

Hepatitis B virus surface antigen drives T cell immunity through non-canonical antigen presentation in mice

Corresponding Author: Dr Jianhua Li

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this elegant study, Li et al use human samples as well as different preclinical models, such as hydrodynamic injection of a HBV genome containing plasmid or a recombinant cccDNA precursor plasmid into the tails of mice or infection of mice with a recombinant adenovirus expressing HBV, to address the different aspects of intrahepatic priming of HBV specific CD8⁺ T cells. They found that a specific subset of intrahepatic resident dendritic cells (DC1) are the primary antigen-presenting cells in shaping CD8⁺ T cell immunity against HBsAg in the liver.

Noteworthy, the authors have used different preclinical models to confirm their findings. By excluding other types of presentation, they suggest that DC1s acquire and present HBsAg by MHC-I cross-dressing but not cross-presentation. They also highlight an important role of B cells in the initial priming of CD4⁺ T cell responses against HBsAg that occurs sequentially in follicular and interfollicular areas. Finally, they also mapped the landscape of ligand-receptor signaling networks between antigen-presenting cells and T cells. The study is quite comprehensive and complex, uses state of the art methodology and addresses an important area in HBV immunobiology.

I have the following comments:

The authors state at several instances that HBV specific CD8⁺ T cells are primed in the liver (e.g. page 5, line36). While this is correct in certain preclinical models, there is no data available for the human situation. Thus, the authors should be more careful with this statement.

The sample size is partly very small, especially in the mice experiments that this reviewer wonders how often the experiments have been repeated. This should be indicated in the figures and methods section.

In Fig1, the control is missing whether certain clusters are only present in one patient, this should be added as supplementary information.

The authors show a lot of negative results without showing positive controls, thus the relevance remains partly obscure. This is especially evident with the conclusion that cross-dressing is active instead of cross-presentation. Can the authors address this directly?

The authors start with a nice description with the cell clusters during acute infection in humans but do not address whether these findings are reflected by the situation in mice. This should at least be discussed.

Reviewer #2

(Remarks to the Author)

The authors Xiaofang Li and Wenxuan Sun submitted a manuscript entitled "Hepatitis B virus surface antigen drives T-cell immunity through non-canonical antigen presentation". Within the manuscript the authors aim to unravel the principles of priming of HBsAg-specific CD8 T cells in the liver. Thereby they identified that HBsAg-MHC-I complexes are transferred to Batf3⁺, XCR1⁺, CCR7⁺ cDC1s by a mechanism termed cross-dressing. Those "cross-dressed" cDC1s in turn can activate

HBsAg-specific CD8 T cells.

There have been controversial discussions for long period, where and how CD8 T cells against hepatotropic viruses are primed. In addition, the outcome of CD8 T cell priming in the liver has been reported to have different outcomes, ranging from T cell deletion to T cell anergy¹⁻³. This manuscript sheds light, how HBV-specific CD8 T cells are primed and by which cells types during acute HBV leading to efficient immune surveillance.

Moreover, the authors also investigated the priming/activation of HBsAg-specific CD4 T cells and B cells in secondary lymphoid tissue. Here the authors identified that B cell take up the antigen in lymphoid tissues and initiate the CD4 T cell response.

Overall, the manuscript is well written and the topic is of great interest. To address the questions the authors used sophisticated technology and generated new models for research. In addition, numerous knockout mice were used to verify the data. Overall, the manuscript should be published, but lacks some prior improvement.

Recent publications indicate that for an anti-HBV response mainly HBcAg and Polymerase-specific T cells are responsible⁴ (and oral presentation on the HBV Meeting 2024). And HBsAg-are often underrepresented. Did the authors had the chance to investigate also the priming of HBcAg and/or Polymerase-specific CD8 T cell in the liver?

Direct points:

Page 4, line 33 onwards: Please specify more the transcription factors identified. There are also interesting transcription factors like STAT2 and IRF2 involved in innate immune signaling, which are avoided by HBV during infection.

Page 6, line 12, and suppl. Figure 2g: Besides anti-CD62L antibody, does FTY720, which is used later in the manuscript, show similar results?

Page 6, line 12: The CD8 T cells are against the OVA-Peptide SIINFEKL (OT-I T cells). Those CD8 T cells most likely have a higher affinity to the TCR than HBsAg-specific CD8 T cells which might severely affect CD8 T cell priming and response. So, the conclusion "...corroborating the essential role of the liver in priming CD8+ T cells against HBsAg." is not supported by the data. May the wording should be adjusted here.

This lower affinity is seen later by comparing Figure 2g and 2i. Env-specific CD8 T cells proliferate much less compared to OT-I T cells. However, it would be interesting to see the proliferation index in Figure 2K, similar to Figure 2e.

Page 6, lines 17-20: The statement should be toned down: The assay used, detects the response of CD8 T cells against an OVA-peptide not against HBsAg-specific CD8 T cells.

Page 7, line 20 onwards: Although the model is very sophisticated and impressive, the CD8 T cell response is against the OVA-peptide and not directed against HBsAg. The authors should think about an alternative statement.

Page 8, line 26-28, and specifically Figure 3h: I am confused about the statement, specifically in the figure legend 3h: the Figure legend states: "h Representative Violet tracer dilution in OT-I T cells (left) and summary data showing proliferation index (right) of Env28 T cells...". Why are left OT-I T cells and for quantification Env28-specific CD8 T cells? This might affect the interpretation as OT-I are H2-Kb restricted and Env28 are H2-Kd-restricted.

Page 8, line 29-30, specifically suppl. Figure 3p+: The authors have transferred C57BL/6 bonemarrow (H2-Kb) and measure the proliferation index of Env28 T cells which are H2-Kd-restricted. Can the authors explain?

Page 8, line 35-37: "suggesting that the HBsAg peptide MHC-I complexes...HBsAg-producing hepatocytes". In Figure 2c, the authors have demonstrated that LSEC also present SIINFEKL-MHC-I complexes. As LSEC might have more intimate contact with DCs, it may very likely be possible that LSEC cross-dressed the DCs. Could the authors exclude that cross-dressing occurs from LSEC? This might also be shown, e.g. with coculture of LSEC and/or primary hepatocytes and cCD1 and check for transfer of MHC-I molecules.

Page 10, lines 22-33: Addressing the importance of the size of the antigen, did the authors had the chance to compare the uptake of the particular HBsAg by B cells to the uptake of the more soluble HBeAg?

Page 11, lines 1-3: The authors conclude that half of the OT-II had migrated from the T cell zone to follicular regions of the spleen and dLN. It might be argued that the OT-II already had been in follicular region. To make the conclusion that they migrated, I am missing the negative control, uninfected or timepoint 0.

Minor:

Page 2, line 37: "...after their acquiring of antigens."

Page 8, line 23: SIINFEKL, the "S" is missing.

Page 11, line 1: "... B cells and cCD1s drove CD8 T and CD4 T cell..., respectively. It seems that the order is wrong.

Page 15, line 28-31: To me, it would not be surprising that B cells take up the antigen independent of IgG-mediated opsonization. At indicated time points here, I would guess that no IgG are produced, because it is too early for this.

- 1 Bertolino, P., Trescol-Biemont, M. C. & Rabourdin-Combe, C. Hepatocytes induce functional activation of naive CD8+ T lymphocytes but fail to promote survival. *European journal of immunology* 28, 221-236 (1998). [https://doi.org/10.1002/\(SICI\)1521-4141\(199801\)28:01<221::AID-IMMU221>3.0.CO;2-F](https://doi.org/10.1002/(SICI)1521-4141(199801)28:01<221::AID-IMMU221>3.0.CO;2-F)
- 2 Holz, L. E., Warren, A., Le Couteur, D. G., Bowen, D. G. & Bertolino, P. CD8+ T cell tolerance following antigen recognition on hepatocytes. *Journal of autoimmunity* (2009).
- 3 De Simone, G. et al. Identification of a Kupffer cell subset capable of reverting the T cell dysfunction induced by hepatocellular priming. *Immunity* 54, 2089-2100 e2088 (2021). <https://doi.org/10.1016/j.immuni.2021.05.005>
- 4 Heim, K. et al. Attenuated effector T cells are linked to control of chronic HBV infection. *Nat Immunol* 25, 1650-1662 (2024). <https://doi.org/10.1038/s41590-024-01928-4>

Reviewer #3

(Remarks to the Author)

In this manuscript by Li X. et al. the authors investigate the early stages of antigen presentation during Hepatitis B virus infection. After analyzing immune cells in blood of patients with acute HBV infection, the authors used multiple mouse models of acute HBV infection. In these mouse models, the authors convincingly demonstrate the predominance of cDC1 in promoting CD8 T cell response. Interestingly, the authors show that cDC1 were acquiring their antigen. Presentation capabilities through cross-dressing. The authors also show that the CD4 T cell response is independent on cDC but dependent on B cells that acquire the antigen in B cell follicle of Secondary lymphoid organs (SLO). Finally, the authors identified CCR5, EB12 and LFA-1, CD80 for the positioning and the interaction between CD4 T cells and B cells in SLO. Whereas CD8 T cells interact with cDC1 through a CD6 and ALCAM. Overall, the experiments are well conducted, and many approaches were confirmed with complementary ones.

Major points:

- The authors unveil precise mechanisms for CD4 and CD8 T cell activation but it is unclear what pertains to a mechanism occurring during acute human HBV infection rather to their experimental model. For example, it is unclear if ALCAM was also detected in cDC1 or if CD80 is increased on B cells of HBV infected patients.
- It is unclear from this study if these non-canonical ways for CD4 and CD8 T cell activation are necessary for the clearance of the infection or for the establishment of a chronic disease.
- In the manuscript the authors do not specify if the patients were under treatment during sample collection. The (potential) treatment these patients could have received is key for the data interpretation.
- The conclusion of the fig 1 "Overall, our scRNA-seq analysis indicates the potential presence of novel intercellular mechanisms of HBsAg presentation underlying T cell immunity in acute HBV patients..." is not supported by the data. It is unclear what novel intracellular mechanism of antigen presentation can be concluded from the scRNA-seq.
- Is the cross-dressing of cDC1 dependent on the infection? Was Sup Fig 3n done in absence of infection? What time point after reconstitution was this experiment conducted?
- It is unclear if the cross-dressing is also occurring in other hepatic diseases or if specific inflammatory molecules promote it.

Minor points:

- gamma-delta T cells (gd T cells) have been reported to play a role in multiple liver diseases. The authors do not comment on the loss of gd T cells in infected patients. V delta 2 T cells have been reported to be enriched in chronically HBV-infected patients. The decrease in gd T cells found by the authors could favor a predominance of certain gd T cell clones.

Reviewer #4

(Remarks to the Author)

The manuscript Li et al. investigated pathways of HBV antigen presentation using the hydrodynamic injection to stimulate OT-1 CD8 and OT-II CD4 T cell epitopes embedded within HBsAg. The authors investigate cells responsible for CD8 T cell induction and describe the mechanism of MHC cross-dressing through acquisition of peptide-MHC by cDC1 from hepatocytes. The authors also investigated CD4 T cell activation showing that adoptively transferred HBs-specific B cells are the primary cell type responsible for CD4 T cell activation in this system. They investigated mechanisms of B cell and CD4 T cell trafficking within the spleen to demonstrate their interaction.

Overall, these data are difficult to reconcile with natural infection and existing literature. Acute hepatitis B has a long incubation phase (6 – 8w) where there is limited activation of innate immunity or detectable virus in the serum. The hydrodynamic transfection model results in immediate expression of a large amount of antigen. Therefore, this model may reflect the peak of antigen expression during acute hepatitis, but controls are missing to confirm that this is 1) not an artifact of rapid antigen expression in many hepatocytes and inflammation induced by the introduction method (hydrodynamic transfection/AdHBV acute model) and 2) specific to HBV. Furthermore, the authors present data from complicated experiments using splenectomized mice and different knockout mouse strains (irf4 fl/fl mice) that are not clearly explained, making it hard to understand the relevance.

Conceptual Concerns

Multiple studies in mice (some cited by the authors) going back 20+ years, and the ability to transplant human livers without matching HLAs, demonstrated that intrahepatic priming with hepatocellular antigens leads to abortive T cell proliferation and function. The cross-dressing data are convincing but inconsistent with the bulk of this existing data. The only way to get effective hepatocellular antigen priming was with the addition of co-stimulation. Can the authors provide insight into why they see effective priming by cDC1 without added co-stimulation? One would presume that the HBsAg in previous systems of hepatocellular antigen expression would have also been subject to cross-dressing. Is it because of liver damage with hydrodynamic transfection? Similarly, in the acute model of AdHBV, many hepatocytes are transduced rapidly, leading to high amounts of antigen expression. Do the author see cross-dressing of cDC1 if they use the persistent AdHBV model?

The authors show presentation of an ova epitope embedded in HBsAg and presentation of a native HBsAg epitope. However, there is no control antigen to demonstrate that this mechanism of antigen presentation is unique to HBsAg. The authors should use a control antigen that is secreted similar to the HBsAg to demonstrate specificity of a "non-canonical" presentation pathway in HBV with the hydrodynamic transfection system.

Specific points

These patients do not meet the definition of acute/resolving hepatitis B. According to the table, all patients are still HBsAg positive, anti-HBs negative and no HBV DNA data is provided. The definition of acute/resolving hepatitis B is HBV DNA & HBsAg negative before 6 months and anti-HBs, anti-HBc positive. Did these patient clear HBV or was this a hepatitis flare in chronic hepatitis B patients?

What single patient was used for intrahepatic analysis? What was the ALT level at the time of cell capture? Was the comparison done on only the matching PBMC from the patient or across all patient PBMCs. Patient heterogeneity is a major issue in hepatitis B and these data do not have sufficient power to draw conclusions from a single comparison.

In suppl fig 3 panel "O" where the authors use different HBV systems, I may be confused but the dot plots do not make sense to me. I think they were trying to show H-2kd-siinfekl expression but the gating does not show that. I would guess it is tetramer gating based on the figure legend but it is unclear.

In figure 4 shows an uncoupling of activation (CD69) and proliferation Fig 4b. Activation happens similarly in DLNs and spleen but no proliferation occurs. I suppose this is possible but seems unlikely. Do the author have an explanation?

In fig 6h, I don't see a significant difference in EBI2 knockout S129G1 B cells. They still look to be localized at the border. There does seem to be a difference in OT-II cells. The B cell data may be over interpreted.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have carefully addressed all my comments, also by including relevant controls and performing additional experiments. I have no further questions.

Reviewer #2

(Remarks to the Author)

The authors Li and Sun submitted a revised manuscript entitled "Hepatitis B virus surface antigen drives T-cell immunity through non-canonical antigen presentation". The revised manuscript displays impressive efforts to improve the quality of the manuscript and to address the comments from the reviewers. The authors addressed all questions from the reviewers by performing additional sophisticated experiments.

For my concerns, the authors addressed all concern satisfactorily.

I do have only minor suggestions to improve the clarity of the manuscript.

1) Page 2, line 13-15: "Currently the goal of therapy...". Maybe the authors could add: "...therapy of chronic HBV infection...". Otherwise, it is not clear what should be therapied.

2) Page 3, line 22: ...hepatocyte-specific viral gene expression?

In general: The manuscript investigates the activation/priming of immune responses against HBV, particularly HBsAg-specific CD8, CD4 and B cells. However, at different points the authors use models that should induce acute or chronic HBV infection. As the authors do not show an acute HBV infection course (Clearance of HBV by loss of pgRNA, HbcAg-negative hepatocytes, lack of HbeAg...) nor development of chronic HBV, I would suggest to remove the wording acute and chronic. This is anyway irrelevant for the main topic of the manuscript.

Reviewer #3

(Remarks to the Author)

The authors satisfyingly and thoroughly answered my questions.

Reviewer #4

(Remarks to the Author)

The authors have done a great deal of work to address my, as well as 3 other reviewer comments. I appreciate that effort.

I think the additional experiments are very insightful for the mechanism and provide specificity for HBsAg while also addressing the potential impacts of inflammation associated with the models. General readers are not always aware of the limitations of the mouse models for HBV and the authors have done a good job of highlighting their specific observations while also recognizing potential limitations.

I have no other comments.

