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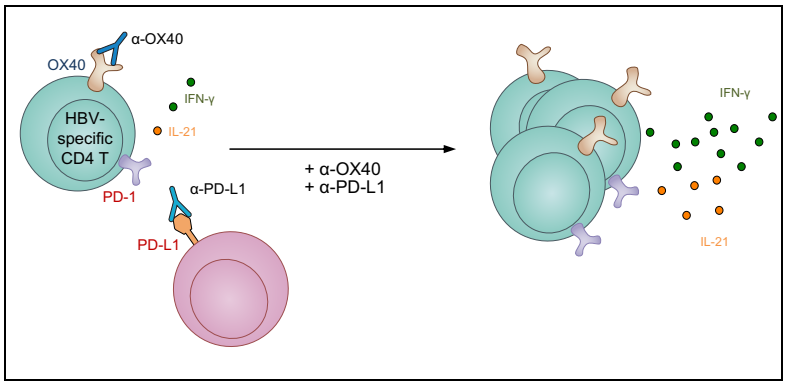
Graphical abstract



Highlights

- OX40 (CD134) and PD-1 are strongly expressed on HBV-specific CD4 T cells *ex vivo.*
- The HBV-specific CD4 T cells predominantly target the polymerase and core proteins.
- Combined OX40 stimulation and PD-L1 blockade functionally augment HBV-specific CD4 T cells.

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Lay summary

CD4 T cells are important in controlling viral infections but are impaired in the context of chronic hepatitis B virus (HBV) infection. Therapeutic approaches to cure chronic HBV infection are highly likely to require an immune-stimulatory component. This study demonstrates that HBV-specific CD4 T cells can be functionally augmented by combined stimulation of the co-stimulatory molecule OX40 and blockade of the inhibitory PD-1 pathway.

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OX40 stimulation and PD-L1 blockade synergistically augment HBV-specific CD4 T cells in patients with HBeAg-negative infection

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Background & Aims: Current antiviral therapies lack the potential to eliminate persistent hepatitis B virus (HBV) infection. HBV-specific T cells are crucial for HBV control and have recently been shown to be protective in patients following discontinuation of antiviral therapy. Thus, T cell-based approaches may greatly improve the therapeutic landscape of HBV infection. We aimed to augment HBV-specific CD4 T cells from chronically infected patients by targeting different immunological pathways.

Methods: Expression of various co-stimulatory and inhibitory receptors on HBV- and influenza-specific CD4 T cells was analyzed directly *ex vivo* by MHC class II-tetramers. Patients infected with HBV genotype D were screened for CD4 T cell responses by IFN-γ ELISpot and intracellular cytokine staining following stimulation with overlapping peptides (OLPs) spanning the HBV-polyprotein. Stimulation with recombinant IL-7, an agonistic OX40-antibody or blockade of PD-L1 was performed in antigen-specific *in vitro* cultures. Cytokine secretion and expression of transcription factors were analyzed by flow cytometry. Responses targeting influenza, Epstein-Barr virus and tetanus toxoid served as controls.

Results: Tetramer-staining revealed that the IL-7 receptor-alpha (CD127), OX40 and PD-1 constitute possible therapeutic targets as they were all strongly expressed on HBV-specific CD4 T cells *ex vivo*. The HBV-specific CD4 T cell responses identified by OLP screening targeted predominantly the HBV-polymerase and core proteins. Combined OX40 stimulation and PD-L1 blockade significantly augmented IFN-γ and IL-21 producing HBV-specific CD4 T cells *in vitro*, suggesting active T helper type 1 cell and follicular T helper cell programs. Indeed, transcription factors T-bet and Bcl6 were strongly expressed in cytokine-producing cells.

Conclusions: Combined OX40 stimulation and PD-L1 blockade augmented secretion of the helper T cell signature cytokines IFN-γ and IL-21, suggesting that immunotherapeutic approaches can improve HBV-specific CD4 T cell responses.

Lay summary: CD4 T cells are important in controlling viral infections but are impaired in the context of chronic hepatitis B virus (HBV) infection. Therapeutic approaches to cure chronic HBV infection are highly likely to require an immune-stimulatory component. This study demonstrates that HBV-specific CD4 T cells can be functionally augmented by combined stimulation of the co-stimulatory molecule OX40 and blockade of the inhibitory PD-1 pathway.

Introduction

Persistent infection with the hepatitis B virus (HBV) is a major risk factor for the development of chronic liver injury, cirrhosis and hepatocellular carcinoma and affects an estimated 350 million people worldwide.[1] While there is a prophylactic vaccination to prevent chronic infection, therapeutic approaches for persistent infection are limited and mostly fail to eliminate the virus.[2] Promising therapeutic approaches include T cell-based immunotherapy as both HBV-specific CD4 and CD8 T cells have been shown to be required for viral clearance but are functionally impaired in the context of chronic infection.[3–5]

Blockade of the inhibitory programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway has been shown to significantly enhance T cell responses in various settings and has already been implemented in clinical practice in the treatment of various solid cancers.[6] However, data from clinical trials in patients with both hepatocellular carcinoma and chronic HBV infection suggest that PD-1 blockade alone is insufficient to improve viral control as none of the included patients achieved HBV surface antigen (HBsAg) seroconversion during the course of therapy.[7] Therefore, additional approaches are required to improve T cell responses in chronic HBV infection. The co-stimulatory molecule OX40 (CD134) could be one such approach as it has also been shown to serve as a target for immunotherapeutic approaches.[8] In a recent phase I trial in late stage cancer patients, it has safely been administered with remarkable effects on tumor burden in some patients.[9]

Keywords: Viral hepatitis; Antiviral immunity; Immunotherapy; Follicular T helper cells; Th1 cells; Cytokines.
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Moreover, OX40 signaling has been shown to facilitate immunological control against a variety of viruses, including HBV.[10–12] Similarly, it has been shown that IL-7 can substantially improve the antiviral CD4 T cell response in a mouse model of persistent viral infection.[13] Thus, targeting PD-1, OX40 or the IL-7 axis might improve the functionality of HBV-specific CD4 T cells.

Studies of HBV-specific CD4 T cell responses in humans chronically infected with the virus have been hampered by the small frequency of circulating HBV-specific CD4 T cells. Consequently, very few studies were able to shed light on the *ex vivo* phenotype of HBV-specific CD4 T cells, their transcriptional activity or their functional properties. While high levels of PD-1 expression on HBV-specific CD4 T cells have been reported, PD-1 blockade did not significantly resuscitate IFN-γ secretion in these cells.[14] Although increasing IFN-γ secretion is important, functional recovery of IL-21 producing HBV-specific CD4 T cells might be similarly relevant as IL-21 has been shown to be centrally involved in successful viral immunity. Indeed, IL-21 has been implicated in the clearance of HBV infection in humans and in a humanized mouse model of HBV infection.[15] In addition, IL-21 facilitates antiviral CD8 T cell immunity which is required for immune control of persistent lymphocytic choriomeningitis virus (LCMV) infection in mice.[16–18] Thus, IL-21 is a central player in antiviral immunity and should be utilized to improve HBV-specific CD4 T cells. Therefore, in this study, we aimed to analyze whether blockade of PD-1 signaling, ligation of OX40 or addition of recombinant IL-7 would be feasible approaches to improve the functionality of HBV-specific CD4 T cell responses.

Materials and methods

Study participants

This study was approved by the ethics committee (344/13 and 227/15_161217) of the University Hospital Freiburg. Written informed consent was obtained from all individuals recruited in this study.

