

Engineering a dual-antigen mRNA vaccine to restore immune control in chronic hepatitis B

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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)

The manuscript by Zhang et. al. investigates the use of mRNA vaccines encoding different HBV antigens +/- IFN- α as an adjuvant, for efficacy in the AAV-HBV mouse model. They find that inclusion of the PreS1 mRNA/protein in combination with HBsAg significantly enhanced the antiviral efficacy of vaccination compared to core or X. the PreS1-HBs combination enhanced anti-HBs production, appeared to increase APC activation as an adjuvant effect and increase HBV-specific T cell responses. Combination of the PreS1-HBs vaccine with IFN- α promoted the formation of memory HBV-specific T cells and appeared to have greater antiviral efficacy than the vaccine alone.

Use of mRNA vaccine technologies for therapeutic vaccination in CHB patients is an expanding field. The need for coordinated induction of HBV-specific CD4 and CD8 T cells is likely to be critical for a successful therapeutic vaccine. While the investigators attempt to address the potential in this study, I have multiple conceptual issues with application of the observations to data from CHB patient and numerous technical questions regarding the experiments that raise concerns.

First, use of PreS1 in vaccines has been tested in CHB patients with no success. A recent study tested PreS1+HBs containing vaccine co-administered with IFN- α as an adjuvant and showed no antiviral activity (pmid: 34661089). Therefore, it is a concern that the efficacy observed in this model does not translate.

Second, there is no evidence that the HBsAg possesses adjuvant activity in CHB patients. In vitro studies showing HBsAg induces cytokines is likely a result of recombinant antigen contaminants. Even if it does, the effect is so modest that it has no biological effect. Otherwise, CHB patients would suffer from chronic inflammatory syndromes that correlate with HBsAg levels and that is not the case. Additionally, antigen presenting cells in the PBMC of CHB patients do not show markers of constitutive activation.

Third, I do agree that there is some effect of the PreS1 addition to the vaccine combination. However, the experiments performed do not define what that is. They did not map T cell responses to the PreS1 region to see how they differed and enhanced immune responses. To me, this would be the novel observation in this study. Instead, they combined PreS1 into different regimens and looked for outcomes. Thus, I don't know why PreS1 inclusion helps the immune response.

Technical questions:

Since PreS1 contains the HBsAg sequence, why did the investigators not test PreS1 with antigens known to induce CD8 T cells like HBcAg?

The investigators mention that vaccines contained an equimolar ratio of mRNAs. Did the HBsAg only vaccine contain 2x mRNA to match antigen production to the PreS1-HBsAg vaccine? Since the PreS1 will include the HBsAg coding sequence, the amount of HBsAg in the PreS1-HBsAg would be double. Could the differences observed between HBsAg only and PreS1-HBsAg just due to a higher dose of HBsAg?

Fig 3a - As mentioned above, it is not clear whether the HBsAg vaccine used 2x the HBsAg mRNA. Therefore, infiltration could be due to antigen load delivered by the vaccination. What does splenic infiltration look like with the HBsAg+HBcAg?

Fig 3B - are the significance comparisons only to empty LNPs or are they between vaccine conditions as well?

Fig 3F - this is a poor representation of the flow data. It is not clear where the gates are, CD4/CD8 are not easily distinguished. Simplified dot plots/density plots would improve interpretation of the data. The same is true for subsequent analyses of HBV-specific T cells by flow cytometry. Supplemental figures of flow plots would enable accurate interpretation of the gating and frequencies.

Fig 4B - I don't see any NK cells in the stacked bar plots. Is that an error? They are typically found at high frequencies in the liver.

Suppl Fig 5A-C - The Ven diagrams are difficult to tell what the comparison is. They need to be labeled better or explained better in the legend. Same for the heatmap. There is no key to indicate what the colored bar at the top of the heat map represents. Overall, the heatmap data looks relatively weak, which would not be surprising since the HBV-specific T cells will be less than 1% of T cells and much less of total CD45.

Fig 4C - did the immune activation coincide with any ALT elevation?

Fig 5D - the heatmap is a poor representation of T cell response data. There does not appear to be any difference and it is not clear what the shading scale represents. Given the very low frequencies of HBV-specific T cells in chronic models, these heatmaps are highly sensitive to minor variations in gating. The investigators should show flow plot and summary graphs to determine if the frequency of HBV specific T cells is believable.

Fig S8 - Intracellular staining for GzmB goes down after CD8 T cell activation in vitro by peptides because of degranulation. Why does GzmB go up in these T cells? And, are the peptides used in this pool specific for the PreS1 region? Or, do they contain peptides from the HBsAg region as well?

Fig 5F - similar to my question above, is it a double dose of HBsAg in the PreS1-sAg vaccine that is necessary for anti-HBs production? If you gave a double dose of HBsAg vax would that induce anti-HBs?

Fig 6E - it is highly unusual that macrophages would cross-present the most OVA peptides vs. DCs in the lymph nodes. Is HBsAg-OVA secreted to make it to macrophages? How do the authors interpret/explain this observation?

Fig 7i - Why would the PreS1-HBs vax not induce any T cell responses in this experiment? The investigators claimed the importance of T cell induction above but now the vaccine has become ineffective? And, the responses are specific to what peptides, PreS1, HBs?

Reviewer #2

(Remarks to the Author)

Zhang et al screened antigen combinations paired with the HBsAg in a mRNA format for efficacy as a therapeutic vaccine in a mouse model of chronic hepatitis B (CHB). They identified preS1-HBsAg as the optimal pairing as the dual-antigen mRNA vaccine elicited robust HBV-specific immune responses leading to near-complete viral genome clearance, reduction in multiple HBV antigens in the serum and liver and production of anti-HBs antibodies.

A significant body of work exists illustrating the advantages of using the PreS region , including PreS1 , both for preventative and therapeutic vaccine design. The previous work has been mostly focused on PreS1-specific Th cell (CD4+) and B cell (antibody)-mediated protective mechanisms . For example, inclusion of the PreS region enhances the immunogenicity of the HBsAg in mice (DOI:10.1126/science.2408336) and humans (DOI.org/10.1007/s00430-014-0374-x) ; a 10-residue PreS1-peptide can prime T cell help for antibody production to the preS2 and HBsAg regions (doi.org/10.4049/jimmunol.138.12.4457) , which can bypass no responsiveness to the preS2 and S regions of HBsAg (J. Immunol.1986 Jul 1;137(1):315-22) ; PreS1 B cell epitopes inserted onto HBcAg or WHcAg carriers elicit neutralizing anti-PreS1-specific antibodies and bypass immune tolerance DOI:10.1016/j.hep.2010.10.019 and DOI.org/10.1080/21645515.2019.1689745). This body of work should be acknowledged for perspective . The authors only cite a single reference (#17).

The novelty of the current study is the focus on targeting PreS1-specific , CD8+ CTL induction elicited via mRNA immunization , which has not been previously examined in any detail. The authors report very efficient viral clearance and reduction in HBsAg , but weak or absent anti-HBs production using the preS1-HBsAg or preS1 only mRNA vaccines .These results and a favorable safety profile suggest that this may be a promising therapeutic strategy going forward .

Specific Comments :

1. The text often refers to the superiority of the preS1-HBsAg construct over HBsAg alone , yet a number of characteristics (ie., serum HBsAg (fig.2c) ; Tregs (fig.3e) ; lymph node CD69+ T cells (fig.3c) ; lymph node PD1+ CD8+ T cells (fig.3d) are not significantly different . Also , several of the other dual-constructs were significantly different from the HBsAg alone construct and these were not mentioned in the text . Perhaps explanations in the text should be more carefully phrased to reflect this complexity .

2. There seems to be some disparity in anti-HBs production after preS1-HBsAg immunization. In Fig.2j it appears to be 1/5

positive mice before the rHBsAg boost and in fig.5f it appears to be 4/5 mice positive for anti-HBs ?

3. Did the anti-HBs assay used detect anti-PreS1 antibodies ? Did the authors measure preS1-specific Abs ?

4. In the in vitro stimulated cellular assays the authors used a panel of peptides specific for HBsAg or preS1 , but do not describe the panel . Did the peptide panel span the entire amino acid sequence of both antigens ?

5. Was there an attempt to identify the specific peptide responsible for the activation ? This is important because a single mouse strain (B6) was used for all the studies . Theoretically , the authors may have identified a " super " strong CTL epitope on the preS1 region restricted to MHC class I molecules of B6 mice rather than the preS1-HBsAg construct outperforming the HBc-HBsAg or HBx-HBsAg constructs ?

6. Did the authors perform any classical in vitro CTL assays to determine if cytotoxic effector T cells explain the antiviral effects of the preS1-HBsAg vaccine ?

7. Granzyme B + CD8+ and CD4+ T cells were detected and presumed to play a pivotal role in viral clearance. This mechanism would presumably result in granzyme B -mediated cytotoxicity against infected hepatocytes , yet no increases in liver enzymes were reported . In fact , the only mention of ALT/AST levels was in Fig. S11c , which showed no elevations in liver enzymes at study endpoint. The authors also show a modest level of T cell IFN γ production , which suggests that the common non-cytopathic pathway for viral elimination is not likely the explanation for viral clearance . Perhaps further discussion of possible clearance mechanisms is warranted .

2.

Reviewer #3

(Remarks to the Author)

In this study, Zhang, Wang, and colleagues investigate the potential of an mRNA vaccine co-expressing preS1 and HBsAg for treating hepatitis B virus (HBV) infection and evaluate the effects of vaccination on viral parameters and virus-specific immunity in a mouse model persistently replicating HBV. Authors demonstrate that combining preS1 and HBsAg in the mRNA vaccine induces virus-specific humoral and cellular immunity, resulting in a significant decrease in markers of infection. In addition, they demonstrate that the therapeutic effect of vaccination can be enhanced by combining it with IFN- α treatment. The authors conclude that the preS1-HBsAg mRNA vaccine represents a promising and easily translatable treatment strategy for chronic hepatitis B.

The work adds insights into developing novel strategies to achieve a functional cure for chronic hepatitis B and is of clinical relevance. Overall, the study is well described and presents analyses of the immune response induced by mRNA vaccination, including transcriptome analyses of immune cells at the site of infection. The manuscript is well-written, the experiments are in majority well-designed, and the results are overall interesting, but several issues and limitations need to be addressed to further improve the manuscript.

Major comments:

1. The authors demonstrate an adjuvant effect of HBsAg using the ovalbumin as a model. This is an interesting result, but the authors' attempt to develop therapy against chronic hepatitis B and to examine the immunostimulatory effect of HBsAg on other HBV antigens is more relevant. The lack of these data is puzzling, as in their initial experiments, they had already combined HBsAg with HBV core or x antigens and shown a decrease in HBV parameters.

2. Following that line, would a combination of preS1+HBsAg with other HBV antigens for vaccination additionally improve the overall antiviral effect? Since alternative therapeutic HBV vaccines that utilize a combination of several HBV antigens for vaccination are currently being investigated (Kosinska et al, Mol Ther Nucleic Acids. 2025), this matter should at least be discussed.

3. Overall, the analysis of the T cell response in the manuscript could be improved to significantly strengthen the authors' findings. 1) The authors claim that the preS1 is the main inducer of T cell response and is responsible for the antiviral effect of the vaccine, but do not show the results for HBsAg-specific stimulation, although they mention the stimulation with HBsAg peptide in the Methods section. Including data on HBsAg-specific T cell responses could substantiate the authors' conclusions. 2) Analysis of the specific T cell response in the liver, the site of infection, using either multimers or peptide pool stimulation, would be more informative than in the spleen and link the authors' findings with the antiviral effect. 3) In Figure 3, is the granzyme B and TGF- β expression measured after the specific pool stimulation or directly ex vivo? I struggle to find this information. Moreover, authors should explain why they do not perform analysis of the T cell activation markers such as CD69 and CD44 on specific CD8 T cells, but total CD8 population?

4. The authors vaccinate the mice with the mRNA vaccine in a one-week interval. This is unusual, as most mRNA vaccinations in preclinical and clinical settings are typically performed at least 2 weeks apart, and such a short interval may interfere with the induction of efficient immunity after vaccination. Did the authors optimize the vaccination regimen before conducting the experiments, and do they have any supporting data?

5. I appreciate the authors' assessment of the treatment's safety, in particular in the context of mRNA vaccination. Although the description of the results appears in the main text, all the figures addressing these issues are currently in the supplement. I believe that at least some of these figures should be presented in the main text. Moreover, the authors examine the longitudinal effect of the treatment on liver damage 6 weeks after it is completed, detecting high levels of granzyme B in vaccine-induced T cells and clearance of almost all infected hepatocytes. Monitoring the levels of liver enzymes during the experiment might be more informative at assessing the risks of the treatment.

6. The authors vaguely mention the AAV-HBV model limitations in the discussion, but should specify the main drawbacks of

the model in relation to their experimental setup and results. The authors plan to develop different models in the future, which "better mimic chronic infection cycles", but these models also pose serious limitations in studying immune therapies against chronic hepatitis B, such as using immunodeficient mice in vaccination studies. It somehow diminishes the authors' findings. I would recommend revising this part of the discussion with a focus on the limitations of the model used or clearly stating what would be the benefit of using an alternative model – this is now not clear to me.

7. Figure 1 – The figure reminds me of a graphical abstract of the manuscript. The author should reconsider keeping it as a main figure. I think their rationale behind the study is clearly described in the introduction.

Minor comments:

1. The opening sentence of the abstract should be modified. It appears that immune tolerance is crucial for anti-HBs seroconversion.

2. Introduction, line 40 – persistent high circulating... The authors should revise this sentence, e.g. persistently high?

3. Introduction, line 41 – T cell exhaustion in CHB – there is piling evidence that HBV-specific T cells do not behave as classically exhausted T cells. The authors might consider an alternative description.

4. eLNP abbreviation should be explained in the figure legends.

5. Figure 5 – The authors follow the same experimental scheme as in Figure 2a. To be consistent and clearer, authors should rephrase "8 weeks after the first vaccine treatment" to "6 weeks after the vaccination". Alternatively, they should stick to the first option throughout the manuscript.

6. Figure 7 – the authors do not specify when IFN- α is applied in relation to mRNA vaccination, neither in the figure legend nor in the main text, and the experimental scheme of IFN- α treatment is now shown in the supplement. This is important information and should be clearly explained to the reader. The authors should also consider presenting the experimental timeline in the main figure.

7. Discussion, line - better mimic chronic infection cycles – I am not sure what authors mean by infection cycles – phases? please rewrite.

8. Methods – the results of several methods such as H&E staining does not appear in the main text, only in the supplement. These methods should also be described in the supplement section.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I was quite skeptical on many aspects of the original manuscripts. However, clarifications added by the authors, and additional data on PreS1-specific T cells and the effects of mRNA delivered HBsAg vs. recombinant HBsAg have addressed my primary concerns. I have no further comments.

Reviewer #2

(Remarks to the Author)

The authors have responded to my original comments in a direct and effective manner. I have no further comments.

Reviewer #3

(Remarks to the Author)

I really appreciate the effort the authors put into revising the manuscript and addressing most of my comments. However, I still have a few questions concerning some of the newly added data.

1. Staining of HBsAg-specific CD8 T cells with tetramers in the spleen (Supplementary Figure 8 f-h): The percentage of tetramer+ cells is very low and does not differ significantly from the eLNP control. Is there a specific signal detected for the eLNP group, or is it only background? I am simply wondering whether, with this result, any analysis of the memory phenotype or the activation markers MFI is reliable. How many events were acquired in the tetramer+ gate? Can the authors show representative dot plots of the tetramer+ cells in the spleen for each experimental group?

2. ALT and AST kinetics data in Figure 7b: It is not clear to me what the experimental group called 'modelling' is and why the X-axis is named 'weeks after modelling', but not the usually used experimental time line. What represents the dotted line in the middle of the graph? I cannot find any information about this analysis anywhere in the text. Can authors explain it?

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Reviewer #1 (Remarks to the Author):

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Author reply: We thank this reviewer for his/her positive assessment on this work and we have carefully addressed the issues raised by the reviewer.

First, use of PreS1 in vaccines has been tested in CHB patients with no success. A recent study tested PreS1+HBs containing vaccine co-administered with IFN- α as an adjuvant and showed no antiviral activity (pmid: 34661089). Therefore, it is a concern that the efficacy observed in this model does not translate.

Author reply: We thank the reviewer for raising this important translational concern. We agree that PreS1-containing therapeutic vaccines have been evaluated clinically, including the phase Ib/IIa BRII-179 (Pre-S1/Pre-S2/S) study with or without low-dose IFN- α co-administration in nucleos(t)ide analogue-treated, virally suppressed CHB patients. In that trial, BRII-179 was safe and immunogenic (including anti-HBs induction in a subset of participants and restoration of HBV surface antigen-specific T-cell responses), but overall did not yield a notable reduction in circulating HBsAg.

We respectfully note that this outcome is more accurately interpreted as immunogenicity without a corresponding HBsAg decline rather than evidence that the PreS1/PreS2/S antigen composition is ineffective. Indeed, the BRII-179 report discusses that the magnitude/kinetics of vaccine-elicited cellular immunity and baseline antigen burden may have limited the ability to translate immunogenicity into measurable antigen decline, and proposes that additional dosing and/or application in lower-HBsAg settings and combination with antigen-lowering therapies may be required. Notably, a recent phase 2a study combining an HBV-targeting siRNA with BRII-179 further supports the rationale of pairing antigen-lowering therapy with vaccination, showing robust anti-HBs induction in a substantial fraction of participants, although sustained HBsAg loss remained uncommon at the group level (*Gastroenterology*, 2025, 169, 136-149).

In addition, an earlier clinical study of Sci-B-Vac, which contains the same envelope

antigen set (Pre-S1/Pre-S2/S) as BR11-179, reported that vaccination combined with lamivudine enhanced short-term viral suppression and induced anti-HBs responses, although HBsAg loss was rare and the benefit was not sustained after discontinuing vaccination (*Antimicrob. Agents Chemother.*, 2009, 53, 5134-5140). Together, these data support the rationale for PreS1-containing antigen combinations while highlighting the need for optimized regimens and combination strategies in CHB.