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Response to the Reviewers' Concerns

We sincerely thank the reviewers for their thoughtful and constructive feedback, as well as for recognizing the significance of our work. The reviewers have raised important points that have helped us clarify and strengthen the manuscript. Below, we provide a detailed point-by-point response to each comment, with our responses highlighted in blue for clarity. The revised manuscript incorporates all suggested changes and is marked with red highlighting to indicate the modifications made. While the major conclusions of our study remain unchanged, the reviewers' insightful comments have allowed us to refine the presentation and better substantiate our findings. We are grateful for the opportunity to improve the manuscript and hope that the revised version meets the expectations of the editor and reviewers for publication.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this elegant study, Li et al use human samples as well as different preclinical models, such as hydrodynamic injection of a HBV genome containing plasmid or a recombinant cccDNA precursor plasmid into the tails of mice or infection of mice with a recombinant adenovirus expressing HBV, to address the different aspects of intrahepatic priming of HBV specific CD8⁺ T cells. They found that a specific subset of intrahepatic resident dendritic cells (DC1) are the primary antigen-presenting cells in shaping CD8⁺ T cell immunity against HBsAg in the liver. Noteworthy, the authors have used different preclinical models to confirm their findings. By excluding other types of presentation, they suggest that DC1s acquire and present HBsAg by MHC-I cross-dressing but not cross-presentation. They also highlight an important role of B cells in the initial priming of CD4⁺ T cell responses against HBsAg that occurs sequentially in follicular and interfollicular areas. Finally, they also mapped the landscape of ligand-receptor signaling networks between antigen-presenting cells and T cells. The study is quite comprehensive and complex, uses state of the art methodology and addresses an important area in HBV immunobiology.

Response:

We sincerely thank the reviewer for the positive and encouraging evaluation of our manuscript. We have carefully revised the manuscript to incorporate your suggestions, which we believe further enhance the clarity and robustness of our findings.

I have the following comments:

The authors state at several instances that HBV specific CD8⁺ T cells are primed in

the liver (e.g. page 5, line36). While this is correct in certain preclinical models, there is no data available for the human situation. Thus, the authors should be more careful with this statement.

Response:

We sincerely appreciate the Reviewer's insightful comment. To address this concern, we have revised the manuscript to avoid absolute statements regarding the priming location of CD8⁺ T cells in HBV infection, particularly in the human context. Specifically, we have modified the statement on page 5, line 36 of the original manuscript to: "*As the location of CD8⁺ T cell priming in HBV infection remains poorly understood, we began investigating the sites of CD8⁺ T cell responses following HBsAg expression in hepatocytes.*" Additionally, we have revised the statement on page 6, line 14 to: "*This corroborates the essential role of the liver in priming CD8⁺ T cells against HBsAg, at least in mice.*" These revisions have been incorporated into the Results section of the revised manuscript (page 6, lines 3-4; page 6, lines 23-24).

The sample size is partly very small, especially in the mice experiments that this reviewer wonders how often the experiments have been repeated. This should be indicated in the figures and methods section.

Response:

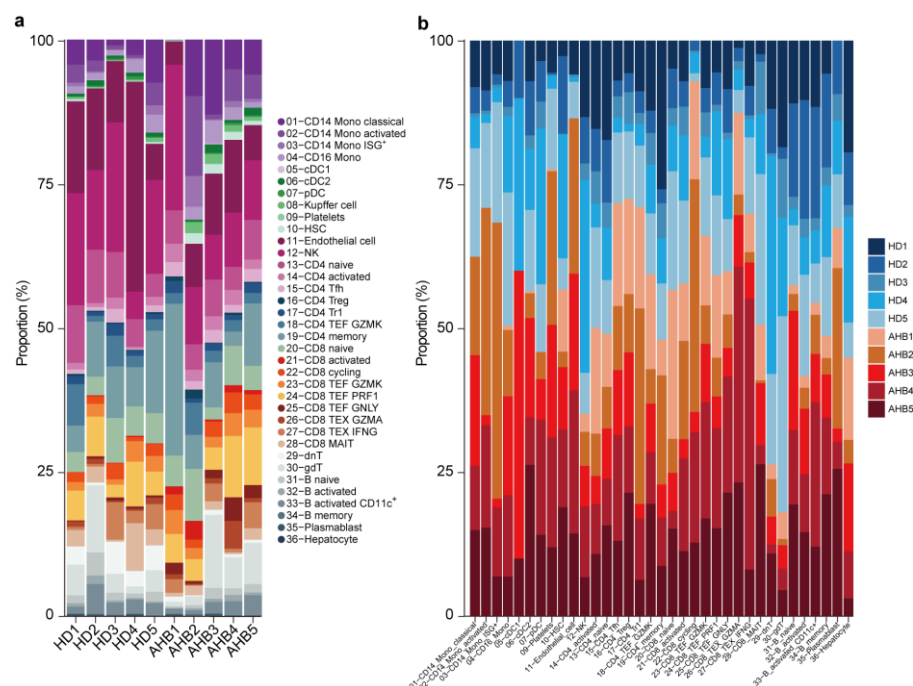
We sincerely appreciate the Reviewer's valuable comment. In the original manuscript, each experiment included at least three mice per group and was repeated at least twice. To further strengthen the robustness of our findings, we have now repeated key experiments and increased the sample size in critical analyses. Details on the number of animals used and experimental repetitions have been clearly specified in the figure legends of the revised manuscript. Specifically, the following figures now reflect these revisions, including expanded sample sizes and additional experimental replicates:

- Figure. 2: e-h, k
- Figure. 3: b, e-g, k
- Figure. 4: b-f
- Figure. 6: g
- Figure. 7: e-g
- Supplementary Figure. 3: d-i
- Supplementary Figure. 4: b-g, l, m
- Supplementary Figure. 6: b, c, j, k, m,
- Supplementary Figure. 7: a-g
- Supplementary Figure. 10: l-n

In Fig1, the control is missing whether certain clusters are only present in one patient, this should be added as supplementary information.

Response:

We sincerely appreciate the Reviewer's insightful suggestion. In response, we have provided additional analyses: (1) the proportion of each cluster in each subject (Reviewer Fig. 1a) and (2) the contribution of each subject to each cluster (Reviewer Fig. 1b). Upon reviewing the data, we confirmed that no clusters were exclusive to a single subject. Reviewer Fig. 1b has been incorporated into Supplementary Fig. 1b of the revised manuscript, and this clarification has been added to the Results section (page 4, lines 18-19).



Reviewer Fig 1. Distribution of cell clusters across subjects. **a** Stacked bar plots displaying the proportions of cell clusters in individual healthy donors (HD) and acute HBV infection (AHB) patients. Each cell cluster is represented by a distinct color and labeled according to its specific subset. **b** Contribution of individual patients to each cell cluster across all HD and AHB subjects. Each color represents a specific sample group (HD1-5 and AHB1-5).

The authors show a lot of negative results without showing positive controls, thus the relevance remains partly obscure. This is especially evident with the conclusion that cross-dressing is active instead of cross-presentation. Can the authors address this directly?

Response:

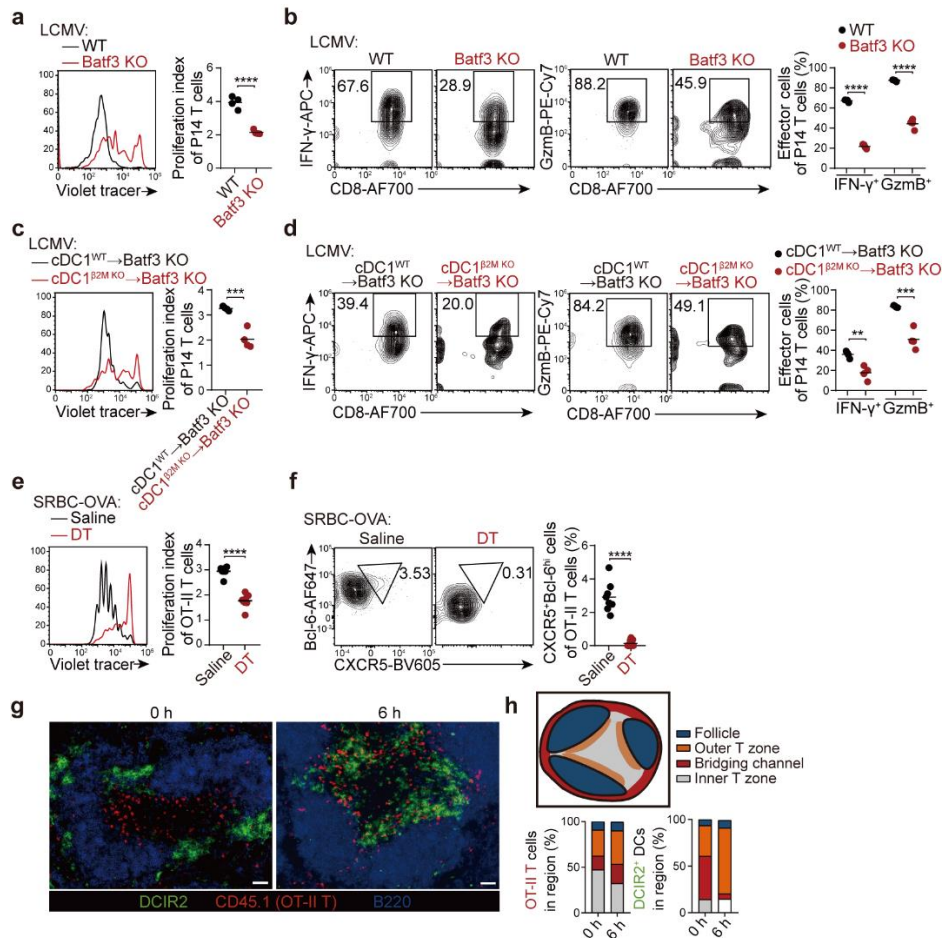
We thank the Reviewer for highlighting the importance of positive controls in supporting our conclusions. In response, we have included appropriate positive controls for key experiments to validate our findings.

For CD8⁺ T cell responses, we used lymphocytic choriomeningitis virus (LCMV) Armstrong as a positive control for cross-presentation^{1,2}. Specifically, we demonstrated that cDC1s are essential for LCMV Armstrong-induced CD8⁺ T cell

responses (Reviewer Fig. 2a, b) and confirmed that MHC-I expression by cDC1s is required for CD8⁺ T cell priming during LCMV Armstrong infection (Reviewer Fig. 2c, d). For CD4⁺ T cell responses, we employed OVA-conjugated sheep red blood cells (SRBC-OVA) as a positive control for classical cDC-mediated CD4⁺ T cell responses. Consistent with our prior findings³, we show that cDCs are required for initiating CD4⁺ T cell responses to SRBC-OVA (Reviewer Fig. 2e, f). After SRBC-OVA immunization, splenic cDCs (DCIR2⁺) migrated from marginal zone bridging channels to the outer T zone, where nearly all activated WT OT-II T cells co-localized with activated DCIR2⁺ cDCs (Reviewer Fig. 2g, h), supporting the traditional view that CD4⁺ T cells are primed by cDCs in T cell zones^{2,4}. These positive controls confirm the validity of our experimental systems and reinforce the robustness of our conclusions regarding cDC1 cross-dressing and B cell-mediated follicular and interfollicular priming mechanisms. The new data have been incorporated into:

- Supplementary Fig. 3j, k and Supplementary Fig. 5a, b (CD8⁺ T cell responses)
- Supplementary Fig. 6g, h and Supplementary Fig. 9f, g (CD4⁺ T cell responses)

Corresponding updates have been added to the Results section (page 7, lines 12-14; page 8, lines 25-27 for CD8⁺ T cell responses; and page 10, lines 26-29; page 13, lines 4-8 for CD4⁺ T cell responses).



Reviewer Fig 2. Positive controls using the LCMV Armstrong and SRBC-OVA models for classical antigen presentation pathways. **a, b** Violet tracer-labeled P14 T cells were transferred into WT or Batf3 KO mice, followed by LCMV Armstrong infection for 3 days. **a** Representative Violet tracer dilution in P14 T cells (left) and summary data showing their proliferation index (right, $n = 4$ mice per group). **b** Representative FACS plots and summary data showing percentages of IFN- γ^+ or GzmB $^+$ P14 T cells ($n = 4$ mice per group). **c, d** Violet tracer-labeled P14 T cells were transferred into Batf3 KO mice reconstituted with β 2M KO versus WT cDC1s, followed by LCMV Armstrong infection for 3 days. **c** Representative Violet tracer dilution in P14 T cells (left) and summary data showing their proliferation index (right, $n = 4$ mice per group). **d** Representative FACS plots (left) and summary data showing percentages of IFN- γ^+ or GzmB $^+$ P14 T cells (right, $n = 4$ mice per group). **e, f** Violet tracer-labeled OT-II T cells were transferred into Zbtb46-DTR chimeras treated with saline or DT, followed by SRBC-OVA immunization for 3 days. **e**, Representative Violet tracer dilution in OT-II T cells (left) and summary data (right) showing their proliferation index ($n = 7$ mice for saline and 8 mice for DT treatment). **f** Representative FACS plots (left) and summary data (right) showing percentages of CXCR5 $^+$ Bcl6 ^{hi} OT-II T cells ($n = 8$ mice per group). **g** Immunofluorescence of OT-II T cells (CD45.1, red), cDC2 cells (DCIR2, green), and endogenous B cells (B220, blue) in the spleens from WT recipients at the indicated time points after SRBC-OVA immunization. **h** Anatomical schematic representation of the spleen (top), percentages of OT-II T cells (bottom, left) and DCIR2 $^+$ DCs (bottom, right) in the specified regions stained in (g). At least two independent experiments were performed. Statistical analysis was performed using two-tailed Student's t -tests. Statistically significant results are labeled (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Scale bars in (g): 50 μ m.

The authors start with a nice description with the cell clusters during acute infection in humans but do not address whether these findings are reflected by the situation in mice. This should at least be discussed.

Response:

We thank the Reviewer for raising this important point. While our study initially focuses on human data to elucidate cell cluster dynamics during acute infection, we recognize the importance of assessing whether these findings are reflected in mice. In fact, in the original manuscript, we had already performed scRNA-seq on liver cells sorted by FACS from mice at days 0 and 2.5 following hydrodynamic injection of pHBV1.3/OVA257-264 (Supplementary Fig. 3a), identifying various DC and CD8 $^+$ T cell subsets in the liver (Fig. 2a, b; Supplementary Fig. 3b; Supplementary Table 3). Additionally, Fig. 1b and Fig. 2a, which depict liver immune landscapes in humans and mice, respectively, were already included in the original manuscript. However, we did not explicitly discuss their comparability in the previous version. To address this, we have now emphasized the similarities between human and mouse liver immune landscapes in the revised manuscript (Fig. 1b and Fig. 2a), as previously reported⁵, suggesting potentially shared features in HBV immune responses. These updates have been incorporated into the Results section (page 6, line 33) and further discussed in the Discussion section (page 19, lines 22-24).

Reviewer #2 (Remarks to the Author):

The authors Xiaofang Li and Wenxuan Sun submitted a manuscript entitled “Hepatitis B virus surface antigen drives T-cell immunity through non-canonical antigen presentation”. Within the manuscript the authors aim to unravel the principles of priming of HBsAg-specific CD8 T cells in the liver. Thereby they identified that HBsAg-MHC-I complexes are transferred to Batf3⁺, XCR1⁺, CCR7⁻ cDC1s by a mechanism termed cross-dressing. Those “cross-dressed” cDC1s in turn can activate HBsAg-specific CD8 T cells.

There have been controversial discussions for long period, where and how CD8 T cells against hepatotropic viruses are primed. In addition, the outcome of CD8 T cell priming in the liver has been reported to have different outcomes, ranging from T cell deletion to T cell anergy¹⁻³. This manuscript sheds light, how HBV-specific CD8 T cells are primed and by which cells types during acute HBV leading to efficient immune surveillance.

Moreover, the authors also investigated the priming/activation of HBsAg-specific CD4 T cells and B cells in secondary lymphoid tissue. Here the authors identified that B cell take up the antigen in lymphoid tissues and initiate the CD4 T cell response.

Overall, the manuscript is well written and the topic is of great interest. To address the questions the authors used sophisticated technology and generated new models for research. In addition, numerous knockout mice were used to verify the data. Overall, the manuscript should be published, but lacks some prior improvement.

Response:

We sincerely thank the Reviewer for the positive and encouraging comments, as well as the constructive feedback. We greatly appreciate the recognition of the significance of our study and the application of sophisticated technologies and innovative models to address these critical questions.

