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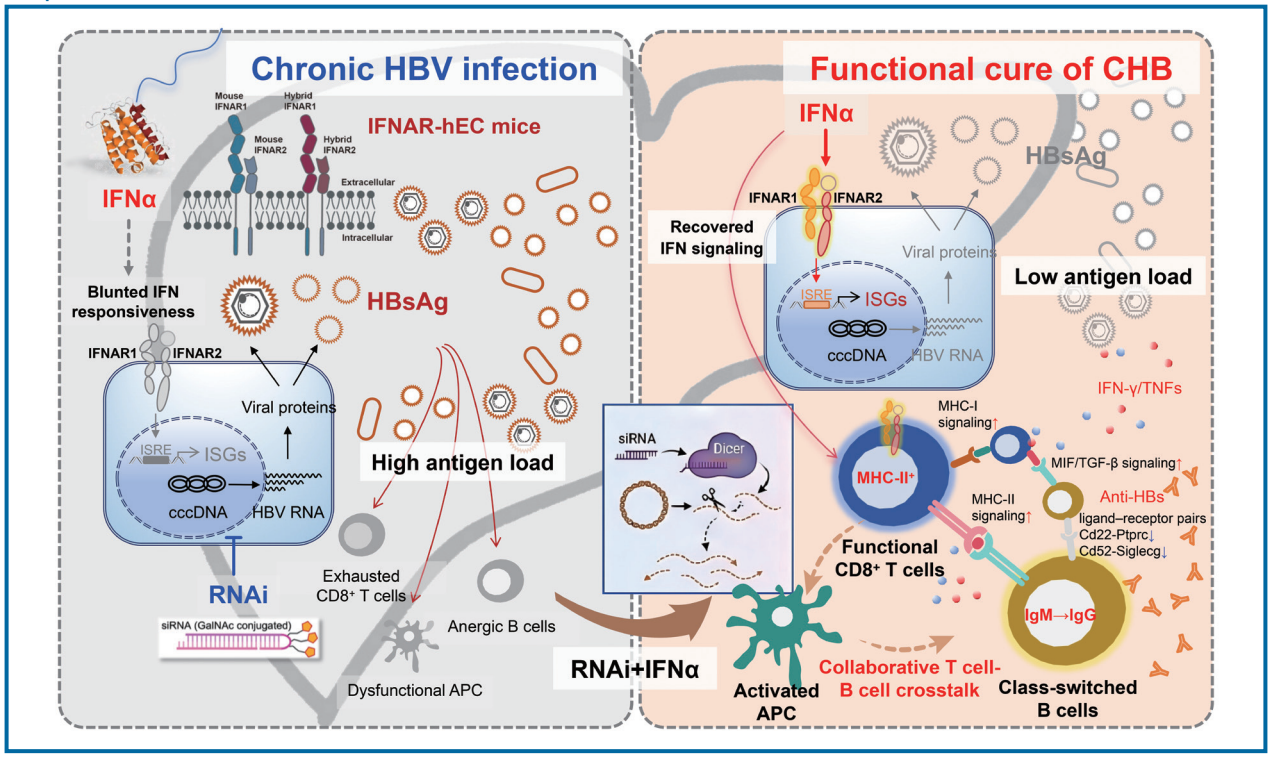
Original Article

RNA interference-mediated hepatitis B virus antigen shutdown enhances the antiviral immune effects of PEGylated interferon-α via T and B cell crosstalk remodeling

Wenjing Zai[1,2,3,*], Kongying Hu[4,*], Mengying He[1,*], Ziyang Song[1,2], Pan Gao[2], Chen Luo[1], Minxiang Xie[1,2], Asha Ashuo[1], Min Yang[1], Jieliang Chen[1], and Zhenghong Yuan[1,2]

[1]Key Laboratory of Medical Molecular Virology (MOE/NHC/CAMS), Research Unit of Cure of Chronic Hepatitis B Virus Infection (CAMS), Shanghai Frontiers Science Center of Pathogenic Microbes and Infection, School of Basic Medical Sciences, Shanghai Medical College Fudan University; [2]Shanghai Institute of Infectious Disease and Biosecurity; [3]Liver Cancer Institute, Zhongshan Hospital, Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Fudan University; [4]National Key Laboratory of Immunity & Inflammation, Department of Pathogen Biology, Naval Medical University, Shanghai, China

Graphical Abstract



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Study Highlights

- Shutting down HBsAg through RNA interference augmented the antiviral effects and the immune responsiveness of PEGIFNα in chronic HBV-carrier IFNAR-hEC mice via augmenting HBV-specific T and B cell functionality.
- Combined RNAi and PEGIFNα therapy reshapes the immune landscape by reducing inhibitory B cell-B cell interactions, enhancing MHC-I signaling among T cells, and improving T cell-B cell crosstalk.
- Enhanced MHC-II signaling networks across B cells and hepatocytes/ $CD8^+$ T cells promoted HBsAg seroconversion in mice receiving RNAi plus PEGIFNα combination treatment.
- Reinforcing MHC-I/II signaling with agonistic antibodies (α4-1BB, αCD40) or IL-2-Fc further potentiates antiviral immune responses, offering a promising adjunctive strategy to improve functional cure rate of CHB.

Background/Aims: PEGylated interferon-α (PEGIFNα) shows promise in treating chronic hepatitis B (CHB), yet patient response remains suboptimal. While suppressing hepatitis B virus (HBV) antigens by RNA interference (RNAi) could enhance PEGIFNα efficacy in CHB patients, the underlying immunological mechanisms remain obscure.

Methods: Using our newly established extracellular humanized IFNAR mouse model of chronic HBV infection, we evaluated the efficacy of a GalNac-conjugated siRNA alone or in combination with PEGIFNα. Phenotypic and functional characteristics of immune cells were assessed by flow cytometry, ELISpot, and single-cell RNA sequencing (scRNA-seq).

Results: High circulating HBsAg reduced antiviral effects and immune responsiveness of PEGIFNα. Combined PEGIFNα and RNAi therapy synergistically and durably suppressed HBsAg (~4$\log_{10}$ IU/mL, vs. PBS) and achieved HBsAg seroconversion in ~30% of mice, outperforming either monotherapy. Mechanistically, PEGIFNα enhanced global T and B cell function, whereas combined therapy further amplified HBV-specific T and B cell responses. scRNA-seq analysis indicated that combined therapy attenuated inhibitory B cell-B cell interactions, strengthened MHC-I-mediated T cell crosstalk, and enhanced MHC-II signaling networks across B cells and hepatocytes/$CD8^+$ T cells, collectively improving the lymphocyte functionality and facilitating HBsAg seroconversion. Reinforcing MHC-I/II signaling with agonistic antibodies (α4-1BB, αCD40) or IL-2-Fc further potentiated antiviral immune responses of combinational regimen.

Conclusions: Combined RNAi and PEGIFNα therapy exerts synergistic antiviral effects by relieving HBV antigen-induced immune tolerance and orchestrating a functional T cell-B cell crosstalk network centered on MHC-I/II signaling. Enhancing antigen presentation pathways represents a promising adjunctive strategy to improve functional cure rate of CHB. **(Clin Mol Hepatol 2026;32:e2026.0385)**

Keywords: Chronic hepatitis B; RNA interference; Interferon-α; Combination therapy; T cell-B cell crosstalk

INTRODUCTION

Over 292 million people worldwide are suffering chronic hepatitis B (CHB),[1] which is a major risk that leads to liver fibrosis, cirrhosis and hepatocellular carcinoma, and current therapeutic options are limited.[2] The persistence of covalently closed circular DNA (cccDNA) and integrated HBV DNA together makes the goal of "complete cure" unachievable.[3] The "functional cure" of CHB, which is characterized by the clearance of seral HBsAg and HBV DNA, is still a challenging endpoint.[4] Nucleos(t)ide analogues (NUCs) are the most widely used clinical therapies that reduce HBV replication, but HBsAg loss is rarely observed even after decades of therapy.[5] PEGylated interferon-α (PEGIFNα) possess both antiviral and immunomodulatory effects, whereas efficacy is moderate, and sustained viral control is uncommon.[6,7] Besides, repeated PEGIFNα treatment can cause systemic toxicity and immunosuppression.[8] Recent

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studies showed that PEGIFNα alone does not improve peripheral HBV-specific CD8$^+$ T cell responses even upon successful treatment,[9] with no significant improvement of T cell proliferation, cytokine production or cytotoxicity.[9,10] To improve therapeutic outcomes, combinations of IFNs and NUCs have been assessed in several clinical studies, showing that NUCs may enhance HBV-specific T cell proliferation, and combined PEGIFNα and NUCs therapy could induce effector cell activation and differentiation, achieving higher proportion of HBsAg loss (~9.1%) within finite duration.[11,12] Whereas merely suppressing HBV replication by NUCs is insufficient to enhance adaptive immunity or improve IFNs response, the curative rate is still far from satisfactory, highlighting the importance of exploring novel curative modalities.

Accumulating evidences indicate that extraordinarily high circulating viral antigens prevent the production of immune responses and contribute to HBV-specific immune tolerance and viral persistence.[13-15] Thus, relieving antigen burden and reconstitution of antiviral immune function represent key factors for CHB therapy. RNA interference (RNAi), which can potently reduce viral mRNA, antigen load and viremia, has the potential to relieve HBsAg-related immunotolerance, thus representing a promising therapeutic strategy.[16-19] It has also been validated to benefit viral burden-induced cytopathology in our previous study.[20] However, functional cure rate is fairly low in CHB patients receiving RNAi monotreatment, since RNAi could not trigger protective antiviral immune responses directly.[21,22] With the advantage of the AAVHBV-based HBV carrier mice and HBV transgenic mice that mimics viral persistence and immune tolerance, recent studies have demonstrated that lowering HBV antigen load by RNAi accompanied with immune stimulation such as therapeutic vaccines is sufficient to overcome virus-specific T/B cell immune tolerance and promote immune reawakening.[23-25] Additional combinational strategies are warranted to obtain better reconstitution of antiviral immune functions and to deepen our understanding on viral persistence and immune tolerance.

Recent clinical studies showed that combining small interfering RNAs (siRNAs) with interferons can achieve deeper HBsAg suppression and higher rates of HBsAg seroclearance and seroconversion, representing a promising strategy.[26-28] However, little is known about the behavior and mechanisms of combined PEGIFNα plus RNAi therapy in the elimination of viral products and restoration of im-

Corresponding author : **Zhenghong Yuan**
Key Laboratory of Medical Molecular Virology (MOE/NHC/CAMS), School of Basic Medical Sciences, Shanghai Medical College, Fudan University, Fuxin Building, DongAn Road 131, Shanghai 200032, People's Republic of China
Tel: +86-21-54237116, Fax: +86-21-64227201, E-mail: zhyuan@shmu.edu.cn
https://orcid.org/0000-0003-0268-4891

Jieliang Chen
Key Laboratory of Medical Molecular Virology (MOE/NHC/CAMS), School of Basic Medical Sciences, Shanghai Medical College, Fudan University, Fuxin Building, DongAn Road 131, Shanghai 200032, People's Republic of China
Tel: +86-21-54237669, Fax: +86-21-64227201, E-mail: jieliangchen@fudan.edu.cn
https://orcid.org/0000-0002-9647-0239

Wenjing Zai
Liver Cancer Institute, Zhongshan Hospital, Key Laboratory of Carcinogenesis and Cancer Invasion, Ministry of Education, Fudan University, 180 Fenglin Rd, Xuhui District, Shanghai, PR China
Tel: +86-21-54237669, Fax: +86-21-64227201, E-mail: zaiwjing@163.com
https://orcid.org/0000-0003-2550-331X

*These authors contributed equally to this work.

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Abbreviations:

cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; DC, dendric cells; ETV, entecavir; FACS, flow cytometry; GalNac-siHBV, GalNac-conjugated HBV siRNA; GZMB, granzyme B; HBV, hepatitis B virus; IFNAR-hEC, extracellular humanized IFNAR; IHC, immunohistochemical; IHL, intrahepatic lymphocytes; ISG, interferon-stimulated gene; NK, natural killer; NUCs, Nucleos(t)ide analogues; PBMC, peripheral blood mononuclear cells; PEGIFNα, PEGylated interferon-α; RNAi, RNA interference; RT-qPCR, real-time quantitative PCR; scRNA-seq, single-cell RNA sequencing; siRNA, small interfering RNA; Treg, regulatory T

mune responses since there are lack of adequate animal models. Recently, an extracellular humanized IFNAR (IFNAR-hEC) mouse model was constructed by our group, has been validated to be responsive to human IFNs with intact endogenous immune system, and has been used to evaluate different effects of human IFN subtypes.[29] With this model, and the in-house established GalNAc-conjugated HBV siRNA (GalNac-siHBV),[30] we were able to comprehensively investigate the effects and clarify the underlying mechanisms of combined PEGIFNα plus RNAi therapy, and to identify novel strategies to improve therapeutic outcomes.

