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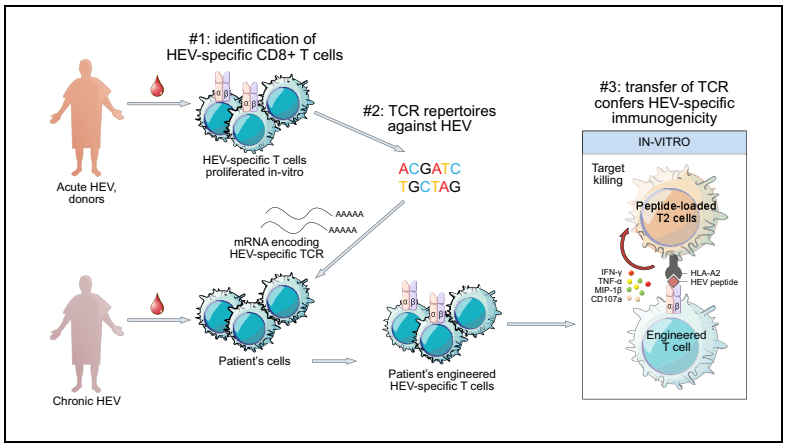
Defining virus-specific CD8+ TCR repertoires for therapeutic regeneration of T cells against chronic hepatitis E

Graphical abstract



Highlights

- Identified 2 HEV-specific CD8+ T cell epitopes located at the RNA helicase and RNA-dependent RNA polymerase.
- Identified and sequenced T cell receptor (TCR) repertoires against HEV.
- Transfer of engineered TCRs to T cells of chronically infected patients confers HEV-specific immunogenicity and cytotoxicity.

Authors

Chai Fen Soon, Patrick Behrendt, Daniel Todt, ..., Heiner Wedemeyer, Margaret Sällberg Chen, Markus Cornberg

Correspondence

Cornberg.Markus@mh-hannover.de
(M. Cornberg)

Lay summary

Patients who are immunosuppressed are vulnerable to developing chronic liver disease following infection with hepatitis E virus (HEV). To-date, there is no approved therapy for chronic hepatitis E. Interferon-α and ribavirin are off-label treatment options, but their applications are limited by side effects. Thus, immunotherapy, more specifically T cell-based therapy, may be an alternative approach. We designed T cell receptor-engineered T cells that effectively conferred immune cells, taken from patients with chronic hepatitis E, with the ability to recognize virus-specific epitopes and mediate killing of target cells *in vitro*.

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Defining virus-specific CD8+ TCR repertoires for therapeutic regeneration of T cells against chronic hepatitis E

Chai Fen Soon[1], Patrick Behrendt[1,2,3], Daniel Todt[4], Michael Peter Manns[1], Heiner Wedemeyer[1,2,5], Margaret Sällberg Chen[6,7,†], Markus Cornberg[1,2,8,9,*,†]

[1]*Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany;* [2]*German Center for Infection Research (DZIF), Partner Site Hannover-Braunschweig, Germany;* [3]*Institute for Experimental Virology, TWINCORE Centre for Experimental and Clinical Infection Research, Germany;* [4]*Department for Molecular and Medical Virology, Ruhr-Universität Bochum, Bochum, Germany;* [5]*Department of Gastroenterology and Hepatology, University Clinic Essen, Essen, Germany;* [6]*Department of Dental Medicine and Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden;* [7]*Shanghai Tenth People's Hospital, Tongji University, Shanghai, China;* [8]*Centre for Individualised Infection Medicine (CIIM), Hannover, Germany;* [9]*Helmholtz Centre for Infection Research (HZI), Braunschweig, Germany*

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Background & Aims: Immunosuppressed patients with chronic hepatitis E virus infection (cHEV), who are ineligible or have failed current treatment with off-label ribavirin, are a potential target population for T cell-based therapy. T cell responses are important for viral control. Herein, we aimed to identify human leukocyte antigen (HLA)-A2 restricted HEV-specific CD8+ T cell epitopes and T cell receptors (TCR) targeting these epitopes, as the basis for a redirected TCR treatment approach for patients with cHEV.