Peripheral blood samples were obtained from a total of 127 patients chronically infected with HBV (cHBV) (66 patients with cHBV for overlapping peptide [OLP] screening, 32 patients with cHBV for HBV core tetramer screening and a further 29 patients cHBV were only used for control responses) and 71 healthy donors (HDs). Detailed characteristics of study participants are provided in Tables S1 and S2. Chronic HBV infection was defined as seropositivity for HBsAg and anti-HBV core for at least 6 months. Patients with hepatitis C virus (HCV) or human immunodeficiency virus coinfection were excluded. Human leucocyte antigen (HLA)-typing was performed by next generation sequencing using commercially available primers (GenDx, Utrecht, The Netherlands) and run on a MiSeq system. Data were analyzed using the NGSengine® Software (GenDx).

Magnetic-bead-based enrichment of antigen-specific CD4 T cells

Phycoerythrin (PE)-labeled major histocompatibility complex (MHC) class II-tetramers of HLA-DRB1* 0101 HBV-derived epitope $Core_{60\text{-}75}^{PE}$ (aa-sequence: LCWGELMTLATWVGVN) and influenza (Flu)-derived epitope $HA_{306\text{-}318}^{PE}$ (aa-sequence: PKYVKQNTLKLAT) were obtained from MBL, Woburn, United States. The tetramer enrichment protocol was performed as described for antigen-specific CD8 T cells by Alanio *et al.*[19] Peripheral blood mononuclear cells (PBMCs) from HLA-DRB1* 0101-positive donors were incubated with the respective tetramer. According to the manufacturer's protocol, enrichment was performed using anti-PE MACS technology (Miltenyi Biotech, Bergisch Gladbach, Germany). Enriched antigen-specific CD4 T cells were detected by flow cytometry. Naïve CD45RA+CCR7 +CD4 T cells and samples with less than 5 antigen-specific CD4 T cells (considered below limit of detection) were excluded from the final analysis. Enriched virus-specific CD4 T cells were calculated as follows: Absolute number of virus-specific CD4 T cells (enriched sample) divided by the absolute number of CD4 T cells (pre-enriched sample) times 100.

Synthetic peptides

A total of 225 OLPs were designed, spanning the entire HBV genotype D-proteome in 18-mers. For technical reasons OLPs 154 and 155 could not be synthesized. Previously described MHC class II-restricted peptides derived from Flu type A virus, Epstein-Barr virus (EBV) and tetanus toxoid (TT) are listed in Table S3. All peptides were synthesized by Genaxxon Bioscience (Ulm, Germany) with >70% purity.

In vitro expansion (OLP Screening)

The *in vitro* expansion was performed with $1.5x10^7$ PBMCs. According to previously described protocols,[20] 20% of the cells were pulsed with all HBV overlapping peptides for 1 h. After 10 days of culture, an ELISpot-assay was performed as screening with pooled peptides, followed by a validation with single peptides via intracellular cytokine staining (ICCS).

Elispot (OLP screening)

After 10 days of in vitro expansion, $5x10^4$ cells per well incubated with pooled OLPs for 24 h on IFN-γ ELISpot capture antibody coated (BD Bioscience, San Jose, USA) polyvinylidene fluoride plates. Negative controls remained without peptide; positive controls were incubated with phorbol myristate acetate (PMA) (50 ng/ml) and ionomycin (Iono) (1 μg/ml). Plates were blocked and developed using IFN-γ ELISpot detection antibody, streptavidine-HRP and 3-Amino-9-ethylcarbazole Substrate Set (all BD Bioscience, San Jose, USA). After blocking, washing and drying, plates were analyzed by ELISpot-Reader and ImmunoSpot software (both Cell. Tech. Lmt., Shaker Heights, USA). Wells with a number of spots at least twice as high as the negative control were considered as possible candidates, followed by ICCS confirmation (see below).

ICCS (OLP screening)

After 13 days of *in vitro* expansion (OLP screening), 2.5×10^5 cells per well were re-stimulated with single HBV_OLPs (15 μg/ml) or PMA and Iono as positive control under the presence of Brefeldin A. Negative controls were incubated with Brefeldin A only. After 5 h of incubation at 37 °C, IgG1 Fc receptors were blocked, cells were stained for surface markers, fixed, permeabilized and stained with cytokine antibodies.

Co-stimulation

In selected experiments, PBMCs were analogously pulsed with peptide. Cells were then incubated with IL-7 (50 ng/ml) (R&D Systems, Minneapolis, USA), agonistic OX40 mononuclear antibody (α-OX40) (300 ng/ml) (clone L106, BD Bioscience, San Jose, USA), inhibitory PD-L1 mononuclear antibody (α-PD-L1) (10 μg/ml) (clone MIH1; eBioscience, San Diego, USA) or a combination of both. Controls remained untreated. On day 3, 7 and 10, half

the volume of medium was replaced with fresh RPMI medium (RPMI1640 supplemented with 10 mM HEPES; 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum, ThermoFischer, Germany) and co-stimulation conditions. Cells were cultured for a total of 13 days.

ICCS and transcription factor staining after co-stimulation

After 13 days of *in vitro* expansion (co-stimulation), 2.5×10^5 cells per well were re-stimulated with single HBV_OLP (15 μg/ml) or control peptide (EBV, Flu and TT) (15 μg/ml). PMA and Iono were used as positive control. Negative controls were incubated with Brefeldin A only (neg). IMDM (including 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, ThermoFischer, Germany) were used as stimulation media. After 5 h of incubation IgG1 Fc receptors were blocked, cells were stained for surface markers, fixed, permeabilized and stained with cytokine antibodies.

Ex vivo stimulation and ICCS

PBMCs were seeded into 96 well plates and incubated overnight. 0.8×10^6 PBMCs were used for peptide stimulation and the co-stimulation with α-OX40 (300 ng/ml) and α-PD-L1 (10 μg/ml). For the control condition $0.4x10^6$ PBMCs were used. After 12 h peptide stimulation with 15 mM peptide (±co-stimulation) cells were incubated with Brefeldin A and Monensin for 5 h at 37 °C. PMA and Iono was used as positive controls. After 5 h of incubation IgG1 Fc receptors were blocked, cells were stained for surface markers, fixed, permeabilized and stained with cytokine antibodies.

Control responses

For comparison with other pathogens, similar assays with PBMCs from 49 cHBV and from 59 HDs and predescribed epitopes of EBV, Flu and TT were performed, with equal *ex vivo* stimulation, *in vitro* expansion, intracellular staining and co-stimulation.

Flow cytometry

Following tetramer enrichment, cells were stained with the following antibodies: anti-$CD4^{FITC}$, $CCR7^{BUV395/BV421}$, anti-$PD1^{BV786}$, anti-$CCR6^{BUV737}$, anti-$CD137(4\text{-}1BB)^{BUV395,}$ anti-$CD134(OX40)^{PE\text{-}Cy7}$, anti-$CD38^{BUV737}$, anti-$ICOS^{BV711}$, anti-$CD127^{BV421}$ (BD Bioscience, San Jose, CA), anti-$CD45RA^{PerCP\text{-}Cy5.5}$, anti-$CD14^{eFluor780}$, anti-$CD19^{eFluor780}$, fixable viability $dye^{eFlour780}$, anti-$CD154(CD40L)^{APC}$ (eBioscience, San Diego, USA), anti-$CXCR5^{APC/BV711}$, anti-$CXCR3^{BV510}$ (Biolegend, San Diego, CA). In order to characterize the antigen-specific CD4 T cells, 2 separate panels were established. After surface staining cells were fixed with 2% PFA/PBS and were analyzed by flow cytometer on a FACS LSRFortessa (BD Bioscience) machine using DIVA software.

