Research article

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Control of APOBEC3B induction and cccDNA decay by NF-κB and miR-138-5p

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Background & Aims: Immune-mediated induction of cytidine deaminase APOBEC3B (A3B) expression leads to HBV covalently closed circular DNA (cccDNA) decay. Here, we aimed to decipher the signalling pathway(s) and regulatory mechanism(s) involved in A3B induction and related HBV control.

Methods: Differentiated HepaRG cells (dHepaRG) knocked-down for NF-κB signalling components, transfected with siRNA or micro RNAs (miRNA), and primary human hepatocytes ± HBV or HBVΔX or HBV-RFP, were treated with lymphotoxin beta receptor (LTβR)-agonist (BS1). The biological outcomes were analysed by reverse transcriptase-qPCR, immunoblotting, luciferase activity, chromatin immune precipitation, electrophoretic mobility-shift assay, targeted-bisulfite-, miRNA-, RNA-, genome-sequencing, and mass-spectrometry.

Results: We found that canonical and non-canonical NF-κB signalling pathways are mandatory for A3B induction and anti-HBV effects. The degree of immune-mediated A3B production is independent of A3B promoter demethylation but is controlled post-transcriptionally by the miRNA 138-5p expression (hsa-miR-138-5p), promoting A3B mRNA decay. Hsa-miR-138-5p over-expression reduced A3B levels and its antiviral effects. Of note, established infection inhibited BS1-induced A3B expression through epigenetic modulation of A3B promoter. Twelve days of treatment with a LTβR-specific agonist BS1 is sufficient to reduce the cccDNA pool by 80% without inducing significant damages to a subset of cancer-related host genes. Interestingly, the A3B-mediated effect on HBV is independent of the transcriptional activity of cccDNA as well as on rcDNA synthesis.

Conclusions: Altogether, A3B represents the only described enzyme to target both transcriptionally active and inactive cccDNA. Thus, inhibiting hsa-miR-138-5p expression should be considered in the combinatorial design of new therapies against HBV, especially in the context of immune-mediated A3B induction.

Keywords: APOBEC3B; Hepatitis B virus; NF-κB; miRNA; cccDNA; HBx.
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Lay summary: Immune-mediated induction of cytidine deaminase APOBEC3B is transcriptionally regulated by NF-κB signalling and post-transcriptionally downregulated by hsa-miR-138-5p expression, leading to cccDNA decay. Timely controlled APOBEC3B-mediated cccDNA decay occurs independently of cccDNA transcriptional activity and without damage to a subset of cancer-related genes. Thus, APOBEC3B-mediated cccDNA decay could offer an efficient therapeutic alternative to target hepatitis B virus chronic infection.

Introduction

HBV is a major global health burden with more than 250 million people chronically infected and about 900,000 related deaths per year (WHO, 2017). Patients with chronic hepatitis B (CHB) are at high risk of developing end-stage liver disease and hepatocellular carcinoma (WHO, 2017). Current treatments (*e.g.* nucleos(t)ides analogues such as tenofovir or pegylated-interferon alpha) allow the control of the infection but not its complete eradication owing to the persistence of the viral minichromosome, called covalently closed circular DNA (cccDNA).[1] Upon stopping treatment the infection can relapse, as a result of side effects or development of resistance.[1] Therefore, new treatments are urgently needed to cure chronic HBV infection.

We and others previously showed that the cytidine deaminase apolipoprotein B mRNA editing enzyme catalytic subunit 3B (APOBEC3B, A3B) is upregulated upon immune-mediated lymphotoxin-β receptor (LTβR) agonisation.[2,3] A3B induction subsequently leads to cccDNA hypermutation and viral decrease in a non-hepatotoxic manner *in vitro*.[2] Notably, it is the extent and quality of hepatic inflammation that can contribute to HBV elimination (*e.g.* in the setting of an acute HBV infection).[3,4] These results opened the door for new R&D strategies to improve *functional cure* in CHB.[2] LTβR is expressed on different hepatic cells (*e.g.* hepatocytes, endothelial cells, hepatic stellate cells)[5] and direct, chronic agonisation in CHB patients with the current tools available (*e.g.* BS1 — an LTβR agonist with non-hepatocyte-specific targeting) might affect liver biology. Thus, understanding the mechanisms of A3B regulation in hepatocytes is an important first step towards the targeted development of new therapies aiming at hepatocyte-specific cccDNA decay.

In distinct cancer types, A3B induction has been shown to be mediated by the nuclear factor-kappa B (NF-κB) pathways.[6] However, whether NF-κB signalling is mandatory for A3B induction in non-cancerous hepatocytes or in the context of a chronic HBV infection has remained unknown. The NF-κB-signalling pathway can be divided into 2 arms: the classical/canonical and the alternative/non-canonical pathways.[7] The canonical pathway, commonly activated by tumour necrosis factor (TNF) family members, signals through the IKK complex (inhibitor of NF-κB kinase complex, consisting of NEMO/IKKα/IKKβ), triggering the phosphorylation and ubiquitination of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) and the release of p50/RelA heterodimer.[7] In addition to the canonical pathway, LTβR agonisation signals through the non-canonical pathway by activating the NF-κB inducing kinase (NIK). This leads to phosphorylation of IKKα and p100 and its processing into p52 forming p52/RelB heterodimers which translocate to the nucleus to activate target genes such as immune mediators.[8]

The liver displays an overall immunosuppressive environment.[9] To regulate the immunosuppressive state, and to prevent inappropriate and/or chronic inflammation induced by pathogen recognition, a large number of immune factors can additionally be regulated at the post-transcriptional level by micro RNAs (miRNAs).[10] miRNAs are small non-coding RNAs involved in mRNA silencing and post-transcriptional regulation through base-pairing with complementary RNA sequences.[10] Protein synthesis of mRNAs targeted by miRNAs is then reduced, either because of the cleavage of the mRNA strand, destabilisation of mRNAs by shortening of the poly(A) tail, or reduced translation of the mRNA.[10]

Here we describe the regulatory mechanisms of A3B induction upon immune-mediated LTβR agonisation at the transcriptional and post-transcriptional level, and identify the regulation of hsa-miR-138-5p as a novel antiviral strategy against HBV.

Materials and methods

Cell culture

HepaRG, a non-transformed progenitor cell line that can be differentiated into hepatocytes, were cultured as described previously.[11] HEK293T cells (ATCC® CRL-1573™, for lentivirus production) and HEK293T/17 cells (293T/17; ATCC CRL-11268, for luciferase assays) were cultured in DMEM (Gibco, Paisley, United-Kingdom) supplemented with 10% foetal calf serum (Gibco) and 50 U/ml penicillin/streptomycin (Gibco). Primary human hepatocytes (PHHs) were isolated were isolated and cultured as previously described.[12] Work with primary cells was approved by the local ethics committee (French ministerial authorisations [AC 2013-1871, DC 2013–1870, AFNOR NF 96 900 Sept 2011]). Written consent was obtained from all patients. HBV, HDV, or HIV chronically infected specimens were excluded.

Transgenic cell line preparation

Knockout HepaRG cell lines were generated by lentiviral transduction of a double-sgRNA containing construct into HepaRG-iCas9-TR (David Durantel, unpublished). Briefly, HepaRG cells were transduced with pLenti6-TR to introduce the tetracyclin repressor (TetR), and subsequently with pLenti4/TO/V5 (Invitrogen, Carlsbad, United-States), in which the coding sequence of an N-terminally 3× FLAG-tagged Cas9 was inserted between the EcoRI and XhoI sites of the vector. The generation of double-sgRNA containing vectors for the knockout cell line generation was described previously.[13] In short, sgRNAs were chosen based on high scoring and no high scoring off-targets using the CHOPCHOP version 2 web tool.[14] These sgRNAs were inserted into pUSEPR (generous gift from Dr. Tscharaganeh, DKFZ, Heidelberg, unpublished) based on methods as described elsewhere.[15]

Preparation of lentiviral particles and transduction of HepaRG cells were performed based on protocols from Addgene. After

each transduction step, HepaRG were selected with blasticidin (Invitrogen; 5 μg/ml; TetR), Zeocin (Invitrogen; 300 μg/ml; Cas9) and puromycin (Sigma Aldrich, Taufkirchen, Germany; 10 μg/ml; sgRNAs) until non-transduced cells have died.

Additional material and methods can be found in the supplementary material.

