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Combined GS-4774 and Tenofovir Therapy Can Improve HBV-Specific T-Cell Responses in Patients With Chronic Hepatitis

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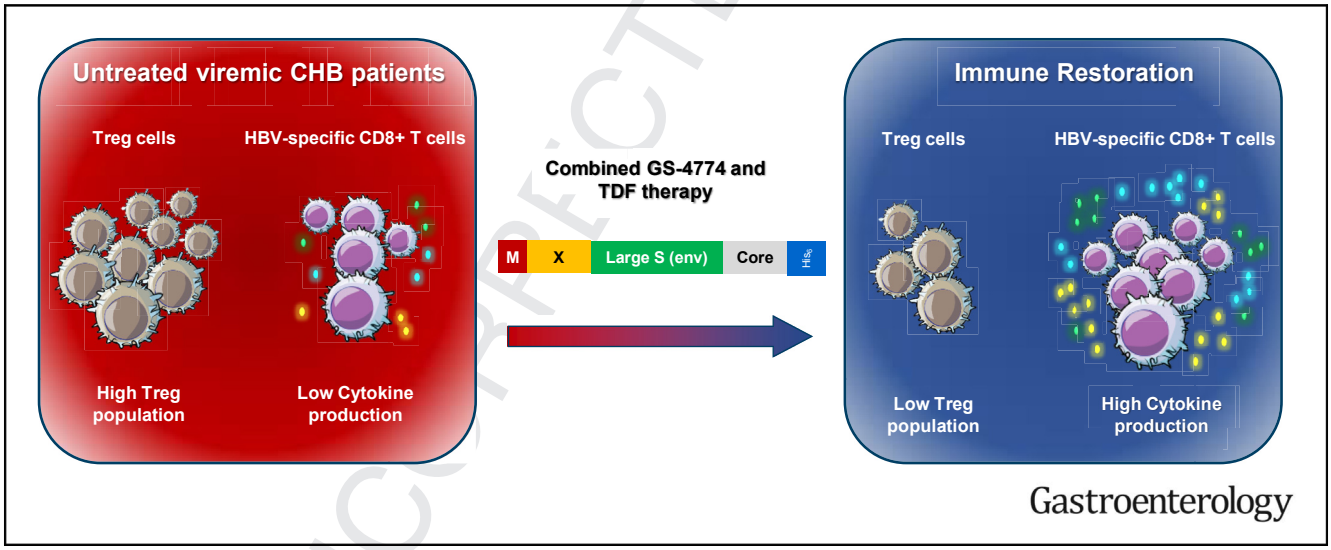

BACKGROUND & AIMS: One strategy to treat chronic hepatitis B virus (HBV) infection could be to increase the functions of virus-specific T cells. We performed a multicenter phase 2 study to evaluate the safety and efficacy of GS-4774, a yeast-based therapeutic vaccine engineered to express HBV antigens, given with tenofovir disoproxil fumarate (TDF) to untreated patients with chronic HBV infection. **METHODS:** We performed an open-label study at 34 sites in Canada, Italy, New Zealand, Romania, South Korea, and United States from July 2014 to August 2016. Adults who were positive for HB surface antigen (HBsAg) > 6 months and levels of HBV DNA ≥2000 IU/mL who had not received antiviral treatment for HBV within 3 months of screening were randomly assigned (1:2:2:2) to groups given oral TDF 300 mg daily alone (n = 27; controls) or with 2, 10, or 40 yeast units GS-4774 (n = 168), administered subcutaneously every 4 weeks until week 20 for a total of 6 doses. Blood samples were collected and analyzed and patients received regular physical examinations. Efficacy was measured by decrease in HBsAg from baseline to week 24. Specific responses to HBV (production of interferon gamma [IFNG], tumor necrosis factor [TNF], interleukin 2 [IL2], and degranulation) were measured in T cells derived from 12 HBeAg-negative patients with genotype D infections, after overnight or 10 days of stimulation of peripheral blood mononuclear cells with peptides from the entire HBV proteome. T-regulatory cells were analyzed for frequency and phenotype. Data from studies of immune cells were compared with data on reductions in HBsAg, HBV DNA, and alanine aminotransferase in blood

samples from patients. **RESULTS:** GS-4774 was safe and well tolerated but did not produce significant decreases in levels of HBsAg. Production of IFNG, TNF, and IL2 increased significantly at weeks 24 and 48, compared with baseline, in HBV-specific CD8+ T cells from patients given GS-4774 but not from controls. GS-4774 had greater effects on CD8+ than CD4+ T cells, which were not affected at all or very weakly by TDF with or without GS-4774. GS-4774 did not affect responses of T cells to other viruses tested. HBV core peptides induced the greatest production of IFNG by T cells following overnight stimulation, whereas HBV envelope antigens did not induce a response. Following 10 days of stimulation, production of IFNG and TNF increased with time of exposure to GS-4774; the greatest levels of responses were to HBV envelope antigens followed by core and polymerase peptides. We observed a correlation in patients given GS-4774 between increased T-cell functions and reductions in numbers of T-regulatory cells. **CONCLUSIONS:** In a phase 2 study of patients with chronic HBV infection given TDF with or without GS-4774, we found that vaccination can increase production of IFNG, TNF, and IL2 by CD8+ T cells exposed to antigenic peptides, with little effect on CD4+ T cells. Although GS-4774 did not reduce levels of HBsAg in patients, its strong immune stimulatory effect on CD8+ T cells might be used in combination with other antiviral agents to boost the antivirus immune response. Clinicaltrials.gov no: NCT02174276.

Keywords: CHB; Treg Cell; Tolerance; Immunotherapy.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

One strategy to treat chronic hepatitis B virus (HBV) infection could be to increase the functions of virus-specific T cells.

NEW FINDINGS

GS-4774 was well tolerated and induced simultaneous restoration of multiple T-cell functions in viremic HBeAg-negative patients with hepatitis B. The largest effects were on CD8+ T cells, associated with a significant reduction in T-regulatory cells.

LIMITATIONS

Despite a strong immune modulatory effect, GS-4774 did not produce clinically significant reductions in HBsAg.

IMPACT

The GS-4774 vaccine can break immune tolerance to HBV in patients with chronic infections and might be used in combination with other antiviral agents to boost the anti-virus immune response.

Chronic hepatitis B virus (HBV) infection represents a worldwide public health concern with approximately 250 million people chronically infected and at risk of developing liver cirrhosis and hepatocellular carcinoma.[1] Nucleos(t)ide analogues (NUC), the most widely used therapies for HBV infection, are very effective in reducing HBV replication, but loss of hepatitis B surface antigen (HBsAg), which is considered functional cure of HBV infection, is observed in fewer than 10% of patients even after many years of therapy.[1] This is due to the integration of HBV DNA sequences in the host genome[2,3] and to the persistence of covalently closed circular DNA, that acts as a reservoir for viral replication, which are not significantly affected by NUC therapies.[4,5]

The host immune response to HBV is a key determinant of the outcome of infection.[6] HBV-specific T cells are deeply exhausted in untreated patients with chronic hepatitis B (CHB): reconstitution of their antiviral function represents a major goal of HBV immune therapies.[6] Therapeutic compounds designed to restore an effective HBV-specific T-cell response represent promising tools for improving the rate of HBsAg loss and seroconversion in subjects with CHB compared with what is currently achievable with NUC alone.[7] In particular, stimulation of virus-specific T-cell responses by specific T-cell vaccines represents a rational immune modulatory approach for a therapeutic reconstitution of protective immunity.[8]

GS-4774 is a yeast-based therapeutic vaccine containing HBV S, X, and core proteins and designed to elicit efficient HBV-specific immune responses[9–11] by promoting antigen processing and presentation in the context of both major histocompatibility complex class I and class II pathways.[12] The yeast component has also been shown to reduce frequency and inhibitory activity of T-regulatory cells (Tregs), likely due to its natural ability to elicit interleukin (IL)-1B production and to favor Th17 over Tregs cell differentiation,[13,14] and to act as an adjuvant for HBV-specific immune responses.[13] Induction of HBV-specific T-cell responses with the oral GS-4774 vaccine has previously been reported in mouse models and healthy volunteers.[9,10]

In this phase 2 study, we assessed the efficacy and safety of GS-4774 in patients with viremic CHB not currently on oral antiviral therapy. Further, we tested the immune modulatory effect of treatment on HBV-specific T-cell responses and Treg cells in a subset of treatment-naïve hepatitis B e-antigen (HBeAg)-negative patients with CHB.

Materials and Methods

Patients

This phase 2, multicenter, randomized, controlled open-label study was conducted at 34 sites in Canada, Italy, New Zealand, Romania, South Korea, and the United States from

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Abbreviations used in this paper: **AE, adverse event; ALT, alanine aminotransferase; APC, antigen-presenting cells; CHB, chronic hepatitis B; CMH, Cochran-Mantel-Haenszel; CMV, cytomegalovirus; EBV, Epstein-Barr virus; ELISpot, enzyme-linked immunosorbent spot assay; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; NUC, nucleos(t)ide analogues; PBMC, peripheral blood mononuclear cells; TDF, tenofovir disoproxil fumarate; Treg, regulatory T cells; TNF, tumor necrosis factor; ULN, upper limit of normal.**

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July 2014 to August 2016 (clinicaltrials.gov: NCT02174276). Patients were at least 18 years old with CHB (documented HBsAg positive > 6 months) with detectable HBV DNA at screening (≥2000 IU/mL) who had not received antiviral treatment for HBV within 3 months of screening. Major exclusion criteria included advanced bridging fibrosis and cirrhosis. Full eligibility criteria are provided in the Supplementary Materials.

All patients provided written informed consent before enrollment. The study was approved by the institutional review boards at participating sites and conducted in compliance with the Declaration of Helsinki, Good Clinical Practice guidelines, and local regulatory requirements.

Study Design and Treatment

Eligible patients were randomized in a 1:2:2:2 ratio to receive oral tenofovir disoproxil fumarate (TDF) 300 mg daily only, or along with GS-4774 at doses of 2, 10, or 40 yeast units (YU). Patients were stratified by HBeAg status (positive vs negative) and alanine aminotransferase (ALT) level (>19 vs ≤19 IU/L for women; >30 vs ≤30 IU/L for men) according to American Association for the Study of Liver Diseases guidelines.[15]

GS-4774 (Gilead Sciences, Inc., Foster City, CA) was administered subcutaneously every 4 weeks until week 20 for a total of 6 doses (GS-4774 2 YU as 1 injection; GS-4774 10 YU as 2 injections; GS-4774 40 YU as 4 injections). All patients received TDF 300 mg once daily at least until the end of the study (week 48) after which, on the investigator's discretion, patients either entered treatment-free follow-up (24 weeks) or an optional treatment extension phase with TDF only (144 weeks) (details in Supplementary Materials).

Study Assessments

Safety was evaluated by assessment of clinical laboratory tests (including measurements of serum HBsAg, anti-HBs, anti-HBe, HBV DNA, and human leukocyte antigen [HLA]), physical examinations, vital signs measurements, and by documentation of adverse events (AEs) according to the schedule provided in Supplementary Table 1. All safety data were collected from the time of first dose of study drug to 30 days after the last dose of study drug.

Endpoints

The primary endpoints were safety and tolerability; the primary efficacy endpoint was the mean change in quantitative serum HBsAg ($\log_{10}$ IU/mL) from baseline to week 24 by least-squares mean. Secondary efficacy endpoints included the mean change in $\log_{10}$ IU/mL from baseline to weeks 12 and 48; the proportion of patients with HBsAg and/or HBeAg loss and seroconversion at weeks 24 and 48 (Supplementary Material); the proportion of patients with ≥0.5 or ≥1 $\log_{10}$ decline in HBsAg at weeks 12, 24, and 48; the proportions of patients with HBV DNA <LLOQ (<20 IU/mL) at weeks 24 and 48; and the proportion of patients experiencing viral breakthrough (ie, 2 consecutive occurrences of HBV DNA ≥69 IU/mL after a HBV DNA <69 IU/mL or an increase in HBV DNA ≥1.0 $\log_{10}$ IU/mL from nadir).

Measurement of HBV-specific T-cell Responses

HBV-specific T-cell responses were measured in a subset of 12 treatment-naïve viremic HBeAg-negative patients infected with HBV genotype D enrolled in 4 Italian centers (Supplementary Table 2). Because only 1 patient was randomized to the tenofovir arm, 9 additional patients with CHB who were not on treatment served as controls and received NUC only, so that the total number of patients in the control cohort was equal to 10. Fifteen patients who had spontaneously recovered from an acute HBV infection who were recruited in the same Italian geographical areas served as an additional control population. The demographic, clinical, and virological characteristics of each individual patient are described in Supplementary Tables 2 and 3.

In Vitro Expansion and Intracellular Cytokine Staining of HBV-specific T Cells

Peripheral blood mononuclear cells (PBMCs) were stimulated either with a panel of 315 15-mer peptides, overlapping by 10 residues, covering the overall HBV genotype D sequence, pooled in 8 mixtures, as previously described,[16] or with a pool of immunodominant HLA class I and II peptides from cytomegalovirus (CMV), Epstein-Barr virus (EBV), and influenza sequences. Immunological assays were performed on day 10 using anti-interferon (IFN)-γ, anti-IL-2 (BD Biosciences, San Jose, CA) and anti-tumor necrosis factor (TNF)-α (Miltenyi, Bergisch Gladbach, Germany) conjugated monoclonal antibodies for the detection of intracellular cytokines, and using an anti-CD107a antibody for the study of the cytotoxic potential. Cells were acquired on a FACSCANTO II flow cytometer and were analyzed with the DIVA software (BD Biosciences).