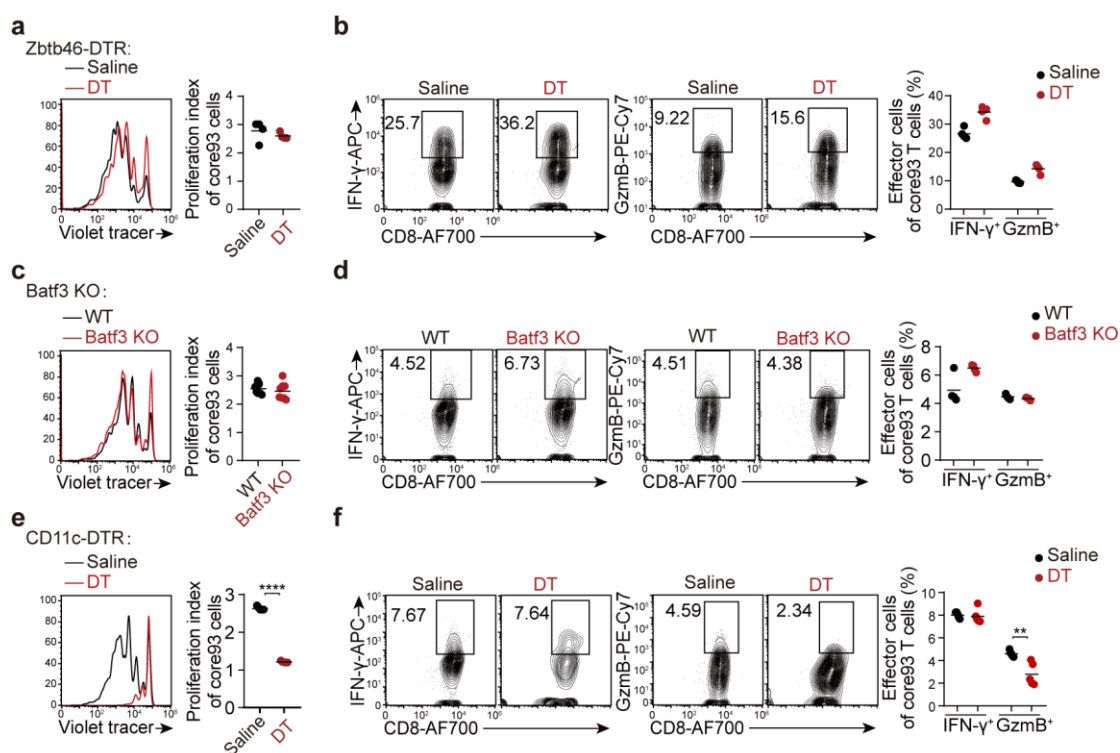
Recent publications indicate that for an anti-HBV response mainly HBcAg and Polymerase-specific T cells are responsible⁴ (and oral presentation on the HBV Meeting 2024). And HBsAg-are often underrepresented. Did the authors had the chance to investigate also the priming of HBcAg and/or Polymerase-specific CD8 T cell in the liver?

Response:

We thank the Reviewer for this insightful question. It is well-established that in chronic HBV infection, persistent high levels of HBsAg may contribute to the deletion or exhaustion of high-affinity T cells, making it challenging to detect HBsAg-specific T cells using conventional methods such as tetramers or peptide pools. This may give the impression that HBsAg does not effectively induce T cell

responses. However, in acute HBV infection, HBsAg-specific CD8⁺ T cells are induced alongside HBcAg- and polymerase-specific T cells, as shown in previous studies^{5, 6, 7, 8}. Given the therapeutic importance of HBsAg clearance in HBV infection, our study primarily focuses on the priming of HBsAg-specific T cell responses.

As suggested by the Reviewer, we investigated the priming of HBcAg-specific CD8⁺ T cells in the liver using Core93 TCR transgenic T cells in Zbtb46-diphtheria toxin (DT) receptor (DTR) mice and cDC1-deficient Batf3 KO mice. Our findings revealed that neither total cDC ablation in Zbtb46-DTR mice via DT nor cDC1 deficiency in Batf3 KO mice affected the proliferation and differentiation of HBcAg-specific CD8⁺ T cells at day 3 following hydrodynamic injection of pHBV1.3, as assessed by flow cytometry (Reviewer Fig. 3a-d). However, consistent with a previous report⁹, we observed that the ablation of CD11c-expressing cells in CD11c-DTR mice via DT significantly impaired these responses (Reviewer Fig. 3e, f). These findings suggest that CD11c-expressing, but non-cDC, cells play a critical role. The identity and mechanisms of these cells are currently under further investigation in our lab. Regarding polymerase-specific CD8⁺ T cells, we were unable to investigate these responses due to the unavailability of polymerase-specific CD8⁺ TCR transgenic mice, which restricts accurate assessments in mice. Given the space limitations and the complexity of this topic, we plan to compile these preliminary findings into a separate manuscript for future independent publication. Although no new data were added to the revised manuscript, we have expanded the Discussion section to address the priming of HBcAg- and polymerase-specific CD8⁺ T cells (page 17, lines 13-28).



Reviewer Fig 3. Identification of antigen-presenting cells involved in priming HBcAg-specific

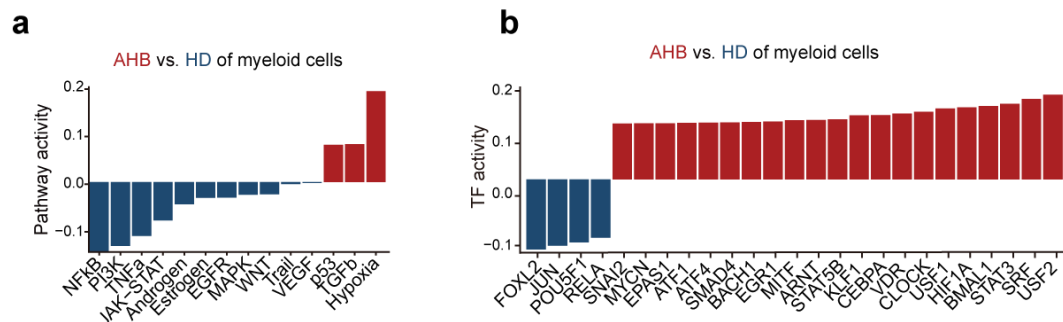
CD8⁺ T cell responses in the pHBV1.3 mouse model. **a, b** Violet tracer-labeled Core93 T cells were transferred into Zbtb46-DTR bone marrow chimeras pretreated with DT versus saline, followed by pHBV1.3 hydrodynamic infection (HDI) for 3.5 days. **a** Representative Violet tracer dilution in Core93 T cells (left) and summary data (right) showing their proliferation index ($n = 4$ mice per group). **b** Representative FACS plots (left) and summary data (right) showing percentages of IFN- γ^+ or GzmB⁺ Core93 T cells ($n = 4$ mice per group). **c, d** Violet tracer-labeled Core93 T cells were transferred into Batf3 KO versus WT mice, followed by pHBV1.3 HDI for 3.5 days. **c** Representative Violet tracer dilution in Core93 T cells (left) and summary data (right) showing the proliferation index of these cells ($n = 8$ mice per group). **d** Representative FACS plots (left) and summary data (right) showing percentages of IFN- γ^+ or GzmB⁺ Core93 T cells ($n = 4$ mice per group). **e, f** Violet tracer-labeled Core93 T cells were transferred into CD11c-DTR chimeras pretreated with DT versus saline, followed by pHBV1.3 HDI for 3.5 days. **e** Representative Violet tracer dilution in Core93 T cells (left) and summary data (right) showing the proliferation index of these cells ($n = 5$ mice per group). **f** Representative FACS plots (left) and summary data (right) showing percentages of IFN- γ^+ or GzmB⁺ Core93 T cells ($n = 5$ mice per group). At least two independent experiments were performed. Statistical analysis was performed using two-tailed Student's *t*-tests. Statistically significant results were labeled (** $p < 0.01$, **** $p < 0.0001$).

Direct points:

Page 4, line 33 onwards: Please specify more the transcription factors identified. There are also interesting transcription factors like STAT2 and IRF2 involved in innate immune signaling, which are avoided by HBV during infection.

Response:

We thank the Reviewer for this insightful suggestion. Discriminant regulon expression analysis (DoRothEA) predicted that the enhanced T cell responses in acute hepatitis B (AHB) patients are driven by specific signaling pathways and transcription factors. Notably, this includes key innate immune modulators such as JAK-STAT pathways, as well as the transcription factors IRF2 and RELA^{10, 11}, suggesting a complex regulatory network shaping T cell dynamics. However, the analysis also revealed downregulation of key innate immune modulators in myeloid cells, including NF- κ B, JAK-STAT pathways, and the transcription factor RELA (Reviewer Fig. 4a, b). These findings are consistent with previous reports showing that patients with acute HBV infection do not mount a robust innate immune response to the virus^{12, 13}. We have specified this information in the Results section (page 4, lines 33-35) and incorporated the new data into Supplementary Fig. 1g, h, with corresponding updates in the Results section (page 5, lines 16–21).

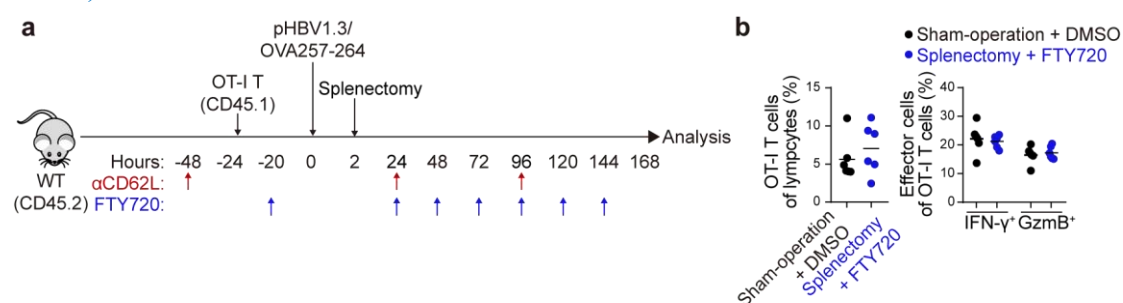


Reviewer Fig 4. DoRothEA analysis of signaling pathways and transcription factor activity in myeloid cells from patients with AHB compared to HDs. **a, b** Bar plots illustrating the activities of highly variable pathways (**a**) and transcription factors (**b**) in myeloid cells for AHB patients versus HDs. Bars represent TF activity levels, with AHB shown in red and HD in blue.

Page 6, line 12, and suppl. Figure 2g: Besides anti-CD62L antibody, does FTY720, which is used later in the manuscript, show similar results?

Response:

We thank the Reviewer for this suggestion. Indeed, splenectomy combined with FTY720 treatment yielded similar results to splenectomy with anti-CD62L antibody treatment. The CD8⁺ T cell response in the liver remained unchanged after splenectomy and FTY720 treatment (Reviewer Fig. 5a, b), which inhibits T cell egress from lymph nodes (LNs). This finding further supports that the liver serves as the primary priming site for CD8⁺ T cells responding to HBsAg, at least in mice. These new data have been incorporated into Supplementary Fig. 2g, i, with corresponding updates in the Results section of the revised manuscript (page 6, lines 19-20).



Reviewer Fig 5. Confirmation of the liver as the primary priming site for CD8⁺ T cells against HBsAg through splenectomy and FTY720 treatment. **a** Overview of the experimental design. **b** Percentages of total (**b**, left), IFN-γ⁺, or GzmB⁺ (**b**, right) OT-I T cells in the livers from WT recipients pretreated with splenectomy and FTY720 versus sham-operation and DMSO, at day 7 post-pHBV1.3/OVA257-264 HDI ($n = 6$ mice per group). At least two independent experiments were performed. Statistical analysis was performed using two-tailed Student's *t*-tests.

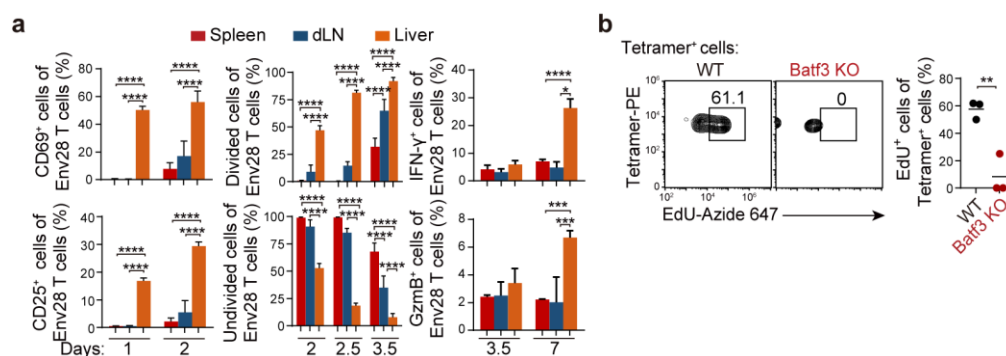
Page 6, line 12: The CD8 T cells are against the OVA-Peptide SIINFELK (OT-I T cells). Those CD8 T cells most likely have a higher affinity to the TCR than HBsAg-specific CD8 T cells which might severely affect CD8 T cell priming and response.

So, the conclusion “...corroborating the essential role of the liver in priming CD8⁺ T cells against HBsAg.” is not supported by the data. May the wording should be adjusted here.

This lower affinity is seen later by comparing Figure 2g and 2i. Env-specific CD8 T cells proliferate much less compared to OT-I T cells. However, it would be interesting to see the proliferation index in Figure 2K, similar to Figure 2e.

Response:

We thank the Reviewer for this thoughtful input. As noted, HBV Env28 TCR T cells exhibit lower affinity for the HBsAg-specific peptide (IPQSLDSWWTSL) than OT-I T cells do for the OVA peptide (SIINFEKL). To assess whether TCR affinity affects CD8⁺ T cell priming and response, we examined HBV Env28 TCR T cells and found that, similar to OT-I cells, they underwent early activation, increased division, and enhanced effector function in the liver compared to the spleen and liver-draining lymph nodes (dLNs) (Reviewer Fig. 6a). These findings indicate that the liver's essential role in priming CD8⁺ T cells against HBsAg is independent of TCR affinity differences. Additionally, as suggested by the Reviewer, we examined endogenous HBsAg-specific CD8⁺ T cell proliferation using EdU incorporation assays in WT and Batf3 KO mice following pHBV1.3 hydrodynamic injection. The results showed a significant reduction in EdU-positive HBV ENV353 tetramer-specific CD8⁺ T cells in Batf3 KO mice (Reviewer Fig. 6b), further supporting cDC1s' critical role in priming HBsAg-specific CD8⁺ T cell responses. These new data have been incorporated into Supplementary Fig. 2j and Fig. 2l, with corresponding updates in the Results section of the revised manuscript (page 6, lines 20-23; page 7, lines 17-21).



Reviewer Fig 6. The response of HBV Env28 TCR T cells in liver and EdU incorporation assays. **a** Percentages of CD69⁺ or CD25⁺ activated cells, divided or undivided cells, and IFN-γ⁺ or GzmB⁺ differentiated cells among Env28 T cells in the specified tissues from WT recipients at the indicated time points following pHBV1.3 HDI (*n* = 3-6 mice per group). **b** Representative FACS plots and summary data showing percentages of EdU⁺ cells among HBV ENV353 tetramer-specific CD8⁺ T cells in the livers of Batf3 KO versus WT mice at day 5 post pHBV1.3 HDI (*n* = 3 mice per group). At least two independent experiments were performed. Statistical analysis was performed using two-tailed Student's *t*-tests. Statistically significant results were labeled (***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

Page 6, lines 17-20: The statement should be toned down: The assay used, detects the

response of CD8 T cells against an OVA-peptide not against HBsAg-specific CD8 T cells.

Response:

We thank the Reviewer for this valid comment. In this study, we used pHBV1.3/OVA257-264, a recombinant HBV replicon that expresses OVA257-264 within HBsAg, to induce OT-I T cell responses in the context of HBV replication. While this assay primarily detects responses to OVA peptide rather than HBsAg itself, it serves as a model for studying HBsAg-related T cell responses. As suggested by the Reviewer, we have revised the wording from “HBsAg-specific CD8⁺ T cells” to “HBsAg-related CD8⁺ T cells” to more accurately reflect the experimental design and tone down the expression. This revision is reflected in the Results section of the revised manuscript (page 6, line 28).

Page 7, line 20 onwards: Although the model is very sophisticated and impressive, the CD8 T cell response is against the OVA-peptide and not directed against HBsAg. The authors should think about an alternative statement.