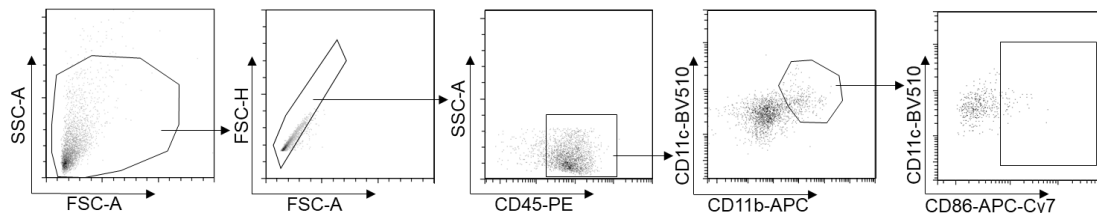
Our current study is a preclinical mechanistic and proof-of-concept investigation. We have now explicitly acknowledged these clinical findings in the revised Discussion and tempered our wording accordingly. Importantly, our approach differs in both modality and mechanism: the LNP-mRNA platform enables intracellular antigen production and is expected to better support endogenous MHC-I presentation and CD8⁺ T cell priming than exogenous protein vaccination in a tolerogenic CHB setting. In our CHB mouse model, we observed robust vaccine-elicited cellular responses together with anti-HBs induction and a reduction in HBsAg, supporting the therapeutic potential of this platform; nevertheless, we emphasize that clinical translation will require optimized patient context (e.g., lower antigenemia) and/or rational combination regimens (e.g., antigen-lowering agents and/or immune modulators). We have added this perspective to the Discussion (page 25-26, line 558-570).

Second, there is no evidence that the HBsAg possesses adjuvant activity in CHB patients. In vitro studies showing HBsAg induces cytokines is likely a result of recombinant antigen contaminants. Even if it does, the effect is so modest that it has no biological effect. Otherwise, CHB patients would suffer from chronic inflammatory syndromes that correlate with HBsAg levels and that is not the case. Additionally, antigen presenting cells in the PBMC of CHB patients do not show markers of constitutive activation.

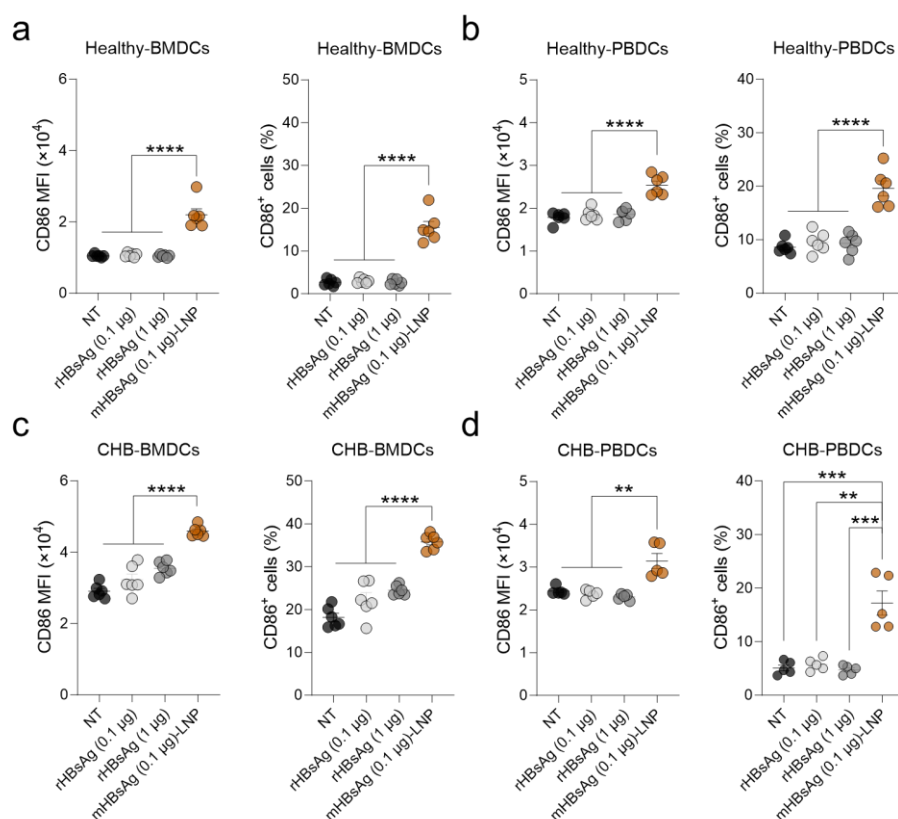
Author reply: We thank the reviewer for this important point. We agree that in CHB patients, HBsAg is predominantly present as a circulating, soluble antigen and there is no evidence that serum HBsAg levels act as a systemic adjuvant that drives constitutive activation of antigen-presenting cells (APCs) of PBMCs. Accordingly, our manuscript does not intend to claim that free HBsAg in CHB functions as a clinically relevant adjuvant. Instead, our “adjuvant-like” terminology refers to a platform-specific context: in our vaccine, HBsAg is delivered as LNP-mRNA, enabling intracellular antigen production within APCs and thereby potentially promoting APC activation and antigen presentation in a manner that differs from exposure to soluble recombinant HBsAg protein.

To directly address concerns about recombinant-protein contaminants and biological relevance, we performed a head-to-head comparison using bone marrow-derived cells (BMDCs) and peripheral blood dendritic cells (PBDCs) generated from healthy and CHB mice. APCs were exposed either to mHBsAg-LNP (mRNA delivery) or recombinant HBsAg (rHBsAg) protein, and activation was assessed by CD86 upregulation. We observed that mHBsAg-LNP robustly increased CD86 expression in both BMDCs and PBDCs, whereas recombinant HBsAg showed rare to weaker effects (Supplementary Fig. 23 and Supplementary Fig. 24). These data support that the immunostimulatory effect we describe is context-dependent and is most evident when HBsAg is delivered and expressed via

mRNA in APCs, rather than implying that circulating HBsAg in CHB patients acts as a systemic adjuvant. Accordingly, we have revised the manuscript to use the more conservative phrasing “potential adjuvant-like effect” and clarified that this refers to the mRNA delivery and expression context. These data were included as Supplementary Fig. 24 in the revision and relevant discussion was added in the revised manuscript (page 17, line 380-387).



Supplementary Fig. 23 Gating strategy for analyzing CD86 expression on PBDCs or BMDCs.



Supplementary Fig. 24 Potential adjuvant-like effects of HBsAg on APC activation. **a-d** Quantification of CD86 expression by BMDCs and PBDCs from healthy (**a, b**) or CHB (**c, d**) mice 24 h after treatment with rHBsAg or mHBsAg-LNP. NT, untreated. $n=5$ or 6. Data are shown as the mean $\pm$ s.e.m. Statistical analysis was performed by a two-tailed unpaired t -test. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Third, I do agree that there is some effect of the PreS1 addition to the vaccine combination. However, the experiments performed do not define what that is. They did not map T cell responses to the PreS1 region to see how they differed and enhanced immune responses. To me, this would be the novel observation in this study. Instead, they combined PreS1 into different regimens and looked for outcomes. Thus, I don't know why PreS1 inclusion helps the immune response.

Author reply: We thank the reviewer for this insightful comment. We agree that defining which sequences within preS1 are targeted by vaccine-elicited T cells is important for understanding why preS1 inclusion enhances the response. In response, we performed additional epitope-mapping experiments using an ELISpot assay with individual 15-mer peptides overlapping by 10 amino acids spanning the entire preS1 domain (peptide list in Supplementary Table 2). Splenocytes collected after preS1-HBsAg mRNA vaccination were stimulated with each peptide individually, enabling peptide-level resolution of the preS1-directed response. These experiments revealed broad preS1 reactivity across multiple peptides, with a clearly dominant hotspot: peptides #4–#6 elicited the strongest responses across animals (Fig. 4e and Supplementary Fig. 19). This mapping provides a mechanistic explanation for the benefit of preS1 inclusion that the vaccine induces robust preS1-directed cellular immunity with an immunodominant region that likely drives the enhanced T-cell response and therapeutic outcomes observed. We have incorporated these data and updated the Results accordingly (page 13, line 282-293).

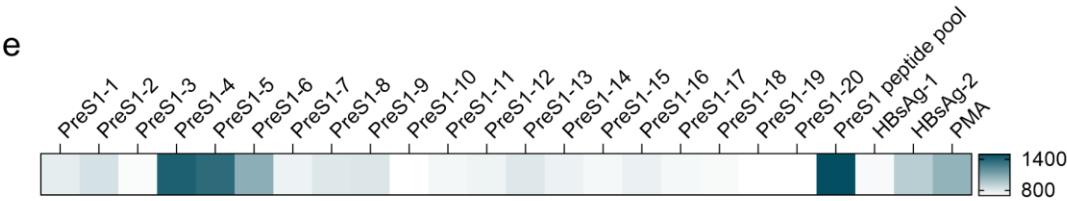
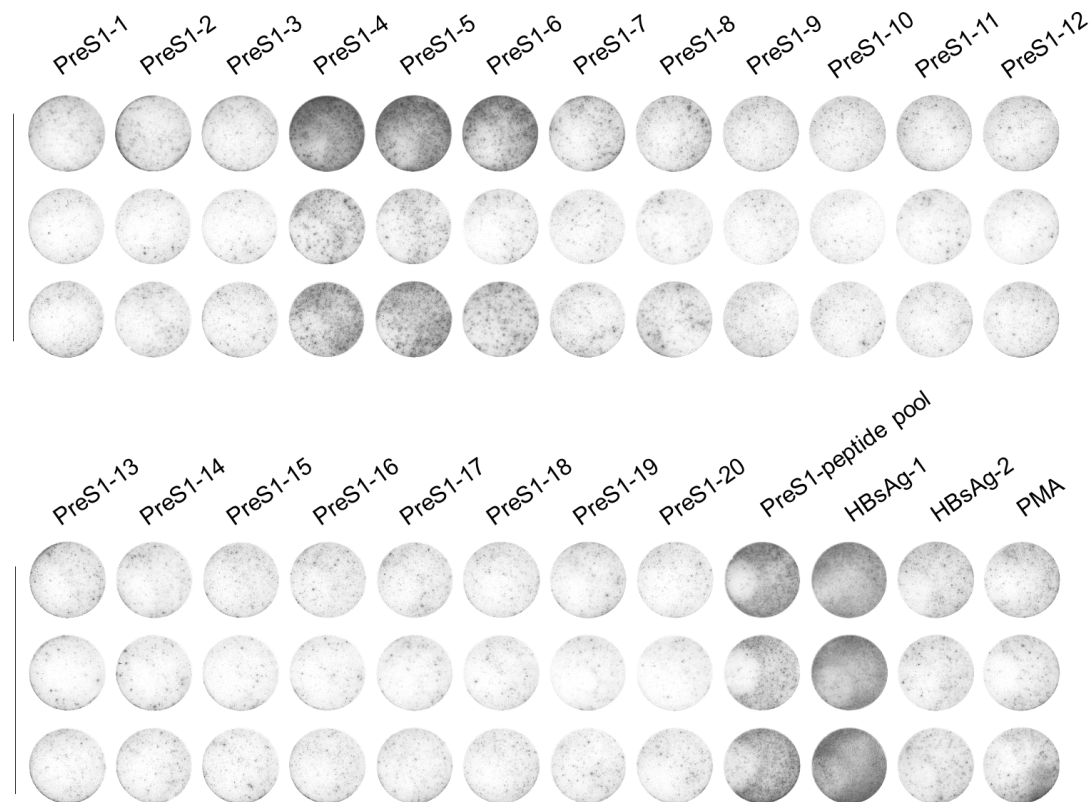


Fig. 4 PreS1 and HBsAg synergistically act to achieve HBsAg seroconversion. e, Quantitative analysis of granzyme B ELISpot results. The colour scale indicates the number of spots per well. Quantification of granzyme B ELISpot results. The colour scale indicates the number of spots per well. Splenocytes (5×10^5 cells per well) from mice immunized three times with the preS1-HBsAg mRNA vaccine were stimulated for 24 h with individual overlapping 15-mer peptides spanning preS1 (10-aa overlap), the full preS1 peptide pool, or two HBsAg epitopes, as indicated. PMA (phorbol 12-myristate 13-acetate) served as a positive control. $n=3$.



Supplementary Fig. 19 Mapping of preS1-specific CTL responses by granzyme B ELISpot. Splenocytes (5×10^5 cells per well) from mice immunized three times with the preS1–HBsAg mRNA vaccine were stimulated for 24 h with individual overlapping 15-mer peptides spanning preS1 (10-aa overlap), the full preS1 peptide pool, or two HBsAg epitopes, as indicated. PMA (phorbol 12-myristate 13-acetate) served as a positive control. $n=3$.

Technical questions:

Since PreS1 contains the HBsAg sequence, why did the investigators not test PreS1 with antigens known to induce CD8 T cells like HBcAg?

Author reply: We thank the reviewer for this suggestion. First, we would like to clarify that the preS1 used in our vaccine is derived solely from the preS1 domain of the HBV large surface protein (L-HBs; preS1-preS2-S) and therefore does not include the preS2 or S domain (HBsAg); we have clarified this in the revised manuscript (page 4, line 91).

Our primary goal was to identify a combination that can overcome CHB-associated HBsAg tolerance to restore protective anti-HBsAg responses toward functional cure-oriented endpoints. In the antigen-combination screen (Fig. 1), we tested multiple HBsAg-containing candidates, including HBcAg-HBsAg, but this construct did not outperform preS1-HBsAg in our therapeutic efficacy readouts. Therefore, we focused subsequent mechanistic studies on the preS1-HBsAg candidate, in which HBsAg provides an adjuvant-like effect and antigenic determinants to support anti-HBsAg induction, while preS1 contributes T cell immunity. Importantly, in our platform preS1 functions as an immunogen: we observed that vaccination elicits preS1-specific T cell responses, including CD8⁺ T cells (Fig. 4e, Supplementary Fig. 11c, and Supplementary Fig. 19). Thus, our design does

incorporate a cellular immunity component through preS1.

We agree that adding HBcAg may further broaden CD8⁺ T-cell targets; however, multi-antigen optimization (format, dose ratio, and immunodominance) requires dedicated investigation and is beyond the scope of the current manuscript. We have added this as an important future direction in the Discussion (page 25-26, line 558-570).

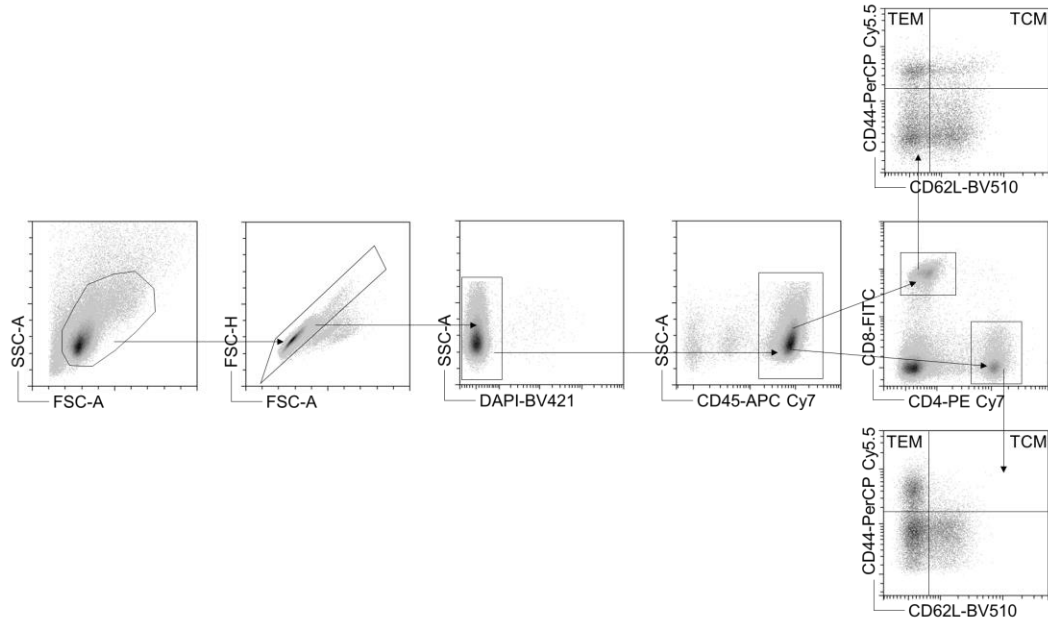
The investigators mention that vaccines contained an equimolar ratio of mRNAs. Did the HBsAg only vaccine contain 2x mRNA to match antigen production to the PreS1-HBsAg vaccine? Since the PreS1 will include the HBsAg coding sequence, the amount of HBsAg in the PreS1-HBsAg would be double. Could the differences observed between HBsAg only and PreS1-HBsAg just due to a higher dose of HBsAg?

Author reply: We thank the reviewer for this important question. The concern assumes that preS1 contains the HBsAg coding sequence; however, our preS1 encodes only the preS1 domain of L-HBs (preS1-preS2-HBsAg) and does not include the preS2 or HBsAg domain. Therefore, the HBsAg coding sequence is not duplicated in the preS1-HBsAg mRNA and is present only once per mRNA molecule. Both the HBsAg-only and preS1-HBsAg vaccines were administered at the same molar dose of mRNA (i.e., the same number of mRNA molecules), and we did not administer a 2× mRNA dose in the HBsAg-only group. Thus, the observed differences are unlikely to be explained by a higher HBsAg dose at the mRNA level in the preS1-HBsAg group.

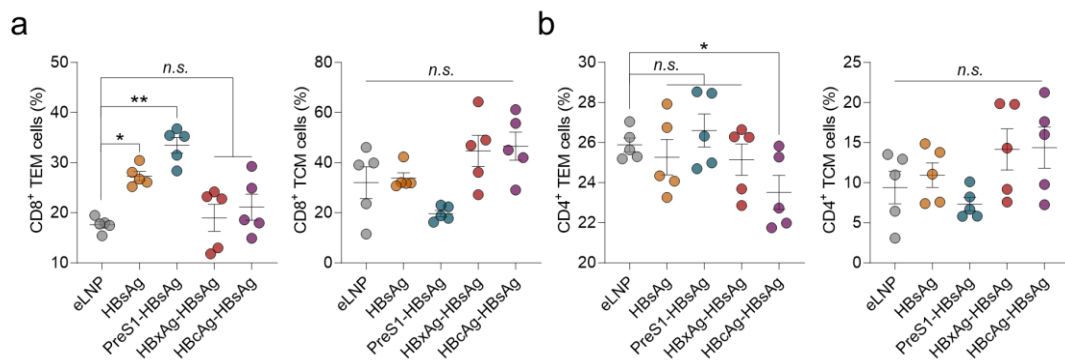
Fig 3a - As mentioned above, it is not clear whether the HBsAg vaccine used 2x the HBsAg mRNA. Therefore, infiltration could be due to antigen load delivered by the vaccination. What does splenic infiltration look like with the HBsAg+HBcAg?

