23 February 2021

Dr. Lian-Sheng Ma
Founder and Chief Executive Officer
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Dear Dr. Ma,

Re: Invitation to submit revised manuscript (ID: 62926)

Thank you for the conditional acceptance of our manuscript as a review in *World Journal of Gastroenterology*.

We have addressed all the comments from the 4 reviewers and are thankful for their rigorous evaluation of our manuscript. Please find enclosed the point-by-point response to the reviewer's questions. Their comments had been useful in improving our manuscript, which incorporates all their suggestions.

Of note, we have changed the manuscript title to "Intracellular interferon signalling pathways as potential regulators of cccDNA in the treatment of chronic hepatitis B" in keeping with the 18-word limit, and to better reflect its content as had been advised.

We hope you would approve this revised manuscript favourably.

Thank you.
Sincerely,

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Response to Reviewer #1:

As the authors discuss, intrahepatic HBV cccDNA is the key replicative intermediate driving HBV replication. Nucleos(t)ide analogue (NA)-based therapy is the mainstay of treatment for chronic hepatitis B but can only indirectly affect the pre-existing pool of HBV cccDNA, as HBV does not replicate using a semi-conservative mechanism. The inability to eradicate HBV cccDNA is the reason for failure of viral clearance and for relapse if antiviral therapy is discontinued. A full understanding of the molecular mechanisms regulating HBV cccDNA should reveal targets for therapeutic intervention. The authors have constructed an excellent overview discussing these aspects, with an emphasis on the interferon response. To review the role of this pivotal HBV molecule and the strategies to suppress or eliminate cccDNA is a difficult task but the authors have managed to provide a comprehensive summation without being drawn into unnecessary meticulous detail. I could find no major flaws in the review, nevertheless, I do have some suggestions which I believe will enhance the manuscript.

Minor editing recommendations:

1. P4. "However, NAs do not target cccDNA well and therefore HBV re-activation persist." This would read better as "However, NAs do not directly target cccDNA and therefore HBV re-activation persists."

> ***Response:*** We thank the reviewer for pointing this out and have made the corresponding recommended edit in P4.

2. P5. "druggable targets". I'm not sure there is such a word as druggable. It is preferable to simply have "targets". The abbreviation rcDNA is used – rc should be introduced (relaxed circular). "where all HBV transcripts are transcribed" should be "from which all HBV transcripts are transcribed".

> ***Response:*** We agree with the reviewer's comments and have made the recommended changes to P5. The word "druggable" has been removed; rcDNA is introduced as "<u>double-stranded rcDNA (relaxed circular DNA)</u>"; the statement "has been changed to

"cccDNA contains 4 overlapping open reading frames (ORFs) from which all HBV transcripts are transcribed".

3. P6. HBs seroconversion needs to be expanded.

Response: We thank the reviewer for pointing this out and have expanded on "HBs seroconversion". The statement in P6 now reads: "cccDNA is found in every phase of the natural course of HBV infection, even in patients who underwent HBs seroconversion to produce protective anti-HBs antibodies after effective antiviral treatment. Seroconversion or the loss of HBs is an important end goal of HBV therapy as it is associated with positive long-term clinical outcomes such as improvement in liver function and reducing the risk of HCC[33]."

4. P9. The abbreviation for ISGs is introduced here but was previously introduced on P4.

Response: We thank the reviewer for this observation and have removed the repeated introduction of the abbreviation for ISGs in this revision. The sub-heading in this revision in P11 has been changed from "Activation of interferon-stimulated genes (ISGs)" to "Activation of ISGs", and the statement "The antiviral effects of IFNs are achieved through IFN-stimulated genes (ISGs),…" changed to "The antiviral effects of IFNs are achieved through ISGs[46,68],…"

5. P11. "These studies indicate that A3A and A3B are one of the key proteins responsible for IFN and LTβR mediated cccDNA clearance." A3A and A3B are two proteins, not one. I suggest: "A3A and A3B are key proteins responsible for IFN and LTβR mediated cccDNA clearance."

Response: We have amended the statement in P13 of this revision according to the reviewer's recommendation.

6. P16. "Of note, as HBV disruption of IFN response is multi-factorial and affects multiple factors," should be: "Of note, as HBV disruption of IFN response is multi-factorial,".

Response: We thank the reviewer for the comment and have deleted the words "and affects multiple factors" in the mentioned statement in accordance to recommendation in P18 of this revision.

