFP was assessed by FACS-FRET in HuH7.5 hepatoma cells (right panel). Cells cotransfected with CFP and YFP served as controls to exclude false positive FRET and subtract background signals. A CFP-YFP fusion construct was used as positive control. Mean values \pm standard deviation of FRET-positive cells from 3-4 independent experiments are given. Black boxes indicate shared regions of HBc mutants giving a FRET signal. (D) Model of cccDNA degradation induced by IFN- α treatment or LT β R-activation.







mock

BS1

IFN-α

mock

BS1

mock

IFN-α



В















+

+

0

PHH

+

+

IFN-α

150 Normalized suvival rate (%) 100 50

1000

2000

IFN-α (IU/ml)

3000

4000

0

0



+

+

Figure S1. (A) The indicated cells were treated with IFN- α for 6 hours and levels of Mx1 and OAS1 were assessed by qRT-PCR. (B, C) dHepaRG cells were infected with HBV and treated 10 days later with IFN-α or Lamivudine (LAM). (B) Viability of cells was assessed by XTT test. (C) Levels of HBeAg, intracellular HBV-DNA, HBV-RNA or HBV-DNA in the supernatants were analyzed and compared to untreated cells (mock). Values given are the mean +/- standard deviation of biological triplicates and were analyzed by t-test. ** p<0.01 and *** p<0.001. (D) HBV cccDNA levels were analyzed by Southern blot 10 days post-treatment. Supercoiled cccDNA bands were identified by their expected size and ability to be linearized upon EcoRI digestion (3.2 kb). (E) Dose response curve of viral markers after IFN- α treatment for 7 days.

В



Α

Figure S2. HBV-infected dHepaRG cells were treated with BS1. After 10 days, we analyzed (A) expression of LTBR and TNFR1 by Western blot and (B) by FACS analyses as well as (C) p100/p52 processing and (D) phosphorylation of ReIA (pReIA) by Western blot. (D) Nuclear translocation of RelB and of (F) RelA by immunohistochemistry (white arrow heads indicate nuclear translocation in hepatocytes). (G) Expression analysis of indicated genes upon BS1 treatment by qRT-PCR. (H) Assessment of cell viability under different concentration of BS1 by XTT tests.



Figure S3. dHepaRG cells were infected with HBV and treated with LT β R-agonist BS1. (A) Dose response curve of viral markers upon BS1 treatment. (B) cccDNA signal detected by Southern blot analysis was quantified using a phosphorimager and compared to quantification of cccDNA of the same extract by qPCR. (C, D) HBV-infected dHepaRG cells were treated with BS1 as indicated in the schemes. cccDNA was quantified by qPCR relative to untreated cells (mock). Values are the mean +/- standard deviation of different replicates and data were analyzed by t-test. ** p<0.01 and *** p<0.001.

Α



Figure S4. dHepaRG cells or primary human hepatocytes (PHH) were infected with HBV and treated with BS1 for 11 days and assayed for apoptosis induction. As positive control, cells were treated 12h before the end of the experiment with 1 μ g/mL of Staurosporine (Stauro). ALT (A) and HBeAg levels (B, right panel) were quantified in the supernatant. Cleaved caspase 3 activity was analyzed (B, left panel).



Figure S5. To exclude induction of IFN responses, HepaRG cells expressing a dominant negative version of the IFN regulated factor 3 (IRF3) were generated. (**A**) dHepaRG and dHepaRGIRF3DN cells were stimulated with poly-IC during 8h and expression of IFN- β was analyzed by qRT-PCR (relative to GAPDH). (**B**) dHepaRG or dHepaRGIRF3DN cells were infected with HBV and treated with BS1 24h later and for 10 days. cccDNA content was analyzed and expressed in % of untreated cells (mock). Values are given as mean +/- standard deviation of three replicates and data were analyzed by t-test. ** p<0.001 and *** p<0.001.







Figure S7. HBV-infected dHepaRG cells were treated with (A) IFN- α or (B) LT β R-agonist BS1, for 10 days. 3D-PCRs analyses were performed on cccDNA. (A, B) 3D-PCR products obtained at indicated temperatures were cloned, sequenced and numbers of G/A mutations were evaluated. (C) 3D-PCR of cccDNA after 10 days of treatment with IFN- α or nucleoside analogues lamivudine (LAM) or entecavir (ETV). (D) Treatment with IFN- α alone was compared to combined IFN- α +ETV treatment.