Methods: HEV genotype 3 overlapping peptide pools were used to screen HEV-specific CD8+ T cell immune responses in HLA-A2+ patients with acute HEV infection and healthy donors, by intracellular cytokine staining. CD8+ T cells targeting the identified epitopes were sorted for sequencing of the TCR repertoires by next generation sequencing. Messenger RNA encoding these TCRs were introduced into lymphocytes of healthy donors and patients with cHEV through TCR redirection. TCR-engineered lymphocytes were evaluated for Dextramer®-binding capacity, target sensitivity and cytotoxicity against peptide-loaded T2 cells.

Results: HEV-specific responses were observed across open reading frame (ORF)1 and ORF2 of the HEV genome in patients with acute resolving HEV infection. HLA-A2-restricted HEV-specific CD8+ T cell epitopes targeting the HEV RNA helicase and RNA-dependent RNA polymerase were selected for functional studies. Introduction of HEV-specific TCRs into lymphocytes of immunocompetent donors and patients with chronic hepatitis E enabled the lymphocytes to bind HEV Dextramers, secrete multiple cytokines and exert cytotoxicity in a target-specific manner.

Conclusion: We identified TCRs that target HEV-specific CD8+ T cell epitopes, and characterized their immune properties, which may have clinical potential in future T cell-based therapy.

Lay summary: Patients who are immunosuppressed are vulnerable to developing chronic liver disease following infection with hepatitis E virus (HEV). To-date, there is no approved therapy for chronic hepatitis E. Interferon-α and ribavirin are off-label treatment options, but their applications are limited by side effects. Thus, immunotherapy, more specifically T cell-based therapy, may be an alternative approach. We designed T cell receptor-engineered T cells that effectively conferred immune cells, taken from patients with chronic hepatitis E, with the ability to recognize virus-specific epitopes and mediate killing of target cells *in vitro*.

Introduction

Hepatitis E virus (HEV) infection is the most common cause of acute viral hepatitis in the global population. HEV is an emerging public health risk found in both the developed and developing countries.[1,2] There are several genotypes of HEV but only one serotype. Genotypes 1 and 2 only infect humans, and are distributed mainly in endemic regions where poor hygiene is the main cause of transmission via the fecal-oral route. Genotypes 3 and 4 are zoonotic; their hosts include both humans and animals, and are found predominantly in urbanized countries. Genotype 3 virus is transmitted by consumption of raw or improperly cooked meat or viscera of infected animals,[2] or through transfusion of contaminated blood products.[3] Usually, patients with acute hepatitis E spontaneously clear the virus. However, immunosuppressed patients, such as solid organ transplant patients, may develop chronic hepatitis E in around 50% of cases.[4,5] Reversion or reduction of the immunosuppressive status may induce spontaneous viral clearance.[4–6] If this is not effective or possible, treatment choices are limited. There is no approved therapy for chronic hepatitis E, but interferon-α or ribavirin are off-label treatment options. Unfortunately, interferon-α is contraindicated in patients receiving lung, heart or kidney transplants as it can trigger graft rejection.[6] Therefore, for these patients, ribavirin is their only option. However, side effects of ribavirin such as hemolytic anemia limit its use.

Keywords: Hepatitis E virus; Chronic hepatitis E; HEV-specific T cell responses; CD8+ T cell receptor; Transient redirection; Immunotherapy; T cell-based therapy.
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* Corresponding author. Address: Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany. Tel: +49 5115326821.
E-mail address: Cornberg.Markus@mh-hannover.de (M. Cornberg).
† Both authors contributed equally.

Ribavirin is effective in approximately 80% of patients who are able to tolerate its use.[7] For those who fail treatment, chronic hepatitis E can progress to liver cirrhosis.[6,8] Additionally, a recent study has shown that ribavirin induces mutations in HEV, [9] leading to enhanced viral replication and eventually drug failure.[10]

Thus, there is an unmet need for alternative treatment options. Previous findings demonstrated that CD8+ T cells play an important role in mediating immunity against HEV infection.[11] We hereby propose the pioneering idea of using T cell therapy as the alternative treatment for patients with chronic hepatitis E.

Similar to findings in chronic hepatitis B or C, HEV-specific T cells are in paucity and usually associated with exhausted phenotypes due to constant exposure to viral antigens.[12] Concepts to either restore or induce HEV-specific T cell responses may be a feasible approach to clear chronic HEV infection. With the recent development of immunotherapy using redirected T cells specific for hepatitis B virus (HBV) to target HBV-related hepatocellular carcinoma,[13] herein, we investigate the possibility of generating HEV-cytotoxic T cells by transfer of engineered T cell receptors (TCRs).