7. P23. In reference 27, the authors are designated using upper case letters.

Response: We thank the reviewer for this observation. This reference has been removed in this revision.

Reviewer suggestions:

1. P6. In the section referring to "cccDNA copy number and persistence", it may be worthwhile pointing out that there is no International Standard for HBV cccDNA or universally endorsed HBV cccDNA assay, hence copy numbers cited in publications reflect the assay used making direct comparisons difficult.

Response: We agree with the reviewer and have included this information in the section of "cccDNA copy number and persistence" in P6.

P6: "cccDNA is found in every phase of the natural course of HBV infection, even in patients who underwent HBs seroconversion to produce anti-HBs antibodies after effective antiviral treatment. Seroconversion or the loss of HBs is an important end goal of HBV therapy as it is associated with positive long-term clinical outcomes such as improvement in liver function and reducing the risk of HCC[33]. Surprisingly, there is currently no international standard for HBV cccDNA, and a universally endorsed HBV cccDNA assay is also lacking. Thus, the kinetics and amount of cccDNA in infected cells are not clearly defined as the cccDNA copy numbers reported merely reflect the assay used in a publication and does not facilitate comparison between studies. Cell culture models suggest that cccDNA persists up to 40 days ...."

2. P7. Similarly, under "Nucleoside/nucleotide analogues" it could include the results of studies on the cessation of long term NA therapy in selected subsets of patients. Patients generally experience a virological and biochemical reactivation and a proportion undergo HB surface antigen (HBsAg) loss.

Response: We thank the reviewer for this good suggestion. We have improved the manuscript by adding in the new Table 1 which summarizes information from multiple clinical datasets, showing the proportion of patients with virological and biochemical reactivation, as well as proportion of patients who lose HBs after cessation of long-term NA therapy. The corresponding description of this is also included in the section "Nucleoside/nucleotide analogues" in P7.

P7: "By suppressing HBV load, NAs can alleviate HBV-associated liver diseases, regress fibrosis[39] and reduce the risk of developing HCC[40]. However, NAs cannot cure HBV infection as loss of virological markers such as HBs is rarely achieved, and seroconversion rates are negligible (Table 1). As a result, HBV re-activation rate is high, with >50% patients showing flares in virological markers (e.g. HBV DNA) and biochemical markers for liver damage (e.g. ALT, alanine aminotransferase). This is often accompanied with irreversible liver decompensation, resulting in death even when re-introduced to lamivudine[41]. Thus, to avoid HBV re-activation, CHB patients are often put on long-term (often >10 years) or even life-long NA therapy.''

3. P8. In the section on "Interferons", measurement of viral markers, such as HBV genotype and quantitative HBsAg, can provide an indication of outcome to interferon therapy and this could be mentioned.

Response: We thank the reviewer for pointing this out and have included relevant information about this in a new paragraph in the section "Interferons" in P9-P10. We have also included a statement about how differences in ISRE sequences between HBV genotypes affect ISRE functionality hence patient sensitivity towards IFN treatment in the section "Epigenetic Silencing and Transcriptional Repression" in P16.

P9-P10: "…, rendering combination therapy efficacious only in selected patients[59,60] but redundant in others[61]. Multiple factors including type of combination therapy, HBV genotype and level of HBV replication greatly affect IFN treatment efficacy[62]. Some

studies suggest that sequential NA and IFN therapy is more effective than simultaneous combination therapy[63], a phenomenon which warrants further confirmatory clinical investigation to enhance clinical response rates. HBV itself also significantly affects patient response to IFN therapy, as treatment outcomes are more efficacious in patients carrying the genotype A virus than the genotype D virus[64], and patients are also thrice more responsive to IFN treatment if they carry the genotype B virus than genotype C virus[65]. As further proof to the extent in which HBV alters patient sensitivity to IFN treatment, 30.4% of patients with low end-of-treatment HBs levels (<10 IU/mL) achieve HBs clearance in a 5-year follow-up, in stark contrast to <10% of patients achieving HBs clearance[66] when end-of-treatment HBs levels are ≥10 IU/mL. This is further supported by the association of greater PEG-IFN response in patients with HBV DNA levels of <9 log10 copies/mL sera[67]. Clearly, the full potential of IFN therapy has yet to be harnessed and directed towards HBV and its cccDNA."

