Research Article
Viral Hepatitis

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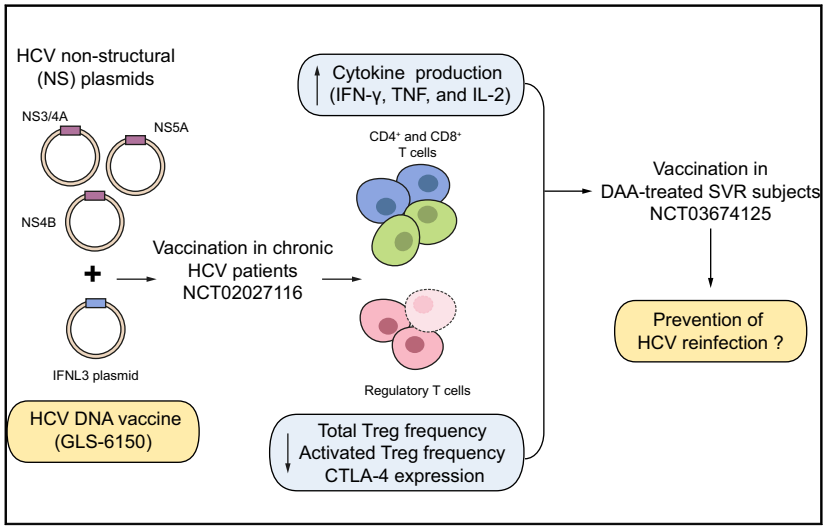
IFNL3-adjuvanted HCV DNA vaccine reduces regulatory T cell frequency and increases virus-specific T cell responses

Graphical abstract



Highlights

- DAAs successfully induce SVR in chronic HCV infection, but a prophylactic HCV vaccine is still warranted.
- We report here the first-in-human study demonstrating safety of an IFNL3-adjuvanted HCV DNA vaccine.
- IFNL3-adjuvanted HCV DNA vaccine reduces Treg cell frequency and increases virus-specific T-cell responses.
- *Ex vivo* IFN-λ3 treatment decreases Treg cell frequency in pre-vaccinated PBMCs, which might be an indirect effect by pDCs.

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Lay summary

Although direct-acting antivirals (DAAs) are successfully used for the treatment of chronic hepatitis C virus (HCV) infection, a prophylactic HCV vaccine needs to be developed, especially for patients who achieve a sustained virologic response. In the current study, we show that a DNA vaccine (GLS-6150) was safe and increased HCV-specific T cell responses. A clinical trial is underway to test this vaccine in patients with a sustained virologic response following DAA therapy.

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IFNL3-adjuvanted HCV DNA vaccine reduces regulatory T cell frequency and increases virus-specific T cell responses

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Background & Aims: Although direct-acting antiviral (DAA) treatment results in a sustained virologic response (SVR) in most patients with chronic HCV infection, they are at risk of re-infection. Moreover, the immune system is not completely normalized even after SVR (*e.g.* increased regulatory T [Treg] cell frequency). We developed a DNA vaccine, GLS-6150, to prevent re-infection of patients with DAA-induced SVR and evaluated its safety and immunogenicity in individuals with chronic HCV infection.

Methods: GLS-6150 consists of plasmids encoding HCV non-structural proteins (NS3-NS5A) and adjuvant *IFNL3*. The vaccine was administered 4 times at 4-weekly intervals to 3 groups (1, 3, or 6 mg/vaccination; n = 6 per group), followed by a 6 mg boost at 24 weeks (n = 14). Peripheral blood T cell responses were evaluated by interferon (IFN)-γ enzyme-linked immunospot assays, intracellular cytokine staining, and major histocompatibility complex class-I (MHC-I) dextramer staining. Treg cell frequency was assessed by flow cytometry.

Results: Severe adverse events or vaccine discontinuation were not reported. The IFN-γ spot-forming cells specific to NS3-NS5A were increased by GLS-6150. Both $CD4^+$ and $CD8^+$ T cells produced multiple cytokines. However, the frequency and phenotype of HCV-specific MHC-I dextramer$^+CD8^+$ T cells were not changed. Interestingly, the frequency of Treg cells, particularly activated Treg cells, was decreased by GLS-6150, as expected from previous reports that *IFNL3* adjuvants decrease Treg cell frequency. *Ex vivo* IFN-λ3 treatment reduced Treg frequency in pre-vaccination peripheral blood mononuclear cells. Finally, Treg cell frequency inversely correlated with HCV-specific, IFN-γ-producing T cell responses in the study participants.

Conclusions: We demonstrate that GLS-6150 decreases Treg cell frequency and enhances HCV-specific T cell responses without significant side effects. A phase I clinical trial of GLS-6150 is currently underway in patients with DAA-induced SVR.

Clinical trial number: NCT02027116.

Lay summary: Although direct-acting antivirals (DAAs) are successfully used for the treatment of chronic hepatitis C virus (HCV) infection, a prophylactic HCV vaccine needs to be developed, especially for patients who achieve a sustained virologic response. In the current study, we show that a DNA vaccine (GLS-6150) was safe and increased HCV-specific T cell responses. A clinical trial is underway to test this vaccine in patients with a sustained virologic response following DAA therapy.

Introduction

Approximately 71 million patients worldwide are chronically infected with HCV.[1] Chronic HCV infection increases the risk of liver cirrhosis and hepatocellular carcinoma (HCC).[1] Treatment of chronic HCV infection with direct-acting antiviral (DAA) agents results in a sustained virologic response (SVR) in >90% of patients and has become the standard of care, replacing pegylated-interferon (PEG-IFN)-based treatment.[1] However, the cost of HCV treatment with DAAs remains high, and access to DAA treatment is limited.[2,3] Furthermore, many patients who achieve SVR by DAA treatment remain at risk of re-infection[4–6] due to continued high-risk behaviors. In addition, HCV clearance by DAA treatment does not induce the protective immunity that may prevent HCV re-infection in chimpanzees.[7] In patients with chronic HCV infection, DAA treatment does not sufficiently induce $CD8^+$ and $CD4^+$ antiviral T cell immunity.[8,9] Therefore, prophylactic vaccines are required in the era of DAAs, particularly for patients who have been treated successfully.

T cells play a crucial role in the immune response against HCV infection. Strong and broad T cell responses are elicited in patients with spontaneously resolving acute HCV infection, in

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contrast to those with acute HCV infection progressing to chronic persistent infection who exhibit weak and narrow T cell responses.[10] Moreover, a robust memory T cell response is observed following spontaneous recovery from HCV infection.[11,12] Previous studies using a chimpanzee model demonstrated the importance of T cell immunity in preventing HCV infection by *in vivo* depletion of $CD4^+$ T cells or $CD8^+$ T cells.[13,14] Effective memory T cell responses significantly reduce the establishment of chronic persistent HCV infection upon re-challenge in previously recovered chimpanzees.[15–17] In addition, T cell responses persist for 2 decades, but humoral responses decline after the spontaneous resolution of HCV infection.[18] Based on these findings, T cells have been a major target for the development of HCV vaccines.

HCV vaccines have been developed to prevent HCV infection by eliciting virus-specific adaptive immune responses. In a chimpanzee model, DNA vaccines encoding HCV non-structural (NS) proteins were demonstrated to induce substantial T cell responses and the subsequent development of memory T cells.[19,20] Furthermore, human trials of prophylactic vaccines were performed in HCV infection-naïve individuals using HCV NS3-5A in human and chimpanzee adenoviral vectors.[21] In addition, a prime-boost strategy combining chimpanzee adenoviral vector and modified vaccinia virus Ankara (MVA) vector was also tried.[22] Currently, the efficacy of the latter vaccine is being tested in a phase I/II trial in naïve individuals who are injection drug users (NCT01436357). However, HCV vaccines preventing re-infection of individuals who have achieved DAA-induced SVR require special consideration because they exhibit unique features, even after HCV clearance by DAAs,[8,9,23–25] including persistently increased regulatory T (Treg) cells.[26]

The relative frequency of $CD4^+CD25^+FoxP3^+$ Treg cells is increased in the blood and liver in patients with chronic persistent HCV infection compared to non-infected controls,[10] indicating that Treg cells contribute to the attenuation of HCV-specific $CD4^+$ and $CD8^+$ T cell responses.[27] Recently, changes in the Treg cell population during and after successful DAA treatment were examined in patients with chronic HCV infection.[26] The increased Treg cell frequency persists even after successful DAA treatment,[26] which may be linked to incomplete immune-reconstitution after DAA treatment. Given that Treg cells attenuate vaccine-induced protective T cell responses,[28–33] Treg cells must be controlled to enhance the prophylactic efficacy of HCV vaccines in patients who achieve DAA-induced SVR.

A novel HCV vaccine, GLS-6150 is a mixture of DNA plasmids encoding the HCV NS3/NS4a, NS4b, and NS5a genes with consensus sequences of genotype 1a and 1b. In addition, GLS-6150 includes DNA plasmids encoding *IFNL3*, which was previously shown to enhance the efficacy of a DNA-based HIV vaccine by decreasing Treg cell frequency in a murine model.[34] The T cell immunogenicity of GLS-6150 was previously demonstrated in a preclinical murine study.[35] In the present study, we performed multicenter, open-label, dose-escalation phase I trial of GLS-6150 (NCT02027116) with a prime-boost regimen in patients with chronic HCV infection to evaluate the safety and immunogenicity. In addition, we evaluated vaccine-induced changes in the Treg cell population and analyzed the relationship between Treg cell frequency and vaccine-induced T cell responses.