Patients and methods

Study cohorts

Twelve patients with acute autochthones HEV infection (aHEV), 9 patients with chronic HEV infection (cHEV) and 9 healthy donors were recruited between 2014–2019 at Hannover Medical School. The diagnosis of acute infection was based on the detection of HEV RNA by quantitative reverse transcription PCR (RT-qPCR; in-house diagnostic laboratory) using primers as described,[14] anti-HEV IgM/IgG seroprevalence, and classical symptoms of acute hepatitis. HEV serology was tested using ELISA-based method (Wantai Biological Pharmacy, China). All patients with cHEV were solid organ transplanted, with more than 3 months of viremia. Four out of 9 chronic patients were included in the T cell receptor redirection assay. HEV seronegative donors are defined by either HEV-naïve (not exposed to HEV) or spontaneously recovered from an asymptomatic HEV infection (resolved and lost anti-HEV IgG). Characteristics of patients are summarized in Table S1; all individuals were human leukocyte antigen (HLA)-A2+, as confirmed by antibody staining using mouse anti-human-HLA-A2 (AlexaFluor647; Bio-Rad Laboratories, USA). Individuals enrolled in this study gave their written informed consent for participating in immunological research projects. This study was reviewed and approved by the local ethics committee of Hannover Medical School (approval number 2315–2014).

HEV overlapping peptide pools

HEV genotype 3c overlapping peptides were synthesized by ProImmune (Oxford, UK), GenBank accession number AF455784. Peptides were made up of 15 amino acids, overlapped by 5 amino acids and spanned the entire 3 HEV open reading frames (ORFs). Overlapping peptide pools (OLPs) 1 to 8 covered ORF1, OLPs 9 to 11 covered ORF2; ORF3 was combined with ORF2 within OLP 11. Peptides were dissolved in sterile DMSO (Sigma-Aldrich, USA) before being grouped into respective peptide pools consisting of 21 to 26 peptides each. The amino acid sequences of all 248 peptides are listed in Table S2.

HEV-specific Dextramers bearing HLA-A*02:01-restricted T cell epitopes

HEV-specific 15-mer epitopes were first identified through positive peptide pools using cytokine readout in intracellular cytokine staining. Next, all the overlapping peptides within that pool were recombined into different matrix sub-pools for a second re-stimulation of the T cell line, to pinpoint a 15-mer target epitope. The amino acid sequences of the positive 15-mer targets were then retrieved from Table S2. These 15-mer epitopes were further narrowed down to 9-mer using IEDB analysis resource Consensus tool,[15] before being sent for major histocompatibility complex class I HLA-A*02:01 Dextramer® synthesis and then labeled with PE fluorochrome (Immudex, Copenhagen, Denmark). Dextramer staining of ≥0.2% was considered positive.

T cell culture and feeder T2 cell line

Isolation of peripheral blood mononuclear cells (PBMCs) from blood was carried out as per standard density gradient separation protocol using Biocoll Separating solution (Biochrom GmbH, Germany). PBMCs were frozen and stored in liquid nitrogen until use. Isolation of CD8+ T cells from PBMCs was done using magnetic MACS CD8+ T cell isolation beads following the protocol (Miltenyi Biotec, USA). Media for CD8+ T cell culture and TAP-deficient T2 cell line (ATCC CRL-1992) maintenance were described previously.[16] T2 cells were loaded with HEV OLPs at a final concentration of 1 μg/ml and irradiated with Caesium-137 (Cs-137) at 40 Gy prior to co-culture at 1/5 of the T cell number. The medium was changed every 3–4 days, while irradiated peptide-loaded T2 cells were replenished every week. The entire culture lasted for 3 weeks.

CD8+ T cell functional assay using intracellular cytokine staining

At the end of 3-week culture, 0.2×10^6 CD8+ T cells were seeded for intracellular cytokine staining (ICS). The cells were re-stimulated with the same pool of HEV peptides (5 μg/ml) added to the medium, in the presence of Brefeldin A (Sigma-Aldrich, USA) at 2 μg/ml for 6 h incubation. Dextramer staining was performed at room temperature for 20 min. Thereafter, the cells were stained with surface antibodies, fixed and permeabilized, and finally stained with intracellular cytokine antibodies as described separately.[16] The cells were acquired using BD LSRFortessa flow cytometer (BD Bioscience); results were analyzed using FlowJo (FlowJo, LLC) and SPICE (NIAID, USA). The gating strategy is outlined in Fig. S1 and antibody information is detailed in the supplementary CTAT Table.