Author reply: We thank the reviewer for this important question. As clarified above, our preS1 encodes only the preS1 domain and does not include the HBsAg sequence; therefore, the HBsAg-coding sequence is not duplicated in the preS1-HBsAg mRNA. The HBsAg-only and preS1-HBsAg vaccines were administered at the same molar dose of mRNA (i.e., the same number of mRNA molecules), and we did not administer a 2× mRNA dose in the HBsAg-only group. Thus, the differences observed in splenic TEM/TCM in original Fig. 3a are unlikely to be explained by a higher HBsAg dose at the mRNA level in the preS1-HBsAg group.

Regarding the HBcAg-HBsAg group, we have now included the splenic TEM/TCM data for this group in Supplementary Fig. 7 (with gating strategy shown in Supplementary Fig. 5). In this CHB mouse model, the preS1-HBsAg vaccine increased the frequency of CD8⁺ TEM relative to the eLNP control, whereas the HBcAg-HBsAg group did not show a significant increase relative to the eLNP control. In contrast, CD8⁺/CD4⁺ TCM did not show a significant increase across groups. The data and related description have been added to the revised manuscript (page 5, line 123-126).



Supplementary Fig. 5 Gating strategy for analyzing TEM and TCM in CD8⁺ T cells and CD4⁺ T cells.



Supplementary Fig. 7 PreS1-HBsAg vaccination increases splenic effector memory T cells in the CHB mouse model. a, b Frequencies of TEM (CD44⁺ CD62L⁻) and TCM (CD44⁺ CD62L⁺) subsets among CD8⁺ (a) and CD4⁺ T (b) cells in spleen. $n=5$. eLNP, empty LNP. Data are shown as the mean $\pm$ s.e.m. Statistical analysis was performed by a two-tailed unpaired t -test. $*P < 0.05$ and $**P < 0.01$; $n.s.$, not significant.

Fig 3B - are the significance comparisons only to empty LNPs or are they between vaccine conditions as well?

Author reply: We thank the reviewer for this question. In original Fig. 3b, all statistical significance annotations reflect comparisons only to the eLNP control; no direct statistical comparisons between vaccine groups are shown in this panel. We have clarified this explicitly in the revised figure legend (page 9, line 191).

Fig 3F - this is a poor representation of the flow data. It is not clear where the gates are, CD4/CD8 are not easily distinguished. Simplified dot plots/density plots would

improve interpretation of the data. The same is true for subsequent analyses of HBV-specific T cells by flow cytometry. Supplemental figures of flow plots would enable accurate interpretation of the gating and frequencies.

Author reply: We thank the reviewer for this constructive feedback. As suggested, in the revised manuscript, we have updated the plot presentation to improve readability (using simplified dot plots) and have added the complete gating strategies for all relevant flow cytometry analyses in the Supplementary Information. The updated plots and gating schemes are provided in Fig. 2f and Supplementary Fig. 10 in the revised manuscript.

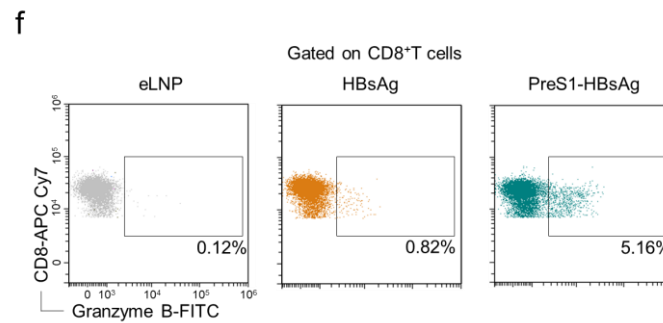
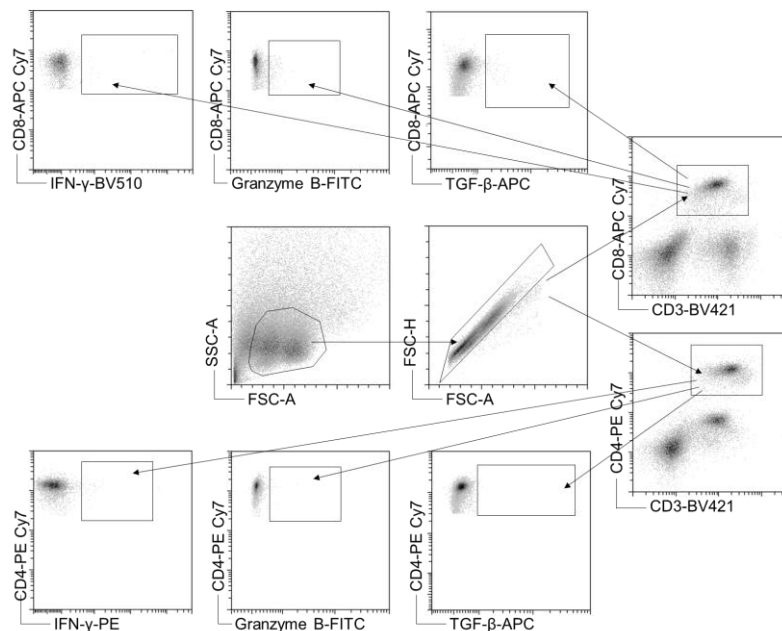


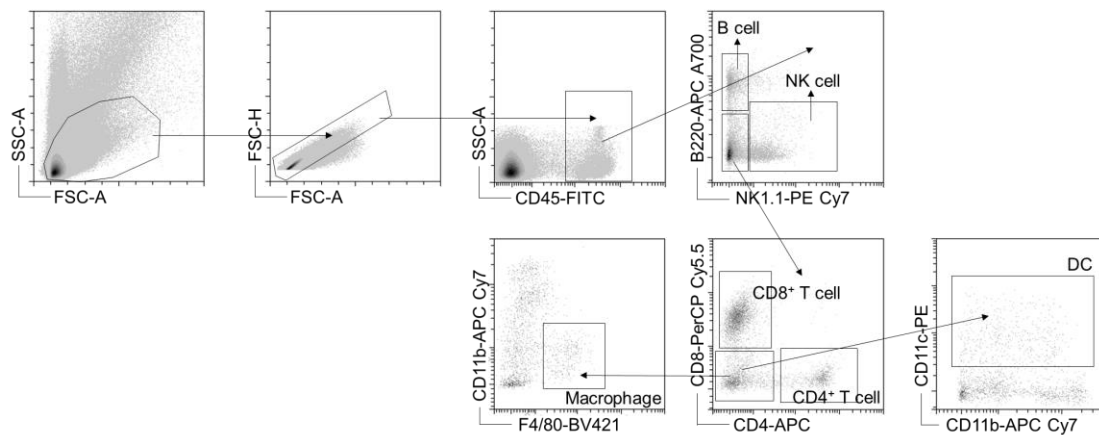
Fig. 2 PreS1-HBsAg mRNA vaccine-mediated reconstitution of HBV-specific immunity. f Representative flow cytometry plots of granzyme B expression by splenic T cells after ex vivo stimulation with a mixed peptide set comprising two HBsAg peptides (HBsAg_{190–197}, VWLSVIWM; and HBsAg_{208–215}, ILSPFLPL) and an overlapping preS1 peptide pool spanning the full preS1 domain (15-mers with 10 aa overlap).



Supplementary Fig. 10 Gating strategy for analyzing IFN- γ , granzyme B, and TGF- β expression in CD8⁺ T cells and CD4⁺ T cells.

Fig 4B - I don't see any NK cells in the stacked bar plots. Is that an error? They are typically found at high frequencies in the liver.

Author reply: We thank the reviewer for this comment. Because NK-lineage cells are typically enriched in the liver, we re-validated the flow cytometry annotation and gating for this compartment. As CD3 was not included in this panel, conventional NK cells and NKT cells cannot be reliably distinguished; we therefore report them collectively as NK1.1⁺ cells. With the updated analysis, hepatic NK1.1⁺ cells account for ~30% of lymphocytes, consistent with the high abundance of NK and NKT cells typically observed in mouse liver. The updated gating strategy is provided in Supplementary Fig. 13, and the revised immune cell composition analyses are shown in Fig. 3a, b in the revised manuscript.



Supplementary Fig. 13 Gating strategy for analyzing CD8⁺ T cells, CD4⁺ T cells, B cells, NK1.1⁺ cells, macrophages, and DCs.

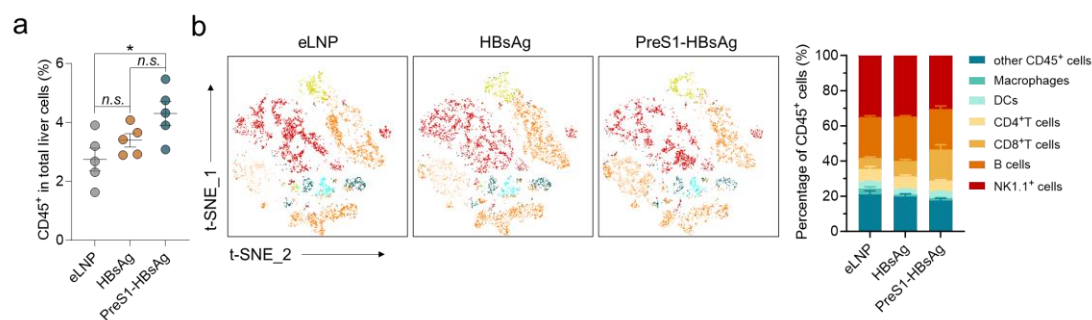


Fig. 3 PreS1-HBsAg mRNA vaccine reprograms the hepatic immune microenvironment in chronic HBV infection. **a** Frequency of CD45⁺ immune cells in the liver by flow cytometry 7 weeks after virus injection (i.e. one week after the third vaccination). *n*=5. **b** t-SNE visualization of various types of CD45⁺ cells in the liver.

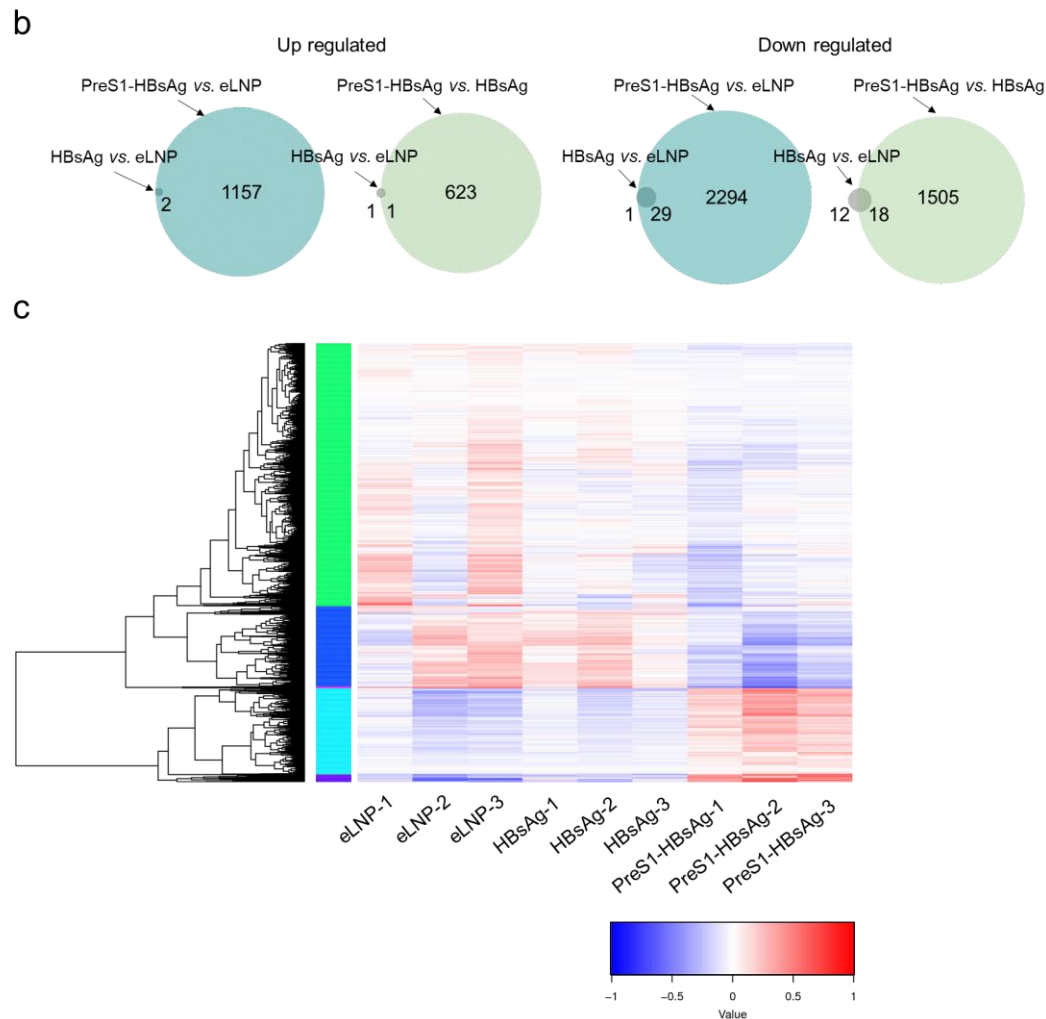
Suppl Fig 5A-C - The Ven diagrams are difficult to tell what the comparison is. They need to be labeled better or explained better in the legend. Same for the heatmap. There is no key to indicate what the colored bar at the top of the heat map represents. Overall, the heatmap data looks relatively weak, which would not be surprising since the HBV-

specific T cells will be less than 1% of T cells and much less of total CD45.

Author reply: We thank the reviewer for these constructive comments. We have revised the presentation and legends for original Fig. S5a-c to improve clarity and interpretation. Specifically, we now explicitly label each Venn diagram with the exact pairwise comparison (e.g., preS1-HBsAg vs eLNP, HBsAg vs eLNP, and preS1-HBsAg vs HBsAg) and clarify in the figure legend that overlaps denote shared significantly upregulated or downregulated DEGs.

For the heatmap, we have added a key and clarified what the colored annotation bar represents (gene modules/clusters defined by unsupervised clustering, while treatment group information is indicated separately on the side) in the updated figure legend.

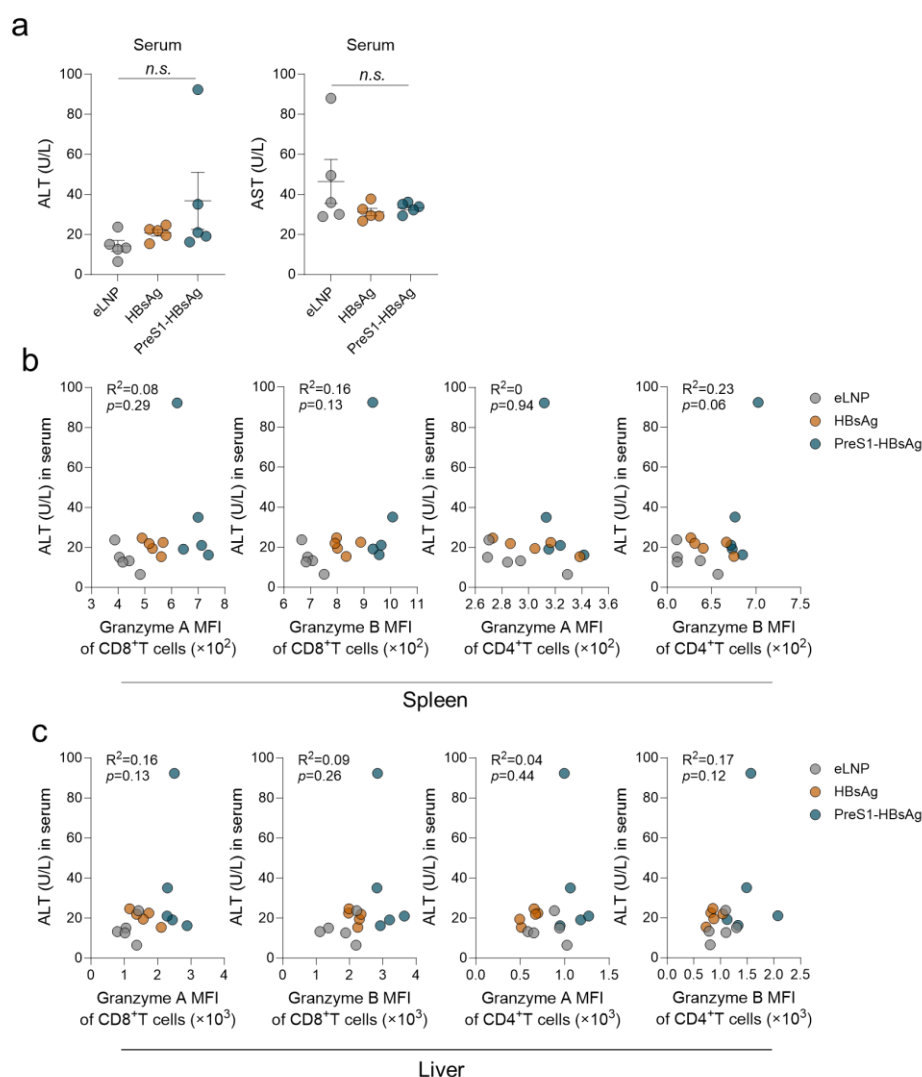
We agree with the reviewer that effect sizes can appear modest when profiling bulk hepatic CD45⁺ immune cells, where HBV-specific T cells represent only a small fraction of the total population and their signals may be diluted. Accordingly, we now state explicitly in the figure legend that transcriptomic profiling was performed on total hepatic CD45⁺ cells, and we provide complementary summaries (volcano plots and DEG counts) alongside the heatmap to contextualize effect size and statistical significance. Importantly, our conclusion that preS1-HBsAg reshapes hepatic immunity is supported by multiple orthogonal analyses presented in the main text (e.g., immune cell composition shifts and immune response-related signatures), rather than relying solely on this heatmap. The revised panels and expanded legends are provided in Supplementary Fig. 14b, c.