Materials and Methods

Study participants

From January 2014 through December 2015, 18 participants were enrolled and followed at 2 locations in South Korea: Severance Hospital and Pusan National University Hospital. Eligible participants were adults between 19 and 65 years of age with genotype 1a or genotype 1b chronic HCV infection and no history of decompensated cirrhosis. Study participants failed prior treatment with either PEG-IFN plus ribavirin (RBV) or a DAA regimen. The study was reviewed and approved by the institutional review board at each hospital. All participants provided written informed consent before enrollment.

DNA vaccine

The GLS-6150 (formerly VGX-6150) vaccine was produced at VGXI, Inc. under cGMP conditions. GLS-6150 contains 4 DNA plasmids (pGX8005, pGX8006, pGX8007, and pGX6005 at mass ratio of 3:3:3:1, respectively) formulated in sterile water for injection. Plasmids pGX8005, pGX8006, and pGX8007 encode HCV NS3/NS4a, NS4b, and NS5a, respectively. Each of the plasmids encoding HCV NS genes contain consensus sequences of the genotype 1a and 1b strains of HCV cloned into the pGX0001 plasmid backbone. Plasmid pGX6005 encodes *IFNL3*.

Study procedures

Eighteen study participants were assigned to 1 of 3 doses of GLS-6150: 1, 3, or 6 mg/dose (n = 6 per group). Each dose group was recruited sequentially. Vaccine was administered as a 1 ml injection into the deltoid muscle and followed immediately by intramuscular (IM) electroporation (EP) as a 4-dose series given at baseline and weeks 4, 8, and 12 as a prime-vaccination (Fig. 1A, B). Long-term follow-up was scheduled through week 36. In September 2014, the study was amended to allow for a boost-vaccination of GLS-6150 at 36 weeks. Fourteen participants were enrolled in the follow-on study and received a 6 mg dose of GLS-6150 as a booster. Follow-up continued for an additional 24 weeks to week 60 (Fig. 1A and B). Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation medium (Corning). PBMCs were cryopreserved immediately after isolation from whole blood. T cell assays were performed using PBMCs collected pre-vaccination and at weeks 16, 36, and 40 (Fig. 1B). Study enrollment in each dose group was equal across the 2 institutions.

Safety evaluations

Vital signs were examined and adverse events reviewed at each study visit. After each vaccination, participants recorded pain and discomfort using a 100-point visual analog scale (VAS). Laboratory assessments included complete blood count, comprehensive metabolic panels, lipid panels, urinalysis, and measurements of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, prothrombin time, and creatinine kinase. Quantitative assessment of HCV RNA was performed at each study visit using a commercial PCR assay (Roche Diagnostics). *IFNL3* genotyping was performed at baseline using the SNaPshot ddNTP Primer Extension Kit (Applied Biosystem) to detect polymorphisms at rs12979860.

Synthetic peptides

One hundred ninety-nine pentadecamer peptides (JPT) overlapping by 8 amino acids each and spanning the HCV NS3/4A,

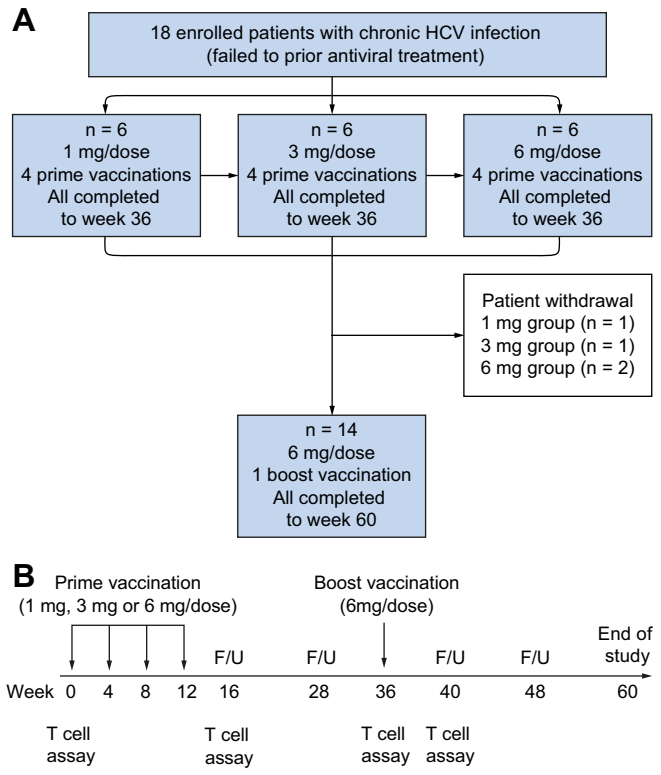

Fig. 1. Flow chart of a phase I trial of GLS-6150. (A) Trial design. Eighteen participants with chronic HCV infection were included in the trial. In the dose-escalation-prime phase, participants were divided into 3 groups according to the dose of vaccination (1, 3, and 6 mg/dose, n = 6 each), and received 4 doses of prime-vaccination. After the dose-escalation phase, 14 participants received 6 mg of boost-vaccination 24 weeks after the last prime-vaccination. (B) Time schedule of the trial. T cell assays were performed using PBMCs obtained prior to the prime-vaccination (pre), 4 weeks after prime-vaccination (week 16), prior to the boost-vaccination (week 36), and 4 weeks after boost-vaccination (week 40) in the 14 participants who received a boost-vaccination. Follow-up (F/U) comprised clinical observation and laboratory tests without any intervention or vaccination. PBMCs, peripheral blood mononuclear cells.

NS4B, and NS5A proteins (vaccine sequence) were resuspended at 20 mg/ml in DMSO. Aliquots of each peptide suspension were pooled and further diluted with PBS to obtain 6 mixtures: NS3/4A-1, NS3/4A-2, NS3/4A-3, NS4B, NS5A-1, and NS5A-2. The final concentration of each peptide in enzyme-linked immunospot (ELISpot) and intracellular cytokine staining (ICS) assays was 1 μg/ml.

Ex vivo IFN-γ ELISpot assay

Direct *ex vivo* IFN-γ ELISpot assays were performed as follows: before the experiments, PBS, BSA-containing PBS, and FBS-containing medium were sterile filtered. The plates were coated with anti-IFN-γ coating antibody (2 μg/ml; Thermo scientific) overnight at 4°C. After washing, they were blocked with PBS containing 1% BSA for 2 h and RPMI medium containing 5% FBS for 1 h at room temperature (RT). Thawed PBMCs (300,000 cells per well) were cultured for 24 h in RPMI medium containing 5% FBS in the presence of DMSO as a negative control, phytohemagglutinin (PHA, 1:100, Thermo scientific) as a positive control, or overlapping peptide (OLP) pools. After washing, biotinylated anti-IFN-γ detection antibody (0.25 μg/ml) (Thermo scientific) was added to the plates. After 2 h of incubation at RT and washing, streptavidin-alkaline phosphatase (SA-AP, 1:5,000, Thermo scientific) containing 1% BSA/PBS/Tween was added to the plates. After 1 h incubation at RT, development solution from the AP Conjugate Substrate Kit (Bio-Rad) was added to the plates and incubated for 5 min at RT. Finally, the plates were washed with distilled water and dried in the dark room at RT overnight. The spots in each well were counted using an automated ELISpot reader (CTL). The number of specific spots was calculated by subtracting the mean number of spots in the DMSO control well from the mean number of spots in the OLP pool-stimulated well. Total responses or responses specific to each NS protein were determined by summing the responses to each OLP pool. We performed IFN-γ ELISpot assays in triplicate and calculated an average. In all assays, the PHA wells had an uncountable colored area, and the maximum spot number in DMSO wells was 22. CD4 microbeads (Miltenyi Biotec) were used for $CD4^+$ T cell isolation or depletion, and CD8 microbeads (Miltenyi Biotec) were used for $CD8^+$ T cell depletion.

ICS

Thawed 1,000,000 PBMCs were stimulated with the HCV NS3/4A-2 OLP pool and anti-CD28 antibody (BD Biosciences). One hour later, brefeldin A (BD Biosciences), monensin (BD Biosciences), and anti-CD107a-FITC (BD Bioscience) were added, followed by an 11 h incubation. Then the PBMCs were harvested, stained with antibodies for surface markers, permeabilized using a Foxp3 staining buffer kit (eBioscience), and additionally stained for intracellular cytokines.

