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CD40 stimulation activates CD8+ T cells and controls HBV in CD4-depleted mice

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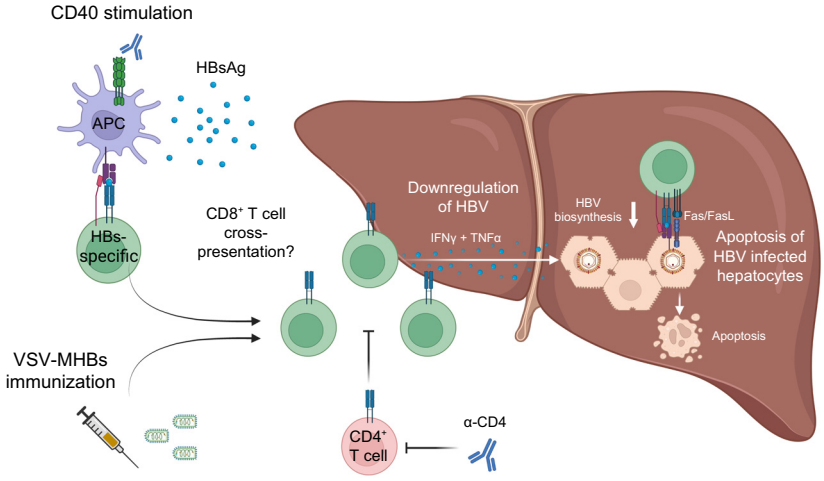
Graphical abstract



Highlights:

- Virus-specific $CD8^+$ T cell responses are desirable for effective HBV immunotherapy.
- CD40 has emerged as a promising target for inducing $CD8^+$ T cell responses.
- α-CD40 orchestrates sustained HBV control and is enhanced through co-immunization.
- HBV-specific $CD8^+$ T cells mediate HBV control following CD40 stimulation.
- HBV control is lost when $CD4^+$ T cells are present during CD40 treatment.

Impact and implications:

Immunotherapy has the potential to overcome immune dysfunction in chronic HBV infection. Using a mouse model of HBV replication, this study shows that CD40 stimulation can induce sustained HBV control, which is dependent on $CD8^+$ T cells and further enhanced by co-immunization. Unexpectedly, CD40-mediated HBV reduction was improved by the depletion of $CD4^+$ cells. These findings suggest potential strategies for reversing HBV persistence in infected individuals.

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CD40 stimulation activates CD8+ T cells and controls HBV in CD4-depleted mice

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Background & Aims: HBV treatment is challenging due to the persistence of the covalently closed circular DNA replication pool, which remains unaffected by antiviral intervention. In this study, we determined whether targeting antigen-presenting cells via CD40 stimulation represents an appropriate therapeutic approach for achieving sustained HBV control in a mouse model of HBV replication.

Methods: Mice were transduced with an adeno-associated virus encoding the HBV genome (AAV-HBV) to initiate HBV replication and were administered agonistic CD40 antibody. CD4-depleting antibody was administered in addition to the CD40 antibody. Viral antigens in the blood were measured over time to determine HBV control. HBV-specific $CD8^+$ T cells were quantified in the spleen and liver at the experimental endpoint.

Results: CD40 stimulation in CD4-depleted AAV-HBV mice resulted in the clearance of HBsAg and HBeAg, along with a reduction in liver HBV mRNA, contrasting with CD4-competent counterparts. $CD8^+$ T cells were indispensable for CD40-mediated HBV control, determined by HBV persistence following their depletion. In CD4-replete mice, CD40 stimulation initially facilitated the expansion of HBV-specific $CD8^+$ T cells, which subsequently could not control HBV. Finally, α-CD4/CD40 treatment reduced antigenemia and liver HBV mRNA levels in chronic AAV-HBV mice, with further enhancement through synergy with immunization by VSV-MHBs (vesicular stomatitis virus expressing middle HBsAg).

Conclusions: Our findings underscore the potential of CD40 stimulation as a targeted therapeutic strategy for achieving sustained HBV control and reveal a $CD4^+$ T cell-dependent limitation on CD40-mediated antiviral efficacy.

Introduction

Despite the availability of an efficacious preventative vaccine, HBV continues to pose a substantial global health concern. Mother-to-child transmission is cited as a primary reason for its endemic nature in some regions.[1] Healthy adults typically resolve HBV naturally, yet most children exposed to HBV face a lifelong infection linked with a heightened likelihood of hepatocellular carcinoma.[2,3] Current therapeutic interventions encompass the use of pegylated-interferon-α[4] as well as nucleos(t)ide analogues that inhibit viral polymerase activity.[5] Nonetheless, these treatments seldom lead to a sterilizing cure, denoted by the total loss of the HBV covalently closed circular DNA transcriptional template from the liver.[6]

The immune system is responsible for viral control and liver pathogenesis in HBV infection. Acute HBV is resolved by a multispecific $CD8^+$ T-cell response that inhibits viral replication through non-cytolytic antiviral cytokines (interferon [IFN]-γ and tumor necrosis factor-α [TNF-α]) and cytolytic killing of infected hepatocytes.[7,8] Immunological dysfunction, the process by which the immune system becomes tolerant to HBV, is considered a significant factor in the progression to chronic HBV (CHB). As a robust cytotoxic T lymphocyte response is paramount in preventing HBV persistence,[9] most experimental CHB immunotherapeutic interventions have targeted $CD8^+$ T cells.

The tumor necrosis factor receptor superfamily member CD40 has sparked interest owing to its potential to trigger antigen-specific $CD8^+$ T-cell responses.[10] Found largely on the surface of antigen-presenting cells (APCs) like dendritic cells, macrophages, and B cells, CD40 is a critical costimulatory molecule. Its natural ligand, CD40L (or CD154), is typically expressed by $CD4^+$ T cells. CD40 ligation is vital for activating and maturing B cells[11] and dendritic cells. This process holds significant value for licensing dendritic cells to cross-present antigens to $CD8^+$ T cells.[12,13] Artificial CD40 stimulation mimics the natural function of CD40L, resulting in enhanced APC antigen cross-presentation[14] and IL-12 production[15] that ultimately promotes the development of cytotoxic $CD8^+$ T cells. In cancer therapy particularly, $CD8^+$ T-cell responses have been generated by exploiting antibodies that crosslink CD40.[16–18] CD40 stimulation was previously shown to induce HBV control in HBV-transgenic mice, though HBV-specific $CD8^+$ T cells were not found.[19] However, in another study, CD40 stimulation reversed PD-1-mediated exhaustion in

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adoptively transferred transgenic CD8$^+$ T cells with the caveat that the *de novo* generation of endogenous HBV-specific T cells was not identified.[20]

Given the therapeutic potential of CD40 stimulation in promoting CD8$^+$ T-cell responses as observed in cancer and viral infections,[21] we postulated that it would have a similar efficacy in overcoming HBV persistence in mice. We discovered a novel mechanism to induce sustained HBV control in mice transduced with an adeno-associated virus encoding the HBV genome (AAV-HBV). Mice successfully resolved HBV following CD40 stimulation, but the presence of CD4$^+$ cells restricted this effect. CD40-mediated control was dependent on CD8$^+$ T cells and their associated effector functions. Finally, HBV persistence was reversed by combining CD40 stimulation with immunization by recombinant vesicular stomatitis virus expressing middle HBsAg (VSV-MHBs). These findings reveal CD40 as a clinically relevant target for controlling HBV and uncover an unexpected regulation by CD4$^+$ cells.

Materials and methods

Mice

BALB/cJ (stock # 000651), C57BL/6 (stock # 000664), B6.129-*Ido1*^{tm1Alm}/J (*Ido1*$^{-/-}$)[51] (stock # 005867), and B6.129S(C)-*Batf3*^{tm1Kmm}/J (*Batf3*$^{-/-}$)[42] (stock # 013755) mice were purchased from The Jackson Laboratory. Male mice were used at 6- to 8-weeks of age. FTY720 (2 μg/ml; Sigma Aldrich) was added to drinking water for lymph node egress inhibition studies. Mice were housed in the Animal Resource Facility at Albany Medical College. All experiments followed protocols approved by the Albany Medical College Institutional Animal Care and Use Committee.

