Efficacy of an Inhibitor of Hepatitis B Virus Expression in Combination With Entecavir and Interferon-α in Woodchucks Chronically Infected With Woodchuck Hepatitis Virus

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RG7834 is a small-molecule inhibitor of hepatitis B virus (HBV) gene expression that significantly reduces the levels of hepatitis B surface antigen (HBsAg) and HBV DNA in a humanized liver HBV mouse model. In the current study, we evaluated the potency of RG7834 in the woodchuck model of chronic HBV infection, alone and in combination with entecavir (ETV) and/or woodchuck interferon-α (wIFN-α). RG7834 reduced woodchuck hepatitis virus (WHV) surface antigen (WHsAg) by a mean of 2.57 $\log_{10}$ from baseline and WHV DNA by a mean of 1.71 $\log_{10}$. ETV + wIFN-α reduced WHsAg and WHV DNA by means of 2.40 $\log_{10}$ and 6.70 $\log_{10}$, respectively. The combination of RG7834, ETV, and wIFN-α profoundly reduced WHsAg and WHV DNA levels by 5.00 $\log_{10}$ and 7.46 $\log_{10}$, respectively. However, both viral parameters rebounded to baseline after treatment was stopped and no antibody response against WHsAg was observed. Effects on viral RNAs were mainly seen with the triple combination treatment, reducing both pregenomic RNA (pgRNA) and WHsAg RNA, whereas RG7834 mainly reduced WHsAg RNA and ETV mainly affected pgRNA. When WHsAg was reduced by the triple combination, peripheral blood mononuclear cells (PBMCs) proliferated significantly in response to viral antigens, but the cellular response was diminished after WHsAg returned to baseline levels during the off-treatment period. Consistent with this, Pearson correlation revealed a strong negative correlation between WHsAg levels and PBMC proliferation in response to peptides covering the entire WHsAg and WHV nucleocapsid antigen. *Conclusion:* A fast and robust reduction of WHsAg by combination therapy reduced WHV-specific immune dysfunction in the periphery. However, the magnitude and/or duration of the induced cellular response were not sufficient to achieve a sustained antiviral response. (*Hepatology Communications* 2020;4:916-931).

Approximately 257 million individuals worldwide are chronically infected with the hepatitis B virus (HBV), and over 880,000 people die each year due to HBV-associated liver conditions, such as cirrhosis and hepatocellular carcinoma (HCC).[(1)] The goal of any new therapy is to achieve sustained loss of HBV surface antigen (HBsAg) when treatment is discontinued; this is also defined as a "functional cure".[(2)] Current treatment options for chronic HBV infection include nucleos(t)ides

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; cccDNA, covalently closed circular DNA; CD, cluster of differentiation; CHB, chronic hepatitis B; ETV, entecavir; GGT, gamma-glutamyl transferase; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IFN, interferon; ISG, interferon-stimulated gene; LPS, lipopolysaccharide; NK, natural killer; PAPD5/7, poly(A) RNA polymerase-associated domain-containing protein 5/7; PBMC, peripheral blood mononuclear cell; PEG-IFN, pegylated interferon; pgRNA, pregenomic RNA; uPA-SCID, urokinase-type plasminogen activator/severe combined immunodeficiency; WHcAg, woodchuck hepatitis virus nucleocapsid antigen; WHsAg, woodchuck hepatitis virus surface antigen; WHV, woodchuck hepatitis virus; wIFN-α, woodchuck interferon-alpha.

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(e.g., entecavir [ETV]) and interferon (IFN) (e.g., pegylated IFN [PEG-IFN]), but both have a very low cure rate.[2] The cure rate is higher for patients who undergo treatment with a combination of nucleos(t)ide and PEG-IFN, although it is still limited to less than 10% of patients.[2,3] Therefore, novel therapies are needed that can be incorporated into new therapeutic strategies with finite treatment duration to increase the HBV cure rate.

In chronic HBV infection, continuous exposure to viral proteins, such as HBsAg in the periphery and liver, is thought to contribute to the exhaustion of antiviral cluster of differentiation (CD)8+ T cells.[4,5] Furthermore, several lines of evidence suggest that viral proteins influence virus-specific immunity by directly modulating immune cells in both the innate and adaptive arms of the immune system.[6-8] These studies are further supported by observations demonstrating that HBV interferes with innate antiviral immune responses in patients with chronic HBV infection.[9] Therefore, future HBV cure strategies may need to include therapeutic agents that reduce or eliminate viral antigens, such as HBsAg, to restore antiviral immunity and control HBV infection. Although the current potent nucleos(t)ide replication inhibitors are expected to remain the backbone of future therapy, this class of inhibitors does not reduce the HBsAg levels sufficiently.

Effective treatment of viral diseases involves the combination of multiple therapeutic strategies targeting various key steps in the viral replication cycle.[10] These combination strategies have proven to be more efficient and effective than monotherapy for treatment of chronic viral diseases, such as infections with human immunodeficiency virus and hepatitis C virus. Similarly, an effective HBV cure may involve a combination of antiviral drugs and immunomodulators to further improve antiviral immunity and control viral infection.[11,12]

We recently reported a novel, orally available, small-molecule HBV expression inhibitor, RG7834, that significantly reduces HBV DNA and HBsAg levels in both *in vitro* and *in vivo* models of chronic HBV infection.[13,14] Another group has also described a structurally similar molecule that reduces HBV expression levels.[15] RG7834 was shown to reduce viral messenger RNA and to accelerate RNA degradation by targeting the host proteins noncanonical poly(A) RNA polymerase-associated domain-containing protein 5 and 7 (PAPD5 and PAPD7).[16]

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Both PAPD5 and PAPD7 are essential host components that are required for HBV RNA stabilization.

Infection of woodchucks with woodchuck hepatitis virus (WHV) is a well-established immunocompetent model of chronic HBV infection.[17] The woodchuck PAPD5 and PAPD7 amino acid sequences are highly similar to those of their human counterparts, suggesting that RG7834 may also be active against WHV.[16] Previous studies in this model have shown that monotherapy or combination therapies that include immunomodulators improve WHV-specific B-cell and T-cell responses, leading to sustained control of WHV infection.[18-20] Furthermore, treatment with woodchuck IFN-α (wIFN-α) reduces viral markers in the serum and liver, with viral rebound typically observed following cessation of treatment.[21] In the current study, we investigated antiviral and immunomodulatory therapeutic strategies with the potential to cure chronic WHV infection in woodchucks. We evaluated the potency of RG7834 alone and in combination with the nucleoside inhibitor ETV and the immunomodulator wIFN-α in woodchucks chronically infected with WHV. RG7834 reduced viral DNA and WHV surface antigen (WHsAg) to a degree similar to that observed for HBV in the urokinase-type plasminogen activator/severe combined immunodeficiency (uPA-SCID) humanized mouse model.[13] Combination treatments reduced WHV DNA and especially WHsAg levels more than RG7834 or ETV alone. The reduction in WHsAg levels correlated with elevated WHV T-cell-specific proliferation in the periphery, although a sustained antiviral response was not achieved.

Materials and Methods

INVESTIGATIONAL DRUGS

RG7834 and ETV were manufactured by F. Hoffmann-La Roche, Ltd., and provided as a powder. The drugs were dissolved in ultrapure water, mixed with woodchuck diet (Dyets, Inc., Bethlehem, PA), and orally administered to woodchucks within 30 minutes after preparation. wIFN-α was manufactured by F. Hoffmann-La Roche, Ltd., and provided as a frozen aqueous solution; this is the same protein formulation used in a previous study.[21] The wIFN-α was thawed on ice and brought to room temperature before subcutaneous administration to woodchucks.

DRUG TREATMENT

The animal protocol and all procedures involving woodchucks were approved by the Institutional Animal Care and Use Committee of Georgetown University. Woodchucks received humane care as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Woodchucks enrolled in the study were born in captivity at the animal facilities of Northeastern Wildlife, Inc. (Harrison, ID) and experimentally infected at 3 days of age with WHV. Following transfer to the animal facilities of Georgetown University, adult woodchucks with chronic WHV infection were confirmed positive for serum WHV DNA and WHsAg and negative for antibodies to WHsAg. Most animals had low levels of gamma-glutamyl transferase (GGT), an established oncofetal marker of HCC in woodchucks,[22] and the absence of liver tumors was confirmed by ultrasonography. Woodchucks were assigned to six treatment groups (n = 4-5/group; Fig. 1), stratified by sex, body weight, pretreatment serum WHV DNA and WHsAg loads, and serum GGT activity. Woodchucks of the monotherapy and combination therapy groups were treated for 14 weeks with ETV (0.1 mg/kg orally, once daily), RG7834 (10 mg/kg orally, twice daily), and/or wIFN-α (0.1 mg/animal subcutaneously, initially 3 times per week and then twice per week after a 16-day dose holiday due to the development of IFN-related adverse events). Woodchucks were followed until the end of the study at week 24. Due to limited availability of woodchucks with established chronic WHV infection, we were unable to include groups treated with a vehicle control or wIFN-α alone.