Supplementary Fig. 14 Transcriptomic profiling of hepatic immune cells post vaccination. **b** Venn diagrams showing the comparison of up-regulated genes and down-regulated genes across groups. Overlapping regions denote genes that are shared as significantly upregulated or downregulated in both comparisons. **c** Clustered heatmap showing differences in gene expression levels across treatment groups. Columns represent samples (labels shown at the bottom), and rows represent genes. The row dendrogram on the far left and the adjacent color annotation bar indicate gene modules identified by hierarchical clustering; genes within the same module exhibit similar expression patterns across samples.

Fig 4C - did the immune activation coincide with any ALT elevation?

Author reply: We thank the reviewer for this question. To assess whether immune activation was accompanied by hepatocellular injury, we measured serum transaminases at the same time point (1 week after completion of immunization). Serum AST was unchanged across groups, and ALT showed only a modest increase in the preS1-HBsAg mRNA vaccine group. In addition, correlation analyses did not reveal a significant association between ALT levels and the measured immune activation parameters in spleen or liver. These data are now provided in Supplementary Fig. 27. The data and related description have been added to the revised manuscript (page 22, line 473-480).



Supplementary Fig. 27 Correlation analysis of immune response and ALT changes. **a** Levels of serum ALT and AST one week after doses of vaccination. $n=5$. **b, c** Correlation analysis of T cell immune response in spleen (**b**) or liver (**c**) and ALT changes. $n=5$. eLNP, empty LNP. Data are shown as the mean $\pm$ s.e.m. Statistical analysis was performed by a two-tailed unpaired *t*-test. *n.s.*, not significant. The Pearson correlation coefficient and a two side Pearson's correlation *P* values are indicated.

Fig 5D - the heatmap is a poor representation of T cell response data. There does not appear to be any difference and it is not clear what the shading scale represents. Given the very low frequencies of HBV-specific T cells in chronic models, these heatmaps are highly sensitive to minor variations in gating. The investigators should show flow plot and summary graphs to determine if the frequency of HBV specific T cells is believable. **Author reply:** We thank the reviewer for this constructive feedback and agree that a heatmap is not optimal for evaluating low-frequency HBV antigen-specific T cell responses, which can be sensitive to gating. To enable direct assessment of gating and response frequencies, we now provide representative flow cytometry plots together with per-mouse

quantification (showing individual animals) for T/NK-cell functional readouts. Key quantifications are shown in the main text (Fig. 4d), and the corresponding representative plots and additional cytokine readouts are provided in the Supplementary Information (Supplementary Fig. 18).

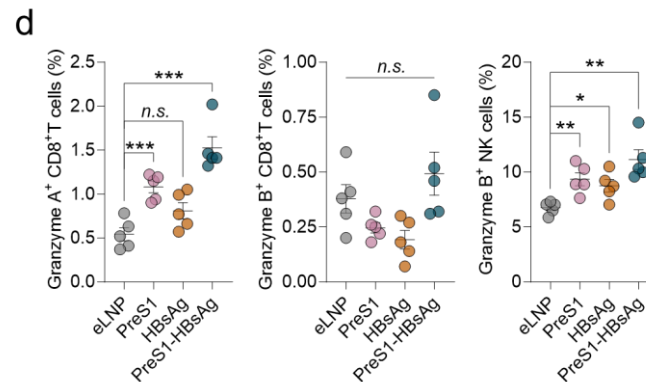
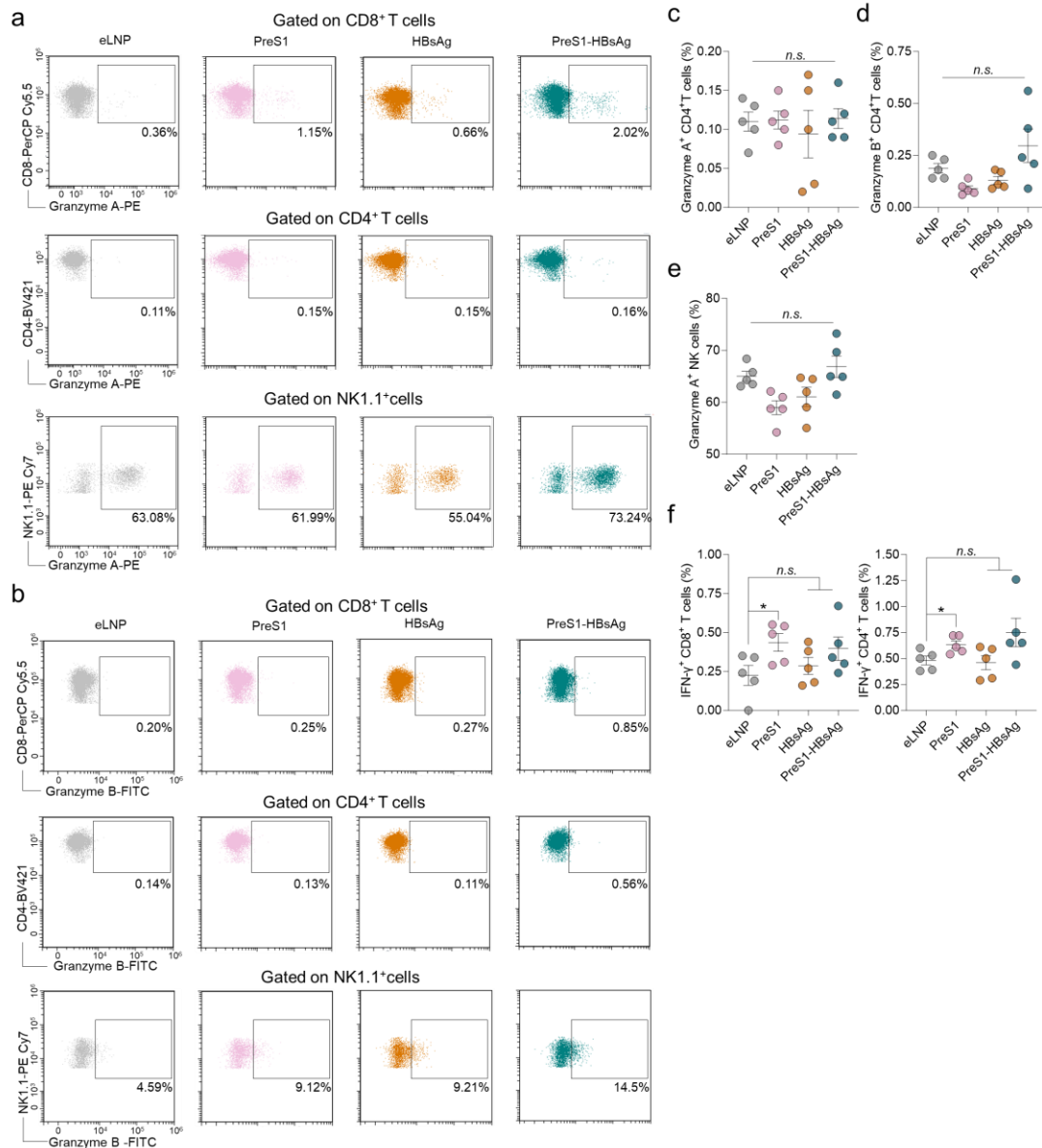


Fig. 4 PreS1 and HBsAg synergistically act to achieve HBsAg seroconversion. d Quantification of granzyme A or granzyme B expression by CD8⁺ T cells and granzyme B expression by NK cells in spleen after ex vivo stimulation with a mixed peptide set comprising two HBsAg peptides (HBsAg₁₉₀₋₁₉₇, VWLSVIWM; and HBsAg₂₀₈₋₂₁₅, ILSPFLPL) and an overlapping preS1 peptide pool spanning the full preS1 domain (15-mers with 10 aa overlap). $n=3$. All data are shown as the mean $\pm$ s.e.m. Statistical analysis was performed by a two-tailed unpaired t -test. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$; $n.s.$, not significant.



Supplementary Fig. 18 Flow cytometry plots and quantification of splenic T/NK-cell responses. Detection of splenic T-cell cytokines is conducted after ex vivo stimulation with a mixture of two HBsAg peptides (HBsAg_{190–197}, VWLSVIWM; and HBsAg_{208–215}, ILSPFLPL) and an overlapping preS1 peptide pool spanning the full preS1 domain (15-mers with 10 aa overlap). **a, b** Representative flow cytometry plots showing granzyme A (**a**) or granzyme B (**b**) expression by CD8⁺ T cells, CD4⁺ T cells, and NK cells. **c, d** Quantification of granzyme A (**c**) or granzyme B (**d**) expression by CD4⁺ T cells. **e** Quantification of granzyme A expression by NK cells. **f** Quantification of IFN- γ expression by CD8⁺ T and CD4⁺ T cells. $n=5$. eLNP, empty LNP. All data are shown as the mean $\pm$ s.e.m. Statistical analysis was performed by a two-tailed unpaired t -test. * $P < 0.05$; n.s., not significant.

Fig S8 - Intracellular staining for GzmB goes down after CD8 T cell activation in vitro by peptides because of degranulation. Why does GzmB go up in these T cells? And, are

the peptides used in this pool specific for the PreS1 region? Or, do they contain peptides from the HBsAg region as well?

Author reply: We thank the reviewer for these insightful questions. (1) We agree that intracellular GzmB can decrease immediately after strong degranulation. In our assay, cells were stimulated for intracellular staining under standard conditions including brefeldin A (BFA), which was used to facilitate intracellular detection of cytokines by limiting their secretion. Importantly, the readout shown here reflects intracellular GzmB content (cellular reservoir/expression state) rather than degranulation per se. An increase in intracellular GzmB in this setting is consistent with the acquisition of a more cytotoxic effector program upon peptide stimulation. We have clarified the staining conditions in the revised Materials and Methods section (page 30, line 691-696). (2) The peptide pool used for this experiment was specific to the preS1 region only and did not include HBsAg-derived peptides.

Fig 5F - similar to my question above, is it a double dose of HBsAg in the PreS1-sAg vaccine that is necessary for anti-HBs production? If you gave a double dose of HBsAg vax would that induce anti-HBs?

Author reply: We thank the reviewer for this important question. Our preS1 sequence encodes only the preS1 domain of L-HBs and does not include the HBsAg domain; therefore, the preS1-HBsAg mRNA contains the HBsAg coding sequence only once, not twice. Because the HBsAg and preS1-HBsAg vaccines were administered at the same molar dose of mRNA (same number of mRNA molecules), both constructs deliver an equivalent 1× HBsAg sequence at the mRNA level, making a double-dose HBsAg explanation unlikely.

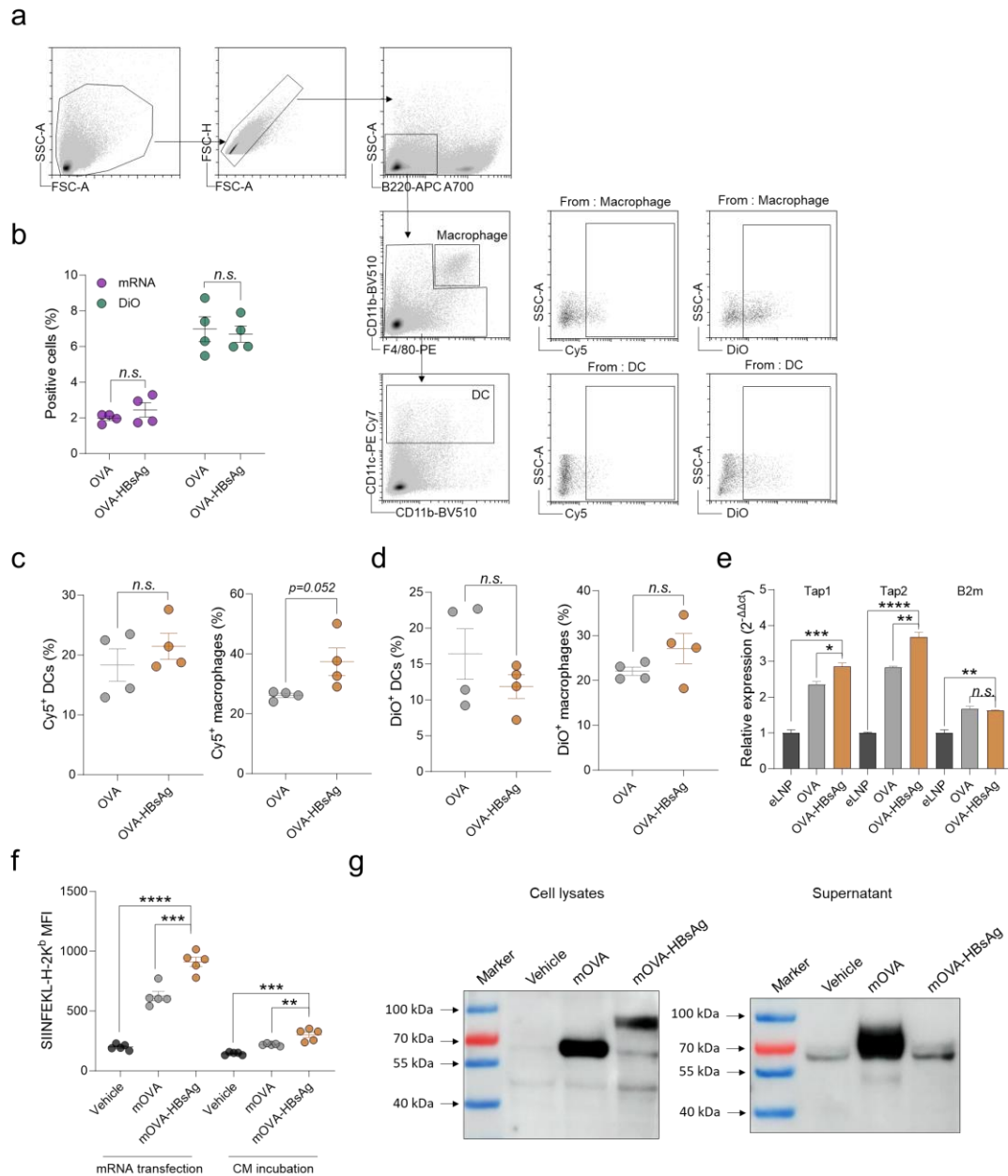
We agree that whether increasing the dose of HBsAg-only mRNA could improve anti-HBsAg induction is an interesting question; however, systematic dose-escalation and optimization are beyond the scope of the current study and will be pursued in future work.

Fig 6E - it is highly unusual that macrophages would cross-present the most OVA peptides vs. DCs in the lymph nodes. Is HBsAg-OVA secreted to make it to macrophages? How do the authors interpret/explain this observation?

Author reply: We thank the reviewer for raising this important point. Although DCs are classically considered the most efficient antigen-presenting cells, our data in Fig. 6e indicate that lymph node (LN) macrophages display higher levels of SIINFEKL-H-2K^b complex following OVA-HBsAg mRNA vaccination. We interpret this finding as reflecting efficient direct uptake and intracellular translation of the mRNA vaccine by macrophages, leading predominantly to endogenous (classical) MHC-I presentation, rather than enhanced cross-presentation of exogenous secreted antigen.

First, using dual-labeled vaccine (DiO-labeled carrier and Cy5-labeled mRNA), we observed that both LN macrophages and DCs internalized the mRNA vaccine, with macrophages showing greater uptake in the OVA-HBsAg group (Supplementary Fig. 22a-d). This supports the possibility that macrophages can act as direct mRNA recipients and generate intracellular antigen for MHC-I loading. Consistent with this, *Tap1/Tap2* (and *β2m*) transcripts were increased in LN macrophages after OVA-HBsAg vaccination (Supplementary Fig. 22e), supporting upregulation of the MHC-I antigen-processing and peptide-loading machinery.

To directly distinguish endogenous presentation from cross-presentation of secreted antigen, we performed *in vitro* experiments with RAW264.7 macrophages. Direct transfection with OVA or OVA-HBsAg mRNA induced markedly higher surface SIINFEKL-H-2K^b presentation than incubation with the matched conditioned medium (CM), and OVA-HBsAg mRNA consistently yielded higher SIINFEKL-H-2K^b levels than OVA mRNA (Supplementary Fig. 22f). Immunoblotting showed that OVA protein was readily detectable in both cell lysates and conditioned medium, whereas OVA-HBsAg protein in the conditioned medium was minimal (Supplementary Fig. 22g). Notably, despite abundant OVA in the conditioned medium, conditioned-medium incubation induced only weak SIINFEKL-H-2K^b presentation (Supplementary Fig. 22f). These data suggest that cross-presentation of secreted antigen is not the dominant contributor in our system. Collectively, these results support the conclusion that, in the context of OVA-HBsAg mRNA vaccination, LN macrophages predominantly present antigen via the classical endogenous MHC-I pathway following direct mRNA uptake and intracellular translation. These data were included as Supplementary Fig. 22 in the revision and relevant discussion was added in the revised manuscript (page 16-17, line 362-377).



Supplementary Fig. 22 Endogenous MHC-I presentation in lymph node macrophages following OVA-HBsAg mRNA vaccination. **a** Gating strategy for analyzing mRNA-Cy5⁺ or DiO⁺ macrophages and DCs. **b** Quantification of mRNA-Cy5⁺ or DiO⁺ cells in lymph node. **c, d** Percentage of mRNA-Cy5⁺ (**c**) or DiO⁺ (**d**) macrophages and DCs in lymph node. $n=4$. **e** Relative expression of *Tap1*, *Tap2*, and *B2m* in lymph node macrophages 24 h after vaccination. $n=3$. **f** Levels of SIINFEKL-H2K^b on RAW264.7 cells 24 h after the indicated treatments. CM, conditioned medium. $n=5$. **g** Western blot analysis of OVA or OVA-HBsAg protein in cell lysates and conditioned medium (supernatant) collected 24 h after mRNA transfection. eLNP, empty LNP. Data are shown as the mean $\pm$ s.e.m. Statistical analysis was performed by a two-tailed unpaired *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; *n.s.*, not significant.