AAV-HBV transduction

AAV (serotype 8) encoding a 1.2-mer HBV genome (Genotype D) was obtained from SignaGen. Mice were transduced via retro-orbital injection of 2-4 × 10^{10} genome copies of AAV-HBV diluted in 200 μl of sterile 1x PBS to initiate HBV replication.

In vivo antibody administration

Agonistic α-CD40 (FGK4.5, Bioxcell) was administered once per week for 4 weeks (100 μg/dose). Depleting [α-CD4 (GK1.5); α-CD8β (Lyt 3.2); α-CD25 (PC-61.5.3); α-IFN-γ (XMG1.2); α-TNF-α (XT3.11); α-IL-10 (JES5-2A5); α-TGF-β (1D11.16.8)] and blocking [α-FasL (MFL3)] antibodies were administered twice per week for a total of 10 doses (225 μg/dose).

Immunizations

For immunizations, VSV-MHBs was diluted in 50 μl PBS, and 1 × 10^6 plaque-forming units/mouse were administered intramuscularly at the designated times.[22]

ELISA

Serum HBsAg, HBs antibody, and HBeAg were measured by ELISA (International Immunodiagnostics) according to manufacturer recommendations. Recombinant HBsAg (subtype ayw) and HBeAg proteins (Fitzgerald Industries) were used to generate a standard curve.

IFN-γ ELISPOT

ELISPOT plates were coated with α-IFN-γ overnight before adding 2 × 10^5 cells. Cells were stimulated with 10 μg/ml HBV peptides overnight. Enumeration of IFN-γ spot-forming cells was achieved using an automated spot counter (Immunospot, Cellular Technology Ltd). Peptides used for restimulation are as follows: H-2^b: HBs 353 [VWLSVIWM], HBs 371 [ILSPFLPL], Core 93 [MGLKFRQL]; H-2^d: HBs 191 [IPQSLDSWWTSL], HBs 364 [WGPSLYSIL], Core 87 [SYVNTNMGL], Core 131 [AYRPPNAPI].

sALT assay

Serum alanine aminotransferase (sALT) was measured with Infinity ALT reagent (ThermoFisher) using a SpectraMax iD3 spectrophotometer (Molecular Devices) and enzyme standards (Verichem Laboratories). Briefly, 10 μl of serum or known standards were combined with 100 μl of Infinity ALT reagent, and the plate was immediately analyzed.

Flow cytometry

Liver and spleen tissues were mechanically dissociated by pressing through a 70 μm filter. For blood analysis, samples were collected in a 10 mM EDTA solution to prevent clotting, followed by RBC lysis with ACK lysing buffer (Lonza). For perfused liver samples, a Percoll gradient was utilized to isolate intrahepatic leukocytes. Briefly, Percoll solution was generated by combining 9 parts Percoll (GE Healthcare) with 1-part 10x PBS, and 40% Percoll was then prepared by diluting with 1x PBS. Samples were resuspended in 40% Percoll and centrifuged for 15 min. For regulatory T cell (Treg) analysis, FoxP3 Transcription Factor Staining Set (ThermoFisher) was used according to manufacturer recommendations in conjunction with α-CD25 (APC, 1:100; Biolegend). FoxP3$^+$ Tregs were gated on single cells/lymphocyte size exclusion/CD4$^+$/CD25$^{+/-}$. For HBs 353-specific CD8$^+$ T cells, samples were stained with α-CD3 (AF700, 1:100; Biolegend), α-CD8 (FITC, 1:100; Biolegend), α-CD19 (PE-Cy5, 1:100; Invitrogen), and HBs 353 pentamer (PE, 1:33; ProImmune). HBs 353-specific CD8$^+$ T cells were gated on single cells/lymphocyte size exclusion/CD19$^-$/CD3$^+$/CD8$^+$/HBs 353 pentamer$^+$. All samples were run on a BD FACSymphony A3 cytometer, and data were analyzed using FlowJo version 10.

Immunohistochemistry

Liver samples were fixed in 10% neutral buffered formalin overnight and then transferred to 70% EtOH. Fixed samples were embedded in paraffin, and 8 μm sections were prepared. For H&E staining, an autostainer was used. For HBcAg staining, slides were deparaffinized with xylene and rehydrated in decreasing concentrations of EtOH followed by 1x PBS. Endogenous peroxidase activity was quenched by incubation with 3% peroxide for 15 min. Slides were blocked with 10% goat serum to reduce staining background. α-HBcAg (1:500, Dako) was diluted in a PBS solution containing 3% BSA by volume (3% PBSA) and was incubated with tissue sections overnight at 4 °C. The following day, slides were washed and incubated with goat α-rabbit biotinylated secondary antibody (1:500) diluted in 3% PBSA for 1 h at room temperature. Slides were washed and incubated with streptavidin-HRP (1:100, BD

Biosciences) diluted in 3% PBSA, and developed using the ImmPACT DAB Substrate Kit (Vector Laboratories) according to the manufacturer's instructions. Sections were counterstained with hematoxylin QS (Vector Laboratories). Images were acquired using an Olympus BX41 microscope and processed with cellSens software.

Liver gene expression (RT-qPCR)

Frozen liver specimens obtained at the specified experimental conclusion were homogenized in RLT buffer supplemented with 2-mercaptoethanol. Subsequently, RNA was extracted from the liver samples using an RNeasy mini kit (Qiagen), following the guidelines provided by the manufacturer. A High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) was employed to synthesize cDNA from 100 ng total RNA, adhering to the manufacturer's protocols. For quantitative PCR, TaqMan Fast Advanced Master Mix (ThermoFisher) was utilized. The reactions were carried out using 1 μl of the cDNA reaction for 40 cycles of 95 °C – 60 °C using a QuantStudio 6 real-time PCR system (ThermoFisher) and analyzed using QuantStudio Design and Analysis software v2. Primers used in this study were as follows: HBV probe 5'-CCT CTT CAT CCT GCT GCT ATG CCT CAT C-3', antisense 5'-GAC AAA CGG GCA ACA TAC CTT-3', sense 5'- GTG TCT GCG GCG TTT TAT CA-3'.[23] Taqman assays (ThermoFisher) include IFNγ (Mm01168134_m1), CD3ε (Mm01179194_m1), CD8α (Mm01182107_g1), and Fas ligand (Mm00438864_m1). RNA expression was normalized to GAPDH (Mm99999915_g1).

HBV DNA qPCR

A High Pure Viral Nucleic Acid Kit (Roche) was used to isolate HBV DNA from serum samples following the manufacturer's instructions. In brief, 20 μl of serum was diluted in 1x PBS and combined with proteinase K, followed by incubation at 72 °C for 30 min. Afterward, the samples were subjected to spin column purification, and the resultant purified DNA was eluted using water. Quantitative PCR (qPCR) for HBV was carried out to establish a standard curve using a plasmid containing the HBV genome. For HBV quantitative PCR reactions, the TaqMan Fast Advanced Master Mix (ThermoFisher) was employed following the methodology described above.

Results

CD40 activation prevents HBV establishment and is restricted by CD4$^+$ T cells

Previously, we highlighted the integral role of CD4$^+$ T cells and the CD40/CD40L axis in resolving HBsAg through antibody seroconversion in AAV-HBV-transduced BALB/c mice.[24] Subsequently, we sought to determine whether CD40 activation could replace T-cell help in HBV regulation utilizing the same model. Mice received either PBS, α-CD4, or α-CD4 and α-CD40 (α-CD4/CD40). We initiated CD4 depletion before AAV-HBV transduction and α-CD40 treatment 2 days after transduction. Antigenemia at week 1 was similar among the groups, suggesting that depletion antibodies do not impact AAV transduction efficiency (Fig. 1A,C). As expected, AAV-HBV mice administered PBS spontaneously resolved HBsAg, while HBsAg persisted in CD4-depleted mice (Fig. 1A). Intriguingly, α-CD4/CD40-treated mice mirrored PBS mice, resolving HBsAg by week 5 post-transduction (Fig. 1A). Since HBsAg clearance in BALB/c mice is antibody-dependent, we explored whether α-CD4/CD40 mice exhibited a similar antibody-mediated resolution. However, HBs antibody was limited to PBS-treated mice (Fig. 1B), suggesting an alternative mechanism of HBsAg decline in the α-CD4/CD40 group. While CD4-depleted mice showed partial HBeAg reduction, it was below the detection limit in α-CD4/CD40 mice (Fig. 1C). In addition, increased sALT levels at week 3 and the presence of functional HBV-specific CD8$^+$ T cells at week 7 in α-CD4/CD40 mice (Fig. 1D,E) suggested that CD8$^+$ T-cell activity was critical for clearance. Finally, intrahepatic HBV gene expression measured at the experiment's end revealed a considerable reduction of HBV RNA in the α-CD4/CD40-treated mice (Fig. 1F). CD4$^+$ T-cell depletion alone was also associated with a reduction in both HBeAg and HBV RNA compared to PBS controls (Fig. 1C,F), likely attributed to their role in regulating antiviral cytokine expression within the liver.[24]