The following antibodies were used for ICCS and transcription factor staining (co-stimulation) after the respective expansion or stimulation protocol: anti-$CD4^{APC}$, anti-$CD4^{PE}$, anti-IFN-γ^{FITC}, anti-$IgG1^{pure}$, anti-$IL\text{-}2^{BV421}$, anti-$IL\text{-}21^{PE}$, anti-$Bcl\text{-}6^{AlexaFlour647}$, (all BD Bioscience, San Jose, CA), anti-$CD4^{eFlour450}$, anti-T-$bet^{PE\text{-}Cy7}$, fixable viability $dye^{eFlour780}$, (all eBioscience, San Diego, USA). After surface staining cells were fixed and then permeabilized by using cytofix/cytoperm (BD Bioscience) for intracellular cytokine staining and FoxP3 Fixation/Permeabilization Concentrate and Diluent kit (eBioscience Foxp3, Thermo Fisher Scientific, Massachusetts, USA) for transcription factor staining and stained intracellular cytokines. For staining of transcriptions factors, fluorescence minus one control was stained as a full panel without the respective transcription factor antibody. Cells were measured in a FACSCanto II (BD Bioscience) flow cytometer and analyzed with FlowJo software (Flowjo, LLC, Ashland, USA). Percentages of cytokine-producing cells are always presented after subtraction of the unspecific background in the negative control.

Statistical analysis

Statistical analyses were performed, and figures were designed using GraphPad Prism 6 software (GraphPad Software, San Diego, USA). For specific information on the statistical tests, please refer to the figure legends. Values of $p < 0.05$ were considered significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

Results

High expression levels of PD-1, OX40 and CD127 on HBV-specific CD4 T cells

The *ex vivo* phenotype of HBV-specific CD4 T cells is not well defined. While some information is available on the hierarchy of inhibitory receptor expression,[14] much less is known about the expression levels of stimulatory receptors that could be utilized therapeutically. Thus, in order to determine the expression levels of several potential therapeutic targets on HBV-specific CD4 T cells directly *ex vivo*, we used MHC class II tetramer-technology with an HBV core protein derived epitope (HLA-DRB1*0101-restricted HBV core 61–80, aa-sequence: CWGELMTLATWVGVNLEDPA). Bead-based enrichment was performed on all samples as has been described previously for HCV-specific CD4 T cells.[21] This approach was also used to identify Flu-specific CD4 T cells (HLA-DRB1*0101-restricted Flu hemagglutinin 307–319, aa-sequence: PKYVKQNTLKLAT). HBV-specific CD4 T cells were detected in 14 out of 32 (44%) HLA-DRB1*0101-positive patients with cHBV, and Flu-specific CD4 T cells were detected in 11 out of 12 (92%) HDs. Both populations, however, were present at comparably low frequencies (Fig. 1A). For phenotypic analyses of inhibitory and activation markers, antigen-specific CD4 T cells were compared to non-naïve bulk CD4 T cells as these markers are typically not expressed on naïve cells. Thus, inclusion of naïve cells would artificially enhance the observed differences between HBV-specific CD4 T cells and bulk CD4 T cells. In agreement with observations from Raziorrouh *et al.*,[14] the inhibitory marker PD-1 and the IL-7 receptor α-chain (CD127) were strongly expressed on HBV-specific CD4 cells (Fig. 1B,C). Compared to HBV-specific CD4 T cells, Flu-specific CD4 T cells expressed less PD-1 and higher levels of CD127 suggesting a less exhausted and more memory-like differentiation status (Fig. 1B,C). Analyses of activation markers and co-stimulatory receptors (Fig. 1D, H) revealed high expression levels of OX40 (CD137, tumor necrosis factor receptor superfamily member 4, TNFRSF4) on HBV-specific CD4 T cells and to a lesser extent also on Flu-specific CD4 T cells (Fig. 1D). In contrast, CD38 (Fig. 1E), 4-1BB (CD137) (Fig. 1F), ICOS (Fig. 1G) and CD40 ligand (CD154) (Fig. 1H) were scarcely expressed on HBV- and Flu-specific CD4 T cells directly *ex vivo*. As OX40 and PD-1 both constitute promising targets for immune-therapeutic interventions, we analyzed co-expression of both receptors on virus-specific CD4 T cells. Interestingly, the large majority of HBV-specific CD4 T