Enzyme-Linked Immunosorbent Spot Assay

Enzyme-linked immunosorbent spot assays (ELISpot) were performed using the panel of 315 15-mer peptides pooled in 8 mixtures; 2 to 4 × 10^5 PBMCs per well were seeded in triplicate and HBV-specific T-cell responses were analyzed after overnight incubation with individual peptide mixtures (1 μM) for IFN-γ production according to the manufacturer's instruction (BD ELISpot, ELISpot Set; Becton Dickinson, Franklin Lakes, NJ). Spots were counted using an automated ELISpot reader (AID ELISpot Reader System). IFN-γ-secreting cells were expressed as spot-forming cells per 1 × 10^6 cells after subtraction of the background. Positive controls consisted of PBMCs stimulated with CMV, EBV, and influenza peptides mixture. ELISpot was considered positive if the number of spots in the stimulated wells was at least 3 standard deviations above background and the difference between the number of spots in the stimulated and unstimulated wells was above 10.

Cell Surface Staining and Flow Cytometry Analysis

For ex vivo Treg cell phenotypic analysis, PBMCs were stained with the following antibodies: Live and Dead (ThermoFisher, Waltham, MA), CD4 (Miltenyi), FoxP3 (eBioscience, San Diego, CA), CD45RA (Miltenyi), CD3 and CD25 (both from BD Biosciences). To assess the proliferation capability, Tregs were permeabilized and stained with anti-Ki67, whereas to measure the activation status were stained with anti-HLA-DR

(both by BD Biosciences). For the phenotypic analysis of the CD8 T-cell population, PBMCs were stained with CD8, CD3, CD127 (all from BD Biosciences), PD-1 (BioLegend, San Diego, CA), TIGIT (eBioscience), CD39 (Miltenyi). Cells were acquired on a FACSCANTO II flow cytometer and analyzed with the DIVA software. For ex vivo analysis of dendritic cells and monocytes, the following monoclonal antibodies were used: CD3, CD16, CD80, CD14, CD11c, CD86 (all from BD Biosciences) and CD123, CD83, CD40, CD56, CD20, HLA-DR (all from eBioscience). Cells were acquired on a BD FACSLyric flow cytometer (BD Bioscences) and analyzed with the FlowJo software (Tree Star, Ashland, OR).

Statistical Analysis

Clinical study. All patients who received at least 1 dose of study medication were included in the safety and efficacy analyses. Safety data were analyzed by treatment group and included all data collected from the date of first dose of study drug up to the last dose date (ie, treatment-emergent). Mean changes in serum HBsAg from baseline were analyzed using mixed-effect model repeat measurement using unstructured, within-patient covariance matrix. Estimated least-square means of treatment effects and differences in treatment effects between GS-4774 groups and the TDF-only group at week 24 were calculated with 95% confidence intervals and unadjusted *P* values. A stratified Cochran-Mantel-Haenszel (CMH) test with ALT levels (greater than upper limit of normal [ULN] or ≤ULN) and HBeAg status (positive or negative) at baseline, as stratification variables, was used to compare the treatment effect between each of the GS-4774 groups and the TDF-only group for HBsAg and HBeAg loss and seroconversion and the proportion of patients with a ≥0.5- or ≥1-log decline in HBsAg. Two-sided CMH test and Fisher's exact *P* values were presented. The association of HLA class I and II antigens to clinical response (≥0.5 $\log_{10}$ IU/mL decline in HBsAg at week 24) was examined using Fisher's exact test. Bonferroni corrected *P* values and false discovery rate–corrected q-values were also calculated for all HLA types with at least 10 patients. To assess the relationship between ΔHBsAg and specific baseline demographic and disease characteristics, univariate and multivariate analysis was performed (Supplementary Materials).

Immunological study. Data were analyzed by GraphPad Prism (GraphPad Software, La Jolla, CA). Statistical significance was assessed by the Mann-Whitney *U* test for nonpaired samples and the Wilcoxon signed rank test for paired data; frequencies were compared by χ^2 and Fisher *F* tests. Correlations were analyzed by the Pearson's correlation test. Multiple linear regression analysis was performed by JASP Software to assess the difference in clinical and virological baseline values in relation to immunological parameters. Hierarchical-clustering of T-cell parameters was performed by GeneSpring-GX (Agilent, Santa Clara, CA). Data were median-normalized before clustering. The clustering was obtained by Canberra Average similarity measure.

Results

Baseline Characteristics

Of the 254 patients screened, 195 were randomized to receive TDF plus GS-4774 (n = 168) or TDF alone (n = 27). Overall, the mean age (range) of patients was 45 (18–69) years and most were men (61%), Asian (80%), and HBeAg-negative (61%) (Table 1). The mean HBsAg baseline titer range across groups was 3.7 to 3.8 $\log_{10}$ IU/mL with a mean baseline HBV DNA level of 5.8 to 6.0 $\log_{10}$ IU/mL. At baseline, 78%, 74%, 63%, and 78% of patients receiving the TDF only, 2-, 10-, and 40-YU GS-4774 doses, respectively, had ALT above the ULN (Table 1).

Efficacy

Mean changes in $\log_{10}$ IU/mL serum HBsAg from baseline through week 48 are shown in Supplementary Figure 1*A*. The mean declines at the primary endpoint (week 24) for the 2-, 10-, and 40-YU GS-4774 groups were −0.096, −0.016, and −0.135 $\log_{10}$ IU/mL, respectively, and statistically no different from the TDF-only group (−0.079 $\log_{10}$ IU/mL). Similar results were also observed at weeks 12 and 48. There were no apparent differences in HBsAg decline between groups by baseline ALT levels or HBeAg status. The greatest mean declines occurred in the patients who were HBeAg-positive and with baseline ALT>ULN (Supplementary Figure 1*B–F*).

Although the proportion of patients who experienced categorical declines never reached statistical significance at any evaluated week (weeks 12, 24, and 48), at week 24, 11 GS-4774-treated patients had ≥0.5 $\log_{10}$ reductions in HBsAg compared with no patients in the TDF-only group. A trend toward significance ($P = .076$ by CMH test) can be observed in the GS-4774 40-YU treatment group compared with the TDF group after adjusting for the baseline stratification factors (HBeAg status and ALT level) (Supplementary Figure 1*G*). No patient achieved HBsAg loss or anti-HBs seroconversion through week 48. Of the 76 patients who were HBeAg-positive at baseline, 1 (4.3%) in the GS-4774 10-YU group achieved HBeAg loss and seroconversion by week 24. At week 48, 5 patients (1 receiving GS-4774 2 YU [5%], 2 receiving GS-4774 10 YU [9%], and 2 receiving GS-4774 40 YU [10%]) achieved HBeAg loss, 3 of whom achieved HBeAg loss and seroconversion (1 in the GS-4774 10-YU group and 2 in the GS-4774 40-YU group).

HBV DNA suppression and Virologic Resistance Analysis as well as HLA allele associations with ΔHBsAg decline are described in the Supplementary Materials and in Supplementary Figure 1*H* and Supplementary Figure 2, respectively.

Safety

GS-4774 was generally well tolerated. Two (1.0%) patients did not complete GS-4774 treatment (both withdrew consent). Five patients did not complete TDF treatment through week 48 (3 withdrew consent, 1 due to pregnancy and 1 was lost to follow-up). One patient in the TDF + GS-4774 2-YU group interrupted TDF due to pyrexia from days 141 to 147. No serious AEs and no deaths were reported.

In the GS-4774 treatment groups, 40% to 80% of patients experienced AEs related to GS-4774 treatment; most were Grade 1 or Grade 2 in severity. Only 5 patients who received GS-4774 10 YU and 3 patients who received GS-

Table 1. Baseline Demographics and Disease Characteristics

	TDF, n = 27	TDF + GS-4774 2 YU, n = 57	TDF + GS-4774 10 YU, n = 56	TDF + GS-4774 40 YU, n = 55	Total, n = 195
Age, y Mean, range	44 (24–67)	46 (18–63)	44 (22–69)	43 (19–62)	44 (18–69)
Male, n (%)	18 (67)	34 (60)	33 (59)	33 (60)	118 (61)
Race, n (%)					
Asian	24 (89)	42 (74)	44 (79)	45 (82)	155 (80)
Native Hawaiian or Pacific Islander	0	0	2 (4)	1 (2)	3 (2)
White	1 (4)	13 (23)	8 (14)	6 (11)	28 (14)
Black	2 (7)	2 (4)	2 (4)	3 (6)	9 (5)
Other	0	0	0	0	0
HBeAg Status Negative, n (%)	17 (63)	35 (61)	33 (59)	34 (62)	119 (61)
HBV DNA (log_{10} IU/mL) Mean (SD)	6.0 (1.64)	5.8(1.99)	5.8 (1.97)	6.0 (1.80)	5.9 (1.88)
HBsAg (log_{10} IU/ mL) Mean (SD)	3.8 (0.78)	3.7 (0.82)	3.7 (0.94)	3.7 (0.80)	3.7 (0.84)
ALT (U/L) Mean (SD)	49.7 (44.50)	48.1 (36.39)	38.4 (29.32)	60.7 (62.31)	49.1 (45.21)
Baseline ALT >ULN (%)	21 (78)	42 (74)	35 (63)	43 (78)	141 (72)
Prior Interferon Experience Yes, n (%)	2 (7)	7 (12)	8 (14)	10 (18)	27 (14)

4774 40 YU experienced Grade 3 AEs related to GS-4774, including injection site pain (n = 5); fatigue (n = 3); and nausea, vomiting, myalgia, and headache (n = 1 each). Overall, the most common treatment-emergent AEs associated with the injection site were pain (28%–82%), erythema (25%–62%), and swelling (16%–40%) (Table 2). The most common non–injection site AEs in the GS-4774 groups were fatigue (18%–38%), headache (16%–35%), and myalgia (11%–36%). AEs appeared to be directly correlated with dose level. Fatigue (19%) and cough (11%) were the most common AEs in the TDF-only treatment group. A total of 43 treatment-emergent Grade 3 or 4 laboratory abnormalities were experienced by 33 patients (1 TDF only, 8 TDF + GS-4774 2 YU, 5 TDF + GS-4774 10 YU, and 19 TDF + GS-4774 40 YU) (Table 2).

Four patients, 2 each in the GS-4774 2-YU and 40-YU groups, had an ALT flare (ALT >2 × baseline and >5 × ULN); 3 patients experienced a flare within the first 4 weeks of treatment and in the fourth patient it occurred at week 8. ALT levels for all 4 patients subsequently normalized. Of these 4 patients, only 1 (TDF + GS-4774 2 YU group) had a ≥0.5 log_{10} decline in HBsAg within 4 weeks after the ALT flare.

Univariate and Multivariate Analysis of ≥0.5 log_{10} IU/mL HBsAg Decline Association

Univariate analysis showed that significant baseline predictors of a ≥0.5 log_{10} IU/mL decline in HBsAg observed at week 24 were age, ALT, log_{10} HBV DNA, HBeAg status, HBsAg, HLA B*52:01, HLA C*12:02, HLA DPB*09:01, and HLA DRB1*15:02. Multivariate analysis was then performed to assess baseline factors associated with ≥0.5 log_{10} IU/mL decline. Higher baseline ALT, HLA DRB*15:02 allele, and HBeAg positivity were determined to be associated with a higher probability of ΔHBsAg (Supplementary Table 4).

In Vitro Analysis of HBV-specific CD4+ and CD8+ T-cell Responses

IFN-γ, TNF-α, and IL2 production by HBV-specific CD8+ T cells improved significantly at weeks 24 and 48 (red lines, Figure 1*A*) compared with baseline in patients treated with GS-4774, whereas no significant changes were observed in the control group of patients receiving NUC alone (green lines, Figure 1*A*). The effect of vaccine was significantly better on CD8- than CD4-mediated responses, which were not affected at all or very weakly by both therapy regimens (Figure 1*A* and *B*). Moreover, no modulation of CMV/EBV/Flu-specific T-cell responses was induced by therapy (Figure 1*C* and Supplementary Figure 3).