Flow cytometry

Fluorochrome-conjugated antibodies used in flow cytometry analysis are presented in Supplementary CTAT table. Dead cells were excluded using LIVE/DEAD red fluorescent dye (Invitrogen). Human leukocyte antigen (HLA)-A2 was determined by staining of anti-human HLA-A2-FITC (BD Biosciences). For intracellular marker staining, surface-stained PBMCs were permeabilized using the FoxP3 staining buffer kit (eBioscience), and then stained for intracellular markers for 30 min at 4°C. Stained cells were analyzed using a BD LSRII flow cytometer (BD Biosciences). White blood cell (WBC) counts and the percentage of lymphocytes were provided by the clinics for each participant. In FACS analysis, we calculated the percentage of $CD3^+$ T cells among the lymphogate and the percentage of $CD25^+CD127^-FoxP3^+CD4^+$ Treg cells among the T cell population.

Major histocompatibility complex class-I (MHC-I) multimer staining and *in vitro* expansion

PE-conjugated-dextramer corresponding to HLA-A2 HCV $NS3_{1073\text{-}1081}$ CVNGVCWTV, $NS3_{1273\text{-}1282}$ GVDPNIRTGV and FITC-conjugated-dextramer corresponding to HLA-A2 HCV $NS3_{1406\text{-}1415}$ KLVALGINAV was purchased from Immudex. Thawed PBMCs (>2,500,000 cells) were stained with surface marker antibodies and the dextramer for 10 min at RT and analyzed by flow cytometry. In an *in vitro* expansion assay, $NS3_{1073\text{-}1081}$- and $NS3_{1406\text{-}1415}$-dextramer$^+CD8^+$ T cells were cultured as previously described.[36] Briefly, 2,000,000 PBMCs were stimulated with 10 μg/ml of a $NS3_{1073\text{-}1081}$ CVNGVCWTV and $NS3_{1406\text{-}1415}$ KLVALGINAV epitope peptides and 0.5 μg/ml of an anti-CD28 antibody (BD Biosciences) in 1 ml of medium containing 10% FBS and 1% penicillin/streptomycin. The PBMCs were incubated at 37°C and 5% CO_2. Fresh medium containing 20 IU/ml interleukin-2 (IL-2) (Peprotech) was added on days 3, 7,

and 10. Epitope-containing medium was washed out by IL-2 containing medium on day 3. On the day 14, cultured PBMCs were stained with fluorochrome-conjugated antibodies and the dextramers, and were analyzed by flow cytometry.

Ex vivo IFN-λ3 treatment

Recombinant human IFN-λ3 was purchased from R&D systems. PBMCs (1×10^6 cells) obtained at baseline were treated with IFN-λ3 (100 ng/ml). The IFN-λ3-treated or PBS-treated PBMCs were cultured in medium containing 10% FBS for 48 h at 37°C in a 5% CO_2 incubator. For measuring Treg cell frequency, cultured PBMCs were harvested and stained with surface marker antibodies, followed by fixation and permeabilization using the FoxP3 staining buffer kit (eBioscience). The cells were then stained with anti-FoxP3-PE antibody, followed by flow cytometric analysis. For the apoptosis assay, cultured PBMCs were harvested and stained with surface marker antibodies. After washing, stained cells were stained with annexin V-PE (BD Biosciences) just before flow cytometric analysis.

Analysis of STAT1 and phosphorylated STAT1 (pSTAT1)

PBMCs (1,000,000 cells) were stained with surface marker antibodies. After washing, they were treated with IFN-λ3 (100 ng/ml) or PBS for 10 min at 37°C, followed by incubation with IC fixation buffer (Invitrogen) for 10 min at RT and fixation with 100% cold methanol for 40 min at 4°C. The cells were then stained with anti-human STAT1 and pSTAT1 antibodies for 1 h at RT, followed by flow cytometric analysis.

Statistical analysis

GraphPad Prism 6 (GraphPad) was used for statistical analyses. To compare data between 2 paired groups, we used the Wilcoxon matched paired *t* test. To compare data between 2 unpaired groups, we used the Mann-Whitney *U* test. We used one-way ANOVA analyses when performing multiple comparisons. To examine the correlation between 2 parameters, we used Spearman's rank correlation test.

Results

GLS-6150 is safe and well-tolerated

The baseline characteristics of the participants are given in Table 1. All participants had well-preserved liver function with no risk behaviors, such as injection drug use. All participants had at least 9.9 years of HCV genotype 1b infection, without an unfavorable *IFNL3* TT genotype. All patients had failed prior antiviral treatment with PEG-IFN and RBV, with some failing multiple treatment courses (Table 1). Vaccination-emergent adverse events (AEs) are presented in Table 2. No serious AEs occurred after vaccination. The grade 3 AE of elevated AST was observed in 1 patient (1/18, 5.6%), but it resolved without treatment; this individual also had multiple pre-study grade 3 elevations of liver enzymes in the 2 years prior to enrollment. Vaccine-related AEs were observed in 38.9% (7/18) of the participants, with the most common events being elevated liver enzymes, dry mouth, headache, fatigue, mucosal inflammation, and nausea. No individual discontinued the study due to AEs (Fig. 1A and Table 2). Collectively, GLS-6150 was safe and well tolerated without serious AEs.

We examined if anti-IFN-λ3 autoantibodies are developed by GLS-6150. In direct ELISA studies using serum samples from healthy donors and the study participants pre-vaccination, at week 16, and at week 40, the optical density was slightly increased after the boost-vaccination but was not different between healthy donors and the study participants at any timepoints (Fig. S1), suggesting that anti-IFN-λ3 autoantibodies can be minimally induced by GLS-6150.

Table 1. Baseline characteristics of the study participants.

	GLS-6150 1 mg (n = 6)	GLS-6150 3 mg (n = 6)	GLS-6150 6 mg (n = 6)
Age, mean ± SD, years	58.0 ± 7.2	56.0 ± 10.4	58.3 ± 2.3
Male, n (%)	2 (33.3)	3 (50.0)	3 (50.0)
BMI, mean ± SD, kg/m^2	25.5 ± 1.5	26.0 ± 2.9	24.2 ± 2.0
ALT at baseline, mean ± SD, U/L	40.8 ± 28.7	19.7 ± 12.8	46.3 ± 37.4
Liver cirrhosis, n (%)	0 (0)	4 (66.7)	3 (50.0)
Decompensated cirrhosis, n (%)	0 (0)	0 (0)	0 (0)
IV drug abuse, n (%)	0 (0)	0 (0)	0 (0)
HCV genotype 1b, n (%)	6 (100)	6 (100)	6 (100)
IFNL3 genotype			
CC, n (%)	1 (16.7)	6 (100)	2 (33.3)
CT, n (%)	5 (83.3)	0 (0)	4 (66.7)
TT, n (%)	0 (0)	0 (0)	0 (0)
Period from primary HCV infection, mean ± SD, years	10.4 ± 3.5	9.9 ± 6.8	10.2 ± 8.9
HCV RNA, mean ± SD, $\log_{10}$ IU/ml	6.2 ± 0.3	6.1 ± 1.4	6.3 ± 0.4
Prior antiviral treatment, n (%)	6 (100)	6 (100)	6 (100)
Peg-IFN + RBV, n (%)	4 (66.7)	5 (83.3)	5 (83.3)
Peg-IFN + RBV & DAA, n (%)	2 (33.3)	1 (16.7)	1 (16.7)
*Response to the prior antiviral treatment			
Partial response, n (%)	0 (0)	0 (0)	2 (33.3)
Non-response, n (%)	3 (50)	1 (16.7)	1 (16.7)
Relapse, n (%)	3 (50)	5 (83.3)	3 (50)

ALT, alanine aminotransferase; BMI, body mass index; DAA, direct-acting antiviral; IFN, interferon; RBV, ribavirin.
*A partial response was defined as > 2 log reduction in HCV RNA from baseline at 12 and 24 weeks of treatment but with detectable HCV RNA. A non-response was defined as <2 log reduction in HCV RNA from baseline at 12 weeks of treatment. Relapse was defined as an initial reduction in HCV RNA to undetectable levels (<50 IU/ml) and the reappearance of HCV RNA after the end of treatment.

Table 2. Vaccination-emergent adverse events in the study participants.

Patients, N (%)	Total (n = 18)	GLS-6150 1 mg (n = 6)	GLS-6150 3 mg (n = 6)	GLS-6150 6 mg (n = 6)
Any AE, n (%)	13 (72.2)	6 (100)	3 (50)	4 (66.7)
AE after primary vaccination, n (%)	13 (72.2)	6 (100)	3 (50)	4 (66.7)
AE after boosting vaccination, n (%)	2 (11.1)	2 (33.3)	0 (0)	0 (0)
Serious AE, n (%)	0 (0)	0 (0)	0 (0)	0 (0)
Discontinuation due to AE, n (%)	0 (0)	0 (0)	0 (0)	0 (0)
Grade 3-4 AE, n (%)	1 (5.6)	*1 (16.7)	0 (0)	0 (0)
Death, n (%)	0 (0)	0 (0)	0 (0)	0 (0)
Vaccine-related AE, n (%)	7 (38.9)	3 (50)	1 (50)	3 (50)
Elevated liver enzyme, n (%)	3 (16.7)	1 (16.7)	0 (0)	2 (33.3)
Dry mouth, n (%)	1 (5.6)	1 (16.7)	0 (0)	0 (0)
Headache, n (%)	1 (5.6)	1 (16.7)	0 (0)	0 (0)
Fatigue, n (%)	2 (11.1)	1 (16.7)	1 (16.7)	0 (0)
Mucosal inflammation, n (%)	1 (5.6)	1 (16.7)	0 (0)	0 (0)
Nausea, n (%)	1 (5.6)	1 (16.7)	0 (0)	0 (0)
Myalgia, n (%)	1 (5.6)	0 (0)	0 (0)	1 (16.7)

AE, adverse event; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
*AST/ALT: 220/121 IU/ml.