Our previous study identified varying immune responses to AAV-HBV based on mouse genetic background.[24] Hence, we also examined the response of C57BL/6 mice to AAV-HBV administration and CD40 stimulation and included an additional group receiving only α-CD40. By week 5 post-transduction, α-CD4/CD40-treated C57BL/6 mice had similarly resolved HBsAg (Fig. 1G), yet α-CD40 stimulation alone could not control HBsAg (Fig. 1G). HBeAg was partially reduced in α-CD40 mice but not cleared as seen in α-CD4/CD40 mice (Fig. 1H). sALT elevation was restricted to α-CD4/CD40 mice at week 3 post-transduction (Fig. 1I). Functional HBV envelope-specific CD8$^+$ T cells were detectable in α-CD4/CD40 mice but not in α-CD40 mice (Fig. 1J), suggesting that CD4$^+$ T cells preclude HBV-specific CD8$^+$ T-cell activation following CD40 stimulation. HBV gene expression was nearly undetectable in α-CD4/CD40 mice, while individually administered α-CD4 and α-CD40 had minimal effect on gene expression (Fig. 1K). Finally, CD8 and Fas ligand gene expression were significantly upregulated in α-CD4/CD40 mice, while neither α-CD4 nor α-CD40 alone had any effect on the expression of these genes in the liver (Fig. 1K). Together, these results suggest that HBV control is achieved independently of genetic background by CD40 activation at the time of transduction and is tightly regulated by CD4$^+$ T cells.

CD4$^+$ T cells restrict CD40-mediated HBV control independently of traditional regulatory T cells and MHCII

Because CD4 depletion was critical for CD40-mediated HBV reduction, we next asked whether Tregs are responsible for inhibiting HBV control, as Tregs are believed to promote HBV persistence in humans.[25] To test this, we utilized α-CD25 to deplete Tregs before AAV-HBV transduction. Again, antibody depletion began prior to AAV-HBV transduction while α-CD40 was administered starting 2 days following transduction. Using BALB/c mice, Treg depletion coupled with α-CD40 failed to reduce HBsAg (Fig. 2A) or HBeAg (Fig. 2B). HBsAg seroconversion was lost following α-CD40 administration, presumably due to competitive inhibition of endogenous CD40/CD40L interactions that facilitate this process.[26] α-CD25/CD40 resulted in increased liver inflammation at week 3 compared to the PBS

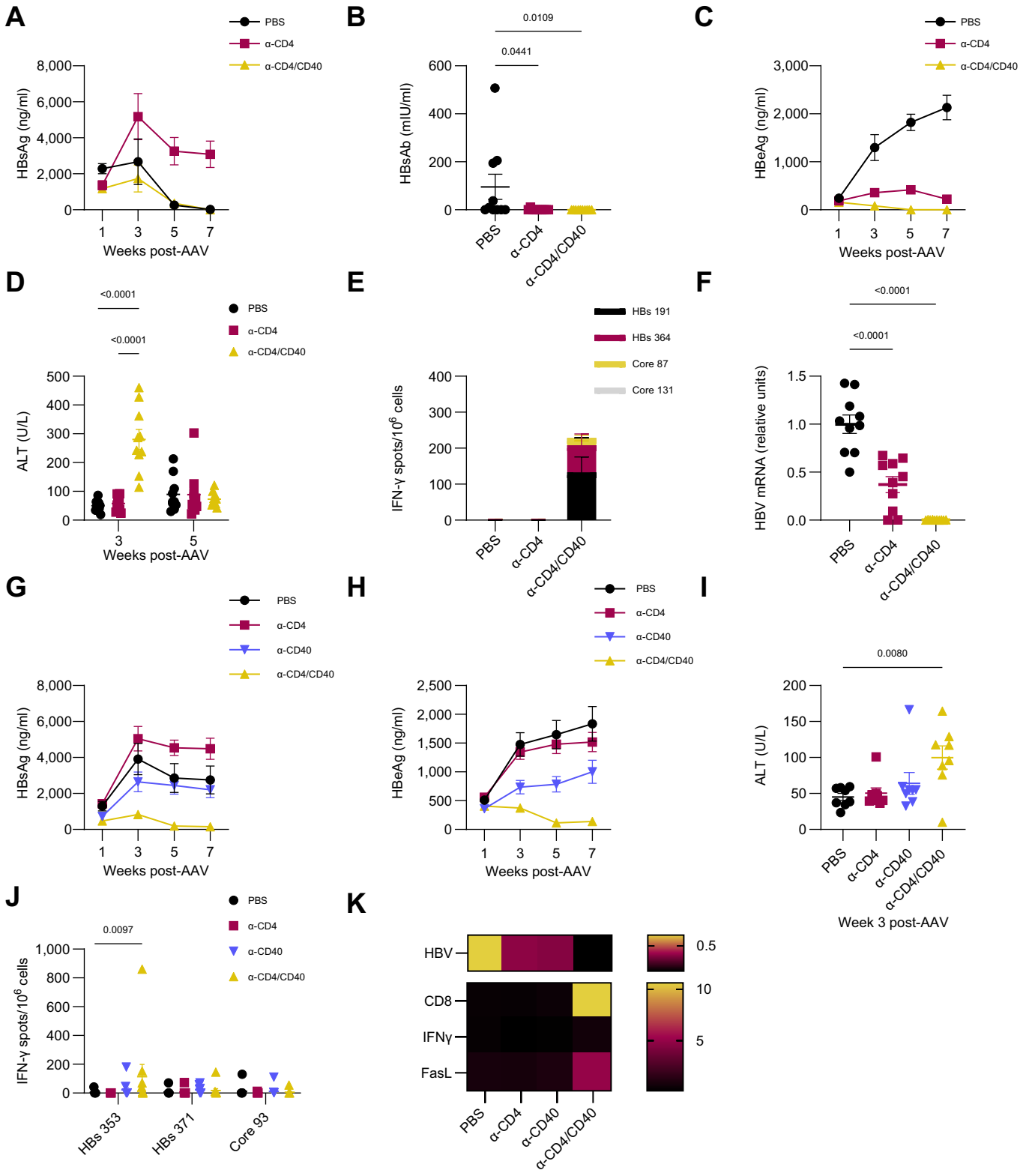

Fig. 1. CD40 activation in CD4-depleted mice promotes antibody-independent HBV control. (A-F) BALB/c mice were administered CD4-depleting antibody before AAV-HBV transduction and CD40 stimulation. Serum (A) HBsAg and (C) HBeAg measured by ELISA over time in CD4-depleted (α-CD4), CD4-depleted with CD40 stimulation (α-CD4/CD40), or control (PBS) AAV-HBV mice. (B) Serum HBsAb was measured by ELISA at the experimental endpoint (week 7). (D) Liver injury was assessed by serum ALT at week 3 and week 5 post AAV-HBV transduction. (E) HBV-specific $CD8^+$ T cells from the spleen were measured by IFN-γ ELISPOT at week 7. Splenocytes were stimulated overnight with BALB/c H-2^d restricted peptides HBs 191, HBs 364, Core 87, or Core 131, and the background from unstimulated controls was subtracted. (F) Liver HBV mRNA expression normalized to endogenous GAPDH was measured at week 7. (G-K) C57BL/6 mice were administered CD4-depleting antibody prior to AAV-HBV transduction and CD40 stimulation. Serum (G) HBsAg and (H) HBeAg were measured over time in CD4-depleted (α-CD4), CD40 stimulated

control group, though sALT returned to normal levels by week 5 (Fig. 2C). Increased sALT did not correspond to functional HBV-specific $CD8^+$ T cells, as α-CD25/CD40 treatment produced comparable levels of $CD8^+$ T cells to the PBS control group (Fig. 2D). Treg depletion was confirmed by flow cytometry, and $CD4^+FoxP3^+CD25^+$ cells were absent in mice receiving α-CD25/CD40 (Fig. 2E). Consistent with antigenemia, liver HBV gene expression in mice receiving α-CD25/CD40 mirrored PBS (Fig. 2F). CD8 and Fas ligand gene expression was increased only in animals receiving α-CD4/CD40 (Fig. 2F), consistent with the T-cell ELISPOT results (Fig. 2D). Histological analyses were performed to identify liver inflammation and HBV-infected hepatocytes by HBV core antigen (HBcAg). Little inflammation was detected, though distinct inflammatory foci were identified in α-CD4/CD40 livers (Fig. 2G, top). Strikingly, HBcAg was undetectable in α-CD4/CD40 livers while abundant in the remaining groups (Fig. 2G, bottom). These findings suggest that $CD25^+$ Tregs are not responsible for restricting HBV control following CD40 stimulation.