Results

Canonical and non-canonical NF-κB signalling induces APOBEC3B upon LTβR agonisation

We and others have shown that agonisation of LTβR triggers A3B transcription.[8] However, in non-transformed hepatocytes, the signalling pathways activated remain to be identified. First, we confirmed that of among APOBEC3 family members, A3B displayed the strongest upregulation (2-fold increase by mRNA sequencing and 3-fold increase by mass spectrometry [MS], respectively) at 1 day post-treatment with BS1, an antibody agonising LTβR (Fig. S1A,B). In addition to the MS results, we also observed an enrichment of A3B mRNA in the polysomes fractions of BS1-treated dHepaRG cells compared with untreated cells (Fig. S1C,D). Of note, this increase was mostly based on signals located in heavy polysomes, indicative of strong translational activity (Fig. S1E). EDTA release control[16] confirmed that the A3B transcript signal was of polysomal origin (Fig. S1F).

To decipher the pathway activated by BS1, RNA sequencing was performed and highlighted that constant BS1 treatment up to 40 days downregulated metabolic pathways (*e.g.* cytochrome P450 mediated detoxification of drugs and xenobiotics) and 'complement and coagulation cascades', whereas pathways usually activated during virus-infection were upregulated (Fig. S2A). In-depth analysis of specific pathways highlighted a strong induction of many of the NF-κB signalling proteins by LTβR agonisation (Fig. S2B), and upregulation of many transcripts of proteins involved in MAPK, NOD-like receptor, IL17, and TNF-signalling pathways (Fig. S2C–F).

Because LTβR activates the 2 NF-κB signalling pathways (canonical and non-canonical),[8] we performed *in silico* analyses of the proximal A3B promoter region to find putative NF-κB binding sites (Fig. 1A). Two κB sites (κB1 and κB2) were identified and tested in electrophoretic mobility shift assays with nuclear extracts of BS1-treated dHepaRG cells (Figs. 1A and S3A). Both κB probes displayed NF-κB binding activities, although with different patterns. In addition, the contribution of both κB sites to the A3B transcriptional activity was monitored with a luciferase vector containing the proximal A3B promoter with wild-type and/or mutated κB1/2 sites. We observed that all NF-κB heterodimers tested were able to induce a luciferase activity with varying efficacy, but p50/RelA and p50/RelB showed the poorest activity (Fig. 1B). Our mutagenesis analysis of each κB site revealed that the κB1 site was the major active site, as its mutation strongly decreased luciferase activity (Fig. 1B). Chromatin immunoprecipitation of the A3B proximal promoter in HepaRG cells highlighted an increase of binding for RelB, p52, and p50 but not RelA, upon BS1 treatment (Figs. 1C and S3B). Of note, binding of p52 as well as polymerase II (a marker of active transcription) was constant from Day 1 to 6 post treatment (Fig. 1C,D).

Furthermore, siRNAs against NIK, or specific IKKβ inhibitors,[17,18] or a combination of both, blocked BS1-induced A3B upregulation at the mRNA and protein level (Figs. 1E and S3C). Results were confirmed using other NF-κB inducing agents (Fig. S3D,E). In addition, HepaRG knockout lines of genes involved in canonical (*i.e.* IKKβ, RelA) and non-canonical (*i.e.* NIK, RelB) NF-κB pathways were generated (Fig. S3F–H). A significant impairment of BS1-induced A3B upregulation was observed in all tested cell lines (Figs. 1F and S3I). Knockdown of NIK in combination with IKKβ was most efficient to prevent A3B upregulation.

Taken together, these data highlight that both arms of NF-κB signalling play an important role in the induction of immune-mediated APOBEC3B expression, and confirmed our previous findings on the crucial role of RelB for A3B promoter activation.[19]

APOBEC3B is as an atypical NF-κB target

Unlike prototypic C-X-C motif chemokine 10 (CXCL10) or A20 (typical NF-κB target genes), which have an induction peak shortly after treatment start, A3B mRNA was significantly, but only weakly induced at 24 h after BS1 exposure (Figs. 2A and S4A). Whereas continued BS1 treatment led to decreased CXCL10 and A20 expression, A3B mRNA levels remained low during a 4-day 'lag phase' followed by a constant rise after 4 days of constant or pulse-chase BS1 treatments (Figs. 2A and S4A, respectively). Similarly, A3B protein level remained low during a 4-day lag phase and increased after 4 days of BS1 treatment (Fig. 2B). RelA phosphorylation, p100 processing to p52, and RelB protein levels were elevated from 18 h post-treatment (Fig. 2B). Notably, similar results for A3B expression were obtained with other NF-κB inducing cytokines (Fig. S4B). The lag phase was not linked to delayed A3B transcription because a constant binding of p52 and polymerase II on A3B promoter were detected from 1 day post treatment onwards (Fig. 1C,D). As promoter demethylation is a key factor of gene expression and could play a role in the observed lag phase, targeted bisulfite sequencing was performed.[20] Over a 12-day period of constant BS1 treatment, no change in A3B promoter methylation was detected (Fig. S4C).

These data highlighted that A3B is an atypical NF-κB target which displays a lag phase profile upon NF-κB activation.

Hsa-miR-138-5p post-transcriptionally regulates APOBEC3B mRNA

Our results suggest that A3B lag phase induction might be a result of post-transcriptional regulation by miRNAs. Therefore, we hypothesised that miRNAs might be negative regulators of A3B mRNA to buffer A3B induction upon short-time LTβR stimulation, which potentially leads to genomic DNA damage.[21] However, a sustained stimulation would lead to a repression of the miRNAs leading to high A3B mRNA levels, needed for an efficient antiviral effect (Fig. 2C).

Combined unbiased small RNA sequencing, RT-qPCR, and *in silico* target prediction algorithms[22] revealed 3 clusters of dysregulated miRNAs in untreated compared with BS1-treated HepaRG cells (Fig. 2D): (i) miRNAs highly expressed at Day 2 post-treatment (when A3B mRNA is low) but downregulated at Day 4 (when A3B mRNA is higher). This group includes the