GLS-6150 enhances HCV-specific T cell responses

We first evaluated the T cell immunogenicity of GLS-6150 by IFN-γ ELISpot assays following *ex vivo* stimulation of PBMCs

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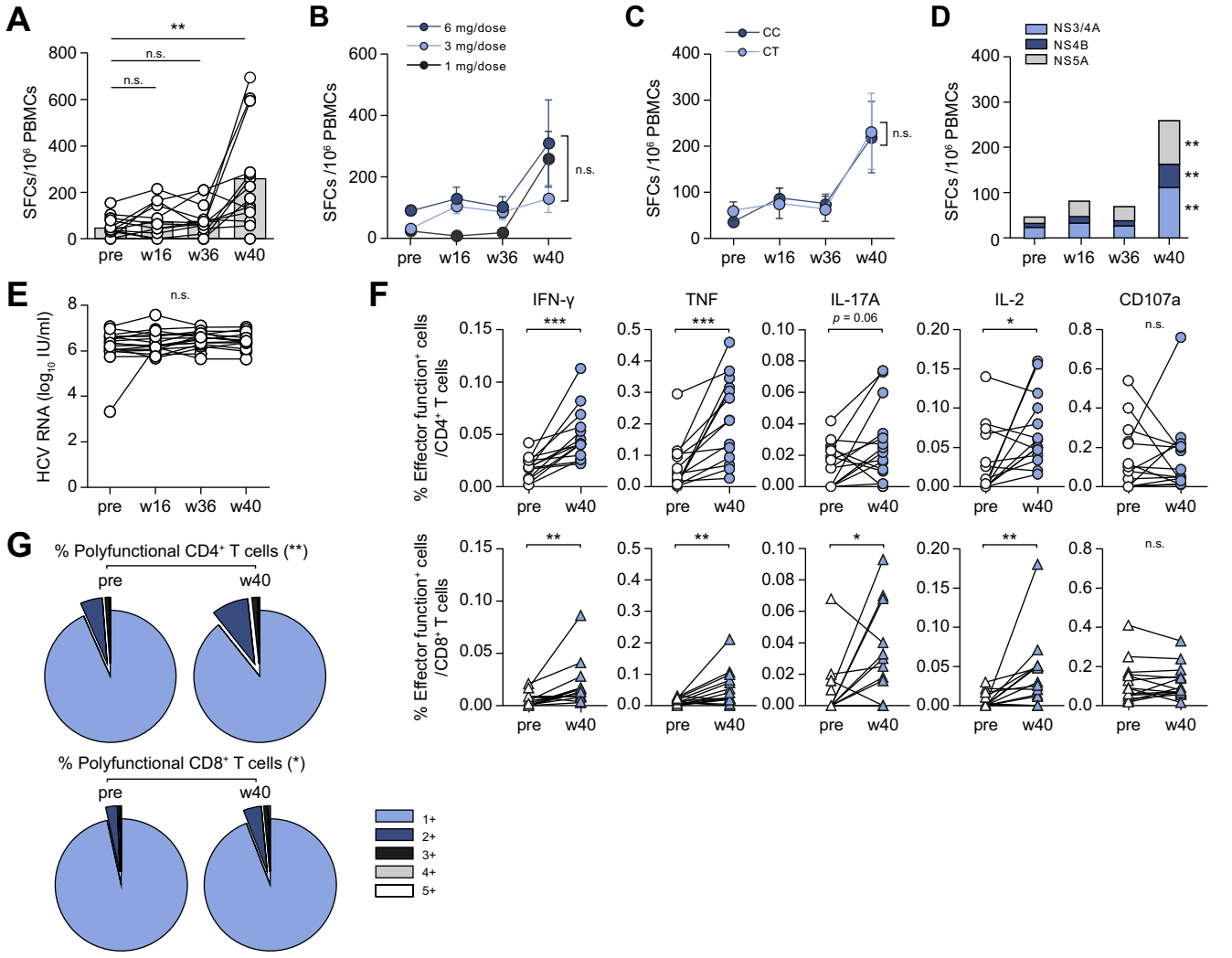

Fig. 2. Enhanced HCV-specific T cell responses by GLS-6150. (A-D) PBMCs from 14 participants who completed the prime-boost-vaccination were analyzed by ELISpot assays following *ex vivo* stimulation of PBMCs with OLP pools (NS3/4A-1, NS3/4A-2, NS3/4A-3, NS4B, NS5A-1, and NS5A-2). Specific SFCs were calculated by subtracting the spot number of the DMSO well from the spot number of the OLP pool-stimulated well. SFCs for each HCV NS protein were calculated by summing SFCs specific to each OLP pool. HCV NS protein-specific SFCs in individual patients (lines and circles) and mean SFCs (boxes) (A). Mean SFCs according to the dose of prime-vaccination: 1 mg (n = 5, gray point and line), 3 mg (n = 6, blue point and line), and 6 mg (n = 5, red point and line) (B). Mean SFCs according to the *IFNL3* genotype: CC (n = 7, red line) and CT (n = 7, blue line) (C). SFCs specific to each HCV NS protein: NS3/4A, blue box; NS4B, red box; and NS5A, yellow box (D). (E) HCV RNA titer in each participant (n = 14) before prime-vaccination and at w16, w36, and w40. (F and G) PBMCs obtained before prime-vaccination and at w40 from 14 participants were analyzed by ICS following *ex vivo* stimulation with the NS3/4A-2 OLP pool. Percentage of effector function-positive cells was calculated by subtracting the percentage in DMSO-treated cells from the percentage in NS3/4A-2 OLP pool-stimulated cells. (F) IFN-γ, IL-2, TNF, IL-17A, and CD107a were examined in $CD4^+$ and $CD8^+$ T cells. (G) Percentage of $CD4^+$ and $CD8^+$ T cells expressing single or multiple effector molecules. Wilcoxon matched paired t-test was used to compare data between 2 paired groups. Mann-Whitney test was used to compare data between 2 unpaired groups. One-way ANOVA analysis was used to perform multiple comparisons. Error bars represent SD. n.s., not significant; $^*p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001. ICS, intracellular cytokine staining; IFN, interferon; IL, interleukin; NS, non-structual; OLP, overlapping peptide; PBMCs, peripheral blood mononuclear cells; SFCs, spot-forming cells; TNF, tumor necrosis factor.

with OLP pools spanning HCV NS3/4A, NS4B, and NS5A. When all participants (n = 14) in the 3 subgroups were analyzed, HCV-specific, IFN-γ-producing T cell responses were significantly increased by the boost-vaccination, but not by the prime-vaccinations (Fig. 2A). IFN-γ-producing T cell responses after the boost-vaccination were not dependent on priming doses across the 6-fold dosing range (Fig. 2B) and were not associated with *IFNL3* genotype (Fig. 2C). To determine the breadth of T cell responses, IFN-γ-producing T cell responses against each HCV protein were analyzed; IFN-γ-producing T cell responses were significantly enhanced by the boost-vaccination for each of the 3 NS regions (NS3/4A, NS4B, and NS5A) used in GLS-6150 (Fig. 2D). However, the enhancement of T cell responses did not reduce HCV RNA titers (Fig. 2E). Next, IFN-γ ELISpot assays were performed following depletion of either $CD4^+$ or $CD8^+$ T cells. The $CD8^+$ T cell-depleted PBMCs had higher IFN-γ spot numbers than $CD4^+$ T cell-depleted PBMCs, though both T cell populations produced IFN-γ (Fig. S2).

We next analyzed $CD4^+$ and $CD8^+$ T cell responses using ICS for IFN-γ, IL-2, tumour necrosis factor (TNF), IL-17A, and CD107a after the vaccination. In both $CD4^+$ and $CD8^+$ T cells, we observed

cytokine production including IFN-γ, IL-2, TNF, and IL-17A (Fig. S3A and B). In addition, the expression of degranulation marker CD107a, was also observed in both $CD4^+$ and $CD8^+$ T cells (Fig. S3A and B). Moreover, for both $CD4^+$ and $CD8^+$ T cells, production of IFN-γ, IL-2, TNF, and IL-17A was significantly increased at week 40 whereas the frequency of $CD107a^+$ cells was not changed (Fig. 2F and Fig. S3C). In addition, the polyfunctionality of $CD4^+$ and $CD8^+$ T cells was increased at week 40 (Fig. 2G). Taken together, these findings indicate that GLS-6150 enhances HCV-specific $CD4^+$ and $CD8^+$ T cell responses with multiple cytokine production.