Fig 7i - Why would the PreS1-HBs vax not induce any T cell responses in this experiment? The investigators claimed the importance of T cell induction above but now the vaccine has become ineffective? And, the responses are specific to what peptides, PreS1, HBs?

Author reply: We thank the reviewer for raising this important point. The lack of IFN- γ increase in the original Fig. 7i does not indicate vaccine ineffectiveness, but reflects the functional bias and timing of the readout in this model. In our therapeutic CHB setting, the preS1-HBsAg vaccine response is preferentially skewed toward a cytotoxic granule program rather than sustained IFN- γ production. Specifically, at 1 week after the third immunization (original Fig. 3g), we observed a significant increase in granzyme B, whereas IFN- γ showed only a modest increase with low absolute magnitude. At 4 weeks after the third immunization (original Fig. 7i), IFN- γ was not increased. Importantly, in an independent repeat experiment at the same 4-week timepoint, we again observed no significant increase in IFN- γ in the preS1-HBsAg vaccine-alone group (Fig. R1), while granzyme A/B production was significantly increased (Fig. 6h and Supplementary Fig. 26e). These updated data are now included in revised Fig. 6h and Supplementary Fig. 26e.

Regarding antigen specificity, cells were stimulated ex vivo with a preS1 peptide pool together with two selected HBsAg peptides (details now explicitly stated in the revised figure legend). Therefore, the measured responses reflect preS1/HBsAg peptide-specific T cell reactivity under the indicated assay conditions.

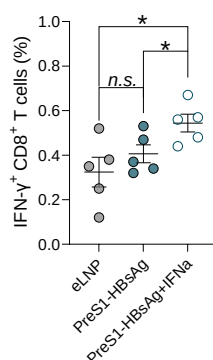


Fig. R1. Frequencies of splenic CD8⁺ T cells producing IFN- γ after preS1-HBsAg vaccination with or without IFN- α co-treatment. Splenocytes were stimulated ex vivo with a mixed peptide set comprising two HBsAg peptides (HBsAg₁₉₀₋₁₉₇, VWLSVIWM; and HBsAg₂₀₈₋₂₁₅, ILSPFLPL) and an overlapping preS1 peptide pool spanning the full preS1 domain (15-mers with 10 aa overlap). $n=5$.

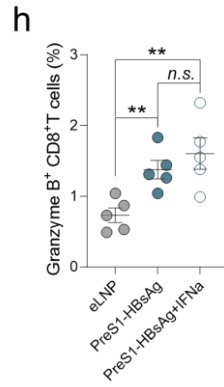
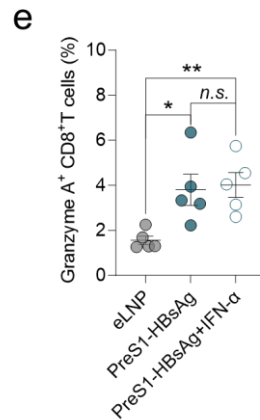


Fig. 6 Enhanced antiviral effects of preS1-HBsAg mRNA vaccine and IFN- α in chronic HBV infection. h Frequencies of CD8⁺ T cells producing granzyme B in the spleen after ex vivo stimulation with a mixed peptide set comprising two HBsAg peptides (HBsAg₁₉₀₋₁₉₇, VWLSVIWM; and HBsAg₂₀₈₋₂₁₅, ILSPFLPL) and an overlapping preS1 peptide pool spanning the full preS1 domain (15-mers with 10 aa overlap). $n=5$.



Supplementary Fig. 26 Enhanced therapeutic effect of preS1-HBsAg vaccine with IFN- α in CHB. e Flow cytometry analysis of the proportion of granzyme A⁺ CD8⁺ T cells in spleen after ex vivo stimulation with a mixture of two HBsAg peptides (HBsAg₁₉₀₋₁₉₇, VWLSVIWM; and HBsAg₂₀₈₋₂₁₅, ILSPFLPL) and an overlapping preS1 peptide pool spanning the full preS1 domain (15-mers with 10 aa overlap). $n=5$.

Reviewer #2 (Remarks to the Author):

Zhang et al screened antigen combinations paired with the HBsAg in a mRNA format for efficacy as a therapeutic vaccine in a mouse model of chronic hepatitis B (CHB). They identified preS1-HBsAg as the optimal pairing as the dual-antigen mRNA vaccine elicited robust HBV-specific immune responses leading to near-complete viral genome clearance, reduction in multiple HBV antigens in the serum and liver and production of anti-HBs antibodies.

A significant body of work exists illustrating the advantages of using the PreS region , including PreS1 , both for preventative and therapeutic vaccine design. The previous work has been mostly focused on PreS1-specific Th cell (CD4+) and B cell (antibody)-mediated protective mechanisms . For example, inclusion of the PreS region enhances the immunogenicity of the HBsAg in mice (DOI:10.1126/science.2408336) and humans (DOI.org/10.1007/s00430-014-0374-x) ; a 10-residue PreS1-peptide can prime T cell help for antibody production to the preS2 and HBsAg regions (doi.org/10.4049/jimmunol.138.12.4457) , which can bypass no responsiveness to the preS2 and S regions of HBsAg (J. Immunol.1986 Jul 1;137(1):315-22) ; PreS1 B cell epitopes inserted onto HBcAg or WHcAg carriers elicit neutralizing anti-PreS1-specific antibodies and bypass immune tolerance DOI:10.1016/j.hep.2010.10.019 and DOI.org/10.1080/21645515.2019.1689745). This body of work should be acknowledged for perspective . The authors only cite a single reference (#17).

Author reply: We thank the reviewer for this important suggestion and agree that the extensive prior work on preS/preS1-based HBV vaccines should be acknowledged for context. In the revised manuscript, we have added the key references cited by the reviewer and expanded the Discussion to briefly summarize how inclusion of preS regions can enhance HBsAg immunogenicity and support helper/antibody-mediated protection and tolerance-bypassing strategies (page 24-25, lines 533-543; refs. 38-43).

The novelty of the current study is the focus on targeting PreS1-specific , CD8+ CTL induction elicited via mRNA immunization , which has not been previously examined in any detail. The authors report very efficient viral clearance and reduction in HBsAg , but weak or absent anti-HBs production using the preS1-HBsAg or preS1 only mRNA vaccines .These results and a favorable safety profile suggest that this may be a promising therapeutic strategy going forward .

Author reply: We thank this reviewer for his/her positive assessment on this work and we have carefully addressed the issues raised by the reviewer.

Specific Comments :

1. The text often refers to the superiority of the preS1-HBsAg construct over HBsAg alone , yet a number of characteristics (ie., serum HBsAg (fig.2c) ; Tregs (fig.3e) ; lymph node CD69+ T cells (fig.3c) ; lymph node PD1+ CD8+ T cells (fig.3d) are not

significantly different . Also , several of the other dual-constructs were significantly different from the HBsAg alone construct and these were not mentioned in the text . Perhaps explanations in the text should be more carefully phrased to reflect this complexity .

Author reply: We thank the reviewer for this careful comment. We agree that not all readouts show statistically significant differences between preS1-HBsAg and HBsAg, and that several endpoints show only non-significant trends (e.g., serum HBsAg in Fig. 2c; Tregs in Fig. 3e; LN CD69⁺ T cells in Fig. 3c; LN PD-1⁺ CD8⁺ T cells in Fig. 3d). We have therefore revised the text to avoid overstatement and to explicitly distinguish statistically significant effects from trends, emphasizing that the advantages of the preS1-HBsAg construct are most evident in key intrahepatic virologic readouts.

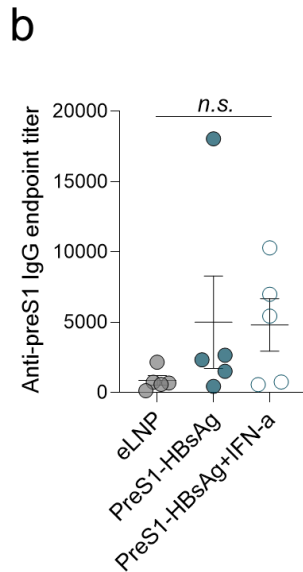
We also agree that some other dual-construct candidates show endpoint-dependent effects. In particular, HBcAg-HBsAg significantly reduced intrahepatic HBV DNA compared with HBsAg alone (Fig. 2d), whereas HBxAg-HBsAg did not show comparable benefits across the other readouts evaluated. We have incorporated this clarification and adjusted the narrative accordingly in the Results (page 5, lines 113–122; page 7, lines 163–167).

2. There seems to be some disparity in anti-HBs production after preS1-HBsAg immunization. In Fig.2j it appears to be 1/5 positive mice before the rHBsAg boost and in fig.5f it appears to be 4/5 mice positive for anti-HBs ?

Author reply: We thank the reviewer for pointing out this apparent difference. The anti-HBsAg measurements in original Fig. 2j and Fig. 5f were performed at different time points relative to the last immunization. Fig. 2j was assessed 1 week after completion of immunization, whereas Fig. 5f was assessed 4 weeks after completion. Consistent with the kinetics of vaccine-induced seroconversion in this chronic setting, anti-HBsAg responses were delayed and variable across individual mice, such that only a subset of animals were above the detection threshold at the earlier time point, while additional mice became detectable at the later time point. These panels derive from independent experiments with the same vaccine regimen, and we have clarified the sampling time points in the revised figure legends (page 7, line 149-150; page 15, line 330).

3. Did the anti-HBs assay used detect anti-PreS1 antibodies ? Did the authors measure preS1-specific Abs ?

Author reply: We thank the reviewer for this careful comment. The anti-HBs assay used in this study (Abbott kit) is specific for anti-HBs and does not detect anti-preS1 antibodies. To directly address this point, we performed an additional anti-preS1 IgG ELISA. In this assay, anti-preS1 antibodies were detectable in mice vaccinated with preS1-HBsAg, whereas the eLNP control group remained at low/near-background levels. Co-treatment with IFN- α did not significantly further increase anti-preS1 titers compared with preS1-HBsAg alone, likely due to relatively large within-group variance. These data were included as Supplementary Fig. 26b in the revised manuscript, and the relevant discussion has been added (page 19, line 434).



Supplementary Fig. 26 Enhanced therapeutic effect of preS1-HBsAg vaccine with IFN- α in CHB. b Serum anti-preS1 IgG titer at 10 weeks after virus injection. $n=5$.

4. In the in vitro stimulated cellular assays the authors used a panel of peptides specific for HBsAg or preS1 , but do not describe the panel . Did the peptide panel span the entire amino acid sequence of both antigens ?

Author reply: We thank the reviewer for this important question. The peptide reagents were designed differently for HBsAg and preS1. For HBsAg, we used two defined MHC-I-restricted epitopes (HBsAg₁₉₀₋₁₉₇, VWLSVIWM; and HBsAg₂₀₈₋₂₁₅, ILSPFLPL) rather than a full-length overlapping peptide set. For preS1, to ensure unbiased coverage, we used an overlapping peptide pool spanning the entire preS1 domain (15-mers overlapping by 10 amino acids). The full peptide information is now provided in Supplementary Table 2, as noted in the revised manuscript (page 13, line 282-293).

Supplementary Table 2. Peptides.

Antigen	Number	Peptide pool/ Peptide
PreS1	PreS1-1	GQNLSTSNPLGFFPD
	PreS1-2	TSNPLGFFPDHQLDP
	PreS1-3	GFFPDHQLDPAFRAN
	PreS1-4	HQLDPAFRANTANPD
	PreS1-5	AFRANTANPDWDFNP
	PreS1-6	TANPDWDFNPNKDTW
	PreS1-7	WDFNPNKDTWPDANK
	PreS1-8	NKDTWPDANKVGAGA
	PreS1-9	PDANKVGAGAFGLGF
	PreS1-10	VGAGAFGLGFTPPHG
	PreS1-11	FGLGFTPPHGGLLGW
	PreS1-12	TPPHGGLLGWSPQAQ
	PreS1-13	GLLGWSPQAQGILQT
	PreS1-14	SPQAQGILQTLPANP
	PreS1-15	GILQTLPANPPPAST
	PreS1-16	LPANPPPASTNRQSG
	PreS1-17	PPASTNRQSGRQPTP
	PreS1-18	NRQSGRQPTPLTPPL
	PreS1-19	RQPTPLTPPLRDTHP
	PreS1-20	PTPLTPPLRDTHPHM
HBsAg-1	HBsAg ₁₉₀₋₁₉₇	VWLSVIWM
HBsAg-2	HBsAg ₂₀₈₋₂₁₅	ILSPFLPL
HBcAg		DIDPYKEFGATVELL
		KEFGATVELLSFLPS
		TVELLSFLPSDFFPS
		SFLPSDFFPSVRDLL
		DDFFPSVRDLLDTASA
		VRDLLDTASALYREA
		DTASALYREALESPE
		LYREALESPEHCSPH
		LESPEHCSPHHTALR
		HCSPHHTALRQAILC
		HTALRQAILCWGELM
		QAILCWGELMTLATW
		WGELMTLATWVGVLN
		TLATWVGVLNLEDPAS
		VGVLNLEDPASRDLLV
		EDPASRDLLVSVYNT

	RDLVVS YVNTNMGLK
	SYVNTNMGLKFRQLL
	NMGLKFRQLLWFHIS
	FRQLLWFHISCLTFG
	WFHISCLTFGRETVI
	CLTFGRETVIEYLV
	RETVIEYLVSFVWI
	EYLVSFVWIRTTPA
	FGVWIRTTPAYRPPN
	RTPPAYRPPNAPILS
	YRPPNAPILSTLPET
	APILSTLPETTVVRR
	TLPETTVVRRRGRSP
	TVVRRRGRSPRRRTP
	RGRSPRRRTPSPRRR
	RRRTPSPRRRRSQSP
	SPRRRRSQSPRRRS
	RSQSPRRRRSQSRES
	QSPRRRRSQSRESQC
HBxAg	AARLCCQLDPARDVL
	CQLDPARDVLCLRPV
	ARDVLCLRPVGAESR
	CLRPVGAESRGRPFS
	GAESRGRPFSGSLGT
	GRPFSGSLGTLSSPS
	GSLGTLSSPSPSAVP
	LSSPSPSAVPTDHGA
	PSAVPTDHGAHLSLR
	TDHGAHLSLRGLPVC
	HLSLRGLPVCAFSSA
	GLPVCAFSSAGPCAL
	AFSSAGPCALRFTSA
	GPCALRFTSARRMET
	RFTSARRMETTVNAH
	RRMETTVNAHQILPK
	TVNAHQILPKVLHKL
	QILPKVLHKRTLGLS
	VLHKRTLGLSAMSTT
	TLGLSAMSTTDLEAY

		AMSTTDLEAYFKDCL
		DLEAYFKDCLFKDWE
		FKDCLFKDWEELGEE
		FKDWEELGEEIRLKV
		ELGEEIRLKVFLGG
		IRLKVFLGGCRHKL
		FVLGGCRHKLVCAPA
		CRHKLVCAPAPCNFF
		KLVCAPAPCNFF TSA
OVA	OVA ₂₅₇₋₂₆₄	SIINFEKL
	OVA ₃₂₃₋₃₃₉	KISQAVHAHA EINEAG

5. Was there an attempt to identify the specific peptide responsible for the activation ? This is important because a single mouse strain (B6) was used for all the studies . Theoretically , the authors may have identified a " super " strong CTL epitope on the preS1 region restricted to MHC class I molecules of B6 mice rather than the preS1-HBsAg construct outperforming the HBc-HBsAg or HBx-HBsAg constructs ?

Author reply: We thank the reviewer for this important question. We agree that all in vivo studies were performed in C57BL/6 (H-2^b) mice and that immunodominant MHC-I-restricted CTL epitopes within preS1 could contribute to the efficacy observed in this model. To address this directly, we performed peptide-level mapping of preS1-directed cellular responses following vaccination. Splenocytes from mice immunized three times with the preS1-HBsAg vaccine were stimulated individually with each 15-mer peptide (10-aa overlap) spanning the full preS1 domain, and antigen-specific responses were quantified by GzmB ELISpot. These experiments revealed broad reactivity across multiple preS1 peptides, with a clear immunodominant hotspot: peptides #4-#6 consistently elicited the strongest responses, comparable to stimulation with the full preS1 peptide pool (Fig. 4e and Supplementary Fig. 19). This mapping indicates that the preS1 region contains a dominant CTL determinant in the C57BL/6 (H-2^b) setting within HQLDPAFRANTANPDWDFNPNKDTW sequence, which likely contributes to the enhanced cellular immunity observed with preS1-containing constructs in our model. We have incorporated these mapping data into the revised Results and explicitly noted the strain/MHC restriction as a limitation and a rationale for future validation across additional genetic backgrounds (page 13, line 282-293).