Evolution of T-cell responses in patients treated with combined GS-4774 and tenofovir is well illustrated by hierarchical-clustering analysis of all data derived from all time points of therapy and from the reference acute hepatitis B control group for the definition of an efficient immune response able to control infection spontaneously (Figure 1*D*). A progressive functional improvement involving primarily IFN-γ and TNF-α production by CD8 cells was detected also by this analysis, as shown by the gradual transition from light to dark intensity of color, indicating progressive changes in quality and intensity of T-cell responses induced by therapy, which make chronic

Table 2. Treatment- Emergent AEs (TEAEs) and Grade 3 or 4 Laboratory Abnormalities

	TDF, n = 27	TDF + GS-4774 2 YU, n = 57	TDF + GS-4774 10 YU, n = 56	TDF + GS-4774 40 YU, n = 55
Any AEs, n (%)	13 (48)	41 (72)	51 (91)	53 (96)
Grade 3 or 4 AEs, n (%)	1 (4)	0	8 (14)	6 (11)
Serious AEs, n (%)	0	0	0	0
TEAEs (any grade) in ≥10% of patients in any treatment group, n (%)				
Injection site pain	0	16 (28)	32 (57)	45 (82)
Injection site erythema	0	14 (25)	20 (36)	34 (62)
Injection site swelling	0	9 (16)	11 (20)	22 (40)
Injection site pruritus	0	4 (7)	18 (32)	18 (33)
Injection site induration	0	4 (7)	5 (9)	18 (33)
Fatigue	5 (19)	10 (18)	21 (38)	20 (36)
Headache	1 (4)	9 (16)	17 (30)	19 (35)
Myalgia	1 (4)	6 (11)	13 (23)	20 (36)
Nausea	1 (4)	5 (9)	10 (18)	11 (20)
Cough	3 (11)	5 (9)	7 (13)	7 (13)
Chills	0	2 (4)	6 (11)	9 (16)
Pyrexia	1 (4)	3 (5)	1 (2)	7 (13)
Nasopharyngitis	2 (7)	0	2 (4)	6 (11)
Laboratory abnormalities				
Hemoglobin Grade 3, 7.0 to <9.0 g/dL or any decrease ≥4.5 g/dL from baseline	0	1 (2)	2 (4)	3 (5)
Occult blood Grade 3	0	2 (4)	0	4 (7)
Creatine kinase Grade 3, 10 to <20 × ULN	1 (4)	1 (2)	1 (2)	2 (4)
Urine erythrocytes Grade 3	0	1 (2)	0	4 (7)
Alanine aminotransferase Grade 3, >5 to 10 × ULN	0	1 (2)	0	2 (4)
Aspartate aminotransferase Grade 3, >5 to 10 × ULN	0	1 (2)	0	2 (4)
Creatine kinase Grade 4, ≥20 × ULN	0	1 (2)	0	2 (4)
Urine glucose Grade 3	0	0	1 (2)	2 (4)
Prothrombin time Grade 3, >1.5 to 3 × ULN	0	0	1 (2)	2 (4)
Alanine aminotransferase Grade 4, >10 × ULN	0	1 (2)	0	1 (2)
Aspartate aminotransferase Grade 4, > 10 × ULN	0	1 (2)	0	0
Bilirubin Grade 3, >2.5 to 5.0 × ULN	0	1 (2)	0	0
Hyperglycemia Grade 3, >250 to 500 mg/dL	0	0	0	1 (2)
Internationalized normalized ratio of prothrombin time Grade 3, >2.0 to 3 × ULN	0	0	0	1 (2)

treated patients progressively more similar to acute self-limited patients.

Because levels of response in individual patients were widely variable, we then analyzed longitudinally each individual CD4 and CD8 T-cell function (IFN-γ, TNF-α, IL-2 production, and CD107a degranulation) in all patients. Notably, in patients receiving GS-4774 and tenofovir, a simultaneous improvement of multiple functions was detected in 10 of 11 patients (Figure 2). Improvement of CD8 responses was significantly better in patients treated with combined therapy than in patients treated with NUC alone ($P = .0003$ by χ^2 test). In addition, increase of responses was significantly greater among CD8 compared with CD4 T-cell subsets in GS-4774 plus TDF-treated patients ($P = .006$ by χ^2 test). This was also confirmed by the study of double IFN-γ+/TNF-α+ and triple IFN-γ+/TNF-α+/IL-2+ HBV-specific CD8 T cells ($P = .0009$ by χ^2 test; Supplementary Figure 4). Although some functional improvements were also observed among CD4 cells, they were less common and no difference was found between patients receiving GS-4774 plus TDF and those receiving NUC alone (Figure 2).

The major contribution to the overall HBV-specific T-cell responses was given by polymerase at all time points and in both patient cohorts (Supplementary Figure 5), followed by envelope and core antigens. During vaccine therapy, a progressive increase of CD8-mediated responses was induced primarily by envelope followed by core and polymerase; minor changes were instead observed for CD4-mediated responses (Figure 3, statistics by Wilcoxon matched-pairs test). Weaker T-cell modulation was detected during treatment with NUC but only at the CD4 T-cell level (Figure 3, green bars). Moreover, phenotypic analysis of dendritic cells and monocytes was performed to assess their activation state, but no clear modulation of frequency and activation was detected during therapy (Supplementary Figure 6). Some baseline differences in age, ALT, and HBV DNA levels were observed between the 2 groups of vaccinees and control patients. To clarify whether this baseline difference may have contributed to the different T-cell responses,

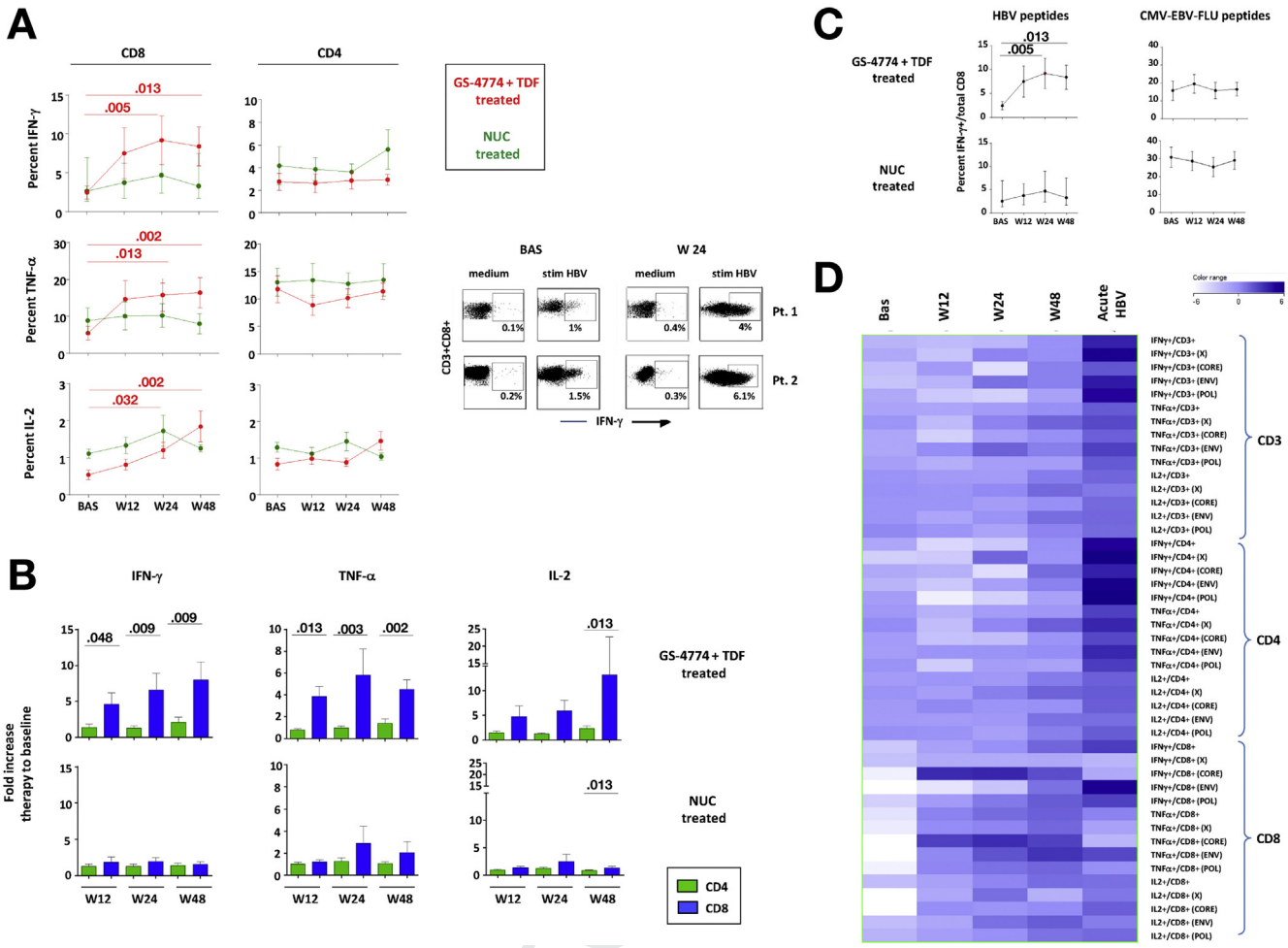
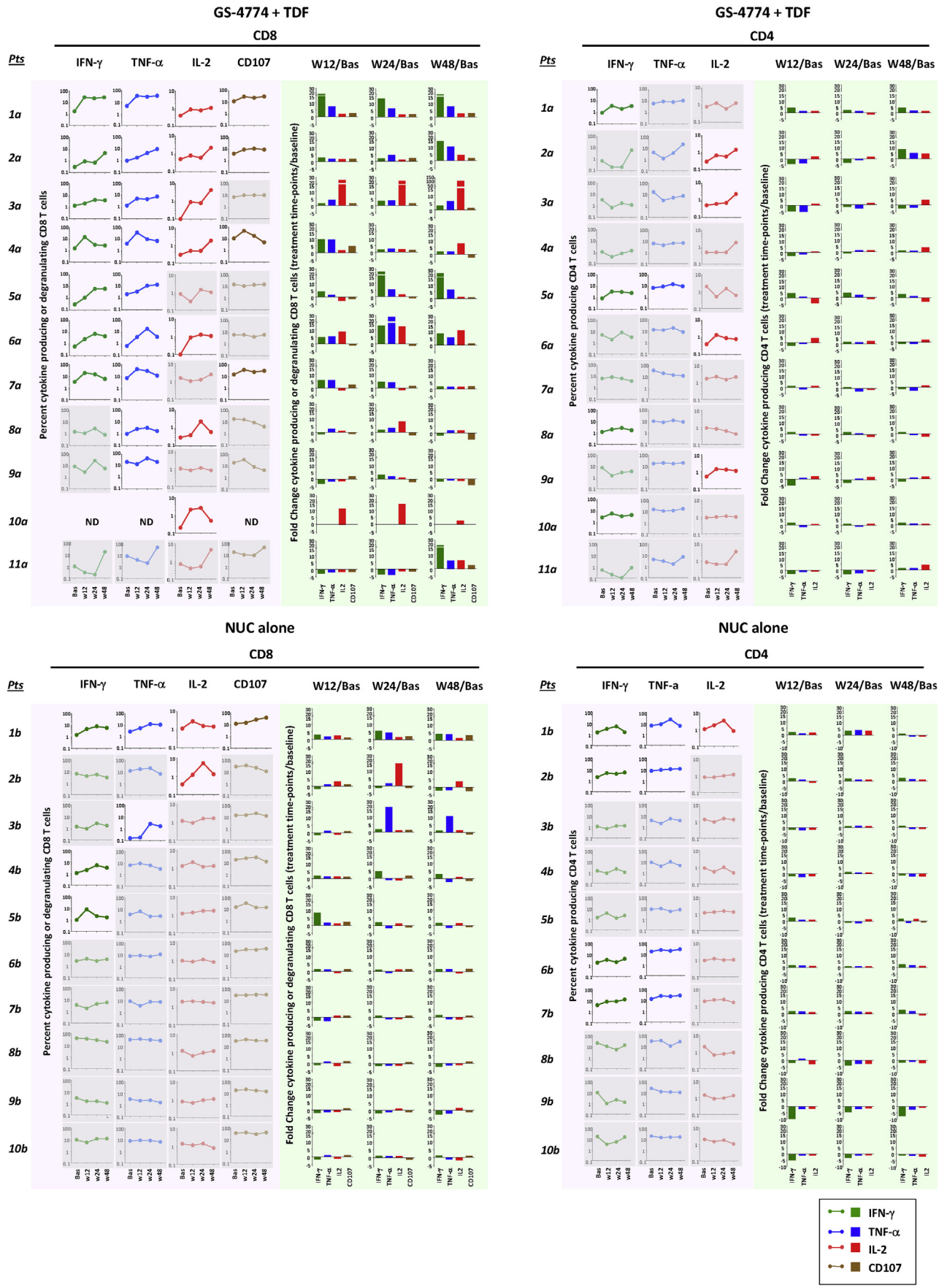


Figure 1. Effect of GS-4774 and TDF therapy on virus-specific T-cell responses. (*A*) Each line shows mean frequency values plus standard error of CD8 and CD4 T cells able to produce IFN-γ, TNF-α, and IL2 following 10 days of stimulation with overlapping peptides covering the overall HBV genotype D sequence. Statistical significance was assessed by the Wilcoxon signed rank test for paired data. Dot-plots of IFN-γ-positive HBV-specific CD8 T cells from 2 representative patients at baseline and week 24 are illustrated on the right of the panel. (*B*) Mean fold-increase plus standard error in the frequency of HBV-specific CD4+ and CD8+ T cells able to produce the indicated cytokines. Ratio between GS-4774 + TDF-treated or NUC-treated patients at weeks 12, 24, and 48 and the corresponding baseline values are illustrated (*P* values by the Wilcoxon signed rank test compare the ratio between CD4 and CD8 responses at that indicated time point with baseline). (*C*) Mean percentage plus standard error of IFN-γ–producing T cells in the global CD8+ T-cell population after 10 days of stimulation with CMV+EBV+FLU peptides (*right*) and with HBV peptide pools (*left*) in chronic naïve patients undergoing GS-4774 +TDF or NUC treatment (n = 11 and n = 10, respectively). (*D*) Hierarchical-clustering representation of IFN-γ, TNF-α, and IL2-positive HBV-specific CD3, CD4, and CD8 T-cell responses induced by HBV antigen stimulation in acute (n = 15) and chronic patients before and during GS-4774 and TDF therapy at sequential time points (Bas, week 12, week 24; week 48; n = 11). Data were analyzed with the software for gene expression analysis (GeneSpring, Agilent Technologies). Results represented in each row were first normalized on the median of all sets of data (baseline, each individual GS-4774 treatment time point and acute patients, the latter tested at a single time point, 3 to 6 months after the ALT peak) for each single function. The color gradation (from the lightest to the darkest) is proportional to the level of functional down- or up-regulation, respectively.

clinical and virological parameters at baseline were assessed by multiple linear regression analysis in relation to immunological results, showing that HBsAg, ALT, and HBV DNA titers of the 2 patient cohorts did not significantly influence their different immunological behaviors, although age showed a weakly positive correlation with the T-cell function. Despite a strong immune modulatory effect, GS-4774 did not result in clinically significant declines in HBsAg levels in patients with CHB.