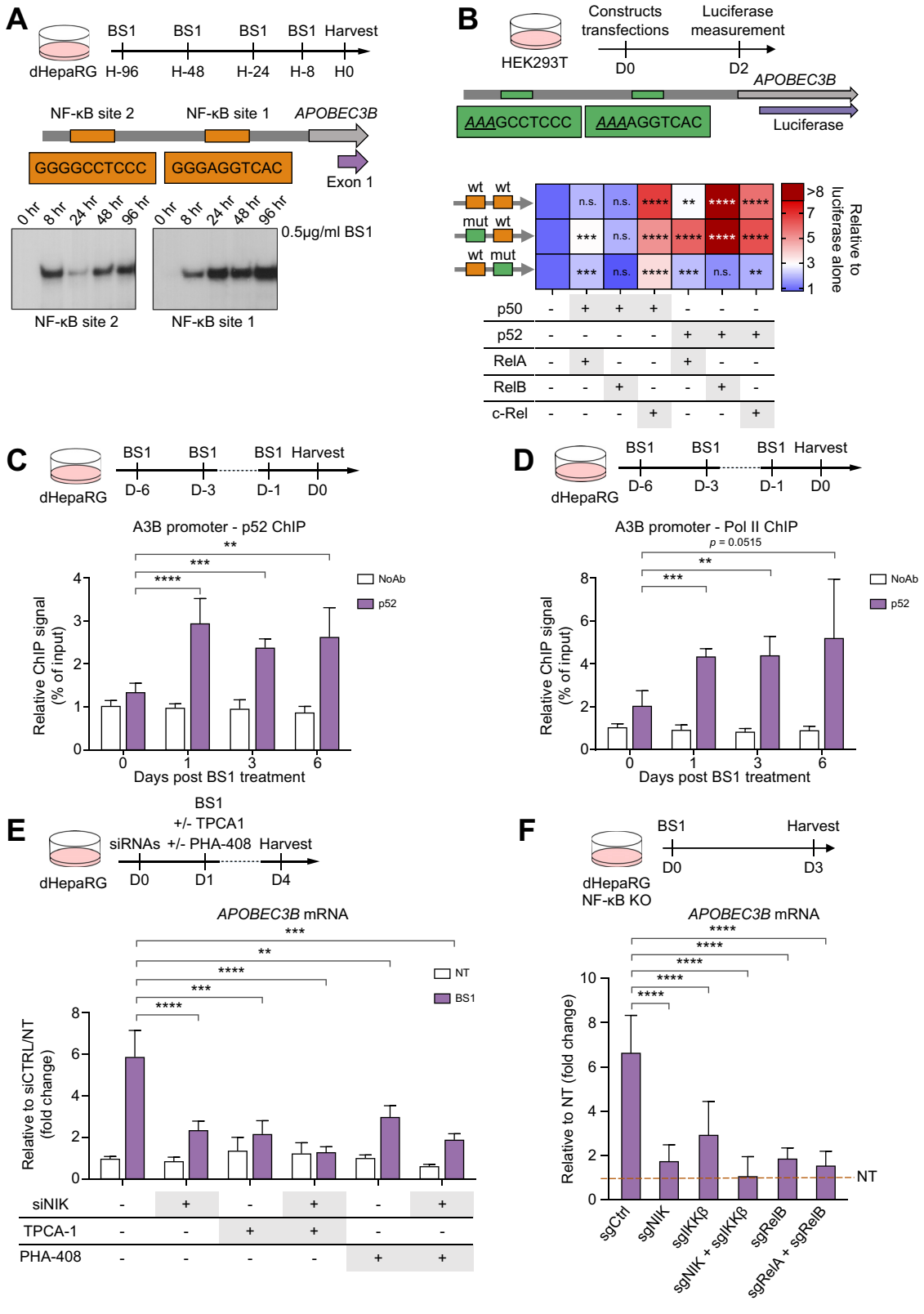

Fig. 1. Lymphotoxin beta receptor agonisation induces APOBEC3B through NF-κB signalling. (A) dHepaRG were treated for indicated times with 0.5 µg/ml BS1. Upper panel: schematic representation of the experiment. Lower panel: labelled probes containing the NF-κB binding sites were analysed by EMSA. (B) HEK293T cells were co-transfected with a luciferase construct containing APOBEC3B promoter (-230, +18, distance to transcription start site) wt or mutated for each NF-κB binding sites, together with NF-κB-transcription-factors-expressing plasmids. Upper panel: schematic representation of the experiment. Lower panel: schematic representation of the downstream promoter region with the inserted mutations of the NF-κB sites. Luciferase activity was assessed 48 h post-transfection. Heat map represents the mean of 1 experiment performed in triplicate. (C,D) dHepaRG were treated for the indicated time with 0.5 µg/ml BS1.

candidates of interest; (ii) miRNAs lowly expressed at Day 2 but with increased expression at Day 4; (iii) miRNAs downregulated under BS1 treatment.

Our *in-silico* analysis revealed that among the 30 miRNAs identified in cluster I, only hsa-miR-138-5p was predicted to have a high binding affinity within the 3′-UTR of A3B (Fig. 2E). Next, we confirmed by RT-qPCR the reduced expression of hsa-miR-138-5p between Day 2 (*i.e.* during the lag phase) and Day 4 (*i.e.* after the lag phase) post BS1-treatment (Fig. 2F).

To assess the functional activity of miR-138-5p on A3B, we fused the luciferase gene upstream of the 3′-UTR of A3B and co-transfected the plasmid together with, either a miRNA ctrl or a miRNA-138-5p expression vector. We observed that expression of miRNA-138-5p decreased luciferase activity whereas expression of miRNA control did not (Fig. 2G,H; 3B-3′-UTR-138). Conversely, insertion of point mutations within the miRNA-138-5p binding site of the 3′-UTR of A3B abrogated the sensitivity to the miRNA-138-5p (Fig. 2G,H; 3B-3′-UTR-138 mut). We next extended our *in-silico* analysis for the presence of miR-138 binding site in the 3′-UTR of other APOBEC3 family members. We found that APOBEC3G (A3G) displays 1 single site whereas APOBEC3A (A3A) contains a pseudo-miR-138-5p binding site with a single point mutation. Interestingly, an A3A variant with a matching miR-138-5p binding site in its 3′-UTR has been identified in a low percentage of the population (single nucleotide polymorphism [SNP] rs1367248965). We confirmed that A3G (3G-3′-UTR-138) and A3A (3A-3′-UTR SNP) variant containing of intact miR-138 binding site were responsive to the expression of miR-138 as opposed to their mutant counterpart (3G-3′-UTR-138 mut and 3A-3′-UTR) (Fig. 2G,H). The sensitivity of these APOBEC mRNAs to miRNA-138 were similar when the full coding sequences (CDS) of the APOBEC3 genes (Fig. S5A,B) or the minimal miRNA-138-5p binding site was cloned downstream of the luciferase gene (Fig. S5C,D).

Noteworthy, in the human genome, two different loci encode hsa-miR-138-5p genes (Fig. S5E,F). However, dHepaRG cells mainly express the hsa-miR-138-1 located on chromosome 3 (Fig. S5G). We observed that BS1-mediated hsa-miR-138-5p repression was prevented either by inhibiting IKK kinase activities (*i.e.* by using TPCA, an IKK inhibitor) or by depleting NIK in HepaRG cells (Fig. S5H,I). Altogether, these results suggest that activation of NF-κB acts as a positive regulator of A3B transcription while inhibiting hsa-miR-138-5p transcription.

Inhibition of NF-κB signalling or forced expression of hsa-miR-138-5p mimics abolish A3B-mediated anti-HBV activity

We next investigated how NF-κB and miRNA-138-5p modulate HBV viraemia. HepaRG control and knockout for different NF-κB signalling proteins were treated with BS1 or tenofovir (Teno) for either 6 or 12 days and cccDNA and viraemia were monitored (Fig. 3A). We observed that BS1-mediated anti-HBV effects (on cccDNA and viraemia) was significantly reduced (Figs. 3B and S6A). Teno, a nucleoside analogue that reduces secreted DNA but not the cccDNA content, was used as control. These results correlate with the absence of BS1-mediated A3B induction and hsa-miR138-5p repression in NIK or IKKβ-deficient cells (Figs. 3C and S6B,C) and was not the consequence of induced cell death (Fig. S6D).

These results were also phenocopied using a miRNA mimic approach (see experimental timeline Fig. 3D). Indeed, transfection of hsa-miR-138-5p mimics reduced A3B levels (Fig. 3E,F) and prevented antiviral effects on cccDNA (Fig. 3G). These results further validate our observations in Fig. 2H showing the effect of the miR-138 binding site in the 3′-UTR of A3B and A3G.

Of note, from the 704 hsa-miR-138-5p predicted targets genes (Table S3), only 6 genes were related to NF-κB signalling or hepatocyte function (HIF1α, HNF4α, JMJD8, MAPKBP1, RelA, UBE2V1). None of the latter was significantly affected by hsa-miR-138-5p mimics or BS1 treatment (Fig. S6E; UBE2V1 could not be detected in dHepaRG), highlighting that the effect of the 138-5p-mimic was most probably limited to A3B.

Interestingly, 7 days post infection, even though both non-infected and infected cells (HepaRG or PHH) showed a significant upregulation of A3B mRNA upon BS1 treatment, HBV-infected cells displayed a 50% reduction of A3B mRNA expression as compared with the non-infected counterpart, which was (i) independent of an increase of hsa-miR-138-5p expression levels and (ii) not sufficient to prevent the antiviral effect on HBV secreted protein (Fig. 4A–E). Chromatin immune precipitation on the activating epigenetic mark H3K4Me3 highlighted that the increase of H3K4Me3 on the A3B promoter induced by BS1 treatment was lost when the cells were infected with HBV (Fig. S6F).

In summary, disrupting A3B induction prevents the immune-mediated effect on HBV cccDNA levels. Moreover, HBV infection itself partially counteracts A3B upregulation during persistent infection.