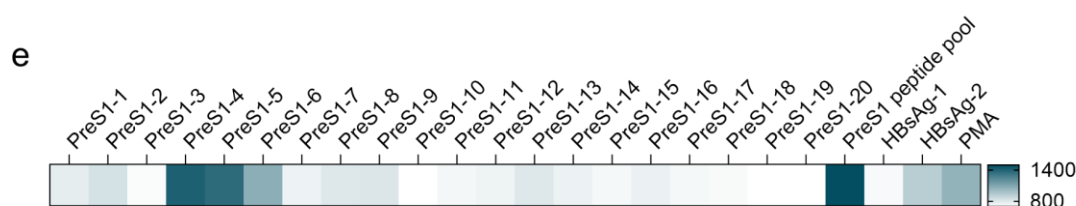
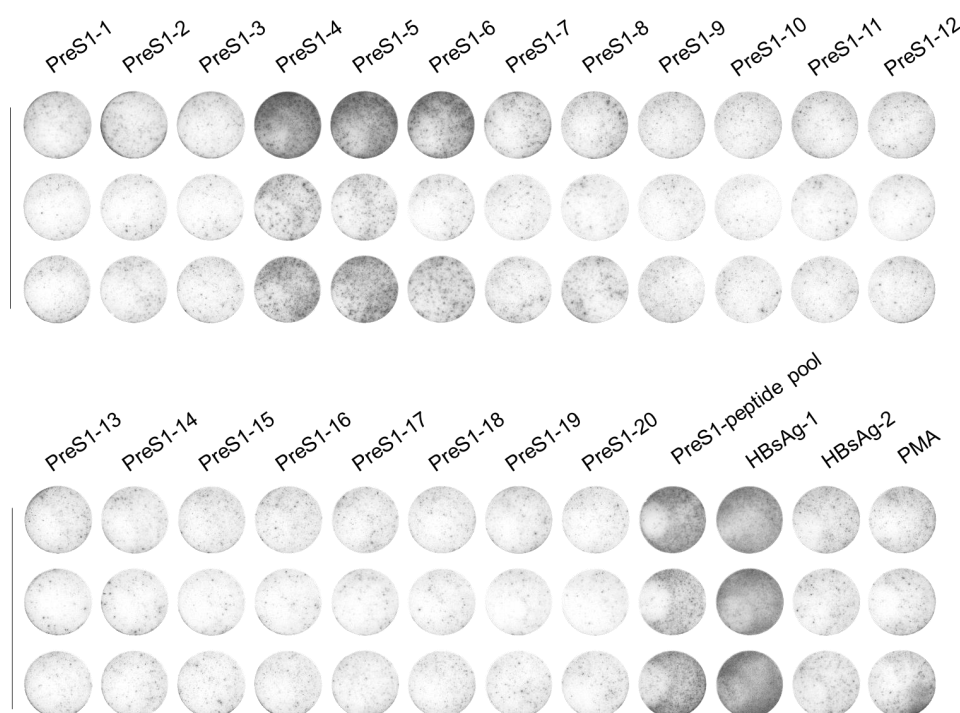


Fig. 4 PreS1 and HBsAg synergistically act to achieve HBsAg seroconversion. e, Quantitative analysis of granzyme B ELISpot results. The colour scale indicates the

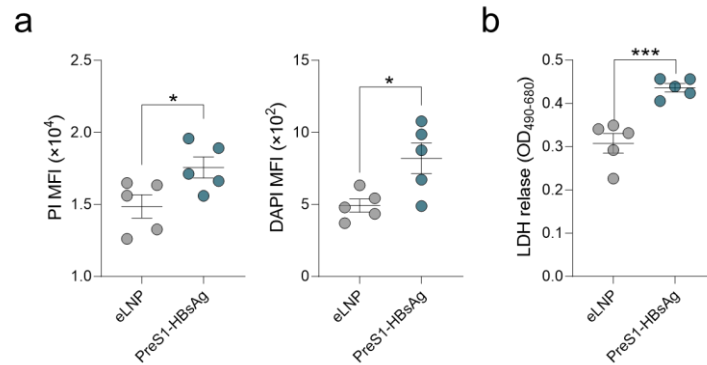
number of spots per well. Splenocytes (5×10^5 cells per well) from mice immunized three times with the preS1-HBsAg mRNA vaccine were stimulated for 24 h with individual overlapping 15-mer peptides spanning preS1 (10-aa overlap), the full preS1 peptide pool, or two HBsAg epitopes, as indicated. PMA (phorbol 12-myristate 13-acetate) served as a positive control. $n=3$.



Supplementary Fig. 19 Mapping of preS1-specific CTL responses by granzyme B ELISpot. Splenocytes (5×10^5 cells per well) from mice immunized three times with the preS1-HBsAg mRNA vaccine were stimulated for 24 h with individual overlapping 15-mer peptides spanning preS1 (10-aa overlap), the full preS1 peptide pool, or two HBsAg epitopes, as indicated. PMA (phorbol 12-myristate 13-acetate) served as a positive control. $n=3$.

6. Did the authors perform any classical in vitro CTL assays to determine if cytotoxic effector T cells explain the antiviral effects of the preS1-HBsAg vaccine ?

Author reply: We thank the reviewer for this important question. To directly test whether vaccine-elicited cytotoxic effector T cells can kill infected target cells, we performed a classical in vitro cytotoxicity assay using splenocytes from vaccinated mice as effectors and hepatocytes isolated from our CHB model mice as target cells. After 24 h of co-culture, hepatocyte death was significantly increased in the preS1-HBsAg mRNA vaccine group compared with the eLNP control, as quantified by increased PI/DAPI staining in hepatocytes and by elevated LDH release in the supernatant. These results support that vaccination induces cytotoxic effector activity capable of killing CHB hepatocytes, consistent with a CTL contribution to the antiviral effects observed in vivo. The data are now provided in Supplementary Fig. 12 (page 8, line 180-182).



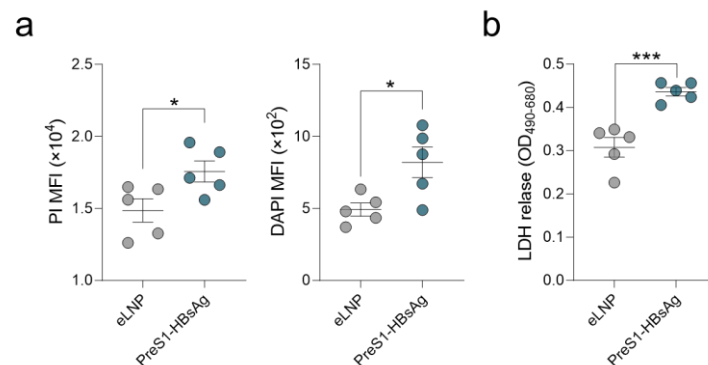
Supplementary Fig. 12 Vaccine-elicited effector cells mediate cytotoxicity against CHB hepatocytes in vitro. Effector splenocytes were isolated from mice immunized with preS1-HBsAg vaccine and co-cultured with hepatocytes isolated from CHB model mice at an effector-to-target ratio of 50:1 for 24 h. **a** Quantification of target-cell death by PI and DAPI staining. $n=5$. **b** LDH release in co-culture supernatants. $n=5$. eLNP, empty LNP. Data are shown as the mean $\pm$ s.e.m. Statistical analysis was performed by a two-tailed unpaired t -test. * $P < 0.05$ and *** $P < 0.001$.

7. Granzyme B + CD8⁺ and CD4⁺ T cells were detected and presumed to play a pivotal role in viral clearance. This mechanism would presumably result in granzyme B - mediated cytotoxicity against infected hepatocytes , yet no increases in liver enzymes were reported . In fact , the only mention of ALT/AST levels was in Fig. S11c , which showed no elevations in liver enzymes at study endpoint. The authors also show a modest level of T cell IFN γ production , which suggests that the common non-cytopathic pathway for viral elimination is not likely the explanation for viral clearance . Perhaps further discussion of possible clearance mechanisms is warranted .

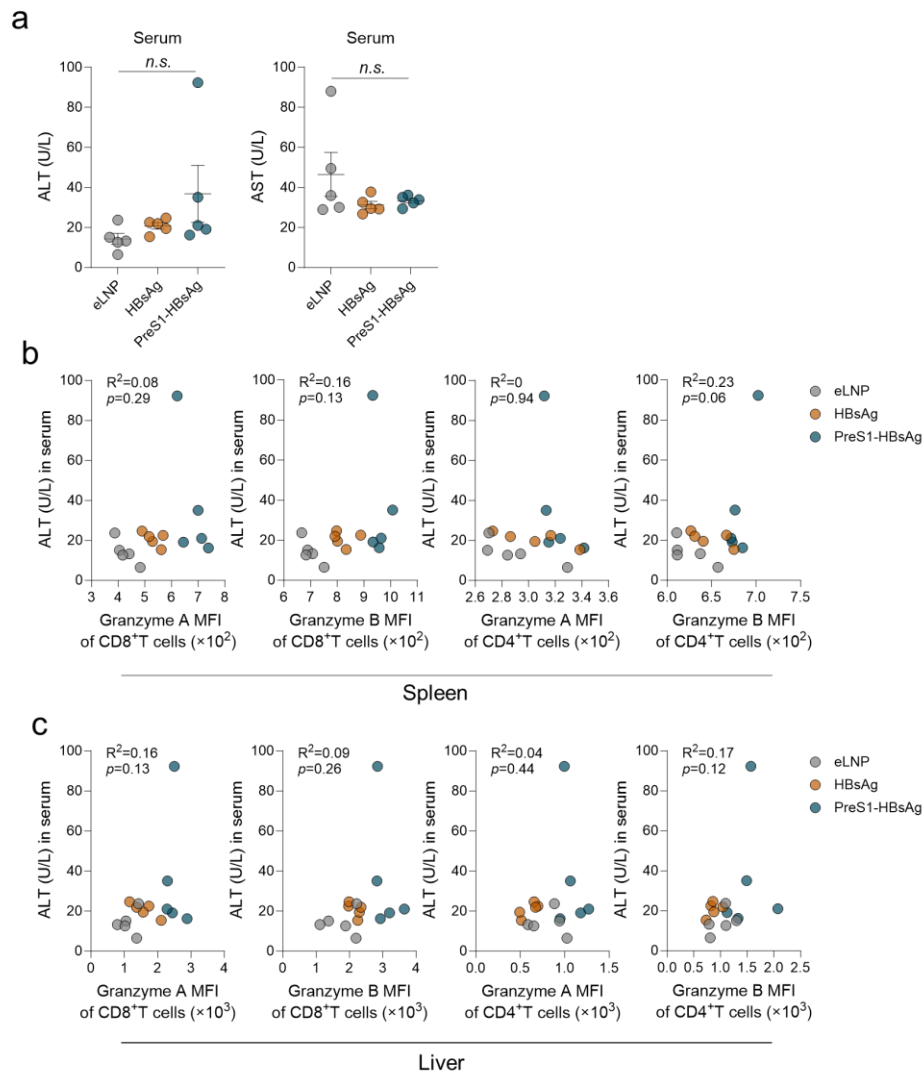
Author reply: We thank the reviewer for this important and insightful comment. We agree that endpoint ALT/AST alone may not fully capture transient cytolytic events during antiviral clearance. To address this point, we added new mechanistic and safety-related evidence. First, in a newly performed ex vivo cytotoxicity assay, effector cells from vaccinated mice directly killed primary hepatocytes isolated from CHB mice (Supplementary Fig. 12), supporting granzyme-associated cytotoxic capacity. Second, beyond endpoint measurements, we assessed serum ALT at an earlier post-immunization timepoint (1 week after completion of the 3-dose regimen) and observed a mild increase in the preS1-HBsAg group, although not statistically significant (Supplementary Fig. 27), consistent with limited/transient cytolytic activity rather than overt liver injury. At endpoint, ALT/AST remained without significant elevation (Fig. 7b), indicating no sustained hepatotoxicity.

Regarding the IFN- γ pathway, we further evaluated efficacy in IFN- γ knockout (GKO) mice. Antiviral activity was preserved in GKO mice, together with increased effector T cells and granzyme-positive CD8⁺ T cells (Fig. R2). Combined with intrahepatic granzyme readouts (Fig. 3g, h) and the ex vivo killing data (Supplementary Fig. 12), these findings support an IFN- γ -independent, granzyme-associated cytotoxic component of antiviral control in this model.

We have revised the Discussion accordingly (page 26, line 571-582). We now explicitly state that our data support a granzyme-associated mechanism, while not excluding additional non-cytopathic or cooperative pathways.



Supplementary Fig. 12 Vaccine-elicited effector cells mediate cytotoxicity against CHB hepatocytes in vitro. Effector splenocytes were isolated from mice immunized with preS1-HBsAg vaccine and co-cultured with hepatocytes isolated from CHB model mice at an effector-to-target ratio of 50:1 for 24 h. **a** Quantification of target-cell death by PI and DAPI staining. *n*=5. **b** LDH release in co-culture supernatants. *n*=5. eLNP, empty LNP. Data are shown as the mean $\pm$ s.e.m. Statistical analysis was performed by a two-tailed unpaired *t*-test. **P* < 0.05 and ****P* < 0.001.



Supplementary Fig. 27 Correlation analysis of immune response and ALT changes.
a Levels of serum ALT and AST one week after the third vaccination. $n=5$. **b, c** Correlation analysis of T cell immune response in spleen (**b**) or liver (**c**) and ALT changes. $n=5$. eLNP, empty LNP. Data are shown as the mean $\pm$ s.e.m. Statistical analysis was performed by a two-tailed unpaired t -test. *n.s.*, not significant. The Pearson correlation coefficient and a two side Pearson's correlation P values are indicated.

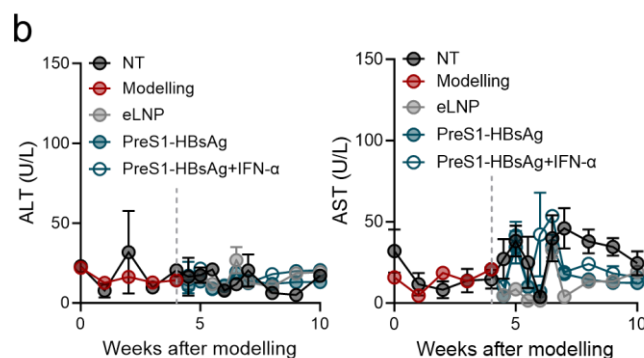


Fig. 7 Safety profile of preS1-HBsAg vaccine and IFN- α combination therapy. b Longitudinal monitoring of ALT/AST in the serum of AAV-HBV1.3 carrier mice receiving indicated treatments. NT, Healthy mice. $n=3$ or 5. eLNP, empty LNP. Data are shown as the mean $\pm$ s.e.m.

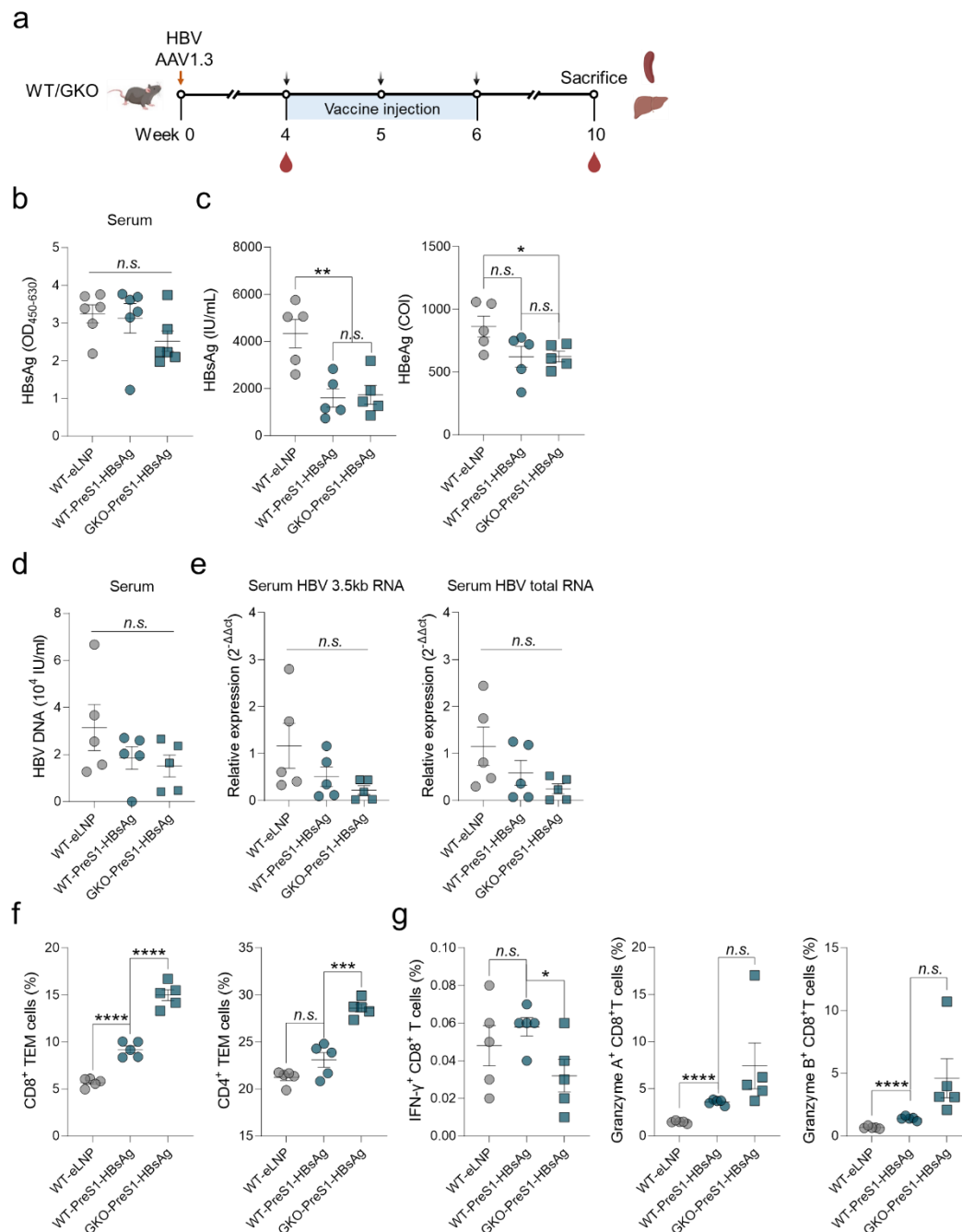


Fig. R2 PreS1-HBsAg vaccine-induced immune responses in wild-type and IFN- γ knockout mice. a Experimental timeline. WT, wild-type C57BL/6 mice. GKO, IFN- γ knockout mice. **b** Serum HBsAg levels quantified by ELISA at 4 weeks after virus injection. **c** Serum HBsAg and HBeAg levels quantified by ELISA at 10 weeks after virus injection. **d** Serum HBV DNA levels quantified by qPCR. **e** Serum HBV RNA levels quantified by qPCR. **f** Flow cytometry analysis of CD8⁺ or CD4⁺ TEMs in spleen.

g Flow cytometry analysis of cytokine-positive CD8⁺ T cells in spleen after ex vivo stimulation with a mixture of two HBsAg peptides (HBsAg_{190–197}, VWLSVIWM; and HBsAg_{208–215}, ILSPFLPL) and an overlapping preS1 peptide pool spanning the full preS1 domain (15-mers with 10 aa overlap). *n*=5. eLNP, empty LNP. All data are shown as the mean ± s.e.m. Statistical analysis was performed by a two-tailed unpaired *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001; *n.s.*, not significant.