DRUG SAFETY AND MORTALITY

Clinical observations were made daily, and measurements for body weight and body temperature were obtained weekly. Clinical chemistry and hematology parameters were determined at regular intervals in serum and whole-blood samples, respectively, at the Animal Health Diagnostic Center of Cornell University (Ithaca, NY). No mortality associated with ETV, RG7834, and/or wIFN-α treatment was observed. Three animals died during the treatment period: Woodchuck M4033 (ETV group) was found

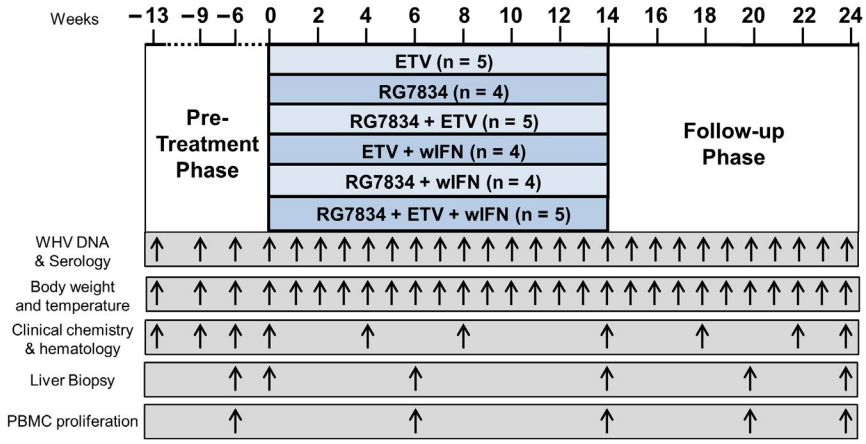

FIG. 1. Woodchuck study design. Woodchucks chronically infected with WHV were dosed with RG7834 (10 mg/kg orally, twice per day) either alone or in combination with ETV (0.1 mg/kg orally, once daily) and/or wIFN (0.1 mg subcutaneous administration, 3 times/week [weeks 0 to 4], no treatment [weeks 4 to 6], and twice weekly [weeks 6 to 14]) for 14 weeks and followed up for another 10 weeks. The approximately 2-week wIFN treatment interruption was due to observed IFN-related adverse events that dissipated by switching to twice weekly dosing. Arrows indicate the time of measurements for specific parameters listed.

dead during week 8, and death was attributed to end-stage HCC; woodchuck F4043 (ETV + wIFN group) experienced severe complications following the anesthesia procedure in week 2 and died shortly thereafter; woodchuck F4052 (RG7834 + ETV + wIFN group) died due to uncontrollable internal hemorrhage after the liver biopsy procedure in week 14. In addition, woodchucks F4029 and M4019 were euthanized during weeks 20 and 23, respectively, due to the development of liver tumors/HCC.

VIRAL AND HOST BIOMARKERS

Please refer to supplementary materials and methods for details of methods for determining serum WHV DNA and WHsAg and liver WHV RNA, DNA and cccDNA. Induction of IFN-stimulated genes (ISGs) in PBMCs was determined by using RT-PCR as described in supplementary materials and methods.

Results

STUDY DESIGN

The antiviral efficacy of RG7834 either alone or in combination with wIFN-α and/or ETV was evaluated in a repeat-dose study in adult woodchucks with established chronic WHV infection (Fig. 1).

Twenty-seven WHV-infected woodchucks were stratified and assigned to treatment with ETV (n = 5), RG7834 (n = 4), RG7834 + ETV (n = 5), ETV + wIFN-α (n = 4), RG7834 + wIFN-α (n = 4), or RG7834 + ETV + wIFN-α (n = 5) (Fig. 1). The oral dosage of RG7834, 10 mg/kg twice daily, was selected to match the exposure used in previous preclinical studies performed in the HBV-infected uPA-SCID mouse model,[13] and pharmacokinetic sampling was performed throughout the study to confirm that the exposure was similar to or higher than that in the uPA-SCID mice (data not shown). The dosages of wIFN-α and ETV were initially selected as subcutaneous injections of 0.1 mg/animal wIFN-α 3 times weekly and oral ETV 0.1 mg/kg daily, based on reported studies.[20,21] In line with a previous study,[21] wIFN-α induced the expression of IFN-stimulated genes (ISGs) in peripheral blood mononuclear cells (PBMCs) isolated from wIFN-α-treated woodchucks after the first dose, and the expression was similar in all woodchucks treated with wIFN-α (Fig. 2) but not present in animals from the other treatment groups. However, due to observed IFN-related adverse events, including anemia, neutropenia, and thrombocytopenia, wIFN-α treatment was interrupted for approximately 2 weeks, followed by a reduction of the wIFN-α dosing frequency from 3 times weekly to twice weekly for the remainder of the dosing period. Dosing of both RG7834 and ETV was uninterrupted throughout the 14-week dosing period.

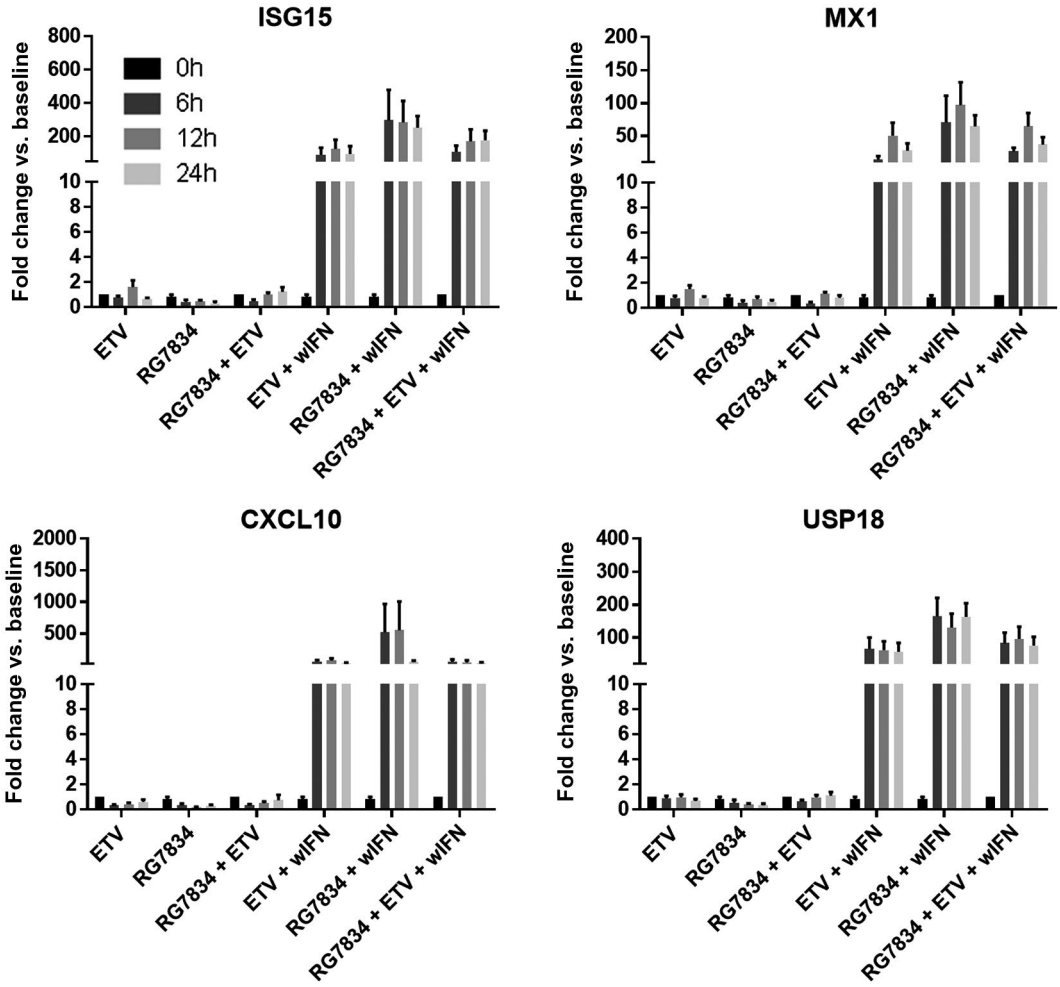

FIG. 2. Induction of PBMC ISG expression by RG7834 alone and in combination therapy. Kinetics of ISG (ISG15, MX1, CXCL10, and USP18) gene expressions were measured by reverse-transcription polymerase chain reaction prior to (0 hours) and 6, 12, and 24 hours postsubcutaneous injection of the first wIFN dose. Fold-change values were calculated relative to baseline (0 hours). Data show mean + SEM. Abbreviations: CXCL10, chemokine (C-X-C motif) ligand 10; h, hour; MX1, MX dynamin-like guanosine triphosphatase 1; USP18, ubiquitin specific peptidase 18.