Transient APOBEC3B induction triggers cccDNA decay without inducing damage to cancer-related genes

One of the major risks in the induction of A3B to eliminate cccDNA might possibly be a DNA-modifying effect. Indeed, A3B

Upper panel: schematic representation of the experiment. Lower panel: binding of (C) p52 and (D) polymerase II to APOBEC3B promoter was analysed by ChIP and qPCR. (E) dHepaRG were transfected with 10 nM of control or NIK-targeting siRNAs for 24 h before being left untreated (NT) or treatment with 0.5 μg/ml of BS1 ± 10 μM of TPCA-1 or 5 μM PHA-408. Upper panel: schematic representation of the experiment. Lower panel: mRNAs were isolated and analysed by RT-qPCR. (F) Knockout dHepaRG lines for NIK (sgNIK), IKKβ (sgIKKβ), NIK and IKKβ (sgNIK + sgIKKβ), RelB (sgRelB), or RelA and RelB (sgRelA + sgRelB), as well as control dHepaRG (sgCtrl) were left untreated (NT) or treated with 0.5 μg/ml of BS1 for 3 days. Upper panel: schematic representation of the experiment. Lower panel: mRNAs were isolated and analysed by RT-qPCR. Bars represent the mean ± SD of (E) 2, (F) 3, or (C,D) 4 independent experiments. Data were submitted to (C–F) 1-way ANOVA. $^{**}p$ <0.01; $^{***}p$ <0.001; $^{****}p$ <0.001. APOBEC3B, apolipoprotein B mRNA editing catalytic polypeptide-like A; ChIP, chromatin immune precipitation; EMSA, electrophoretic mobility-shift assay; IKKβ, inhibitor of nuclear factor kappa B kinase subunit beta; NF-κB, nuclear factor kappa B; NIK, NF-κB inducing kinase; n.s., not significant; wt, wild-type.

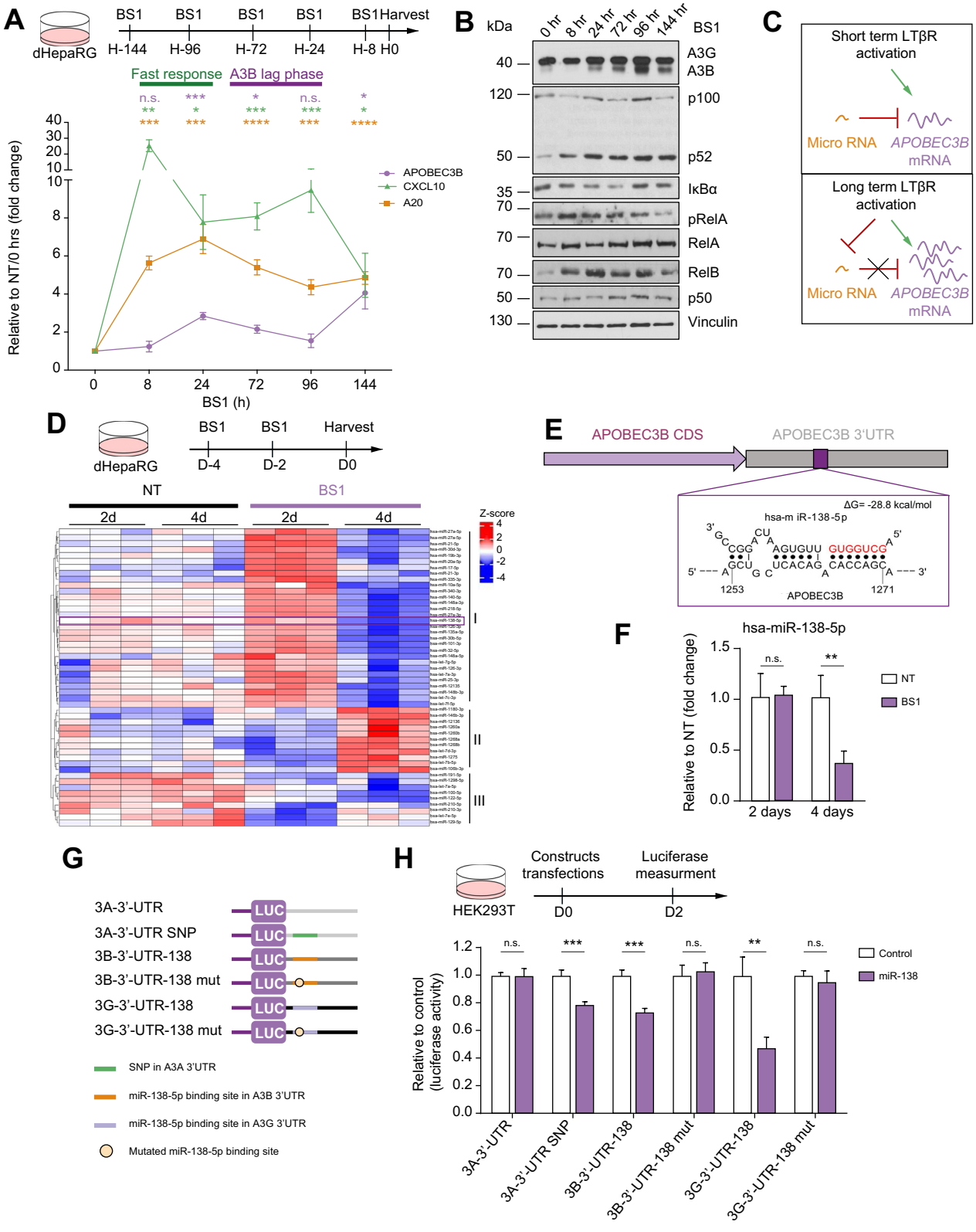

Fig. 2. APOBEC3B is post-transcriptionally regulated by the miRNA-138-5p. (A,B) Treatment of dHepaRG with 0.5 μg/ml BS1 was started sequentially and stopped altogether at the indicated time points. (A) Upper panel: schematic representation of the experiment. Lower panel: mRNAs of interest were extracted and analysed by RT-qPCR. (B) Proteins were analysed by immunoblotting. (C) Schematic representation of the working hypothesis. (D) dHepaRG were treated for 2 or 4 days with 0.5 μg/ml of BS1. Upper panel: schematic representation of the experiment. Lower panel: RNAs were extracted and small RNA were sequenced. Top 50 significantly dysregulated miRNAs of combined sequencing and RT-qPCR data was unbiased clustered and plotted. Cluster I represents miRNAs highly expressed at Day 2 and lowly expressed at Day 4 (*i.e.* miRNAs of interest); Cluster II represents miRNAs lowly expressed at Day 2 and highly expressed at Day 4; Cluster III