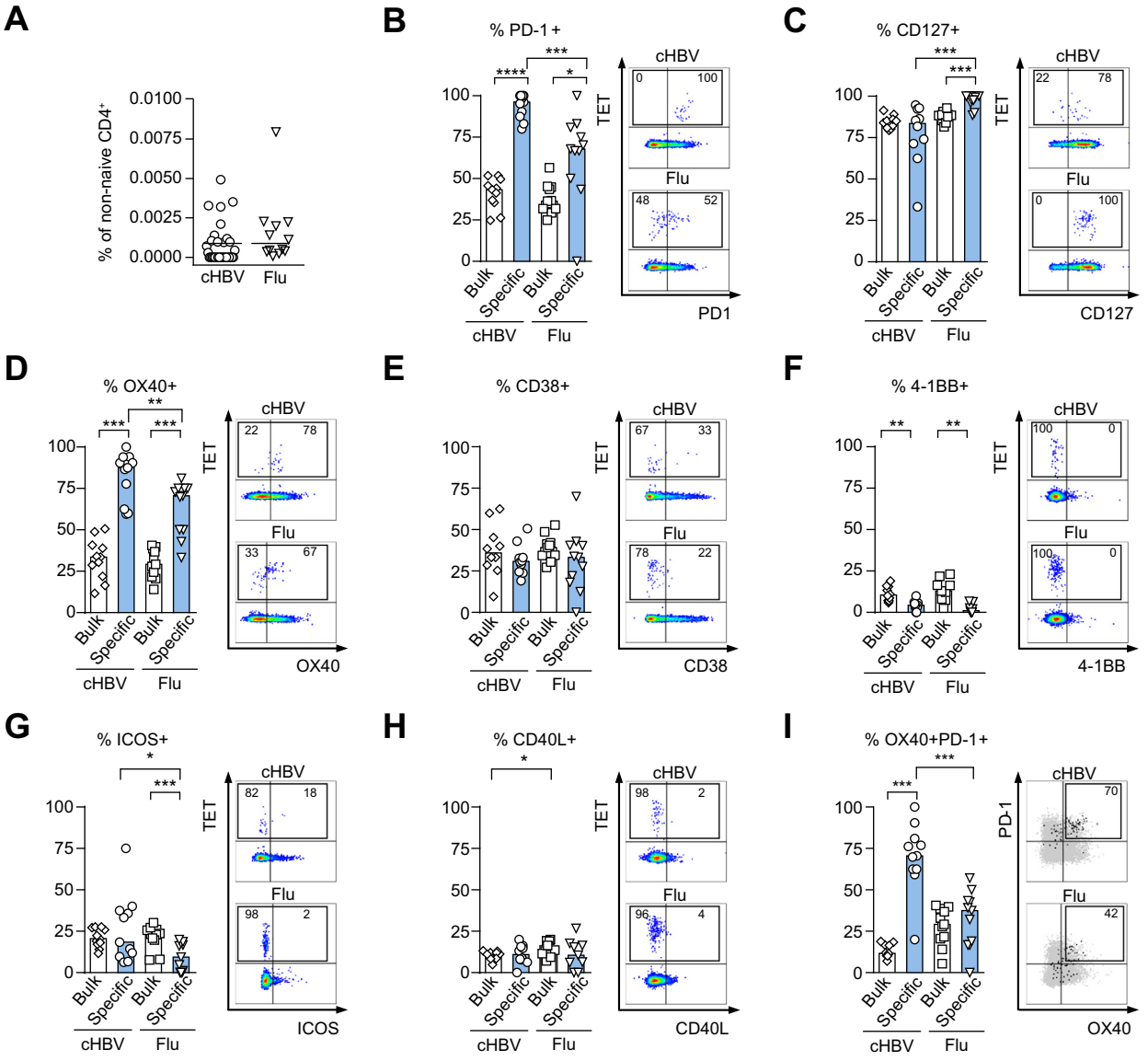

Fig. 1. *Ex vivo* phenotype of HBV- and Flu-specific CD4 T cells. (A) HBV-specific CD4 T cells were detectable in 14 (out of 32) patients with cHBV. Flu-specific CD4 T cells were detectable in 11 (out of 12) HDs. Mann-Whitney rank sum test ($p = 0.05357$). Lines indicate medians of detectable responses. (B-H) Expression of PD-1, CD127, OX40 (CD134), CD38, 4-1BB (CD137), ICOS and CD40L (CD154) on virus-specific CD4 T cells in comparison to bulk non-naïve CD4 T cell population and representative pseudocolor plots showing expression of the indicated protein on virus-specific CD4 T cells. (I) Co-expression of OX40 and PD-1 on virus-specific CD4 T cells in comparison to bulk non-naïve CD4 T cell population. Representative dot plots showing OX40 and PD-1 co-expression on virus-specific CD4 T cells (black dots) and bulk non-naïve CD4 T cells (grey dots). (B-I) Bars indicate medians. Differences between 2 populations were analyzed using paired *t* test (bulk *vs.* TET) and unpaired *t* test (HBV *vs.* Flu). *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001. cHBV, chronic HBV; Flu, influenza type A; HBV, hepatitis B virus; HDs, healthy donors; TET, enriched virus-specific CD4 T cells. (This figure appears in colour on the web.)

cells co-express both receptors, while co-expression levels on Flu-specific CD4 T cells were significantly lower (Fig. 1I).

Analysis of HBV-specific CD4 T cell responses in a large cohort of chronically infected individuals

Previous studies analyzing HBV-specific T cells have provided conflicting results with regards to the viral proteins being targeted by CD4 T cells. Indeed, it has been demonstrated that CD4 T cell responses are largely restricted to the core and polymerase proteins[22,23] while another study suggested reactivity also towards the surface protein.[24] Thus, in order to identify HBV-specific CD4 T cell responses in individual patients and to analyze the breadth of the HBV-specific CD4 T cell response, we performed *in vitro* stimulation assays using OLPs spanning the entire HBV proteome. OLP stimulation was performed in 66 HBeAg-negative patients chronically infected with HBV genotype D (Table S1). Stimulation and culture were performed as described in the methods section. Readout was performed using ELISpot as well as intracellular cytokine staining to confirm each individual ELISpot response. Of note, OLP stimulation was also performed in 11 HDs in order to determine HBV-unspecific background. Importantly, 6 of 11 HDs (55%) mounted responses against 2 regions within the polymerase protein (*i.e.* OLP #16, 17, 105 and 106). Therefore, these OLPs were excluded from the following analyses in HBV-infected patients.

A total of 48 of 66 patients (73%) did not mount detectable HBV-specific CD4 T cell responses and 8 (12%), 7 (11%) and 3 (4%) patients mounted responses against 1, 2 or 3 HBV OLPs,

respectively (Fig. 2A). Interestingly, HBV-specific CD4 T cell responses were only detectable in patients with low to moderate viral titers up to 15,000 IU/ml. Indeed, patients with higher viral loads (>20,000 IU/ml) did not mount HBV-specific CD4 T cell responses (Fig. 2B). In our cohort, the presence of detectable CD4 responses was unrelated to treatment status (Fig. 2B, patients undergoing antiviral therapy displayed as red symbols). In agreement with previous reports,[22,23,25] we detected the majority of responses directed against the core and polymerase proteins. Few responses targeted the X protein and no HBs-specific CD4 T cell responses were detected (Fig. 2C). HBV-specific CD4 T cell responses were generally of low frequency and frequencies were comparable between responses targeting different viral proteins (Fig. 2D). Frequencies of HBV-specific CD4 T cell responses were independent of viral loads and alanine aminotransferase (ALT) levels in our cohort (Fig. S2C).

Combined OX40 stimulation and PD-L1 blockade enhances the expansion of cytokine-producing HBV-specific CD4 T cells

Next, we aimed to determine whether HBV-specific CD4 T cells in chronic HBV infection can be functionally augmented by either blockade of PD-L1 (α-PD-L1), stimulation of OX40 (α-OX40) or addition of recombinant IL-7 as these approaches have been shown to improve antiviral T cell responses in mouse models of viral infection and vaccine studies.[11,13,26] Moreover, we performed blockade of PD-L1 in combination with OX40 stimulation in order to analyze whether both interventions could synergistically enhance HBV-specific CD4 T cell responses. We stimulated PBMCs from chronically infected patients with OLPs identified in Fig. 2D (selected patients for stimulation experiments displayed as white symbols). As shown in Fig. 3, stimulation with IL-7, α-OX40 or α-PD-L1 alone did not increase the percentage of IFN-γ secreting CD4 T cells after *in vitro* culture with HBV-specific epitopes. Interestingly however, combination of α-OX40 and α-PD-L1 resulted in a significant increase of cytokine-producing CD4 T cells. Importantly, this effect was observed for secretion of IFN-γ as well as IL-21 (Fig. 3A,D). We also observed a tendency towards stronger IL-2 production; however, this effect did not reach statistical significance (Fig. 3C,D). As these observations were made following *in vitro* culture, we aimed to assess whether combined α-OX40 and α-PD-L1 treatment was also able to unmask HBV-specific CD4 T cell responses directly *ex vivo*. Therefore, we performed overnight stimulations in patients with a confirmed OLP-response in the presence or absence of α-OX40 and α-PD-L1. This approach, however, was unable to boost HBV-specific CD4 T cell responses after overnight-culture (Fig. S4). Collectively, these data indicate that HBV-specific CD4 T cells are sensitive to immune-therapeutic interventions and that combined targeting of the PD-1 and OX40 pathways significantly increases the percentage of IL-21 and IFN-γ producing CD4 T cells.