In the present study, we found that high circulating HBsAg reduced PEGIFNα responsiveness, but preceding RNAi partially rescued its efficacy. Combined RNAi plus PEGIFNα therapy induced a potent HBsAg reduction of ~4$\log_{10}$ IU/mL, and achieved a HBsAg seroconversion rate of ~30%, outperforming either monotherapy. The synergistic efficacy was mainly attributed to the ablation of high antigen burden-induced immune tolerance by RNAi, the induction of unspecific immune responses by PEGIFNα, and the improved virus-specific immune responses and altered T-B cell communications by combined therapy. Reinforcing MHC-I/II signaling via antibodies or cytokines further amplified HBV-specific T and B cell immunity thus improving therapeutic outcomes. Collectively, our findings uncover a previous unrecognized mechanism by which RNAi and PEGIFNα synergistically remodel intrahepatic immunity through restoration of T-B cell collaboration, providing a scientific rationale for optimizing this combination regimen to achieve functional cure of CHB.

MATERIALS AND METHODS

Animal models

C57BL/6 mice were purchased from GemPharmatech Co., Ltd (Jiangsu, China). IFNAR-hEC mice were bled by Cyagen biotechnology company (Jiangsu, China). Mice experiments were approved by the Biomedical Research Ethics Committee of the Fudan University.

The rAAV8-HBV1.3 (ayw) viruses were purchased from Five Plus Biotech (Beijing, China). Animal models were constructed via intravenously injection with the rAAV8-HBV1.3 viruses (2.5×10^{10} viral genome (v.g.) copies per mice) and were grouped to obtain similar HBsAg levels.[31]

Animal treatment

HBV-specific siRNAs were designed to target all forms of HBV transcripts (GalNac-siHBV). 2.5 mg/kg of GalNac-siHBV were subcutaneously (s.c.) injected every 4 weeks. The IFNAR-hEC mice received 30 μg/kg of PEGASYS (PEGASYS; Roche) by subcutaneous injection (s.c.) twice per week for 20 weeks. Two micrograms of HBsAg (ayw) (Fitzgerald, USA) plus 25 μg of CpG-1826 (MCE, USA) were subcutaneously injected weekly for vaccination. The anti-mouse PD-1 antibody (αPD-1), 4-1BB agonist antibody (α4-1BB) and CD40 agonist antibody (αCD40) (Selleck, USA) were intraperitoneally injected (i.p.) once biweekly at a dose of 4 mg/kg. The recombinant mouse IL-2/Fc fusion protein (rmIL-2-Fc) were purified in-house and were intraperitoneally injected twice per week at the dose of 50 μg/kg.

Statistical analysis

Data were analyzed using an unpaired two-tailed Student's *t*-test, one-way analysis of variance (ANOVA), or two-way ANOVA with Sidak/Dunnett multiple comparison correction, with GraphPad Prism. Numbers in graphs indicate *P*-values.

RESULTS

High circulating HBsAg reduced the immune response to PEGIFNα in mice

To assess how chronic HBV infection alters interferon-α immunogenicity, an in-house constructed IFNAR-hEC mouse model was utilized, and the clinically used PEGIFNα2a (PEGASYS) was evaluated in the rAAV8-HBV1.3 (AAVHBV)-based chronic HBV-carrier mouse model. Chronic infection was confirmed by circulating HBsAg, with mock-infected mice as control. Four weeks post AAVHBV injection, intrahepatic lymphocytes (IHL) and splenocytes were collected for T cell response analysis (Fig. 1A, Supplementary Table 1). PEGIFNα treatment did not increase IFN-γ production upon stimulation with HBV

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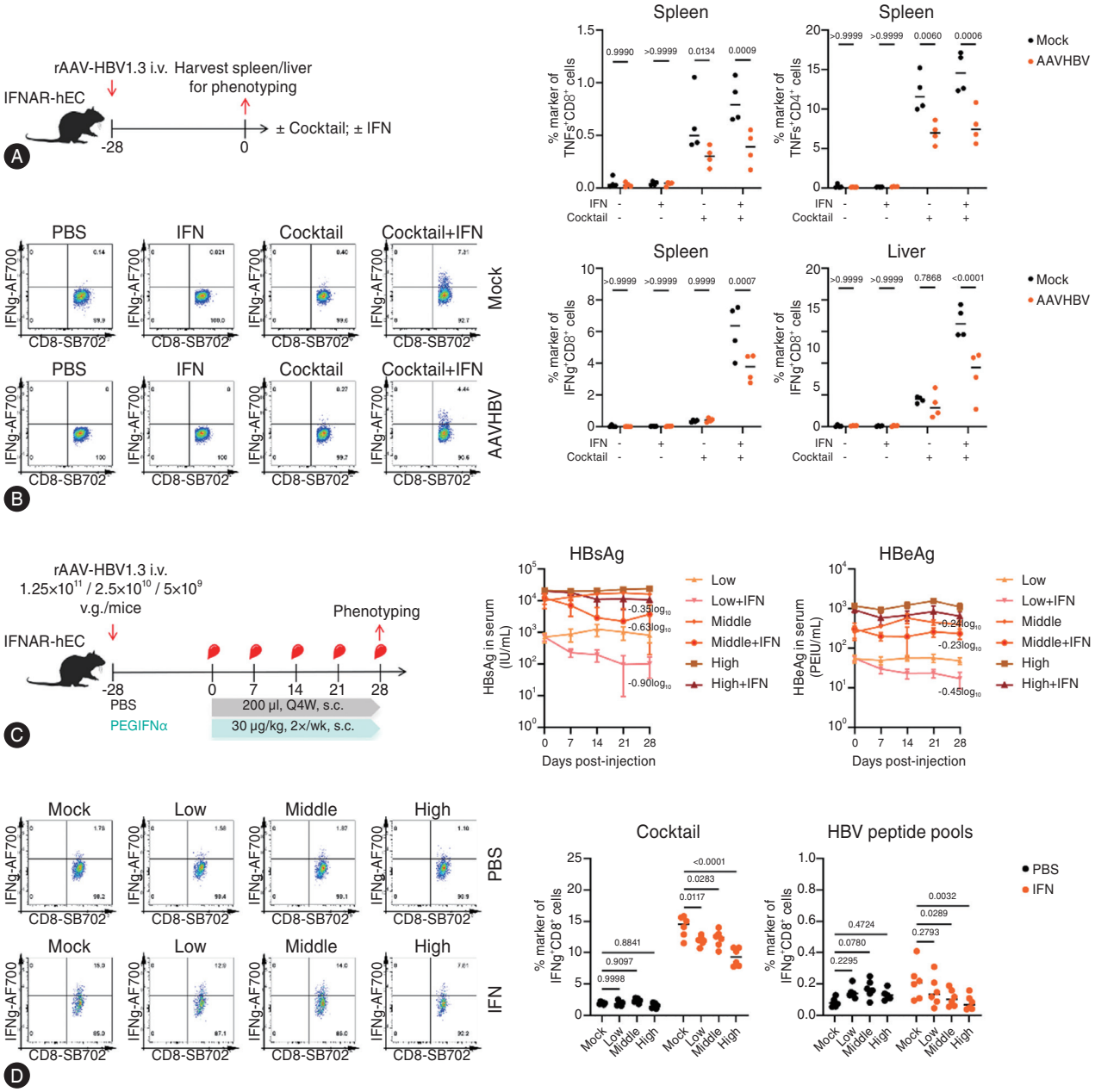


Figure 1. High circulating HBsAg level reduced the immune response to PEGIFNα in HBV-carrier mice. (A) IFNAR-hEC mice were intravenously injected with PBS or rAAV-HBV1.3 (2.5× 10^{10} viral genome (v.g.) copies per mouse), 4 weeks later, intrahepatic lymphocytes (IHL) and splenocytes were harvested and stimulated with Phorbol 12-Myristate 13-Acetate (PMA) & Ionomycin (Cocktail) in the presence or absence of PEGASYS (500 ng/mL) for 16 hours. The proportion of TNFs- and IFNγ-secreting $CD8^+$ and $CD4^+$ T cells in splenocytes were determined via FACS analysis (n=4). (B) Representative density plots and statistical analysis of the proportion of IFNγ-secreting cells in splenic and intrahepatic $CD8^+CD3^+$ T cells (n=4). (C) Schematics of treatment procedures. Mice were intravenously injected with different doses of rAAV-HBV1.3 (High: 1.25×10^{11} v.g./mice, Middle: 2.5×10^{10} v.g./mice, Low: 5×10^9 v.g./mice), then the chronic HBV-carrier IFNAR-hEC mice were treated with PBS or PEGASYS (30 μg/kg, s.c., 2×wk) for 4 weeks. Seral HBsAg and HBeAg levels were determined weekly via commercial kits (n=6). (D) Splenocytes from mice of different groups were stimulated with PMA/ionomycin cocktail or HBV-derived overlapping peptide pools covering the envelope and core protein *in vitro* for 24 hours, then the intracellular IFN-γ secretion were determined via FACS analysis (n=6). (E) Splenic T cell responses to HBV-derived peptide pools or cocktail were tested by a T cell ELISpot assay. Statistical analysis was displayed (n=4). (F) Splenic B cell responses to HBsAg or R848+IL-2 stimulation were tested by a B cell ELISpot assay. Statistical analysis was displayed (n=4). Data were analyzed by two-way ANOVA with Sidak multiple comparison correction (A, B, D–F), and presented as mean±standard deviation. Numbers in graphs indicate *P*-values. FACS, flow cytometry; HBV, hepatitis B virus; IFNAR-hEC, extracellular humanized IFNAR; PEGIFNα, PEGylated interferon-α.

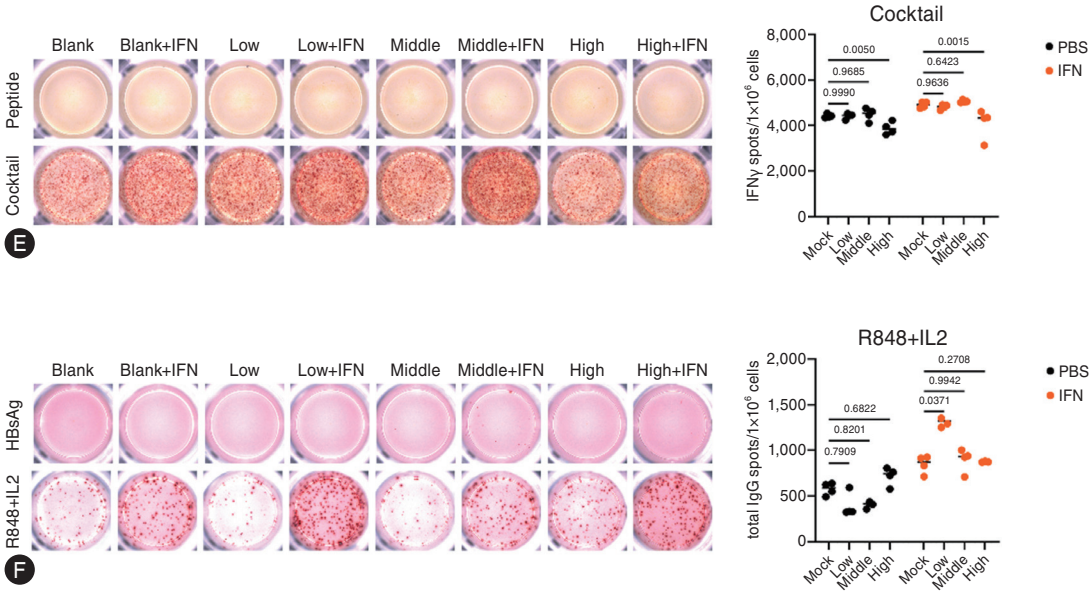


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envelope and core peptide pools, indicating limited HBV-specific T cell responses (data not shown). Stimulation with PMA/ionomycin cocktail with or without PEGIFNα (500 ng/mL, 16 h) showed reduced IFN-γ/TNFs production in HBV-carrier compared with mock mice (Fig. 1A, 1B).

To further investigate the impact of circulating HBsAg levels on PEGIFNα responsiveness, mice were challenged with low (5×10^9 v.g./mice), middle (2.5×10^{10} v.g./mice) or high (1.25×10^{11} v.g./mice) doses of AAVHBV, achieving ~1,000, ~10,000, and ~20,000 IU/mL at week 4 post-injection. Mice were then treated with PBS or PEGIFNα (30 μg/kg, 2×/wk) for 4 weeks (Fig. 1C). Viral parameters were monitored weekly. Mice challenged with the low dose of AAVHBV showed a potent reduction in HBV antigens ($-0.90\log_{10}$ IU/mL for HBsAg, $-0.45\log_{10}$ PEIU/mL for HBeAg), the middle dose showed a modest reduction ($-0.63\log_{10}$ IU/mL for HBsAg, $-0.23\log_{10}$ PEIU/mL for HBeAg), and the high dose a minor reduction ($-0.35\log_{10}$ IU/mL for HBsAg, $-0.24\log_{10}$ PEIU/mL for HBeAg), compared with PBS-treated mice. Splenocytes were stimulated *in vitro* with PMA/ionomycin cocktail or HBV peptide pools. Higher viral antigen load was associated with reduced PEGIFNα response (less production of IFN-γ) under cocktail stimulation (Fig. 1D). ELISplot confirmed reduced IFN-γ secretion in splenocytes of high viral antigen load group mice after 24 hours of cocktail stimulation, and reduced total IgG production after 48 hours of R848&IL-2 stimulation (Fig. 1E, 1F). Whereas the levels of unspecific activation markers on B cells and dendric cells (DCs) were comparable across groups (Supplementary Fig. 1). These results together indicate that high circulating HBsAg levels were associated with reduced interferon-α responsiveness, influencing the function of global T and B cell compartment. However, we didn't detect HBV antigen-related IFN-γ production or anti-HBs IgG production under these conditions.

