Dear Editor,

Deposited in your online submission system, please find our revised manuscript entitled "**ANTI-VIRAL ROLE OF TLR4 IN HEPATITIS B VIRUS (HBV) INFECTION: AN INVITRO STUDY"** accompanied by a point-by-point response to reviewers' comments. We appreciate the helpful comments and insightful suggestions of the Reviewers that have been incorporated in the revised version of our manuscript. We included additional data, and provided clarification on other critical points. We also verified that our digital images and text passed the requirements for publication using the Page length estimate tool. We hope that you will now find the revised manuscript satisfactory and suitable for publication in World journal of Gastroenterology.

Many thanks for handling this submission.

Sincerely,

Runu Chakravarty

Reviewer 1:

Major comments –

1. In material and methods, the section of RT-PCR should be written with more detail: in which samples were performed the experiments? Which primers were used in the study? How was performed the quantification?

Total RNA was extracted from liver biopsy specimens for the relative p53 expression. For the RNA expression from HepG2 and HepG2.2.15 cells, $1x10^6$ cells were plated in 6-well plate for the different experiments. For the mRNA expression of TLR4, different cell cycle regulators and the different TLR4 signaling molecules from HepG2/HepG2.2.15 cells, total RNA was treated with DNase and was reverse transcribed with random hexamers using Revert Aid first-strand cDNA synthesis kit (MBI Fermentas). Real time PCR was performed in ABI 7200 SDS (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green (Applied Biosystems). The target mRNA was relatively quantified and was normalized to the internal control (GAPDH). The PCR cycle number (C_T) at which the exponential growth in the fluorescence from the dye (SYBR Green) passes a certain threshold was used to calculate the relative gene expression. $2^{-\Delta\Delta CT}$ was calculated to represent the relative quantification of the gene, where ΔC_T ($C_{T\text{-target gene}} - C_{T\text{-GAPDH}}$). $\Delta\Delta CT = \Delta C_{T(\text{Experiment})} - \Delta C_{T(\text{Control})}$

Real time PCR was performed in triplicates for all the samples.

Primer sequences used in the paper are as follows:

GAPDH: FP-5'- AATCCCATCACCATCTTCCA -3'
RP-5'- TGGACTCCACGACGTACTCA -3'

TLR4: FP-5'- AGGATGATGCCAGGATGATGTC -3'
RP-5'- TCAGGTCCAGGTTCTTGGTTGAG -3'

p53: FP-5'-CCCAAGCAATGGATGATTTGA-3'

RP-5’-GGCATTCTGGGAGCTTCATCT-3’

p21: FP-5’-GGACAGCAGAGGAAGAC-3’
RP-5’-GGCGTTTGGAGTGGTAGAAA-3’

Rb: FP-5’-CGGGAGTCGGGAGAGGACGG-3’
RP-5’-CGAGAGGCAGGTCCTCCGGG-3’

Cyclin D1: FP-5’-AGCTCCTGTGCTGCGAAGTGGAAAC-3’
RP-5’-AGTGTTCAATGAAATCGTGCGGGGT-3’

Cyclin E: FP-5’-CAGCACTTTCT TG AGCAACACCCTC-3’
RP-5’-TCTCTAT GTCGCACCACTGATACCC-3’

Cyclin B1: FP-5’-AAGAGCTTTAAACTTTGGTCTGGG-3’
RP-5’-CTTTGTAAGTCCTTGATTTACCATG-3’

Cyclin A: FP-5’GCATGTCACCGTTCCTCCTT-3’
RP-5’CAGGGCATCTTCACGCTCTAT-3’

MyD88: FP- 5’-AAGTTATTT GTTTACAAACAGCGACCA-3’
RP- 5’-GGA AGAATGGCAAATATCGGCT-3’

Traf 6: FP- 5'-GCCCAGGCTGTTCATAGTTT-3'
RP-5'-CAAGGGAGGTGGCTGTCATA-3'

TRIF: FP-5’- ACGCCATAGACCACTCAGCTTTCA -3’
RP-5’- AGGTTGCTCATCATGGCTTGGTTC -3’

IRF3: FP-5’- TCTTCCAGCAGACCATCTCC-3’
RP-5’- TGCCTCACGTAGCTCATCAC -3’