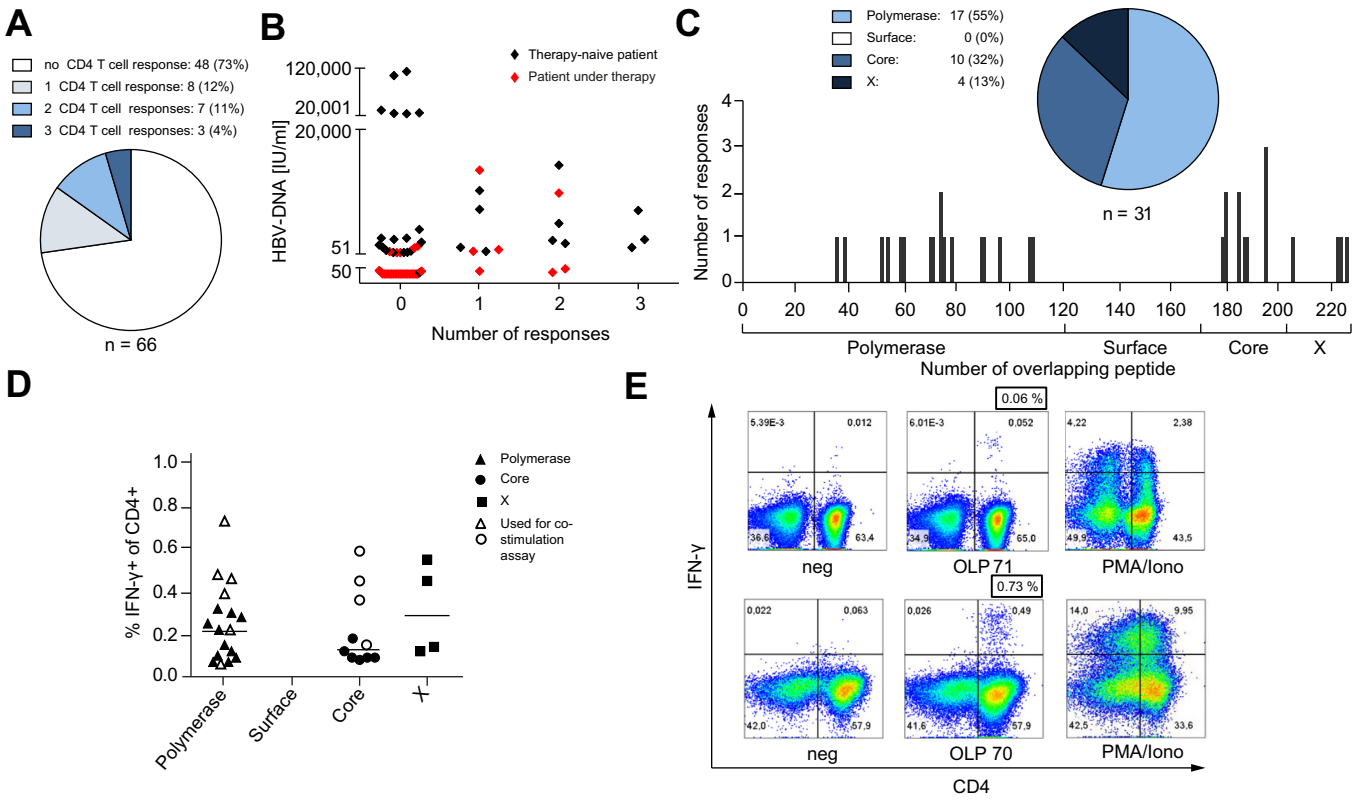

Fig. 2. HBV-specific CD4 T cell responses primarily target epitopes of polymerase, core and X proteins. PBMCs from 66 patients with cHBV were screened for HBV-specific CD4 T cell responses by ELISpot and ICCS for IFN-γ following *in vitro* culture using 223 18-mer HBV OLPs. (A) 18 patients showed positive responses either to 1, 2 or 3 OLPs, while 48 did not mount detectable responses. (B) The number of responses is plotted against HBV-DNA levels in all 66 patients. (C) The total number of 31 responses were directed against viral polymerase (n = 17), core (n = 10) and the X protein (n = 4). No T cell responses against the surface protein were detected. (D) Frequencies of IFN-γ+ CD4 T cells were calculated as outlined in the methods section and ranged from 0.06% (weakest response included) to 0.73% (strongest response included). (E) Representative pseudocolor plots for negative control (neg), OLP-stimulated (OLP) and positive control with PMA/Iono gated on via-dye negative single-cell lymphocytes. cHBV, chronic HBV; HBV, hepatitis B virus; ICCS, intracellular cytokine staining; Iono, ionomycin; OLPs, overlapping peptides; PBMCs, peripheral blood mononuclear cells; PMA, phorbol myristate acetate. (This figure appears in colour on the web.)

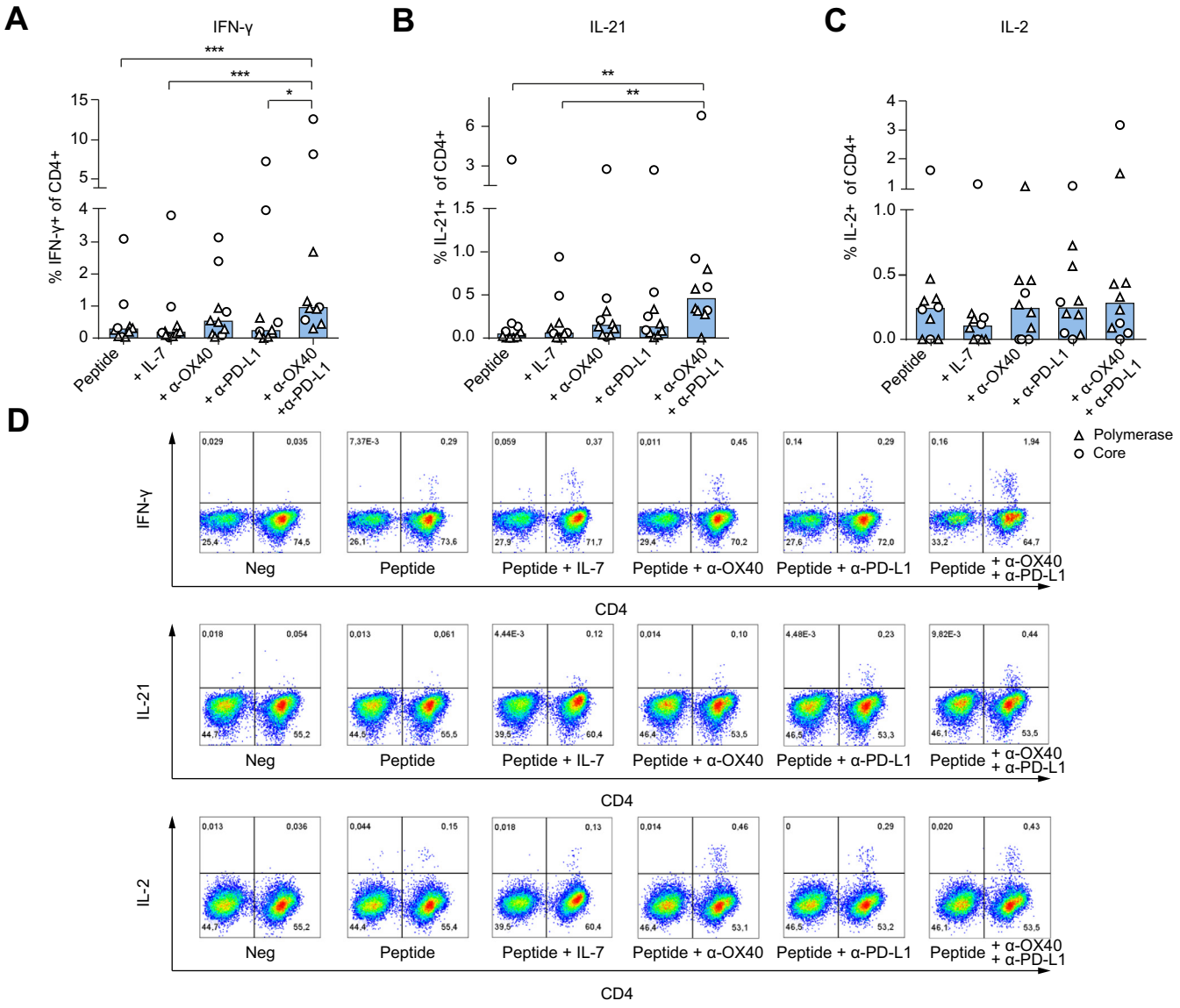

Fig. 3. OX40 stimulation and PD-L1 blockade synergistically enhance HBV-specific CD4 T cells. PBMCs were pulsed with pre-identified reactive HBV OLPs and co-cultured with recombinant IL-7, agonistic OX40-antibody (α-OX40), PD-L1-antibody (α-PD-L1) or a combination of the latter. The control remained without any co-stimulation (peptide). Frequencies were calculated as described in the methods section. Bars indicate medians. (A-C) A significant increase of IFN-γ-secreting cells (A) and IL-21-secreting cells (B) was detectable after co-stimulation with α-OX40 and α-PD-L1 while IL-2-secreting cells were not affected (C). (D) Representative pseudocolor plots for the respective culture conditions. Controls were not re-stimulated with peptide as negative control (neg). Friedman's test and Dunn's test were applied. *p <0.05, **p <0.01, ***p <0.001. HBV, hepatitis B virus; OLPs, overlapping peptides; PBMCs, peripheral blood mononuclear cells. (This figure appears in colour on the web.)