Combined RNAi and PEGIFNα therapy exhibited synergistic antiviral effects in HBV-carrier IFNAR-hEC mice

To investigate whether reducing viral antigen load could help relieve HBV-related suppression on PEGIFNα responsiveness, we employed a GalNAc-conjugated HBV siRNA (GalNac-siHBV) combining two previous optimized siRNA triggers targeting HBsAg (siHBs) and HBx (siHBx), respectively, to knockdown all forms of HBV transcripts (both cccDNA- and intHBV-derived RNAs) with high efficiency and safety (Supplementary Fig. 2). The AAVHBV mouse model was utilized to evaluate the *in vivo* activity of relative agents.[32] GalNAc-siHBV potently and dose-dependently suppressed antigenemia and viremia, whereas entecavir (ETV) blocked HBV replication but failed to reduce antigenemia (Supplementary Fig. 2B). Neither ETV nor RNAi triggered antiviral immune responses (Supplementary Fig. 3A–3D).

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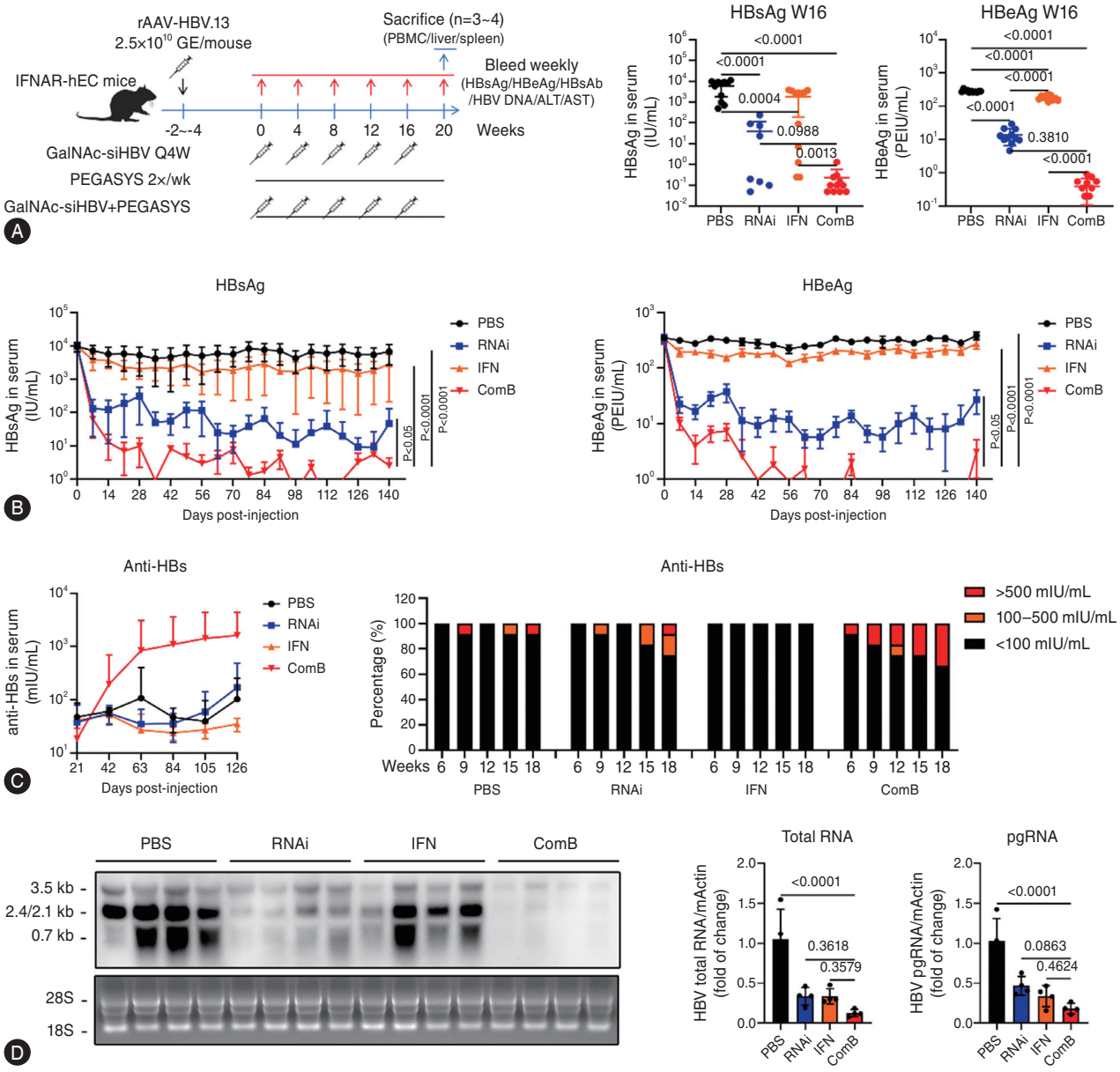


Figure 2. Combined RNAi and PEGIFNα treatment displayed synergistic antiviral efficacy in chronic HBV-carrier IFN-hEC mice. (A) Schematics of treatment procedures. Briefly, chronic HBV-carrier IFNAR-hEC mice were treated with GalNac-siHBV (2.5 mg/kg, s.c., Q4W), PEGASYS (30 μg/kg, s.c., 2×wk), or both concurrently. Seral HBsAg and HBeAg levels at week 16 were determined. (B) Seral HBsAg and HBeAg levels during 20 weeks' treatment were evaluated. (C) Seral anti-HBs levels and the percentage of HBsAg seroconversion in mice of different groups. (D) Intrahepatic HBV RNA levels were determined via Norther Blot analysis and real-time quantitative PCR (RT-qPCR) analysis (n=4). (E) Representative graphs of intrahepatic HBc (left). Scale bar indicates 50 μm. The HBc-positive cells per mm^2 liver sections (n=4) (right). (F) The expression levels of ISGs relative to *actin* mRNA levels (n=4). Data were analyzed using one-way analysis of variance (ANOVA) with Dunnett multiple comparison correction (A, D–F), or two-way ANOVA with Sidak multiple comparison correction (B), and presented as mean±standard deviation. Numbers in graphs indicate *P*-values. GalNac-siHBV, GalNac-conjugated HBV siRNA; HBV, hepatitis B virus; IFNAR-hEC, extracellular humanized IFNAR; ISGs, interferon-stimulated genes; PEGIFNα, PEGylated interferon-α; RNAi, RNA interference.

The effects of PEGIFNα were further evaluated in comparison with RNAi using the AAVHBV IFNAR-hEC mouse model. Unlike RNAi, PEGIFNα led to moderate viral antigen reduction but evident unspecific immune activation (Supplementary Fig. 3E, 3F). No evident HBV DNA reduction was observed due to ~2log_{10} lower HBV DNA levels in

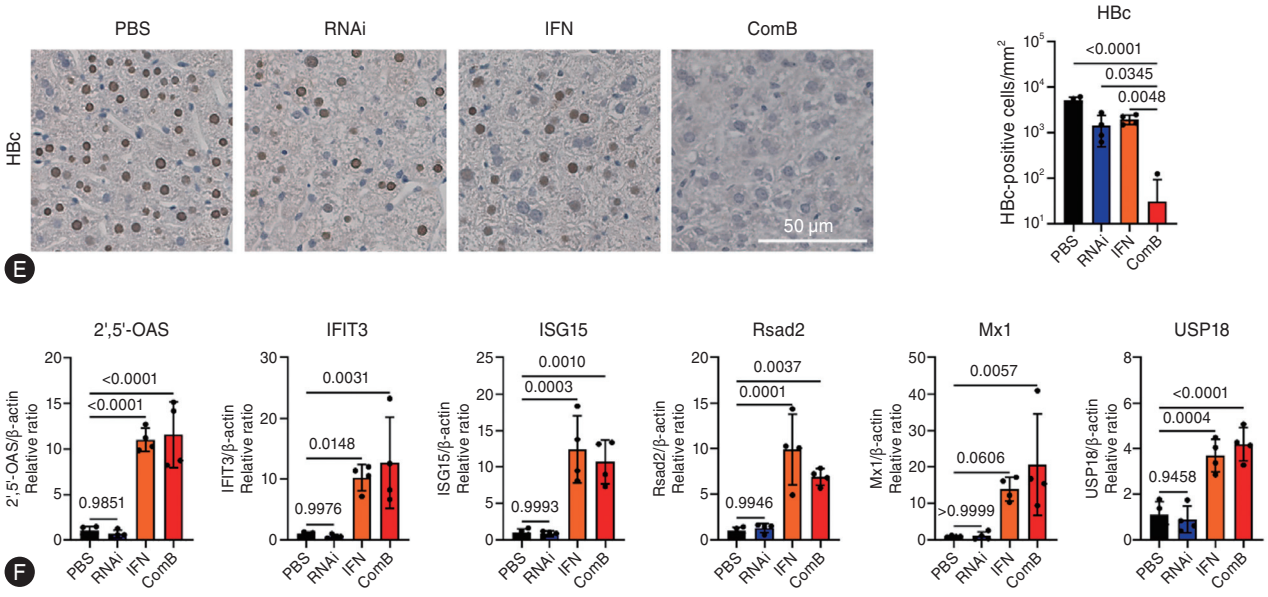


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IFNAR-hEC mouse than that in the wildtype mouse (also reported by Wang et al.[32]).

Chronic HBV-carrier IFNAR-hEC mice were treated with either GalNac-siHBV (RNAi, 2.5 mg/kg, Q4W), PEGIFNα (IFN, 30 μg/kg, 2×/wk), or both (ComB). RNAi alone significantly reduced HBsAg (–2.18log$_{10}$ IU/mL, vs. PBS) and HBeAg at week 16. PEGIFNα alone only showed only a moderate HBsAg reduction trend (–0.51log$_{10}$ IU/mL), with partial mice refractory to treatment. Combined therapy further suppressed HBsAg (–4.42log$_{10}$ IU/mL) and HBeAg (Fig. 2A). Similar results were observed in female mice (Supplementary Fig. 4A, 4B). Specifically, combined treatment induced higher HBsAg seroconversion (~30%, 4/12) and anti-HBs secretion (>1,000 mIU/mL) after 20 weeks of treatment (Fig. 2C). Intrahepatic HBV RNA and DNA loads (Fig. 2D, Supplementary Fig. 4C, 4D) and intranuclear HBc staining (Fig. 2E, Supplementary Fig. 4E) were markedly reduced in the ComB group. Successful induction of typical interferon-stimulated genes was confirmed by real-time quantitative PCR (RT-qPCR) analysis (Fig. 2F, Supplementary Fig. 4F, Supplementary Table 2). This synergistic effect was specifically observed with combined RNAi and IFN treatment, but not in the ETV-containing combination arms (Supplementary Fig. 5). Global transcriptional analysis of liver tissues showed that, RNAi treatment influenced metabolism-related pathways, whereas combined therapy further enhanced immune response-related pathways (Supplementary Fig. 6). Besides, both RNAi and PEGIFNα monotherapy, as well as their combination, displayed satisfactory safety profiles, with no evident ALT/AST elevation, body weight reduction, or organism abnormality, aside from some blood cell reduction attributed to PEGIFNα treatment (Supplementary Fig. 7). Together, combined RNAi and PEGIFNα therapy could be applied as an effective treatment to exhibit synergistic antiviral effects along with high security profiles.