P16: "This is clinically significant, as mutations in the HBV ISRE affects CHB patient response to IFN treatment[95] to render IFN treatment less effective. In addition, the HBV ISRE sequence is HBV genotype dependent, thus its sequence-dependent functionality partially accounts for differences in patient responder rates between carriers of HBV genotypes B and C[99]."

4. P16. Another potential strategy is to target the cccDNA molecule itself using the CRISPR/Cas9 system. There are several reports in the literature examining the potential of this technology to silence HBV cccDNA.

Response: This is a good suggestion. We have modified Figure 3 to include the CRISPR/Cas9 system in targeting cccDNA, and have written a new paragraph in the revised section "Targeted degradation of HBV products" in P20-21 to describe the studies involving this. We have also edited the conclusion in P23 to include the use of CRISPR/Cas9 system as a novel strategy against HBV.

P20-P21: "To avoid drug-induced toxicity, approaches that directly target HBV products (cccDNA, RNA and proteins) are being developed. One such approach is to directly degrade cccDNA using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats)/CRISPR-associated protein 9) DNA editing machinery, where guide RNA-directed gene editing of cccDNA specifically results in its erroneous repair by non-

homologous end-joining (NHEJ) which cleaves and/or mutate cccDNA. This has been shown to effectively reduce cccDNA copies and is followed by concomitant clearance of HBs, HBc and HBe expression in mice with humanized liver[115,116] and multiple human liver cell lines[117,118]. While the cccDNA-targeting system is being developed and improved for clinical use, a major concern for this strategy is the generation of off-target double-strand breaks in the host genome that may facilitate HBV genomic integration to cause liver cancer. A suggested solution for this is to tether impaired Cas9 (dCas9) to APOBECs so that cccDNA may be mutated by base-changes instead of strand-breaks[119], highlighting again the importance of APOBECs in the role of targeted cccDNA degradation."

P23: "These drugs may be used together with current HBV therapies, new anti-HBV drugs in the pipelines such as cell-penetrating antibodies, cccDNA-targeting CRISPR/Cas9 system and even natural compounds that directly target HBV proteins that disrupt IFN signalling for degradation."

5. General. Several strategies designed to target the HBV cccDNA minichromosome will also have an effect on the host cell chromosome and this potential for toxicity should also be touched on.

Response: The reviewer refers to the use of epidrugs that target the epigenetic modifications of HBV cccDNA mini chromosome to result in silencing. In this revised manuscript, we have elaborated on the potential for toxicity in the use of such drugs in P20 of the "Epidrugs" section.

P20: "…, other unexplored epidrugs that inhibit the transcription activation group or activate the transcription repression and silencing group may also be tested for efficacy in directly silencing cccDNA. While epidrugs directly silence cccDNA *in vitro*, their greatest challenge in utility as an anti-HBV therapeutic depends on their ability to specifically target cccDNA in infected cells while sparing host genome to avoid carcinogenesis and toxicity[112,113]. In addition, due to compromised functionality of HBV-infected livers, many drugs that have safe profiles in the treatment of non-liver diseases are contraindicated in CHB. For example, the FDA-approved DNMT inhibitor, 5-azacytidine commonly used in the treatment of hematologic malignancies is contraindicated in patients with advanced liver cancer to control HBV infection due to

hepatotoxicity[114]. Thus, epidrugs need to be carefully evaluated for safety and suitability in the treatment of HBV infections.

Response to Reviewer #2:

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1. Some acute HBV could achieve a complete remission while others turned into chronic HBV. The link of these therapeutic outcomes with cccDNA and the immune microenvironment should be discussed. The levels of local concentration of interferon-gamma was not mentioned throughout the manuscript. Would that be associated with immune exhaustion?

Response: We agree with the reviewer that the immune microenvironment plays an important role in therapeutic outcomes in the treatment of HBV infection, and have added a new paragraph on the immune environment in relation to immune exhaustion and IFN-γ levels the "Interferons" section in P8-P9 of this revised manuscript to discuss this. We have also modified the abstract in P2 to reflect the importance of the immune environment in HBV clearance.

P2: "They have been shown to induce cccDNA clearance, but their use in the treatment of HBV infection is limited as HBV-targeting immune cells are exhausted and HBV has devised multiple mechanisms to evade and suppress IFN signalling."

P8-P9: "However, the use of IFNs in the clinical setting is limited due to the need for high dosage and unpredictably variable patient response, which depends on the status of immune cells, HBV titre and type of HBV. It is also less tolerated in patients due to pleiotropic off-target effects from IFN signalling.