Ex Vivo Analysis of HBV-specific T-cell Responses

To further investigate the effect of therapy on T-cell responses, additional experiments were performed by ex vivo IFN-γ ELISpot assay following 18 hours of PBMC incubation with overlapping peptides covering the overall x, core, polymerase, and envelope sequences. Preliminary experiments were performed to maximally improve the sensitivity of the assay and define the optimal experimental



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conditions for a reliable detection of low-frequency T cells. Combined GS-4774 and TDF therapy significantly improved T-cell responses at week 48, as compared with baseline (Figure 4*A–C*, left graphs). No statistically significant difference was instead detected in the NUC-treated group (Figure 4*A–C*, right graphs). In the vaccine group, improvement at week 48 was predominantly sustained by core- (50% responsive patients) followed by polymerase-specific (30%) responses (Figure 4*D* and *E*, left graphs). Frequencies of peripheral HBV-specific IFN-γ+ T cells were significantly higher and more widely multispecific in the reference group of patients with self-limited acute hepatitis B than in chronic naïve viremic patients ($P = .004$ and $P = .0006$ for vaccine and control groups, respectively; Figure 4*A*). Despite the improvement of the overall T-cell responsiveness at week 48, ex vivo T-cell reactivity to envelope antigens in vaccine-treated patients remained negative even at the end of therapy, suggesting that envelope-specific responses are the most exhausted and more difficult to be restored (Figure 4*D* and *E*).

Phenotypic Analysis of the Total CD8 Population

During chronic viral infection, exhausted T cells can coexpress different inhibitory markers in association with different levels of memory/differentiation molecules.[17–19] Because of the low number of HLA-A*0201+ patients in the vaccine-treated group, we were unable to perform a phenotypic analysis on HBV-specific T cells in the peripheral blood with HBV peptide HLA-A*0201 dextramers. Therefore, the expression of the inhibitory receptors PD-1, TIGIT, CD39, and the differentiation marker CD127 was monitored on total CD8+ T cells throughout the course of vaccine treatment. Combined GS-4774 and TDF therapy significantly reduced the frequency of PD-1+/CD127− CD8 T cells compared with baseline time point, whereas no significant modulation was observed in the NUC-treated control group (Supplementary Figure 7). Expression of TIGIT and CD39 on CD8 cells was not affected by treatment (not shown). These data suggest that CD8 T cells of patients treated with vaccine therapy underwent a progressive phenotypic modulation leading to the expression of a less-exhausted T-cell profile as compared with patients treated with NUC alone.

Impact on Treg Cells

Treg cells can exert a negative regulatory role on HBV-specific T-cell responses.[20,21] Recent work showed that the yeast-based Tarmogen vector can reduce frequency and inhibitory function of Tregs.[13] Thus, we examined the effect of GS-4774 and TDF therapy on the Treg population throughout the course of treatment and the correlation between Treg and T-cell responses. Vaccine treatment significantly reduced the frequency of total Tregs, as indicated by the progressive decline of the $CD25^{hi}FOXP3+CD4+$ Treg cell percentage during GS-4774 therapy, whereas no modulation was observed in the control group of NUC-treated patients (Figure 5*A*). In addition, when we directly compared Treg frequency and IFN-γ or TNF-α production by HBV-specific CD8+ T cells throughout the course of treatment, an inverse significant correlation was observed in vaccine- but not in NUC-alone–treated patients (Figure 5*B*). We then separated the Treg population into 3 phenotypically and functionally distinct T-cell subsets by the expression of CD45RA, CD25, and FOXP3,[22] namely $FoxP3^{hi}CD45RA\text{-}CD25^{hi}$ activated Treg cells, $FoxP3^{low}CD45RA+CD25^{hi}$ resting Treg cells, and $FoxP3^{low}CD45RA\text{-}CD25^{hi}$ nonsuppressive Treg cells (conv-Treg) (Figure 5*C*). Notably, GS-4774 vaccine treatment significantly decreased the frequency of activated Treg cells; although the decline of resting Tregs did not reach statistical significance, the overall phenotypic profile was consistent with a reduced suppressive Treg function (Figure 5*C*). Conv-Treg cells instead remained numerically stable throughout the course of GS-4774 treatment but up-regulated Ki67 and HLA-DR, suggesting a predominant stimulation of memory-like non-Treg cells associated with a contraction of the suppressive Treg cell component (Figure 5*D*). Remarkably, no significant changes were observed in the control group of patients receiving NUC therapy alone (Figure 5*C* and *D*, white dots).

Discussion

In patients with viremic CHB not on oral antiviral therapy, the yeast-based GS-4774 vaccine containing HBV core, envelope, and x proteins was safe and well tolerated but did not result in significant reductions in mean HBsAg levels in any treatment group evaluated through week 48. Only a

Figure 2. Profiles of HBV-specific CD4+ and CD8+ T-cell responses after in vitro expansion in individual patients treated with GS-4774 + TDF or NUC alone. Longitudinal analysis (baseline, week 12, week 24, week 48) of IFN-γ, TNF-α, IL-2 production and CD107a degranulation by CD4+ and CD8+ T cells after in vitro expansion in individual treated patients. Individual T-cell functions were assumed to be improved in a given patient when 2 criteria were met simultaneously, namely responses higher than baseline in at least 2 time points during therapy and follow-up and a fold-increase greater than 2 relative to baseline in at least one of them. T-cell functions that did or did not meet the previously described criteria for the definition of improved responses are illustrated with the *pink and gray* background, respectively. The *y*-axis refers to the percentage of CD8 and CD4 T cells derived from each treated patient able to produce cytokines and to degranulate following 10 days of stimulation with overlapping peptides. The *x*-axis indicates the time points of analysis. Statistical significance was assessed by χ^2 test comparing the following: (a) the numbers of improved T-cell functions between CD8 responses in patients treated with combined therapy and in patients treated with NUC alone ($P = .0003$); (b) CD8 vs CD4 T-cell responses in GS-4774 plus TDF-treated patients ($P = .006$). In the *green area*, improvement of individual T-cell functions in each patient is represented as fold change (*color bars*) between HBV-specific CD8+ and CD4+ T-cell functions at weeks 12, 24, 48 and the corresponding baseline values (statistical significance between CD8 vs CD4 T-cell responses in GS-4774 plus TDF-treated patients by the Wilcoxon signed rank test for paired data; $P = .037$ and $P = .019$ for IFN-γ and TNF-α production at week 12, respectively; $P = .0098$ and $P = .0098$ for IFN-γ and TNF-α production at week 24, respectively; $P = .0098$, $P = .0049$ and $P = .0186$ for IFN-γ, TNF-α, and IL2 production at week 48, respectively).

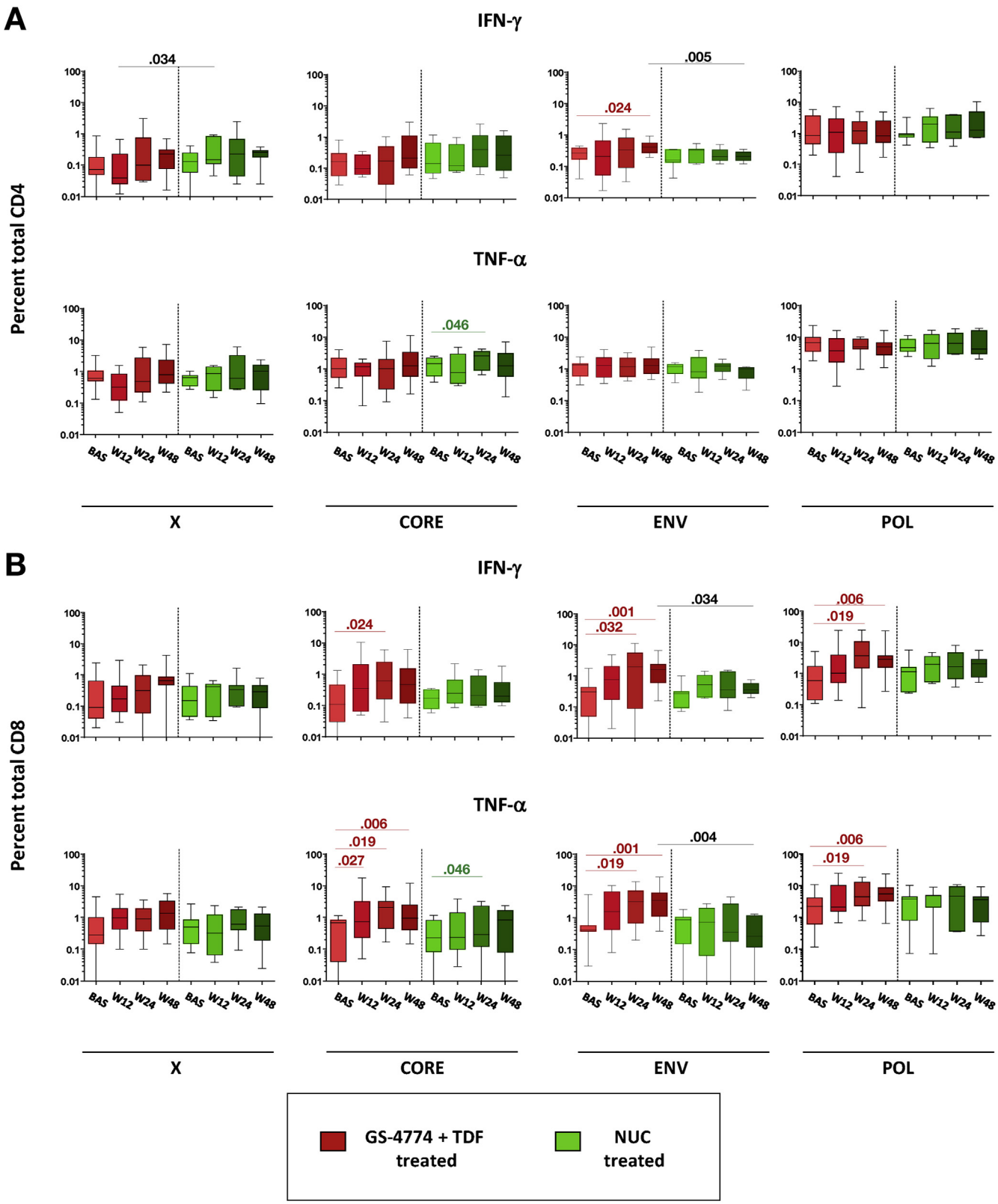

Figure 3. HBV-specific CD4+ and CD8+ T-cell responses to individual HBV antigens. Cytokine production was analyzed after 10 days of stimulation with different HBV peptide pools in patients with chronic HBV undergoing GS-4774+TDF (n = 11, *red bar*) or NUC treatment (n = 10, *green bar*). Each bar represents the median percentage plus 5 to 95 percentile of CD4+ (*A*) and CD8+ (*B*) T cells producing IFN-γ and TNF-α in response to peptide pools spanning distinct HBV regions. Significant improvements at treatment (week 12, week 24) and follow-up (week 48) time points compared with baseline are shown (statistical significance by the Wilcoxon signed rank test for paired data).