IRF7: FP-5’- CAGATCCAGTCCCAACCAAG -3’
RP-5’- GTCTCTACTGCCCACCCGTA -3’

TBK1: FP-5’- AGCGGCAGAGTTAGGTGAAA -3’
RP-5’- CCAGTGATCCACCTGGAGAT -3’

JNK: FP-5'- GTACTTGTATGAAACCACCTTTCT -3'
RP-5'- AGCATCTCTTTCTGAATCTATGAAG -3'

p38: FP-5’- TCTGCTTACCCTTCACCTTTG -3’
RP-5’- CACATCCTCACTCTGCTAGAAAT -3’

PI3K: FP-5'- AAGGGTGCTAAAGAGGAACAC -3’
RP-5'-CATGAGGTACTGGCCAAAGAT -3'
NF-κB: FP-5’-CGCATCCAGACCAACAACA-3’
RP-5’- TGCCAGAGTTTCGGTTCAC-3’

2. In material and methods, in the sections of western blotting and confocal imaging, the reference number of the antibodies and the concentrations used in the study should be indicated.

Western Blot:

- ✓ Anti-H3K27me3 - 1:500(ACTIVE MOTIF: 39155)
- ✓ Anti-H3K39me3 – 1:1000 (ACTIVE MOTIF:39161)
- ✓ Anti-H3K4me3 - 1:500 (ACTIVE MOTIF: 39159)
- ✓ Anti-H3K36me3 - 1:500 (ABCAM: ab9050)
- ✓ Anti-H3K9Ac - 1:1000 (MILLIPORE, 07-352**)**
- ✓ Anti-H3K18Ac - 1:500(ACTIVE MOTIF: 39587)
- ✓ Anti-H3 - 1:10000 (ABCAM: ab10799)
- ✓ Anti-p53 - 1:5000 (SANTA CRUZ: SC-126)
- ✓ Anti-NF-κB- 1:1000 (e-Bioscience14-6731-81)
- ✓ Anti-β-actin - 1:2000 (Sigma: A2228-100UL)
- ✓ Anti-Rabbit IgG-HRP - 1:10000 (SIGMA: A1949
- ✓ Anti-Mouse IgG-HRP - 1:5000 (PROMEGA: W402B)

Confocal Microscopy

- ✓ Anti-NF-κB – 1:1000 (e-Bioscience14-6731-81)
- ✓ Anti-Rabbit Alexa Fluor 488- 1:1000 (INVITROGEN: A11034)

3. The technique used to determine the cytokines is not described in the material and methods.

The protocol used for cytokine bead array is as follows:

Cytokines were screened from the supernatant of the HepG2.2.15 cells treated with 4µg/ml of LPS-B5 Ultrapure (seeded in 6 well plate with a seeding density of $1x10^6$ cells/well) using cytokine bead array (CBA, BD Biosciences). The assay was conducted using 25µl of sample and using a 10-point standard curve (ranging from 0 to 5000 pg/mL) was included for each cytokine measured (IL-6, IL-8, IL-10, IL-1β, IL-12p70 and Human TNF). The samples were analyzed using a BD Accuri C6 flow cytometer (BD Bioscience). FCAP Array software (BD version 3.1) was used to create the standard curves for each cytokine and convert the fluorescent MFI values into cytokine concentrations.

4. In material and methods, in the section of Cell cycle analysis: How many cells were analysed for each sample in BD FACS Calibur platform?

10,000 events were recorded for each sample.

5. In material and methods, in the section of MTT assay. How many cells were seeded in each well?

HepG2.2.15 cells were plated in a 6 well format with a seeding density of $1x10^6$ cells/well.

6. In material and methods, authors should include a section explaining the Statistical analysis used for each technique.

All data are expressed as mean +/- standard deviation (SD) from at least three separate experiments. The differences between groups were analyzed using unpaired 2-tailed Student's *t*-test. Differences were estimated at a statistical significance of $p < 0.05$.

7. The number of experiments performed for each technique is unknown. Please, clarify this point. All the results presented in the paper are without a statistical analysis and that's why it is difficult to know if the changes described in the paper are true or not. Please add the statistical analysis for the data of each technique.

