Sept 09, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: Manuscript 12678-revised.doc).

Title: High Persistence Rate of HBV in Hydrodynamic-Injection-Based Transfection Model of C3H/HeN Mice
Author: Xiuhua Peng, Xiaonan Ren, Lixiang Chen, Bisheng Shi , Chunhua Xu, Zhong Fang, Xue Liu, Jieliang Chen , Xiaonan Zhang , Yunwen Hu , Xiaohui Zhou

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 12678
The manuscript has been improved according to the suggestions of reviewers:
1. Format has been updated according to the format requirements of *World Journal of Gastroenterology.*
2 Revision has been made according to the suggestions of the reviewer

(1) Reviewed by 00069464

Hepatitis B virus infection is a major public health problem. The etiological mechanisms of the host immune responses that lead to HBV persistence or clearance are still to be elucidated integrally yet. Antiviral with IFN-alpha (IFN-α) and nucleoside/nucleotide analogs significantly improve the prognosis of patients with HBV infection, however the efficacy and safety of these drugs, as well as drug-resistances of nucleoside/nucleotide analogs remains unsettled tough issues in clinical practice. The ideal animal model is very important for playing research to resolve the issues above. In this manuscript, Peng et al. construct an ideal HBV infection model in C3H/HeN(H-2k) mice By hydrodynamic injection with pAAV/HBV1.2 plasmid DNA. Work is written correctly. The remaining description, discussion on the topic is clear and correct. And I think this manuscript can be accepted for published in WJG with a revision.

The major and minor points of 00069464 reviewers' criticisms		**Our responses and dispositions**
1. The author state the hydrodynamic injection of HBV genome into C3H/HeN mice could impair the T cells' function in those mice, both globally and specifically (i.e. HBsAg-specific T-cell immunity). To demonstrate the issues, author evaluated the production of IFN-γ after HBsAg protein vaccination in HI C3H/HeN mice and HI C57BL/6 mice. However, the number of animals is low (n=3). And if the immune response (the production of IFN-γ) of HBsAg protein vaccination (S fragment of HBV DNA) in C3H/HeN mice and C57BL/6 mice after HI HBV genome can represent specific T-cell immunity?		Thanks for your suggestions. We really agree with your viewpoints on the number of animals is low (n=3) for T cell function analysis. Because mice were used for other assays, the number for detecting T cells' function was limited. Undoutedly it need take a long time to setup this persistent model again to enlarge the number of animals. Despite all this, the difference between C3H/HeN mice and C57BL/6 mice is obvious according to the currently data. We agree that HBsAg cannot represent all of the HBV specific T-cell immunity. Actually, HBsAg and HBcAg are two major antigens to induce HBV specific T-cell immune responses, which play important roles in clearance of intrahepatic HBV. Thus we rewrote the text in a more accurate way: replacing the "HBV specific T-cell immunity" with "HBsAg specific T-cell immunity".
2 in Fig 4D, the differences in the pictures depicting spots of IFN-γ after PMA and ionomysin stimulation were mild, please further present them with extensional numerous or with bar chart.		We are sorry for not revealing the spots clearly. The data were shown with a bar chart in Fig4D according to the reviewer's suggestion.

3 “Freshly isolated mouse splenocytes” in page 4, splenocytes including lymphocytes, antigen presenting cell, macrophages, dendritic cells, etc, in this manuscript, splenocyte is a mixed liquor of these cells or one of which?		Although splenocyte is a mixture of lymphocytes, antigen presenting cell, macrophages, dendritic cells etc, lymphocytes is the major part among them. Most importantly, the antigen specific memory T lymphocytes in the splenocytes can respond to the specific antigen or epitope peptides upon re-stimulation and produce cytokines such as IFN-gamma prominently, and thus can be detected by ELISPOT or Intracellular cytokine staining (ICS) assays. Actually this is a standard protocol to detect antigen specific T cell responses in the splenocytes, as described in the *Current Protocols in Immunology*.
4 The references 2 is questionable, please check.		We are sorry for the mistake, and the reference has been amended according to your comment.
5 The format of the references should refer to WJG.		Thanks for your kindly reminding. Format has been updated in the format of WJG.

(2) Reviewed by 00004603

The model of hydrodynamic injection of plasmids containing replication component of hepatitis B virus seems to be promissing. It appears that not only the way of HBV plasmid delivery, but also the right choice of mice with immunodefficient features are of importance for successful development of this model. I have no major problems with this manuscript.

The minor points of 00004603 reviewers' criticisms		**Our responses and dispositions**
1. It is not quite clear why such reagimen of IFNa treatment is included: it is difficult to expect that the treatment as it is designed would clear the infection.		Thanks for your question. We treated the HI mice with PBS or IFN-α or entecavir for the purpose to indicate that the model can be used to analyze the therapeutic effect of drugs. IFN-α and entecavir are in common use in the clinical practice. Considering the cost of the IFN-α, we did apply a complete treatment regimen of IFN-α. Therefore, the therapeutic effect of IFN-α is not so effective as entecavir in the manuscript. But we did see a decrease of HBV DNA after the IFN-α treatment.
2. Few misspellings are in the text.		Thank you for careful reading of our manuscrip t. We have careful double checked and tried to correct all the misspellings in the text.