Frequency and phenotype of HCV-specific, MHC-I multimer$^+$CD8$^+$ T cells are not changed by GLS-6150

We used 3 MHC-I dextramers (HLA-A2 $NS3_{1073\text{-}1081}$ CVNGVCWTV, HLA-A2 $NS3_{1273\text{-}1282}$ GVDPNIRTGV, and HLA-A2 $NS3_{1406\text{-}1415}$ KLVALGINAV) to analyze changes in the frequency and phenotype of HCV-specific $CD8^+$ T cells in 7 participants with HLA-A2 positivity using samples obtained at pre-vaccination and week 40. The *ex vivo* frequency of dextramer$^+$CD8$^+$ T cells was not significantly changed by the vaccination (Fig. 3A and B). When dextramer$^+$CD8$^+$ T cells were expanded *in vitro* by stimulation with cognate epitope peptides to examine their proliferative capacity, we observed no enhancement in the expansion of dextramer$^+$CD8$^+$ T cells after vaccination (Fig. 3C).

In addition, the percentages of effector memory cells ($CD45RA^-CCR7^-$), effector memory RA cells ($CD45RA^+CCR7^-$), and $CD127^+$ cells among dextramer$^+$CD8$^+$ T cells were not significantly changed by the vaccination (Fig. 3D and E, Fig. S4A and B). Moreover, the percentages of PD-1^+ cells and activated cells ($CD38^+$ or $HLA\text{-}DR^+$) were not significantly changed by the vaccination (Fig. 3F and G, Fig. S4B and C), although there is a possibility that activation markers were upregulated immediately after boost-vaccination and downregulated 4 weeks later. There was also no change in the frequencies or phenotypes of HCV-specific $CD8^+$ T cells at week 36 compared to pre-vaccination (Fig. S4D-H). Taken together, there was no change in the frequency and phenotype of dextramer$^+$CD8$^+$ T cells by the vaccination even after using 3 MHC-I dextramers.

GLS-6150 reduces the size of the Treg population

To find a mechanism underlying vaccine-induced increases in HCV-specific, IFN-γ-producing T cell responses, we examined changes in the Treg population before and after GLS-6150 vaccination. The gating strategy for $CD25^+CD127^-FoxP3^+CD4^+$ Treg cells is shown in Fig. 4A and Fig. S5A. First, we compared the Treg cell frequency between healthy donors and study participants with chronic HCV infection prior to vaccination, finding that it was significantly higher in the study participants (Fig. 4B). Next, we assessed the change in Treg cell frequency and found that Treg cell frequency was significantly reduced after vaccination, particularly after the boost-vaccination (Fig. 4C and D). When we directly compared the Treg cell frequency between PBMCs from healthy controls and week 40 PBMCs from the study participants, we found no significant difference (Fig. S5B). Thus, the Treg cell frequency was reduced to the normal level in study participants by vaccination. In addition, the absolute number of Treg cells was decreased by the vaccination (Fig. 4E). We further analyzed which Treg cell subpopulation was decreased by the vaccination. Treg cells were divided into 3 phenotypically

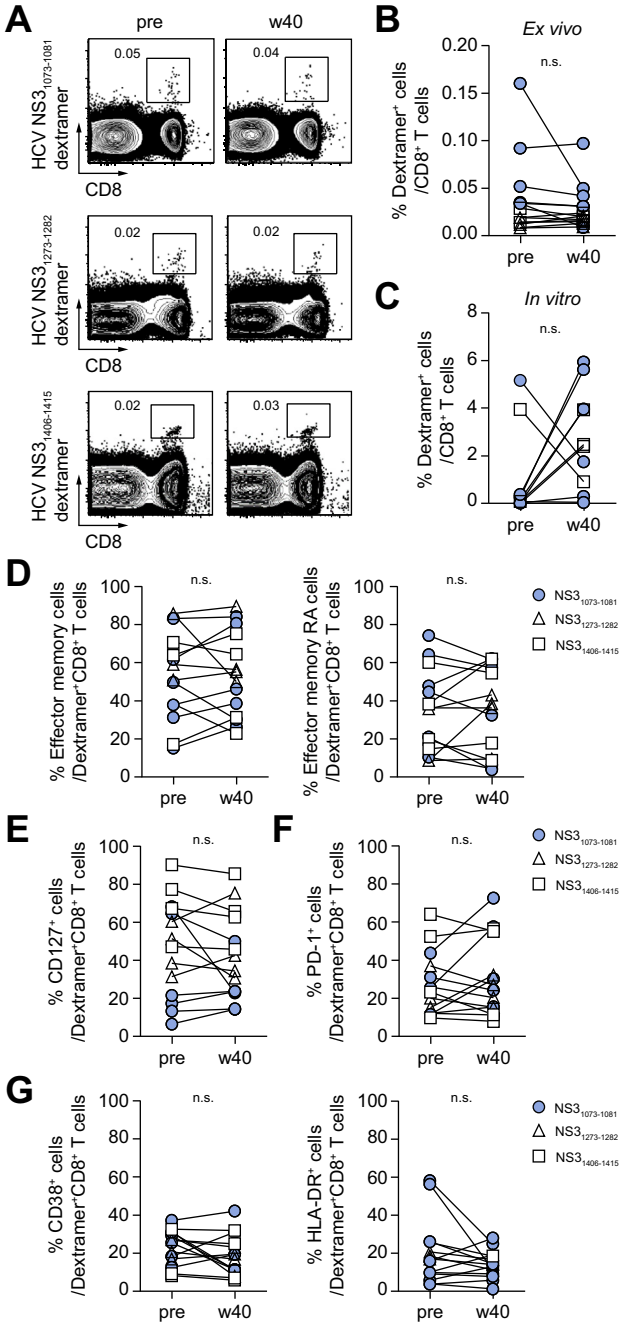

Fig. 3. Characterization of MHC-I multimer$^+$CD8$^+$ T cells after GLS-6150 vaccination. PBMCs from 7 participants with HLA-A2 obtained at baseline and week 40 were stained with MHC-I dextramer corresponding to HCV $NS3_{1073\text{-}1081}$ CVNGVCWTV (n = 6), $NS3_{1273\text{-}1282}$ GVDPNIRTGV (n = 4), $NS3_{1406\text{-}1415}$ KLVALGINAV (n = 4) and were analyzed by flow cytometry. (A and B) *Ex vivo* frequency of dextramer$^+$CD8$^+$ T cells was analyzed. Representative plot (A) and the graph (B). (C) Frequencies of $NS3_{1073\text{-}1081}$ and $NS3_{1406\text{-}1415}$ dextramer$^+$CD8$^+$ T cells after *in vitro* expansion with a corresponding epitope peptide for 14 days were analyzed. (D-G) Phenotypic analysis of dextramer$^+$CD8$^+$ T cells was performed by flow cytometry. Frequency of effector memory cells ($CD45RA^-CCR7^-$) and effector memory RA cells ($CD45RA^+CCR7^-$) (D), frequency of $CD127^+$ cells (E), frequency of PD-1^+ cells (F), frequency of $CD38^+$ and $HLA\text{-}DR^+$ cells (G) among dextramer$^+$CD8$^+$ T cells were analyzed. Wilcoxon matched paired t-test was used to compare data between 2 paired groups. n.s., not significant. HLA, human leukocyte antigen; MHC-I, major histocompatibility complex class-I; NS, non-structual; PBMCs, peripheral blood mononuclear cells.

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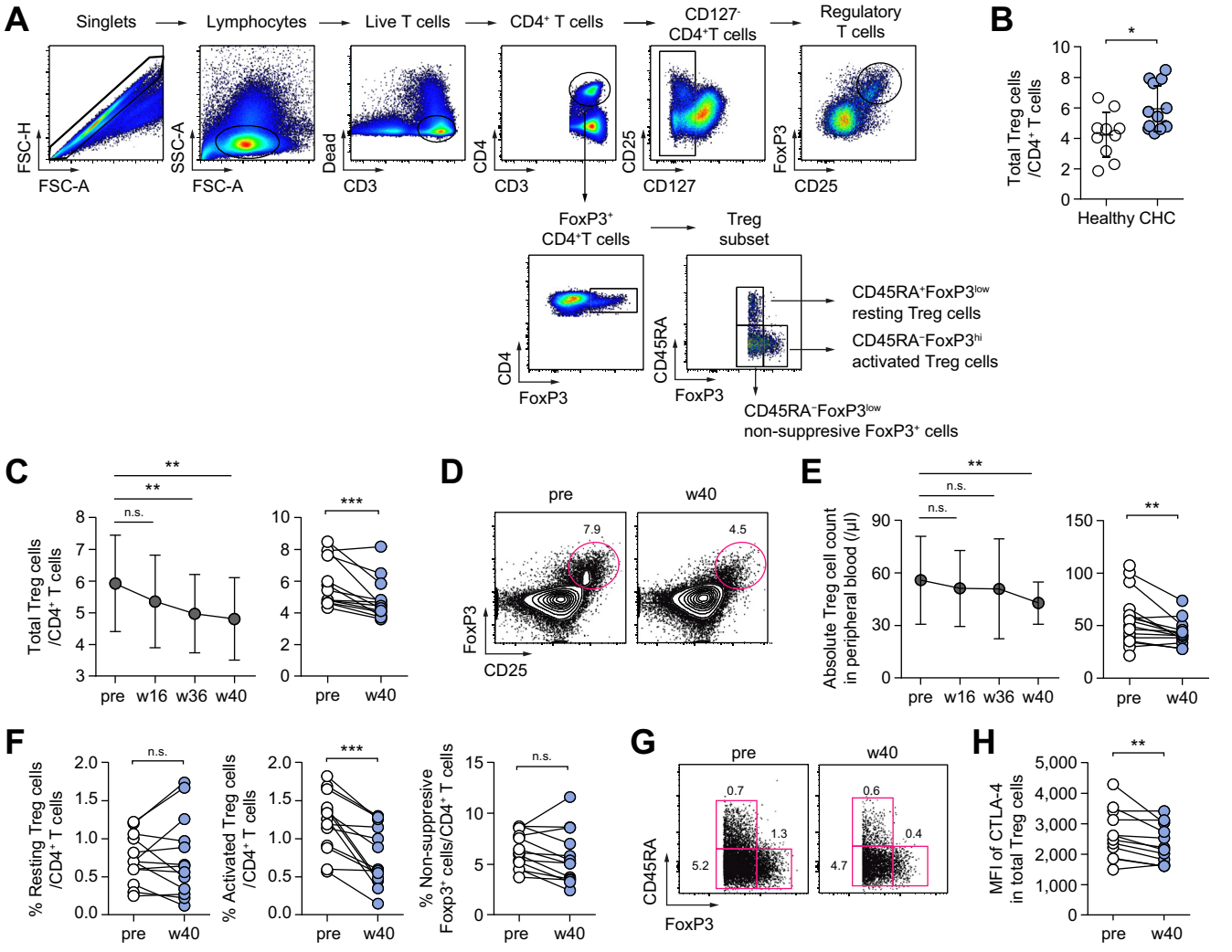