Combined RNAi and PEGIFNα therapy led to augmented HBV-specific T cell and B cell responses

Peripheral blood mononuclear cells (PBMCs) were analyzed by flow cytometry (FACS) during the 20-weeks treatment period. PEGIFNα alone or in combination resulted in comparable increased expression of Ki67, CD69 and CD80 on CD3$^+$ T cells. Ki67 expression was increased on CD8$^+$ but not CD4$^+$ T cells, and also on natural killer (NK) cells, with both regimens (Supplementary Fig. 8A). The expression levels of Ki67, CD69 and CD86 molecule on DCs were also increased in mice receiving PEGIFNα alone or in combination, as well as an increased proportion of circulating Foxp3$^+$CD25$^+$ regulatory T (Treg) cells. (Supplementary Fig. 8B). Whereas the magnitude of T/NK/DC cell activation or proliferation by PEGIFNα treatment did not correlate

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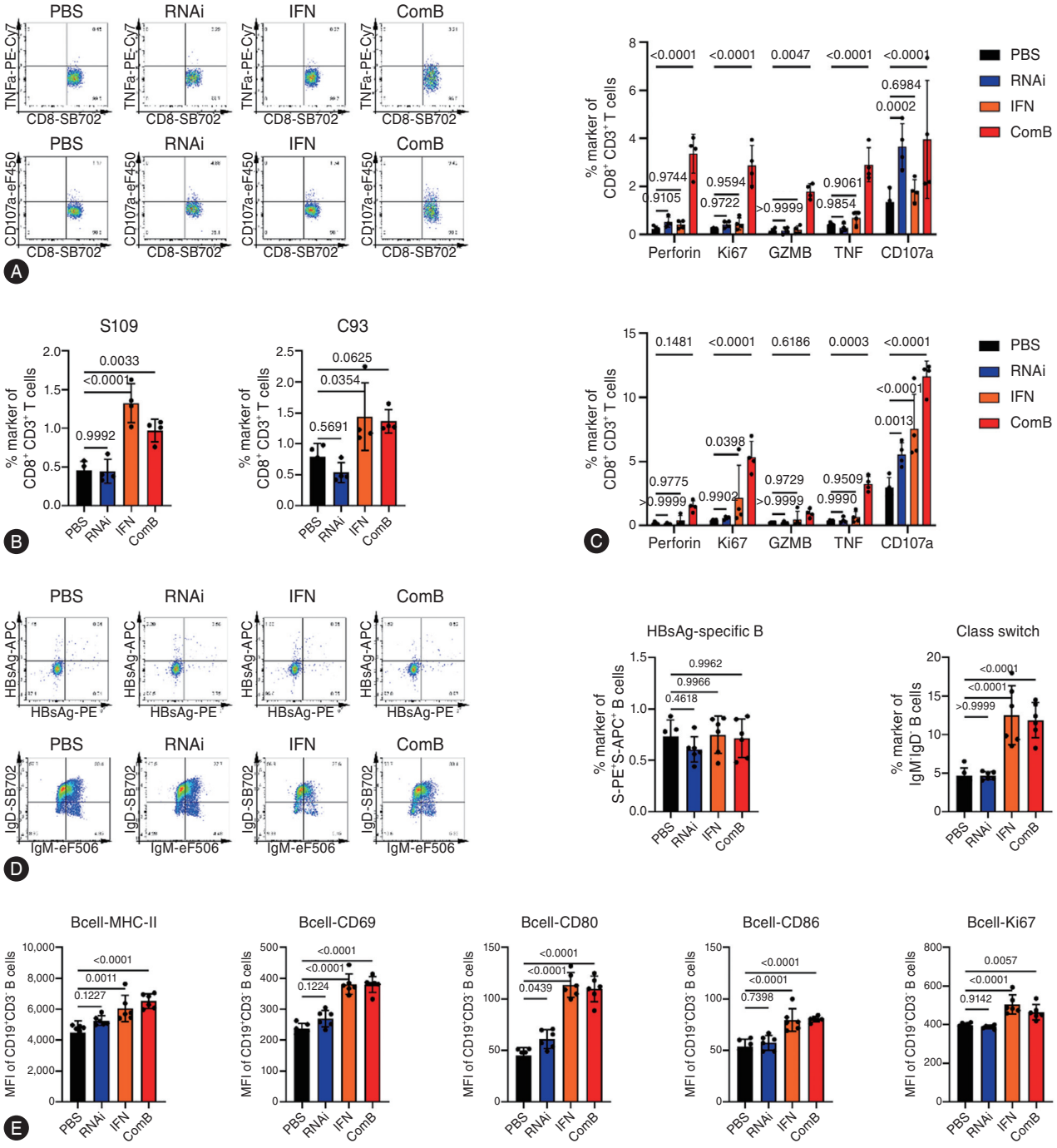


Figure 3. Combined RNAi and PEGIFNα treatment augmented HBV-specific T and B cell responses. (A) Representative density plots and statistical analysis of the proportion of cells in intrahepatic $CD8^+CD3^+$ T cells after *ex vivo* HBV-derived peptide stimulation were displayed (n=4). (B) The proportion of HBsAg-specific (stained by S109 tetramer) and core-specific $CD8^+CD3^+$ T cells (stained by C93 tetramer) in gated liver lymphocytes (n=4). (C) Statistical analysis of the proportion of perforin, Ki67, GZMB, TNFα and CD107a expressing splenic $CD8^+CD3^+$ after *ex vivo* HBV-derived peptide stimulation (n=4). (D) Representative density plots of the proportion of HBsAg-specific and IgD^-IgM^- classed switched B cells in PBMCs. Statistical analysis of the proportion of HBsAg-specific and IgD^-IgM^- classed switched B cells (n=6). (E) Mean fluorescent intensity (MFI) of MHC-II, CD69, CD80, CD86 and Ki67 staining in gated $CD19^+CD3^-$ B cells (n=6). (F) Specific B cell responses to HBsAg were tested in a B cell ELISpot assay. Statistical analysis was applied (n=4). Data were analyzed by one-way ANOVA with Dunnett multiple comparison correction (B, D–F), or two-way ANOVA with Sidak multiple comparison correction (B, C), and were presented as mean±standard deviation. Numbers in graphs indicate *P*-values. HBV, hepatitis B virus; PEGIFNα, PEGylated interferon-α; RNAi, RNA interference.

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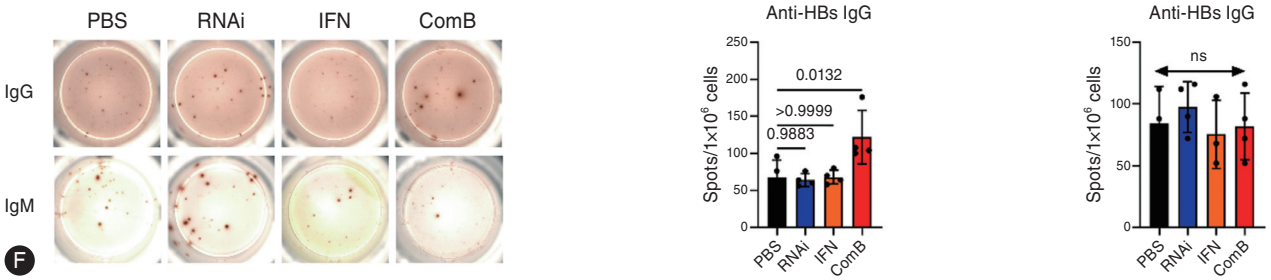


Figure 3. Continued.

with HBsAg levels, this was due to the absence of a significant difference between PEGIFNα only and combination groups. Peripheral T/NK cells exhibited enhanced functionality by PEGIFNα treatment, with more $CD8^+$/$CD4^+$ T and NK cells stained positive with perforin, granzyme B (GZMB) and TNFs (vs. PBS). There was also an increased percentage of $CD62L^-CD44^+$ effector memory (TEM) and $CD62L^+CD44^+$ central memory (TCM) $CD8^+$ T cells, as well as a higher percentage of circulating HBsAg-specific $CD8^+$ T cells in mice receiving PEGIFNα alone or in combination (Supplementary Fig. 8C). Higher percentages of $CD8^+$ T cells were positively stained with perforin, Ki67, GZMB, TNFs and CD107a upon *ex vivo* HBV-derived peptides restimulation in IHL of combined therapy, although the proportions of HBsAg-specific (stained by S109 tetramer) and core-specific $CD8^+$ T cells (stained by C93 tetramer) retained comparable between monotherapy and combination therapy (Fig. 3A, 3B). Similar results were observed in splenocytes, showing enhanced HBV-specific $CD8^+$ T cell functionality only in mice receiving combination therapy (Fig. 3C). Immunohistochemical (IHC) analysis revealed marked intrahepatic infiltration of $CD8^+$ T cells induced by the combination therapy (Supplementary Fig. 8D). These results indicate that PEGIFNα treatment induced unspecific immune activation, while combination therapy further enhanced HBV-specific T cell functionality, which may play a key role in the synergistic mechanisms underlying viral control.

Analysis of B cell phenotypes revealed that both PEGIFNα regimens induced a higher percentage of IgM^-IgD^- class-switched B cells, which is closely correlated with antibody secretion, whereas the percentage of HBsAg-specific B cells remained unchanged (Fig. 3D). Both groups also induced higher expression levels of activation molecule MHC-II, CD69, CD80, CD86, as well as the proliferation marker Ki67, on global B cells (Fig. 3E). ELISpot analysis showed that combination therapy induced an evident elevation of HBsAg-specific IgG isotype but not IgM antibody secretion in splenocytes, surpassing PEGIFNα monotherapy and PEGIFNα plus ETV therapy (Fig. 3F, Supplementary Fig. 5C). RNA-seq analysis of splenic B cells at week 20 and 24 (4 weeks after treatment discontinuation) showed that, PEGIFNα treatment exhibited potent effects on B cell activation (Supplementary Fig. 9), whereas combination therapy induced prolonged B cell responses after treatment cessation (Supplementary Fig. 10). HBV antigens demonstrated a trend of rebound, whereas the percentage of IgM^-IgD^- class-switched B cells retained at higher level in mice of combined therapy at week 24, both in global and HBsAg-specific B cells (Supplementary Fig. 11A–11C). To further questionee whether RNAi treatment could help alleviate HBV-related B cell immune tolerance, mice were treated with RNAi, PEGIFNα, or both for 12 weeks, followed by HBsAg/CpG-1826 vaccination (once weekly, 4 doses in total) (Supplementary Fig. 11D). An evident HBsAg reduction was only observed in vaccinated RNAi and combination groups (Supplementary Fig. 11E). ELISpot analysis revealed that only the combination group mounted a strong anti-HBs IgG response following vaccination (Supplementary Fig. 11F). Together, these results demonstrate that concurrent RNAi treatment could help breach the impairment of PEGIFNα responsiveness, activating both global and HBV-specific B cell compartments, thus increasing the likelihood of HBsAg seroconversion.

Single-cell RNA sequencing revealed complex interaction networks between T and B cells after combined RNAi and PEGIFNα therapy

Comprehensive unbiased scRNA-seq analyze of intrahe-

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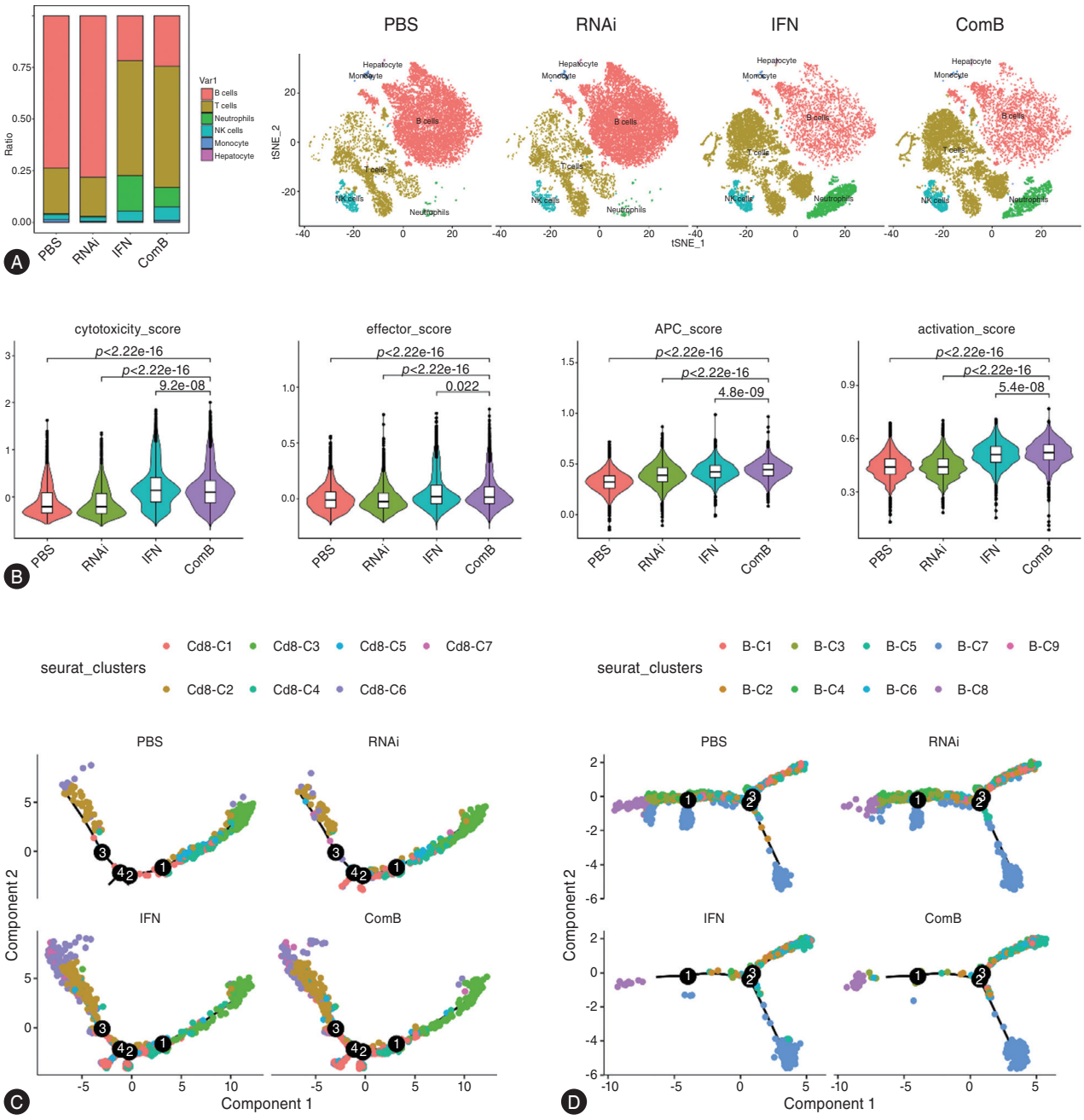