At the tissue level, immune tolerance from chronic infection significantly reduces clinical efficacy of IFN treatment. The extracellular arm of the IFN-mediated antiviral response depends on the activation of HBV-specific $CD4^+$ helper T-cells and $CD8^+$ cytotoxic T-cells to produce cytokines such as IFN-γ and TNF-α (tumour necrosis factor-alpha) that lead to the elimination of HBV-infected cells[50]. However, constant exposure to HBV leads to T-cell exhaustion and an immunotolerant environment[51]. While IFN-γ also induces intracellular antiviral properties, its continued elevation upregulates PD-1 (programmed death-1) immune checkpoint protein on T-cells and also induces its ligand PD-L1 (programmed death-ligand 1) on hepatocytes, leading to immune tolerance as HBV-specific T-cells fail to act on infected cells[52,53]. IFN-γ also promotes the secretion chemokines from hepatic macrophages that retain $CD4^+$ T-cells in the liver and induce apoptosis of HBV-specific T-cells, further contributing to HBV evasion of immune clearance. Many other mechanisms of how HBV-specific immune cells' antiviral activities

have been augmented in chronic HBV infection have been documented and reviewed[54,56], together showing very clearly that IFN treatment alone cannot induce effective clearance of HBV, allowing HBV to persist. Indeed, strategies aimed at restoring extracellular anti-HBV immunity in CHB patients such as with anti-PD1 therapeutics[57] and adoptive T-cell therapy[58] are being investigated. Since immune tolerance in CHB patients render immune cells non-responsive to IFN therapy, the efficacy of IFN therapy lies heavily on the intracellular arm of IFN-mediated immunity brought about by induction of intracellular antiviral proteins from IFN signaling.

2. The extent (or percentage) of the decrease in the interferon signaling as a result of HBV should be discussed. Which step is considered as the rate determining step in the interferon signaling inhibition?

Response: We thank the reviewer for the useful concept about "rate determining step" in interferon signaling inhibition, and have incorporated this into the revised manuscript. In the section "HBV disruption of interferon signaling" on P17-P18, we have included studies which show the percentage loss of ISG expression as a consequence of increased SOCS2 and SOCS3 expression brought about by HBe and HBx. We also suggest in the section "Inducers of APOBECs" that the use of SOCs inhibitors is most likely to strengthen IFN-signaling, and have added a statement in P19 about the use of SOCs inhibitors as being most likely to strengthen IFN-signaling.

P17-P18: "As SOCS2 also prevents STAT1 phosphorylation, STAT1 is prevented from entering the nucleus to induce ISG expression. Since SOCS2 and SOCS3 are the most direct upstream inhibitors of the IFN signaling pathway, their increased expression significantly attenuates IFN-dependent transcription of ISGs to compromise the therapeutic efficacy of IFNs. It was found that overexpression of HBe alone leads to 6-fold reduction in phosphorylated STAT1 nuclear translocation, hence downregulating PKR (protein kinase R) and OAS (oligoadenylate synthetase) gene expression by 50%[20]. Similarly, HBx overexpression alone doubled SOCS3 expression and increased PP2A expression by 5-fold, significantly reducing PKR and OAS expression by 3-fold[101]. It is thus clear to see, that the combined effects of expressing HBx and HBe in infected cells, especially in cells with high viral titre and virological markers, will compromise the clinical efficacy of direct IFN therapy. Other HBV proteins act further down the IFN

signalling cascade to prevent STAT1 nuclear translocation, further disrupting ISG expression."

P19: "In addition, the IFN response may also be strengthened by reducing the action of negative regulators of the IFN signalling pathway, primarily by acting on SOCS2 or SOCS3 and PP2A. This directly inhibits the degradation of IFN signaling, allowing ISGF3 and GAF complexes to form hence carry out transcription of ISGs."