RG7834 ALONE AND IN COMBINATION WITH wIFN-α AND/OR ETV SIGNIFICANTLY REDUCES SERUM WHV DNA AND WHsAg LEVELS

RG7834 reduced serum viral DNA and WHsAg by a mean of 1.71 $\log_{10}$ and 2.57 $\log_{10}$, respectively, from baseline to the end of treatment at week 14 (Table 1). In comparison, ETV reduced WHV DNA and WHsAg levels by 6.63 $\log_{10}$ and 2.23 $\log_{10}$, respectively. The combination of RG7834 and ETV reduced WHsAg levels by a mean of 3.42 $\log_{10}$, a more profound decrease than seen with either drug alone, whereas the DNA level was reduced by 6.62 $\log_{10}$, similar to that with ETV alone. The addition of wIFN-α to either ETV or RG7834 did not significantly enhance the antiviral potency compared with RG7834 or ETV alone. However, the triple combination of RG7834, ETV, and wIFN-α profoundly reduced WHV DNA and WHsAg levels by 7.46 $\log_{10}$ and 5.00 $\log_{10}$, respectively.

The kinetics of the antiviral responses were notably different for the RG7834 treatment groups than for the groups treated without RG7834 (Figs. 3 and 4). Time to reach a reduction of WHV DNA

TABLE 1. ANTIVIRAL ACTIVITY AND EFFECT ON ALBUMIN LEVELS OF RG7834 ALONE AND IN COMBINATION WITH ETV AND/OR wIFN IN WOODCHUCKS CHRONICALLY INFECTED WITH WHV

Group	WHV DNA	WHsAg	Albumin
ETV	–6.63 ± 0.25*	–2.23 ± 0.51*	–0.51 ± 0.13†
RG7834	–1.71 ± 0.25*	–2.57 ± 0.51*	–0.58 ± 0.14†
RG7834 + ETV	–6.62 ± 0.22*	–3.42 ± 0.45*	–0.38 ± 0.12†
ETV + wIFN	–6.70 ± 0.29*	–2.40 ± 0.59*	–1.17 ± 0.15*
RG7834 + wIFN	–2.32 ± 0.25*	–2.65 ± 0.51*	–0.80 ± 0.14 *
RG7834 + ETV + wIFN	–7.46 ± 0.22*	–5.00 ± 0.45*	–0.68 ± 0.12*

Mean $\log_{10}$ reduction values ± SEM from all animals during treatment period (week 14 vs. week 0) are shown. *P* values were adjusted for multiple testing for each parameter separately, using Holm's method.
*$P < 0.001$; †$P < 0.01$.

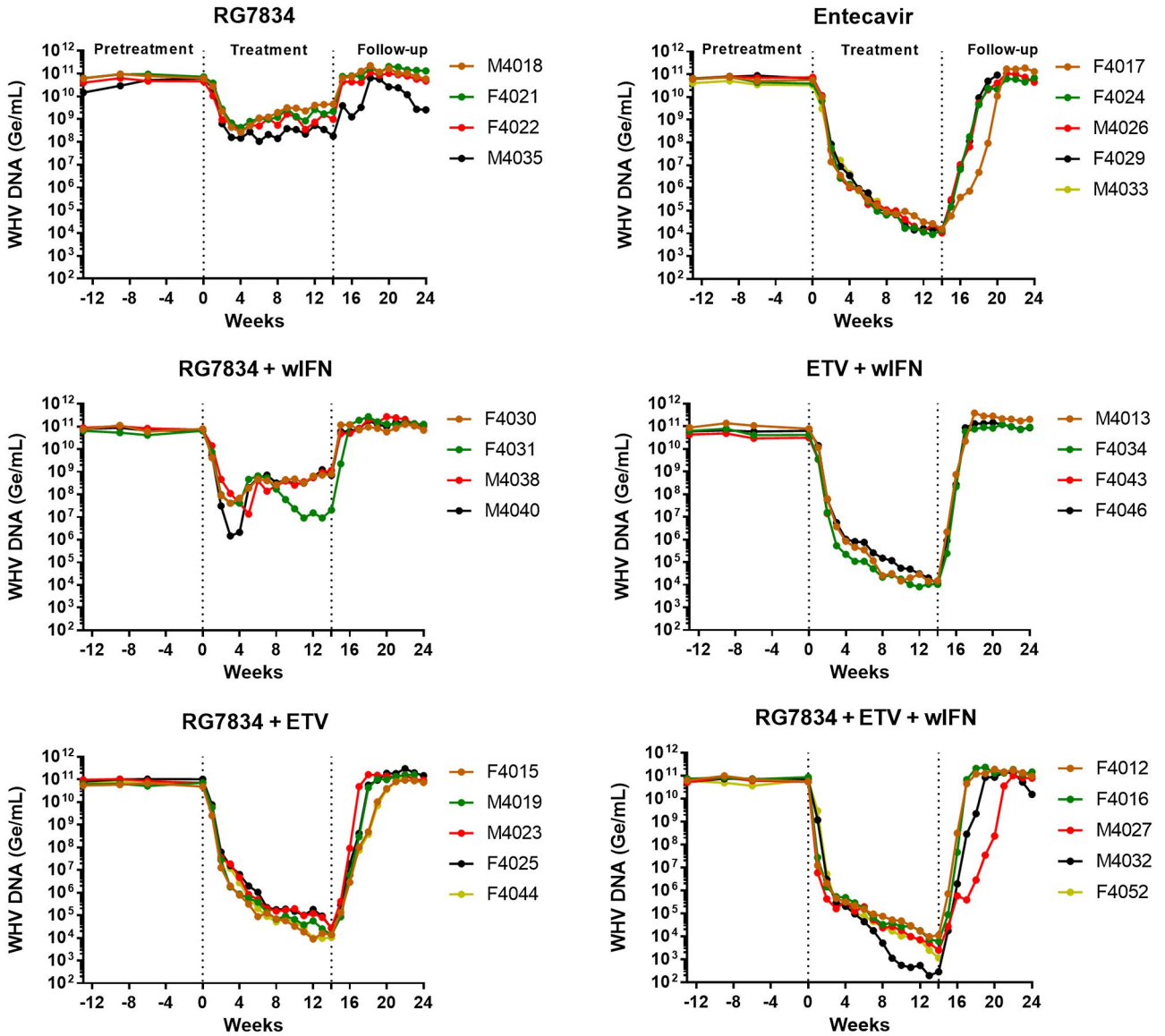

FIG. 3. Effect of RG7834 and ETV alone and in combination therapy on serum WHV DNA levels. Kinetics of WHV DNA load from individual woodchucks was measured by dot blot hybridization or quantitative polymerase chain reaction at the indicated time points. Abbreviation: Ge, genome equivalents.