Fig. 4. Changes in the Treg cell population after GLS-6150 vaccination. The frequencies and phenotypes of Treg cells were analyzed within 24 h after thawing the cryopreserved PBMC samples. (A) Gating strategy for the flow cytometric analysis of Treg cells and subpopulations. Total Treg cells represent $CD25^+FoxP3^+CD127^-CD4^+$ T cells. Treg subpopulations were $CD45RA^+FoxP3^{low}CD4^+$ resting Treg, $CD45RA^-FoxP3^{hi}CD4^+$ activated Treg, and $CD45RA^-FoxP3^{low}CD4^+$ non-suppressive $FoxP3^+$ cells. (B) PBMCs from healthy donors (n = 10) and participants with chronic HCV infection (n = 14, pre-vaccination samples) were analyzed for Treg cell frequency among $CD4^+$ T cells. (C-E) PBMCs from 14 study participants before prime-vaccination and at week 16, week 36, and week 40 were analyzed for Treg cells. The frequency of total Treg cells among $CD4^+$ T cells was analyzed at each timepoint (C, left), and before prime-vaccination and at week 40 in each participant (C, right). Representative plots are presented in (D). The absolute number of total Treg cells was analyzed at each timepoint (E, left), and before prime-vaccination and at week 40 in each participant (E, right). (F and G) The frequencies of resting Treg, activated Treg, and non-suppressive $FoxP3^+$ cells among $CD4^+$ T cells were analyzed. A comparison of the frequency of each subset before prime-vaccination and at week 40 in each participant (F) and representative plots (G) are presented. (H) Comparison of the MFI of CTLA-4 in total Treg cells before prime-vaccination and at week 40 in each participant. Wilcoxon matched paired *t* test was used to compare data between 2 paired groups. Mann-Whitney *U* test was used to compare data between 2 unpaired groups. One-way ANOVA analysis was used to perform multiple comparisons. Error bars represent SD. n.s., not significant; $^*p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001. CTLA-4, cytotoxic T lymphocyte antigen-4; MFI, mean fluorescence intensity; PBMCs, peripheral blood mononuclear cells; Treg, regulatory T.

and functionally different subsets: $CD45RA^+FoxP3^{low}CD4^+$ resting Treg, $CD45RA^-FoxP3^{hi}CD4^+$ activated Treg, and $CD45RA^-FoxP3^{low}CD4^+$ non-suppressive $FoxP3^+$ cells.[37] The gating strategy is presented in Fig. 4A. Although the frequencies of resting Treg or non-suppressive $FoxP3^+$ cells were not significantly changed after the vaccination, the frequency of activated Treg cells was significantly reduced after vaccination (Fig. 4F and G). We also examined whether the phenotype of Treg cells was changed by the vaccination. Cytotoxic T lymphocyte antigen-4 (CTLA-4) is an essential molecule for the suppressive function of Treg cells,[38] and its expression was significantly decreased after vaccination (Fig. 4H and Fig. S5C). Collectively, these findings suggest that GLS-6150 has a negative effect on the Treg cell population, both numerically and qualitatively.

Next, we analyzed the data from the 3 individuals with strong T cell responses in ELISpot at week 40 (strong responders) and the other 11 participants (weak responders) separately. When we compared the $CD4^+$ and $CD8^+$ T cell responses using ICS for IFN-γ, IL-2, TNF, IL-17A, and CD107a, strong responders (n = 3) had a significantly higher frequency of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells than low responders (n = 11), but other effector functions did not differ between the 2 groups (Fig. S6A). In the analysis of $dextramer^+$ cells, a strong responder (n = 1) tended to have a higher frequency of