To evaluate relevant cytokines and pathways associated with immune regulation by $CD4^+$ T cells, TGF-β and IL-10 were depleted from C57BL/6 mice prior to AAV-HBV transduction and CD40 stimulation. Additionally, indoleamine 2,3 dioxygenase 1 (Ido1) knockout mice received CD40 stimulation following AAV-HBV transduction. Using HBeAg as a readout for HBV control, we found that neither mice with combined depletion of TGF-β and IL-10 nor *Ido1*$^{-/-}$ mice controlled HBV (Fig. 2H), and HBs 353-specific $CD8^+$ T cells were identifiable only in mice receiving α-CD4/CD40 (Fig. 2I). Since key regulatory components relating to $CD4^+$ T cells appeared to be relatively unimportant in restricting HBV control following CD40 stimulation, we next asked if restrictive $CD4^+$ T cells were being instructed in an antigen-dependent manner. To determine this, MHCII/TCR interactions were blocked prior to AAV-HBV transduction and CD40 stimulation. MHCII-blocked mice failed to control HBeAg following CD40 stimulation, unlike CD4-depleted mice (Fig. 2J). HBs 353-specific $CD8^+$ T cells were absent in MHCII-blocked mice but abundant in CD4-depleted mice at week 7 (Fig. 2K), underscoring the complexity of $CD4^+$ T cells in CD40-mediated HBV control. Due to the crucial role of type 1 conventional dendritic cells (cDC1s) in CD40-mediated tumor control,[27–29] we examined their role in HBV-transduced *Batf3*$^{-/-}$ mice that lack these cells and found that these mice also effectively controlled HBeAg following CD4 depletion and CD40 stimulation (Fig. 2J).

HBV-specific $CD8^+$ T cells control HBV in CD40-stimulated AAV-HBV mice

$CD8^+$ T cells were found to be the most significant determinant of virus control in chimpanzees.[7] Other animal studies have revealed contributions of cytolytic (Fas/Fas ligand, perforin/granzyme B)[20,30] and non-cytolytic (IFN-γ/TNF-α)[31,32] effector functions of $CD8^+$ T cells in HBV control. Due to their involvement in HBV control as well as mounting evidence that CD40 stimulation generates functional $CD8^+$ T-cell responses,[16,21,33,34] we next asked whether $CD8^+$ T cells mediate the HBV control observed following CD40 stimulation in CD4-depleted AAV-HBV mice. Mice were treated with α-CD4/CD40 as previously described. In addition, α-CD8, α-IFN-γ/TNF-α, or α-FasL antibodies were combined with α-CD4/CD40 to determine the role of $CD8^+$ T cells and their associated effector functions in CD40-mediated HBV control. CD8 and IFN-γ/TNF-α depletion were detrimental to HBV control, as HBsAg (Fig. 3A), HBeAg (Fig. 3B), and serum HBV DNA (Fig. 3E) remained elevated throughout the experimental duration. Fas ligand blockade also impaired HBV control but to a lesser degree. HBsAg (Fig. 3A) and HBeAg (Fig. 3B) were reduced in Fas ligand-blocked mice, but complete clearance was never achieved. Except for mice receiving only α-CD4/CD40, none of the groups experienced an increase in sALT (Fig. 3C) or had detectable HBV-specific $CD8^+$ T cells in the spleen at the experimental endpoint (week 9) (Fig. 3D). These findings implicate $CD8^+$ T cells as the primary mediator of HBV control following CD40 stimulation through both cytolytic and non-cytolytic means.

$CD8^+$ T cell-mediated HBV control by CD40 stimulation in $CD4^+$ T cell-depleted mice is sustained long term

Since one of the defining characteristics of antigen-specific responses is the formation of immunological memory, we sought to understand whether HBV control following CD40 stimulation is sustained, and if so, to determine the recall capacity of liver $CD8^+$ T cells to restimulation. $CD8^+$ T cells were depleted following HBeAg loss at 7 weeks post-α-CD4/CD40 treatment, and HBeAg was tracked following depletion to determine antigen rebound. HBeAg remained undetectable throughout the experiment duration up to 5 weeks following CD8 depletion (Fig. 3F), implying that the antiviral response ceased by week 7 post-treatment. At week 12, $CD8^+$ T cells within the liver were restimulated with HBs 353 peptide to determine their recall capacity. HBs-specific $CD8^+$ T-cell detection was limited to animals receiving α-CD4/CD40, with 40% of the mice in this group responding to HBs 353 stimulation (Fig. 3G). Lastly, we determined the role of lymphoid tissue egress by sphingosine 1-phosphate in CD8-mediated HBV control. Mice treated with α-CD4/CD40 and receiving FTY720 had elevated levels of serum HBeAg compared to those treated with α-CD4/CD40 that did not receive FTY720 (Fig. 3H), which suggests that sphingosine 1-phosphate signaling is required for complete CD40-mediated HBV control. Together, CD40-mediated HBV control is sustained long term and can generate tissue-resident $CD8^+$ T-cell memory.

Although $CD4^+$ T-cell depletion is essential for CD40-mediated HBV control, it was unclear whether $CD8^+$ T-cell

(α-CD40), CD4-depleted with CD40 stimulation (α-CD4/CD40), or control (PBS) AAV-HBV mice. (I) Serum ALT was measured at week 3 post-transduction. (J) HBV-specific $CD8^+$ T cells from the spleen were measured by IFN-γ ELISPOT at week 7. Splenocytes were stimulated overnight with C57BL/6 H-2^b restricted peptides HBs 353, HBs 371, or Core 93, and background was subtracted from unstimulated controls. (K) Liver RNA expression of HBV, CD8, IFN-γ, and Fas ligand was measured by RT-qPCR at week 7. Gene expression was normalized to endogenous GAPDH expression. Fold change in geometric mean was normalized to gene expression in control mice. n = 10 mice per group. Statistical significance was determined by one- or two-way ANOVA and Dunnett's multiple comparison test. AAV, adeno-associated virus; ALT, alanine aminotransferase; HBsAb, HBs antibody; RT-qPCR, quantitative reverse-transcription PCR.