0.055/0.055/0.050

0.055/0.055/0.055

0.20/0.25/0.23

0.060/0.055/0.055

0.11/0.15/0.11

CXCL10 1577 Chr. 4: 1490756 - 1492332 37,67 103 739 0.44/0.44/0.45 0.16/0.16/0.17 CXCL13 1884 Chr. 4: 3074754 - 3076637 37,58 114 082 0.31/0.30/0.30 0.055/0.055/0.055 0.71/0.74/0.74 CCL17 2124 Chr. 16: 11062012 - 11064135 56,36 87 806 0.27/0.30/0.29 CCL20 1767 Chr. 2: 78889580 - 78891346 34,24 121 839 0.32/0.31/0.31 0.055/0.050/0.050 2070 Chr. 7: 22759130 - 22761199 45,17 0.41/0.45/0.41 0.13/0.13/0.12 IL6 118 519

D



Figure S8. (A) dHepaRG cells or PHH were infected with HBV, treated with IFN- α or LT β R-agonist BS1 for 10 days and 3D-PCR analysis was performed on a region of the PrnP gene. (B) 13 different amplicons from different genomic DNA regions from cells treated with IFN-α or BS1 were submitted for deep sequencing. The overall % of base exchanged for each base was analyzed. (C) The table gives the genes analyzed, length and genomic position of the amplicons, the coverage rate as well as mutations rates. (D) dHepaRG cells or primary human hepatocyte (PHH) were infected with HBV or mock-infected and treated with IFN- α or BS1 4 days later for 10 days. Total DNA was extracted and AP sites were quantified. Values given are the mean +/- standard deviation of biological triplicates from one experiment.



Figure S9. dHepaRG cells were infected with HBV and treated with LTβR-agonist BS1. **(A)** Affymetrix gene expression array analysis was confirmed by qRT-PCR analysis of the 50 most strongly up- and down-regulated genes. Results are shown as level of regulation according to the scale bars. **(B, C)** Regulated genes were classified according to their activity and binding properties. Stars indicate proteins for which nucleic acid binding properties have been described.



Figure S10. (A) Indicated cells were infected with HBV and treated with LTβR-agonist BS1 (dHepaRG, dHepaRG-IRF3DN: 10 days; PHH: 7 days; PHH in uPA/SCID mice: 2 weeks). Expression of APOBEC3 family members were analyzed by qRT-PCR and values are shown as mean +/- standard deviation of independent replicates. (B) PHH were infected with HBV and treated with BS1 at 7 dpi for 10 days. Levels of cccDNA were compared to untreated PHH of the same donor (mock). (C) PCR analysis to detect the wild-type A3B allele or a common deletion of the A3B allele in genomic DNA extracted from PHH derived from different donors as well as from HepaRG cells.



Figure S11. dHepaRG cells were infected with HBV and treated with LT β R-agonist BS1. Expression levels of APOBEC3B and APOBEC3G after 10 days or at indicated time points were analyzed by qRT-PCR. Values show mean +/- standard deviation of independent replicates and data and were analyzed by t-test. * p<0.05, ** p<0.01 and *** p<0.001.





С

humans treated with PEG-IFN-α



Figure S12. (A) Indicated cells were infected with HBV and treated with IFN- α for 6 hours. Expression of APOBEC3 family members was analyzed by qRT-PCR and values are shown as mean +/- standard deviation of independent replicates. (B) Chimpanzees X0101 was treated with 10 million IU of recombinant human IFN α -2a, chimpanzee X0233 with consensus IFN. Liver biopsies were obtained 8 hrs post treatment and expression of APOBEC3 genes were analyzed. (C) RNA isolated from livers of HCV-patients (n=3 to 6 at different time points) before and after treatment with 1.5 µg/kg pegIFN α -2b were analyzed for the expression of APOBEC3 family members by gene expression array at indicated time points. Intensity values are given for the different APOBEC3 family members (upper graph) and fold changes were calculated when intensity values were above the threshold (lower graph).

В

chimpanzees treated 8h with IFN-a



PHH

Figure S13. (A) dHepaRG cells or (B) PHH were infected with HBV and treated with IFN- α . Expression of the indicated APOBEC3 mRNA after 6h or at the indicated time point were analyzed by qRT-PCR. Values show mean +/- standard deviation of independent replicates.