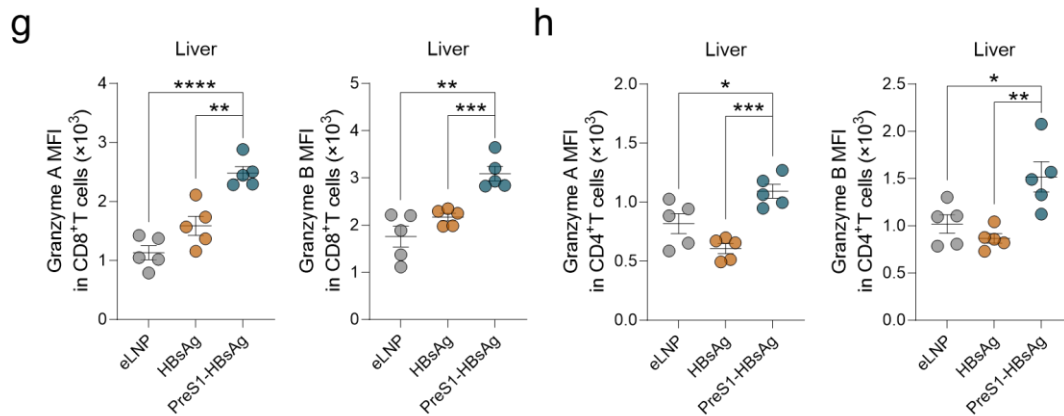


Fig. 3 PreS1-HBsAg mRNA vaccine reprograms the hepatic immune microenvironment in chronic HBV infection. g, h Granzyme A and granzyme B MFI in intrahepatic CD8⁺ T cells (**g**) and CD4⁺ T cells (**h**) after ex vivo stimulation with a mixed peptide set comprising two HBsAg peptides (HBsAg_{190–197}, VWLSVIWM; and HBsAg_{208–215}, ILSPFLPL) and an overlapping preS1 peptide pool spanning the full preS1 domain (15-mers with 10 aa overlap). *n*=5. eLNP, empty LNP. Data are shown as the mean ± s.e.m. Statistical analysis was performed by a two-tailed unpaired *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001; *n.s.*, not significant.

Reviewer #3 (Remarks to the Author):

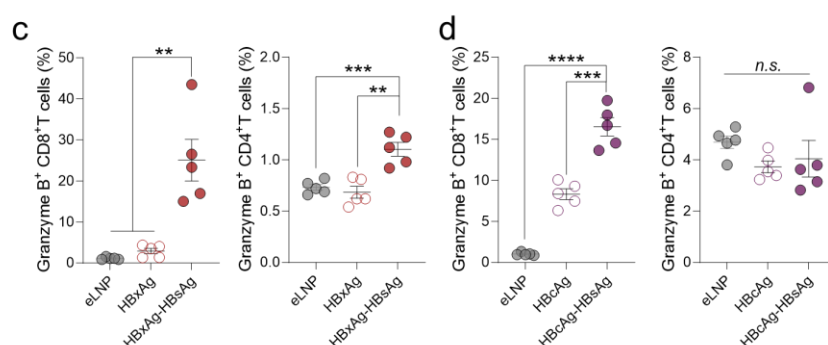
In this study, Zhang, Wang, and colleagues investigate the potential of an mRNA vaccine co-expressing preS1 and HBsAg for treating hepatitis B virus (HBV) infection and evaluate the effects of vaccination on viral parameters and virus-specific immunity in a mouse model persistently replicating HBV. Authors demonstrate that combining preS1 and HBsAg in the mRNA vaccine induces virus-specific humoral and cellular immunity, resulting in a significant decrease in markers of infection. In addition, they demonstrate that the therapeutic effect of vaccination can be enhanced by combining it with IFN- α treatment. The authors conclude that the preS1-HBsAg mRNA vaccine represents a promising and easily translatable treatment strategy for chronic hepatitis B. The work adds insights into developing novel strategies to achieve a functional cure for chronic hepatitis B and is of clinical relevance. Overall, the study is well described and presents analyses of the immune response induced by mRNA vaccination, including transcriptome analyses of immune cells at the site of infection. The manuscript is well-written, the experiments are in majority well-designed, and the results are overall interesting, but several issues and limitations need to be addressed to further improve the manuscript.

Author reply: We thank this reviewer for his/her positive assessment on this work and we have carefully addressed the issues raised by the reviewer.

Major comments:

1. The authors demonstrate an adjuvant effect of HBsAg using the ovalbumin as a model. This is an interesting result, but the authors' attempt to develop therapy against chronic hepatitis B and to examine the immunostimulatory effect of HBsAg on other HBV antigens is more relevant. The lack of these data is puzzling, as in their initial experiments, they had already combined HBsAg with HBV core or x antigens and shown a decrease in HBV parameters.

Author reply: We thank the reviewer for this constructive comment. We agree that demonstrating any immunostimulatory (adjuvant-like) effect of HBsAg in the context of HBV antigens is more directly relevant to the therapeutic intent of this study. We clarify that the readouts in revised Fig. 5g-i (originally Fig. 6g-i) reflect preS1-specific responses that are enhanced in the preS1-HBsAg group (splenocytes were stimulated ex vivo with the preS1 peptide pool; this is now stated in the figure legend). To address whether HBsAg can similarly enhance responses to other HBV antigens, we performed additional experiments evaluating T cell effector readouts after HBxAg or HBcAg peptide-pool stimulation in mice immunized with the corresponding antigen constructs with or without HBsAg. We observed increased frequencies of granzyme B⁺ CD8⁺ (and CD4⁺) T cells in the HBsAg-containing groups, supporting an adjuvant-like immunostimulatory effect of HBsAg in HBV-antigen settings. These new data are provided in Supplementary Fig. 25c, d. We have incorporated these data and updated the Results accordingly (page 17, lines 394-401).



Supplementary Fig. 25 HBsAg confers a potential adjuvant-like effect on HBV antigen immunogenicity. c, d Frequencies of granzyme B⁺ CD8⁺ T cell and CD4⁺ T cell in spleen after ex vivo stimulation with the HBxAg peptide pool (c) or HBcAg peptide pool (d). *n*=5.

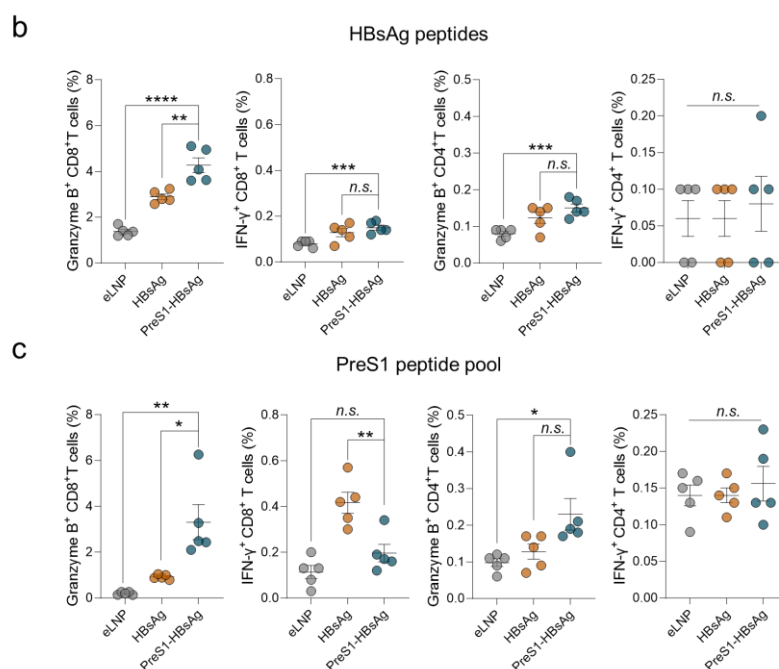
2. Following that line, would a combination of preS1+HBsAg with other HBV antigens for vaccination additionally improve the overall antiviral effect? Since alternative therapeutic HBV vaccines that utilize a combination of several HBV antigens for vaccination are currently being investigated (Kosinska et al, Mol Ther Nucleic Acids. 2025), this matter should at least be discussed.

Author reply: We thank the reviewer for this insightful suggestion. We agree that adding additional HBV antigens to the preS1–HBsAg vaccine could, in principle, broaden epitope coverage and may further enhance antiviral efficacy in CHB. This concept is supported by recent multi-antigen therapeutic vaccination strategies, such as the TherVacB regimen reported by Kosinska et al. (*Mol. Ther. Nucleic Acids*, 2025), which uses a heterologous prime–boost approach to induce strong, multi-specific T-cell responses against multiple HBV proteins and achieves sustained antiviral effects in HBV carrier models across clinically relevant genotypes. Accordingly, we have added a brief Discussion section addressing the rationale and future potential of multi-antigen combinations building on our preS1–HBsAg platform (page 25-26, line 558-570). Systematic optimization (antigen choice, format, and dosing) is beyond the scope of the current study.

3. Overall, the analysis of the T cell response in the manuscript could be improved to significantly strengthen the authors' findings. 1) The authors claim that the preS1 is the main inducer of T cell response and is responsible for the antiviral effect of the vaccine, but do not show the results for HBsAg-specific stimulation, although they mention the stimulation with HBsAg peptide in the Methods section. Including data on HBsAg-specific T cell responses could substantiate the authors' conclusions. 2) Analysis of the specific T cell response in the liver, the site of infection, using either multimers or peptide pool stimulation, would be more informative than in the spleen and link the authors' findings with the antiviral effect. 3) In Figure 3, is the granzyme B and TGF-β expression measured after the specific pool stimulation or directly ex vivo? I struggle to find this information. Moreover, authors should explain why they do not perform analysis of the T cell activation markers such as CD69 and CD44 on specific CD8 T cells, but total CD8 population?

Author reply: We sincerely thank the reviewer for these insightful suggestions. As suggested, we have performed additional experiments and analyses to strengthen the T-cell response data. Our point-by-point responses are provided below.

1) We agree that HBsAg-specific readouts should be shown explicitly. In response to the reviewer's suggestion, we repeated the functional assays using HBsAg-specific stimulation. Specifically, splenocytes were stimulated *ex vivo* with two defined HBsAg MHC-I immunodominant epitopes (HBsAg_{190–197}, VWLSVIWM; and HBsAg_{208–215}, ILSPFLPL), while preS1 responses were assessed using an overlapping peptide pool spanning the full preS1 domain. Under these conditions, we observed readily detectable HBsAg-reactive granzyme B responses in the preS1–HBsAg–vaccinated mice (new Supplementary Fig. 11b, c). These data substantiate that HBsAg-specific effector responses can be induced in our platform, alongside robust preS1-directed cellular responses. We have incorporated these data and updated the Results accordingly (page 8, line 178-180).



Supplementary Fig. 11 b, c Frequencies of granzyme B- and IFN-γ-producing CD8⁺ and CD4⁺ T cells in spleen after *ex vivo* stimulation with two HBsAg peptides (HBsAg_{190–197}, VWLSVIWM; and HBsAg_{208–215}, ILSPFLPL) (b) or an overlapping preS1 peptide pool (15-mers overlapping by 10 aa) spanning the full preS1 domain (c). *n*=5.

2) To address antigen-specific T-cell responses at the site of infection, we extended our analyses to intrahepatic lymphocytes. By HBsAg tetramer staining, HBsAg-specific CD8⁺ T cells were detectable in the liver. And the preS1-HBsAg group showed a numerically higher frequency than the HBsAg-only group, although the difference did not reach statistical significance (Fig. 3f). In addition, functional readouts in the liver revealed enhanced cytotoxic effector activity in the preS1–HBsAg group: following *ex vivo* antigen stimulation, intrahepatic T cells displayed higher granzyme A and granzyme B levels compared with HBsAg-only controls (Fig. 3g, h). These data suggest that preS1-HBsAg vaccination enhances intrahepatic HBV-specific antiviral immunity. We have incorporated

these data and updated the Results accordingly (page 10, lines 234-244).

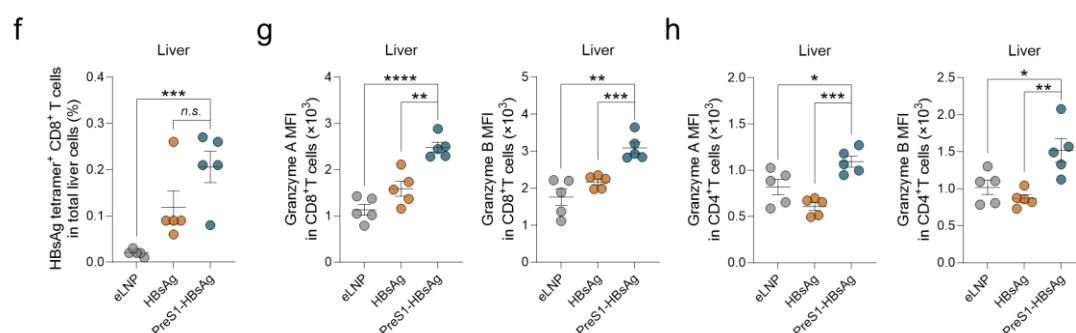
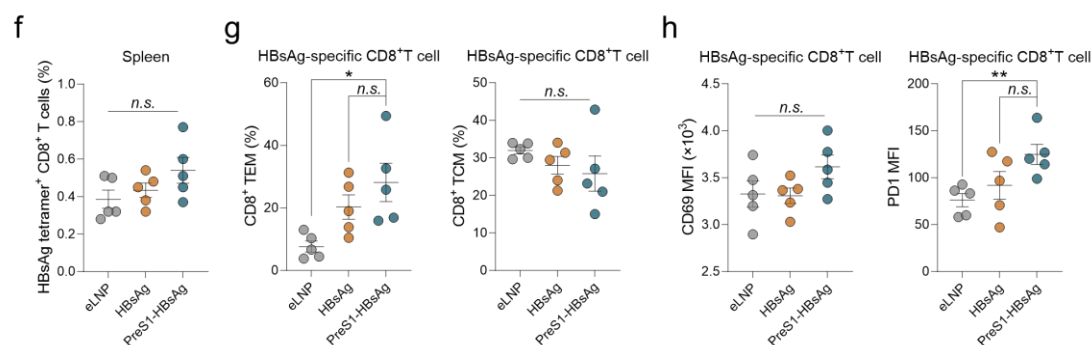


Fig. 3 **f** Frequency of HBsAg tetramer⁺ CD8⁺ T cells in liver. *n*=5. **g, h** Flow cytometry analysis of granzyme A and granzyme B MFI in intrahepatic CD8⁺ T cells (**g**) and CD4⁺ T cells (**h**) after ex vivo stimulation with a mixed peptide set comprising two HBsAg peptides (HBsAg_{190–197}, VWLSVIWM; and HBsAg_{208–215}, ILSPFLPL) and an overlapping preS1 peptide pool spanning the full preS1 domain (15-mers with 10 aa overlap). *n*=5.

3) We apologize for not stating this clearly. In the original Fig. 3, granzyme B and TGF-β were quantified by intracellular staining of splenocytes after ex vivo antigen stimulation, rather than by directly staining without stimulation. Specifically, splenocytes were restimulated with a peptide mixture comprising two defined HBsAg CD8⁺ T-cell epitope peptides and an overlapping preS1 peptide pool. We have now added this information explicitly to the figure legend. Therefore, the results reflect HBV antigen-specific CD8⁺ T cell responses.

We agree that analyzing activation markers on antigen-specific CD8⁺ T cells would be ideal. We therefore performed HBsAg tetramer staining; however, the frequency of HBsAg tetramer⁺ CD8⁺ T cells in spleen was very low and did not differ significantly between groups (Supplementary Fig. 8f), which limits statistical power for detailed phenotyping. For transparency, we now provide the tetramer frequency and activation-marker readouts within this compartment (including CD69 and PD-1) in new Supplementary Fig. 8g, h. In addition, because multimer reagents are not available for preS1 epitopes in our setting, we analyzed total CD8⁺ T cells as a consistent readout across groups. While this does not substitute for antigen-specific quantification, it provides a complementary population-level measure of vaccine-induced effector skewing/activation in the same tissue and time window. We have incorporated these data and updated the Results accordingly (page 7, lines 168-170).