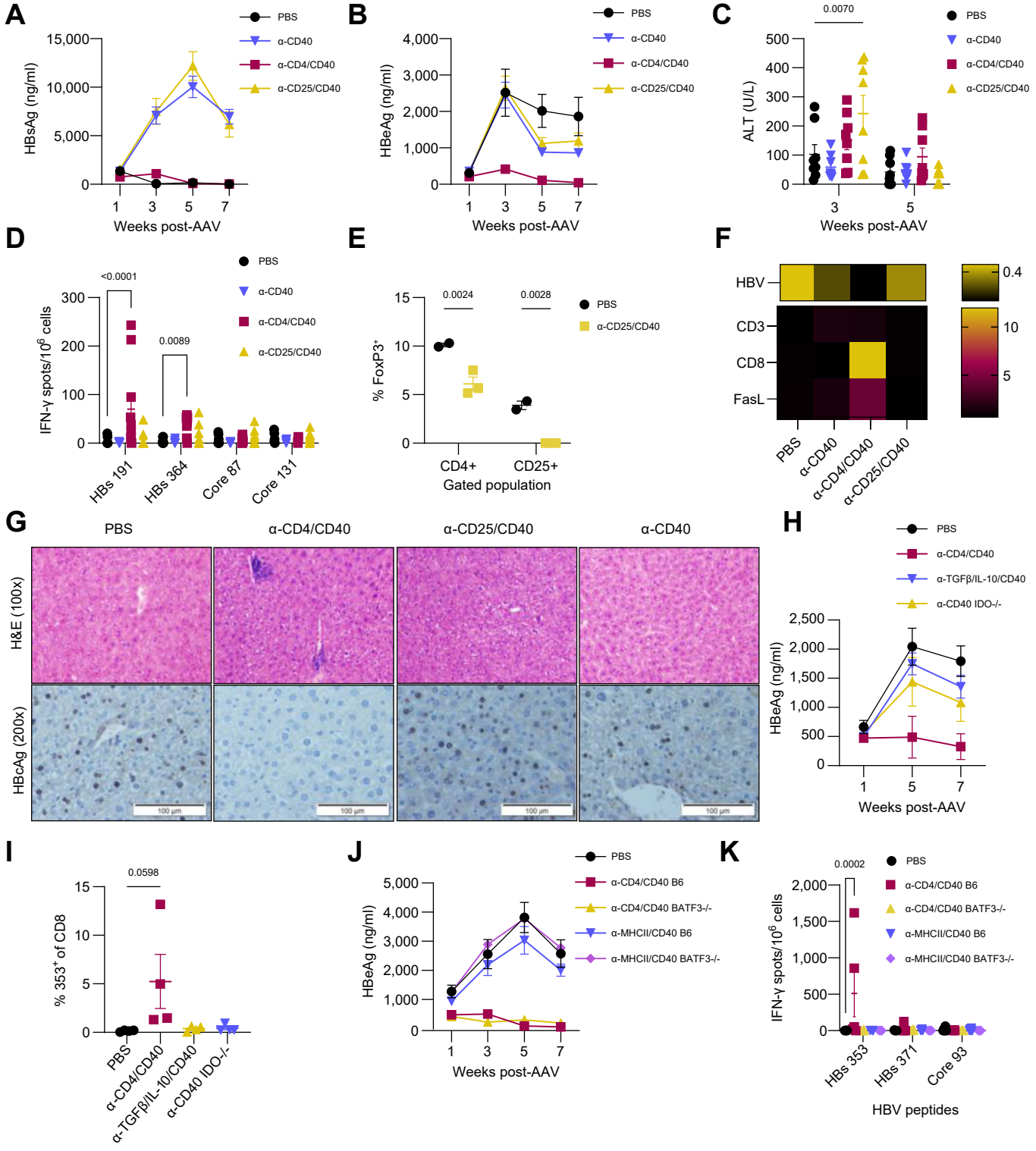

Fig. 2. CD4⁺ T cells restrict CD40-mediated HBV control independently of regulatory T cells and TCR signaling. (A-G) BALB/c mice received CD4- or CD25-depleting antibody prior to AAV-HBV transduction and CD40 stimulation (n = 10). Serum (A) HBsAg and (B) HBeAg were measured over time in CD40-stimulated (α-CD40); CD4-depleted, CD40-stimulated (α-CD4/CD40); CD25-depleted, CD40 stimulated (α-CD25/CD40); and PBS control mice (PBS). (C) Serum ALT was measured at weeks 3 and 5 post-transduction. (D) HBV-specific CD8⁺ T cells were measured by ELISPOT using BALB/c peptides HBs 191, HBs 364, Core 87, and Core 131. (E) Treg depletion was measured by flow cytometry in CD25-depleted mice by gating on $CD4^+/CD25^{+/-}$/FoxP3⁺ events (n = 2-3). (F) Liver HBV, CD3, CD8, and Fas ligand RNA expression was measured at week 7 by RT-qPCR. Gene expression was normalized to endogenous GAPDH expression. Fold change in geometric mean was normalized to gene expression in PBS mice. (G, top) H&E staining and (G, bottom) HBcAg staining of representative formalin-fixed liver sections. (H–I) TGF-β/IL-10 depletion (C57BL/6) and *Ido*$^{-/-}$ in CD40-stimulated AAV-HBV mice. (H) HBeAg was measured over time in mice receiving CD4- or TGF-β/IL-10-depleting antibodies before AAV-HBV transduction and CD40 stimulation. (I) Frequency of liver HBs 353⁺ CD8⁺ T cells gated on single cells/lymphocyte size exclusion/$CD3^+/CD19^-/CD8^+$/353 pentamer⁺ events (n = 4). (J-K) Wild-type or *Batf3*$^{-/-}$ mice were administered CD4-depleting or MHCII (I-A/I-E)-blocking antibodies prior to CD40 stimulation (n = 8). (J) Serum HBeAg was measured over time by ELISA. (K) HBV-specific CD8⁺ T cells were measured by IFN-γ ELISPOT at week 7 post-transduction. Statistical significance was determined by one- or two-way ANOVA and Dunnett's multiple comparison test. AAV, adeno-associated virus; ALT, alanine amino-transferase; RT-qPCR, quantitative reverse-transcription PCR.