Figure 4. Single-cell RNA sequencing revealed the complexity of T and B cells in combined RNAi and PEGIFNα treated mice. (A) Mice intrahepatic Cd45$^+$ immune cells were collected for scRNA-seq at week 20. The proportion of each cell subcluster in different groups. TSNE plot of intrahepatic immune cells from mice of different treatment. (B) Violin plots of gene set enrichment analysis scores of CD8$^+$ T cell subclusters, the cytotoxicity and effector-related scores were displayed. Violin plots of gene set enrichment analysis scores of B cell subclusters, the antigen processing and presenting, and activation -related scores were calculated. (C) Pseudo-time trajectory analysis of CD8$^+$ T and (D) B cells showing the distribution of each cell subset on the development trajectory. (E) Heatmap and pathway enrichment analysis of different expressed genes in CD8$^+$ T and (F) B cells by ClusterGVis. Data were analyzed by unpaired two-tailed Students' *t*-test analysis (B), and were presented as mean±standard deviation. Numbers in graphs indicate *P*-values. PEGIFNα, PEGylated interferon-α; RNAi, RNA interference.

patic CD45$^+$ immune cells after 20 weeks of consecutive treatment yielded high-quality expression data for 44,603 cells after quality control (Supplementary Fig. 12A). Six clusters were assigned based on marker gene expression,

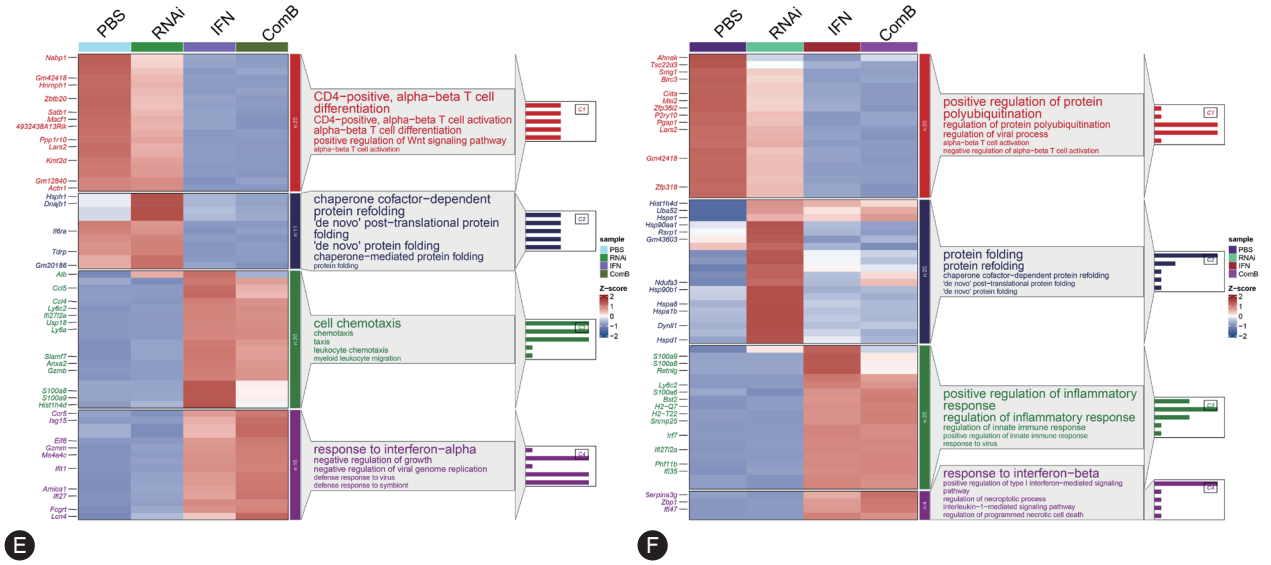


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including hepatocytes, monocytes, neutrophils, T cells, B cells and NK cells (Supplementary Fig. 12B), showing an increased proportion of T cells, NKs, and neutrophils, and a decreased proportion of B cells by PEGIFNα treatment (Fig. 4A). Further sub-clustering revealed the complexity of T and B cells, with much more $CD8^+$ T sub-cluster cells than $CD4^+$ T cells, including naïve-like (Cd8-C1, Cd8-C3, Cd8-C4, Cd8-C5), cytotoxic (Cd8-C2), proliferating (Cd8-C6) and activated $CD8^+$ T cells (Cd8-C7) (Supplementary Fig. 12C, 12D, Supplementary Fig. 13A, 13C). The B cells were sub-clustered into naïve (B-C1, B-C2, B-C3, B-C9), transitional (B-C4), atypical memory (atMBC) (B-C5), marginal zoom (MZB) (B-C6), classical memory (MBC) (B-C7), and activated B cells (B-C8) (Supplementary Fig. 12E, 12F, Supplementary Fig. 13B, 13D). Combination therapy induced a significant elevation of cytotoxic- and effector -related gene scores in $CD8^+$ T cells, as well as antigen processing and presentation, and activation-related gene scores in B cells (Fig. 4B, Supplementary Table 3). This phenomenon was also observed in huIFNAR mice (Supplementary Figs. 16, 17), and in clinical patients.[28] Pseudotime analysis confirmed enhanced development of $Cd8^+$ T cells from naïve-like to proliferating and cytotoxic phenotypes by combination therapy (Fig. 4C, Supplementary Fig. 14A, 14B), as well as enhanced development of B cells from naïve-like to transitional or memory-like states (Fig. 4D, Supplementary Fig. 14C, 14D). Pathway enrichment analysis revealed that, RNAi improved signaling pathways associated with protein folding, PEGIFNα induced inflammatory response and cell chemotaxis-related pathways, whereas combination therapy further enhanced interferon-α/β responses and antiviral responses in T and B cells (Fig. 4E, 4F, Supplementary Fig. 15A).

Communication analysis of individual samples revealed dominant crosstalk between T and B cells, via H2-aa/ab1 ligand and CD4 receptor pairs (MHC-II signaling), PEGIFNα treatment further enhanced T cell-T cell interactions primarily through H2-q7 ligand and CD8a/b1 receptor pairs (MHC-I signaling) (Supplementary Fig. 15B, 15C). Communications across different groups were further evaluated. Negative B cell signaling modulators, including Cd22-Ptprc and Cd52-Siglecg pairs, predominantly medicated B cell-B cell communication, reflecting an inhibitory immune state of B cells.[33,34] PEGIFNα treatment reduced Cd22-Ptprc and Cd52-Siglecg pair interactions by depressing ligand or receptor expression (Fig. 5A, 5B). Combination therapy enhanced Tgfb1-(Tgfbr1+Tgfbr2) and Mif-(Cd74+Cxcr4) interactions in both B cell-B cell and B cell-T cell pairs by promoting ligand or receptor expression. Combination therapy further enhanced MHC-I signaling among T cells, and improved the expression of MHC-I-related molecules, including H2-T23, H2-Q7, H2-Q6, H2-D1, H2-K1, H2-Q4, H2-T22, and Cd8a/b1, promoting antigen presentation (Fig. 5B). MIF signaling, which promotes B cell

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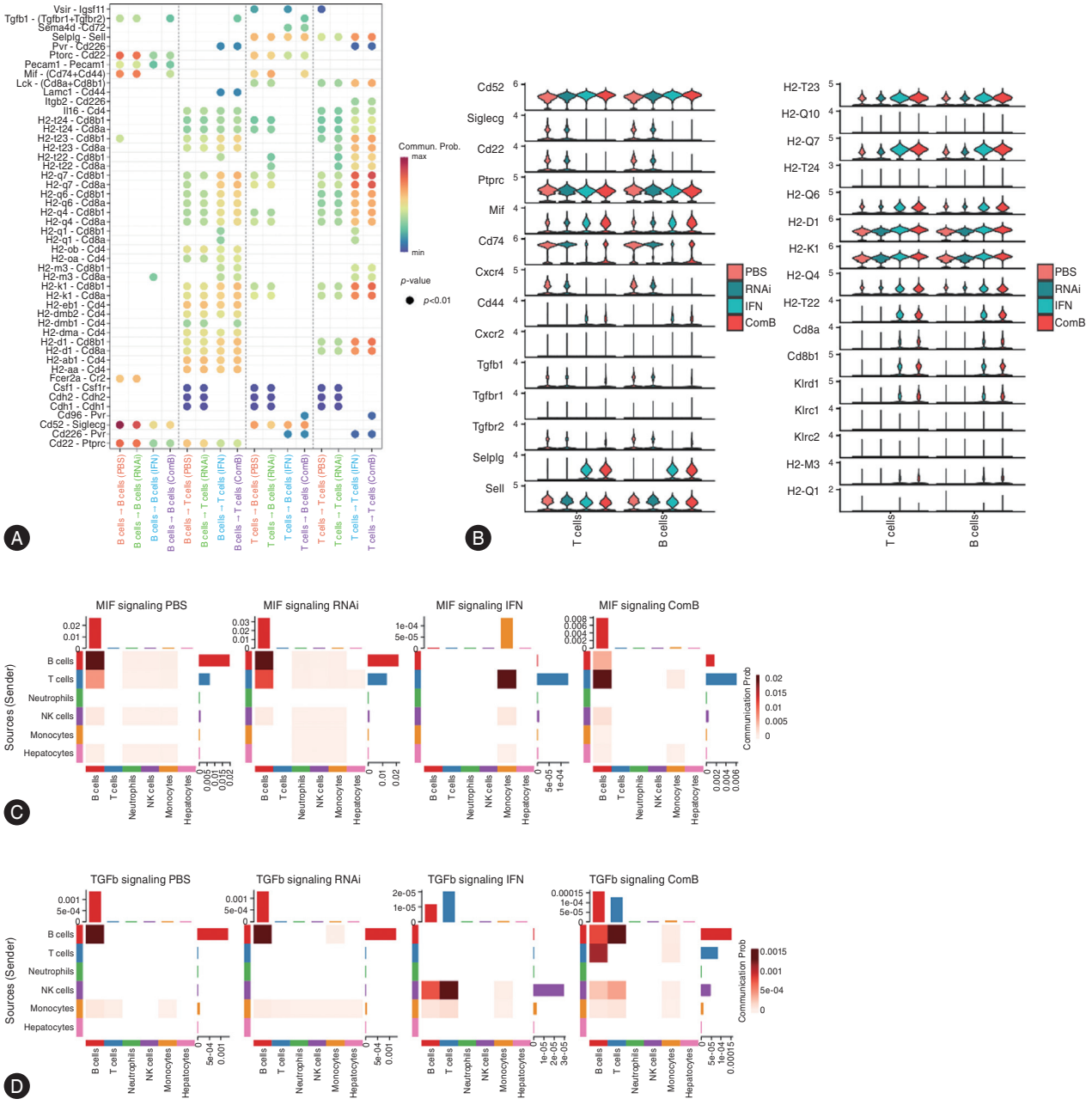


Figure 5. Cell-cell communication analysis indicated the complex interactions between T and B cells after combined RNAi and PEGIFNα treatment. (A) Comparison of the significant ligand-receptor pairs between T and B cells. Dot color represents the communication probability of the specific ligand-receptor pair between sender and receiver clusters. (B) Violin plots showing the expression levels of ligand-receptor pairs associate with T cells and B cells crosstalk in different groups. (C) Heatmap showing communication probability of MIF and (D) TGFb signaling. The top and right bar around the heatmap represent the sum of column values (incoming signaling) and row values (outgoing signaling) respectively. (E) Incoming and outcoming strengths of the cell interaction signals of T and B cell subpopulations. (F) Comparison of the significant ligand-receptor pairs across different groups among B cell subclusters. PEGIFNα, PEGylated interferon-α; RNAi, RNA interference.

migration, shifted from B cell-B cell interaction to T cell-monocyte communication with PEGIFNα, but was enhanced between T and B cells by combination therapy, supporting B cell migration (Fig. 5C). TGFb signaling, which mediates cell proliferation, shifted from B cell-B cell to NK-T cell communication by PEGIFNα treatment, and

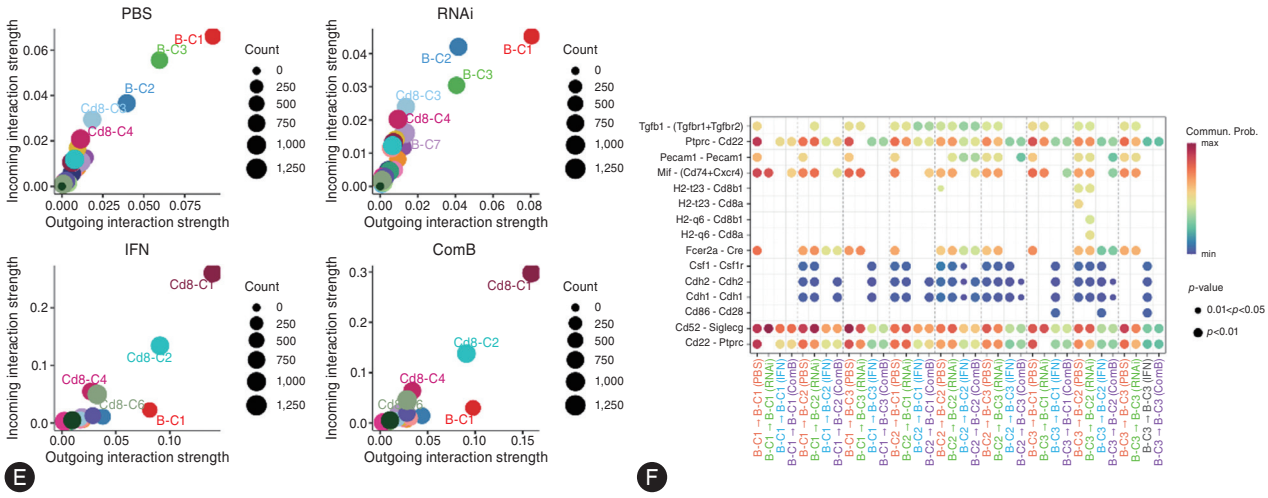


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was increased between B and T cells by combination therapy (Fig. 5D). These results indicate that combination therapy may enhance HBsAg antigen recognition and antibody secretion by promoting B cell migration and T cell proliferation.