CD4 T cells targeting Flu-, EBV- and TT-epitopes are sensitive to combined OX40 stimulation and PD-L1 blockade

In order to analyze whether the observed effects are specific for HBV-specific CD4 T cells, we performed control experiments with CD4 T cell epitopes targeting different antigens in patients with cHBV and HDs. We screened 49 patients with cHBV and 59 HDs for CD4 T cell responses against previously described epitopes derived from EBV, Flu and TT (Tables S1, S2 and S3). Patients and donors with the strongest detectable responses were cultured in the presence or absence of α-OX40 and α-PD-L1 (Fig. 4). Of note, baseline frequencies (peptide only) were slightly higher in HD compared to cHBV which is likely a selection bias, as more HDs were screened and some low frequency responses were not included in the stimulation assays. However, as shown in Fig. 4, T cell responses against viral epitopes and TT epitopes showed comparable patterns of augmentation after combined OX40 stimulation and PD-L1 blockade, independent of the frequency of antigen-specific CD4 T cells in the absence of α-OX40 and α-PD-L1. These observations indicate that immune interventions involving PD-L1 blockade and OX40 stimulation are not only effective in augmenting HBV-specific CD4 T cells but can also enhance CD4 T cells with various other specificities.

Transcription factors T-bet and Bcl6 are strongly expressed on cytokine-secreting HBV-specific CD4 T cells

IL-21 and IFN-γ are the signature cytokines of follicular T helper (Tfh) cells and T helper type 1 (Th1) cells, respectively. In mouse

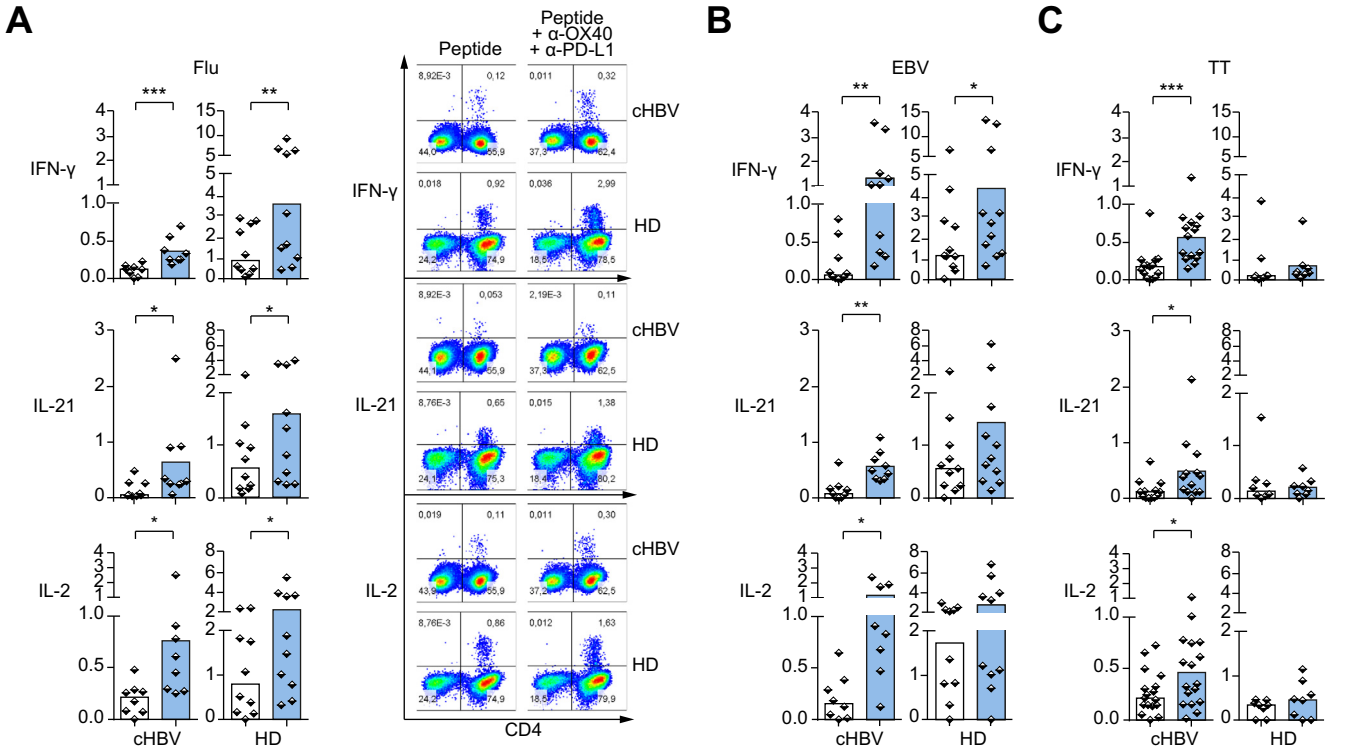

Fig. 4. Combined OX40 stimulation and PD-L1 blockade enhance control responses to Flu, EBV and TT in patients with cHBV and HDs. To analyze the impact of combined OX40 stimulation and PD-L1 blockade on other pathogen-specific CD4 T cells, assays similar to those displayed in Fig. 3 were performed. PBMCs from patients with cHBV and HDs were pulsed with peptides from (A) Flu type A virus, (B) EBV, (C) TT followed by *in vitro* cultures in the absence (light grey bars) or presence of α-OX40 and α-PD-L1 (dark grey bars). Percentages of CD4 T cells secreting IFN-γ (top), IL-21 (middle) and IL-2 (bottom) following culture with the indicated conditions are displayed after correcting for background cytokine production. Bars represent medians. Mann-Whitney *U* test was applied. **p* <0.05, ***p* <0.01, ****p* <0.001. cHBV, chronic hepatitis B virus; EBV, Epstein-Barr virus; Flu, influenza type A; HDs, healthy donors; PBMCs, peripheral blood mononuclear cells; TT, tetanus toxoid. (This figure appears in colour on the web.)

models of viral infection, it has been shown that virus-specific CD4 T cells acquire either a Th1 or Tfh phenotype.[27,28] Thus, we aimed to analyze whether HBV-specific CD4 T cells preferentially display a Tfh or Th1 phenotype. Co-expression of the C-X-C chemokine receptor type 5 (CXCR5) and PD-1 was used to identify CD4 T cells with a Tfh phenotype while expression of the C-X-C chemokine receptor type 3 (CXCR3) in the absence of C-C chemokine receptor type 6 (CCR6) expression was used to identify Th1 cells. Interestingly, we observed that the Tfh and Th1 distribution within HBV-specific CD4 T cells was very heterogeneous in patients with cHBV, with Tfh frequencies ranging from 12.5% to 91.3% (median 40%) and Th1 frequencies ranging from 15.8% to 69.6% (median 35.1%). Importantly however, there was a significant overlap of cells with a Th1 phenotype and a Tfh phenotype, suggesting a lack of a clear Th1 *vs.* Tfh distribution within HBV-specific CD4 T cells (Fig. 5A,B). As the Th1 signature cytokine IFN-γ was the most prominent cytokine produced by HBV-specific CD4 T cells after *in vitro* culture (Fig. 3), we aimed to analyze whether *in vitro* culture preferentially gives rise to cells with functional and transcriptional Th1 properties. Therefore, we analyzed whether IL-21 and IFN-γ were produced by different cells following HBV-specific *in vitro* culture in the presence of α-OX40 and α-PD-L1. Interestingly, we observed that IL-21 was almost exclusively secreted by IFN-γ co-expressing cells (Fig. 5C), On the transcriptional level, T-bet facilitates Th1 differentiation and IFN-γ secretion while Bcl6 functions as the transcriptional regulator for Tfh differentiation,[29,30] under certain circumstances even repressing IFN-γ secretion.[31] Therefore, we analyzed whether expression of these transcription factors mirrored the cytokine expression patterns in HBV-specific CD4 T cells. Interestingly, we observed that expression levels of T-bet and Bcl6 were upregulated in HBV-specific cells producing either IFN-γ alone (IFN-γ+IL-21-) or both IFN-γ and IL-21 (IFN-γ+IL-21+) (Fig. 5D,E). These observations further support the notion that HBV-specific CD4 T cells display a lack of a specific lineage commitment towards Th1 or Tfh differentiation. As it has been suggested that T-bet expression is required for T cells to improve their effector functions following checkpoint blockade,[32] we sought to analyze whether the expression levels of T-bet impacted the augmentation of HBV-specific CD4 T cells after combined PD-L1 blockade and OX40 stimulation. Interestingly, the expression of T-bet in IFN-γ+IL-21- CD4 T cells following HBV-specific culture in the presence of α-OX40 and α-PD-L1 correlated with an increase of IFN-γ producing T cells under these conditions compared to peptide stimulation alone (Fig. 5D). In contrast, Bcl6 expression tended to be negatively associated with the fold change of IFN-γ +IL-21- CD4 T cells following combined PD-L1 blockade and OX40 stimulation, although this association did not reach statistical significance (Fig. 5E). In sum, our observations show that cytokine-producing HBV-specific CD4 T cells cannot be separated into Th1 and Tfh subsets based on their transcription