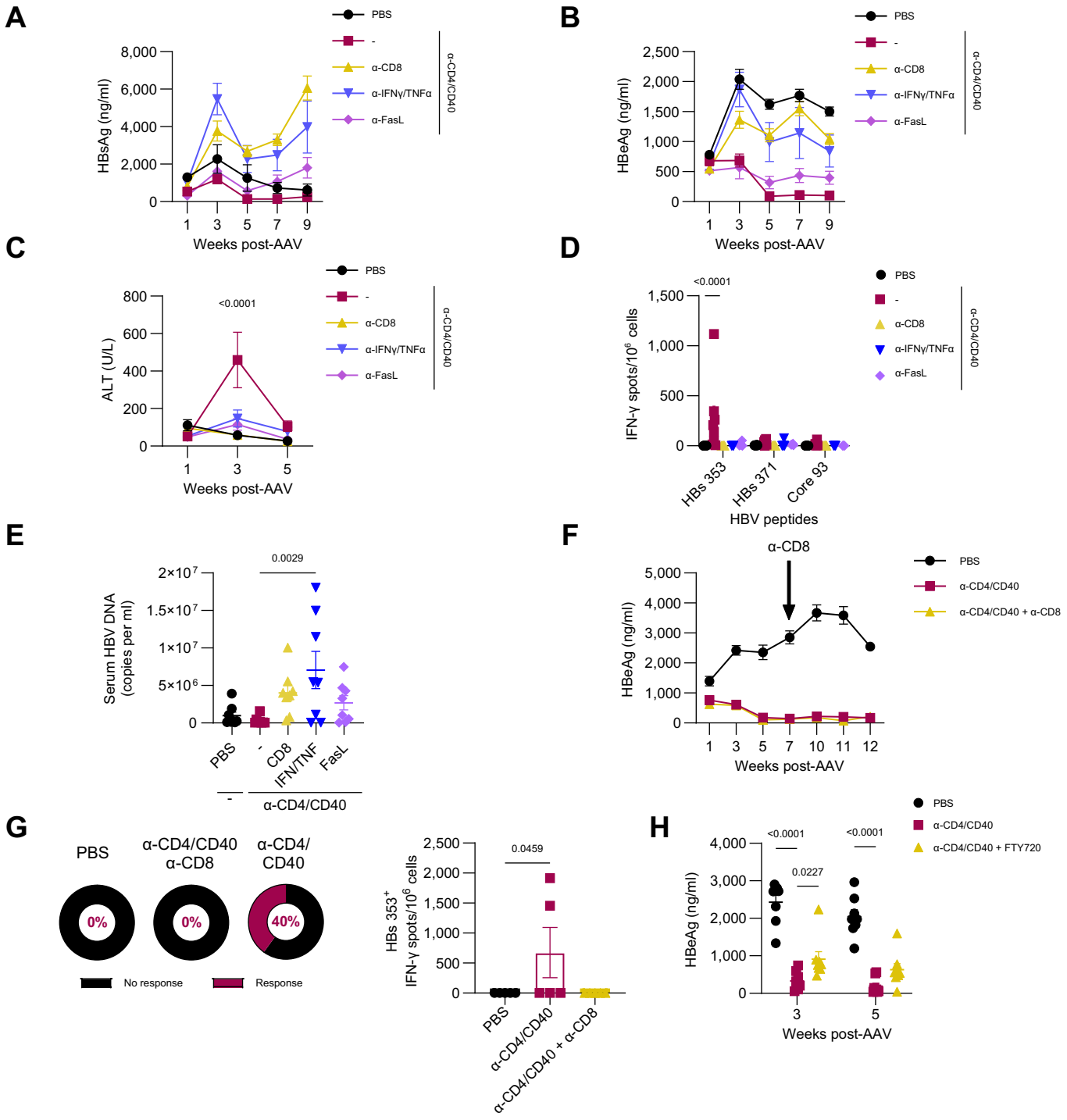

Fig. 3. Virus-specific CD8⁺ T cells control HBV following CD40 stimulation. (A-E) AAV-HBV-transduced C57BL/6 mice received PBS, α-CD4/CD40, or α-CD4/CD40 in combination with α-CD8, α-IFNγ/α-TNFα, or α-FasL. Serum (A) HBsAg and (B) HBeAg were measured by ELISA over time. (C) Serum ALT was measured at weeks 1, 3, and 5 post AAV-HBV transduction. (D) HBV-specific CD8⁺ T cells were measured by IFNγ ELISPOT assay. (E) HBV DNA in the serum was measured at week 9 post-transduction by qPCR. (F, G) CD8⁺ T cells were depleted from mice treated with α-CD4/CD40 following HBeAg clearance (week 7). (F) HBeAg was measured over time by ELISA. (G, left) Percentages of responders following CD8⁺ T cell restimulation by HBs 353 peptide. (G, right) T cell ELISPOT of intrahepatic leukocytes following restimulation with HBs 353. Counts were normalized by subtracting the background from unstimulated wells. (H) Temporal HBeAg ELISA analysis of mice treated with FTY720 beginning prior to CD40 stimulation and continuing throughout the experimental duration. n = 8 mice per group. Statistical significance was determined by one- or two-way ANOVA and Dunnett's or Kruskal-Wallis (G) multiple comparison test. AAV, adeno-associated virus; ALT, alanine aminotransferase; qPCR, quantitative PCR.

expansion occurs in CD4-competent mice following CD40 stimulation. To address this, we examined HBs-specific CD8⁺ T-cell responses in the liver of mice receiving CD40 stimulation at weeks 1, 2, and 4 post-treatment. Antigenemia (HBsAg and HBeAg) was lowered following CD40 stimulation compared to control mice at weeks 2 and 4, though CD4-depleted mice

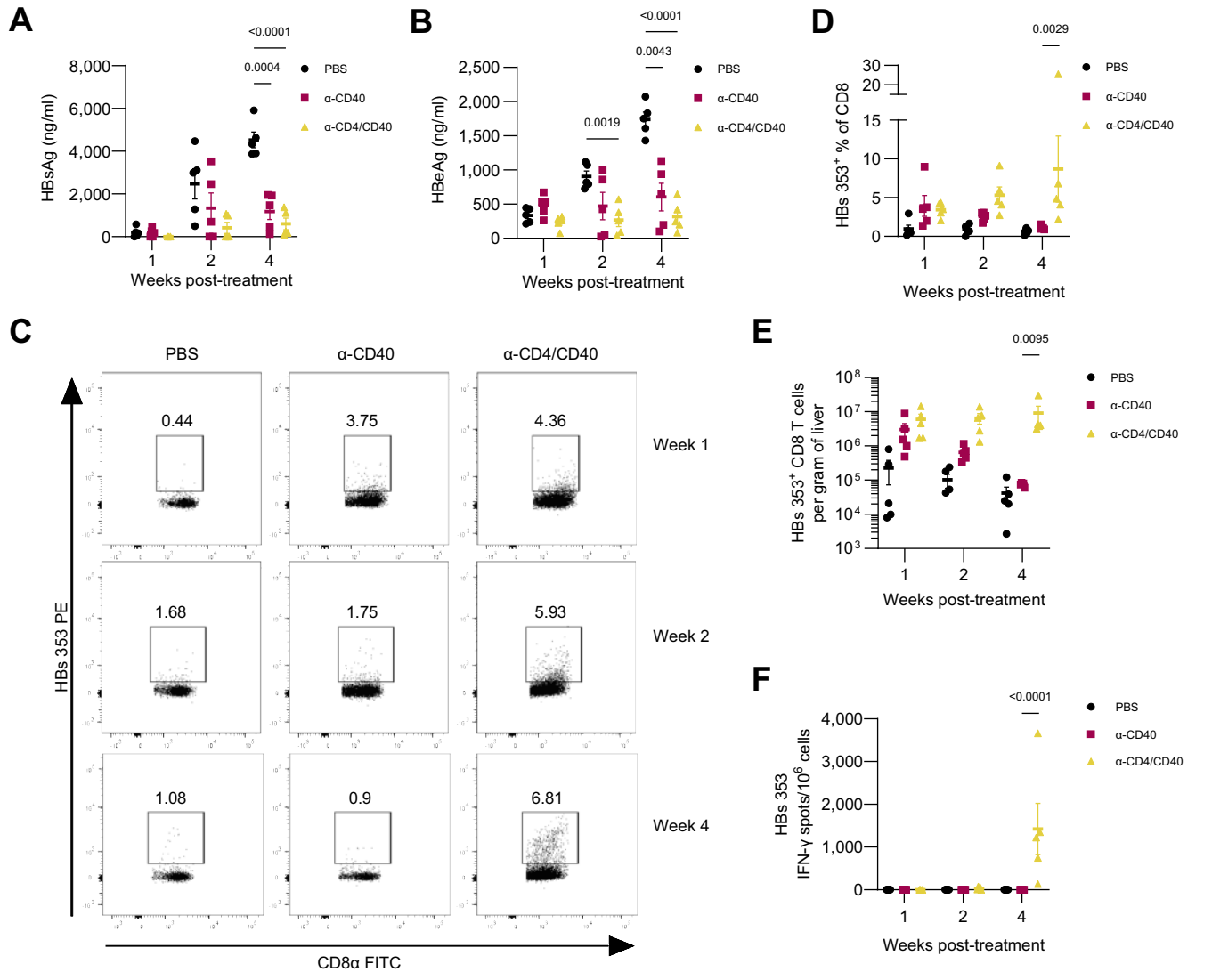

Fig. 4. HBs-specific CD8⁺ T cells expand in CD40-stimulated, CD4-competent mice but are deleted over time. AAV-HBV mice received PBS, α-CD40, or α-CD4/CD40 and were euthanized at weeks 1, 2, and 4 post-treatment to examine HBV-specific CD8⁺ T cell responses. (A) HBsAg and (B) HBeAg were measured at each time point by ELISA. (C) Representative flow cytometry plots of HBs 353-specific CD8⁺ T cells at each timepoint. Cells were gated on CD45⁺/CD19⁻/CD3⁺/CD8α⁺/HBs 353⁺ events. (D) HBs 353⁺ events graphed as a percentage of total intrahepatic CD8⁺ T cells at weeks 1, 2, and 4 post-treatment. (E) HBs 353-specific CD8⁺ T cells were quantified and normalized per gram of liver. Liver mass was recorded at harvest, and purified leukocytes were enumerated before downstream flow cytometry analysis. (F) CD8⁺ T cell response to HBs 353 stimulation was determined by IFN-γ ELISPOT assay. Results were normalized to unstimulated controls. n = 5 mice per group. Statistical significance was determined by two-way ANOVA and Dunnett's multiple comparison test. AAV, adeno-associated virus.

receiving CD40 stimulation showed the greatest reduction (Fig. 4A,B). Most HBV-specific CD8⁺ T cells generated following CD40 stimulation in CD4-depleted mice were specific for HBs 353. HBs 353-specific CD8⁺ T cells were increased in both CD4-depleted and CD4-competent CD40-stimulated mouse livers at week 1 (Fig. 4C). However, the percentage of HBs 353-specific CD8⁺ T cells declined over time at weeks 2 and 4 post-treatment in mice receiving CD40 stimulation (Fig. 4D). CD4-depleted/CD40-stimulated mice, on average, displayed an increase in the percentage of HBs 353-specific CD8⁺ T cells over time (Fig. 4D). This trend was consistent in the CD8⁺ T-cell count from the liver in that CD40-stimulated mice had similar numbers of HBs 353-specific CD8⁺ T cells as CD4-depleted/CD40-stimulated mice at week 1 that declined over the 4-week experimental duration (Fig. 4E). *In vitro* stimulation of intrahepatic leukocytes with HBV peptide at each time point showed that CD8⁺ T cells were responsive to restimulation only in the CD4-depleted/CD40-stimulated group as determined by IFN-γ ELISPOT (Fig. 4F). These findings indicate that HBV-specific CD8⁺ T cells expand following CD40 stimulation at the time of AAV-HBV transduction in CD4-competent mice but are unable to control HBV and are eventually subject to deletion.