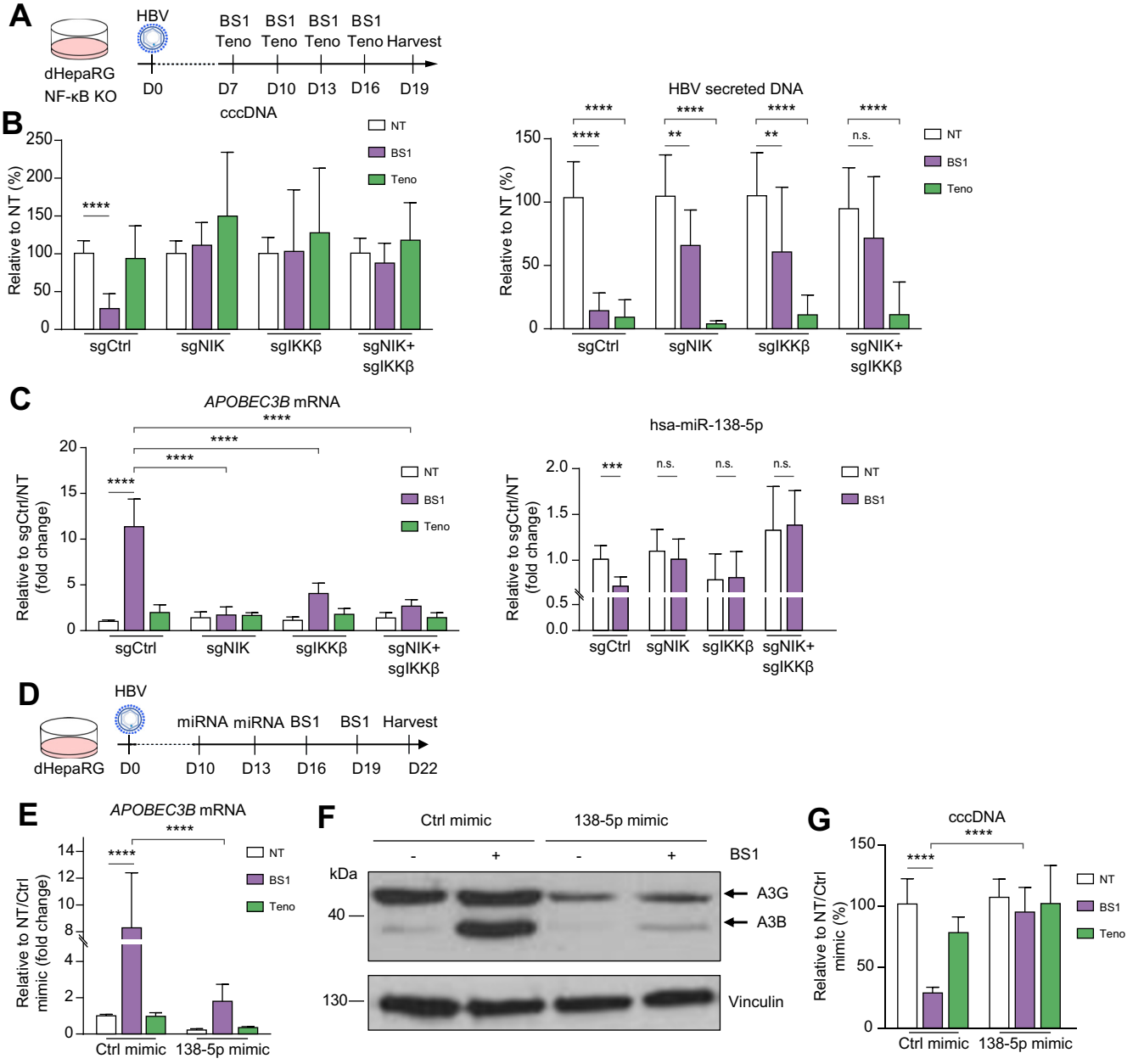

Fig. 3. Dysregulation of APOBEC3B expression by disruption of NF-κB signalling and mi-RNA 138-5p prevent the antiviral effect. (A) Schematic representation of the experiment presented in panels B and C. (B,C) Knockout dHepaRG lines for NIK (sgNIK), IKKβ (sgIKKβ), or NIK and IKKβ (sgNIK + sgIKKβ), as well as control dHepaRG (sgCtrl) were infected with HBV. Seven-d.p.i. cells were left untreated (NT) or treated with 0.5 μg/ml of BS1 or 0.5 μM of tenofovir for 12 days. (B) DNA and (C) RNAs were isolated and analysed using RT-qPCR or qPCR. (D–G) dHepaRG were infected with HBV and 10 and 13 d.p.i. transfected with 10 nM microRNA (miR)-138-5p or control mimics. Cells were then left untreated (NT) or treated for 6 days with 0.5 μg/ml of BS1 or 0.5 μM of tenofovir. (D) Schematic representation of the experiment presented in panels E–G. (E) RNAs, (F) proteins, and (G) DNA were isolated and analysed using RT-qPCR, immunoblotting, and qPCR, respectively. Bars represent the mean ± SD of (B,C) 3 or (E, G) 6 independent experiments. Data were submitted to (B, C, E, G) 1-way ANOVA. *p <0.05; **p <0.01; ***p <0.001; ****p <0.001. APOBEC3B, apolipoprotein B mRNA editing catalytic polypeptide-like A; d.p.i., days post infection; IKKβ: inhibitor of nuclear factor kappa B kinase subunit beta; NF-κB, nuclear factor kappa B; NIK, NF-κB inducing kinase; n.s., not significant.

represents miRNA lowly expressed at Day 2 and Day 4. (E) Schematic illustration of the miRNA-138-5p binding site on the APOBEC3B 3′-UTR. (F) dHepaRG were left untreated (NT) or treated with 0.5 μg/ml of BS1 for 2 or 4 days (see schematic representation of the experiment in D). miRNAs were extracted and analysed by RT-qPCR. (G,H) HEK293T cells were co-transfected with luciferase-3′-UTR fusion constructs and either miR-138-5p-expressing plasmids or control miR-expressing plasmids. (G) Schematic representations of luciferase-3′-UTR fusions used. (H) Upper panel: schematic representation of the experiment. Lower panel: luciferase activity was assayed 48 h post transfection. Bars, respectively points, represent the mean ± SD of (F, H) 1 experiment, or (A) 3 experiments performed in triplicate. Data were submitted to (A, F, H) unpaired Student's t test. *p <0.05; **p <0.01; ***p <0.001; ****p <0.001. APOBEC3B, apolipoprotein B mRNA editing catalytic polypeptide-like A; n.s., not significant; UTR, untranslated transcribed region.

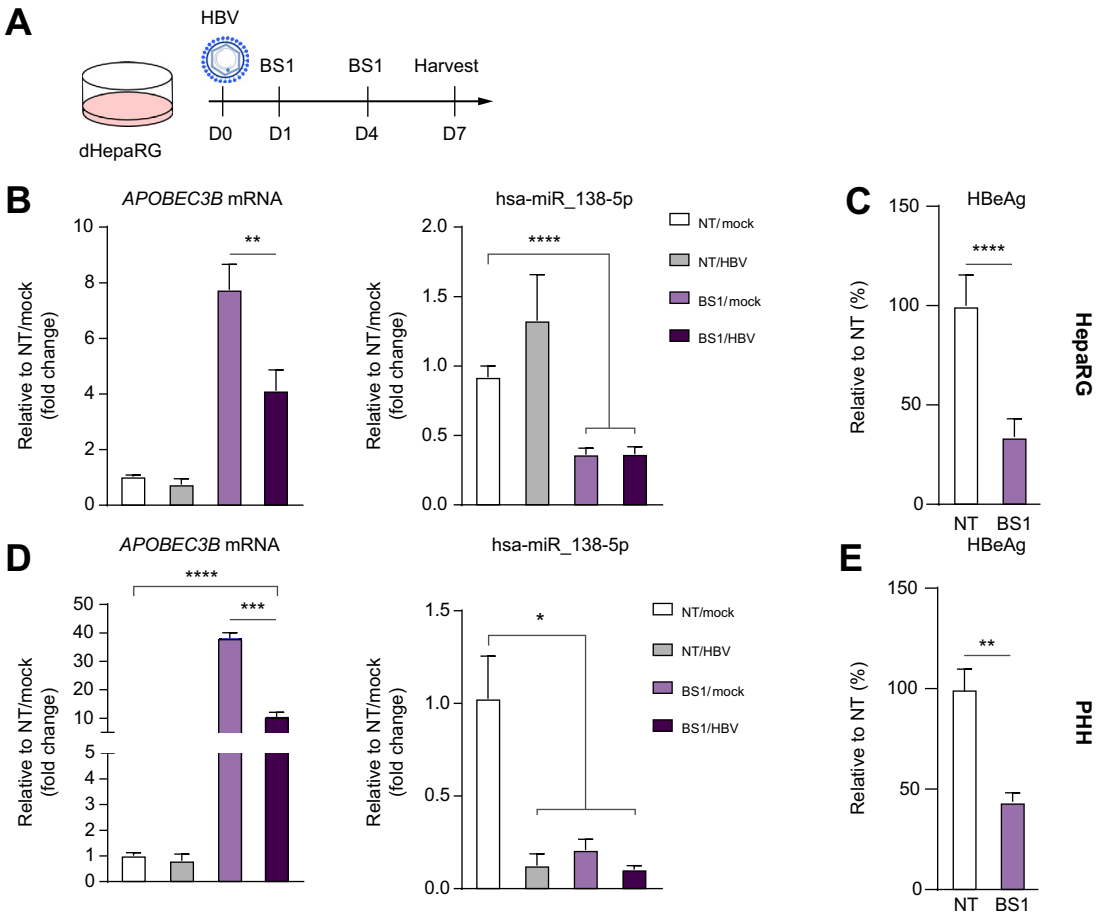

Fig. 4. HBV-mediated inhibition of APOBEC3B expression is not sufficient to prevent the antiviral effect. (A) Schematic representation of the experiment presented in panels B–E. (B,C) dHepaRG or (D,E) PHH were infected with HBV and left untreated (NT) or treated with 0.5 μg/ml of BS1 starting 1 d.p.i, for 6 days. (B,D) RNAs were isolated and analysed using RT-qPCR or qPCR. (C,E) Levels of HBeAg were detected in the cell culture supernatant via ELISA. Bars represent the mean ± SD of (D,E) 1 experiment, or (B,C) 3 independent experiments. Data were submitted to (B-E) unpaired Student's *t* test. **p* <0.05; ***p* <0.01; ****p* <0.001; *****p* <0.001. d.p.i., day post infection; n.s., not significant; PHH, primary human hepatocytes.

expression has been described to be associated with cancer development.[21] As we have previously described that A3B-induced cccDNA decay does not lead to rebounds of HBV infection *in vitro*,[2] we hypothesised that short-term A3B induction could be sufficient to ensure viral decay without affecting genomic DNA, in line with the observation that high A3B levels are present in acute, self-limiting HBV infection in patients.[4]

Whereas 12 days of BS1-treatment led to an ∼80% decrease of cccDNA (Figs. 3A,B and 5A), no significant mutational load was observed on a subset of genes related to cancer development, as analysed by targeted deep-sequencing of 766 genes associated with somatic mutations in tumours (*e.g.* tumour suppressors; oncogenes) (Table S4). Of 2,868 detected SNPs (compared with the human reference genome hg19), only 13 were shared by all BS1-treated cells, whereas 12 were also shared in non-treated cells above the cut-off level (Fig. 5B). Closer analyses of SNPs in the tri-nucleotide context revealed no significant differences in SNP frequencies between non-treated and BS1-treated cells (Fig. 5C–E).