The interactions between T and B cell subclusters were analyzed, showing that the incoming and outgoing interaction strength of the B-C1, B-C2 and B-C3 cells was the most prominent in PBS/RNAi- treated groups, PEGIFNα treatment reduced the interaction strength among B cells but enhanced interaction signaling in Cd8-C1 and Cd8-C2 T cells (Fig. 5E). Cell-cell communication probability analysis further confirmed reduced inhibitory Cd22-Ptprc and Cd52-Siglecg interactions among B cells by combination therapy, leading to B cell activation (Fig. 5F). In addition, the Mif-(Cd74+Cxcr4) pair interaction was enhanced in combination therapy group, compared with PEGIFNα monotherapy. Reduced inhibitory interactions among B cells and enhanced MHC-I signaling among $CD8^+$ T cells by PEGIFNα were also observed in huIFNAR mice (Supplementary Fig. 17C–17E). Collectively, these results demonstrate that combination therapy reduces inhibitory interactions between B cells and B cells, enhanced MHC-I signaling among T cells, and improved T cell-B cell crosstalk, thereby facilitating B cell migration and T cell proliferation to support antigen clearance and immune control of the virus.

Enhanced MHC-II-signaling networks promoted HBsAg seroconversion after combined RNAi and PEGIFNα treatment

To interrogate the potential causes that mediating HBsAg seroconversion and prolonged HBV control, serum markers of typical mice during the treatment cessation were displayed. HBV antigen rebounded evidently in HBsAg-untransformed mice, but retained at undetectable levels one month after treatment cessation in HBsAg-transformed mice (Fig. 6A). Persistent control of HBeAg were not observed, indicating that sustained HBsAg loss was attributed to immune control, but not cccDNA silence or clearance. Whereas anti-HBs levels in transformed mice maintained for at least 1 month after treatment cessation. We further evaluated the intrahepatic $CD45^+$ immune phenotypes after 20 weeks of combination treatment in untransformed (ComB1) and transformed (ComB2) mice (Supplementary Fig. 18A, 18B). Mice of $HBsAb^+$ displayed a marked reduction of infiltrated neutrophils (Fig. 6B). Further sub-clustering of T and B cells revealed an increased percentage of naïve like $CD8^+$ T cells and activated B cells, alongside a decreased percentage of cytotoxic and proliferating $CD8^+$ T cells, as well as reduced transitional B cells and atMBCs (Supplementary Fig. 18C–18F). The expression levels of MHC-I-related molecules were comparable, whereas the expression levels of MHC-II-related molecules were increased, including H2-Aa, H2-Ab1, H2-Eb1, H2-DMb2, H2-Oa and H2-Ob, in HBsAg-transformed mice (Fig. 6C). The

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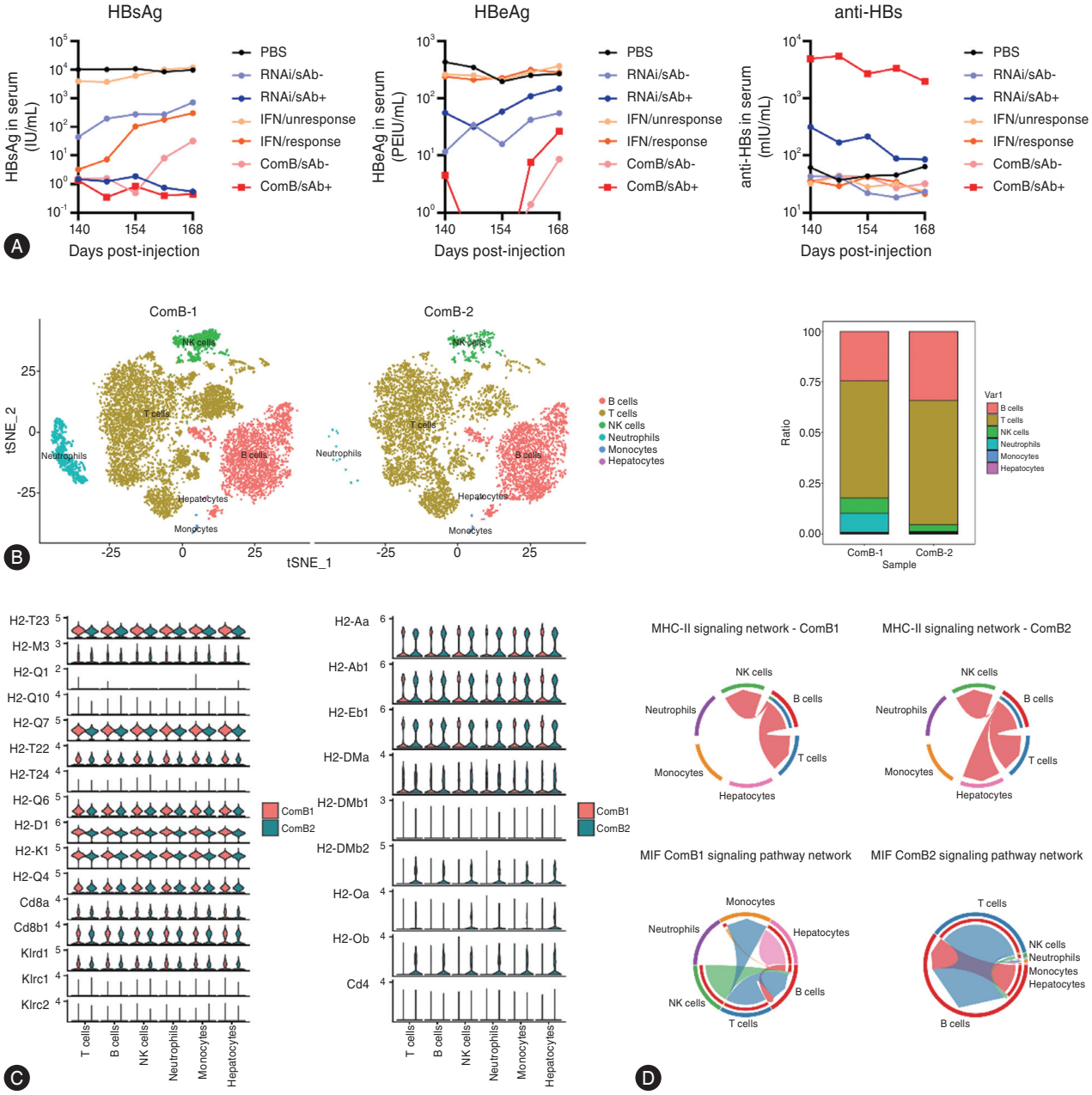


Figure 6. Enhanced MHC-II-signaling networks mediated HBsAg seroconversion after combined RNAi and PEGIFNα treatment. (A) Serum HBsAg, HBeAg and anti-HBs levels of typical mice in different groups (one line represented one mice). (B) Single-cell RNAseq of intrahepatic CD45$^+$ immune cells (ComB-1: HBsAg-untransformed; ComB-2: HBsAg-transformed). The proportion of each cluster in different groups. (C) Violin plots showing the expression levels of ligand-receptor pairs associate with MHC-I (left) and MHC-II (right) signaling. (D) Interaction networks emphasize cell-to-cell interactions via MHC-II (top) and MIF (bottom) pathways. (E) Up (left) and down (right) regulated genes in CD8$^+$ T and (F) B cells between HBsAg-transformed and untransformed mice were subjected to pathway enrichment analysis using Gene Ontology (GO) function analysis (BP: biological process; CC: cellular component; MF: molecular function).

MHC-II signaling network between B cells and hepatocytes, as well as the MIF signaling between T and B cells, were enhanced in mice of HBsAb$^+$ (Fig. 6D). This phenomenon was consistent with that observed in clinical samples, showing an enhanced MHC-II-expressing hepatocytes in patients of functional cure.[35]

Pathway enrichment analysis showed that, the CD8$^+$ T cells of transformed mice exhibited enhanced signaling

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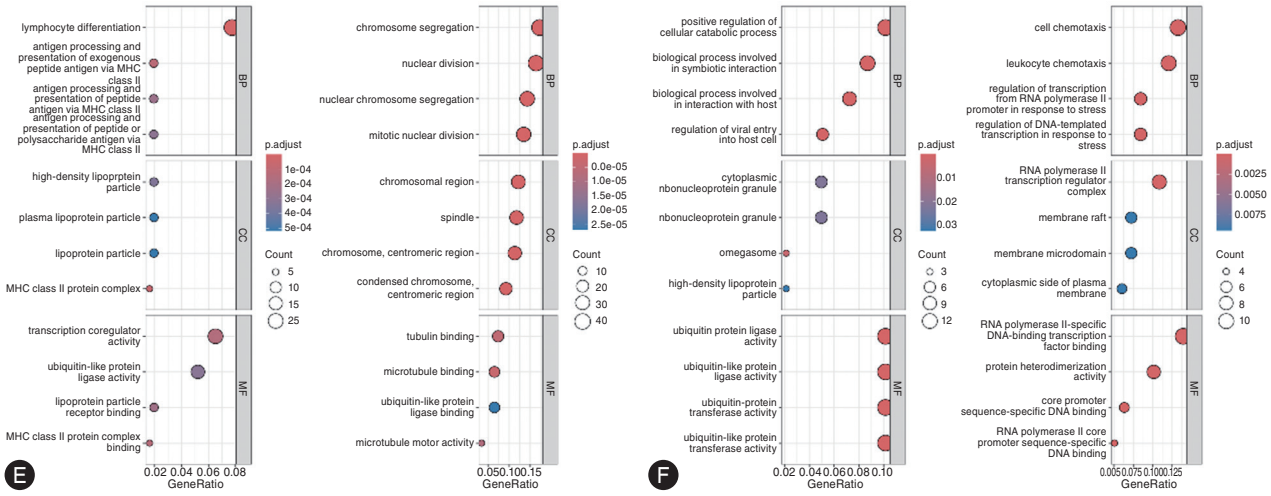


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pathway in "lymphocyte differentiation", and "antigen processing and presentation of peptide via MHC-II", and reduced "chromosome segregation" and "nucleic division" signaling (Fig. 6E). Further analysis of B cells showed an increased signaling in "positive regulation of cellular catabolic process", and "ubiquitin protein ligase activity", representing enhanced B cell metabolic activity, and a depressed signaling in "cell chemotaxis" and "signaling related to RNA polymerase II-specific transcription", suggesting reduced trend of B cell migration and transcription (Fig. 6F). Together, these results suggested that enhanced MHC-II signaling networks between B cells and hepatocytes/CD8$^+$ T cells, along with enhanced antigen processing and presentation pathways in CD8$^+$ T cells, promoted HBsAg seroconversion after long-term RNAi plus PEGIFNα treatment.

Reinforcing MHC-I/II signaling augmented anti-HBV efficacy of combined RNAi plus PEGIFNα therapy

The importance of antigen processing and presentation pathway in the synergy of combination therapy led us to hypothesize that reinforcing MHC-I/II signaling could further boost HBV-specific T and B cell immunity. Previous studies have shown that interleukin-2 (IL-2) and 4-1BB agonist antibody can promote MHC-I signaling-mediated effector T cell responses, whereas PD-1 blockage or CD40 agonist antibody can facilitate B cell maturation and differentiation into antibody-secreting cells.[13,36,37] PD-1/PD-L1 blockage has also been reported to break immune tolerance in chronic HBV-carrier mice.[13] Mice were treated with RNAi, PEGIFNα, or both, in the presence or absence of mouse PD-1 antibody (αPD-1), CD40 agonist antibody (αCD40), 4-1BB agonist antibody (α4-1BB) (i.p., 4 mg/kg, once biweekly), or recombinant mouse IL-2-Fc (rmIL-2-Fc) (50 μg/kg, twice weekly) (Fig. 7A). Combination therapy induced a synergistic reduction, and additional treatment with antibodies or rmIL-2-Fc further led to an additive suppression of HBV antigens, with α4-1BB eliciting the most potent and sustained effect (Fig. 7B). FACS analysis revealed that these adjunctive agents increased the percentages of HBV-specific T and B cells (Fig. 7C), promoting T cell proliferation and activation, and enhanced B cell proliferation and class switching (Fig. 7D). The functionality of HBV-specific CD8$^+$ and CD4$^+$ T cells was also improved in both periphery (Fig. 7E) and spleen (Fig. 7F), as evidenced by increased intracellular cytokine staining after *ex vivo* HBV peptide stimulation.