Response:

We thank the Reviewer for this valid comment. To more accurately describe our experimental results, we have revised the wording from “a non-secreted variant of HBsAg” to “a non-secreted variant of recombinant HBsAg.” Additionally, we have added “recombinant” before HBsAg where appropriate to better reflect the experimental model. This revision is reflected in the Results section of the revised manuscript (e.g., page 7, line 36).

Page 8, line 26-28, and specifically Figure 3h: I am confused about the statement, specifically in the figure legend 3h: the Figure legend states: “h Representative Violet tracer dilution in OT-I T cells (left) and summary data showing proliferation index (right) of Env28 T cells...”. Why are left OT-I T cells and for quantification Env28-specific CD8 T cells? This might affect the interpretation as OT-I are H2-Kb restricted and Env28 are H2-Kd-restricted.

Response:

We apologize for the confusion caused by the error in the figure legend. Both the left and right panels in Figure 3h represent HBV Env28 CD8⁺ T cells. This has been corrected in the figure legend of the revised manuscript (page 45, line 3-6).

Page 8, line 29-30, specifically suppl. Figure 3p+r: The authors have transferred C57BL/6 bone marrow (H2-Kb) and measure the proliferation index of Env28 T cells which are H2-Kd-restricted. Can the authors explain?

Response:

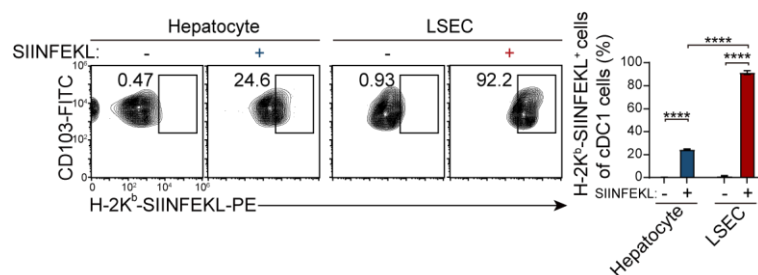
We apologize for the insufficient description of the experimental setup in our original

manuscript. The goal of these experiments was to confirm that C57BL/6 (H-2K^b)-derived cDC1s could mediate an H-2K^d-restricted Env28 T cell response in the pcccDNA and AdHBV models, similar to the approach used in Fig. 3h for the pHBV1.3 model. To clarify, we transferred Batf3 KO or C57BL/6 (H-2K^b) bone marrow into lethally irradiated F1 (C57BL/6 × BALB/c) mice. After successful reconstitution, we transferred HBsAg-specific Env28 CD8⁺ T cells (H-2K^d-restricted) into these mice. The data showed that C57BL/6-derived cDC1s successfully mediated the H-2K^d-restricted response of Env28 T cells following pcccDNA and AdHBV infection (now presented in Supplementary Fig. 5f, g). These findings suggest that cDC1s acquired the H-2K^d-Env28 peptide complex through cross-dressing. We have expanded the Results section to clarify this point in the revised manuscript (page 9, lines 7-12).

Page 8, line 35-37: “suggesting that the HBsAg peptide MHC-I complexes...HBsAg-producing hepatocytes”. In Figure 2c, the authors have demonstrated that LSEC also present SIINFEKL-MHC-I complexes. As LSEC might have more intimate contact with DCs, it may very likely be possible that LSEC cross-dressed the DCs. Could the authors exclude that cross-dressing occurs from LSEC? This might also be shown, e.g. with coculture of LSEC and/or primary hepatocytes and cCD1 and check for transfer of MHC-I molecules.

Response:

We sincerely thank the Reviewer for this thoughtful and insightful suggestion. Indeed, as the Reviewer noted, LSECs can uptake dying hepatocytes (Supplementary Fig. 4j) and present H-2K^b-SIINFEKL complexes at higher levels than hepatocytes (Fig. 2c), as shown in our original manuscript. This led us to hypothesize that LSECs, in addition to HBsAg-producing hepatocytes, may contribute to cDC1 cross-dressing. To test this, we loaded SIINFEKL onto both LSECs and hepatocytes and cocultured them with cDC1s to assess H-2K^b-SIINFEKL complex transfer. Flow cytometry analysis revealed that both LSECs and hepatocytes transferred these complexes to cDC1s, with LSECs demonstrating greater efficiency (Reviewer Fig. 7). These results suggest that cDC1s likely acquire HBsAg peptide-MHC-I complexes from irradiation-resistant cells, particularly LSECs, to drive the CD8⁺ T cell response against HBsAg. However, further studies are needed to elucidate the exact mechanisms of MHC-I-peptide complex transfer from LSECs or hepatocytes to cDC1s, potentially using imaging and genetic approaches. We have included these new data in Fig. 3l, with corresponding updates in the Results section (page 10, lines 1-10) and an expanded discussion (page 16, lines 27-30) of the revised manuscript.

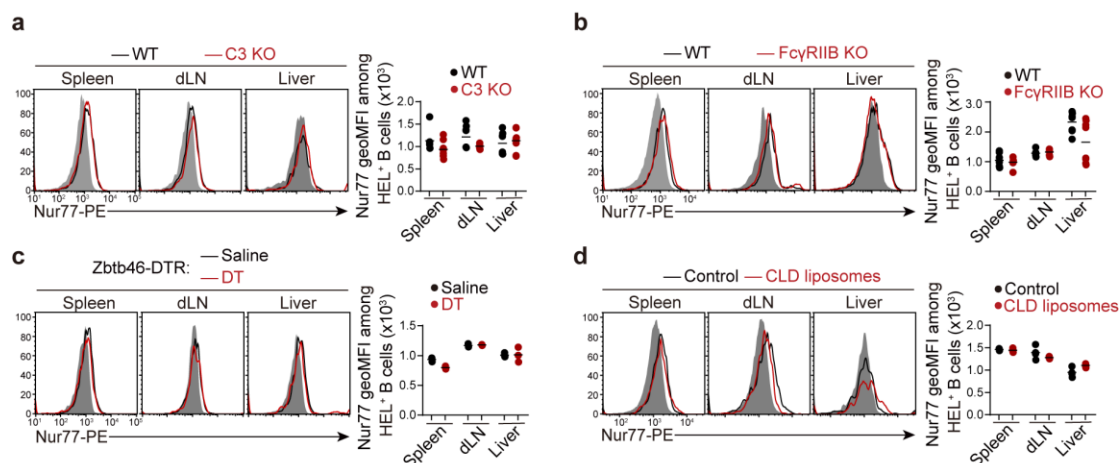


Reviewer Fig 7. Hepatocytes and LSECs present SIINFEKL-MHC-I complexes to cDC1s. Representative FACS plots (left) and summary data (right) showing percentages of H-2K^b-SIINFEKL expressing cDC1s after a 1-day co-culture with unpulsed or SIINFEKL-pulsed hepatocytes or LSECs ($n = 3$ biological replicates). At least two independent experiments were performed. Statistical analysis was performed using two-tailed Student's t -tests. Statistically significant results were labeled (**** $p < 0.0001$).

Page 10, lines 22-33: Addressing the importance of the size of the antigen, did the authors had the chance to compare the uptake of the particular HBsAg by B cells to the uptake of the more soluble HBeAg?

Response:

We thank the Reviewer for this helpful suggestion. Due to the lack of HBeAg-specific BCR knockin mice, directly comparing HBeAg uptake to HBsAg in our experiments is challenging. However, we addressed the importance of antigen size by using hen egg lysozyme (HEL) (~14.4 kDa) as a small soluble antigen control, leveraging HEL-specific Hy10 BCR knockin mice available in our lab. To do this, we constructed a soluble HEL-expressing plasmid (pALB-Ig κ /HEL) under the hepatocyte-specific ALB promoter, incorporating an Ig κ leader sequence to facilitate secretion. Following hydrodynamic injection, we observed that HEL induced intact BCR signaling in HEL-specific Hy10 B cells even after knockout of C3 or Fc γ RIIB expression (Reviewer Fig. 8a, b), or depletion of cDCs or macrophages (Reviewer Fig. 8c, d). These control experiments further support our conclusion that HBsAg behaves similarly to small soluble antigens in accessing lymphoid follicles to activate B cells. We have included these data in Supplementary Fig. 8a-d, with corresponding updates in the Results section of the revised manuscript (page 12, lines 12-15).

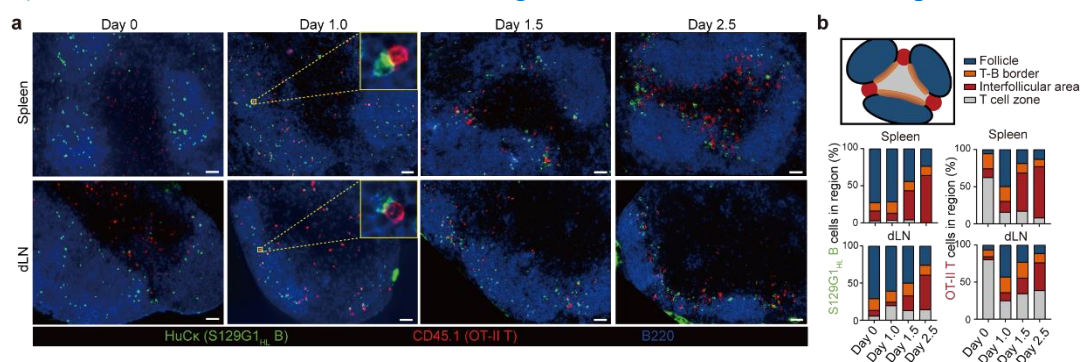


Reviewer Fig 8. Soluble HEL accesses follicular B cells independently of opsonization. a, b Representative histograms (left) and summary geoMFI data (right) showing Nur77 expression in transferred Hy10 B cells in the spleens, dLNs, and livers of C3 KO (a) and FcγRIIB KO (b) compare to their respective WT control, at 18 hours post- pALB-Igk/HEL HDI ($n = 8$ mice per group). **c, d** Representative histograms (left) and summary geoMFI data (right) showing Nur77 expression in transferred Hy10 B cells in the spleens, dLNs, and livers of Zbtb46-DTR chimeras treated with DT (c, $n = 3$ mice per group) or CLD liposomes treated WT mice (d, $n = 4$ mice per group), compared to their respective controls, at 18 hours post- pALB-Igk/HEL HDI. At least two independent experiments were performed. Statistical analysis was performed using two-tailed Student's *t*-tests.

Page 11, lines 1-3: The authors conclude that half of the OT-II had migrated from the T cell zone to follicular regions of the spleen and dLN. It might be argued that the OT-II already had been in follicular region. To make the conclusion that they migrated, I am missing the negative control, uninfected or timepoint 0.

Response:

We thank the Reviewer for this valid comment. To clarify OT-II cell positioning before migration, we have included day 0 (0-hour) as a negative control. The 18-hour time point was deemed redundant and therefore not included. The results show that at day 0, the majority of OT-II cells were located in the T cell zone (Reviewer Fig. 9a, b). These data have been included in Fig. 6a, b of the revised manuscript.



Reviewer Fig 9. Interactions of S129G1_{HL} B cells and OT-II T cells following hydrodynamic injection of pHBV1.3/OVA323-339. a Immunofluorescence of S129G1_{HL} B cells (HuCk, green), OT-II T cells (CD45.1, red), and endogenous B cells (B220, blue) in the spleens and dLNs from WT recipients at the indicated time points post -pHBV1.3/OVA323-339 HDI. **b** Anatomical schematic representation of the spleen and dLN (top), percentages of S129G1_{HL} B cells (bottom, left) and OT-II T cells (bottom, right) in the specified regions stained in (a) (Top: $n = 6$ white pulp from 3 mice; Bottom: $n = 4-6$ LN slices from 4-6 mice). At least two independent experiments were performed. Scale bars: 50 μ m.

Minor:

Page 2, line 37: "...after their acquiring of antigens."

Response:

We thank the reviewer for this suggestion. We have revised the phrase to "after their acquisition of antigens," which aligns better with formal scientific writing conventions. This change has been incorporated into the revised manuscript (Page 2, line 32).

Page 8, line 23: IINFEKL, the "S" is missing.

Response:

We thank the reviewer for pointing out this oversight. The missing "S" in "SIINFEKL" has been corrected in the revised manuscript (Page 8, line 38).

Page 12, line 1: "... B cells and cDC1s drove CD8 T and CD4 T cell..., respectively. It seems that the order is wrong.

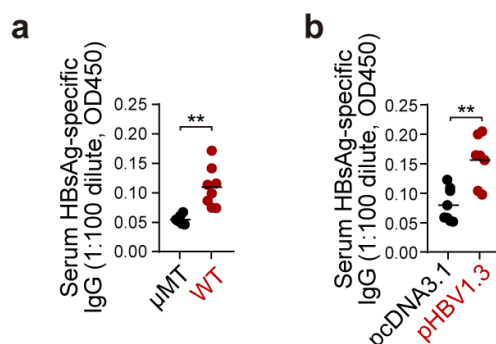
Response:

We thank the reviewer for pointing out this error. The order has been corrected to ensure clarity and consistency in the revised manuscript (Page 13, line 37).

Page 15, line 28-31: To me, it would not be surprising that B cells take up the antigen independent of IgG-mediated opsonization. At indicated time points here, I would guess that no IgG are produced, because it is too early for this.

Response:

We thank the Reviewer for this thoughtful comment. We acknowledge the concern regarding the timing of IgG production. However, our data show that natural IgG binding to HBsAg was detectable in WT mice but absent in B cell-deficient μ MT mice (Reviewer Fig. 10a). Furthermore, HBsAg-specific IgG was detected as early as 18 hours after hydrodynamic injection of pHBV1.3 in WT mice (Reviewer Fig. 10b). These findings suggest that HBsAg's ability to bypass opsonization or conduit system size constraints is not due to a lack of HBsAg-specific antibodies. We have included these new data in Supplementary Fig. 8e, f, with corresponding updates in the Results section of the revised manuscript (page 12, lines 19-23).



Reviewer Fig 10. Detection of naturally occurring and infection-induced HBsAg-specific IgG. a Serum HBsAg-specific IgG levels from naïve WT and μ MT mice. **b** Serum HBsAg-

specific IgG levels from mice that underwent HDI with either pcDNA3.1 or pHBV1.3 for 18 hours. At least two independent experiments were performed. Statistical analysis was performed using two-tailed Student's *t*-tests. Statistically significant results are labeled (***p* < 0.01).

1 Bertolino, P., Trescol-Biemont, M. C. & Rabourdin-Combe, C. Hepatocytes induce functional activation of naive CD8⁺ T lymphocytes but fail to promote survival. *European journal of immunology* 28, 221-236 (1998). [https://doi.org/10.1002/\(SICI\)1521-4141\(199801\)28:01<221::AID-IMMU221>3.0.CO;2-F](https://doi.org/10.1002/(SICI)1521-4141(199801)28:01<221::AID-IMMU221>3.0.CO;2-F)

2 Holz, L. E., Warren, A., Le Couteur, D. G., Bowen, D. G. & Bertolino, P. CD8⁺ T cell tolerance following antigen recognition on hepatocytes. *Journal of autoimmunity* (2009).

3 De Simone, G. et al. Identification of a Kupffer cell subset capable of reverting the T cell dysfunction induced by hepatocellular priming. *Immunity* 54, 2089-2100 e2088 (2021). <https://doi.org/10.1016/j.immuni.2021.05.005>

4 Heim, K. et al. Attenuated effector T cells are linked to control of chronic HBV infection. *Nat Immunol* 25, 1650-1662 (2024). <https://doi.org/10.1038/s41590-024-01928-4>

Response:

We sincerely thank the Reviewer for these valuable and insightful references.

Reviewer #3 (Remarks to the Author):

In this manuscript by Li X. et al. the authors investigate the early stages of antigen presentation during Hepatitis B virus infection. After analyzing immune cells in blood of patients with acute HBV infection, the authors used multiple mouse models of acute HBV infection. In these mouse models, the authors convincingly demonstrate the predominance of cDC1 in promoting CD8 T cell response. Interestingly, the authors show that cDC1 were acquiring their antigen. Presentation capabilities through cross-dressing. The authors also show that the CD4 T cell response is independent on cDC but dependent on B cells that acquire the antigen in B cell follicle of Secondary lymphoid organs (SLO). Finally, the authors identified CCR5, EBI2 and LFA-1, CD80 for the positioning and the interaction between CD4 T cells and B cells in SLO. Whereas CD8 T cells interact with cDC1 through a CD6 and ALCAM. Overall, the experiments are well conducted, and many approaches were confirmed with complementary ones.

Response:

We sincerely thank the Reviewer for the positive and encouraging feedback. We appreciate your thoughtful review and are pleased to address your suggestions in the revised manuscript.