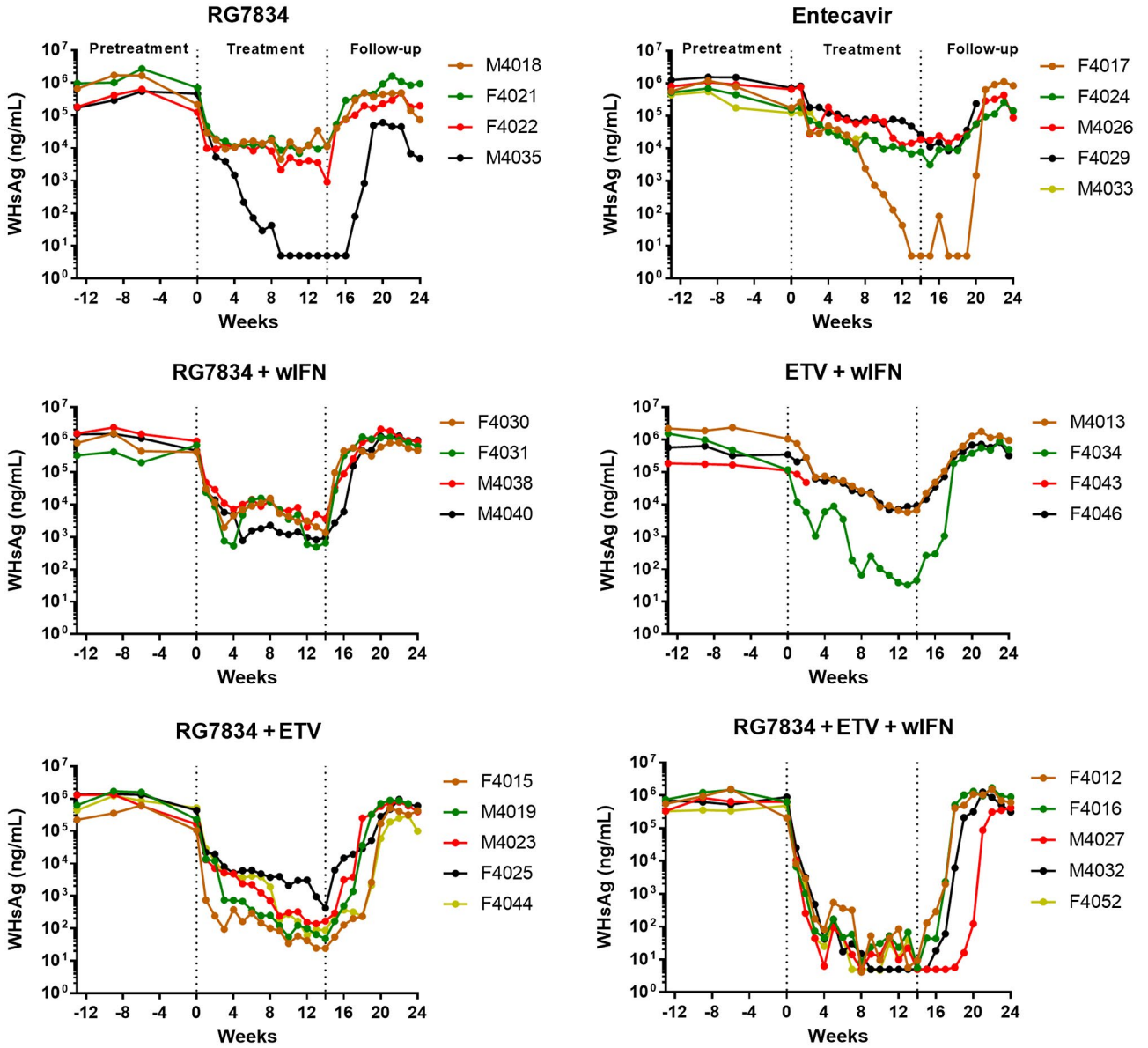

FIG. 4. Effect of RG7834 and ETV alone and in combination therapy on serum WHsAg levels. Kinetics of WHsAg load from individual woodchucks was measured at the indicated time points by enzyme-linked immunosorbent assay.

and WHsAg by a mean 1.5 $\log_{10}$ from baseline is shown in Supporting Fig. S1. RG7834 reduced WHsAg from baseline in a mean of 20 days after treatment compared with a mean of 76 days for the ETV group. In contrast, ETV reduced WHV DNA more quickly than RG7834 (means of 6 and 11 days, respectively). The kinetics of the initial antiviral responses for ETV + wIFN-α were similar to those of ETV for both WHV DNA and WHsAg. However, the responses were fastest for the triple combination (means of 3 and 6 days for WHV DNA and WHsAg, respectively).

To monitor the durability of the antiviral response, we measured viral parameters for another 10 weeks following the cessation of treatment. Both WHV DNA and WHsAg rebounded in all treatment groups, and the relapse was comparable among the treatment regimens (Figs. 3 and 4). Consistent with this observation, we did not detect anti-WHsAg antibodies throughout the treatment and follow-up periods

(data not shown). In line with the results of a previous study in the uPA-SCID mouse model, the antiviral activity of RG7834 specifically reduced WHsAg more than ETV (Fig. 4) and levels of serum albumin were not significantly modulated when compared to ETV (Supporting Fig. S2).[13] In addition, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and sorbitol dehydrogenase (SDH) levels were not significantly modulated in any group during the treatment period (Supporting Figs. S3-S5).

COMBINATION THERAPY TRANSIENTLY REDUCES INTRAHEPATIC VIRAL DNA, RNA, AND COVALENTLY CLOSED CIRCULAR DNA LEVELS

To monitor changes of intrahepatic viral markers, we performed sequential biopsies at baseline (weeks –6 and 0), on treatment (weeks 6 and 14), and during the off-treatment follow-up (weeks 20 and 24). There were no significant changes to intrahepatic viral DNA levels in the groups treated with RG7834 or RG7834 + wIFN-α (Fig. 5). In contrast, all groups treated with ETV, whether alone or in combination, had significantly reduced viral intracellular DNA on week 14.

Although total intracellular viral RNAs were reduced from baseline in the group treated with RG7834 alone, these changes were not statistically significant. Furthermore, intrahepatic WHV pregenomic RNA (pgRNA) and WHsAg RNA molecules were separately analyzed for individual animals (Supporting Figs. S2 and S3). Animals treated with RG7834 alone showed reduction in WHsAg RNA levels during treatment without impacting pgRNA levels. ETV also did not induce significant changes to total intracellular viral RNA levels, but the reduction levels were more profound for pgRNA compared to WHsAg RNA. However, the groups treated with drug combinations tended to have reduced viral RNA levels, and the triple combination group exhibited a significant reduction in total viral RNAs, including both pgRNA and WHsAg RNA.

Next, we measured intrahepatic covalently closed circular DNA (cccDNA) levels (Fig. 6). RG7834 alone or in combination with wIFN-α had no impact on the hepatic WHV cccDNA pool. In contrast, ETV either alone and more so in combination with RG7834 and/or wIFN-α reduced the cccDNA pool significantly by the end of the treatment period. Consistent with its effects on the other parameters tested, the triple combination treatment had the strongest effect on cccDNA levels during the treatment period. Thus, the triple combination treatment significantly reduced intrahepatic viral DNA, RNA, and cccDNA levels. However, these changes were transient in nature, and all parameters returned to baseline levels following the cessation of treatment.

THE COMBINATION OF RG7834, ETV, AND wIFN-α TRANSIENTLY IMPROVES VIRUS-SPECIFIC CELLULAR IMMUNE RESPONSES

To evaluate WHV-specific T-cell responses, we used a modified PBMC proliferation assay, as described.[23] We investigated the response of woodchuck PBMCs to peptides covering the entire WHV nucleocapsid antigen (WHcAg) or WHsAg. RG7834 or ETV alone had no significant impact on PBMC proliferation. However, we observed a trend toward increased PBMC proliferation and decreased viral WHsAg in the combination groups by week 14 (Fig. 7A). Although the changes were not statistically significant for most of the groups, PBMC proliferation significantly increased in the triple combination group. Interestingly, the increase in PBMC proliferation was inversely correlated with WHsAg levels, and the levels of proliferation returned to baseline in all of the combination groups after the cessation of treatment. As a no-peptide control, we showed that PBMCs stimulated with lipopolysaccharide (LPS) instead of WHV-specific peptides were not significantly affected by any treatment in our study (Supporting Fig. S8). Altogether, the triple combination group had a higher degree of WHsAg reduction than the other groups and a pronounced increase in PBMC proliferation in response to stimulation with either viral antigen.

WHsAg LEVELS INVERSELY CORRELATE WITH WHV ANTIGEN-SPECIFIC PROLIFERATION OF PBMCs

To investigate whether any variables in our study were correlated, we used pairwise Pearson correlation analyses on variable levels in individual animals