Supplementary Fig. 8 **f** Proportion of HBsAg tetramer⁺ in CD8⁺ T cells in spleen. **g**

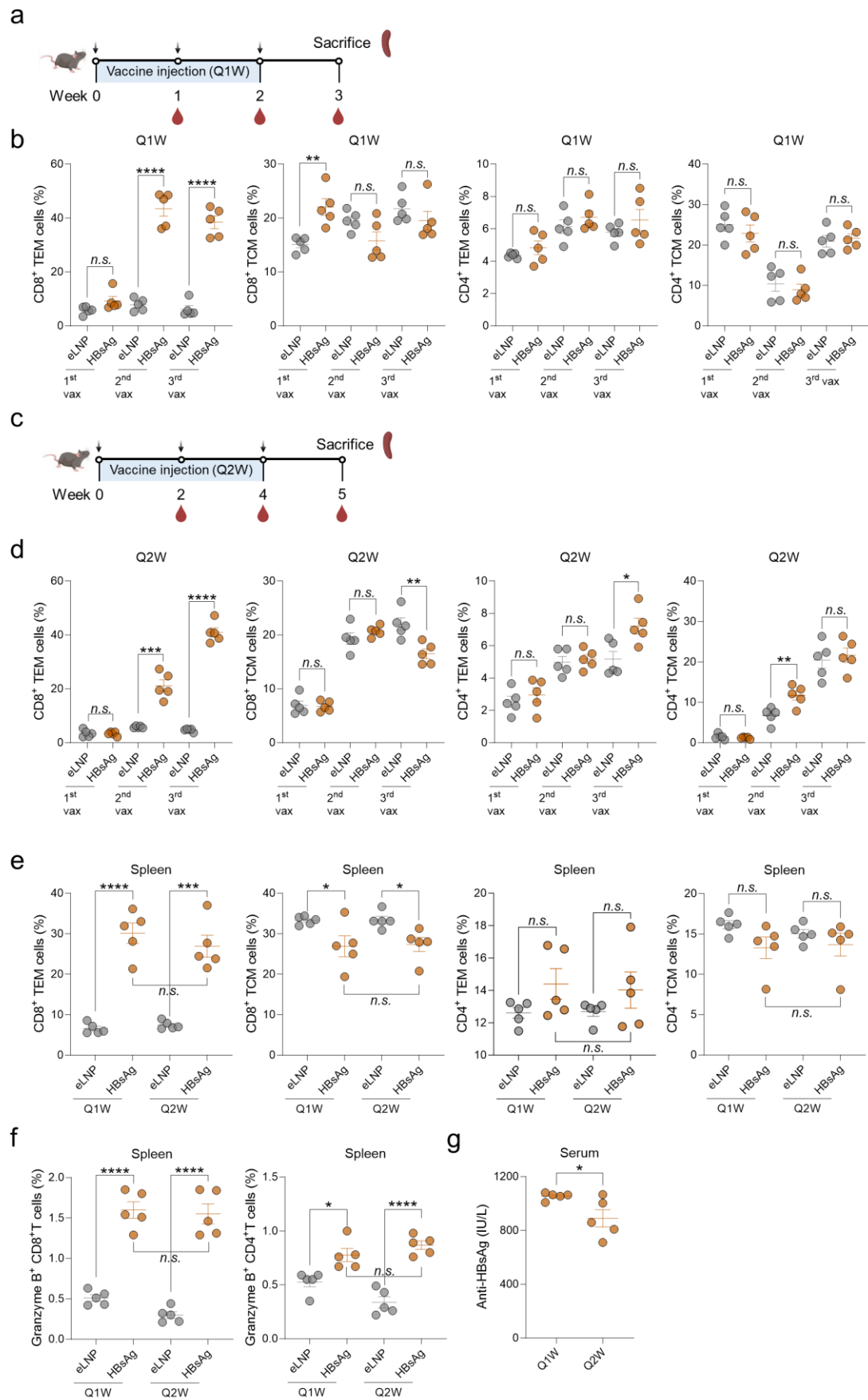
Proportion of TEM and TCM in HBsAg tetramer⁺ CD8⁺ T cells in spleen. **h** MFI of CD69 and PD1 in HBsAg tetramer⁺ CD8⁺ T cells in spleen. *n*=5.

4. The authors vaccinate the mice with the mRNA vaccine in a one-week interval. This is unusual, as most mRNA vaccinations in preclinical and clinical settings are typically performed at least 2 weeks apart, and such a short interval may interfere with the induction of efficient immunity after vaccination. Did the authors optimize the vaccination regimen before conducting the experiments, and do they have any supporting data?

Author reply: We thank the reviewer for this important question. We agree that many prophylactic mRNA vaccine studies use ≥ 2 -week intervals to favour durable memory formation. Here, however, our goal was therapeutic vaccination in a tolerogenic CHB setting, and we therefore tested whether a more intensive schedule could better maintain antigen-specific effector activity during the treatment window.

To address whether the 1-week interval affects immune induction and whether the regimen was optimized, we performed a head-to-head comparison of dosing schedules (weekly vs every 2 weeks) while keeping the total number of immunizations constant. For longitudinal peripheral-blood monitoring, samples were collected at the end of each dosing interval (immediately prior to the next injection)—i.e., 1 week after injection in the weekly arm and 2 weeks after injection in the every-2-week arm (Supplementary Fig. 4a-d). These data therefore reflect interval-end levels rather than time-aligned post-dose kinetics, and were used to assess how well responses are sustained between doses. Overall, memory T-cell readouts were broadly comparable between regimens, with a trend toward higher interval-end levels under the weekly schedule during the treatment period.

Importantly, for a direct, time-matched comparison, terminal analyses were performed 1 week after completion of the full immunization course for both regimens at sacrifice (Supplementary Fig. 4e-g). Under these matched endpoint conditions, the two regimens elicited comparable T-cell phenotypes/function (including effector-memory and granzyme B⁺ readouts), whereas the weekly regimen yielded higher serum anti-HBsAg levels at this matched endpoint. These data are now provided in Supplementary Fig. 4 and described in the revised Results (page 4-5, line 104-109).



Supplementary Fig. 4 Influence of different dosing cycles on the immune response

induced by the vaccine. a Experimental timeline for weekly immunization (Q1W; injections at weeks 0, 1, and 2). **b** Frequencies of TEM (CD44⁺ CD62L⁻) and TCM (CD44⁺ CD62L⁺) subsets among CD8⁺ and CD4⁺ T cells in blood measured 1 week after each injection. *n*=5. **c** Experimental timeline for immunization every two weeks (Q2W; injections at weeks 0, 2, and 4). **d** Frequencies of TEM and TCM subsets among CD8⁺ and CD4⁺ T cells in blood measured 2 weeks after the first and second injections and 1 week after the third injection. *n*=5. **(e-g)** Terminal analyses at 1 week after completion of the full immunization course. **e** Frequencies of TEM and TCM subsets among CD8⁺ and CD4⁺ T cells in spleen. **f** Frequencies of CD8⁺ and CD4⁺ T cells producing granzyme B in spleen upon ex vivo stimulation with two HBsAg epitopes HBsAg_{190–197} (VWLSVIWM) and HBsAg_{208–215} (ILSPFLPL). **g** Serum anti-HBsAg levels. *n*=5. eLNP, empty LNP. Data are shown as the mean ± s.e.m. Statistical analysis was performed by a two-tailed unpaired *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001; *n.s.*, not significant.

5. I appreciate the authors' assessment of the treatment's safety, in particular in the context of mRNA vaccination. Although the description of the results appears in the main text, all the figures addressing these issues are currently in the supplement. I believe that at least some of these figures should be presented in the main text. Moreover, the authors examine the longitudinal effect of the treatment on liver damage 6 weeks after it is completed, detecting high levels of granzyme B in vaccine-induced T cells and clearance of almost all infected hepatocytes. Monitoring the levels of liver enzymes during the experiment might be more informative at assessing the risks of the treatment.

Author reply: We sincerely appreciate the reviewer's insightful suggestions. We agree that longitudinal liver-enzyme monitoring during treatment is more informative for assessing potential hepatotoxicity than a single late timepoint. We have therefore added serial ALT/AST measurements throughout the dosing period and follow-up. Despite robust vaccine-induced cytotoxic T-cell activity, ALT levels remained largely stable, and AST showed only transient fluctuations during treatment without sustained elevation. Both parameters remained largely within the normal range, indicating no treatment-associated hepatotoxicity. These data are now presented in Fig. 7b in the main text together with body-weight tracking and representative liver H&E histology. Additional systemic safety parameters (e.g., food/water intake and peripheral blood counts) are provided in the Supplementary Fig. 28. We have incorporated these data and updated the Results accordingly (page 22, line 484-486).

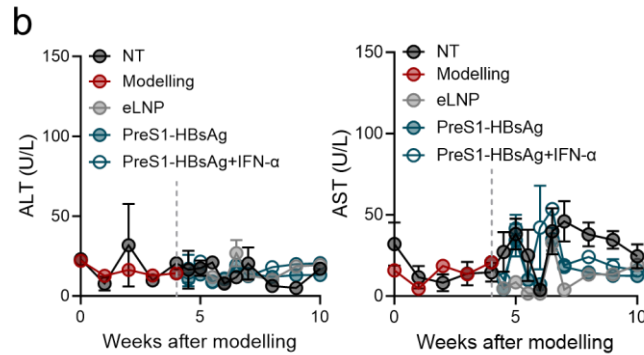


Fig. 7 Safety profile of preS1-HBsAg vaccine and combination therapy. b Longitudinal monitoring of ALT/AST in the serum of AAV-HBV1.3 carrier mice receiving indicated treatments. $n=3$ or 5. Data are shown as the mean $\pm$ s.e.m.

6. The authors vaguely mention the AAV-HBV model limitations in the discussion, but should specify the main drawbacks of the model in relation to their experimental setup and results. The authors plan to develop different models in the future, which “better mimic chronic infection cycles”, but these models also pose serious limitations in studying immune therapies against chronic hepatitis B, such as using immunodeficient mice in vaccination studies. It somehow diminishes the authors’ findings. I would recommend revising this part of the discussion with a focus on the limitations of the model used or clearly stating what would be the benefit of using an alternative model – this is now not clear to me.

Author reply: We appreciate the reviewer’s valuable suggestion. In response, we have revised the Discussion to explicitly clarify the limitations of the current model and the rationale for using complementary models. The revised text is provided below and has been incorporated into the manuscript (page 27, line 601-612).

However, the translational potential of these strategies depends on addressing key model limitations. Our current AAV-based system, while enabling stable HBV persistence, represents a simplified setting. First, the use of a single genotype (ayw) does not capture the global diversity of HBV strains, which differ in prevalence and pathogenicity. Future studies should therefore include genotype-diverse settings (e.g., B and C, which are prevalent in Asia) to assess breadth across viral variants. Moreover, AAV vectors do not fully recapitulate the recurrent hepatocyte infection dynamics seen in natural HBV disease: HBV infection is initiated through NTCP-dependent entry and sustained by reinfection cycles, whereas AAV-mediated transduction bypasses this receptor-dependent viral life cycle. Consequently, this model primarily evaluates immune control of HBV-antigen-expressing hepatocytes by the vaccine, but does not directly resolve effects on entry blockade, de novo cccDNA reservoir dynamics, or durability under continuous reinfection pressure. To strengthen translational inference, transgenic models with humanized livers and immune systems can provide complementary insight into therapeutic vaccine efficacy by more closely modeling natural HBV infection, persistence, and reinfection dynamics, as well as host immune responses.

7. Figure 1 – The figure reminds me of a graphical abstract of the manuscript. The author should reconsider keeping it as a main figure. I think their rationale behind the study is clearly described in the introduction.

Author reply: We sincerely appreciate the reviewer's valuable suggestion. We agree that the original Fig. 1 primarily functions as a schematic overview/graphical abstract. Accordingly, we have moved it to the Supplementary Information as Supplementary Fig. 1 and revised the main figures/figure numbering in the manuscript.

Minor comments:

1. The opening sentence of the abstract should be modified. It appears that immune tolerance is crucial for anti-HBs seroconversion.

Author reply: We thank the reviewer for this valuable comment and have revised the opening sentence of the Abstract accordingly for clarity. The revised text is provided below and has been incorporated into the manuscript (page 2, line 19-20).

Revised text: Therapeutic vaccination for chronic hepatitis B (CHB) remains challenging, as persistent immune tolerance to HBsAg impedes anti-HBsAg seroconversion.

2. Introduction, line 40 – persistent high circulating... The authors should revise this sentence, e.g. persistently high?

Author reply: We sincerely appreciate the reviewer's valuable suggestion. We have revised the phrasing of this sentence. The updated text now reads: "This therapeutic gap stems from the failure to restore virus-specific immune responses in CHB, where persistently high levels of circulating HBsAg induce immune tolerance and T cell dysfunction, key immunological hallmarks preventing functional cure." (page 2, line 42)

3. Introduction, line 41 – T cell exhaustion in CHB – there is piling evidence that HBV-specific T cells do not behave as classically exhausted T cells. The authors might consider an alternative description.

Author reply: We sincerely appreciate the reviewer's valuable suggestion. As suggested, we have revised the wording to in the Introduction from "T cell exhaustion" to "T cell dysfunction". (page 2, line 43)

4. eLNP abbreviation should be explained in the figure legends.

Author reply: We sincerely appreciate the reviewer's valuable suggestion. We have now defined "eLNP" as empty LNP in figure legends.

5. Figure 5 – The authors follow the same experimental scheme as in Figure 2a. To be consistent and clearer, authors should rephrase "8 weeks after the first vaccine treatment" to "6 weeks after the vaccination". Alternatively, they should stick to the first option throughout the manuscript.

Author reply: We sincerely appreciate the reviewer's valuable suggestion. To improve consistency and clarity, we have standardized timepoint descriptions throughout the manuscript to a single reference framework (weeks after virus injection). Accordingly, in revised Fig. 4 (original Fig. 5), "8 weeks after the first vaccine treatment" has been updated to "10 weeks after virus injection", consistent with Fig. 1a.

6. Figure 7 – the authors do not specify when IFN- α is applied in relation to mRNA vaccination, neither in the figure legend nor in the main text, and the experimental

scheme of IFN- α treatment is now shown in the supplement. This is important information and should be clearly explained to the reader. The authors should also consider presenting the experimental timeline in the main figure.

Author reply: We sincerely appreciate the reviewer's valuable suggestion. We agree that the timing of IFN- α relative to mRNA vaccination should be clearly stated. In this experiment, IFN- α was administered on the same day as each mRNA vaccination dose. We have moved the experimental timeline to the main figure (revised Fig. 6a).

7. Discussion, line - better mimic chronic infection cycles – I am not sure what authors mean by infection cycles – phases? please rewrite.

Author reply: We sincerely appreciate the reviewer's valuable suggestion. We have revised the sentence for clarity.

Updated Discussion text: To strengthen translational inference, transgenic models with humanized livers and immune systems can provide complementary insight into therapeutic vaccine efficacy by more closely modeling natural HBV infection, persistence, and reinfection dynamics, as well as host immune responses. (page 27, line 608-612)

8. Methods – the results of several methods such as H&E staining does not appear in the main text, only in the supplement. These methods should also be described in the supplement section.

Author reply: We thank the reviewer for this valuable suggestion. We have moved the Methods descriptions for assays whose results are presented only in the Supplementary Information to the Supplementary Methods section.

Reviewer #1 (Remarks to the Author):

I was quite skeptical on many aspects of the original manuscripts. However, clarifications added by the authors, and additional data on PreS1-specific T cells and the effects of mRNA delivered HBsAg vs. recombinant HBsAg have addressed my primary concerns. I have no further comments.

Author reply: We sincerely thank the reviewer for the positive assessment of our revised manuscript. We are pleased that the clarifications and additional data have addressed the reviewer's primary concerns.

Reviewer #2 (Remarks to the Author):

The authors have responded to my original comments in a direct and effective manner. I have no further comments.

Author reply: We sincerely thank the reviewer for the positive assessment of our revision. We are pleased that our responses to the original comments were considered direct and effective, and we appreciate that the reviewer has no further comments.

Reviewer #3 (Remarks to the Author):

I really appreciate the effort the authors put into revising the manuscript and addressing most of my comments. However, I still have a few questions concerning some of the newly added data.

Author reply: We sincerely thank the reviewer for the thoughtful evaluation of our revised manuscript. We appreciate the reviewer's recognition of our efforts to address most of the comments, and we have carefully responded to the remaining questions concerning the newly added data below.

1. Staining of HBsAg-specific CD8 T cells with tetramers in the spleen (Supplementary Figure 8 f-h): The percentage of tetramer⁺ cells is very low and does not differ significantly from the eLNP control. Is there a specific signal detected for the eLNP group, or is it only background? I am simply wondering whether, with this result, any analysis of the memory phenotype or the activation markers MFI is reliable. How many events were acquired in the tetramer⁺ gate? Can the authors show representative dot plots of the tetramer⁺ cells in the spleen for each experimental group?

Author reply: We thank the reviewer for this important and insightful comment. We agree that the frequency of HBsAg tetramer⁺ CD8⁺ T cells detected in the spleen was very low. In our analysis, 20,000 CD8⁺ T cells were acquired per sample, yielding only around 160 events in the tetramer⁺ gate. The signal observed in the eLNP group was minimal and likely close to background. As already noted in the manuscript, the frequencies were low and not significantly different across groups, which limits detailed phenotypic analysis of this population. For the sake of accuracy, we have removed the further phenotypic analysis of this group of cells. In response to the reviewer's suggestion, we now provide representative dot plots of splenic HBsAg tetramer⁺ CD8⁺ T cells for each experimental group in Fig. R1.

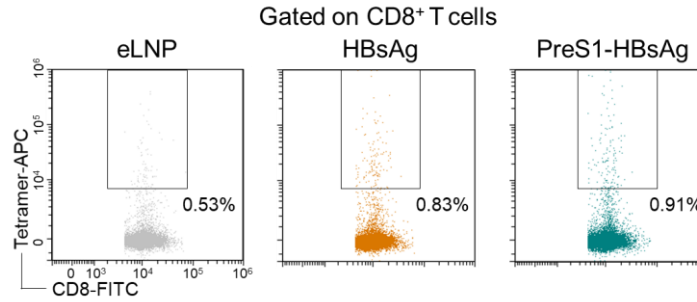


Fig. R1 Representative flow cytometry plots showing HBsAg tetramer⁺ CD8⁺T cells in spleen.

2. ALT and AST kinetics data in Figure 7b: It is not clear to me what the experimental group called 'modelling' is and why the X-axis is named 'weeks after modelling', but not the usually used experimental time line. What represents the dotted line in the middle of the graph? I cannot find any information about this analysis anywhere in the text. Can authors explain it?

Author reply: We thank the reviewer for this important comment. In this experiment, “modelling” refers to CHB model establishment by AAV-HBV1.3 injection. The day of AAV-HBV1.3 administration was defined as week 0. The dotted line indicates the time point (week 4) at which CHB model mice were allocated into the different experimental groups after confirmation of model establishment, with group assignment balanced according to serum HBsAg levels. We agree that the original labeling was unclear. To avoid causing any misunderstandings among readers, we have revised the presentation format of this data.

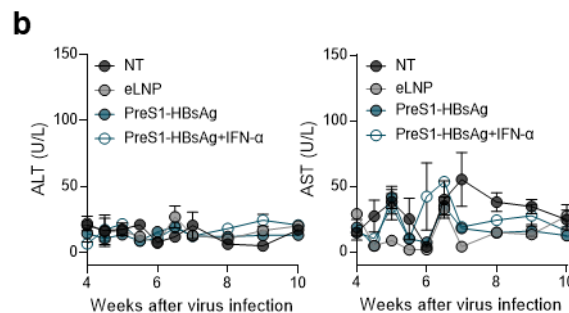


Fig. 7 Safety profile of preS1-HBsAg vaccine and IFN- α combination therapy. b Longitudinal monitoring of ALT/AST in the serum of AAV-HBV1.3 carrier mice receiving indicated treatments. $n=3$ biologically independent mice in the NT group, $n=5$ biologically independent mice in other groups. Source data are provided as a Source Data file.