Response to Reviewer #3:

This manuscript provides a comprehensive review of the difficulties and recent progress in the process of HBV viral therapy, especially the mechanism by which HBV produces interferon resistance, which is of great value for a comprehensive understanding of the progress of interferon therapy for HBV and further basic research. To improve the quality of the manuscript, it can be partially modified from:

(1) The manuscript reviewed the content of HBV versus and NAs, the title can't represent the content of the article well

> ***Response:*** We thank the reviewer for the comment and have amended the manuscript title to reflect its content more accurately. The new title "Intracellular interferon signalling pathways as potential regulators of cccDNA in the treatment of chronic hepatitis B" states explicitly that the manuscript describes the intracellular mechanisms of IFN signaling which regulate cccDNA levels, and how these mechanisms may be exploited for the potential treatment of HBV infections. Even though we have included a section on "Nucleoside/nucleotide analogues" (NAs), this is not the focus of our review. Instead, the description of the flaws in NA therapy was necessary to orientate readers on the need for non-NA therapeutics in the treatment of HBV infections, for which our review suggests that enhancing intracellular IFN signaling is one such alternative approach.

(2) Increasing the number of references in the last 5 years, reviewing more recent advances

> ***Response:*** We thank the reviewer for the comment, and have improved the manuscript by citing more work from references in the last 5 years. In this revision, there are a total of 160 references. Amongst which, 71 of them are published within the past 5 years from 2016-2021. We have included the latest research and review articles, with 17 references published in 2020-2021.

(3) Add a paragraph to review the progress of clinical research on interferon therapy for HBV.

Response: The reviewer has made a good suggestion. We have improved the manuscript accordingly to include a new Table 3, which summarizes recent clinical research on interferon therapy for HBV. We have also added a new section "Clinical updates on the use of interferons" in P22. Recent clinical studies on the use of other IFN subtypes such as IFN-λ3 have also been included in P19, in the section "Inducers of APOBECs".

P19: "… including activation of IFN signalling using other IFN sub-types such as IFN-γ and IFN-λ, and alternative pathways involving TNF-α and LTβR activation. IFN-λ3 was found to be specifically upregulated in patients treated with adefovir or tenofovir, and further shown in cell culture models to be effective in reducing HBs by inducing ISGs[106]. Studies in HepaRG differentiated hepatocytes also show that IFN-β, IFN-λ1 and IFN-λ2 induce longer-lasting APOBEC expression than IFN-α2, and are just as efficient in mediating cccDNA degradation[107]. IFN-γ and TNF-α have also been shown to upregulate APOBEC expression. In particular, the utility of IFN-γ in the treatment of HBV infections can be explored as it is currently used clinically for the treatment of hepatic fibrosis[108]. An LTβR agonist antibody has already been developed and was shown to be safe in mice at lower dosing requirements than clinical IFN-α[15]."

P22: "Neither NAs nor IFNs alone can achieve effective HBV elimination in majority of HBV carriers. However, their mechanisms of action complement one another to potentially achieve viral elimination. NAs are effective in suppressing viral titre in most patients, allowing IFNs to effectively mount a cellular immune response in a less immunotolerant environment when HBV titre is decreased, and concurrently allow IFN-mediated intracellular antiviral mechanisms to effectively act against HBV and its cccDNA with less antagonistic effects from decreased HBV titre. As such, combination therapy is increasingly explored, with many experimenting the types of combinations that can be administered (Table 3). Combinations explored include NA monotherapy followed by IFN monotherapy or vice versa, periods of monotherapy followed by periods of IFN and NA co-administration, or co-therapy followed by monotherapy. Interestingly, the efficacies of combination therapy differ greatly. When NA monotherapy is switched to IFN monotherapy, higher rates of HBe and HBs seroconversion are observed together with lower relapse rates. Simultaneous administration of NAs and IFNs for more than 24 weeks, followed by sustained NA

treatment gives very high HBe seroconversion rate of 50%, accompanied by the loss of HBs expression in 16% patients. This is a remarkable feat considering the loss of HBs is usually less than 5% with NA or IFN monotherapy (Table 1) In contrast, multiple reports show that simultaneous administration of NAs and IFN yield conflicting results, with many studies showing little benefit from adding IFNs into the regime of NA treatment[129,130]. Further large-scale clinical studies are needed to ascertain the differences in these findings."

Response to Reviewer #4:

This review manuscript has summarized the therapeutical methods for HBV infections clinically.

1. Although IFN signaling is mentioned by authors specially to be the better candidate for curing HBV compared to other ways such as NAs or drugs, their basement lacks enough supporting in this manuscript using review. Authors can select research or mini-review tools to declare this point with appropriate and convincing ways.