We have provided the statistical analysis for all the experiments. All data are expressed as mean +/- standard deviation (SD) from at least three separate experiments. The differences between groups were analyzed using unpaired 2-tailed Student's *t*-test. Differences were estimated at a statistical significance of $p < 0.05$.

8. Figure 1. Why did the authors choose to do the experiments with the cell line HEPG2.2.15 instead of HEPG2? Authors described that HEPG2 has a higher level of TLR4 than HEPG2.2.15. Please explain the reasons.

HepG2.2.15 are well established and widely used cell line derived from human hepatoma cell line HepG2. This cell line is transfected with a full length HBV genome and constitutively expresses HBV. This is well recognized as a stable infection model. HepG2.2.15 is a more suitable model compared to HepG2 since in HepG2.2.15 cells the interaction of HBV with hepatoma cells is reflected better. It is also expected that the expression of TLR4 is lowered in HepG2.2.15 cells, probably due to the viral infection. Thus, HepG2.2.15 cell line is a more relevant model to understand the role of TLR4 in HBV infection in hepatocyte microenvironment. Moreover, HepG2 will only reveal the effect of transient infection after transfection of HBV genome.

9. Figure 1B ,C, D. How do the authors explain a decrease in the viral load but not in the viral proteins for the concentrations of 1 and 2 ug/ml of LPS?

It can be explained that doses of 1 and 2 ug/ml of LPS are sufficient to target the viral genome, but the viral proteins are repressed only at higher doses of LPS. The viral DNA load is estimated by qRT-PCR, while the surface antigens (HBsAg and HBeAg) are measured by ELISA. The qRT-PCR being a highly sensitive technique can effectively detect the minor changes in viral DNA load at intermediate LPS concentrations, whereas ELISA being a colorimetric assay has its own limitations.

10. Figure 1C, D. Authors should check if there is a concentration-dependent repression of viral protein by statistical analysis, because only the concentration of 4 ug/ml of LPS seems to reduce these proteins.
    The statistical analysis has been performed for Figs. 1C and 1D.

11. Figure 2 A, B. Authors should check if there are differences between control and LPS by statistical analysis, because the graphs of the FACS seem quite similar.

As mentioned in the manuscript, there is no significant change in the cell cycle upon TLR4 activation (Fig. 2A, 2B), although there are certain cell cycle regulators (Fig. 3A) which gets modulated.

12. In Figure 5A, authors describe a downregulation of p53 and upregulation of NF-kB. However, in the picture of western blot, the levels of these proteins do not seem to be modified.

The band intensity of p53 and NF-κB was derived after normalizing it with β-Actin. Image J software was used to analyze the data. The band intensity was calculated for p53, NF-κB and β-Actin. The normalized intensity of p53 and NF-κB was then calculated from that of β-Actin. This was then used to calculate the fold change of the LPS treated samples with respect to the control samples.
After re-analyzing the data for quantitative alterations, it was observed that though NF-KB protein expression was higher in LPS treated samples compared to control HepG2.2.15 cells, p53 expression was also up regulated on stimulating TLR4. This suggests that the p53 independent pathway plays a significant role in the release of G1/S arrest and hence the viral elimination.

Minor comments –

1. There are some spelling mistakes in the manuscript.

We have corrected the minor spelling mistakes in the manuscript.

2. In Material and Methods, section Cell cycle analysis, in the first line, remove Phosphate Buffered Saline, leave only PBS.

PBS has been mentioned as suggested.

3. Graph bar of Figure 1B present a different style (letter, colour of bars) respect to graph bars of Figure 1C and 1D.

We have represented Figure 1C and D in different colour, since they signify the HBV specific proteins (HBsAg and HBeAg) , while 1B represents the HBV DNA. They have been analysed using different tests and hence we chose to represent the HBV DNA load (analysed by qPCR) and HBV proteins (analysed by ELISA) in different colours.

4. Figure 2D, 3A, 5A the titles on the Y axes are not present.

We have added the titles.

Reviewer 2:

Major comments:

1. In figure-1B, the difference between LPS (1ug/ml) and LPS (2ug/ml) is very high compare to the difference observed in figure 1C & D. Please explain this discrepancy.