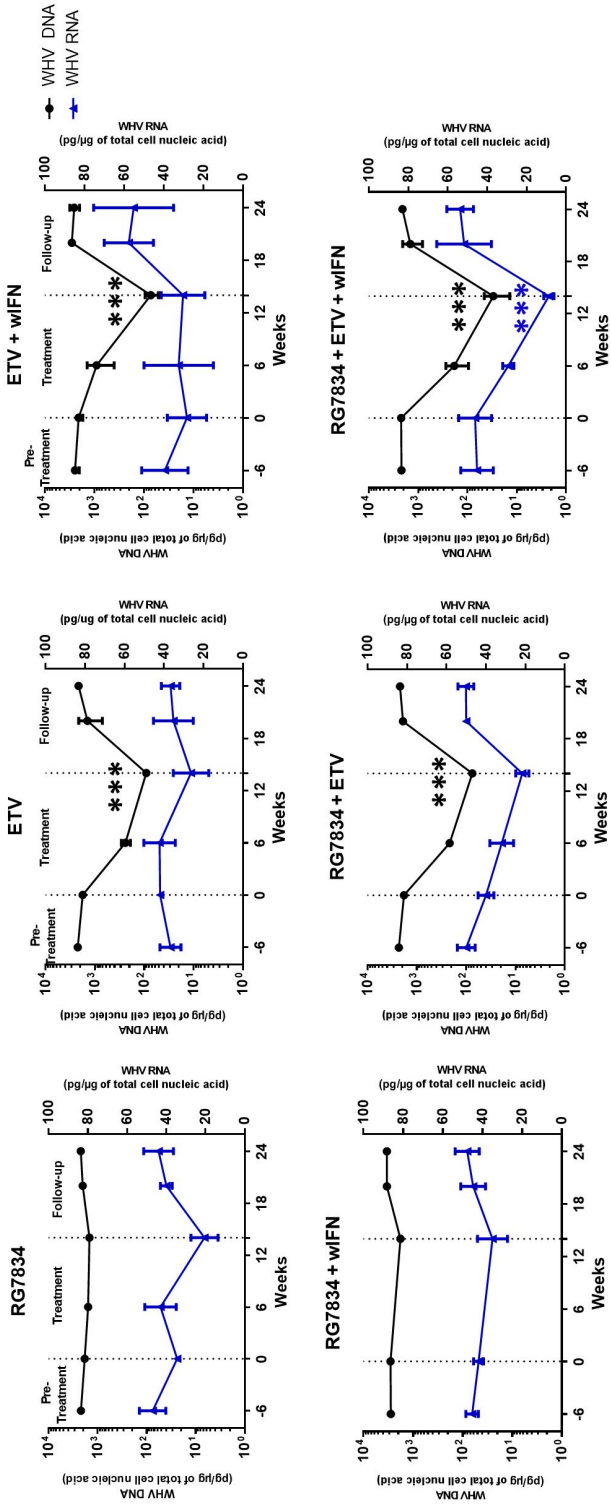

FIG. 5. Effect of RG7834 and ETV alone and in combination therapy on intrahepatic WHV DNA and WHV RNA levels. Changes in mean WHV DNA load (black line, left axis) and mean WHV RNA levels (blue line, right axis) were measured by Southern and northern hybridization, respectively, and are shown for the various treatment groups. Data show single data points or mean ± SEM; ***$P < 0.001$ compared to week 0.

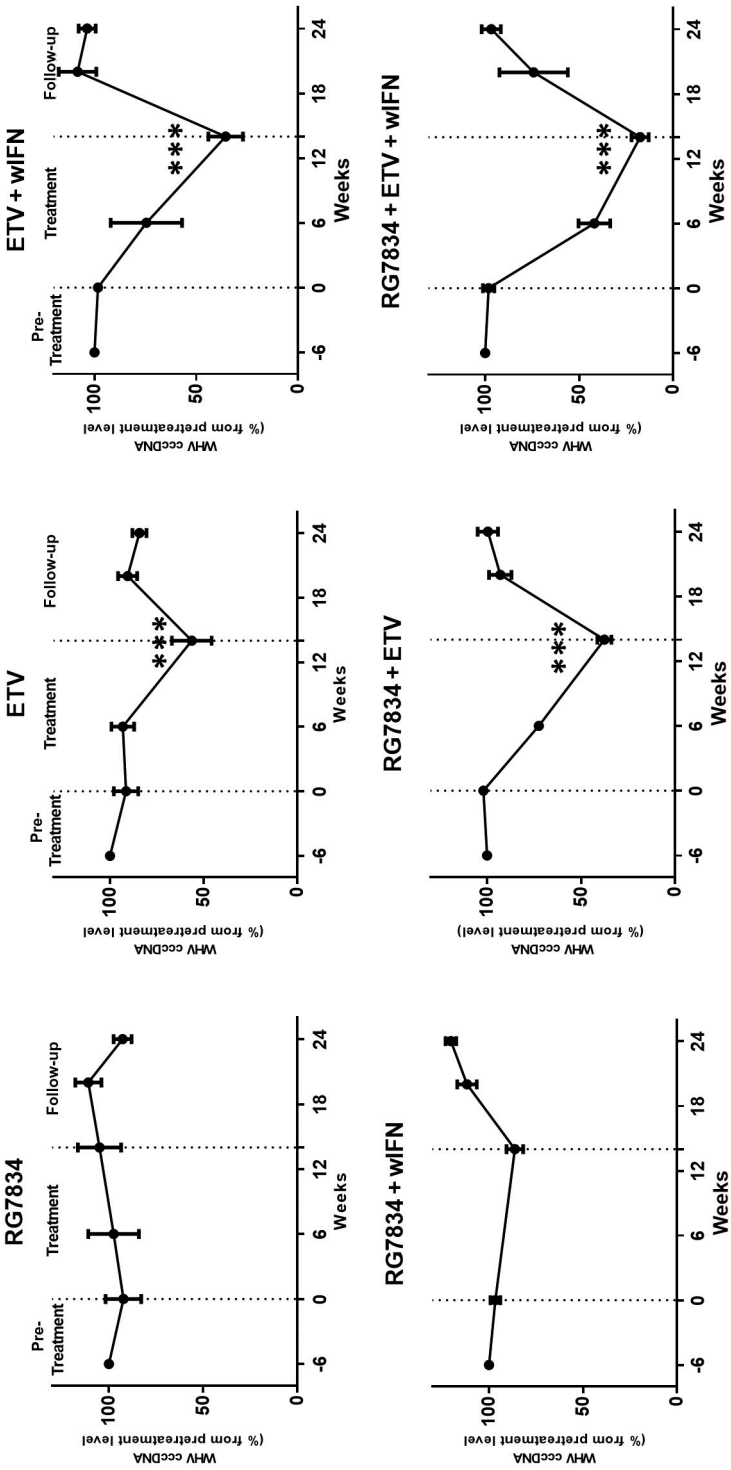

FIG. 6. Effect of RG7834 and ETV alone and in combination therapy on intrahepatic WHV cccDNA levels. Percentage changes in mean intrahepatic WHV cccDNA levels were measured by Southern hybridization and are shown for the various treatment groups. Data show single data points or mean ± SEM; *** $P < 0.001$ compared to week 0.