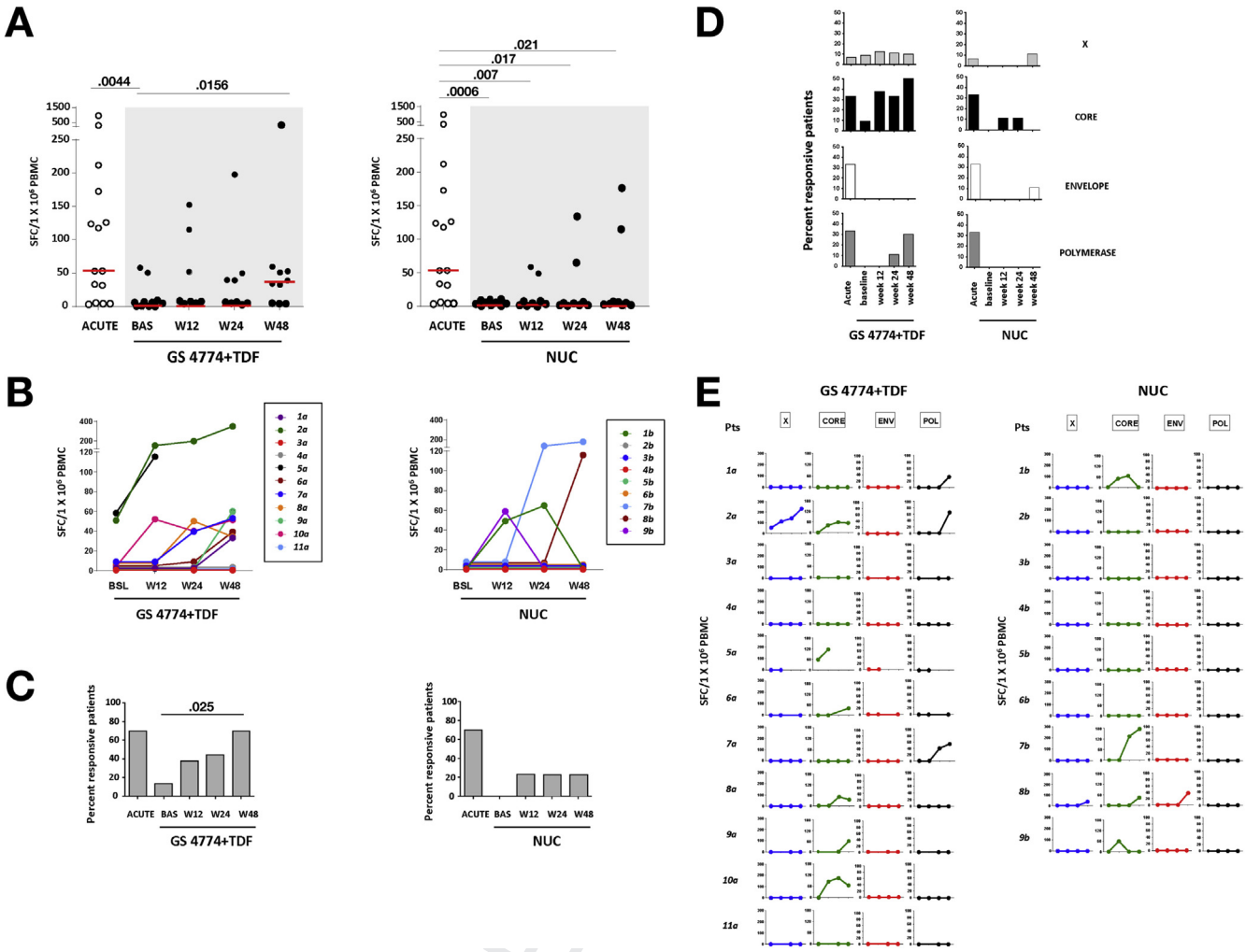

Figure 4. Ex vivo functional profile of HBV-specific T-cell responses. (*A*) Frequencies of IFN-γ secreting cells after 18 hours of stimulation with the overall HBV peptide panel, assessed by ELISpot assay in patients with acute (n = 15) and chronic HBV under GS-4774 and TDF therapy (n = 11) or under therapy with NUC alone (n = 9) at sequential time points (Bas, week 12, week 24; week 48). Each symbol represents the total frequency of IFN-γ–secreting cells calculated in each patient by summing positive responses to individual peptide pools after subtraction of the background; *red lines* indicate the median values of IFN-γ–secreting cells in the indicated time points. ELISpot was considered positive if the number of spots in the stimulated wells was at least 3 standard deviations above background and the difference between the number of spots in the stimulated and unstimulated wells was above 10. Statistical significance was assessed by the Mann-Whitney *U* test for nonpaired samples and the Wilcoxon signed rank test for paired data. (*B*) Longitudinal analysis of IFN-γ production in each treated patient (GS-4774+TDF on the *left* and NUC alone on the *right*) represented as line graphs. (*C*) Percentage of responsive patients is illustrated; a subject was considered responder when 1 or more peptide pool could elicit a positive response. Statistical significance was assessed by χ^2 and Fisher F tests. (*D*) Percentage of responsive patients to individual HBV antigens in the indicated populations (acute, GS-4774+TDF-treated and NUC-treated patients). (*E*) Longitudinal analysis of IFN-γ production to individual HBV antigens in individual treated patients. The *x*-axis indicates the time points (Bas, week 12, week 24 and, week 48) whereas the *y*-axis illustrates the spot-forming cells (SFC) per 1×10^6 cells.

small proportion of patients in our study demonstrated HBsAg declines of ≥0.5 $\log_{10}$ IU/mL and no statistical significance was reached at any week evaluated (weeks 12, 24, and 48). However, at the end of GS-4774 therapy (week 24), the only patients observed to have ≥0.5 $\log_{10}$ IU/mL reductions in HBsAg were those who received GS-4774. Furthermore, patients treated with the highest GS-4774 dose showed a trend toward significance in HBsAg decline compared with the TDF-only group (Supplementary Figure 1*G*, $P = .076$).

GS-4774 in combination with TDF was able to induce a significant improvement of IFN-γ, TNF-α, and IL2 production by HBV-specific CD8+ T cells in a subset of treatment-naïve viremic HBeAg-negative patients, which was not observed in the control group of patients treated with NUC alone. This effect was more pronounced on HBV-specific CD8 than on CD4 T cells. Interestingly, when breadth and quality of T-cell responses were analyzed in each individual patient, at least 2 of the analyzed CD4 and CD8 functions appeared to be always improved by therapy with the single

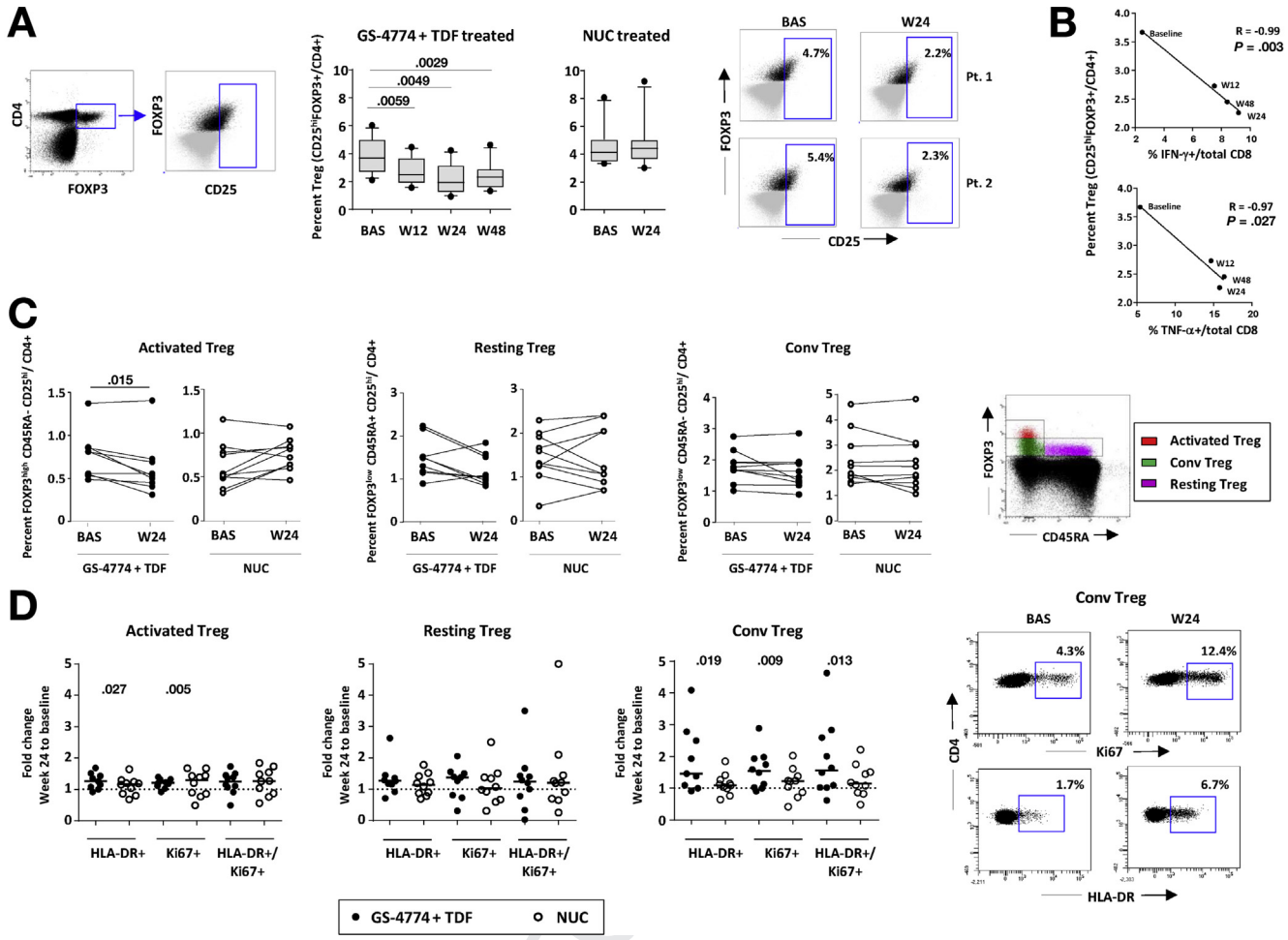

Figure 5. Effect of combined GS-4774 and TDF therapy on Treg cells. (*A*) CD3, CD4, CD25, and FoxP3 were used to identify Treg cells. The frequency of CD4+FoxP3+CD25^{hi} Tregs was evaluated in PBMCs by flow cytometry before (baseline), during (week 12 and week 24) and after (week 48) combined GS-4774+TDF treatment. Results are expressed as median percentage of Tregs in chronic patients undergoing GS-4774+TDF or NUC treatment (n = 11 and n = 10, respectively) at the indicated time points. The Wilcoxon signed rank test was used to analyze paired samples. Fluorescence-activated cell sorter (FACS) plots from 2 representative patients at baseline and week 24 are illustrated on the *right*. (*B*) Inverse correlation between mean Treg frequency and mean IFN-γ or TNF-α production by HBV-specific CD8+ T cells in GS-4774 plus TDF-treated patients throughout the course of therapy by the Pearson's correlation test. (*C*) Separation of the FoxP3+CD4+CD25^{hi} T-cell population into 3 phenotypically distinct subsets by the expression of CD45RA: FoxP3^{hi}CD45RA-CD25^{hi} activated Treg cells, FoxP3^{low}CD45RA+CD25^{hi} resting Treg cells, FoxP3^{low}CD45RA-CD25^{hi} nonsuppressive Treg cells. The different Treg subsets were analyzed longitudinally in GS-4774 + TDF-treated (*black dots*) or NUC-treated (*white dots*) patients (n = 9 and n = 10, respectively); statistics by the Wilcoxon signed rank test. A representative FACS plot is illustrated on the *right graph*. (*D*) Isolated and combined expression of HLA-DR and Ki67 on Treg cells of each subset (activated, resting, nonsuppressive Tregs) before (baseline) and during (week 24) combined GS-4774+TDF or NUC treatment (n = 9 and n = 10, respectively); each *dot* represents the fold change between week 24 of treatment and the corresponding baseline value; statistics by the Wilcoxon matched-paired test. FACS plots from a representative patient showing HLA-DR and Ki67 expression by Conv-Treg cells at baseline and week 24 are represented on the *right*.

exception of a patient who was totally refractory to the modulatory effect of GS-4774. Magnitude of T-cell restoration did not correlate with decline of HBsAg levels, which remained almost totally unchanged in all patients, irrespective of the level of their HBV-specific T-cell reactivity. A delayed effect on HBsAg loss cannot be excluded because follow-up was limited to 24 weeks. Moreover, better efficacy should be likely achievable with higher GS-4774 doses, but safety data from earlier studies limit dosage levels.

HBV polymerase was the predominant specificity among the circulating T-cell pool detectable following 10 days of expansion in vitro before and during therapy, whereas increase of T-cell responses induced by vaccine therapy was sustained by all HBV antigens, with the exception of x, even if the GS-4774 vaccine does not contain polymerase. Although we were unable to show a clear modulation of the activation state of circulating dendritic cell and monocytes by therapy, the enhancement of polymerase-specific T-cell responses during therapy may be related to the described adjuvant effect exerted by the yeast component of the GS-4774 vaccine[12] allowing to improve antigen presentation by in vivo HBV antigen preloaded dendritic cells.[23]

Moreover, lack of phenotype modulation of peripheral antigen-presenting cells does not exclude changes in their activation state within lymph nodes and liver at the site of antigen presentation.