Immunization enhances the therapeutic effect of CD40 stimulation in chronic HBV

The studies above have applied CD40 stimulation at the time of AAV-HBV transduction. However, an essential consideration for therapeutic intervention is that individuals seeking HBV

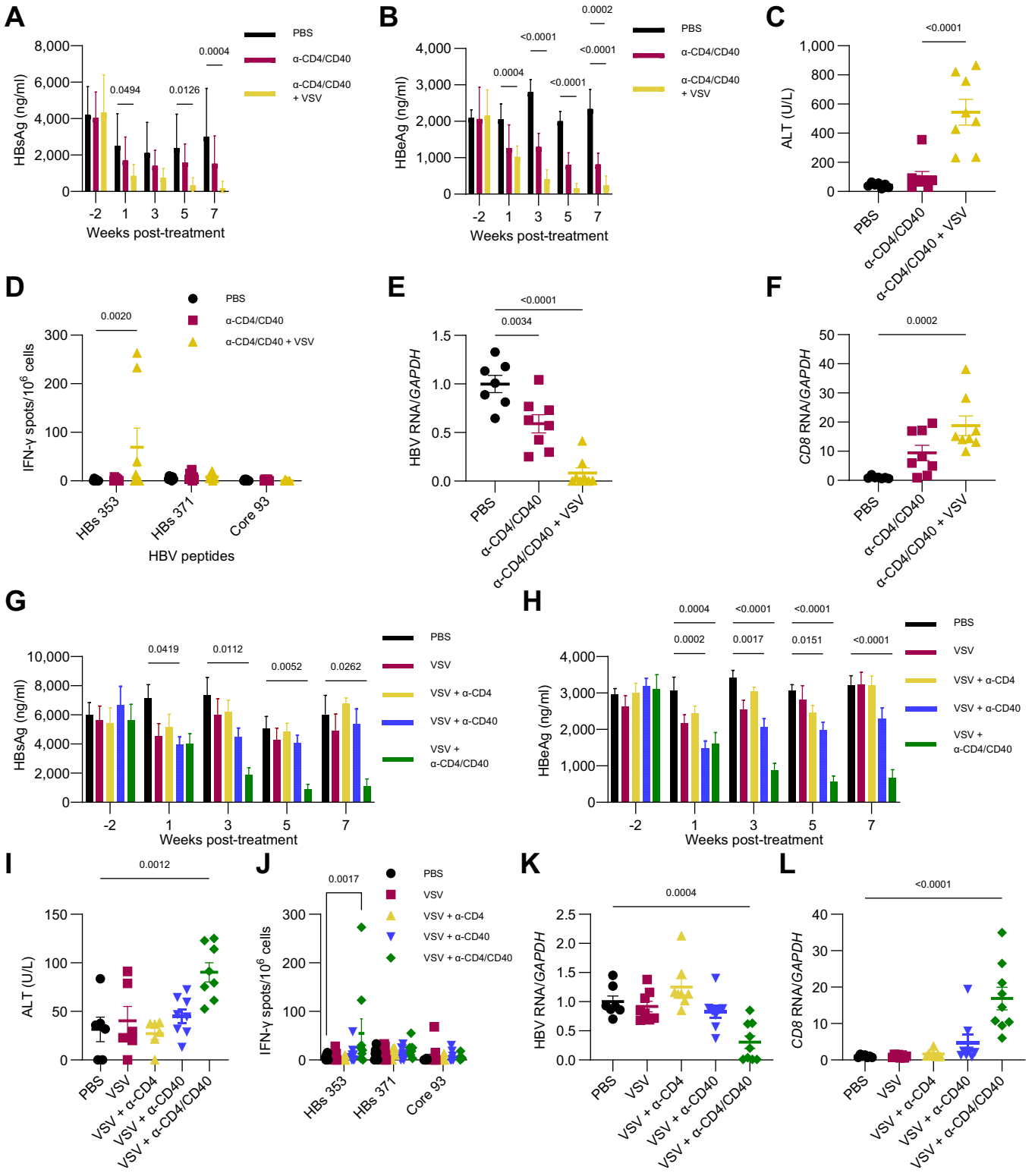

Fig. 5. A combination of CD40 stimulation and VSV-MHBs immunization reverses HBV persistence. (A-F) AAV-HBV mice were treated with PBS, α-CD4/CD40, or α-CD4/CD40 plus VSV-MHBs immunization 8 weeks post-transduction. (G-L) AAV-HBV mice were treated with PBS, VSV-MHBs, α-CD4 + VSV-MHBs, α-CD40 + VSV-MHBs, or α-CD4/CD40 + VSV-MHBs. Serum (A, G) HBsAg and (B, H) HBeAg were measured over time by ELISA. (C, I) Serum ALT was measured at week 3 post-treatment. (D, J) HBV-specific $CD8^+$ T cells were measured from splenocytes at week 7 post-treatment by IFNγ ELISPOT. Results were normalized to unstimulated splenocytes. Liver (E, K) HBV and (F, L) *CD8* RNA expression were measured by RT-qPCR at week 7 post-treatment. Gene expression was normalized to endogenous GAPDH expression. n = 8 mice per group. Statistical significance was determined by one- or two-way ANOVA and Dunnett's multiple comparison test. AAV, adeno-associated virus; VSV, vesicular stomatitis virus; ALT, alanine aminotransferase; RT-qPCR, quantitative reverse-transcription PCR.

therapy would likely be experiencing a chronic presentation of HBV (CHB). A key feature of CHB is immune tolerance, characterized in part by the absence of a functional HBV-specific CD8$^+$ T-cell response. Additionally, most hepatocytes are infected with HBV in CHB, placing the feasibility of immunotherapy by CD8$^+$ T cells under question. To understand how CD40 stimulation affects CHB, mice were transduced with AAV-HBV to generate high levels of HBV antigen production, and on week 8 post-transduction CD4-depletion began followed by CD40 stimulation 1 week later. Because we expected an additional barrier of immune tolerance in high-antigen chronic mice that would not be present during initial HBV establishment (*i.e.*, at the time of transduction), we included an experimental group that received intramuscular immunization with VSV-MHBs in addition to the α-CD4/CD40 regimen. Mice were grouped by HBsAg levels at week 7 post-transduction (week 2 pre-treatment). α-CD4/CD40-treated mice showed a reduction in serum HBsAg (Fig. 5A) and HBeAg (Fig. 5B) at week 1 post-treatment, but antigen decline ceased after week 1. Mice receiving both α-CD4/CD40 and VSV-MHBs exhibited a profound decline in both HBsAg (Fig. 5A) and HBeAg (Fig. 5B) that plateaued at week 5 post-treatment. sALT was significantly elevated in mice receiving α-CD4/CD40 and VSV-MHBs but unchanged in mice receiving α-CD4/CD40 alone (Fig. 5C). Corresponding with elevated sALT was the detection of splenic HBs 353-specific CD8$^+$ T cells only in mice receiving α-CD4/CD40 and VSV-MHBs (Fig. 5D), indicating that α-CD4/CD40 alone is insufficient to induce an HBV-resolving CD8$^+$ T-cell response following prolonged HBV persistence. Lastly, liver gene expression was examined and revealed a 50% reduction in HBV RNA in α-CD4/CD40 mice and a 92% reduction in HBV RNA in mice receiving a combination of α-CD4/CD40 and VSV-MHBs (Fig. 5E). Liver CD8 RNA was also examined and corresponded with HBV gene expression in that α-CD4/CD40 mice experienced a slight and statistically insignificant increase while mice receiving VSV-MHBs immunization additionally experienced a 20-fold increase in CD8 expression (Fig. 5F).