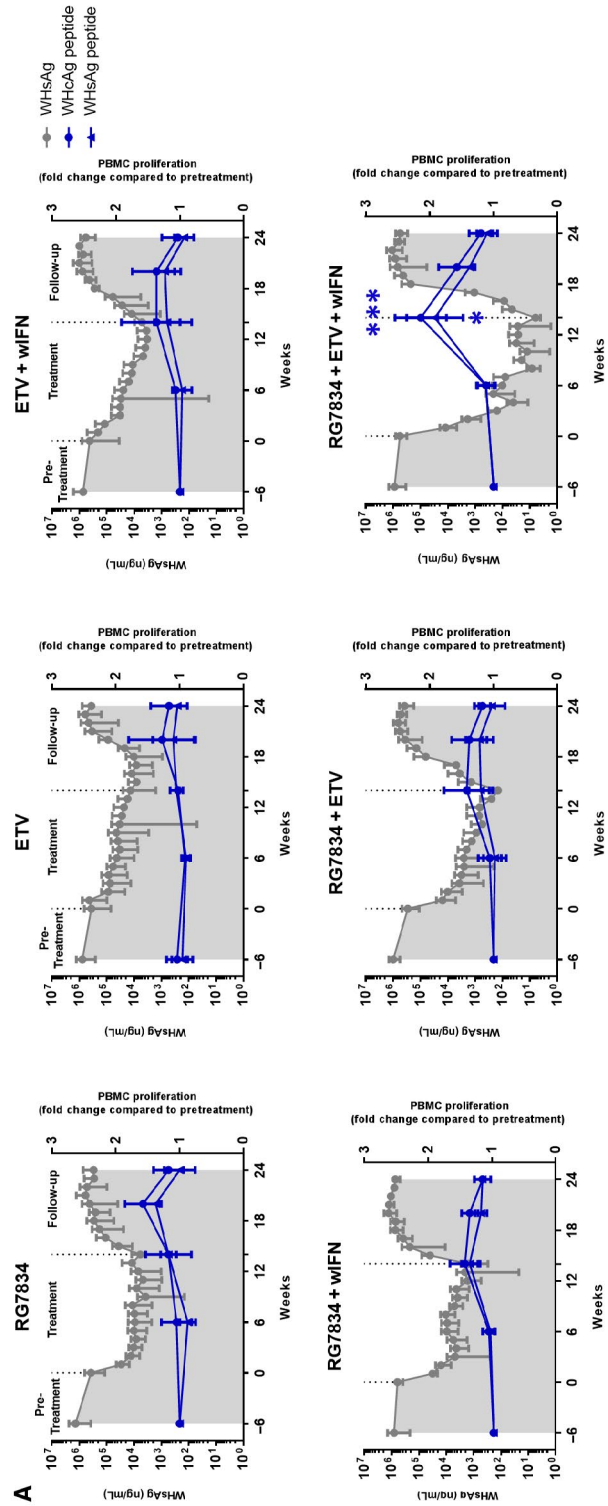

FIG. 7. Effects of treatment and correlation of viral and host parameters. (A) Effect of RG7834 and ETV alone and in combination therapy on the kinetics of serum WHsAg levels and viral antigen-specific proliferation of PBMCs. Changes in mean serum WHsAg levels (gray line, left axis) are shown for the various treatment groups. PBMCs were stimulated with WHsAg- or WHcAg-derived peptides, and proliferation was measured by the amount of adenosine triphosphate present in cells (blue lines, right axis). Mean fold-change values compared to medium control stimulation are shown. Data show mean ± SD. $^{*}P < 0.001$ compared to week –6. $^{***}P < 0.001$ for both WHsAg peptide and WHcAg peptide. (B) Correlation of viral and host parameters measured in all treated woodchucks. Pairwise Pearson correlations were calculated from pooled time points from all treated animals in the study. Positive correlations are marked green and negative ones red (range from –1 to +1). PBMCs were stimulated with WHsAg- and WHcAg-derived peptides and with LPS as a no-peptide control. (C) Correlation of WHsAg decline and PBMC proliferation in all treated woodchucks. WHsAg log reduction values and PBMC proliferation changes after stimulation with WHsAg- and WHcAg-derived peptides (left and middle panels, respectively) and with LPS as a no-peptide control (right panel) were determined based on values from week 14 and week –6. Pearson correlations were calculated, and values are indicated in the figure.

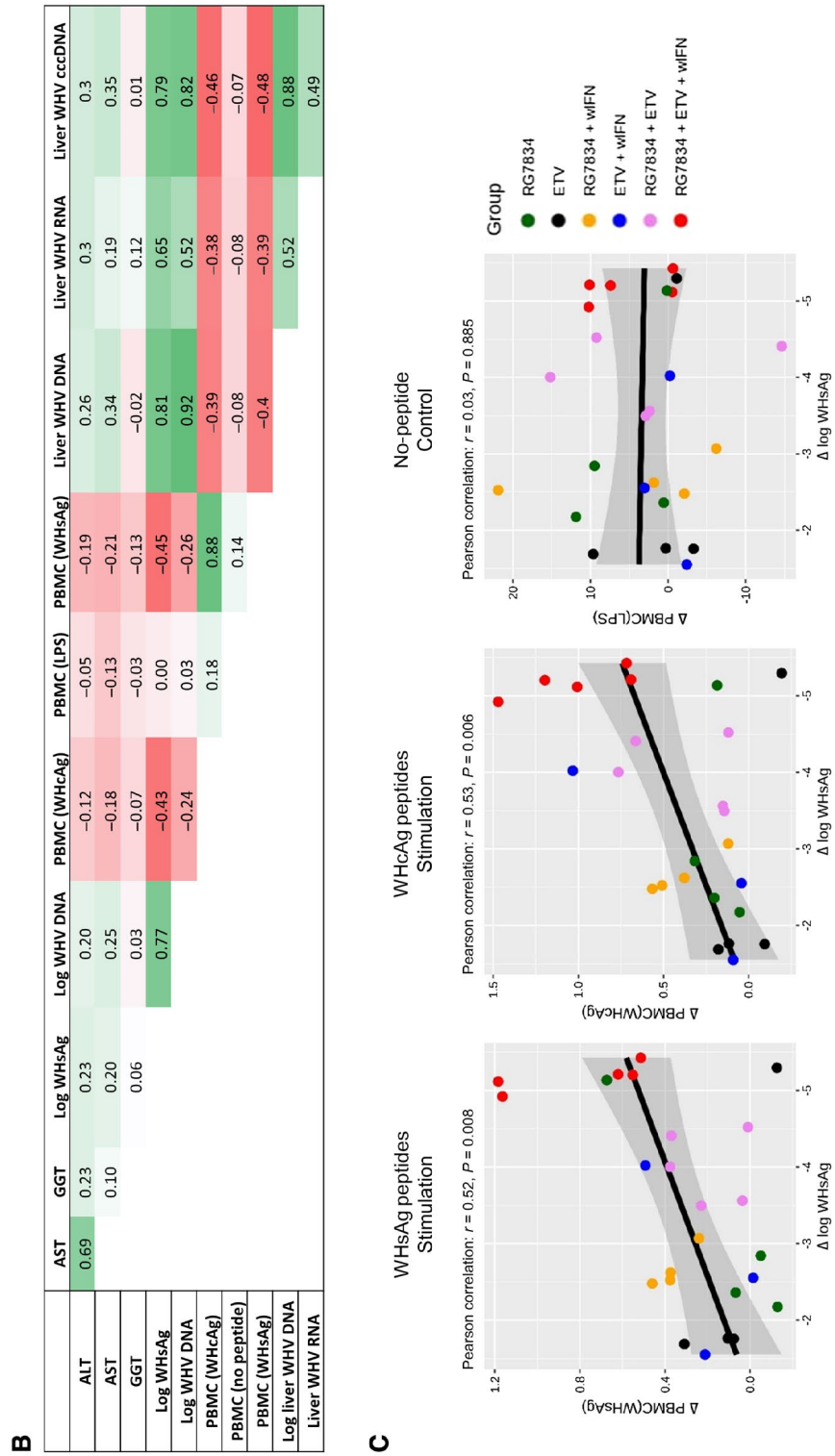

FIG. 7. (Continued).

from each group (Fig. 7B). Our analysis suggested a weak negative correlation for WHV DNA levels and PBMC proliferation in response to stimulation with WHsAg- (–0.26) and WHcAg-derived peptides (–0.24). In contrast, we observed a strong negative correlation for WHsAg levels and PBMC proliferation in response to WHsAg (–0.45) and WHcAg-derived peptides (–0.43). As expected, PBMCs stimulated with LPS instead of WHV-specific peptides did not show a significant correlation with WHsAg or WHV DNA levels (0.0 and 0.03, respectively). Furthermore, although PBMC proliferation after stimulation with WHsAg-derived peptides was positively correlated with that after stimulation with WHcAg-derived peptides (0.88), there were no significant associations between unstimulated PBMC and WHsAg- or WHcAg-stimulated PBMC proliferation (0.14 and 0.18, respectively). Furthermore, this correlation was also shown at the individual animal level (Fig. 7C). Taking these findings together, our analysis suggests a strong negative correlation between WHsAg levels and virus-specific cellular immunity in chronically infected woodchucks.

Discussion

In our study, combining RG7834 with both wIFN-α and ETV enhanced its efficacy in chronically WHV-infected woodchucks. We showed a faster response and more profound suppression of viral parameters in serum and liver with the triple combination regimen than with any other regimen. However, despite marked suppression of WHsAg and WHV DNA (5.00 $\log_{10}$ and 7.46 $\log_{10}$, respectively) for 14 weeks, both viral parameters returned to baseline after the cessation of treatment. Thus, there was no functional cure induced in this study. Interestingly, we found that reduced WHsAg levels resulted in enhanced virus-specific T-cell responses. However, the magnitudes and/or durations of the induced cellular responses were not sufficient to control the WHV infection.

ETV therapy has shown histologic, virologic, and biochemical benefit in patients with chronic hepatitis B (CHB).[24] ETV is a guanosine nucleoside analogue that potently inhibits HBV DNA polymerase, leading to suppression of HBV replication.[25] However, ETV does not significantly modulate HBV proteins or cccDNA levels in uPA-SCID mice at the selected dose.[13] By contrast, ETV has been shown in multiple studies to reduce both WHV DNA and WHsAg levels in WHV-infected woodchucks when using a relatively high dose.[20,26] Other nucleoside analogues, including telbivudine (a thymidine nucleoside analogue) and clevudine (a fluorinated l-arabinofuranosyl nucleoside analogue), also reduce WHsAg levels in WHV-infected woodchucks, especially at high doses and/or during prolonged treatment.[27] In line with these studies, ETV also reduced WHV DNA and WHsAg as well as intrahepatic viral DNA and cccDNA levels in our study. These observations may signify differences in antiviral effects among replication cycles of hepadnaviruses. For example, it is possible that the WHV cccDNA pool in woodchuck hepatocytes is more sensitive to ETV treatment than HBV cccDNA is in human hepatocytes in the uPA-SCID mouse model because it may require more replenishment by viral replication to support persistent infection. In this scenario, potent inhibitors of viral polymerase, such as ETV in our study, would prevent adequate replenishment of the WHV cccDNA pool, leading to a significant reduction of cccDNA and other viral markers over time.