С



Figure S14. dHepaRG cells were treated with IFN- α or mock-treated and fixed three days later. A3A, A3B, A3F or A3G localization were determined by **(A, C, D)** immunofluorescence staining or **(B)** Western blot analysis.



Figure S15. HBV-infected dHepaRG-tA-Vif cells were treated with (A) IFN- α or (B, C) LT β R-agonist BS1 for 10 days. 24h prior to HBV infection, HIV-Vif expression has been induced by doxycycline (dox). (A, B) Western blot analysis and (C) cccDNA qPCR were performed at the end of treatment. Values show the mean +/- standard deviation of independent replicates and data were analyzed by t-test.** p<0.01. (D) A3A and (E) A3B were overexpressed in HepG2-H1.3 cells. 3D-PCR experiments were performed 5 days later and the resulting PCR products were cloned and sequenced. Content of each base is given.



Figure S16. dHepaRG-pEpi-H1.3 cells were treated with IFN- α (500 IU/ml) or BS1 (0.5 µg/ml) for 11 days. (A) pEpi-H1.3 DNA, cccDNA and total intracellular HBV DNA were quantified by specific qPCR. (B) 3D-PCR specific for pEPIH.13 or cccDNA are shown. (C) HBV cccDNA 3D-PCR products obtained at indicated temperatures were cloned, sequenced and numbers of G/A mutations were evaluated.



Figure S17. HBV-infected dHepaRG and PHH were treated with IFN- α at 7 dpi for 3 days. A3A and HBc were analyzed by immunofluorescence staining and quantification (mean ± SD) of A3A/HBc colocalisation in the nuclei from 5 different vision fields are given.



Figure S18. HepG2H1.3-A3A cells were analyzed by chromatin immunoprecipitation with anti-A3A, anti-HBc or using protein A/G beads only (negative control) and by qPCR using selective primers for (A) cccDNA (B) GAPDH, tumor suppressor p53, cellular/sarcoma tyrosine kinase (Src) and myelocytomatosis (c-myc).



Figure S19. (A) HepG2H1.3 cells were transfected with a plasmid expressing A3A. (**B**) Huh-7 cells were cotransfected with a plasmid expressing A3A-Flag and one containing a 1.1-fold HBV genome expressing all HBV genes. After 72h, cells were lysed and the indicated immunoprecipitations (IP) followed by Western blot for the A3A, Flag tag and HBV core (HBc) were performed.



Figure S20. Proximity Ligation Assay (PLA) staining in HepaRG cells with antibodies against HBc and A3A. Imaging was done by spinning disc microscopy and single cells were identified based on DAPI staining. PLA spots were automatically counted with a fixed threshold in all measurements.





Figure S21. (A) Interaction of serial HBV Core (HBc)-YFP deletion mutants with A3G-CFP assessed by FACS-FRET in Huh7.5 hepatoma cells. Indicated are percentages of cells scoring FRET positive for each co-transfection. CFP-only and YFP-only as well as co-transfection of a highly membrane incorporated MEM fusion protein served as negative controls. A CFP-YFP fusion protein was used as positive control. Mean values and standard deviations were calculated from three to four independent experiments. **(B)** The potential interaction sites of modeled A3A (red) and HBV core protein (blue) are depicted: amino acids of HBV core locating within a distance of 3.1 Å from A3A, which are mainly located at residues 110 to 149, are highlighted in green indicating interaction. The red ball indicates the zinc atom embedded in the catalytic site of A3A. Our model indicates an interaction of HBV core α -helix 4 with A3A.

mouse number	treatment	viremia (copies/mL)		total intracellular HBV DNA (copies/cell)	cccDNA (copies/cell)
		before	end	end	end
5839	hu-IgG	1,4E+07	3,3E+07	570	1,79
1118	CBE11	1,2E+07	3,2E+06	84	0,84
1129	CBE11	6,0E+06	5,3E+06	105	0,96

Table S1. HBV-infected humanized uPA/SCID mice were treated 2 times with chlodronate (day 0 and day 5) and one time with hu-IgG or CBE11 (day 3). Mice were sacrified at day 10 and HBV replication markes were analyzed.