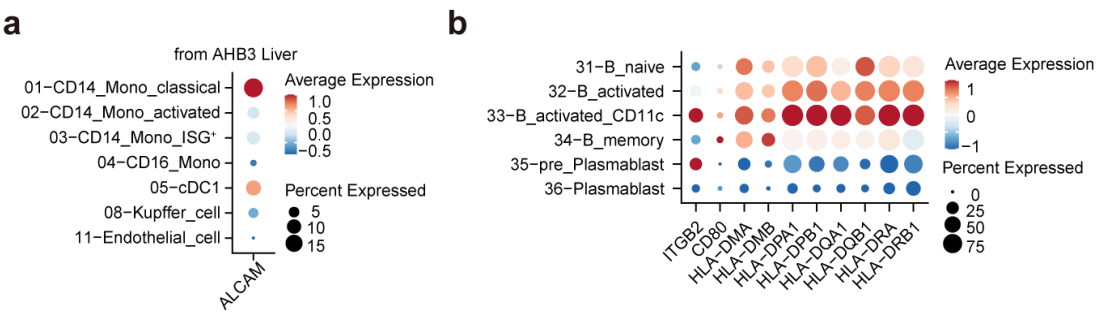
Major points:

-The authors unveil precise mechanisms for CD4 and CD8 T cell activation but it is unclear what pertains to a mechanism occurring during acute human HBV infection rather to their experimental model. For example, it is unclear if ALCAM was also detected in cDC1 or if CD80 is increased on B cells of HBV infected patients.

Response:

We sincerely thank the Reviewer for this important question. We acknowledge the need to clarify how our findings in HBV mouse models relate to acute human HBV infection. In this study, we used the pHBV1.3, prcccDNA, AdHBV, and AAV8-HBV mouse models to investigate HBsAg presentation mechanisms relevant to T cell responses. These models support HBV gene expression, genome replication, and progeny virus production specifically in hepatocytes, closely mirroring key virological aspects of human HBV infection^{14, 15}. While they do not fully replicate human HBV infection dynamics, they provide valuable insights into viral immunopathogenesis^{16, 17, 18, 19}.

To address the Reviewer’s concern, we analyzed scRNA-seq data from PBMCs and liver biopsies of a patient with acute HBV infection and found ALCAM mRNA expression in cDC1s (Reviewer Fig. 11a). Additionally, scRNA-seq analysis of sorted HBsAg-specific B cells from four patients revealed increased expression of *Itgb2* (encoding LFA-1) and CD80 mRNA in activated B cells, along with upregulation of HLA-II genes (Reviewer Fig. 11b). These findings suggest that ALCAM, LFA-1, and CD80 may contribute to T cell responses during acute human HBV infection. We have incorporated these new data into Supplementary Fig. 10h, o, with corresponding updates in the Results section of the revised manuscript (page 14, lines 25-28; page 15, lines 13-17). Additionally, we have expanded the Discussion section in the revised manuscript to further address the relevance of murine HBV models to human infection (page 19, lines 9-38).

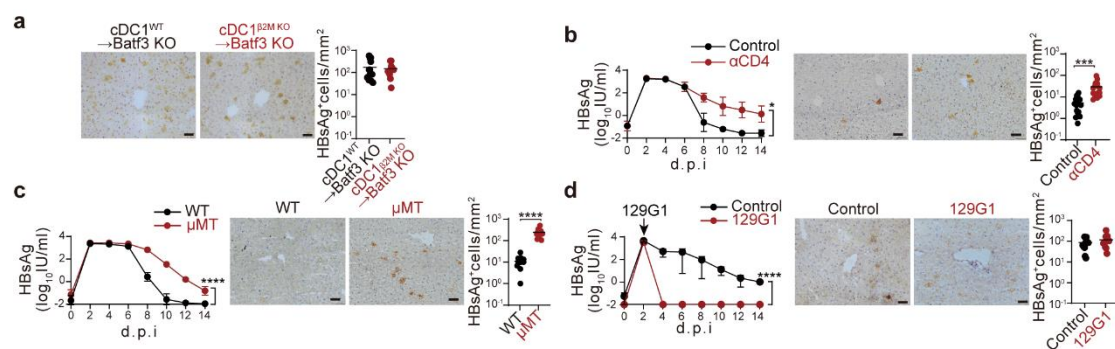


Reviewer Fig 11. ALCAM mRNA expression in human cDC1s, and *Itgb2* and CD80 mRNA expression in human B cells. a Dot plot illustrating ALCAM mRNA expression in liver cells from one AHB patient. **b** Dot plot illustrating *Itgb2*, CD80, and HLA-II mRNA expression in HBsAg-specific B cells from PBMCs of four AHB patients.

-It is unclear from this study if these non-canonical ways for CD4 and CD8 T cell activation are necessary for the clearance of the infection or for the establishment of a chronic disease.

Response:

We thank the reviewer for these important questions. First, we investigated whether the two non-canonical antigen presentation pathways contribute to HBsAg clearance in the pHBV1.3 mouse model. To assess the role of MHC-I cross-dressing of cDC1s in intrahepatic HBsAg clearance, we examined whether MHC-I expression on cDC1s was required for this function. While our data confirm that cDC1s play an important role in intrahepatic HBsAg clearance (Fig. 2m, previously shown in the original manuscript), MHC-I expression on cDC1s was not required (Reviewer Fig. 12a). This suggests that cDC1 cross-dressing may contribute to the clearance of intrahepatic HBsAg. Similarly, in B cell-deficient μ MT mice, both circulating and intrahepatic HBsAg clearance were reduced (Reviewer Fig. 12c). However, injection of the HBsAg-specific antibody 129G1 cleared only circulating HBsAg, without affecting intrahepatic HBsAg clearance (Reviewer Fig. 12d). These findings indicate that B cells may not only mediate an antibody response, with the assistance of Tfh cells, to clear circulating HBsAg, but also initiate effector CD4⁺ T cell responses to clear intrahepatic HBsAg. Together, these results suggest that the two non-canonical antigen presentation pathways involving cDC1s and B cells contribute to HBsAg clearance. These data have been incorporated into Fig. 3i, 4h, and Supplementary Fig. 7m, n, with corresponding descriptions added to the Results section of the revised manuscript (page 9, lines 26-29; page 11, lines 16-22).



Reviewer Fig 12. cDC1- and B cell-mediated non-canonical antigen presentation contributes to HBsAg clearance. **a** Intrahepatic HBsAg levels in Batf3 KO mice reconstituted with either β 2M KO or WT cDC1s, measured on day 14 after pHBV1.3 HDI ($n = 15$ view fields from 5 mice). **b** Circulating (left, $n = 4$ mice per group) and intrahepatic (right, $n = 15$ view fields from 5 mice) HBsAg levels in WT mice treated with anti-CD4 or control antibody, with serum collected at the indicated time points and liver tissues on day 14 after pHBV1.3 HDI. **c** Circulating (left, $n = 4$ mice per group) and intrahepatic (right, $n = 15$ view fields from 5 mice) HBsAg levels in WT and μ MT mice, with serum collected at the indicated time points and liver tissues on day 14 after pHBV1.3 HDI. **d** Circulating (left, $n = 4$ mice per group) and intrahepatic (right, $n = 15$ view fields from 5 mice) HBsAg levels in μ MT mice treated with control or 129G1 antibody, with serum collected at the indicated time points and liver tissues on day 14 after pHBV1.3 HDI. At least two independent experiments were performed. Statistical analyses for serum HBsAg (b-d) were conducted using two-way ANOVA, while other comparisons were analyzed using two-tailed Student's t -tests. Statistically significant results are indicated (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$).

0.0001). Scale bars: 50 μ m.

Next, we investigated whether HBV manipulates the two non-canonical antigen presentation pathways to establish chronic infection using a chronic AdHBV mouse model, where a low infection dose (1×10^8 i.u.) was used to induce persistent infection¹⁷. At an early stage of chronic AdHBV infection, we found that cDC1s were required for endogenous CD8⁺ T cell responses in the liver (Reviewer Fig. 13a), but MHC-I expression on cDC1s was not necessary (Reviewer Fig. 13b). Furthermore, by reconstituting F1 mice with either Batf3 KO or C57BL/6 (H-2K^b) bone marrow, we demonstrated that C57BL/6-derived cDC1s could mediate the H-2K^d-restricted response of Env28 T cells (Reviewer Fig. 13c). These results suggest that, similar to acute HBV infection, cDC1s drive CD8⁺ T cell responses against HBsAg in the liver via MHC-I cross-dressing at the early stage of chronic AdHBV infection. To compare T cell responses at early and late stages of chronic AdHBV infection, we transferred HBV Env28 TCR transgenic T cells into mice at both time points (Reviewer Fig. 13d). Compared to the early stage, Env28 T cells exhibited weak proliferation and differentiation at the late stage (Reviewer Fig. 13e-h). Notably, this weak response at the late stage persisted even when cDC1 recruitment was blocked using an anti-XCL1 antibody (Reviewer Fig. 13g, h), suggesting that cDC1s may no longer be required for T cell responses at later stages of infection. The mechanisms underlying this transition remain unclear and warrant further investigation in future studies.

The essential role of B cells in initiating endogenous CD4⁺ T cell responses to HBsAg was also demonstrated in μ MT mice at the early stage of chronic AdHBV infection (Reviewer Fig. 13i, j). To investigate the functional state of S129G1_{HL} B cells during HBV persistence, we crossed S129G1_{HL} mice with HBV-transgenic (HBV-Tg) mice. In this double-transgenic model, HBsAg-specific S129G1_{HL} B cells (HBV Tg-S129G1_{HL} B cells) exhibited impaired antigen uptake and presentation capabilities (Reviewer Fig. 13k, l), suggesting that B cells may lose their ability to initiate CD4⁺ T cell responses as HBV persistence is established. A portion of these findings regarding B cell deficiency was orally presented at the 2023 International HBV Meeting (Abstract book, page 74).

Taken together, these results demonstrate that HBV can manipulate the two non-canonical antigen presentation pathways to establish chronic infection. However, further studies are needed to elucidate the underlying mechanisms. The new data on the early stage of chronic AdHBV infection have been incorporated into Supplementary Fig. 5m-o and Supplementary Fig. 7i, j, with corresponding descriptions added to the Results section (page 9, lines 12-19; page 11, lines 9-12). Given the scope of these findings and space limitations, we believe it is more appropriate to present the data on the late stage of chronic AdHBV infection and the double-transgenic model in a separate manuscript rather than including them here. Nonetheless, we have discussed these findings in the revised Discussion section (page 16, lines 30-32; page 18, lines 23-25).

Representative FACS plots (left) and summary data (right) showing percentages of endogenous CD25⁺OX40⁺ (i, *n* = 4 mice per group) and T-bet⁺GzmB^{hi} (j, *n* = 4 mice per group) CD4⁺ T cells in the spleens, dLNs, and livers from WT or μ MT mice, at day 5 after AdHBV infection with a dose of 1×10^8 i.u. k Representative histograms and summary geoMFI data showing surface (left) and intracellular (right) uptake of iFluor488-labeled HBsAg by S129G1_{HL} and HBV Tg-S129G1_{HL} B cells (*n* = 3 biological replicates). l Summary geoMFI data of CD44 (left) and Nur77 (right) expression in OT-II T cells co-cultured for 24 hours with S129G1_{HL} or HBV Tg-S129G1_{HL} B cells in the presence or absence of HBsAg-OVA (*n* = 3 biological replicates). At least two independent experiments were performed. Statistical analysis was performed using two-tailed Student's *t*-tests. Statistically significant results are labeled (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

-In the manuscript the authors do not specify if the patients were under treatment during sample collection. The (potential) treatment these patients could have received is key for the data interpretation.

Response:

We thank the Reviewer for highlighting the importance of treatment information. We clarify that PBMC samples from four patients were collected between 2-14 days after symptom onset and prior to treatment, except for AHB3, whose PBMC sample and liver biopsy were collected during treatment (Reviewer Table 1). While liver biopsy in acute HBV cases is uncommon, AHB3's biopsy was performed during the typical stabilization phase of acute HBV, when such procedures are generally considered safer. Given that AHB3 was the only patient sampled during treatment, treatment effects are unlikely to have significantly influenced our overall conclusions regarding PBMCs but should be considered when interpreting findings from liver cells. We have updated the sampling and treatment details in the revised Supplementary Table 1.

Reviewer Table 1. Summary of patient treatment details during sample collection.

	AHB1	AHB2	AHB3	AHB4	AHB5
Age (years)	27	58	44	53	32
Sex (Male/Female)	F	M	M	M	M
Date of treatment beginning	11/19/2014	9/23/2021	2/18/2020	12/7/2021	6/17/2021
Date of blood sampling	11/18/2014	9/20/2021	2/28/2020	12/6/2021	6/15/2021
Date of liver sampling	NA	NA	2/28/2020	NA	NA
Treatment	GL GSH PPC	GL GSH AM lamivudine	GL GSH PPC	GL GSH AM Entecavir	GSH MGIG PPC BA
Abbreviations: GL, glycyrrhizin; GSH, glutathione; PPC, polyene phosphatidyl choline; AM, adenosine methionine; MGIG, magnesium isoglycyrrhizinate; BA, bicyclic alcohol; NA, not available.					

-The conclusion of the fig 1 “Overall, our scRNA-seq analysis indicates the potential presence of novel intercellular mechanisms of HBsAg presentation underlying T cell

immunity in acute HBV patients...” is not supported by the data. It is unclear what novel intracellular mechanism of antigen presentation can be concluded from the scRNA-seq.

Response:

We thank the Reviewer for this valuable feedback. Upon reflection, we recognize that our initial statement regarding the identification of novel intercellular mechanisms of HBsAg presentation may have been overstated and not fully supported by the current scRNA-seq data from human samples. Our analysis suggests that cDC2s may not play a significant role in initiating CD4⁺ T cell responses, while cDC1s could be involved in priming CD8⁺ T cells in acute HBV patients. However, elucidating novel mechanisms of antigen presentation requires further investigation, particularly through complementary studies using acute HBV mouse models. Therefore, we have revised the conclusion to: *"Overall, our scRNA-seq analysis suggests that HBsAg presentation in acute HBV patients may involve cDC1s, independent of cDC2s, highlighting a distinct cellular basis for T cell immunity."* This revision is now reflected in the Results section of the revised manuscript (page 5, lines 37-39). Additionally, we have adjusted the corresponding statement in the Introduction section (page 3, lines 25-26) to ensure consistency.

-Is the cross-dressing of cDC1 dependent on the infection? Was Sup Fig 3n done in absence of infection? What time point after reconstitution was this experiment conducted?

Response:

We thank the Reviewer for these important questions. Our study demonstrates cDC1 cross-dressing in the context of HBV replication, but whether this phenomenon occurs under normal, uninfected homeostatic conditions remains unclear. Regarding Supplementary Fig. 3n (now updated as Supplementary Fig. 5c in the revised manuscript), this experiment was conducted before hydrodynamic infection with pHBV1.3/OVA257-264 to confirm that cDC1s in the mice receiving BALB/c bone marrow were exclusively BALB/c-derived. We have clarified this point in the supplementary figure legend of the revised manuscript (page 63, line 11). As for the time point after reconstitution, while we are unsure which specific experiment the Reviewer is referring to, we have made efforts to ensure that time points are clearly indicated in the figure legends where relevant.

-It is unclear if the cross-dressing is also occurring in other hepatic diseases or if specific inflammatory molecules promote it.

Response:

We thank the Reviewer for these insightful questions. Currently, our study focuses on cDC1 cross-dressing in the context of acute HBV infection, and we have not explored whether this phenomenon occurs in other hepatic diseases. While DC cross-dressing

has been implicated in anti-tumor, anti-viral, and anti-allograft immunity in certain experimental contexts^{20, 21, 22, 23}, studies on its role in hepatic diseases remain limited. One report suggests that graft-infiltrating PD-L1^{hi} cross-dressed DCs may play a key role in liver transplant tolerance²⁴. However, the involvement of DC cross-dressing in liver inflammation, fibrosis, cancer, or other hepatic conditions remains largely unexplored. This highlights the need for further investigation, which we have now emphasized in the Discussion section of the revised manuscript (page 17, lines 4-11).