IHC analysis showed synergistic reduction of intrahepatic HBc staining in combination therapy and adjunctive groups (Fig. 8A, 8B), α4-1BB and rmIL-2-Fc also increased intrahepatic CD3$^+$, CD4$^+$, and CD8$^+$ T cell infiltration (Fig. 8C, Supplementary Fig. 19A). Histological and serum biochemical analysis confirmed the security of these combinations, with α4-1BB co-treatment triggered an evident ALT and AST elevation, indicating cytocidal effects of infiltrating T cells (Supplementary Fig. 19B). Immunofluorescence anal-

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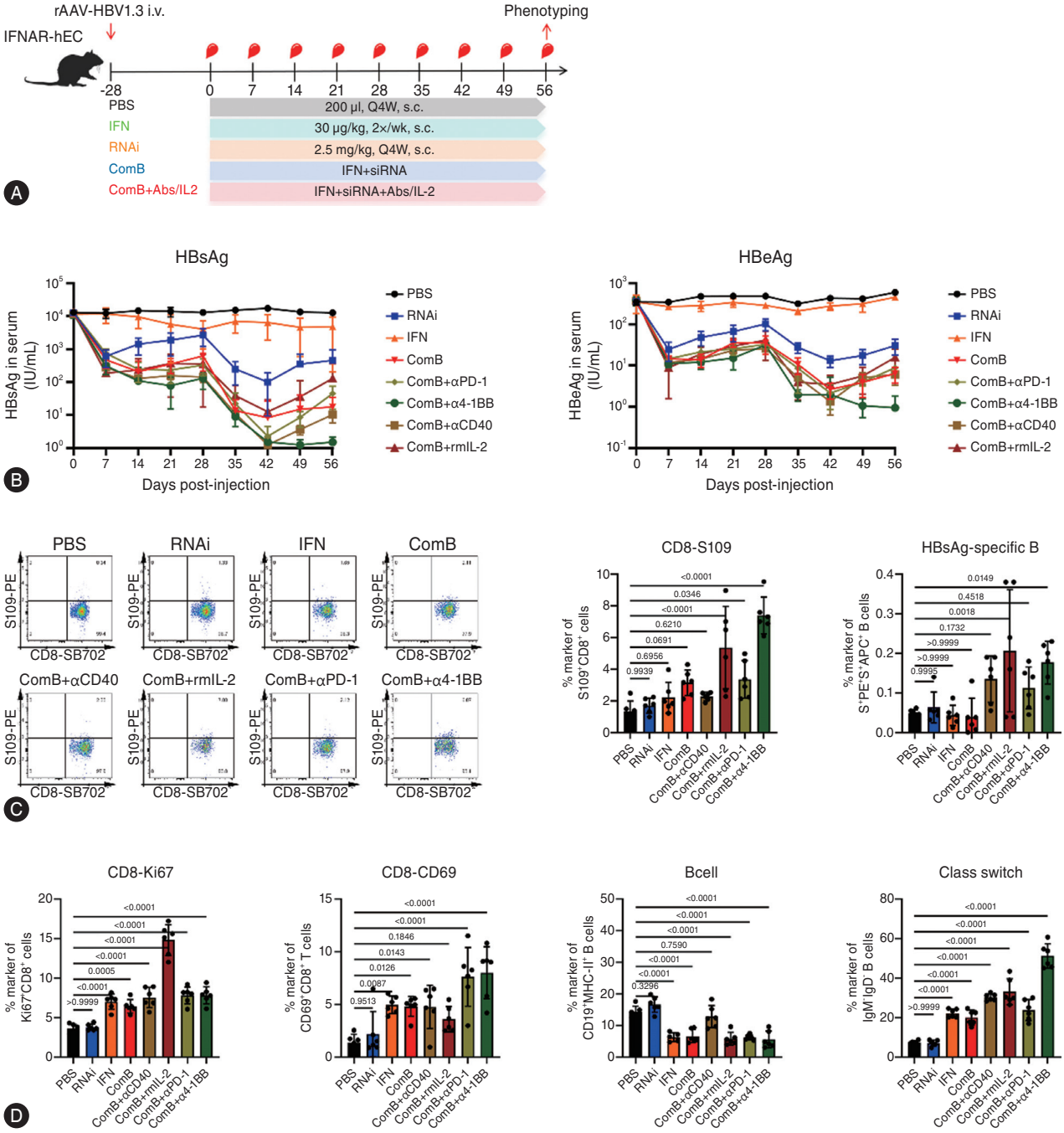


Figure 7. Adjunctive antibody or cytokine treatment augmented the antiviral effects of combined RNAi and PEGIFNα therapy. (A) Schematics of treatment procedures. Briefly, chronic HBV-carrier IFNAR-hEC mice were treated with GalNac-siHBV (2.5 mg/kg, s.c., Q4W), PEGASYS (30 μg/kg, s.c., 2×wk), or both concurrently, with or without the additive treatment of anti-PD-1 (αPD-1), anti-4-1BB (α4-1BB), anti-CD40 antibodies (αCD40) (4 mg/kg, i.p., Q2W) or recombinant mouse IL-2-Fc (rmIL-2) cytokine (50 μg/kg, i.p., 2×wk). (B) Seral HBsAg and HBeAg levels were monitored once weekly (n=6). (C) The proportion of HBsAg-specific $CD8^+CD3^+$ T cells and HBsAg-specific $CD19^+CD3^-$ B cells in gated PBMCs (n=6). (D) The proportion of $Ki67^+$ and $CD69^+$ $CD8^+CD3^+$ T cells in PBMCs. The proportion of $CD19^+MHC\text{-}II^-$ B cells and IgD^-IgM^- classed switched B cells in PBMCs (n=6). (E) Statistical analysis of the proportion of IFNγ- and perforin-expressing $CD8^+CD3^+$ and $CD4^+CD3^+$ T cells in PBMCs after *ex vivo* HBV-derived peptide pools stimulation (n=6). (F) The proportion of Ki67-positive $CD8^+CD3^+$ and $CD4^+CD3^+$ T cells in splenocytes (n=6). The proportion of perforin-expressing $CD8^+CD3^+$ and $CD4^+CD3^+$ T splenocytes after *ex vivo* HBV-derived peptide pools stimulation (n=6). Data were analyzed by one-way ANOVA with Dunnett multiple comparison correction (C–F), and were presented as mean±standard deviation. Numbers in graphs indicate *P*-values. HBV, hepatitis B virus; IFNAR-hEC, extracellular humanized IFNAR; PBMC, peripheral blood mononuclear cell.

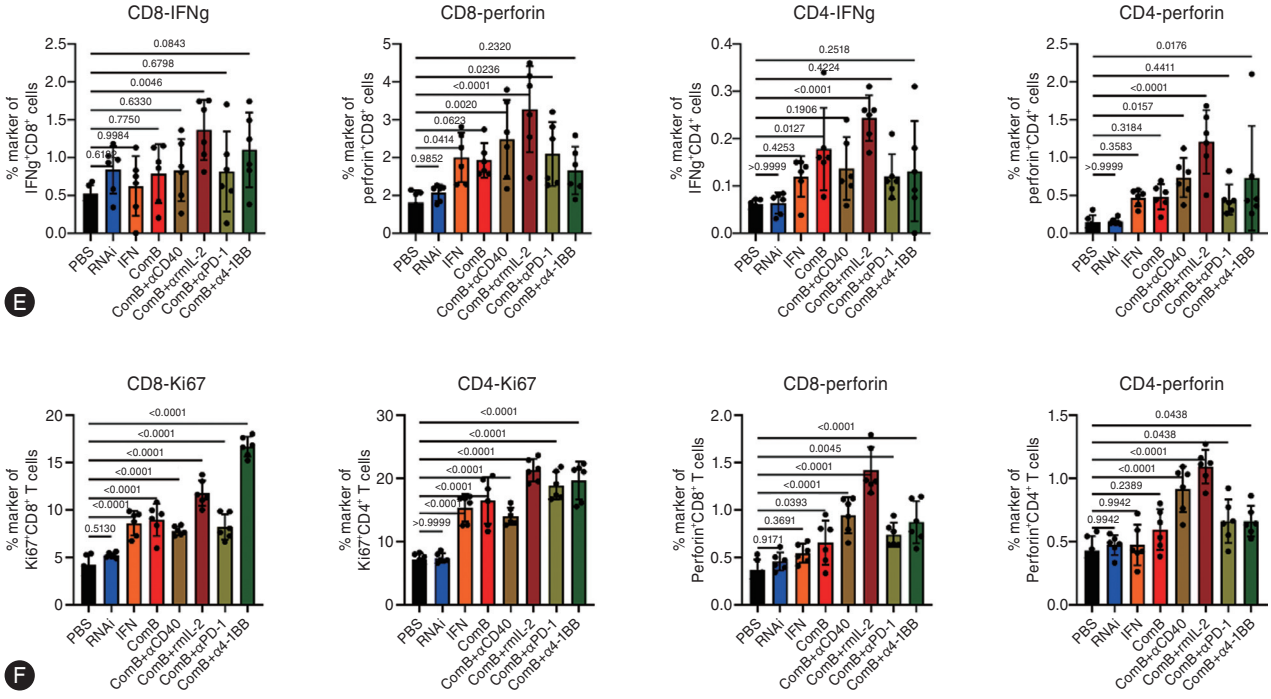


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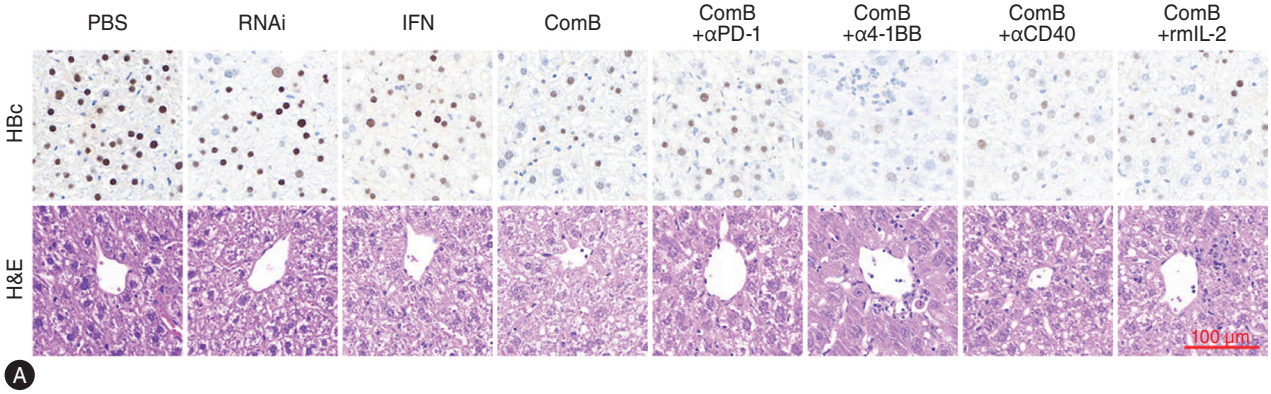


Figure 8. Adjunctive antibody or cytokine treatment enhanced the immune responses of combined RNAi and PEGIFNα therapy. (A) Representative graphs of intrahepatic HBc staining and histological analysis. Scale bar indicates 100 μm. (B) Quantification of the HBc-, (C) CD3-, CD8- and CD4-positive cells per mm^2 liver sections (n=4). (D) Immunofluorescence analysis of liver sections stained for MHC-II (red), CD8 (green) and CD19 (purple). Scale bar indicates 100 μm. (E) IHL or splenocytes were stimulated with HBV-derived peptide pools or cocktail for 24 hours, and the IFNγ-secreting T cells were tested by a T cell ELISpot assay (n=4). (F) Splenic B cell responses to HBsAg or R848+IL-2 stimulation were tested by a B cell ELISpot assay (n=4). Data were analyzed by one-way ANOVA with Dunnett multiple comparison correction (B, C, E, F), and were presented as mean±standard deviation. Numbers in graphs indicate *P*-values. HBV, hepatitis B virus; IHL, intrahepatic lymphocytes.

ysis further revealed pronounced infiltration of $CD8^+$ T cells and B cells in close proximity, forming immune synapse-like structures, along with obvious co-staining of MHC-II and CD8 in the combination plus adjunctive agent groups, suggesting active intrahepatic T cell-B cell crosstalk (Fig. 8D). ELISpot analysis of IHL and splenocytes showed that additive treatment with α4-1BB/rmIL-2-Fc promoted IFN-γ secretion by *ex vivo* HBV peptide pools stimulation, in mice receiving combination therapy, indicating improved HBV-specific T cell functionality (Fig. 8E). Whereas combination therapy plus αCD40 promoted anti-HBs IgG antibody production in splenocytes, and enhanced total IgG secretion