Previous studies utilizing therapeutic immunization indicate that the induction of HBV-controlling CD8$^+$ T-cell responses is ineffective in AAV-HBV-transduced mice expressing high HBV antigen levels.[35] To identify how VSV-MHBs immunization contributes to HBV control, mice transduced with AAV-HBV to generate high HBV antigen levels were treated after 8 weeks with VSV-MHBs alone or combined with α-CD4/CD40 (together or individually). Immunization with VSV-MHBs alone did not affect HBsAg (Fig. 5G) or HBeAg (Fig. 5H). Of the five experimental groups, only VSV + α-CD4/CD40 treatment markedly reduced antigenemia by both HBsAg (Fig. 5G) and HBeAg (Fig. 5H). Consistent with treatment at the time of transduction was the partial reduction in HBeAg following VSV + α-CD40 treatment (Fig. 5H). sALT elevation was restricted to VSV + α-CD4/CD40-treated mice at week 3 post-treatment (Fig. 5I), and this corresponded with the detection of HBV envelope-specific CD8$^+$ T cells which were also restricted to VSV + α-CD4/CD40-treated mice (Fig. 5J). CD8 gene expression in the liver was significantly elevated only in mice receiving VSV + α-CD4/CD40 (Fig. 5L), and HBV gene expression was significantly reduced in the same mice (Fig. 5K). Together, these findings demonstrate that immunization enhances HBV control following CD40 stimulation in CD4-depleted mice with persistent HBV.

Discussion

CD40 activation has previously been shown to induce non-cytolytic, T cell-independent HBV control mainly through the production of IFN-γ and TNF-α by antigen-presenting cells.[19] However, this study was performed using HBV-transgenic mice in which HBV-specific effector cells are limited in number and function. The AAV-HBV model utilized herein is considered less tolerogenic as HBV exposure occurs in adulthood *vs*. prenatally in HBV-transgenic mice. For this reason, the AAV-HBV model is less likely to preclude HBV-specific effector responses including virus-specific CD8$^+$ T cells and antibodies. A subsequent investigation of CD40 activation in HBV revealed that PD-1 exhaustion of adoptively transferred core-specific CD8$^+$ T cells was reversible following FGK4.5 treatment.[20] Effector functions were determined to be restored, suggesting that α-CD40 treatment is effective in reversing PD-1-mediated exhaustion, but *de novo* virus-specific CD8$^+$ T cells were not reported following CD40 stimulation. Our work demonstrates that, in the absence of CD4$^+$ T cells, successive CD40 activation generates HBV-specific CD8$^+$ T cells capable of controlling HBV replication.

T-cell help is considered an essential component of formulating an immune response. Not only are CD4$^+$ T cells indispensable for B cell activation and maturation of the antibody response to HBV,[36] but they are also important for promoting CD8$^+$ T-cell responses. Upon activation, CD4$^+$ T cells differentiate into defined effector subsets (T helper 1, 2, and 17 cells, etc.) with specialized roles. The most described functions of T-cell help in CD8$^+$ T-cell responses include the licensing of antigen-presenting cells for cross-presentation through CD40/CD40L interactions and supporting CD8$^+$ T-cell expansion by producing key cytokines IL-2[37] and IL-21.[38,39] In HBV-infected chimpanzees, CD4-depletion delayed HBV control,[40] suggesting that T-cell help is essential for HBV resolution. Contrastingly, CD4$^+$ Tregs are believed to contribute to HBV persistence, though direct evidence of their contribution is limited. Correlative clinical data suggest that Tregs are more abundant and activated in patients with CHB than healthy controls.[25] Some Treg-associated factors that might promote tolerance include regulatory cytokines IL-10 and TGF-β and the immune checkpoint protein CTLA-4.[41] Our findings support the notion that CD4$^+$ T cells are detrimental to CD8$^+$ T cell-mediated resolution of HBV following CD40 stimulation, though the precise mechanism of restriction remains elusive. While CD4-depletion facilitated CD40-mediated HBV control, combined depletion of IL-10 and TGF-β or Treg depletion failed to control HBV in any capacity when paired with CD40 stimulation. MHCII-blockade also did not recapitulate CD4 depletion, indicating that CD4$^+$ T cells function in a TCR-independent manner to restrict CD40-mediated HBV control. An undefined combination of CD4$^+$ T cell-derived cytokines and immune checkpoints is likely responsible for inhibiting HBV control, and additional research is required to understand how CD4$^+$ cells restrain HBV control in the unique context of CD40 activation.

Cognate CD40 signaling is critical for dendritic cell licensing, promoting cDC1 survival and maturation and eventually CD8$^+$ T-cell priming through cross-presentation.[42] Here, cDC1s were dispensable for CD40-mediated virus control as HBV control was independent of Batf3. However, the downstream pathways that promote APC cross-presentation following CD40 ligation

are incompletely understood. In B cells, CD40-dependent effector functions involve the activity of several signal transduction pathways and transcription factors.[11,43,44] Recently described proteins regulated by CD40 signaling in cDC1 include CD70, 4-1BB ligand, COX-2, and Bcl-xL.[28] Wu *et al.* concluded that CD70, COX-2, and Bcl-xL partially contribute to CD40 help, supporting cDC1 survival and cross-presentation.[28] However, conditional knockdown of these molecules blunted but did not ablate the resulting anti-tumor $CD8^+$ T-cell responses. Thus, CD40 ligation appears to support dendritic cell survival and cross-presentation ability through a combination of CD70, COX-2, and Bcl-xL.

CD40 is currently being targeted as an immunotherapy for persistent viral infections like HIV and as an anti-tumor treatment.[17,45,46] Recent clinical trials of CD40 monoclonal antibodies in cancer have demonstrated moderate anti-tumor responses at best.[47] $CD4^+$ T cells are understood to regulate otherwise productive immune responses in some malignancies like gastrointestinal cancers, particularly by impairing $CD8^+$ T-cell function or reducing the $CD8^+$ TCR repertoire.[48,49] Based on our findings, a plausible solution to improve the efficacy of CD40 activation might be to deplete or transiently block the activation of $CD4^+$ T cells prior to α-CD40 treatment to enhance antigen-specific $CD8^+$ T cell responses. A previous study found that depletion of $CD4^+$ T cells before α-CD40 treatment in the context of immunization induces cytotoxic T lymphocyte responses similar to CD4-replete mice, to which the authors rationalized CD40 activation as a therapeutic for persistent viral infection where $CD4^+$ T cells are absent or nonfunctional.[12] Further studies with CD40 stimulation in which $CD4^+$ T cells are blocked or depleted are required to evaluate this approach.

In interpreting the results of our study, it is crucial to consider a few limitations that may have impacted our findings. First, the use of AAV-HBV transduction is notably restricted due to the absence of a functional receptor for HBV entry in mice, effectively preventing the reinfection of the liver. Second, HBV covalently closed circular DNA does not efficiently form in mice, and an AAV episome serves as the transcriptional template for virus replication in this model.[50] This undoubtedly introduces caution when extrapolating our findings clinically, where the dynamics of HBV infection might differ. Furthermore, although we observed robust HBV protection following CD40 stimulation in CD4-deficient scenarios, these outcomes could depend on the AAV-HBV dose. Nevertheless, these findings reveal a potential immunoregulatory mechanism that may be exploited to develop new therapies for CHB.

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Abbreviations

AAV, adeno-associated virus; APCs, antigen-presenting cells; CHB, chronic hepatitis B; cDC1, type 1 conventional dendritic cells; IFN, interferon; IL, interleukin; MHBs, middle hepatitis B virus surface antigen; sALT, serum alanine aminotransferase; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; Treg, regulatory T cell; VSV, vesicular stomatitis virus.

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Conflict of interest

M.D.R. reports financial relationships with CaroGen Corporation, royalties from a Yale University patent, and prior research funding from Gilead Sciences outside this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Bailey, J.T.: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, and Writing – review and editing. Cangialosi, S: Investigation and Methodology. Moshkani, S: Investigation, Methodology, and Writing – review and editing. Rexhouse, C: Investigation, Methodology, and Writing – review and editing. Cimino, J.L.: Investigation, Methodology, and Writing – review and editing. Robek, M.D.: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, and Writing – review and editing.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT3.5/4 from OpenAI in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Supplementary data

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Author names in bold designate shared co-first authorship

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