Additionally, HBV transgenic mice, a non-inflamed model of chronic HBV infection, demonstrate that hepatocytes can directly present antigens to virus-specific naïve CD8⁺ T cells, triggering initial proliferation but ultimately failing to induce IFN- γ production or cytolytic effector function due to insufficient co-stimulatory signals^{25, 26}. In contrast, our study provides strong evidence that intrahepatic resident Batf3⁺XCR1⁺CCR7⁻ cDC1s drive CD8⁺ T cell proliferation and differentiation via MHC-I cross-dressing in acute pHBV1.3, prcccDNA, and AdHBV mouse models (Fig. 3 and Supplementary Fig. 5 in the revised manuscript), which exhibit relatively high levels of inflammation. Similarly, cDC1 cross-dressing was observed at an early stage of chronic AdHBV (established with an infection dose of 1×10^8 i.u.¹⁷) and AAV8-HBV mouse models (Supplementary Fig. 5m-r in the revised manuscript), both of which caused mild yet observable liver inflammation (Supplementary Fig. 5s in the revised manuscript)^{17, 27}. These findings raise the possibility that cDC1 cross-dressing may not occur in the absence of inflammation but is instead triggered under specific inflammatory conditions. However, the precise inflammatory molecules involved and the underlying mechanisms remain to be elucidated. An intriguing avenue for future research would be to investigate how MHC-I-peptide complexes are transferred from LSECs or hepatocytes to cDC1s, utilizing imaging and genetic approaches in an inflammatory context. We have expanded the Discussion section to highlight these points in the revised manuscript (page 16, lines 15-30).

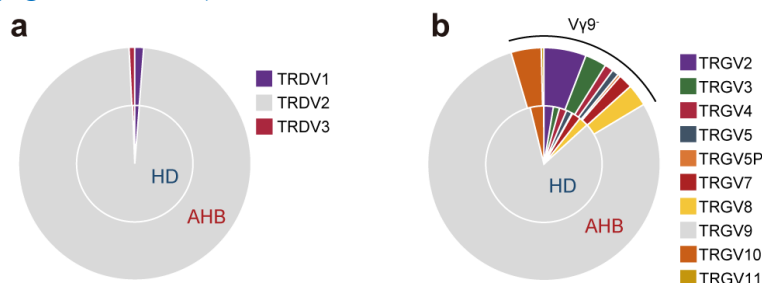
Minor points:

-gamma-delta T cells (gd T cells) have been reported to play a role in multiple liver diseases. The authors do not comment on the loss of gd T cells in infected patients. V delta 2 T cells have been reported to enriched in chronically HBV-infected patients. The decrease in gd T cells found by the authors could favor a predominance of certain gd T cell clones.

Response:

We thank the Reviewer for raising this important point regarding gamma-delta ($\gamma\delta$) T cells. As noted, $\gamma\delta$ T cells, particularly V δ 2⁺ T cells, have been reported to play a role in various liver diseases, including chronic HBV infection. In our analysis, we did not observe significant changes in the overall frequency of V δ 2⁺ T cells within the $\gamma\delta$ T cell population in the PBMCs of patients with acute HBV infection (Reviewer Fig. 14a). However, we found an increased proportion of the V γ 9⁻ subset within the $\gamma\delta$ T cell population in acute HBV patients (Reviewer Fig. 14b), consistent with previous

reports that human V γ 9⁺ T cells undergo dominant clonal expansion and exhibit an effector phenotype in adaptive antiviral immunosurveillance²⁸. These new data (Reviewer Fig. 14b) are now included in Supplementary Fig. 1e, and we have expanded the corresponding description in the Results section of the revised manuscript (page 5, lines 2-5).



Reviewer Fig 14. Comparison of T cell receptor variable gene usage in $\gamma\delta$ T cells from HDs and AHBs. a Distribution of T cell receptor delta variable (TRDV) genes among $\gamma\delta$ T cells between HDs and AHBs. **b** Distribution of T cell receptor gamma variable (TRGV) gene usage among $\gamma\delta$ T cells between HDs and AHBs, highlighting the presence of V γ 9⁺ T cells.

Reviewer #4 (Remarks to the Author):

The manuscript Li et.al. investigated pathways of HBV antigen presentation using the hydrodynamic injection to stimulate OT-I CD8 and OT-II CD4 T cell epitopes embedded within HBsAg. The authors investigate cells responsible for CD8 T cell induction and describe the mechanism of MHC cross-dressing through acquisition of peptide-MHC by cDC1 from hepatocytes. The authors also investigated CD4 T cell activation showing that adoptively transferred HBs-specific B cells are the primary cell type responsible for CD4 T cell activation in this system. They investigated mechanisms of B cell and CD4 T cell trafficking within the spleen to demonstrate their interaction.

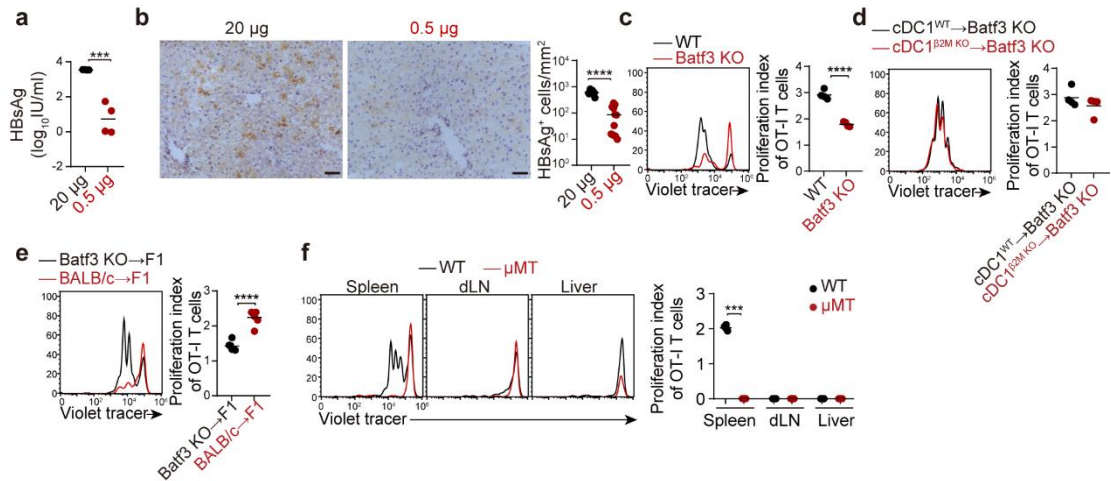
Overall, these data are difficult to reconcile with natural infection and existing literature. Acute hepatitis B has a long incubation phase (6 – 8w) where there is limited activation of innate immunity or detectable virus in the serum. The hydrodynamic transfection model results in immediate expression of a large amount of antigen. Therefore, this model may reflect the peak of antigen expression during acute hepatitis, but controls are missing to confirm that this is 1) not an artifact of rapid antigen expression in many hepatocytes and inflammation induced by the introduction method (hydrodynamic transfection/AdHBV acute model) and 2) specific to HBV. Furthermore, the authors present data from complicated experiments using splenectomized mice and different knockout mouse strains (irf4 fl/fl mice) that are not clearly explained, making it hard to understand the relevance.

Response:

We thank the reviewer for their thoughtful feedback and for highlighting key concerns regarding the relevance of our models. While human samples provide valuable

insights into HBV immune responses, mouse models remain essential for dissecting immune mechanisms that are difficult to study directly in human infections. Although these models do not fully replicate the prolonged incubation phase of acute HBV infection, they serve as powerful tools for investigating specific aspects of viral immunopathogenesis, including antigen presentation and T cell priming^{25, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39}.

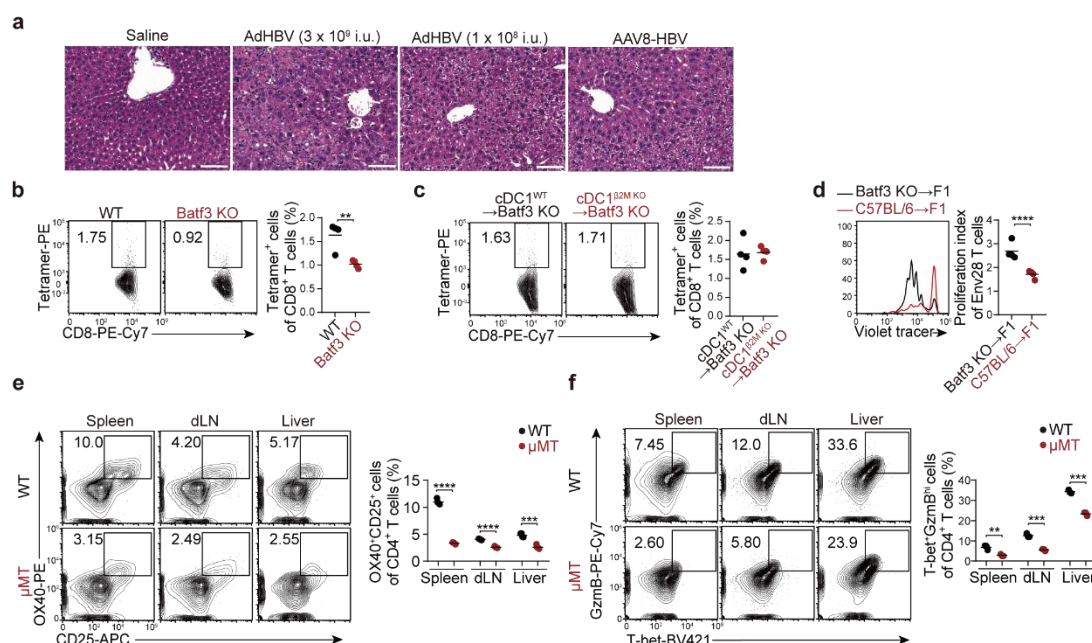
As the reviewer noted, acute HBV infection in humans is characterized by a long incubation period (6-8 weeks) with limited inflammation and undetectable viremia in the early stages. To determine whether the two non-canonical antigen presentation pathways we observed are artifacts of rapid antigen expression induced by hydrodynamic injection or AdHBV infection, we significantly reduced the pHBV1.3 dose from 20 µg to 0.5 µg. This resulted in a ~100-fold decrease in HBsAg levels in sera (from 3000 IU to 30 IU) and a 10-fold decrease in liver tissues (Reviewer Fig. 15a, b). Despite this lower antigen expression, OT-I T cell proliferation remained significantly reduced in Batf3 KO mice at day 3.5 after hydrodynamic injection of 0.5 µg of pHBV1.3/OVA257-264 (Reviewer Fig. 15c). Critically, this proliferation was independent of MHC-I expression on cDC1s, as β 2M KO cDC1s retained the ability to drive OT-I expansion (Reviewer Fig. 15d), demonstrating that MHC-I cross-dressing of cDC1s occurs even at low HBsAg expression levels. To further confirm this, we transplanted bone marrow from Batf3 KO or WT BALB/c (H-2K^d) mice into lethally irradiated F1 (C57BL/6 × BALB/c) recipients. Following low-dose pHBV1.3/OVA257-264 injection, OT-I T cell expansion was observed in mice reconstituted with BALB/c bone marrow but not in those with Batf3 KO bone marrow (Reviewer Fig. 15e). This finding further underscores that the H-2K^b-restricted OT-I response was mediated by BALB/c cDC1s, reinforcing the role of MHC-I cross-dressing of cDC1s even under low HBsAg expression condition. Similarly, at day 3.5 after injection of 0.5 µg of pHBV1.3/OVA323-339, OT-II T cell proliferation was greatly impaired in B cell-deficient μ MT mice (Reviewer Fig. 15f), indicating an important role for B cells in initiating CD4⁺ T cell responses even under low HBsAg expression condition. These results collectively confirm that the two non-canonical antigen presentation pathways remain active even at low HBsAg expression levels, arguing against the idea that they are artifacts of antigen overload. These new data have been incorporated into Supplementary Fig. 5h-l and Supplementary Fig. 7h, with corresponding descriptions added to the Results section of the revised manuscript (page 9, lines 12-19; page 11, lines 9-11).



Reviewer Fig 15. Revealing non-canonical antigen presentation pathways in the low-dose pHBV1.3 mouse model. **a, b** Serum (**a**, $n = 4$ mice per group) and intrahepatic (**b**, $n = 15$ view fields from 5 mice) HBsAg levels in WT mice at day 3 after 20 μg or 0.5 μg pHBV1.3 HDI. **c** Representative Violet tracer dilution in OT-I T cells (left) and summary data showing their proliferation index (right) from the livers of WT and Batf3 KO mice, at day 3.5 after 0.5 μg pHBV1.3/OVA257-264 HDI ($n = 4$ mice per group). **d** Representative Violet tracer dilution in OT-I T cells (left) and summary data showing their proliferation index (right) from the livers of Batf3 KO mice reconstituted with either WT cDC1 or $\beta 2\text{M}$ KO cDC1s, at day 3.5 following 0.5 μg of pHBV1.3/OVA257-264 HDI ($n = 4$ mice per group). **e** Representative Violet tracer dilution in OT-I T cells (left) and summary data showing their proliferation index (right) at day 3.5 in the livers of either BALB/c or Batf3 KO bone marrow-reconstituted F1 chimeras, after 0.5 μg of pHBV1.3/OVA257-264 HDI ($n = 4$ mice per group). **f** Representative Violet tracer dilution in OT-II T cells (left) and summary data (right) showing their proliferation index in the spleens, dLNs, and livers from WT and μMT mice, at day 3.5 after 0.5 μg of pHBV1.3/OVA323-339 HDI ($n = 6$ mice per group). At least two independent experiments were performed. Statistical analysis was performed using two-tailed Student's t -tests. Statistically significant results are labeled (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Scale bars: 50 μm .

To determine whether the observed non-canonical antigen presentation pathways are artifacts of high inflammation induced by hydrodynamic injection of pHBV1.3 or AdHBV infection, we utilized an adeno-associated virus serotype 8 expressing HBV (AAV8-HBV), which induced only mild yet observable liver inflammation compared to AdHBV infection (established with infection doses of 1×10^8 i.u. and 3×10^9 i.u.¹⁷) (Reviewer Fig. 16a)^{17, 27}. Our results demonstrated that both non-canonical antigen presentation pathways remained active at day 5 after AAV8-HBV infection, even under conditions of low inflammation (Reviewer Fig. 16b-f). However, HBV transgenic mice—a non-inflamed model of chronic HBV infection—have previously shown that hepatocytes can directly present antigens to virus-specific naïve CD8⁺ T cells, leading to initial proliferation but ultimately failing to induce IFN- γ production or cytolytic effector function^{25, 26}. Thus, our findings suggest that cDC1 cross-dressing may not occur in the complete absence of inflammation but is instead triggered under specific inflammatory conditions. The exact inflammatory molecules involved and the

underlying mechanism remain to be elucidated. These new data have been incorporated into Supplementary Fig. 5p-s (CD8⁺ T cell response) and Supplementary Fig. 7k, l (CD4⁺ T cell response), with corresponding descriptions added to the Results section of the revised manuscript (page 9, lines 12-19; page 11, lines 9-12). We have also expanded the Discussion section to highlight these points (page 16, lines 15-30).



Reviewer Fig 16. Revealing non-canonical antigen presentation pathways in the AAV8-HBV mouse model. **a** Hematoxylin and eosin (H&E) -stained liver sections from mice treated with saline, infected with different doses of AdHBV (1 × 10⁸ i.u. and 3 × 10⁹ i.u.), or infected with AAV8-HBV for 3 days. Scale bars: 50 μm. **b** Representative FACS plots (left) and summary data (right) showing percentages of ENV353 tetramer-specific CD8⁺ T cells in the livers from Batf3 KO versus WT mice, at day 5 post-AAV8-HBV infection (*n* = 4 mice per group). **c** Representative FACS plots (left) and summary data (right) showing percentages of ENV353 tetramer-specific CD8⁺ T cells in the livers from Batf3 KO mice reconstituted with either β2M KO cDC1 or WT cDC1s, at day 5 post-AAV8-HBV infection (*n* = 4 mice per group). **d** Representative Violet tracer dilution (left) and summary data (right) showing the proliferation index of transferred Env28 T cells in the livers from F1 chimeras reconstituted with either C57BL/6 or Batf3 KO bone marrow, at day 3.5 post-AAV8-HBV infection (*n* = 4 mice per group). **e, f** Representative FACS plots (left) and summary data (right) showing percentages of endogenous CD25⁺OX40⁺ (**e**, *n* = 4 mice per group) and T-bet⁺GzmB^{hi} (**f**, *n* = 3 mice per group) CD4⁺ T cells in the spleens, dLNs, and livers from WT and μMT mice, at day 5 post-AAV8-HBV infection. At least two independent experiments were performed. Statistical analysis was performed using two-tailed Student's *t*-tests. Statistically significant results are labeled (***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

We also examined whether the two non-canonical antigen presentation pathways are specific to HBV by using LCMV and SRBC-OVA as controls (Reviewer Fig. 2a-h), as requested by the first reviewer. Additionally, we expanded the Discussion section to explore the potential relevance of these pathways in other viral infections and immune

contexts (page 16, lines 7-12; page 17, lines 4-11).