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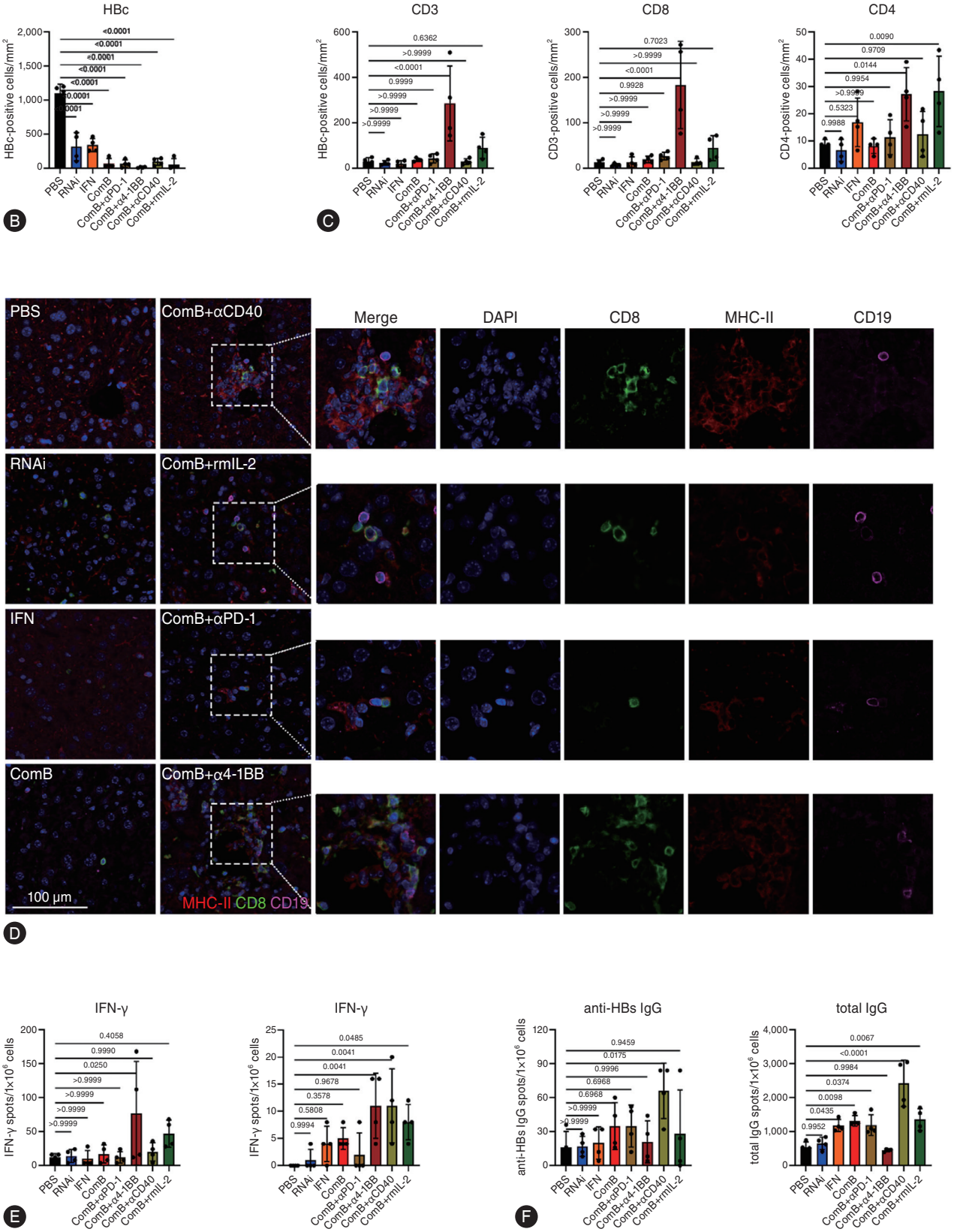


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following *ex vivo* stimulation with R848/IL-2 for 48 hours (Fig. 8F). Together, these results demonstrated that adjunctive treatment with antibodies or cytokine to enhance MHC-I/II signaling may improve the functionality of HBV-specific T and B cells, thus improve the therapeutic outcomes of combined RNAi and PEGIFNα therapy.

DISCUSSION

Potent NUCs suppress viral replication but do not target HBV transcripts or cccDNA reservoir. IFN-α has the benefit of finite treatment duration but the functional cure rate is still low. HBV siRNAs represent a promising therapeutic approach currently under investigation since it can both reduce antigen and viraemia levels.[38,39] However, merely RNAi cannot reconstruct antiviral immune responses. Viral antigens reappeared once upon treatment withdrawal. Recent studies showed that reducing HBV antigens via RNAi followed by immune stimulation with therapeutic vaccines could establish effective antiviral immune responses.[40] In the present study, we found that compared with NUCs and PEGIFNα, siRNA more effectively reduces antigen burden and alleviates high antigen load-related immune tolerance, thus offering the opportunity of immune reawakening. However, direct comparisons among these drugs are limited by differences in administration route, dosing regime, and pharmacokinetic profiles.

PEGIFNα has been demonstrated to exert diverse effects on innate and adaptive immune cells, and has potential to induce HBsAg seroconversion within finite treatment duration in partial CHB patients.[41] It is believed to allow restoration of HBV-specific T cell function by inhibiting viremia and antigenemia and via direct immunomodulation, whereas to what extent IFN-α can restore T cell function remains open questions.[9,42] Previous studies identified that IFN-α can act directly on both $CD4^+$ and $CD8^+$ T cells, increasing ratios of long-lived naïve/memory T cells and enhancing effector T cell cytotoxicity.[43] However, the immunomodulatory effects of IFN-α might be partly blunted by its antiproliferative effects and the inherent immune exhaustion in CHB patients. Using our IFNAR-hEC mouse model, we found that IFN-α can induce significant activation and proliferation of T/B/NK/DC cells, whereas high circulating HBsAg level was associated with reduced IFN-α responsiveness, as reflected by the diminished antiviral efficacy in high-viral-load mice, reduced cytokine production upon PMA/ionomycin stimulation, and suppressed total IgG secretion following R848+IL2 incubation.

The role of T cells in achieving HBV cure has been well-investigated, while little is known about the role of B cells in IFN-α-mediated anti-HBV immune responses. Recent studies indicated that type I IFNs can promote B cell activation and class switching during viral infection.[44] IFNs can also facilitate antigen presentation to B cells, resulting in stronger antigen-specific responses.[45] However, IFNs may also impair the survival and development of precursor and immature B cells, block B cell responses, and induce immunosuppressive molecules.[46] Consistently, our results also showed that PEGIFNα treatment improved the function of global B cells by upregulating MHC-II, CD69, CD80 and CD86 molecules, promoting B cell proliferation, and induced high percentage of IgM^-IgD^- class-switched B cells, but failed to trigger anti-HBs antibody production, even after long-term treatment or following subsequent HBsAg/CpG-1826 vaccination. The percentage of $FoxP3^+CD25^+$ Treg cells was also elevated, indicating an immunoinhibitory facet of PEGIFNα therapy.

Combined RNAi and PEGIFNα therapy have been under clinical study recently (e.g., VIR-2218 plus PEGIFNα), showing deeper suppression of viral parameters, higher rate of HBsAg seroclearance (~14.9%, 11/69), and higher incidence of HBsAg seroconversion (~14.5%, 10/69).[26,27] However, the underlying synergistic mechanisms remain obscure. Our IFNAR-hEC mouse model, which enables human PEGIFNα evaluation with an intact immune system, uniquely reveals the mechanistic T/B network remodeling underlying this synergy, extending beyond clinical observations. Based on this mouse model, we found that GalNac-siHBV exhibited potent and dose-dependent anti-HBV activity, PEGIFNα alone moderately suppressed viral antigens. Their combination induced deeper HBsAg reduction (~4$\log_{10}$ IU/mL, vs. PBS), and achieved anti-HBs seroconversion in ~30% (4/12) of treated mice (titers > 1,000 mIU/mL). Mechanistically, combination therapy elicited synergistic HBV-specific T and B cell activation, improved T cell function upon *ex vivo* HBV peptide stimulation, and enhanced anti-HBs IgG isotype secretion following HBsAg/CpG-1826 vaccination. Specifically, frequencies of circulating HBsAg-specific T and B cells did not change, but their

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functionality was influenced by HBsAg levels and PEGIFNα treatment, consistent with previous reports.[47]

Single-cell RNA-seq analysis showed that PEGIFNα treatment boosted high levels of $CD8^+$ T cell responses, leading to the development of $CD8^+$ T from naïve-like to proliferating and cytotoxic T states, and enhanced communications among $CD8^+$ T cells via MHC-I signaling. Whereas the percentage of naive B cells decreased with the proportions of transitional, atypical and classical memory B cells increased. Besides, the antigen processing and presentation, activation, and humoral immune response-related scores of B cells were improved. Inhibitory Cd22-Ptprc and Cd52-Siglecg pair interactions among B cells were reduced by PEGIFNα treatment through inhibiting relative ligand or receptor expression. Combination therapy enhanced MIF and TGFb signaling between T and B cells, promoting B cell migration and T cell proliferation. Enhanced MHC-II signaling networks between B cells and hepatocytes/$Cd8^+$ T cells further promoted HBsAg seroconversion. Considering the crucial role of MHC-I/II signaling in mediating antiviral immune responses, adjunctive antibodies or cytokines further deepened antigen reduction and strengthened HBV-specific T/B cell responses. Together, these results confirmed that combined RNAi and PEGIFNα treatment exerted synergistic antiviral efficacy and orchestrated protective cellular immune effects in T cell-B cell-dependent manners.

Although this study has some important implications for futural clinical CHB therapy, there are some limitations that need to be addressed in further studies. Since the AAVH-BV-based mouse model is not driven by the cccDNA reservoir and cannot fully recapitulate human immune features, the direct effects of combined treatment on cccDNA were elucidated in another study based on our recombinant cccDNA models and HBV-infected HepG2-NTCP cells, showing additive to synergistic suppression of epigenetic modifications and accessibility compared with either monotherapy.[48] Considering the presence of integrated HBV DNA as a source of HBsAg production, and mice models cannot fully recapitulate human HBV infection, an in-depth analysis of anti-HBV and immunomodulatory mechanisms of combination therapy, based on clinical-derived samples or human liver-chimeric mice, should provide more detailed information. Since downstream signaling and immune regulation are still mediated by the murine immune system in IFNAR-hEC mouse, complementary studies in human-derived systems are necessary to distinguish the effects on hepatocytes from those mediated by immune cells. The inclusion of a NUC-containing arm demonstrated that, unlike combined RNAi and IFN therapy, suppressing viral replication with ETV provided no additive antigen reduction or immune restoration over IFN monotherapy. These findings are consistent with clinical data, reinforcing the notion that HBsAg reduction, rather than DNA suppression, is the key determinant for immune restoration. Nevertheless, future studies incorporating NUC-based combinations are warranted to determine whether long-term viral suppression alongside siRNA-mediated HBsAg reduction can augment durable immunological control. Besides, the HBsAg seroconversion rate was only ~30%, and subsequent stimulation with HBsAg/CpG-1826 vaccination did not further trigger anti-HBs transformation. We speculate that the subtle differences in HBV tolerance extend among individual mice, and the insufficient immunomodulatory effects of existing IFN-α2, are the reasons for the low HBsAg seroconversion rate. Efforts to optimize the dosage and timing of both drugs may help improve the HBsAg seroconversion rate. Although reinforcing MHC-I/II signaling pathway through antibodies or cytokines to some extend improved the suppression of HBV replication and enhanced HBV-specific T cell and B cell responses, the benefits appeared modest, and the immune responses seems to be overactivated. Further development of other complementary immunomodulatory strategies is still necessary.[37] Since our data indicates that there are immunosuppressive effects against B cells after long-term PEGIFNα treatment, for those recipients who did not achieve anti-HBs seroconversion, continued PEGIFNα therapy may offer limited benefits while might instead add to immunosuppressive effects and lead to systemic toxicity. The issues of how to improve the efficacy and reduce the side effects of PEGIFNα have long been questioned. Our recent studies suggest that different subtypes of IFN-α have differential antiviral and immune-modulating activities. Compared with IFN-α2, subtypes of IFN-α with superior activity in activation of antiviral immune responses may improve the therapeutic efficacy.[49] It is reasonable to anticipate that they will also be more effective when used in combination therapy, which is currently under study.

In conclusion, the present study evaluated the antiviral

effects of combined RNAi and PEGIFNα treatment in chronic HBV-carrier IFNAR-hEC mice, showing synergistic antiviral efficacy, with a potent reduction of HBsAg of ~4log10 IU/mL and a HBsAg seroconversion rate of ~30%. Synergy was mainly attributed to the ablation of high antigen burden-induced immune tolerance by RNAi, induction of global immune responses by PEGIFNα, and enhancement of virus-specific T/B cell responses and remodeling of T cell-B cell communications by combination therapy. Adjunctive treatment with antibodies or cytokines to enhance MHC-I/II signaling may provide benefit to improve functional cure rate in CHB.

Authors' Contribution

Zhenghong Yuan, Jieliang Chen and Wenjing Zai conceived the project, designed the experiments and supervised the overall project. Wenjing Zai and Kongying Hu collected the samples and performed the virological experiments. Wenjing Zai and Mengying He analyzed the scRNA-seq data and performed the FACS and ELISpot experiments. Ziyang Song, Pan Gao, Chen Luo and Min Yang helped performing the animal experiment. Minxiang Xie and Asha Ashuo provided the methods for T cell and B cell analysis. Wenjing Zai, Kongying Hu and Mengying He wrote the manuscript. All authors contributed to reading and editing the manuscript.

All authors have read and approved the article.

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Data are available on reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information. Additional data are available upon reasonable request.

Conflicts of Interest

The authors have no conflicts to disclose.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Clinical and Molecular Hepatology website (http://www.e-cmh.org).

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