Altogether, transient upregulation of A3B in hepatocytes is sufficient to eliminate cccDNA without inducing a detrimental mutational load to a subset of cancer-related genes *in vitro*.

The antiviral effects of APOBEC3B expression are independent of cccDNA transcriptional activity and can occur on double-stranded DNA

Finally, two of the suggested limitations of A3B-induced cccDNA decay are that: (i) transcriptionally inactive cccDNA (*i.e.* during occult infection), might escape deamination and lead to HBV relapses further on; (ii) like other members of the APOBEC3 family (*e.g.* A3G), A3B might act only on single-stranded DNA during HBV reverse transcription.[23,24]

X-protein deficient HBV (HBV ΔX) cccDNA has been shown to be transcriptionally inactive and have a condensed chromatin state.[25,26] To address if A3B can target transcriptionally inactive

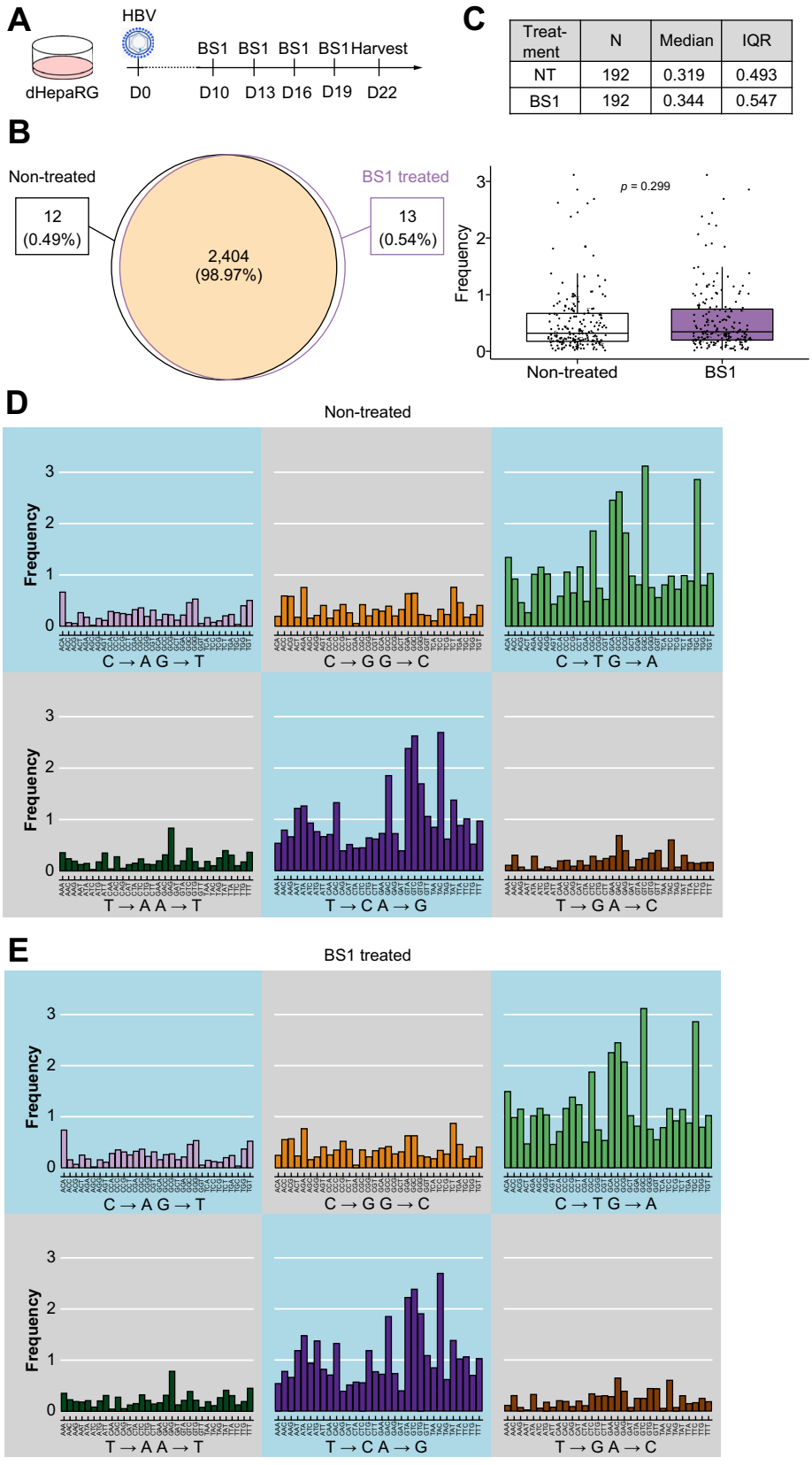

Fig. 5. APOBEC3B induction does not induce *de novo* mutations in a subset of genes related to cancer development. (A–E) dHepaRG were infected and 10 d.p.i. were left untreated (NT) or treated with 0.5 μg/ml BS1 for 12 days. DNA was extracted and subjected to panel sequencing of a panel containing 766 genes (CeGaT CancerPrecision panel). A total of 2,868 single nucleotide variants (SNVs) were detected. (A) Schematic representation of the experiment. (B) These SNVs were then filtered to identify SNVs, that occur in all samples with a number of alleles (NAF) >5% and a coverage >30 (2,404), only in all treated samples (13) and

cccDNA, dHepaRG were infected with HBV wild-type (wt) or HBV ΔX (Fig. 6A). In both HBV wt- and HBV ΔX-infected cells, a similar reduction of cccDNA levels was observed upon BS1-treatment (Fig. 6B). The decrease of cccDNA levels in BS1-treated HBV ΔX-infected dHepaRG cells was confirmed by Southern-blot analysis (Fig. 6C).

Moreover, we infected dHepaRG with a tRFP-NLS recombinant strain of HBV (tRFP-rHBV), in which the Pol/HBsAg ORF was disrupted by the insertion of a TTR promoter driving a tRFP-NLS reporter (*i.e.* there is no reverse transcription, and no produced relaxed circular DNA or rcDNA) (Fig. 6D). Infected cells were positive for RFP (Fig. 6E). BS1 treatment decreased the number RFP-positive cells (Fig. 6F), induced A3B (Fig. 6G), and reduced cccDNA levels (Fig. 6H), as well as pregenomic RNA (pgRNA) and RFP mRNA (Fig. 6I). As no rcDNA can be formed in these cells, the reduction of HBV DNA observed was specific of cccDNA.

Thus, A3B acted directly on inactive cccDNA in a reverse transcription-independent manner.

Discussion

APOBEC3B (A3B) has been proposed to be an antiviral enzyme, targeting a multitude of DNA viruses.[2,27,28] We have previously shown that induction of A3B by LTβR agonisation leads to non-cytolytic degradation of nuclear HBV cccDNA, enabling long-term inhibition of HBV-replication without rebound, even after treatment cessation.[2] These findings were also independently confirmed *in vivo* by T cell-mediated LTβR activation.[3]

Previous studies identified A3B as a NF-κB target gene in cancer cell lines.[29] Here, we describe that both NF-κB pathways (canonical and non-canonical) are involved in LTβR-induced A3B in non-transformed human hepatocytes (dHepaRG). *In silico* analysis identified 2 putative NF-κB binding sites in the proximal promoter of A3B. These sites were bound by NF-κB complexes in mobility shift and chromatin immunoprecipitation assays, as well as activated in luciferase assays. Chemical based-approaches combined with genetic loss of function of IKKβ and NIK further highlighted the involvement of both NF-κB pathways for A3B induction. A time course analysis of NF-κB (p52 and RelB) and polymerase II recruitment to the A3B promoter and the level of A3B transcript highlighted a post-transcriptional mechanism involving the hsa-miR-138-5p. Amongst the miRNAs previously identified to repress A3B *in silico*,[30] only hsa-miR-138-5p was detected in our miRNA analysis. An IKKβ- and NIK-dependent inverse correlation between the expression of hsa-miR-138-5p and A3B was observed in BS1-stimulated cells. These results suggest that NF-κB pathways regulate the expression of a repressor of hsa-miR-138-5p expression. Alternatively, generation of p52/p52 dimers could compete out transcriptionally active NF-κB dimers on the hsa-miR-138-5p promoter region.