Finally, we have expanded the descriptions of the experiments and mouse models in the Results and Methods sections to improve clarity and readability in the revised manuscript.

Conceptual Concerns

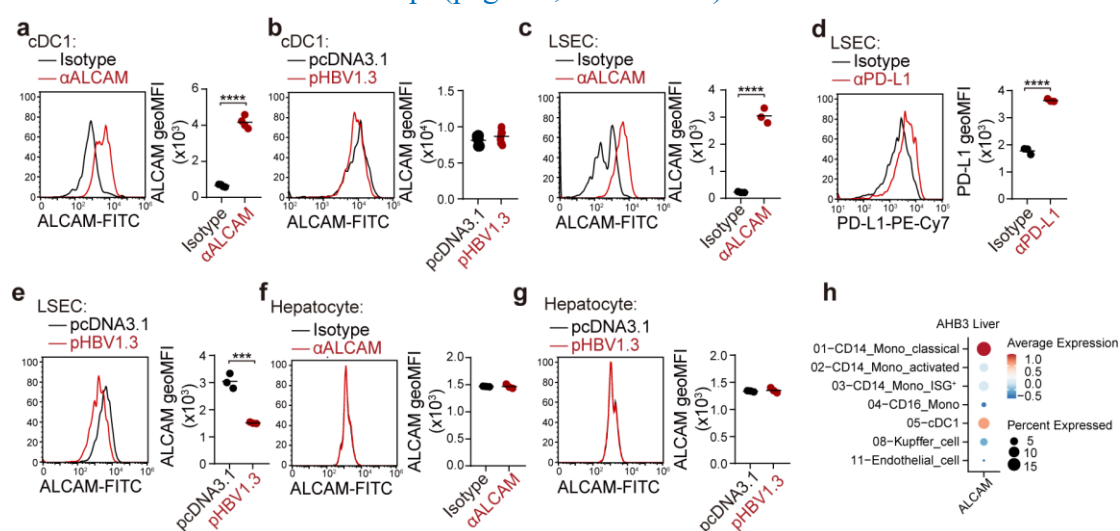
Multiple studies in mice (some cited by the authors) going back 20+ years, and the ability to transplant human livers without matching HLAs, demonstrated that intrahepatic priming with hepatocellular antigens leads to abortive T cell proliferation and function. The cross-dressing data are convincing but inconsistent with the bulk of this existing data. The only way to get effective hepatocellular antigen priming was with the addition of co-stimulation. Can the authors provide insight into why they see effective priming by cDC1 without added co-stimulation? One would presume that the HBsAg in previous systems of hepatocellular antigen expression would have also been subject to cross-dressing. Is it because of liver damage with hydrodynamic transfection? Similarly, in the acute model of AdHBV, many hepatocytes are transduced rapidly, leading to high amounts of antigen expression. Do the author see cross-dressing of cDC1 if they use the persistent AdHBV model?

Response:

We thank the reviewer for highlighting this important conceptual concern. We acknowledge that multiple studies, including those cited in our manuscript, have demonstrated that intrahepatic priming with HBV antigens in non-inflamed HBV transgenic mice often results in abortive T cell proliferation and function, particularly in the absence of additional co-stimulatory signals^{25, 26, 29}. This reflects the liver's tolerogenic environment, where antigen presentation by hepatocytes or liver sinusoidal endothelial cells (LSECs) typically induces tolerance through mechanisms such as T cell anergy, apoptosis, or regulatory T cell induction. Similarly, the tolerogenic nature of the liver is evident in human liver transplantation, where mismatched HLA grafts often exhibit reduced rejection rates compared to other solid organs⁴⁰.

In this study, we provide strong evidence that intrahepatic resident Batf3⁺XCR1⁺CCR7⁻ cDC1s are the primary antigen-presenting cells responsible for priming CD8⁺ T cell responses against HBsAg in the liver via cross-dressing. Our NicheNet analysis and in vitro blocking experiments indicate that an ALCAM-CD6-dependent co-stimulatory mechanism plays a key role in this process (Fig. 7a–c in the revised manuscript). Notably, ALCAM mRNA was highly enriched in cDC1s and moderately expressed in cDC2s and virus-infected hepatocytes, but not in other DC subsets, Kupffer cells, or LSECs (Fig. 7a in the revised manuscript). Moreover, cDC1s constitutively expressed ALCAM protein at homeostasis (Reviewer Fig. 17a), with expression levels remaining stable following pHBV1.3 hydrodynamic injection

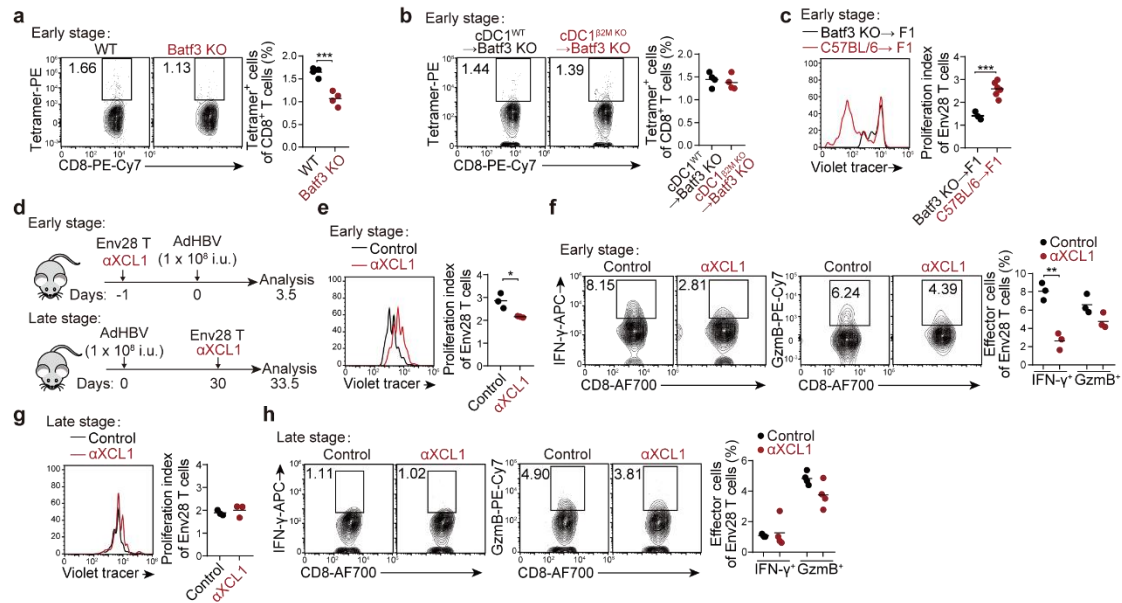
(Reviewer Fig. 17b). In contrast, while LSECs also constitutively expressed ALCAM (Reviewer Fig. 17c), they co-expressed the immuno-inhibitory checkpoint PD-L1 (Reviewer Fig. 17d), consistent with previous reports^{41, 42}, and their ALCAM expression decreased following pHBV1.3 hydrodynamic injection (Reviewer Fig. 17e). Hepatocytes did not express ALCAM under either condition (Reviewer Fig. 17f, g). These findings may partially explain why only cDC1s were capable of priming CD8⁺ T cell responses. Additionally, scRNA-seq data from a liver biopsy of a patient with acute HBV infection revealed ALCAM mRNA expression in cDC1s (Reviewer Fig. 17h), suggesting a potential role for ALCAM in co-stimulating CD8⁺ T cell responses in human hepatic cDC1s. Collectively, our results indicate that cDC1s are uniquely capable of priming effective CD8⁺ T cell responses when cross-dressed with HBsAg peptide-MHC-I complexes, due to their constitutive expression of the co-stimulatory molecule ALCAM. These new data have been incorporated into Supplementary Fig. 10a-h, with corresponding descriptions added to the Results section of the revised manuscript (page 14, lines 14-28).



Reviewer Fig 17. ALCAM expression in liver cell types under homeostatic conditions and following pHBV1.3 HDI. a Representative histogram (left) and summary geometric mean fluorescent intensity (geoMFI) data (right) showing ALCAM expression on cDC1s from the livers of naïve mice compare to the isotype control ($n = 4$ mice per group). **b** Representative histogram (left) and summary geoMFI data (right) showing ALCAM expression on cDC1s from the livers of pHBV1.3 versus pcDNA3.1 HDI mice ($n = 6$ mice per group). **c, d** Representative histogram (left) and summary geoMFI data (right) showing ALCAM (**c**) and PD-L1 (**d**) expression on LSECs from the livers of naïve mice compare to their isotype controls ($n = 3$ mice per group). **e** Representative histogram (left) and summary geoMFI data (right) showing ALCAM expression on LSECs from the livers of pHBV1.3 versus pcDNA3.1 HDI mice ($n = 3$ mice per group). **f** Representative histogram (left) and summary geoMFI data (right) showing ALCAM expression on hepatocytes from the livers of naïve mice compare to the isotype control ($n = 3$ mice per group). **g** Representative histogram (left) and summary geoMFI data (right) showing ALCAM expression on hepatocytes from the livers of pHBV1.3 versus pcDNA3.1 HDI mice ($n = 3$ mice per group). **h** Dot plot illustrating ALCAM mRNA expression in liver cells from one AHB patient. At least two independent experiments were performed. Statistical analysis was performed using two-tailed

Student's *t*-tests. Statistically significant results are labeled (***p* < 0.001, *****p* < 0.0001).

We have already addressed that the two non-canonical antigen presentation pathways we observed are not artifacts of rapid antigen expression or high inflammation induced by hydrodynamic injection or AdHBV infection in the previous response (Reviewer Fig 15, 16), so we will not repeat it here. To determine whether cDC1 cross-dressing occurs in a chronic AdHBV model, we used a low infection dose (1×10^8 i.u.) to establish persistent infection, as previously reported¹⁷. At the early stage of chronic AdHBV infection, cDC1s were required for the endogenous CD8⁺ T cell response in the liver (Reviewer Fig. 18a), but MHC-I expression on cDC1s was not necessary for this response (Reviewer Fig. 18b). Furthermore, by reconstituting F1 mice with either Batf3 KO or C57BL/6 (H-2K^b) bone marrow, we demonstrated that C57BL/6-derived cDC1s could mediate the H-2K^d-restricted response of Env28 T cells at this stage (Reviewer Fig. 18c). These findings suggest that, similar to acute HBV infection, cDC1s drive CD8⁺ T cell responses against HBsAg in the liver via MHC-I cross-dressing during the early stage of chronic AdHBV infection. To further investigate the role of cDC1s at the late stage, we transferred Env28 T cells into mice at both early and late stages of chronic AdHBV infection (Reviewer Fig. 18d). Compared to the early stage, Env28 T cells exhibited weaker proliferation and differentiation at the late stage (Reviewer Fig. 18e-h). Notably, this late-stage response could not be blocked by inhibiting cDC1 recruitment with an anti-XCL1 antibody (Reviewer Fig. 18g, h), suggesting that cDC1s are no longer required for T cell responses at the late stage of infection. Together, these results suggest that while HBsAg-driven CD8⁺ T cell responses rely on cDC1 cross-dressing at the early stage of chronic HBV infection, HBV may manipulate this pathway to establish chronic infection. However, the underlying mechanisms require further investigation. New data on the early stage of chronic AdHBV infection have been incorporated into Supplementary Fig. 5m-o, with corresponding descriptions added to the Results section (page 9, lines 12-19). Given the scope of these findings, the space limitations, and the yet unidentified mechanisms, we believe it is more appropriate to present the late-stage data in a separate manuscript rather than including them here. Nonetheless, we have discussed these findings in the revised Discussion section (page 16, lines 19-34).



Reviewer Fig 18. cDC1 cross-dressing in the chronic AdHBV model. **a** Representative FACS plots (left) and summary data (right) showing the percentages of ENV353 tetramer-specific CD8⁺ T cells in the livers of WT and Batf3 KO mice at day 5 after AdHBV infection with a dose of 1×10^8 i.u. ($n = 3$ mice per group). **b** Representative FACS plots (left) and summary data (right) showing percentages of ENV353 tetramer-specific CD8⁺ T cells in the livers from Batf3 KO mice reconstituted with either WT or $\beta 2M$ KO cDC1s at day 5 after AdHBV infection with a dose of 1×10^8 i.u. ($n = 4$ mice per group). **c** Representative Violet tracer dilution (left) and summary data (right) showing the proliferation index of transferred Env28 T cells in the livers from F1 chimeras reconstituted with C57BL/6 or Batf3 KO bone marrow at day 3.5 after AdHBV infection with a dose of 1×10^8 i.u. ($n = 3$ mice per group). **d** Schematic showing the AdHBV infection (1×10^8 i.u.) and cell transfer timeline. **e, f** Representative Violet tracer dilution (left) and summary data (right) showing the proliferation index of transferred Env28 T cells (**e**, $n = 3$ mice per group) and percentages of IFN- γ ⁺ or GzmB⁺ Env28 T cells (**f**, $n = 3$ mice per group) in the livers of mice as described in (**d**, top). **g, h** Representative Violet tracer dilution (left) and summary data (right) showing the proliferation index of transferred Env28 T cells (**g**, $n = 3$ mice per group) and percentages of IFN- γ ⁺ or GzmB⁺ Env28 T cells (**h**, $n = 3$ mice per group) in the livers of mice as described in (**d**, bottom). At least two independent experiments were performed. Statistical analysis was performed using two-tailed Student's *t*-tests. Statistically significant results are labeled (** $p < 0.001$, **** $p < 0.0001$).

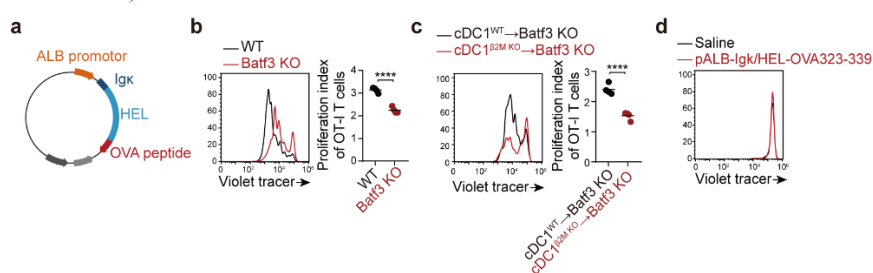
The authors show presentation of an ova epitope embedded in HBsAg and presentation of a native HBsAg epitope. However, there is no control antigen to demonstrate that this mechanism of antigen presentation is unique to HBsAg. The authors should use a control antigen that is secreted similar to the HBsAg to demonstrate specificity of a “non-canonical” presentation pathway in HBV with the hydrodynamic transfection system.

Response:

We appreciate the reviewer's insightful comment regarding the need for a control

antigen to determine whether the “non-canonical” antigen presentation pathway is specific to HBsAg in the hydrodynamic transfection system. To address this, we constructed a secreted hen egg lysozyme (HEL)-OVA257-264 or -OVA323-339 fusion protein (pALB-Igk/HEL-OVA257-264 and pALB-Igk/HEL-OVA323-339, respectively), expressed under the control of the hepatocyte-specific albumin (ALB) promoter and incorporating an Igk leader sequence at the N-terminus to facilitate secretion (Reviewer Fig. 19a). To assess whether cDC1 cross-dressing occurs with this control antigen, OT-I T cells were transferred into WT and Batf3 KO mice, followed by hydrodynamic injection of the pALB-Igk/HEL-OVA257-264 plasmid. The results showed that cDC1s were required for OT-I T cell responses at day 3.5 after injection (Reviewer Fig. 19b). However, in contrast to HBsAg, MHC-I expression on cDC1s was essential for OT-I T cell proliferation in this model (Reviewer Fig. 19c), indicating that cDC1 cross-dressing did not occur. This suggests that cross-dressing may not be a general feature of secreted proteins from hepatocytes but rather a mechanism potentially specific to HBsAg.