IFN-α is a pleiotropic cytokine that has both direct antiviral and immunomodulatory properties.[28] IFN-α has been used to treat patients with HBV, but it generates a sustained response in only a minority of patients. Previous studies in chronically WHV-infected woodchucks showed that wIFN-α reduces both WHV DNA and WHsAg, and the degree of antiviral response shows similarities to the responses observed in patients with CHB treated with PEG-IFN.[3,21] Several studies have indicated that IFN-α induces a direct antiviral response to HBV.[29-34] Consistent with this, we and others have shown direct antiviral activity of IFN-α in HBV-infected uPA-SCID mice, which lack immune cells.[13,35] In the current study, wIFN-α induced the expression of ISGs in PBMCs (Fig. 2), many of which may also have antiviral effector functions.[28] wIFN-α treatment further complemented the antiviral activity of ETV and RG7834 to produce stronger antiviral responses. Similarly, combination treatment with RG7834, ETV, and PEG-IFN in HBV-infected uPA-SCID mice produced a larger reduction in HBV DNA and HBsAg than RG7834 with or without ETV.[13] Thus, it is possible that most if not all wIFN-α responses

in our study are due to the direct antiviral activity of IFN-α. Although IFN-α has been reported to activate the functional responses of immune cells from both the innate and adaptive arms of the immune system, such as natural killer (NK) cells and CD8+ T cells,[36] other studies have demonstrated that IFN-α treatment improves the number and function of NK cells but does not improve the functional responses of HBV-specific CD8+ T cells.[37,38] Furthermore, CD8+ T cells in chronic HBV infection were shown to upregulate a death receptor that makes them susceptible to NK cell-mediated deletion.[39] Taken together, these findings make it unlikely that the improved cellular antigen-specific responses in the triple combination group of our study were due to the immunomodulatory effect of wIFN-α.

Multiple lines of evidence suggest that dominant viral antigens may interfere with viral-specific immune responses in CHB. Continuous exposure of T cells to excessive viral antigens has been suggested to contribute to anti-viral CD8+ T-cell exhaustion in chronic viral infection, and the degree of T-cell dysfunction is related to the level of viral replication.[40,41] Furthermore, effective intrahepatic CD8+ T-cell immune responses are only generated in mouse if the cells are exposed to hepatocytes expressing low levels of antigen.[5] In line with these concepts, a profound reduction of WHsAg levels in our study resulted in significant improvement of WHcAg- and WHsAg-specific T-cell responses. Further supporting this relationship, we also showed an inverse relationship between WHsAg levels and T-cell responses from all the animals in our study, suggesting that a reduction in WHsAg improved the T-cell response, as noted in the triple combination group (Fig. 7B). Nonetheless, the strength and/or duration of the T-cell responses were not sufficient to durably control WHV infection after cessation of treatment. Notably, we did not observe a significant relationship between improved T-cell responses and elevated peripheral woodchuck ALT, AST, and SDH, which are sensitive markers of hepatocyte damage associated with immune clearance of HBV[42] (Supporting Figs. S3-S5); this indicates that the T-cell response was not sufficiently potent to clear WHV-infected hepatocytes. It is unclear to what extent the existing virus-specific T cells can be reinvigorated following the reduction of viral antigens. As an example, in a mouse model of chronic lymphocytic choriomeningitis virus (LCMV) infection, LCMV-specific T cells transferred from an infected animal to a naive uninfected animal failed to persist.[43] Nonetheless, the PBMC proliferation assay used in our study may not have had sufficient sensitivity and was unable to differentiate between CD4+ and CD8+ T cells; thus, additional experiments are needed to further characterize the elicited T-cell responses in our study and their contribution to the observed antiviral effects.

We did not detect any anti-WHsAg antibodies throughout the study. This observation may partly explain the viral and antigen rebound after the cessation of treatment. In line with our data, in a previous combination study with ETV and a retinoic acid-inducible gene I agonist (SB 9200), mainly an activator of innate immunity and IFN-α/β pathways, serum WHsAg levels were transiently reduced by 3.3 $\log_{10}$ but antibodies against WHsAg were not detected.[44] Because lowering the WHsAg levels did not result in detection of antibodies, we can rule out the possibility that antibodies are not detected in chronically WHV-infected woodchucks because they are in WHsAg/anti-WHsAg complexes, as has been reported for HBV in patients with CHB.[46] Consistent with this observation, recent studies have suggested that circulating and intrahepatic antiviral B cells in patients with chronic HBV infection are also defective.[46,47] HBsAg-specific and global B cells in CHB were enriched with CD21- and CD27-positive atypical memory B cells that had high expression of inhibitory receptors. In addition, in chronic LCMV infection, early loss of virus-specific B cells is shown to be dependent on IFN-α/β signaling.[48] Thus, treatment with systemic IFN-α may not be an effective strategy to restore the function of atypical memory B cells in CHB. Supporting this, woodchuck studies that have shown a high rate of seroconversion are limited to those that used therapeutic agents that directly activate B cells, such as toll-like receptor 7 agonists or a therapeutic vaccine containing a combination therapy that was designed to generate profound humoral responses.[19,20,26]

Our data suggest there is potential value in combining multiple therapeutic strategies in order to control viral infection. Therapeutic strategies that directly reduce viral antigen levels may be needed to partially reverse the decline in virus-specific T-cell responses. However, these strategies may also need to be complemented with emerging immunomodulatory

approaches that directly boost the numbers and functions of virus-specific T cells and B cells. Indeed, multiple groups have shown that therapeutic approaches, such as immune checkpoint receptor blockade, can be exploited to improve the responses of both HBV-specific T cells and B cells.[46,47,49] A study in the woodchuck model that included a combination of nucleoside treatment, therapeutic vaccination to generate *de novo* antiviral immune responses, and programmed death-ligand 1 blockade potently suppressed WHV replication, improved virus-specific T-cell responses, and induced anti-WHsAg antibodies, leading to complete viral clearance in one of three animals.[26] Thus, future combination strategies may need to include molecules that lower HBsAg and immunomodulatory approaches that can induce profound virus-specific T-cell and B-cell responses. Together, these approaches may be able to control viral infection, leading to a functional cure.