The peculiar regulation of A3B might be a conserved evolutionary mechanism to avoid a detrimental A3B-mediated genome editing.[21] Several studies have demonstrated a link between hsa-miR-138-5p and tumour development suggesting a tumour suppressor activity for hsa-miR-138-5p.[31] Thus, it will be interesting to assess whether the hsa-miR-138-5p is downregulated in cancer harbouring an A3B signature or high A3B expression.

We observed that interfering with A3B transcriptionally or post-transcriptionally severely impaired BS1-mediated cccDNA decay. Thus, these findings raise important considerations concerning new therapeutic tools involving LTβR activation for the treatment of patients with CHB. As chronic inflammation and tumour development might develop with long-lasting BS1 treatment, a time-restricted administration (*e.g.* 4 weeks) would be mandatory. Indeed, we confirmed *in vitro* that 12 days BS1-treatment was sufficient to strongly decrease cccDNA levels without inducing mutations within a subset of cancer-related genes.

A repression of A3B was observed in infected cells, upon BS1 treatment and independently of hsa-miR-138-5p. We showed that HBV infection inhibited A3B transcription activation at the epigenetic level, as previously described for interferon β.[32] Thus, understanding the full repertoire of HBV-inhibitory mechanisms on hepatic immune responses might reveal promising targets to enable full A3B induction and other immune mediators. Understanding the HBV-mediated A3B expression, the mechanisms of downregulation of the hsa-miR-138-5p, and — as recently published — the inhibition of HIF1α stabilisation, could ensure effective immune-mediated control of the viral infection.[19]

Although A3B has been proposed to deaminate only ssDNA,[23,33] as described for A3A and A3G,[34] we have shown that an X-deficient HBV with a transcriptionally inactive cccDNA and a replication-deficient virus (*i.e.* no reverse transcription) were still susceptible to cccDNA degradation. These results ruled out that the antiviral effects are due to the editing of replicative intermediate of HBV, *i.e.* the relaxed circular DNA, in the cytoplasm, and the nuclear re-import of dysfunctional, mutated HBV genomes. Whether A3B can either induce unwinding of the cccDNA via yet to be described helicase activity, act on ssDNA that naturally occurs in a transcription-independent manner, or act on dsDNA, remains to be determined. Thus, we propose that A3B induction could possibly be used in the treatment of patients with poorly active cccDNA (*i.e.* inactive carrier), to eliminate the virus before any reactivation.

It will be also important to assess the effect of A3B on integrated HBV genomes, as it is a recurrent event which has been described to be involved in liver pathogenesis.[35] Moreover, as integrations risks increase over time, it is important to diagnose and treat the patients early on. Indeed, if patients are treated

only in all 'not treated' samples (12). Four hundred and thirty-nine SNVs were not found in all samples but they were not specific to either of the 2 groups. Inspection of the 13 and 12 genes showed that they have NAFs close to the cut-off of 5% but are detected in the other samples also. (C–E) SNVs in every possible trinucleotide context were analysed for their frequency. (C) Comparison of the frequency of SNVs between non-treated and BS1 treated samples. In the table, the median frequency and the IQR of SNVs are presented. In the box plot, every data point represents a SNV in a trinucleotide context. Data were submitted to the Wilcoxon-signed Rank Sum test. (D) Frequency for all SNVs in a trinucleotide context of non-treated samples. (E) Frequency for all SNVs in a trinucleotide context of non-treated samples. APOBEC3B, apolipoprotein B mRNA editing catalytic polypeptide-like A; d.p.i., days post infection.

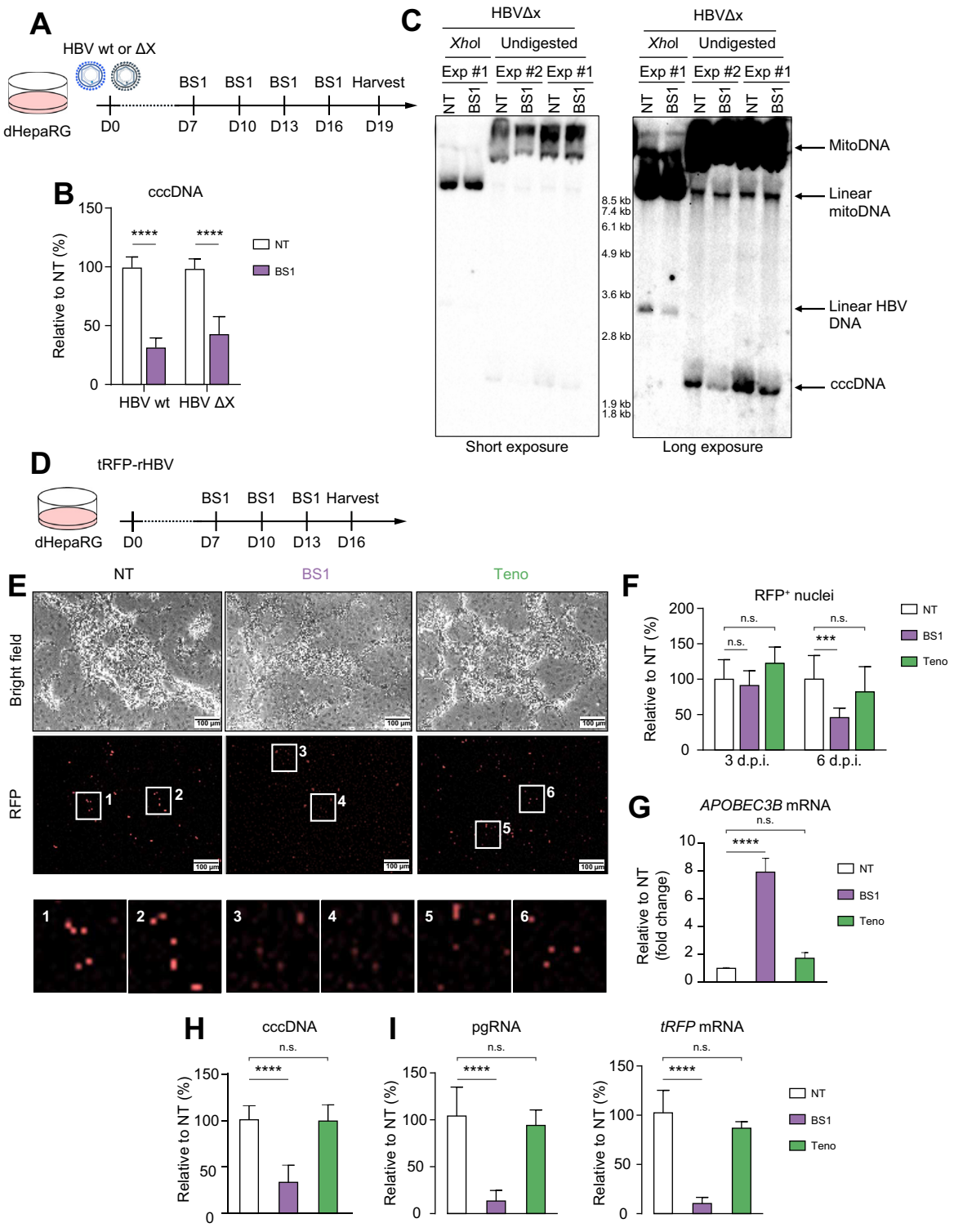

Fig. 6. APOBEC3B effect on double-stranded DNA is independent of transcription. (A–C) dHepaRG were infected with wild-type (wt) HBV or HBx deficient (ΔX) HBV. Seven d.p.i, cells were left untreated (NT) or treated with 0.5 μg/ml BS1 for 11 days. (A) Schematic representation of the experiment. (B,C) DNA were extracted and analysed using (B) qPCR and (C) Southern-blotting. (D–I) dHepaRG were infected with a recombinant tRFP-rHBV virus. Seven d.p.i., cells were left untreated (NT) or treated with 0.5 μg/ml BS1 or 0.5 μM of tenofovir for 9 days. (D) Schematic representation of the experiments presented in panels E–I. (E) Representative photos of bright field and fluorescent microscopy of the different treatments at 6 d.p.i. (F) Quantification of the number of RFP positive cells per view field. (G,I) RNA and (H) DNA were extracted and quantified by RT-qPCR and qPCR. Bars represent the mean ± SD of (F–I) 2 or (B) 4 independent experiments performed in triplicate. Data were submitted to (B) unpaired Student's *t* test or (F–I) 1-way ANOVA. ***p <0.001; ****p <0.001. APOBEC3B, apolipoprotein B mRNA editing catalytic polypeptide-like A; d.p.i., days post infection; n.s., not significant.