To further assess whether a secreted antigen alone is sufficient to drive B cell-mediated CD4⁺ T cell priming, HEL-specific Hy10 B cells and OT-II T cells were transferred into WT mice, followed by hydrodynamic injection of the pALB-Igk/HEL-OVA323-339 plasmid. While the HEL-OVA323-339 protein engaged HEL-specific Hy10 B cells, as indicated by Nur77 expression (Reviewer Fig. 8), it did not induce OT-II T cell proliferation (Reviewer Fig. 19d). This suggests that BCR engagement by antigen alone does not necessarily lead to effective CD4⁺ T cell priming. Notably, B cells have been identified as the predominant antigen-presenting cells initiating naïve CD4⁺ T cell responses when RNA phage Q β -derived virus-like particles are used as model antigens⁴³. Virus-like particles can cross-link BCRs on antigen-specific B cells through multivalent interactions with particulate and highly repetitive protein antigens, thereby strongly inducing the expression of co-stimulatory molecules⁴⁴. These findings suggest that while both subviral particle HBsAg and the soluble HEL-OVA antigen are secreted, they may differ significantly in their ability to activate B cells and provide sufficient co-stimulatory signals for T cell priming, highlighting the specificity of the HBsAg presentation pathway in driving T cell responses. We have incorporated Reviewer Fig. 19a-c into Supplementary Fig. 5t-v, with corresponding descriptions added to the Results section of the revised manuscript (page 9, lines 19-26). Additionally, we have expanded the Discussion section to address the uniqueness of this mechanism to HBsAg (page 17, lines 30-39; page 18, lines 1-11).



Reviewer Fig 19. The uniqueness of non-canonical antigen presentation pathways for HBsAg, revealed by secretable HEL models. **a** Map of pALB-Igk/HEL-OVA peptide construct. **b** Representative Violet tracer dilution of OT-I T cells (left) and summary data (right) showing their proliferation index in WT and Batf3 KO mice, at day 3.5 post- pALB-Igk/HEL-OVA257-264 HDI ($n = 4$ mice per group). **c** Representative Violet tracer dilution of OT-I T cells (left) and summary data (right) showing their proliferation index in the Batf3 KO mice that were reconstituted with either WT cDC1s and β 2M KO cDC1s, at day 3.5 post- pALB-Igk/HEL-OVA257-264 HDI ($n = 4$ mice per group). **d** Representative Violet tracer dilution of OT-II T cells in the WT mice that were HDI with saline or pALB-Igk/HEL-OVA323-339 for 3.5 days. At least two independent experiments were performed. Statistical analysis was performed using two-tailed Student's t-tests. Statistically significant results are labeled (**** $p < 0.0001$).

Specific points

These patients do not meet the definition of acute/resolving hepatitis B. According to the table, all patients are still HBsAg positive, anti-HBs negative and no HBV DNA data is provided. The definition of acute/resolving hepatitis B is HBV DNA & HBsAg negative before 6 months and anti-HBs, anti-HBc positive. Did these patient clear HBV or was this a hepatitis flare in chronic hepatitis B patients?

Response:

We appreciate the reviewer's insightful comment and apologize for the insufficient clarity in our previous description of the patients' clinical information. Upon verification, all patients meet the diagnostic criteria for acute hepatitis B and had no prior history of HBV infection (Reviewer Table 2). Specifically, HBsAg positivity was first detected within 2 to 14 days after symptom onset, with concurrent anti-HBc positivity and absence of anti-HBs antibodies, consistent with acute HBV infection. Longitudinal follow-up (up to four months) confirmed HBV DNA and HBsAg clearance in all cases, accompanied by the development of anti-HBs antibodies while maintaining anti-HBc positivity. Notably, long-term follow-up demonstrated stable antibody levels without reoccurrence of HBsAg positivity, further supporting that these patients successfully resolved acute HBV infection rather than experiencing a hepatitis flare in the context of chronic hepatitis B. To enhance clarity, we have updated Supplementary Table 1 and revised the Methods section in the revised manuscript accordingly (page 21, lines 27-33).

Reviewer Table 2. Clinical characteristics of AHB samples.

	AHB1	AHB2	AHB3	AHB4	AHB5
Age (years)	27	58	44	53	32
Sex (Male/Female)	Female	Male	Male	Male	Male
Date of symptom onset	11/4/2014	9/19/2021	2/11/2020	11/30/2021	6/5/2021
Date of blood sampling	11/19/2014	9/24/2021	2/28/2020	12/7/2021	6/17/2021
Date of liver sampling	NA	NA	2/28/2020	NA	NA
Test results from ALT (U/L)	1244	4447	1845	3729	3300

the date of blood sampling	AST (U/L)	541	4962	670	1471	1585
	TBIL (μmol/L)	167.90	136.30	29.80	244.20	70.50
	DBIL (μmol/L)	145.10	75.40	14.90	167.70	39.70
	HBsAg (IU/mL) ^a	> 250.00 (+)	>250.00 (+)	25.93 (+)	>250.00(+)	1.84(+)
	HBsAb (IU/L) ^b	0.50 (-)	0.00 (-)	0.00 (-)	0.00 (-)	7.62 (-)
	HBeAg (s/co) ^c	73.58 (+)	0.50 (-)	33.75 (+)	0.40 (-)	33.38(+)
	HBeAb (s/co) ^d	4.20 (-)	0.50 (+)	0.58 (+)	0.02 (+)	0.80 (+)
	HBcAb (s/co) ^e	9.60 (+)	3.70 (+)	7.74 (+)	0.02 (+)	4.89 (+)
	HBcAb IgM (s/co) ^f	24.10 (+)	5.06 (+)	29.87 (+)	10.30 (+)	7.56 (+)
Date of first HBsAg negative		2/13/2015	11/18/2021	6/21/2020	1/14/2022	6/30/2021
Test results on the date of first HBsAg serological negativity	HBsAg (IU/mL) ^a	(-)	0.00 (-)	0.00 (-)	0.00 (-)	0.00 (-)
	HBsAb (IU/L) ^b	(-)	3.70 (-)	99.83 (+)	6.30 (-)	6.40 (-)
	HBeAg (s/co) ^c	(-)	0.34 (-)	0.33 (-)	0.31 (-)	0.27 (-)
	HBeAb (s/co) ^d	(+)	0.10 (+)	0.02 (+)	0.00 (+)	0.08 (+)
	HBcAb (s/co) ^e	(+)	7.70 (+)	7.72 (+)	7.80 (+)	8.00 (+)
	HBcAb IgM (s/co) ^f	NA	4.97 (+)	3.08 (+)	9.65 (+)	5.90 (+)
	HBV DNA (copies/mL)	<10	<10	<10	<10	<10
Duration of HBsAg serological conversion (days)		101	60	131	45	15
Date of first serological HBsAb positivity		5/12/2015	1/6/2022	6/21/2020	2/18/2022	8/15/2022
HBsAb (IU/L)		51.23 (+)	148.10 (+)	99.83 (+)	38.50 (+)	15.48 (+)
NA, not available; ^a Negative defined as serum HBsAg < 0.05 IU/ml; ^b Positive defined as serum HBsAb > 10.0 IU/L; ^c Negative defined as serum HBeAg < 1.00 s/co; ^d Positive defined as serum HBeAb < 1.0 s/co; ^e Positive defined as serum HBcAb > 1.0 s/co; ^f Positive defined as serum HBcAb IgM > 1.0 s/co.						

What single patient was used for intrahepatic analysis? What was the ALT level at the time of cell capture? Was the comparison done on only the matching PBMC from the patient or across all patient PBMCs. Patient heterogeneity is a major issue in hepatitis B and these data do not have sufficient power to draw conclusions from a single comparison.

Response:

Thank you for your inquiry regarding the intrahepatic analysis and patient heterogeneity. Due to the rarity of liver biopsy specimens from acute hepatitis B cases, this analysis was conducted on a single patient. At the time of cell capture, the ALT level was markedly elevated (1845 U/L), indicating active liver inflammation. To ensure a controlled comparison, we specifically analyzed intrahepatic cells alongside matched PBMCs from the same patient rather than across all patient PBMC samples. We acknowledge that the conclusions drawn from a single-patient comparison are preliminary and that patient heterogeneity remains a significant challenge in hepatitis B research. We agree that future studies with a larger cohort are necessary to validate

these findings. Our ongoing work aims to strengthen these observations by incorporating additional samples from both acute and chronic HBV-infected patients, with a particular focus on non-canonical antigen presentation pathways in chronic infection. To enhance clarity, we have updated Supplementary Table 1 and tempered the conclusions in the Results section of the revised manuscript (page 4, lines 34-37).

In suppl fig 3 panel "O" where the authors use different HBV systems, I may be confused but the dot plots do not make sense to me. I think they were trying to show H-2kd-siinfekl expression but the gating does not show that. I would guess it is tetramer gating based on the figure legend but it is unclear.

Response:

We thank the reviewer for pointing out this graphic illustration error. The data in Supplementary Fig. 3, panel "O" were intended to show the percentage of endogenous HBsAg-specific CD8⁺ T cells identified using the H-2K^b-ENV353 tetramer. We have now corrected the graphic illustration in Supplementary Fig. 3, panel "O", which is now presented as Supplementary Fig. 5d in the revised manuscript.

In figure 4 shows an uncoupling of activation (CD69) and proliferation Fig 4b. Activation happens similarly in DLNs and spleen but no proliferation occurs. I suppose this is possible but seems unlikely. Do the author have an explanation?

Response:

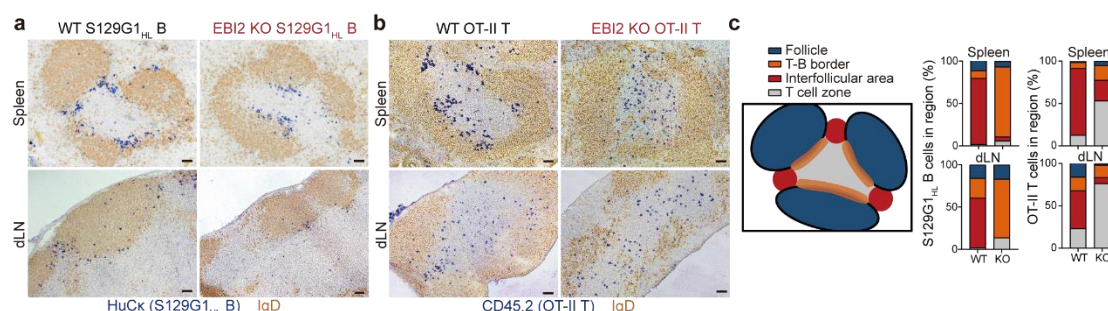
We appreciate the reviewer's thoughtful question. Our data show that OT-II T cell activation was abrogated in the spleen and impaired in the dLNs but remained intact in the liver of B cell-deficient μ MT mice (Fig. 4a). Notably, OT-II T cell proliferation (Fig. 4b) and differentiation (Fig. 4c, d) were nearly absent in all three organs. These findings suggest that B cells are essential for initiating OT-II T cell proliferation and differentiation, while other cells may provide weak activation signals that fail to support complete T cell responses, particularly in the liver. We have clarified this point in the Results section of the revised manuscript (page 10, lines 38-39; page 11, lines 1-2).

In fig 6h, I don't see a significant difference in EBI2 knockout S129G1 B cells. They still look to be localized at the border. There does seem to be a difference in OT-II cells. The B cell data may be over interpreted.

Response:

We apologize for the lack of clarity. The results show that EBI2 knockout S129G1^{HL} B cells failed to concentrate in the interfollicular regions and instead localized to the central T-B border (Fig. 6h, i in the revised manuscript). This mispositioning likely led to decreased interactions between B cells and CD4⁺ T cells in the interfollicular regions, as supported by the finding that EBI2-deficient OT-II T cells proliferated less rapidly than WT OT-II T cells (Fig. 6l in the revised manuscript). Notably, the

interfollicular region and the T-B border are adjacent but distinct niches within secondary lymphoid tissues. To enhance clarity, we have added a positional illustration and statistical analyses to aid in data interpretation (Reviewer Fig. 20c). Additionally, the positional illustration has been incorporated into Figure 6b in the revised manuscript. We have also revised the description for clarity in the Results section of the revised manuscript (page 13, line 23-28).



Reviewer Fig 20. EBI2 guide the interfollicular priming of CD4⁺ T cells by B cells. a

Immunohistochemistry of the spleens and dLNs from WT recipients at day 2.5 post-pHBV1.3/OVA323-339 HDI, showing WT S129G1_{HL} B cells or EBI2 KO S129G1_{HL} B cells (HuCκ, blue) and endogenous B cells (IgD, brown). **b** Immunohistochemistry of the spleens and dLNs from WT recipients at day 2.5 post-pHBV1.3/OVA323-339 HDI, showing WT OT-II T cells or EBI2 KO OT-II T cells (CD45.1, blue) and endogenous B cells (IgD, brown). **c** Anatomical schematic representation of the spleen and dLN (left), percentages of WT S129G1_{HL} B cells and EBI2 KO S129G1_{HL} B cells (middle) in the specified regions stained in (a, top: $n = 6$ white pulp from 3 mice; bottom: $n = 4-6$ LN slices from 4-6 mice), and percentages of WT OT-II T cells and EBI2 KO OT-II T cells (right) in the specified regions stained in (b, top: $n = 6$ white pulp from 3 mice; bottom: $n = 4-6$ LN slices from 4-6 mice). At least two independent experiments were performed. Scale bars: 50 μ m.

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Response to the Reviewers' Comments and Suggestions

We sincerely appreciate the reviewers' time and effort in evaluating our manuscript, as well as their recognition of our revisions. Below, we provide a point-by-point response to each comment, with our replies highlighted in blue for clarity.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have carefully addressed all my comments, also by including relevant controls and performing additional experiments. I have no further questions.

Response:

We sincerely appreciate the reviewer's thorough evaluation and positive feedback. Thank you for your time and consideration.

Reviewer #2 (Remarks to the Author):

The authors Li and Sun submitted a revised manuscript entitled "Hepatitis B virus surface antigen drives T-cell immunity through non-canonical antigen presentation". The revised manuscript displays impressive efforts to improve the quality of the manuscript and to address the comments from the reviewers. The authors addressed all questions from the reviewers by performing additional sophisticated experiments. For my concerns, the authors addressed all concern satisfactorily. I do have only minor suggestions to improve the clarity of the manuscript.

Response:

We sincerely appreciate the reviewer's positive feedback and recognition of our efforts to improve the manuscript. Below, we have carefully addressed the minor suggestions to further enhance clarity.

1) Page 2, line 13-15: "Currently the goal of therapy..." . Maybe the authors could add: "...therapy of chronic HBV infection..." . Otherwise, it is not clear what should be therapied.

Response:

Thank you for your suggestion. We have revised the sentence to specify that the goal of therapy refers to chronic HBV infection, as reflected in the Results section of the revised manuscript (page 2, lines 13).

2) Page 3, line 22: ...hepatocyte-specific viral gene expression?

Response:

Thank you for your suggestion. We have revised 'virus gene expression' to 'viral gene expression' for accuracy, as reflected in the Results section of the revised manuscript (page 3, lines 22).

In general: The manuscript investigates the activation/priming of immune responses against HBV, particularly HBsAg-specific CD8, CD4 and B cells. However, at different points the authors use models that should induce acute or chronic HBV infection. As the authors do not show an acute HBV infection course (Clearance of HBV by loss of pgRNA, HbcAg-negative hepatocytes, lack of HbeAg...) nor development of chronic HBV, I would suggest to remove the wording acute and chronic. This is anyway irrelevant for the main topic of the manuscript.

Response:

Thank you for this great suggestion, which helped resolve our confusion about overemphasizing acute and chronic infections in the previous revision, as they are not directly relevant to the main topic. We have removed these terms when describing preclinical mouse models, as reflected in the Results section of the revised manuscript (page 3, line 36; page 7, lines 11-12; page 6, lines 36-37; page 9, lines 3-4; page 10, lines 35-36, 39; page 16, lines 5-6).

Reviewer #3 (Remarks to the Author):

The authors satisfyingly and thoroughly answered my questions.

Response:

Thank you for your positive feedback. We appreciate your thorough review of our manuscript

Reviewer #4 (Remarks to the Author):

The authors have done a great deal of work to address my, as well as 3 other reviewer comments. I appreciate that effort.

I think the additional experiments are very insightful for the mechanism and provide specificity for HBsAg while also addressing the potential impacts of inflammation associated with the models. General readers are not always aware of the limitations of the mouse models for HBV and the authors have done a good job of highlighting their specific observations while also recognizing potential limitations.

I have no other comments.

Response:

Thank you for your thoughtful review and positive feedback. We appreciate your recognition of our efforts to address all reviewer comments and improve the manuscript.