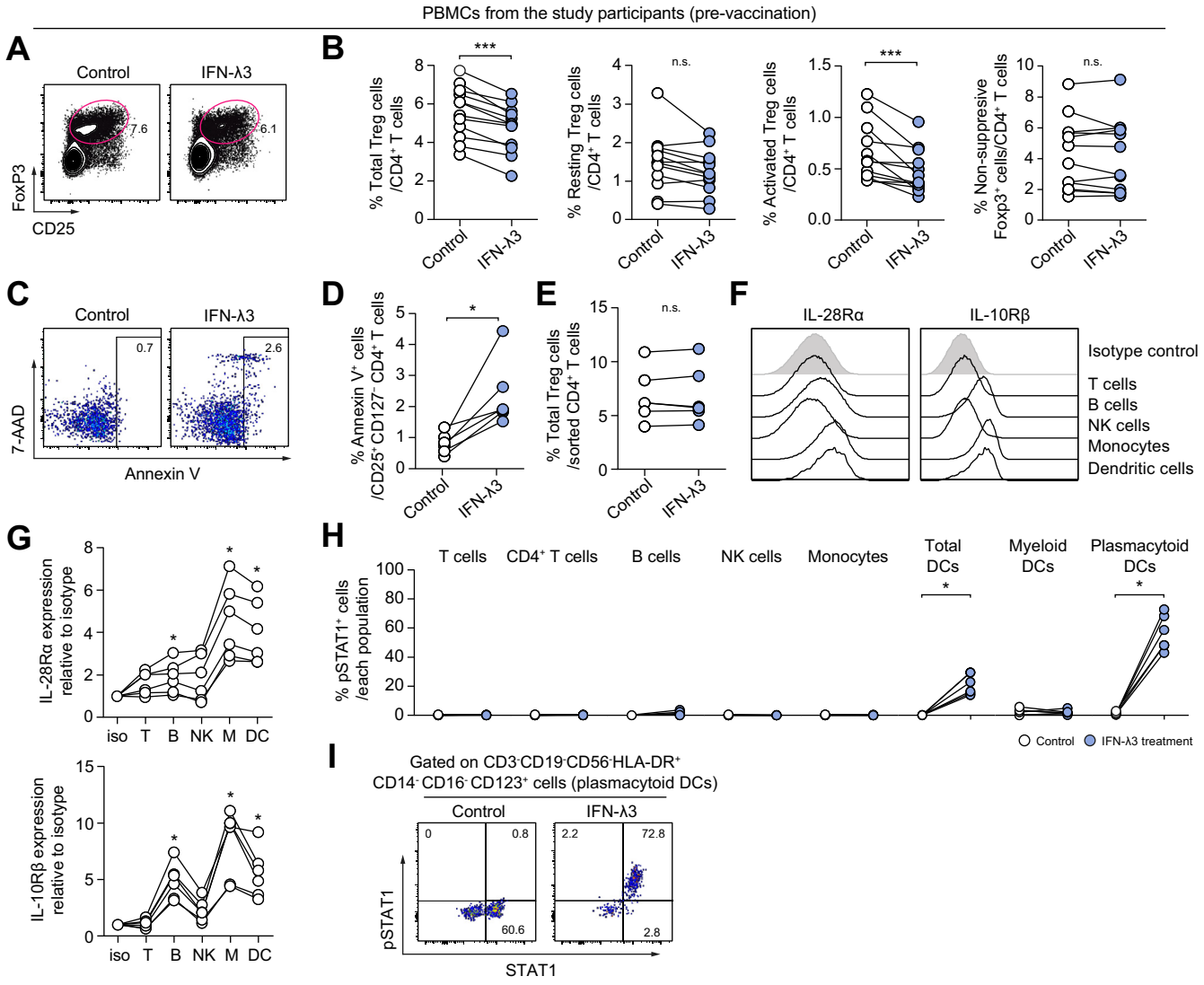

Fig. 5. Reduced Treg cell frequency after *ex vivo* IFN-λ3 treatment in pre-vaccination PBMCs. (A-E) Pre-vaccination PBMCs from the participants were treated with 100 ng/ml of recombinant human IFN-λ3 for 48 h, and analyzed by flow cytometry (A and B) The frequency of total Treg cells, resting Treg cells, activated Treg cells, and non-suppressive FoxP3$^+$ cells were analyzed (n = 12). (A) Representative plots of total Treg cells with and without IFN-λ3 treatment. (B) The frequencies of Treg and Treg subpopulations were analyzed with and without IFN-λ3 treatment. (C) Representative plots and (D) graph of the frequency of annexin V$^+$ cells measured among CD25$^+$CD127$^-$CD4$^+$ T cells (n = 6) with and without IFN-λ3 treatment. (E) CD4$^+$ T cells were isolated from pre-vaccination PBMCs (n = 6) using MACS. The frequency of total Treg cells was analyzed among sorted CD4$^+$ T cells with or without IFN-λ3 treatment. (F and G) The expression of IL-28Rα and IL-10Rβ in pre-vaccination PBMCs (n = 6) was analyzed by flow cytometry. T cells (CD3$^+$), NK cells (CD3-CD56$^+$), B cells (CD3$^-$CD19$^+$), monocytes (CD3$^-$CD19$^-$CD56$^-$HLA-DR$^+$), and DCs (CD3$^-$CD19$^-$CD56$^-$HLA-DR$^+$CD14$^-$CD16$^-$) were analyzed. Representative plots (F) and the relative expression of IL-28Rα and IL-10Rβ to isotype control (G) are presented. (H and I) Pre-vaccination PBMCs (n = 6) were treated with 100 ng/ml of IFN-λ3 for 10 min and analyzed by flow cytometry. (H) STAT1$^+$ and phosphorylated-STAT1$^+$ (pSTAT1$^+$) cells were measured in each population, including CD4$^+$ T cells, myeloid DCs (CD11c$^+$ DCs), and plasmacytoid DCs (pDCs, CD123$^+$ DCs). (I) Representative plots of STAT1 and pSTAT1 expression in pDCs. Wilcoxon matched paired t-test was used to compare data between 2 paired groups. Error bars represent SD. n.s., not significant; *p <0.05; ***p <0.001. DCs, dendritic cells; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; IFN, interferon; IL, interleukin; NK, natural killer; PBMCs, peripheral blood mononuclear cells; pDCs, plasmacytoid DCs; Treg, regulatory T.

$NS3_{1273-1282}$ dextramer$^+$CD8$^+$ T cells than weak responders (n = 3) post-vaccination, but the difference was not significant (Fig. S5B). We also found no significant difference in Treg cell frequency between strong (n = 3) and weak (n = 11) responders (Fig. S6C).

Ex vivo IFN-λ3 treatment reduces Treg cell frequency in pre-vaccinated PBMCs

As previous reports have shown that an *IFNL3* gene adjuvant reduces Treg cell frequency in a murine model of DNA vaccination,[34,39] we questioned whether IFN-λ3 directly reduces the frequency of Treg cells. We treated *ex vivo* PBMCs obtained from the study participants prior to the vaccination with human recombinant IFN-λ3. Cultured PBMCs were harvested 48 h after IFN-λ3 treatment, and the frequency of total Treg cells and each subpopulation of Treg cells was determined. Interestingly, *ex vivo* IFN-λ3 treatment significantly decreased the total Treg cell frequency (Fig. 5A and B). In particular, the activated Treg subpopulation, but not resting Treg or non-suppressive FoxP3$^+$ subpopulations, was significantly decreased by *ex vivo* IFN-λ3 treatment (Fig. 5B). This *ex vivo* experiment successfully recapitulated that GLS-6150, the *IFNL3*-adjuvanted HCV DNA vaccine,

decreased the frequency of Treg cells *in vivo*. Meanwhile, we found that the effect of IFN-λ3 on the frequency of Treg cells was not observed among PBMCs from healthy donors (Fig. S7A and B).

To determine the mechanisms of Treg reduction by IFN-λ3, we performed annexin V staining after *ex vivo* IFN-λ3 treatment for 48 h in pre-vaccination PBMCs from the study participants. The percentage of annexin V^+ apoptotic cells among Treg cells was significantly increased by IFN-λ3 treatment (Fig. 5C and D), indicating that IFN-λ3 induces the apoptotic death of Treg cells.

To examine whether the effect of IFN-λ3 on Treg cells is direct or indirect, we first treated $CD4^+$ T cells sorted from pre-vaccination PBMCs with IFN-λ3, but there was no effect of IFN-λ3 on the frequency of Treg cells (Fig. 5E). Therefore, we examined the expression of IFN-λ receptor in PBMCs from HCV-infected patients. We found that B cells ($CD3^-CD19^+$), monocytes ($CD3^-CD19^-CD56^-HLA\text{-}DR^+$), and dendritic cells (DCs, $CD3^-CD19^-CD56^-HLA\text{-}DR^+CD14^-CD16^-$) express IL-28Rα and IL-10Rβ, components of IFN-λ receptor, but T ($CD3^+$) and natural killer (NK, $CD3^-CD56^+$) cells do not (Fig. 5F and G). When we stained pSTAT1 after *ex vivo* IFN-λ3 stimulation of PBMCs, we found that STAT1 was significantly phosphorylated by IFN-λ3 in DCs, especially plasmacytoid DCs (pDCs, $CD123^+CD11c^-$ DCs), but not in other cells (Fig. 5H and I), indicating that pDCs directly respond upon IFN-λ3 stimulation.

We also performed the same experiments with PBMCs from healthy donors and observed similar results (Fig. S7C and D). However, the expression of IL-28Rα in pDCs was significantly higher in HCV-infected patients compared to healthy donors (Fig. S7E). Accordingly, IFN-λ3-induced STAT1 phosphorylation in pDCs was significantly stronger in HCV-infected patients compared to healthy donors (Fig. S7F).

HCV-specific, IFN-γ-producing T cell responses correlate with the Treg cell frequency

Finally, we examined the impact of the frequency of Treg cells on IFN-γ-producing T cell responses by analyzing the correlation between Treg cell frequency and HCV-specific IFN-γ spot numbers in study participants. We performed a regression analysis of Treg cell frequency and HCV-specific IFN-γ spot numbers using data from all 4 timepoints (before vaccination, week 16, week 36, and week 40) and all 14 participants. We found that the Treg cell frequency among the $CD4^+$ T cell populations inversely correlated with the HCV-specific IFN-γ spot number (Fig. 6A). In addition, the frequency of activated Treg cells among $CD4^+$ T cells inversely correlated with the HCV-specific IFN-γ spot number (Fig. 6B) whereas the frequency of resting Treg (Fig. 6C) or non-suppressive $FoxP3^+$ cells (Fig. 6D) did not. We calculated the fold-change in IFN-γ spots and the Treg frequency from pre-vaccination to week 40 and found a weak tendency for an inverse correlation (R = -0.4505) among the study participants, though it was not significant (Fig. S8). These data indicate that HCV-specific, IFN-γ-producing T cell responses may be affected by the increased frequency of Treg cells in chronically HCV-infected patients. Thus, decreasing the frequency of Treg cells, by using an *IFNL3* gene adjuvant, can enhance vaccine-induced, virus-specific T cell responses in patients with chronic HCV infection or following successful DAA treatment.

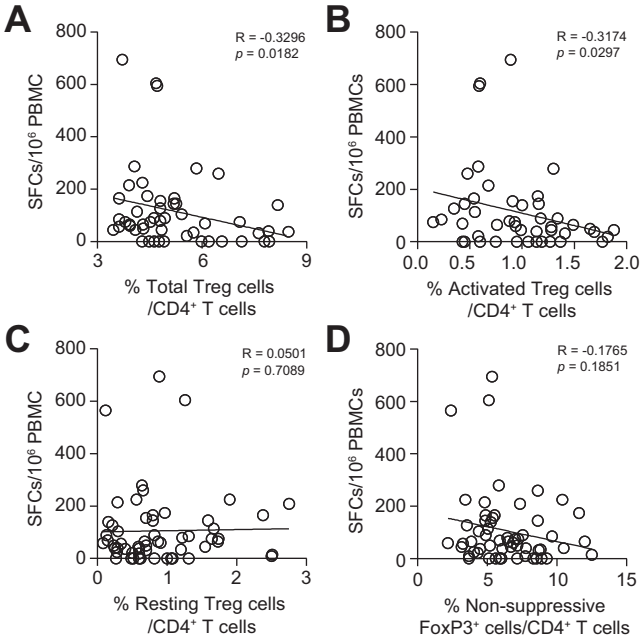

Fig. 6. Correlation between HCV-specific, IFN-γ-producing T cell responses and Treg cell frequency. Correlation analyses were performed between the HCV-specific T cell responses and the frequency of total Treg cells (A), activated Treg cells (B), resting Treg cells (C), or non-suppressive $FoxP3^+$ cells (D) among $CD4^+$ T cells using the data from all 4 timepoints for all 14 participants. Spearman`s rank correlation test was performed. IFN, interferon; Treg, regulatory T.