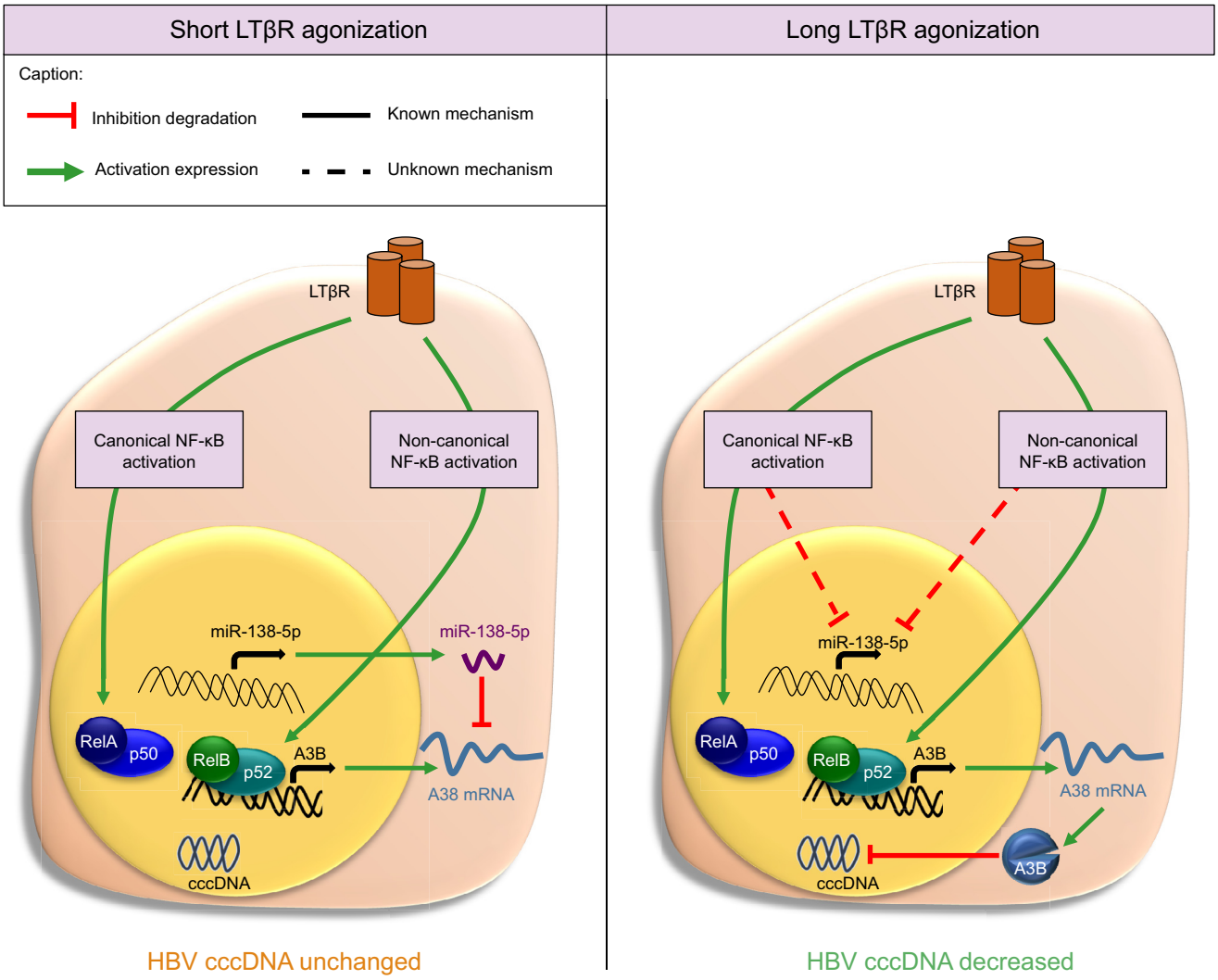

Fig. 7. APOBEC3B induction and subsequent cccDNA decay depend on NF-κB signalling and miR-138-5p decrease. Graphical representation of the main proposed mechanism(s). Upon short-time agonisation of the LTβR, NF-κB signalling induces weak APOBEC3B mRNA expression because of the inhibitory activity of miR-138-5p, thereby preventing cccDNA decay. Upon a prolonged agonisation of LTβR, the miR-138-5p levels is decreased allowing potent induction of APOBEC3B mRNA, and subsequently cccDNA decay that is independent of cccDNA transcriptional activity. APOBEC3B, apolipoprotein B mRNA editing catalytic polypeptide-like A; cccDNA, covalently closed circular DNA; NF-κB, nuclear factor kappa B.

before or soon after integrations, we could hypothesise that both the elimination of HBV cccDNA by A3B, and the natural renewal of hepatocytes within the liver, might lead to elimination of hepatocytes in which the HBV genome has been integrated.

In summary, we have shown that LTβR agonisation and activated NF-κB signalling pathways lead to APOBEC3B induction (Fig. 7). Moreover, hsa-miR-138-5p negatively regulates APOBEC3B expression and aberrant hsa-miR-138-5p expression inhibits A3B-mediated cccDNA decay, as measured by qPCR and Southern-blot analyses. We believe that blocking hsa-miR-138-5p expression or preventing hsa-miR-138-5p binding to A3B might represent a new therapeutic approach (*e.g.* in a combinatorial regiment with other treatments) that should be considered to ensure the full functionality of LTβR agonists-based treatments.

Abbreviations

A20, tumour necrosis factor alpha-induced protein 3; APOBEC3A/A3A, apolipoprotein B mRNA editing catalytic polypeptide-like A; APOBEC3B/A3B, apolipoprotein B mRNA editing catalytic polypeptide-like B; APOBEC3G/A3G, apolipoprotein B mRNA editing catalytic polypeptide-like G; BCA, bicinchoninic acid assay; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; ChIP, chromatin immune precipitation; CXCL10, C-X-C motif chemokine ligand 10; d.p.i., days post infection; EMSA, electrophoretic mobility-shift assay; H3K4Me3, histone 3 lysine 4 trimethylation; IFNα/γ, interferon alpha/gamma; IKKα/β, IκB kinase alpha/beta; JMJD8, jumonji domain containing 8; LPS, lipopolysaccharide; LTβR,

lymphotoxin beta receptor; MAPK, mitogen-activated protein kinase; miRNA, micro RNA; NEMO, NF-κB essential modulator; NF-κB, nuclear factor kappa B; NIK, NF-κB inducing kinase; NT, non-treated; RelA, NF-κB p65 subunit; RT-qPCR, reverse transcription-quantitative PCR; siCTRL, siRNA control; TNF, tumour necrosis factor; UBE2V1, ubiquitin conjugating enzyme E2 V1; UTR, untranslated region.

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Conflicts of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceptualisation: SFD, TR, MR, FR, DD, JL, ED, MH. Methodology: SFD, TR, MR, FR, DD, JL, ED, MH. Formal analysis: SFD, TR, MR, ZH. Investigation: SFD, TR, MR, ZH, FR, KN, SS, CR, NG, MS, MSt, JW, RB, RP, LCS, RF, SP, CL, RO, TE, KR, RR, KU, DT, JL, DBL. Resources: KU, UP, DD, JL, ED, MH. Data curation: SFD, TR, MR, ZH. Writing-original draft: SFD, TR, DD, JL, ED, MH. Visualisation: SFD, TR, MR, ED, MH. Supervision: ED, MH. Project administration: ED, MH. Funding acquisition: DD, JL, ED, MH.

Data availability statement

The data that support the findings of this study are available from the corresponding author, MH, upon reasonable request.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2021.100354.

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Author names in bold designate shared co-first authorship

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