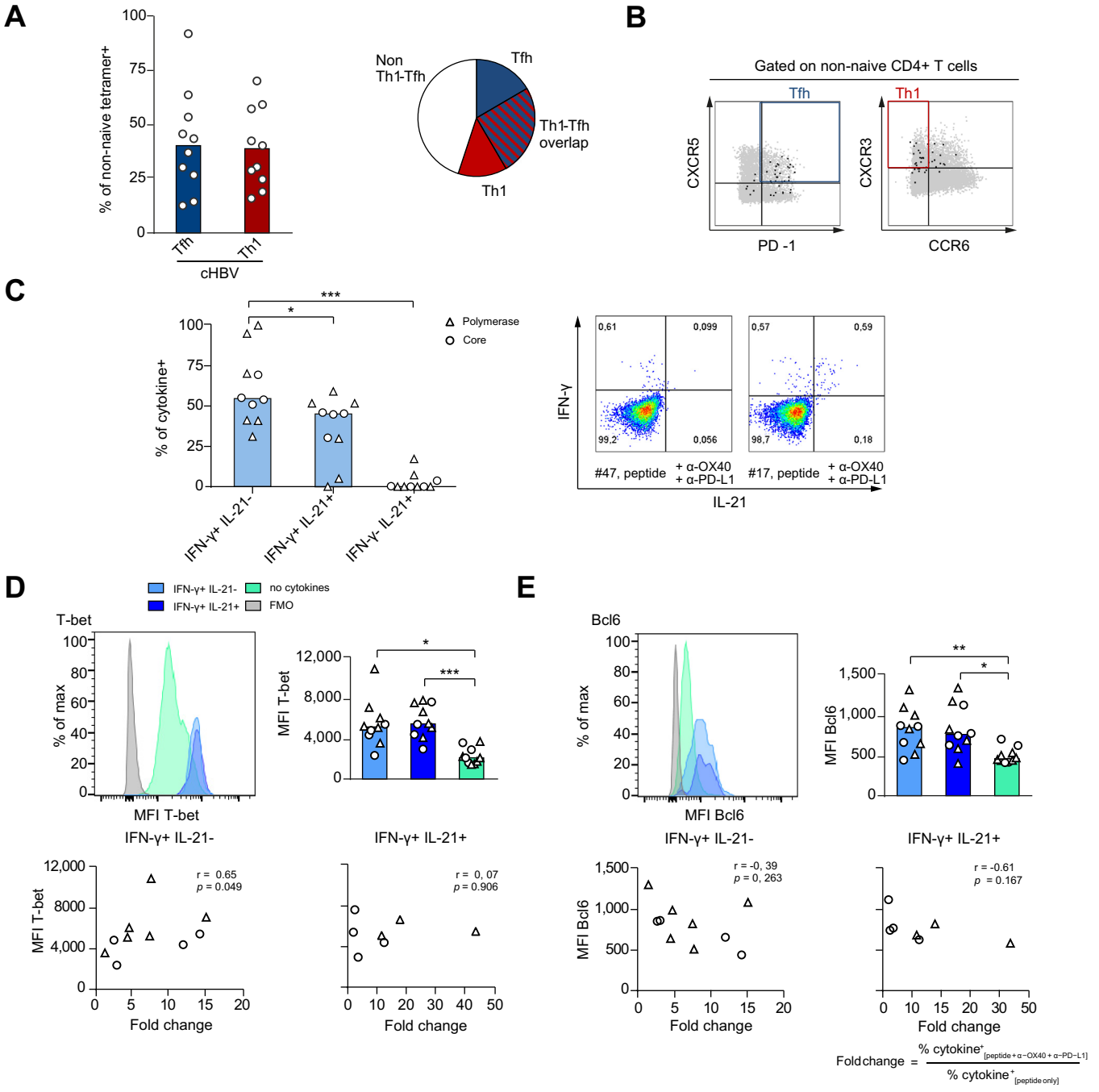

Fig. 5. Overlapping Tfh and Th1 lineage commitments of HBV-specific CD4 T cells. (A) Phenotype of HBV-specific CD4 T cells. Distribution of Tfh (CXCR5+, PD-1+) and Th1 phenotype (CXCR3+, CCR6−). Overlap of Tfh and Th1 phenotype is represented by CXCR5+ Th1: [%TET of Th1 - %TET of (CXCR5− Th1)] as shown in the pie chart. (B) Representative dot plots showing Tfh and Th1 phenotype on virus-specific CD4 T cells (black dots) and bulk non-naïve CD4 T cells (grey dots). (C) Frequencies of all cytokine-producing cells reveal the absence of IL-21+IFN-γ- cells. (D, E) PBMCs of cHBV patients were pulsed with respective OLPs followed by co-stimulation (α-OX40 and α-PD-L1). T-bet and Bcl6 expression was analyzed in IL-21+, IFN-γ+ and IL-21-, IFN-γ+ cells. Upper panel: Representative histograms and MFI of T-bet and Bcl6 expression. Lower panel: Correlation (spearman) of transcription factor expression and fold change of cytokine production. Fold change was calculated as % cytokine+ cells following peptide stimulation in the presence of α-OX40 and α-PD-L1 normalized to % cytokine+ cells following peptide stimulation only. Bars represent medians. Wilcoxon test was applied. *p <0.05, **p <0.01, ***p <0.001. cHBV, chronic hepatitis B virus; FMO, fluorescence minus one; MFI, median fluorescence intensity; OLPs, overlapping peptides; PBMCs, peripheral blood mononuclear cells; Tfh, follicular T helper; Th1, T helper type 1. (This figure appears in colour on the web.)

factor expression and their cytokine secretion pattern after *in vitro* culture in the presence of α-OX40 and α-PD-L1. However, the level of T-bet expression in HBV-specific CD4 T cells correlated with the responsiveness to combined PD-L1 blockade and OX40 stimulation with regards to IFN-γ secretion.