The increased reactivity of HBV envelope-specific T cells detected following 10 days of in vitro expansion contrasts with the lack of response to envelope peptides observed by ex vivo analysis after a few hours of peptide stimulation. Because short-term contact with antigen in ex vivo assays is expected to primarily stimulate in vivo activated effector T cells, which are ready to express their function, lack of ex vivo responses may indicate a partial and incomplete restoration of envelope-specific T-cell functions with a poor capacity to generate terminally differentiated effectors. Although conclusions must be drawn carefully because ex vivo analysis was limited to IFN-γ detection, our data are in line with the concept that envelope-specific responses are the most exhausted and more difficult to be restored, as a possible result of the high amounts of envelope antigens that are constantly present in the circulation and liver of chronic patients.

Despite the improvement induced by vaccination, the overall HBV-specific T-cell response only rarely became comparable to what was observed in acute self-limited infections, which represent the reference for quality and strength of T-cell reactivity associated with successful control of infection. This partial T-cell restoration detected both in vitro and ex vivo may explain the minimal effect on HBsAg levels observed in treated patients. Intensity and poly-functionality of T-cell responses, assessed as percentage of cytokine-producing T cells and number of improved functions induced by therapy, were better in a subgroup of patients, but these differences were not correlated with baseline levels of serum HBsAg and HBV DNA or with baseline efficiency of T-cell responses before starting therapy. Interestingly, a decreased frequency of PD-1+/CD127− CD8 T cells was detected in patients treated with vaccine and TDF, suggesting that therapeutic vaccination can induce a progressive phenotypic modulation toward a less-exhausted T-cell profile, as compared with patients treated with NUC alone.

The effect of vaccination on HBV-specific T-cell responses was associated with a modulation of Tregs. In CHB infection, a suppressive effect of Tregs on T cells has been reported; it can be mediated by direct T-cell-cell contact and by secretion of suppressive cytokines inhibiting the development of an efficient HBV-specific T-cell functionality.[20,21] As a likely effect of the yeast component of the vaccine, GS-4774 therapy significantly reduced the frequency of total Tregs as well as the percentage of activated and resting Treg cell subpopulations, which was not seen in control patients treated with NUC alone. Instead, the number of conventional nonsuppressive Treg cells tended to be constant throughout the course of treatment. Conventional Treg cells, however, up-regulated proliferation and activation markers during GS-4774 therapy, indicating a predominant stimulation of memory-like non-Treg cells on vaccine therapy. Thus, these findings demonstrate that the yeast-based GS-4774 T-cell vaccine can decrease the frequency of Treg subpopulations that are known to express suppressive activity, while favoring the activation of the nonsuppressive conventional Treg cell subset.

In summary, our study indicates that although the GS-4774 vaccine was unable to elicit a significant decline of HBsAg, it can efficiently induce CD8 T-cell responses. This is certainly relevant because the CD8-mediated function is known to be essential for the control of infection. On the other hand, however, lack of effect on HBV-specific CD4 T cells may explain why vaccination was ineffective on HBsAg levels, in view of the essential role played by HBV-specific CD4 T cells in providing help to B cells and CD8+ T cells. The improvement of baseline HBV-specific T-cell responses was sustained by all antigenic specificities. Interestingly, also HBsAg-specific responses, which are expected to be profoundly exhausted in chronic patients, were improved by therapy when assessed after expansion in vitro but not by ex vivo analysis. This implies that, despite some functional improvement, frequency of HBsAg-specific T cells remained quite low in vivo with poor terminal effector differentiation. Together with the lack of CD4 induction, partial restoration of envelope-specific T-cell responses, as suggested by their lack of detection ex vivo, may be another important cause of the vaccine failure in reducing HBsAg load. This is suggested by their known association with complete control of infection and anti-HBs seroconversion,[16] although the opposite possibility that persistent HBsAg may limit functional restoration of HBV envelope-specific T cells cannot be totally ruled out.

In conclusion, our study shows that HBV-specific CD8 T cells of chronic naïve patients are not totally refractory to an exogenous antigen stimulation even when antigen administration through vaccination is not preceded by a prolonged control of virus replication by NUC therapy. If the conclusions of the present study are interpreted in the context of our prior results, a first element of the overall emerging picture is that NUC therapy can induce a progressive improvement in the efficiency of T-cell responses, as a function of the time of treatment, and that at least 2 to 3 years of therapy are needed to induce detectable and durable improvements in HBeAg-negative CHB.[16,24] However, only the limited proportion of NUC-treated patients who succeed in anti-HBs seroconversion can achieve optimal levels of T-cell functional restoration, with quality and strength of responses very similar to what is detectable after spontaneous resolution of an acute infection. Vaccination and TLR7 stimulation can improve the effect of NUC on T-cell responses but they are not sufficient to permit the acquisition of an optimal immune reactivity adequate to provide complete control of infection.[24] Altogether, our data suggest that restoration of an efficient T-cell function may still occur after decades of exposure to high antigen loads, thereby confirming that immune modulation can represent a successful approach to cure HBV infection. However, the way to rapidly reconstitute a completely protective immune response still needs to be defined. In particular, future combination therapies including vaccines should consider the sequential administration of drugs able to improve T-cell responsiveness to

antigen stimulation by lowering the antigen load, such as, for example, silencing RNA compounds or capsid assembly inhibitors, or by inhibiting immune checkpoints or modulating T-cell metabolism, followed by T- and B-cell boosting through vaccination.[19,25]

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2019.03.044.

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Author contributions: CB, MR: immunology study concept and design, execution of experiments, acquisition of data, analysis and interpretation of data; drafting of the manuscript; statistical analysis. HLAJ, SKY, EMY, HT, TR, SF, SHA, XM: acquisition of data, recruitment and characterization of patients during efficacy study. AV, VB, PF, GA: execution of experiments, acquisition of data, analysis and interpretation of data, statistical analysis. DL: administrative support. AA, FB, CC, MM, RS, VP, BC, DC: recruitment and characterization of the patients during immunology study. GP: statistical analysis. PA, MRB, YZ, AJ, AM: interpretation of data, critical revision of the manuscript. AHL, AG, GMS, BM, JW: Clinical trial design, execution of clinical study, and/or clinical study oversight, critical revision of the manuscript. CF: immunology study concept and design, interpretation of data, critical revision of the manuscript, obtained funding, study supervision.

Conflicts of interest

These authors disclose the following: Harry L.A. Janssen: Gilead, AbbVie, BMS, Janssen, Medimmune, MSD, Novartis, Roche, Tekmira. Eric M. Yoshida: Gilead, AbbVie, Merck, Springbank, Janssen, Intercept, Celgene. Huy N. Trinh: Gilead, Intercept, Assembly. Tim C. Rodell: GlobeImmune, Inc. Pietro Andreone: Grant: Roche, MSD, Gilead Sciences, Consultant: Roche, MSD, Janssen Cilag, AbbVie, Boehringer Ingelheim, Gilead Sciences, Intercept, BMS. Alessandra Mangia: Grant: Roche, MSD, Janssen, Gilead, Consultant: Roche, MSD, Janssen Cilag, Boehringer Ingelheim, Gilead Sciences, BMS. Maurizia R. Brunetto: Grant: AbbVie, BMS, MSD, Consultant: BMS, Gilead, Janssen, Roche, AbbVie, MSD. Yang Zhao, Audrey H. Lau, Anuj Gaggar, G. Mani Subramanian, Jacky Woo, Adarsh Joshi, and Benedetta Massetto are employees of Gilead Sciences. Scott Fung: Gilead, Merck, AbbVie, BMS. Sang Hoon Ahn: Gilead Sciences, AbbVie, Green Cross, Janssen, Assembly Biosciences, Arbutus Biopharma. Carlo Ferrari: Grant: Gilead Srl, Bristol Squibb, Roche Spa, AbbVie; Consultant: AbbVie, Arrowhead, Vir Biotechnology Inc., Abivax, Inovio Pharmaceuticals, Transgene. Xiaoli Ma: Gilead. The remaining authors disclose no conflicts.

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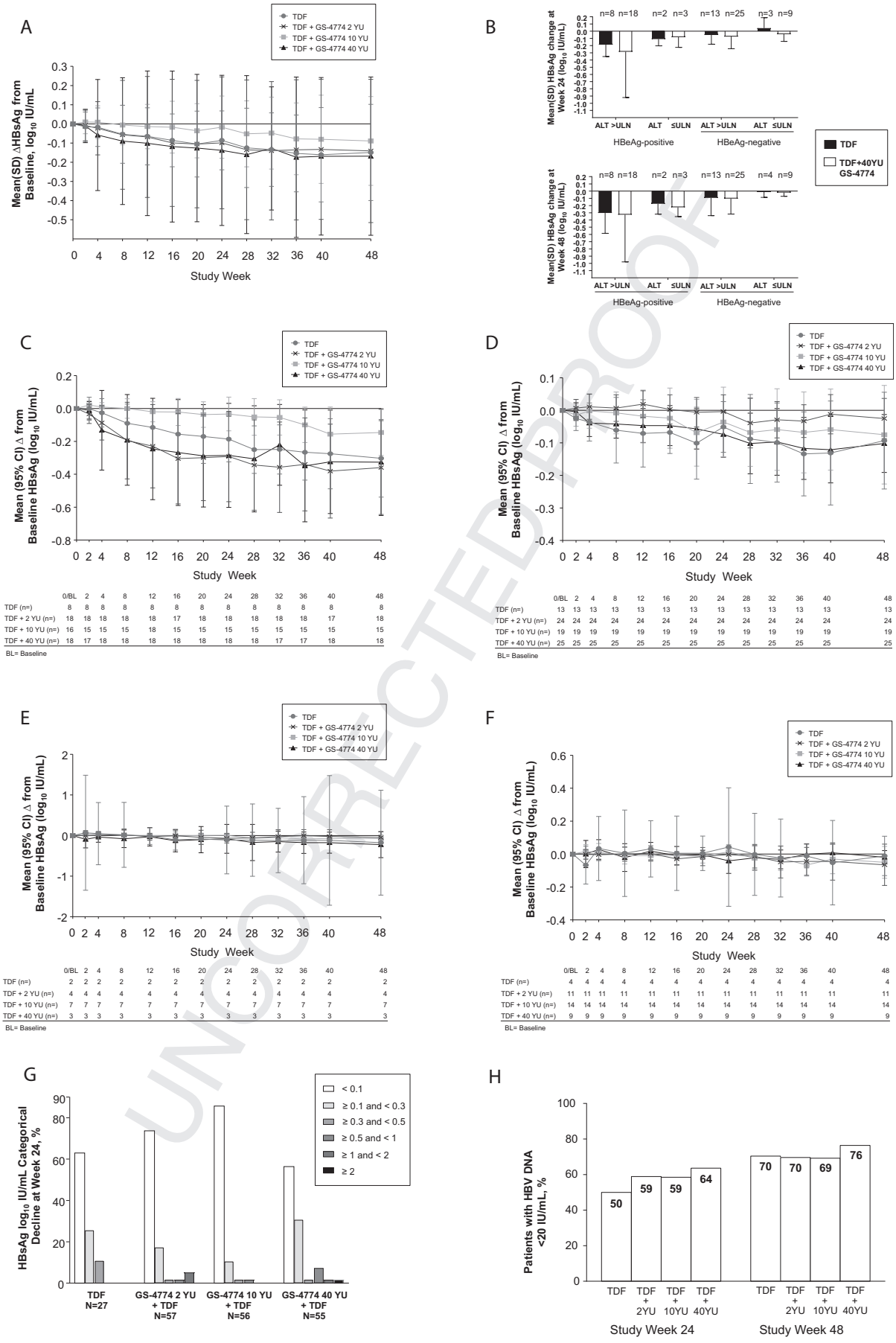
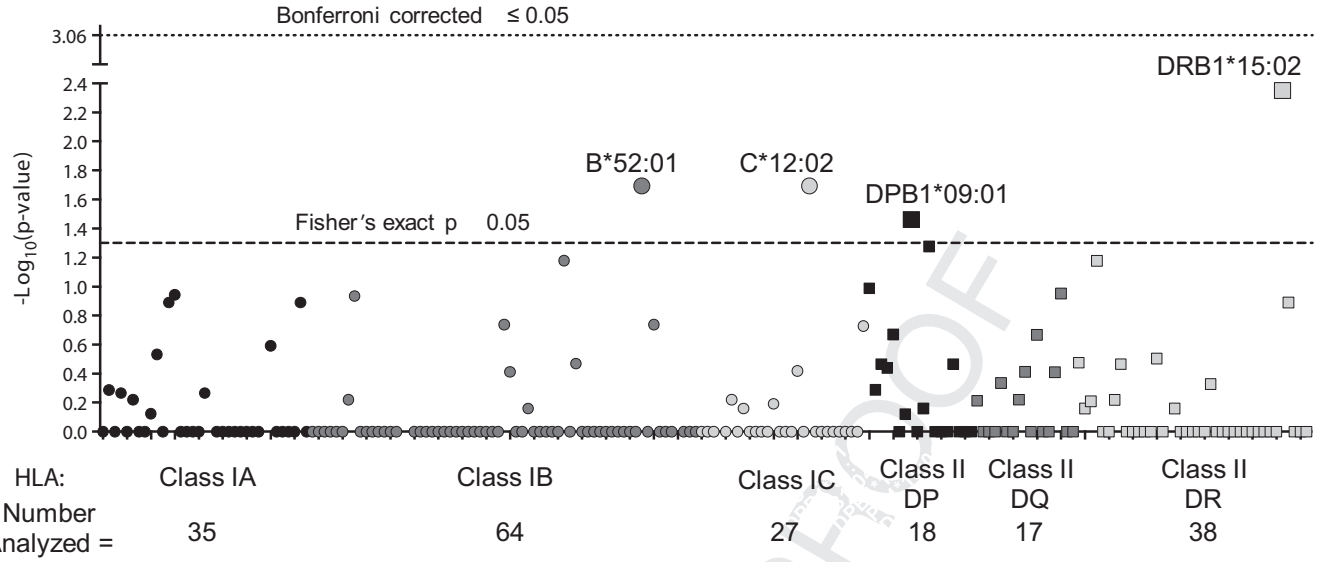
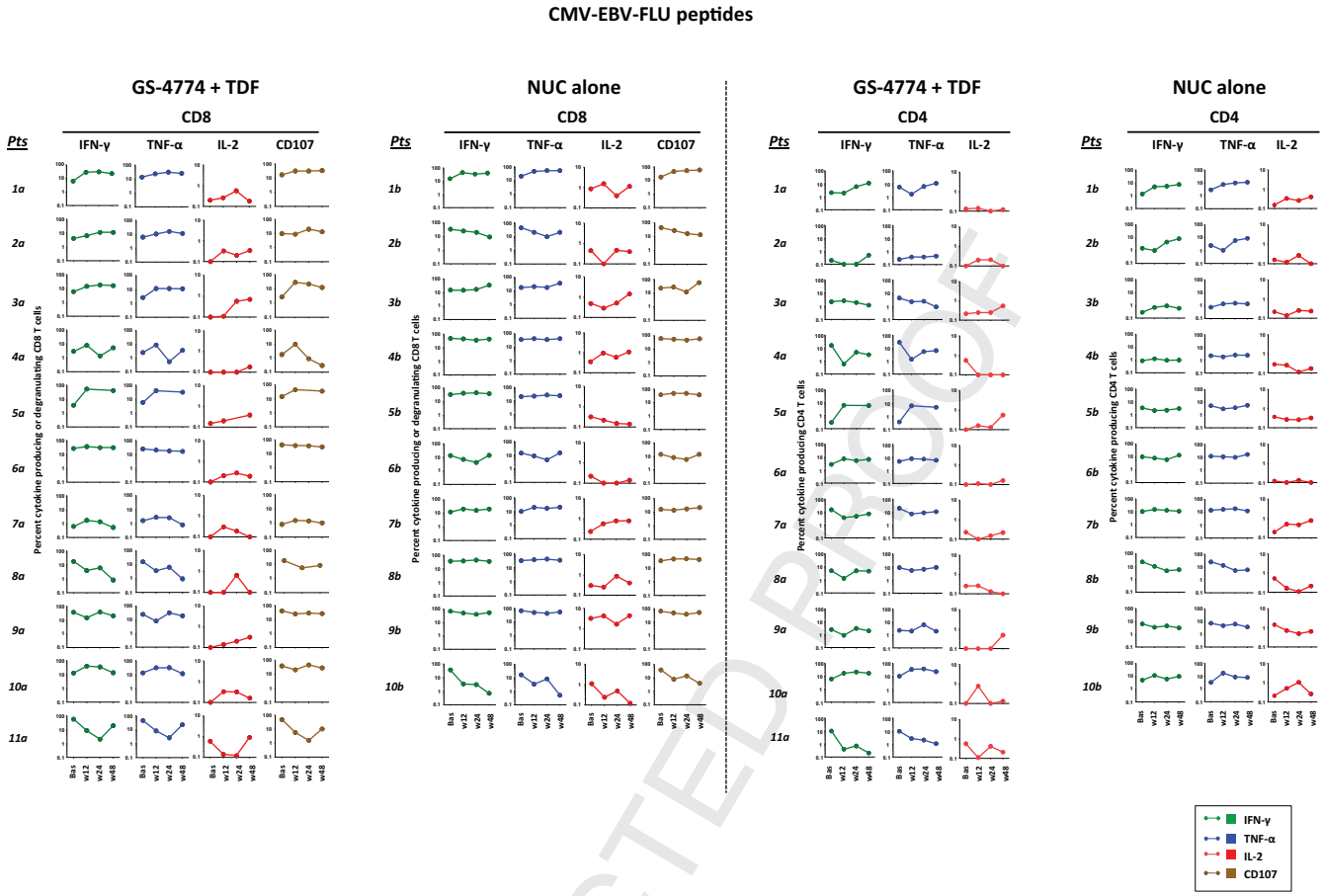