REFERENCES

1) Revill PA, Chisari FV, Block JM, Dandri M, Gehring AJ, Guo H, et al.; Members of the ICE-HBV Working Groups; ICE-HBV Stakeholders Group Chairs; ICE-HBV Senior Advisors. A global scientific strategy to cure hepatitis B. Lancet Gastroenterol Hepatol 2019;4:545-558.
2) Likhitsup A, Lok AS. Understanding the natural history of hepatitis B virus infection and the new definitions of cure and the endpoints of clinical trials. Clin Liver Dis 2019;23:401-416.
3) Kwon H, Lok AS. Hepatitis B therapy. Nat Rev Gastroenterol Hepatol 2011;8:275-284.
4) Frebel H, Richter K, Oxenius A. How chronic viral infections impact on antigen-specific T-cell responses. Eur J Immunol 2010;40:654-663.
5) Ochel A, Cebula M, Riehn M, Hillebrand U, Lipps C, Schirmbeck R, et al. Effective intrahepatic CD8+ T-cell immune responses are induced by low but not high numbers of antigen-expressing hepatocytes. Cell Mol Immunol 2016;13:805-815.
6) Kondo Y, Ninomiya M, Kakazu E, Kimura O, Shimosegawa T. Hepatitis B surface antigen could contribute to the immunopathogenesis of hepatitis B virus infection. ISRN Gastroenterol 2013;2013:935295.
7) Op den Brouw ML, Binda RS, van Roosmalen MH, Protzer U, Janssen HL, van der Molen RG, et al. Hepatitis B virus surface antigen impairs myeloid dendritic cell function: a possible immune escape mechanism of hepatitis B virus. Immunology 2009;126:280-289.
8) Chen Y, Wei H, Sun R, Tian Z. Impaired function of hepatic natural killer cells from murine chronic HBsAg carriers. Int Immunopharmacol 2005;5:1839-1852.
9) **Lebosse F**, **Testoni B**, Fresquet J, Facchetti F, Galmozzi E, Fournier M, et al. Intrahepatic innate immune response pathways are downregulated in untreated chronic hepatitis B. J Hepatol 2017;66:897-909.
10) Moreno S, Perno CF, Mallon PW, Behrens G, Corbeau P, Routy JP, et al. Two-drug vs. three-drug combinations for HIV-1: do we have enough data to make the switch? HIV Med 2019;20(Suppl. 4):2-12.
11) Gehring AJ, Protzer U. Targeting innate and adaptive immune responses to cure chronic HBV infection. Gastroenterology 2019;156:325-337.
12) Martinez MG, Testoni B, Zoulim F. Biological basis for functional cure of chronic hepatitis B. J Viral Hepat 2019;26:786-794.
13) **Mueller H**, **Wildum S**, Luangsay S, Walther J, Lopez A, Tropberger P, et al. A novel orally available small molecule that inhibits hepatitis B virus expression. J Hepatol 2018;68:412-420.
14) Han X, Zhou C, Jiang M, Wang Y, Wang J, Cheng Z, et al. Discovery of RG7834: the first-in-class selective and orally available small molecule hepatitis B virus expression inhibitor with novel mechanism of action. J Med Chem 2018;61:10619-10634.
15) Zhou T, Block T, Liu F, Kondratowicz AS, Sun L, Rawat S, et al. HBsAg mRNA degradation induced by a dihydroquinolizinone compound depends on the HBV posttranscriptional regulatory element. Antiviral Res 2018;149:191-201.
16) Mueller H, Lopez A, Tropberger P, Wildum S, Schmaler J, Pedersen L, et al. PAPD5/7 are host factors that are required for hepatitis B virus RNA stabilization. Hepatology 2019;69:1398-1411.
17) Roggendorf M, Yang D, Lu M. The woodchuck: a model for therapeutic vaccination against hepadnaviral infection. Pathol Biol (Paris) 2010;58:308-314.
18) **Menne S**, **Tumas DB**, Liu KH, Thampi L, AlDeghaither D, Baldwin BH, et al. Sustained efficacy and seroconversion with the Toll-like receptor 7 agonist GS-9620 in the Woodchuck model of chronic hepatitis B. J Hepatol 2015;62:1237-1245.
19) Kosinska AD, Liu J, Lu M, Roggendorf M. Therapeutic vaccination and immunomodulation in the treatment of chronic hepatitis B: preclinical studies in the woodchuck. Med Microbiol Immunol 2015;204:103-114.
20) Korolowizc KE, Li B, Huang X, Yon C, Rodrigo E, Corpuz M, et al. Liver-targeted toll-like receptor 7 agonist combined with entecavir promotes a functional cure in the woodchuck model of hepatitis B virus. Hepatol Commun 2019;3:1296-1310.
21) Fletcher SP, Chin DJ, Gruenbaum L, Bitter H, Rasmussen E, Ravindran P, et al. Intrahepatic transcriptional signature associated with response to interferon-alpha treatment in the woodchuck model of chronic hepatitis B. PLoS Pathog 2015;11:e1005103. Erratum in: PLoS Pathog 2016;12:e1005541.
22) Hornbuckle WE, Graham ES, Roth L, Baldwin BH, Wickenden C, Tennant BC. Laboratory assessment of hepatic injury in the woodchuck (Marmota monax). Lab Anim Sci 1985;35:376-381.
23) Menne S, Tennant BC, Gerin JL, Cote PJ. Chemoimmunotherapy of chronic hepatitis B virus infection in the woodchuck model overcomes immunologic tolerance and restores T-cell responses to pre-S and S regions of the viral envelope protein. J Virol 2007;81:10614-10624.
24) Chang TT, Lai CL, Kew Yoon S, Lee SS, Coelho HS, Carrilho FJ, et al. Entecavir treatment for up to 5 years in patients with hepatitis B e antigen-positive chronic hepatitis B. Hepatology 2010;51:422-430.
25) Rivkin A. Entecavir: a new nucleoside analogue for the treatment of chronic hepatitis B. Drugs Today (Barc) 2007;43:201-220.
26) Liu J, Zhang E, Ma Z, Wu W, Kosinska A, Zhang X, et al. Enhancing virus-specific immunity in vivo by combining therapeutic vaccination and PD-L1 blockade in chronic hepadnaviral infection. PLoS Pathog 2014;10:e1003856.
27) Menne S, Cote PJ. The woodchuck as an animal model for pathogenesis and therapy of chronic hepatitis B virus infection. World J Gastroenterol 2007;13:104-124.
28) Schoggins JW. Interferon-stimulated genes: what do they all do? Annu Rev Virol 2019;6:567-584.

29) Belloni L, Allweiss L, Guerrieri F, Pediconi N, Volz T, Pollicino T, et al. IFN-alpha inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. J Clin Invest 2012;122:529-537.
30) Liu F, Campagna M, Qi Y, Zhao X, Guo F, Xu C, et al. Alpha-interferon suppresses hepadnavirus transcription by altering epigenetic modification of cccDNA minichromosomes. PLoS Pathog 2013;9:e1003613.
31) Wieland SF, Eustaquio A, Whitten-Bauer C, Boyd B, Chisari FV. Interferon prevents formation of replication-competent hepatitis B virus RNA-containing nucleocapsids. Proc Natl Acad Sci U S A 2005;102:9913-9917.
32) Anderson AL, Banks KE, Pontoglio M, Yaniv M, McLachlan A. Alpha/beta interferon differentially modulates the clearance of cytoplasmic encapsidated replication intermediates and nuclear covalently closed circular hepatitis B virus (HBV) DNA from the livers of hepatocyte nuclear factor 1alpha-null HBV transgenic mice. J Virol 2005;79:11045-11052.
33) Xu C, Guo H, Pan XB, Mao R, Yu W, Xu X, et al. Interferons accelerate decay of replication-competent nucleocapsids of hepatitis B virus. J Virol 2010;84:9332-9340.
34) Li N, Zhang L, Chen L, Feng W, Xu Y, Chen F, et al. MxA inhibits hepatitis B virus replication by interaction with hepatitis B core antigen. Hepatology 2012;56:803-811.
35) Allweiss L, Volz T, Lutgehetmann M, Giersch K, Bornscheuer T, Lohse AW, et al. Immune cell responses are not required to induce substantial hepatitis B virus antigen decline during pegylated interferon-alpha administration. J Hepatol 2014;60:500-507.
36) Swiecki M, Colonna M. Type I interferons: diversity of sources, production pathways and effects on immune responses. Curr Opin Virol 2011;1:463-475.
37) Penna A, Laccabue D, Libri I, Giuberti T, Schivazappa S, Alfieri A, et al. Peginterferon-alpha does not improve early peripheral blood HBV-specific T-cell responses in HBeAg-negative chronic hepatitis. J Hepatol 2012;56:1239-1246.
38) Micco L, Peppa D, Loggi E, Schurich A, Jefferson L, Cursaro C, et al. Differential boosting of innate and adaptive antiviral responses during pegylated-interferon-alpha therapy of chronic hepatitis B. J Hepatol 2013;58:225-233.
39) Peppa D, Gill US, Reynolds G, Easom NJ, Pallett LJ, Schurich A, et al. Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion. J Exp Med 2013;210:99-114.
40) Ferrari C. HBV and the immune response. Liver Int 2015;35 (Suppl. 1):121-128.
41) Bertoletti A, Ferrari C. Adaptive immunity in HBV infection. J Hepatol 2016;64(Suppl.):S71-S83.
42) Wong D, Littlejohn M, Edwards R, Jackson K, Revill P, Gaggar A, et al. ALT flares during nucleotide analogue therapy are associated with HBsAg loss in genotype A HBeAg-positive chronic hepatitis B. Liver Int 2018;38:1760-1769.
43) Wherry EJ, Barber DL, Kaech SM, Blattman JN, Ahmed R. Antigen-independent memory CD8 T cells do not develop during chronic viral infection. Proc Natl Acad Sci U S A 2004;101:16004-16009.
44) Suresh M, Korolowicz KE, Balarezo M, Iyer RP, Padmanabhan S, Cleary D, et al. Antiviral efficacy and host immune response induction during sequential treatment with SB 9200 followed by Entecavir in woodchucks. PLoS One 2017;12:e0169631.
45) de Niet A, Jansen L, Zaaijer HL, Klause U, Takkenberg B, Janssen HL, et al. Experimental HBsAg/anti-HBs complex assay for prediction of HBeAg loss in chronic hepatitis B patients treated with pegylated interferon and adefovir. Antivir Ther 2014;19:259-267.
46) Salimzadeh L, Le Bert N, Dutertre CA, Gill US, Newell EW, Frey C, et al. PD-1 blockade partially recovers dysfunctional virus-specific B cells in chronic hepatitis B infection. J Clin Invest 2018;128:4573-4587.
47) Burton AR, Pallett LJ, McCoy LE, Suveizdyte K, Amin OE, Swadling L, et al. Circulating and intrahepatic antiviral B cells are defective in hepatitis B. J Clin Invest 2018;128:4588-4603.
48) Dempsey LA. IFN-α/β-mediated suppression of B cells. Nat Immunol 2016;17:1341.
49) Fisicaro P, Valdatta C, Massari M, Loggi E, Biasini E, Sacchelli L, et al. Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B. Gastroenterology 2010;138:682-693.e1-e4.

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