Response: We thank the reviewer for this comment and we do not mean that IFN pathways are confirmed to be better than other ways. This review gathers statistics from multiple recent clinical studies in the new Table 1 and Table 3. Table 1 summarizes the statistics from 9 different clinical studies on NA monotherapy and IFN or PEG-IFN monotherapy, and studies that directly compare between the 2 treatment options show that a greater proportion of patients treated with IFNs or PEG-IFNs alone lose HBe and rcDNA than patients treated with NAs alone. In addition, a higher proportion of patients treated with IFNs or PEG-IFN achieve normal ALT. Therefore, IFN therapy is not inferior to NA therapy. We have explained this in the text in P8. Table 3 summarizes the new approach of combination therapy, and shows that the addition of IFN into the treatment regimen has additional benefits over NA monotherapy, where significantly higher proportions of patients achieve loss of HBs, HBe and even seroconvert to acquire protective anti-HBs antibodies, a feat rarely achieved with NA monotherapy. **We hope that data presented in Table 1 and Table 3 suggest the possibility that IFN therapy has its benefits and the study and development of drugs based on its underlying antiviral mechanisms against cccDNA may be useful for eliminating HBV, which is the main message for the review.**

P8: "The use of PEG-IFN has superseded standard IFN-α as pegylation improves IFN-α half-life, requiring less frequent dosing[47]. More importantly, PEG-IFN-α is more effective in reducing cccDNA levels and also leads to greater rates of ALT normalization (Table1). When compared to NAs, treatment with IFN and PEG-IFN leads to higher rates of HBe and HBs seroconversion with greater reduction in HBV markers indicative of lower HBV replication rates. Of note, HBs seroconversion is rarely achieved with the use of NAs. Recent clinical studies[48,49] have also confirmed that switching from NA therapy

to IFN therapy sustains more significant HBe and HBs losses for longer periods, demonstrating the potency of IFNs in the suppression of HBV replication. However, the use of IFNs in the clinical setting is limited due to…"

2. In addition, this manuscript has utilized over-citations including one reference with several times (reference 5, 15, 16, 17, etc) and several references in one place (21-25, 49-54, etc) without different explanations.

Response: We thank the reviewer for pointing this out and have re-organized the references such that specific references are not utilized several times, and only a maximum of 3 references may be found in one place.

9 March 2021

Dr. Lian-Sheng Ma
Founder and Chief Executive Officer
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Dear Dr. Ma,

Re: Response to additional comments for manuscript no. 62926

Thank you for the additional comment to our manuscript "Intracellular interferon signalling pathways as potential regulators of cccDNA in the treatment of chronic hepatitis B" in *World Journal of Gastroenterology*.

All our figures are original. They are designed and meticulously illustrated by us, solely for the purpose of publication in WJG !

The editable figures had been submitted into the F6 portal as "62926-Image-File-revision.pptx". Hence, we have not provided any documents for re-publication as this is not applicable.

As requested, we have prepared and uploaded the audio core tip file '62926-Audio-Coretip' through the F6 portal. Please let us know if it is of suitable quality.

Finally, we have replied to the comments uploaded by the science editor through the F6 portal.

We would like to bring to your attention the reviewer has made several erroneous criticisms. The reviewer has clearly skipped several lines of texts, misread and misinterpreted our message. In our response, it is clear to see that **our manuscript is in fact aligned with the reviewer's thoughts, and does not conflict with the reviewer's idea of an improved manuscript.**

We re-iterate and emphasize that the review:

- does not state that interferons are the only means for HBV therapy, as we have summarized other treatment options such as the current use of nucleoside/nucleotide analogues (Pages 7-8), and novel strategies such as CRISPR/Cas9 mediated targeted destruction of cccDNA and intracellular antibodies to target HBV proteins (Pages 19-22).
- We also did not suggest that interferons are "specially" used for the treatment of HBV infections alone, and in fact, agree with the reviewer that interferons have many immune functions (page 8 and 10) and are also used to treat many other viral infections explicitly stated in page 4.

Hence we are totally confused by the way the reviewer has approached this manuscript. All other reviewers are happy with the review except for this particular reviewer. We have responded as best as we can in an unbiased way.

It is important to point out that this is a review, and our purpose is to capture and summarize published peer-reviewed data. The text is totally guided by currently basic and clinical data only, and we have no intention to advance any biased views.

The collection of published data tells us what is known about how interferons and its downstream signalling pathways affect HBV replication by regulating cccDNA structure and function, and how these pathways may be enhanced to potentially achieve the therapeutic outcome of losing cccDNA function or expression.