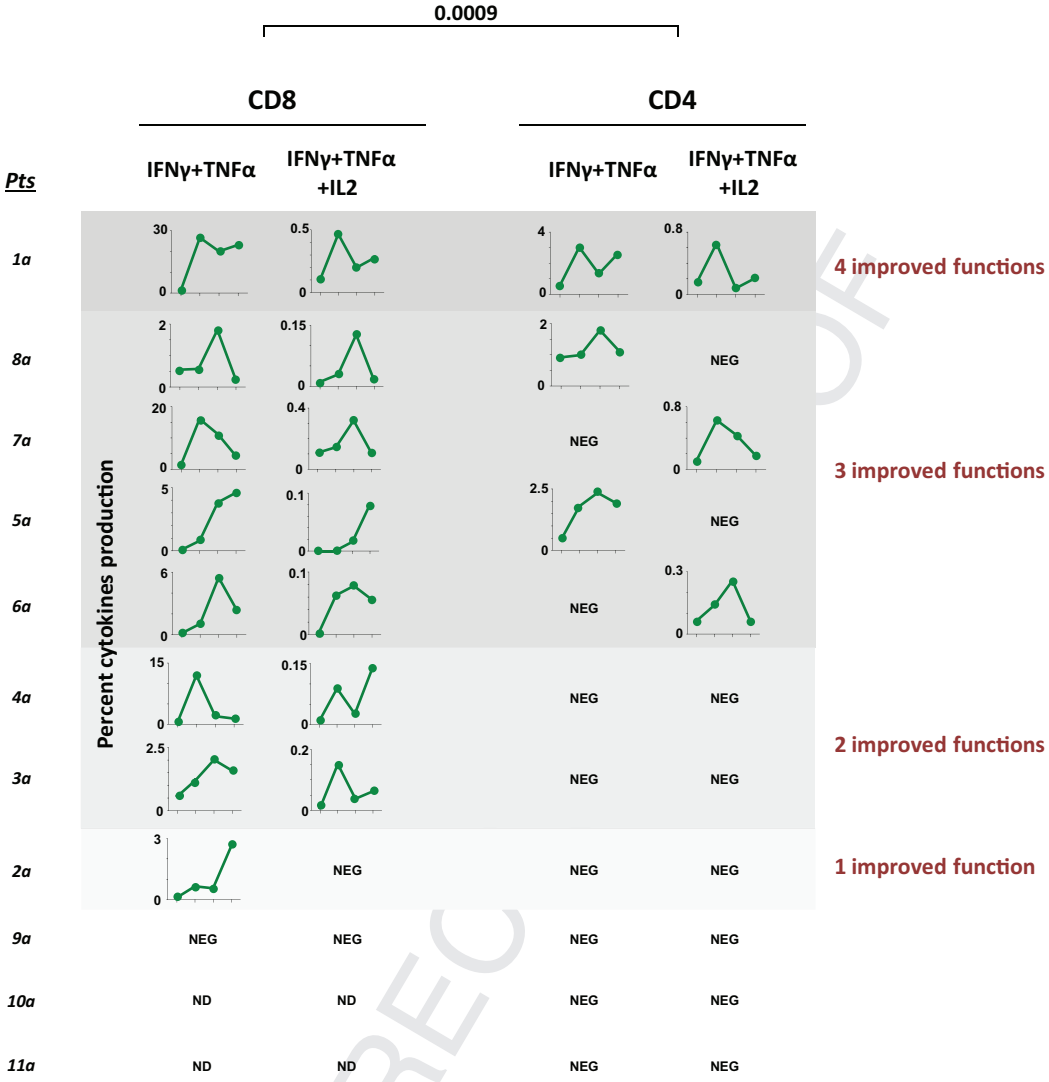


Supplementary Figure 2. GS-4774 HLA Allele Association Assessment. The association of HLA class I and class II antigens to clinical response (≥0.5 log decline) was examined by Fisher's exact test. The dashed line represents a Fisher's exact value of $P \leq 0.05$ and the dotted line represents a Bonferroni corrected value of ≤0.05.

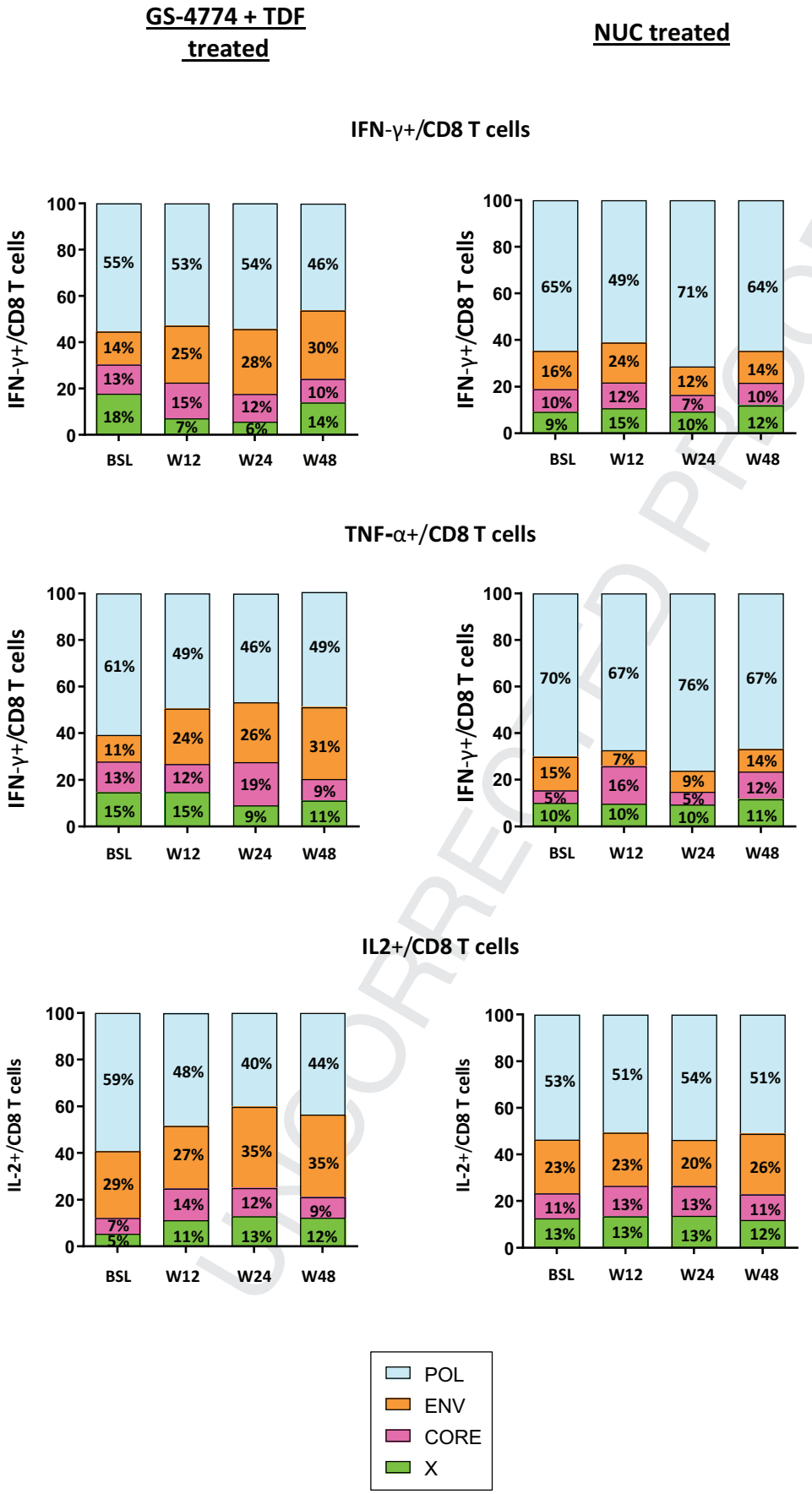
Supplementary Figure 1. GS-4774 Efficacy Assessment. (*A*) Mean HBsAg (log10 IU/mL) levels were measured for changes in comparison to baseline at through Study Week 48; (*B*) Change from baseline to week 24 and 48 in HBsAg (log10 IU/ml) in patient subgroups stratified by baseline ALT level (>ULN or ≤ULN) and HBeAg status (positive or negative); (*C*) Mean (95% CI) HBsAg (Log10 IU/mL) Change from Baseline by Visit: ALT >ULN and HBeAg+ at Baseline; (*D*) Mean (95% CI) HBsAg (Log10 IU/mL) Change from Baseline by Visit: ALT >ULN and HBeAg- at Baseline; (*E*) Mean (95% CI) HBsAg (Log10 IU/mL) Change from Baseline by Visit: ALT ≤ULN and HBeAg+ at Baseline; (*F*) Mean (95% CI) HBsAg (Log10 IU/mL) Change from Baseline by Visit: ALT ≤ULN and HBeAg- at Baseline; (*G*) Categorical percentage decline of HBsAg by treatment after 24 weeks: GS-4774 2 YU vs TDF only (P=.155), GS4774 10 YU vs TDF only (P=.408), GS4774 40 YU vs TDF only (P=.076); (*H*) HBV DNA levels <LLOQ by treatment at Study Weeks 24 and 48.