Discussion

DAA treatment results in SVR in >90% of patients with chronic HCV infection and has replaced PEG-IFN-based treatment as standard of care.[1] However, recent clinical reports showed that patients with high-risk behavior who achieved SVR on DAA treatment are still at risk of re-infection.[4–6] In addition, chimpanzees with DAA-induced viral clearance were re-infected upon HCV re-challenge,[7] and DAA treatment did not sufficiently modulate $CD8^+$ and $CD4^+$ T cell immunity in patients with chronic HCV infection,[8,9] indicating that protective immunity is not elicited even after successful DAA treatment. Thus, prophylactic HCV vaccines need to be developed to prevent HCV infection particularly for patients with high-risk behaviors who have been successfully treated by DAAs.

DAA treatment is known to not induce complete immunological restoration.[8,9,24–26,40] Plasma cytokine and chemokine profiles have revealed that decreased levels of cytokines, such as IFN-γ and IL-17, in patients with chronic HCV infection are not restored by DAA treatment.[24] Moreover, DAA treatment does not induce long-term T cell functional restoration in terms of cytokine secretion in $CD8^+$[8] and $CD4^+$[9] T cells. DAA treatment also does not reverse functional imprinting on the NK cell repertoire induced by chronic HCV infection.[25] Importantly, increased peripheral Treg cell frequency in chronic HCV infection persists even after successful DAA treatment.[26] As Treg cells limit vaccine-induced T cell responses,[28–33] contribute to the progression of fibrosis,[41,42] and are associated with the increased risk of HCC,[43] increased Treg cell frequency should be considered in HCV vaccine development, especially vaccines for patients with successful DAA treatment.

In the present study, GLS-6150, a DNA vaccine including the HCV NS3/NS4a, NS4b, and NS5a genes and an *IFNL3* gene adjuvant, reduced the Treg cell frequency in patients with chronic HCV infection. Moreover, the Treg cell frequency

inversely correlated with IFN-γ-producing T cell responses. However, the increased T cell response was not accompanied by an increase in the frequency and phenotype of HCV-specific, MHC-I multimer$^+$CD8$^+$ T cells as other therapeutic vaccine studies previously reported.[44–46] Interestingly, our *ex vivo* data showed that IFN-λ3 directly reduces Treg cell frequency, which is increased in chronic HCV infection compared to healthy controls. Therefore, our study shows that an *IFNL3* gene adjuvant decreases the frequency of Treg cells and enhances vaccine-induced, virus-specific T cell responses in patients with increased Treg frequency.

A recently developed prophylactic HCV vaccine candidate is currently under clinical investigation (NCT01436357). This vaccine also targets HCV NS protein-specific T cells via chimpanzee adenovirus 3 and MVA, which are used for priming and boosting, respectively, and induces potent CD4$^+$ and CD8$^+$ T cell responses against HCV NS proteins in HCV infection-naïve healthy volunteers without high-risk behavior.[22] However, adenoviral and MVA-vectored vaccines are not known to reduce Treg cell frequency. In contrast, GLS-6150 is aimed to reduce Treg cell frequency using an *IFNL3* gene adjuvant to enhance the protective T cell immunity against HCV re-infection, especially in patients with DAA-induced SVR.

Several studies have reported that Treg cells limit vaccine-induced T cell responses, and thus tried to enhance T cell immunogenicity by decreasing Treg cells.[28–32] Previous studies used adjuvants that target receptors upregulated on Treg cells, such as CD25 or CCR4, to reduce Treg cell frequency and enhance vaccine-induced immune responses.[28,32,33] In addition, some Toll-like receptor agonists, such as Poly(I:C)[29] and CpG-ODN,[29,31] have been used as vaccine adjuvants to amplify the ratio of effector T cells to Treg cells. In the current study, we showed that IFN-λ3 reduces Treg cell frequency among the CD4$^+$ T cells of HCV-infected patients with *ex vivo* and *in vivo* treatments, suggesting that IFN-λ3 is a potential candidate for the vaccine adjuvant, especially in the presence of increased Treg cell frequency.

IFN-λ3 belongs to the IFN-λ family and induces an antiviral state with the expression of interferon-stimulated genes, such as type I IFNs.[47] Although the mechanism is unclear, it was previously described that an *IFNL3* gene adjuvant led to a decrease in Treg cell number.[34,39] In particular, a previous murine study first found that an *IFNL3* gene adjuvant decreases Treg frequency and further increases vaccine-induced T cell responses.[34] Another murine study showed that an *IFNL3* gene adjuvant decreases Treg frequency.[39] In addition, IFN-λ3 decreased Treg frequency, thereby playing a therapeutic role (*e.g.*, decreased tumor size and metastasis) in a cervical cancer model of mice.[48] Use of an *IFNL3* gene adjuvant has been shown to enhance vaccine-induced T cell responses against HIV antigens in mice and macaques.[34,49] Recently, a preclinical study of HCV DNA vaccines in mice[35] reported that vaccine-induced HCV-specific T cell responses are enhanced by the *IFNL3* gene adjuvant.

In this study, GLS-6150 increased T cell responses but did not decrease HCV RNA titer in HCV-infected individuals. Although sequence mapping was not performed, there is a possibility that increased T cell responses recognize the vaccine epitopes but not the endogenous viral sequences as previously described.[45,50] In addition, MHC class-I multimer staining revealed that CD8$^+$ T cells targeting immunodominant epitopes were not expanded by GLS-6150, suggesting that the increased CD8$^+$ T cell response might be caused by *de novo* priming rather than boosting of pre-existing responses.

GLS-6150 could effectively boost T cell responses when given at week 36 but could not induce T cell responses in 3 priming vaccinations. Accordingly, there was no change in the frequencies or phenotypes of HCV-specific CD8$^+$ T cells at week 36 compared to the pre-vaccination. Although the mechanisms underlying the effect of the prime-boost strategy in DNA vaccination are still unclear, a certain period may be required to maximize the effect of boost-vaccination.

In the current study, GLS-6150 preferentially increased CD4$^+$ T cell responses compared to CD8$^+$ T cell responses, demonstrated by IFN-γ ELISpot assays with CD4$^+$ or CD8$^+$ T cell depletion, ICS assays, and analysis with MHC class-I multimers. DNA vaccination in humans commonly increases both CD4$^+$ and CD8$^+$ T cell responses.[51] However, the superior induction of CD4$^+$ T cell responses compared to CD8$^+$ T cell responses could be observed in recent reports of DNA vaccination in humans,[52,53] although its mechanism needs to be elucidated in future studies.

In conclusion, *IFNL3*-adjuvanted GLS-6150 enhances HCV-specific T cell responses and decreases Treg cell frequency in patients with chronic HCV infection. We showed that Treg cell frequency inversely correlates with HCV-specific T cell responses, suggesting that Treg cells need to be targeted to improve vaccine-induced immune responses in chronically infected or DAA-treated patients. In addition, we showed that *ex vivo* IFN-λ3 treatment reduces Treg cell frequency in patients with chronic HCV infection. Therefore, our study shows that decreasing the frequency of Treg cells with an *IFNL3* gene adjuvant can enhance vaccine-induced, virus-specific T cell responses in patients with chronic HCV infection. A clinical trial of GLS-6150 is currently underway in patients who have been successfully treated with DAAs to assess the safety and immunogenicity of the vaccine (NCT03674125).

Abbreviations

AE, adverse events; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CTLA-4, cytotoxic T lymphocyte antigen-4; DAA, direct-acting antiviral; DCs, dendritic cells; ELISpot, enzyme-linked immunospot; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; ICS, intracellular cytokine staining; IFN, interferon; IL, interleukin; MHC-I, major histocompatibility complex class-I; MFI, mean fluorescence intensity; MVA, modified vaccinia virus Ankara; NK, natural killer; NS, nonstructural; OLP, overlapping peptide; PBMCs, peripheral blood mononuclear cells; pDCs, plasmacytoid DCs; PEG-IFN, pegylated-interferon; RT, room temperature; SFCs, spot-forming cells; SVR, sustained virologic response; TNF, tumor necrosis factor; Treg, regulatory T; VAS, visual analog scale; WBC, white blood cell.

Financial support

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Conflict of interest

H.L, J.N.M, and M.J are employees of GeneOne Life Science. S.W is an employee of Inovio Pharmaceuticals, Inc. D.B.W owns stock and stock options of Inovio Pharmaceuticals, Inc. E.C.S is a member of the scientific advisory board of GeneOne Life Science.

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

Authors' contributions

H.L, S.W, J.N.M, D.B.W, M.J, J.H, S.H.A, and E.C.S designed the study. J.H and S.H.A performed the clinical trial and provided patient samples. J.W.H, P.S.S, S.H.H, H.L, J.Y.K, and J.Y.K performed the experiments. J.W.H, P.S.S, S.H.H, S.H.P, and E.C.S analyzed the data. J.W.H, J.H, S.H.A, and E.C.S wrote and edited the manuscript.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2020.02.009.

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Author names in bold designate shared co-first authorship

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