It can be explained that doses of 1 and 2 ug/ml of LPS are sufficient to target the viral genome, but the viral proteins are repressed only at higher doses of LPS. The viral DNA load is estimated by qRT-PCR, while the surface antigens (HBsAg and HBeAg) are measured by

ELISA. The qRT-PCR being a highly sensitive technique can effectively detect the minor changes in viral DNA load at intermediate LPS concentrations, whereas ELISA being a colorimetric assay has its own limitations.

2. In figure legend 1C, the authors have mentioned that the effect is dose pendent; however, the data doesn't show a dose dependent effect between 1ug/ml and 2ug/ml doses.

There was negligible decrease in the O.D. for HBeAg production between the 1ug/ml and 2ug/ml doses (2.896 and 2.792 respectively). We have thus made the correction in the manuscript stating that though a significant dose dependent viral DNA reduction was observed, the viral proteins did not show the same.

3. In figure 2C the labelling for LPS-treated (72 HRS) group has to be corrected

We have made the change.

4. Why results of MT assay (figure 2C) showed less viable cells at 72 hr LPS treatment group? Please explain.

There was a very slight decrease in viability at 72 hours. HepG2.2.15 cells have a transfected HBV genome that leads to stable production of viral particles. The transfected construct poses minor cell stress that probably caused insignificant cell death at 72 hours.

5. In figure 2D, the labelling should be LPS-treated not TLR4-treated

We have made the change.

6. Throughout the manuscript, the p-value for different statistical analysis has to be provided

We have provided the statistical analysis for all the experiments.

7. In figure 5B, what is the difference between the upper two and lower two panels?

Figure 5B is the confocal imaging showing the nuclear translocation of NF-KB from cytoplasm on TLR4 stimulation. The upper panel in figure5B shows a field with number of cells. In the lower panel we have tried to focus on single cells, to make the analysis more confirmatory.

8. In figure 6 B, C & D, all the inhibitors should be labelled with LPS

Necessary corrections have been made in the Fig. 6B-D.

9. 9. In figure 5A, the relative band intensity for p53 doesn't match with the images.

We apologize for the inadvertent mistake. Necessary corrections have been made in the p53 quantification (Fig. 5A).

Reviewer 3:

1. Complementary data should be added in the Results on the calculation of the modifications in the quantitative markers - see below comment for the Figures. Thus, the figures are satisfactory but the calculation of the 'p' values, significant or not, is necessary in the legends (Fig 1-6). Examples: Figure 1 for HBV DNA, HBs Ag, HBe Ag; Figure 2 for Fow cytometry and Epigenetic signatures. Please add a systematic mention of the calculated 'p' value for the modulation in quantified parameters.

All data are expressed as mean +/- standard deviation (SD) from at least three separate experiments. The differences between groups were analyzed using unpaired 2-tailed Student's *t*-test. Differences were estimated at a statistical significance of $p < 0.05$.

2. Concerning the Discussion/Conclusion section: The authors mention that after TLR4 activation, the acetylation signatures H3K9Ac and H3K18Ac, which are transcription activation marks increased. Their observation suggested that a combination therapy, which can target multiple epigenetic factors, can be important to limit HBV infection. It would be relevant for the authors to add here a paragraph devoted to the innovative therapeutic approaches, which are in progress to fight against chronic HBV infection and how epigenetic modulation could help in allowing this research to improve.

Modulation of host epigenetic landscape upon virus infection has been observed in several cases. Adenoviral as well as SV40 virus infection leads to hypoacetylation of H3K18Ac. A recent report shows that H3K18Ac is downregulated upon HBV infection. Thus LPS treatment which further restores the H3K18Ac status could be an ideal futuristic anti-viral therapy. It can also be observed that LPS treatment leads to significant increase of H3K9Ac mark in human genome. Thus epigenetic therapy to hyperacetylate H3K9 or H3K18 at gene promoters which leads to a classical gene activation scenario can be adopted as a novel strategy to eliminate HBV infection.

(Pandey, V. and Kumar, V. Stabilization of SIRT7 deacetylase by viral oncoprotein HBx leads to inhibition of growth restrictive RPS7 gene and facilitates cellular transformation. Sci. Rep. ***5****, 14806; doi: 10.1038/srep14806 (2015).*

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