Supplementary Figure 3. Behaviour of CMV/EBV/FLU-specific CD4+ and CD8+T cells after in vitro expansion in individual patients treated with GS-4774 + TDF or NUC alone. Longitudinal analysis (baseline, w12, w24, w48) of IFN-γ, TNF-α, IL-2 production and cytotoxic potential (CD107a) of CD4+ and CD8+ T-cells after in vitro expansion in individual treated patients. Y-axis refers to the percentage of CD8 and CD4 T-cells derived from each treated patient able to produce cytokines and to degranulate following 10 days stimulation with CMV/EBV/FLU-specific overlapping peptides. The x-axis indicates the time points of analysis.



Supplementary Figure 4. Behaviour of multifunctional HBV-specific CD4+ and CD8+T cells in relation to GS-4774+TDF treatment. Longitudinal analysis (baseline, w12, w24, w48) of double IFN-γ+/TNF-α+ and triple IFNγ+/TNFα+/IL2+ HBV-specific CD8+ and CD4+ T cells after in vitro HBV peptide stimulation in individual GS-4774+TDF treated patients. The definition of improved T cell response in individual patients was based on the simultaneous presence of two criteria: two multifunctional responses higher than baseline during therapy and follow-up with a fold increase greater than 2 in at least one of them. Different multifunctionality profiles were identified according to the different numbers of improved multifunctional responses: one patient able to recover 4 analyzed parameters, four patients able to improve 3 different parameters, three able to improve 1 or 2 parameters and three with no functional improvement. Statistical significance was assessed by the Chi Square test and Fisher F tests by comparing the numbers of improved T cell functions between CD8 and CD4 T cell responses in GS-4774 plus TDF treated patients.



Supplementary Figure 5. Contribution of each HBV antigen to the overall HBV-specific T cell response. Column charts represent the stimulatory activity of each HBV antigen within the total population of IFNγ (top), TNFα (*middle*), IL2 (*bottom*) producing CD8+ T cells in chronic naïve patients under combined GS-4774 and Tenofovir therapy (n=11) and under NUC therapy alone (n=10) at sequential timepoints (Bas, w12, w24; w48). Columns illustrate the contribution (expressed as percentage) of each antigenic region to the global HBV-specific cytokine production before, during and after therapy.

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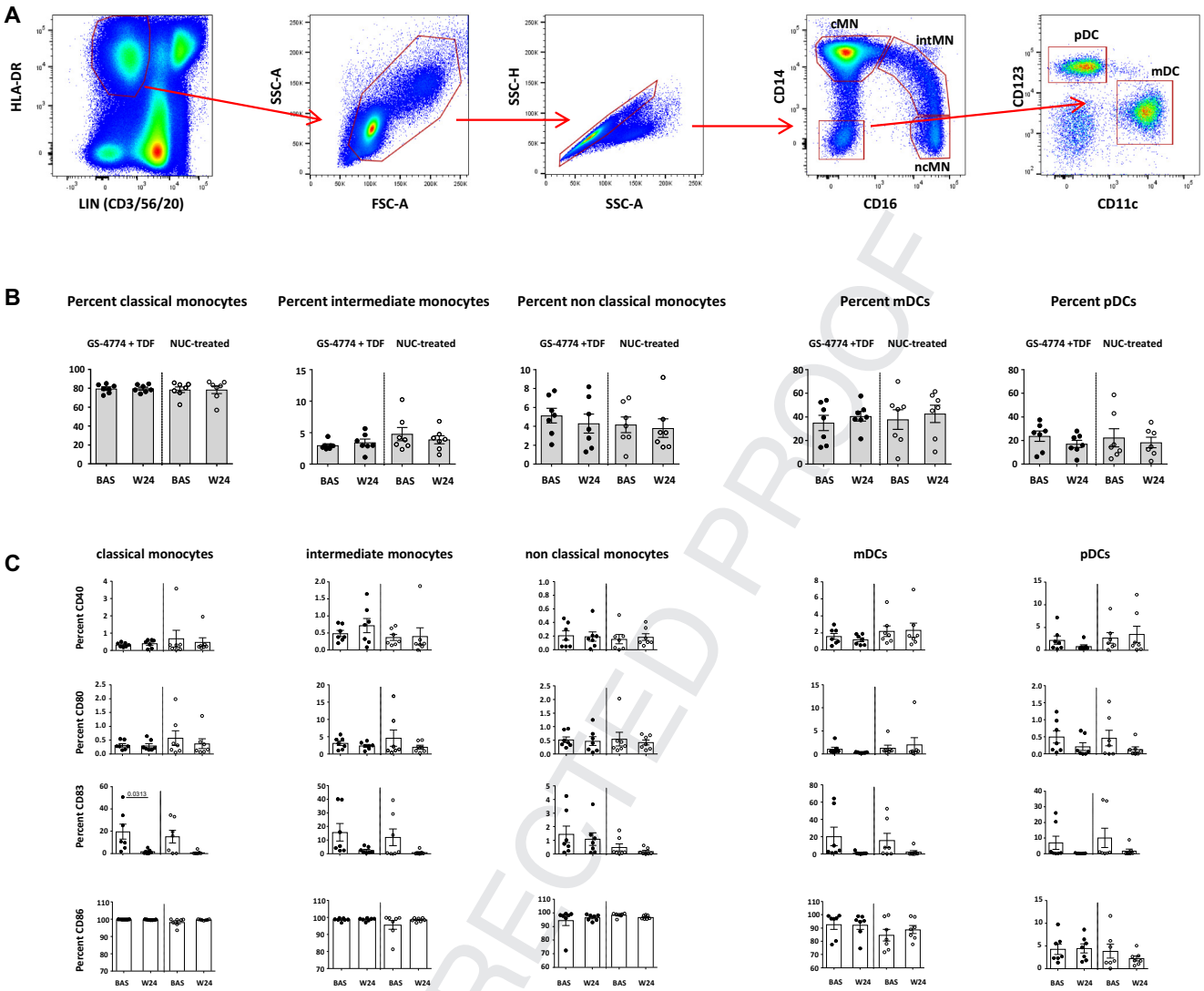
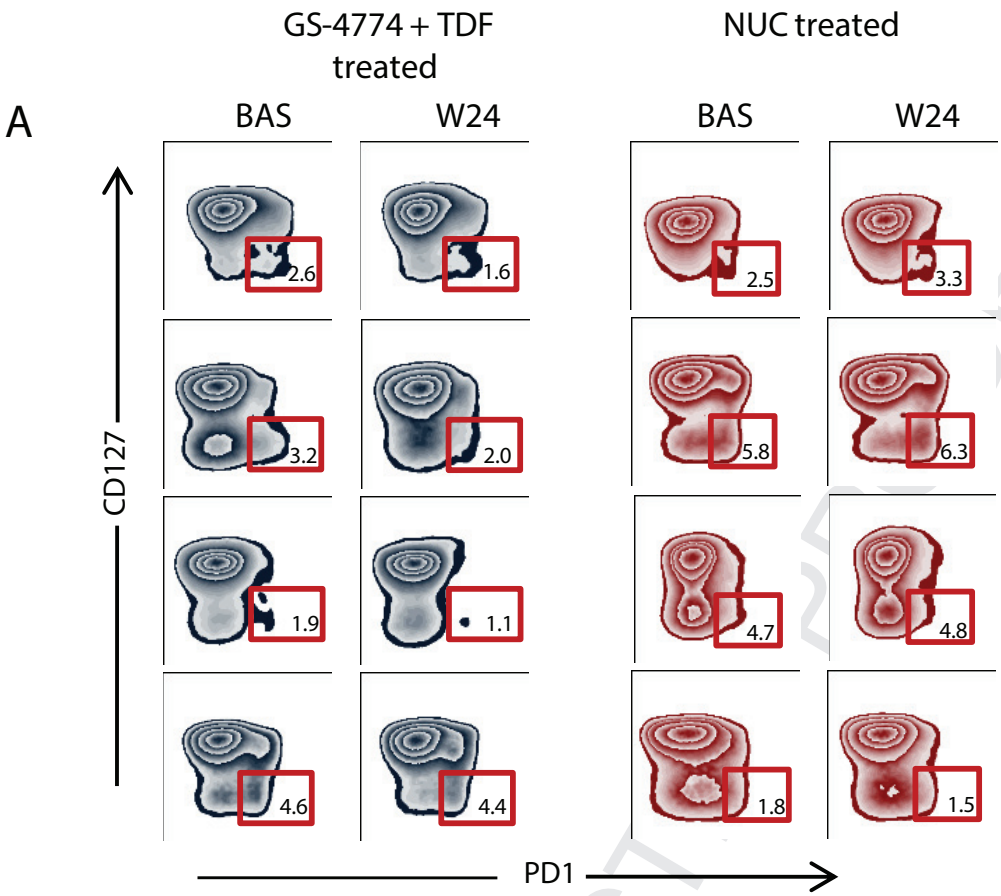
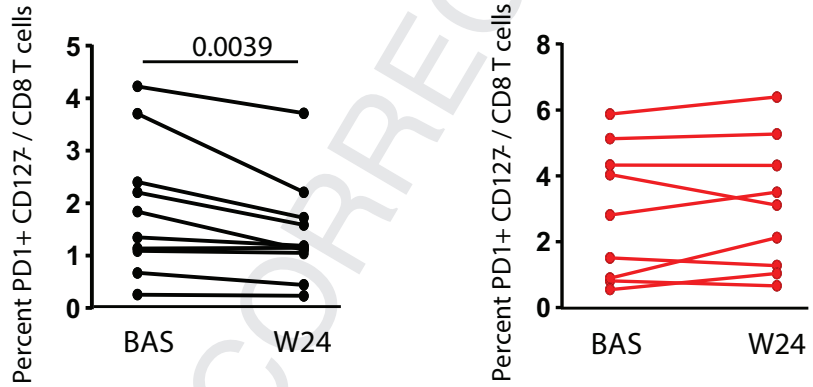
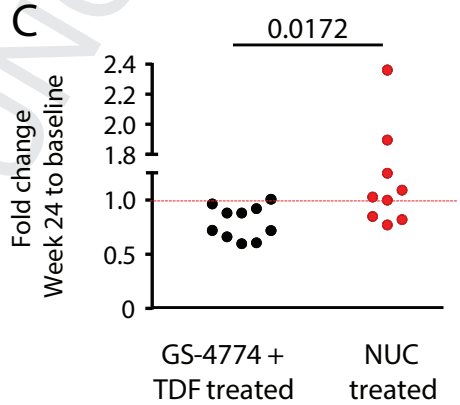


Supplementary Figure 6. Ex vivo analysis of dendritic cells and monocytes in patients treated with GS-4774+TDF or NUC alone. (*A*) Antigen presenting cells (APCs) gating strategy. Lineage-negative (CD3/CD56/CD20) HLA-DR+ APCs were derived from total live PBMCs and gated by forward and side scatter followed by single-cell using area and height parameters. Three distinct subsets of monocytes were identified:"classical" CD14+CD16- (cMN), "non classical" CD14^{low}CD16+ (ncMN), and "intermediate" CD14+CD16+ (intMN). CD11c myeloid DCs (mDC) and CD123 plasmacytoid DCs (pDC) were instead identified from the CD14/CD16 double negative population. *(B)* Graphs show the frequencies of the 5 distinct cell populations before (baseline) and during (w24) treatment in the indicated patient groups. Each symbol indicates a patient and bar shows mean value plus standard error; statistics by the Wilcoxon-signed-rank test. *(C)* Expression of co-stimulatory and activation molecules (CD40, CD80, CD86) as well as maturation markers (CD83) was assessed on each cell populations before (baseline) and during (w24) treatment (n=7 patients for both GS-4774+TDF and NUC alone therapy). Results are expressed as mean frequency plus standard error of each markers detected in the overall patient population at the indicated time points; statistics by the Wilcoxon-signed-rank test.







Supplementary Figure 7. T-cell phenotype in patients treated with GS-4774+TDF or NUC alone. (*A*) Representative plots of PD-1 and CD127 co-expression on total CD8 T cells from chronic HBV patients under GS-4774 and Tenofovir therapy (left panels) or NUC alone (right panels) at sequential time points (Bas and w24; n=10 and n=9, respectively). (*B*) Frequencies of $CD127^{low}$ PD-1+ CD8+ T cells before (baseline) and during (w24) treatment in the indicated patient groups. Each symbol indicates a patient; statistics by the Wilcoxon-signed-rank test. (*C*) Fold-change of $CD127^{low}$ PD-1+ T-cell subsets analyzed in the global CD8+ T-cell population, as the ratio between the frequency of $CD127^{low}$ PD-1+ T cell in GS-4774 + TDF treated or NUC treated patients at w24 with respect to the corresponding baseline values (statistics by the Wilcoxon-signed-rank test).

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