Our new title, "Intracellular interferon signalling pathways as potential regulators of cccDNA in the treatment of chronic hepatitis B" is thus **not in conflict with the reviewer's comment.** Our title is in line with the reviewer's comments, as it implies that it is not interferons *per se* but the downstream effectors, for which there are too many to list, that have potential in regulating HBV replication by acting on cccDNA.

Nevertheless, we thank the reviewer for taking time to read our revised manuscript. We have edited the manuscript according to the reviewer's comment and enhanced its clarity by adding back "missing citations" that had been removed during the previous revision in response to the reviewer's earlier comment on "over-citations".

We have taken the reviewer's comments seriously and hope that you agree that our manuscript does not conflict with the reviewer's suggestions for improvement.
We sincerely hope that you will consider our review favourably.

Thank you.
Sincerely,

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Response to additional comment

1. The revision by author did not basically correct their demonstrations.

Response: We have made major revisions to our original manuscript, and addressed all issues raised by every reviewer. It is therefore unclear what further "demonstrations" need to be corrected.

2. IFN is a general but not special immuno- modulators to treat HBV clinically.

Response: We agree with the reviewer, and in fact **have stated this fact explicitly in our text.** We have introduced interferons to have many roles in immunity in page 8 and that it can be used to treat many types of viral infections in page 4. The immune roles of interferons were re-iterated again in page 10.

P4: "IFNs bind to their cognate receptors to elicit an intracellular signalling cascade that activates a set of interferon-stimulated genes (ISGs) with antiviral, immunomodulatory and anti-proliferative functions[12]. As such, IFNs are often used to treat viral infections against a range of viruses including HBV[13], hepatitis C virus[14], and West Nile virus[15]"

P8: "IFN-α and PEG-IFN are immunomodulators that augment cell-mediated immunity, part of which includes intracellular antiviral activities that can be executed without the aid of immune cells[46]..... "

P10: "Interferons are key mediators of immunity, comprising a group of cytokines with antiviral properties against a wide range of pathogens."

3. The potential target factors of IFN influenced must be mentioned in the title rather than considered IFN as the regulator according to authors' statements.

Response: We thank the reviewer for the suggestion, which is not in conflict with our title "Intracellular interferon signalling pathways as potential regulators of cccDNA in the treatment of chronic hepatitis B". In agreement with the reviewer's comment, our title suggests that signaling from interferons results in the activation and recruitment of factors that would be efficacious in suppressing cccDNA function or reducing cccDNA levels. We are unable to state all the factors in the title, for which there are too many (APOBEC3A, APOBEC3B, IRF9, ISG20, IFI16, STAT1, STAT2, ISGF3, GAF, p300/CBP, Set1A, GCN5, DNMT3A, PRC2 complex, HDAC1, SIRT1 etc) as the word limit for the title is 18 words.

4. And in the epigenetic regulation of cccDNA, the role of IFN is not definite.

Response: We have cited several papers (Refs 10, 84, 85, 90, 91) showing that cccDNA is epigenetically modified under the influence of interferons and its induced gene targets.

5. As mentioned by author to describe that "In studies using cultured cells and HBV-infected chimeric uPA/SCID mice repopulated with PHH, IFN-α reduced trimethylation of H3K4 and acetylation of H3K27 and H3K122 on cccDNA chromatin to inhibit transcription. The dependence on IFN-α was abrogated when the p300/CBP histone acetyltransferase (HAT) complex for H3K27 and H3K122 was specifically inhibited with the HAT inhibitor C646 in a dose-dependent manner." etc, what is the supporting reference? Authors should use proper and cautious expression in the related place of manuscript.

Response: We thank the reviewer for pointing this out, and have removed the statement "The dependence on IFN-α was abrogated when the p300/CBP histone acetyltransferase (HAT) complex for H3K27 and H3K122 was specifically inhibited with the HAT inhibitor C646 in a dose-dependent manner." in P14. We have edited the remaining statement to accurately reflect the citation which demonstrated that the addition of IFN-α specifically modifies epigenetic PTMs on cccDNA but not on the control host promoters for ACTB and Nanog.

P14: "In studies using PHH, IFN-α specifically reduced trimethylation of H3K4 and acetylation of H3K27 and H3K122 on cccDNA chromatin to inhibit transcription of HBV RNA, but had negligible effect on epigenetic modification for the control promoters of ACTB and Nanog in the host genome[10]."