(3) Reviewed by 00503560

General comments: The paper by Xiuhua Peng et al. showed HBV persisted longer in C3H/HeN(H-2k) mice after the hydrodynamic injection(HI) compared with C57BL/6(H-2b) mice, suggesting that host genetic background determines the rate of HBV clearance. The authors suggested that this could be a novel animal model for chronic hepatitis B infection to elucidate the disease pathogenesis and develop new antiviral treatments. Overall, the study showed a clear association between mouse genetic background and the rate of persistence. However, the authors should elucidate the mechanistic basis for the frequent persistence in the C3H/HeN (H-2k) mice.

The major and minor points of 00503560 reviewers' criticisms		**Our views and dispositions**
1 In this study, the authors demonstrated MHC haplotype H-2K, and young age of mice were related to the high rate of HBV persistence after hydrodynamic injections. The authors also claimed that the high persistence rate was associated with impaired HBsAg-specific T cell responses, but only three mice per group were examined with relatively large error bars. A larger number of mice should be examined to substantiate the authors' claim. In addition, the weak HBs-Ag specific T cell responses in H-2K mice could reflect the lack of positive signals required for the induction of HBsAg-specific T cell responses, or it could reflect the presence of negative signals that suppress HBsAg-specific T cell responses. The authors should distinguish these alternatives by hydrodynamically injecting HBV plasmid into F1 hybrid of C3H/HeN and C57BL/6.		Thanks for your comments. We agree with your viewpoint on the number of animals is low (n=3) for T cell function analysis. Because mice were used for other assays, the number for detecting T cells' function was limited. Nevertheless, the difference between C3H/HeN mice and C57BL/6 mice was obvious. Hydrodynamically injecting HBV plasmid into F1 hybrid of C3H/HeN and C57BL/6 to detect T cells' function is an profoundly thoughtful suggestion. We planned to report a good HI model of HBV persistence firstly, and the further studies on the mechanistic basis for the HBV persistence in the C3H/HeN mice will be continued in the next step of our research. Since it takes quite a long time to setup a persistent HI model, and even longer in the F1 hybrid offsprings, we will pay attention to carry out experiments with larger number of mice and to explore the phenotypes in the F1 hybrid offsprings in the further studies.
2 As cited in this manuscript, Huang et al. have previously shown that HBV replication persisted in FEV/N(H-2q) mice after hydrodynamic injection. Authors should explain why C3H/HeN mice are superior to FEV/N mice in studying the disease pathogenesis and HBV persistence.		Huang et al have previously shown that HBV replication persisted in FEV/N (H-2q) mice after hydrodynamic injection. However, their observation were based on a small number of mice, i.e. only 7 mice per group with a 85.7% persistence rate. Our data came from 25 mice per group, and 22 mice in them showed persistence of HBV (88%). Beside, we also expand the genetic background range of mice to study the HBV persistent mechanisms. Comparison of C3H/HeN ($H\text{-}2^k$, mostly chronic/persistent), FEV/N (H-2q, chronic/persistent), C57/BL6 ($H\text{-}2^b$, partially chronic/persistent), and BALB/c ($H\text{-}2^d$, mostly accute/non-persistent) , could help to clarify the influence of genetic factors on the HBV persistent rate. That will be another

		interesting research to be performed.
3 In the Fig.4D, the authors showed three pictures depicting spots of IFN-γ after PMA and ionomysin stimulation. But it is impossible to assess the data because the differences between groups were very modest. The data should be numerically represented.		Thanks for your suggestion. The data were shown with a bar chart in Fig4D.
4 In the Fig5, the label of horizontal axis should be changed to "Weeks after treatment " from "Weeks after hydrodynamic injection."		Sorry for the mistake in the label, and we thanks a lot for your careful reading. The label was amended according to your comment.
5 "mimcs the natural coarse" in the Introduction was a typo.		The text was amended according to your kindly comment.
6 In the Materials and Methods, IFN-γ ELISPOT assay part reads "The splenocytes of C3H/HeN and C57BL/6 mice s.c. injected mice with 10μg OVA protein were stimulated with 10μg/ml OVA protein", but it was unclear why this should be described in this paper.		It was another work of our research group and shouldn't be included in this manuscript. This inconsistency was due to our oversight during writing the methods and materials. We are sorry about it. The related content has been deleted in the edited manuscript.
7 The title of Fig. 4, "Impaired HBsAg-specific T cell response were impaired in HI C3H/HeN mice." was incorrect. It should be either "Impaired HBsAg-specific T cell responses in HI C3H/HeN mice" or "HBsAg-specific T cell responses were impaired in HI C3H/HeN mice."		Thank you very much to point out the sentence structural and grammatical issues in our manuscript. The mistake were corrected in the edited manuscript.

3 References and typesetting were corrected according to the format requirements of

World Journal of Gastroenterology.

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

Xiaohui Zhou Ph.D. & M.D.
Associate Professor of Microbiology and Immunology,
Vice-Director of Animal Facility,
Principal Investigator in Infectious Animal Model Department,
Shanghai Public Health Clinical Center, Fudan University

Animal Facility Building, PI's Office
2901 Caolang Road, Jin Shan District, Shanghai 201508, P.R.China
Tel: 86-21-37990333-5239
Fax:86-21-37990333-5235
Email: zhouxiaohui@shaphc.org