Discussion

It is widely accepted that HBV-specific T cells are crucially required to eliminate HBV infection.[33] Indeed, therapeutic strategies to cure persistent HBV infection almost invariably include approaches to enhance antiviral T cell immunity in

order to eliminate HBV-infected hepatocytes.[34] The recent success of T cell-based cancer-immunotherapies serves as a proof-of-concept that resuscitation of exhausted or dysfunctional T cells is feasible in the clinical setting and might therefore also be a viable option in the context of persistent viral infection.[35] Clinical trials in patients with hepatocellular carcinoma and concurrent HBV infection provide the first glimpse of information on the antiviral effects of checkpoint blockade. In the CheckMate 040-study, a monoclonal antibody against PD-1 was administered to patients with hepatocellular carcinoma and HBV infection. Interestingly, in 3 out of 66 patients a decline of HBsAg-levels >1 log was observed.[36] While these data suggest some antiviral capacity of PD-1 blockade, the results are clearly insufficient to advocate this approach as a mono-therapeutic option for chronic HBV infection. However, so far there is no information from clinical trials on how blockade of PD-1 *in vivo* affects the circulating HBV-specific T cells. Data from Raziorrouh *et al.*, looking at the effects of PD-1 blockade on HBV-specific CD4 T cells *in vitro*, revealed increased levels of HBV-specific IFN-γ, IL-2 or TNF secretion in 4 out of 13 individuals, while the remaining 9 patients displayed unaltered cytokine patterns after PD-1 blockade.[14] Thus, these results are in line with our data on blockade of the PD-1 pathway alone in HBV-specific CD4 T cells (Fig. 3) and might also explain the observation that PD-1 blockade *in vivo* does not significantly improve virological control of chronic HBV infection. This notion underlines the requirement for additional approaches to improve the functionality of HBV-specific CD4 T cells. Importantly, tetramer-based analyses of the virus-specific CD4 T cell phenotype *ex vivo* revealed high expression levels of both OX40 and PD-1 on HBV-specific CD4 T cells compared to Flu-specific CD4 T cells and (Fig. 1), providing a strong rationale to study combined OX40 stimulation and PD-L1 blockade. Indeed, it has been shown for CD8 T cells that combination of PD-1 pathway blockade and OX40 stimulation has the potential to synergistically augment T cell responses in mice.[37] Importantly, our data demonstrate that these synergistic effects can also be observed in CD4 T cell responses, even across different antigen-specificities (HBV, Flu and EBV). The relevance for receptor expression as a prerequisite for successful immune interventions has recently been described in the context of cancer immunotherapy, where strong PD-1 expression on tumor-infiltrating lymphocytes was associated with successful checkpoint blockade with nivolumab.[38] Yet, the presence of the receptor does not necessarily guarantee a successful therapeutic intervention. Indeed, although it has been shown that addition of IL-7 can significantly enhance the numbers of antiviral CD4 and CD8 T cells in chronic LCMV infection in mice[13] and the IL-7 receptor α-chain CD127 is readily expressed on both HBV- and Flu-specific CD4 T cells *ex vivo*, addition of recombinant IL-7 did not improve the virus-specific CD4 T cell responses (Fig. 3). Since beneficial effects of IL-7 on HBV-specific CD4 T cells could not be observed and IL-7 has been implicated in the development and maintenance of autoimmunity,[39] we have not performed combined approaches involving IL-7.

In contrast, both OX40 stimulation and PD-1 pathway blockade are clinically feasible options that deserve further investigation in the context of chronic viral infection. And although the mere improvement of virus-specific T cell responses does not necessarily translate into improved immune control of a persistent pathogen, as has been shown in the murine LCMV model,[40,41] HBV-specific T cells can be of great importance in the context of chronic infection where their relevance appears to reach beyond the immediate effect on viral loads. Indeed, Rivino *et al.* could show that the presence of core- and / or polymerase-specific T cells was associated with an absence of viral flares in patients following discontinuation of nucleos(t)ide-analogues (NUCs).[25] This relevance of pre-existing core- / polymerase-specific T cells in preventing viral flares could be the rationale to target the OX40 and PD-1-pathways in order to boost HBV-specific CD4 T cell responses and improve the outcome of therapeutic strategies that include the cessation of NUC therapy in chronically HBV-infected individuals. Importantly, we did not detect functional CD4 T cell responses in the majority of patients with suppressed viral titers under NUC therapy (Fig. 2B). As these patients would be candidates for discontinuation of NUC therapy, expansion of the existing HBV-specific T cells by combined OX40 stimulation and PD-1 pathway blockade could be a therapeutic option prior to NUC withdrawal. Indeed, although we have analyzed the effects of OX40 stimulation and PD-L1 blockade on T cell responses that have been identified in the absence of these antibodies, the enhancement of low-level IFN-γ and IL-21 producing T cell responses suggests that this approach may increase the number of detectable responses in patients with cHBV.

Of note, this study was performed in HBeAg-negative patients as they constitute the vast majority of patients with cHBV in our clinic. In light of our observation that patients with higher viral titers are less likely to mount HBV-specific CD4 T cell responses (Fig. 2B) it is certainly possible that the HBeAg status influences various aspects of the HBV-specific T cell response. However, in our clinical routine, HBeAg-negative patients represent the cohort with the most pressing need for novel therapeutic approaches.

In response to viral infections in mice, CD4 T cells primarily differentiate into Th1 and Tfh cells, in the context of self-limiting and persistent viral infection.[27,28] It is unclear whether this is also the case in chronic viral infections in humans. In order to be able to identify the lineage commitment of HBV-specific CD4 T cells, we analyzed Tfh and Th1 surface markers directly *ex vivo*, T-bet and Bcl6 as the master regulators of these lineages on the transcriptional level in addition to their signature cytokines IFN-γ and IL-21.[29,30] Interestingly, based on the phenotype, cytokine and transcription factor profile, we could not clearly separate a Th1- and a Tfh-population within HBV-specific CD4 T cells. Indeed, we observed a significant overlap between Tfh and Th1 surface markers directly *ex vivo*, as well as between T-bet and Bcl6 expression in IFN-γ-producing cells and even slightly higher levels of both transcription factors in cells co-producing IFN-γ and IL-21 (Fig. 5). HBV-specific CD4 T cells producing IL-21 but not IFN-γ were not observed. Interestingly, these results are in line with recent observations in the LCMV model, where T-bet was shown to be involved in Tfh cell development and germinal center generation. The expression of T-bet in Tfh cells was also reflected in significant IFN-γ secretion, particularly during early Tfh cell differentiation.[42] Thus, the co-expression of IFN-γ and IL-21 in the presence of both T-bet and Bcl6 as observed in our study could be a sign of follicular helper T cell differentiation, although T-bet and IFN-γ-signatures were much more dominant, suggesting a predominant Th1-commitment. Further studies will have to analyze the transcriptional regulation of HBV-specific CD4 T cells in the context of chronic infection in order to precisely define helper T cell-lineages within HBV-specific CD4 T cells.

Collectively, our data demonstrate that OX40 and PD-1, 2 promising candidate pathways for immunotherapeutic interventions, are strongly expressed *ex vivo* on HBV-specific CD4 T cells in chronically infected patients. Combined ligation of both pathways significantly enhances the percentages of IFN-γ and IL-21 producing CD4 T cells after HBV-specific culture. These observations may prove useful in designing novel strategies to contain or even cure chronic HBV infection.

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Conflict of interest
The authors declare no conflicts of interest that pertain to this work.

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

Authors' contributions
Study concept and design: CNH, TB; acquisition of data: FJ, KW, MS, BC, JL, PE, FE; analysis and interpretation of data: FJ, KW, KZ, TF, CNH, TB; drafting of the manuscript: FJ, KW, TB; critical revision of the manuscript for important intellectual content: KZ, TF, MH, CNH, RT; statistical analysis: FJ, KW, TB; obtained funding: MH, CNH, RT, TB; technical, or material support: MH, CNH, RT; study supervision: RT, TB

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Supplementary data
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