HEV-specific CD8+ T cell sorting and TCR repertoire sequencing

HEV-specific CD8+ T cells were sorted using Dextramer by BD Aria IIu (in-house sorting facility). The cell pellets were resuspended in RNAprotect® Cell Reagent (Qiagen GmbH, Germany) and shipped frozen to Adaptive Biotechnologies (Seattle, USA) for TCR repertoire sequencing of alpha and beta chains (ImmunoSEQ). Based on the sequencing results, the nucleotide sequences of alpha and beta chains of each TCR were reconstituted for the entire V(D)J segment for TCR gene constructs.

TCR plasmid DNA gene construction and transient TCR redirection

TCR gene constructs were designed and murinized by replacing the human constant chain with murine analogues.[17–19]

Synthetic genes were manufactured by Invitrogen and cloned into *E. coli* for amplification. TCR plasmid DNA were purified for linearization (Endofree Plasmid Maxiprep, Qiagen) and the subsequent mRNA synthesis using mMESSAGE mMACHINE® T7 Ultra Kit (Life Technologies) with ARCA capping and poly-A tailing.[18] PBMCs from HLA-A2+ donor or patients with cHEV were expanded for 7 days in AIM-V medium supplemented with 2% human AB serum, 600 IU/ml of IL-2 and 50 ng/ml of anti-CD3 (BioLegend, USA). The day before redirection, IL-2 was increased to 1,000 IU/ml. In preparation for TCR redirection, 20 μg of mRNA encoding the individual TCR construct was added to 10×10^6 cells, and transferred to a cuvette for electroporation using Amaxa Cell Line Nucleofector Kit V and Nucleofector 2b device, program X-01 (both from Lonza, Switzerland). Post-redirection, T cells were kept in AIM-V medium supplemented with 2% human AB serum and 100 IU/ml of IL-2. One day later, engineered T cells were used in co-culture assays, using peptide-loaded T2 cells as target cells. The co-culture lasted for 6 h and was followed by ICS.

Results

Identification of HLA-A2-restricted HEV-specific CD8+ T cell responses in patients with acute hepatitis E and healthy donors

CD8+ T cells from HLA-A2+ donors and patients were cultured in the presence of HEV overlapping peptides (loaded on T2 cells) for 3 weeks to allow sufficient expansion of HEV-specific T cells. At the end of expansion, HEV-specific T cell epitopes were identified based on functional response by the detection of pro-inflammatory cytokines (IFN-γ, TNF-α and MIP-1β) using intracellular cytokine staining. As shown in Fig. 1A,B cytokine responses were more pronounced in patients with acute hepatitis E than control donors. If a specific peptide pool stimulated cytokine production in CD8+ T cells, the target peptide of 15 amino acids (15-mer) was identified from the pool. Fig. 1C shows a simplified workflow outlining this epitope identification process. T cell responses in patients were both stronger (higher percentage of cytokine-producing CD8+ T cells) and broader (present in more peptide pools) than the donor cohort.

Characterization of ORF1-specific T cell epitope

Based on cytokine readout, the identified 15-mer epitopes are listed in Table 1. Three of the epitopes were further narrowed down to 9-mer using IEDB consensus tool, and confirmed by Dextramer staining (Table 1). We found that there is no epitope in ORF3 capable of stimulating T cell immunity in the present cohorts. In addition, the number of responders found in ORF2 is notable, although ORF2 is comparatively smaller in genomic size than ORF1. This observation corresponded to the findings of Brown *et al.*, where the HEV capsid encoded by ORF2 was a highly targeted region,[20] probably due to its crucial role in host cell attachment and eliciting neutralizing antibody.[21] Fig. S2 shows Dextramer staining of epitope YVSDTVTFV (ORF2, peptide pool 10) in a representative patient with acute HEV infection, which we did not pursue further as the T cell responses against this epitope were low.

Nonetheless, our results indicated that the most robust T cells responses with the highest level of cytokine production were against ORF1, the non-structural part of the HEV genome, with sufficient cell proliferation for subsequent analysis. Two prominent epitopes, one located at the helicase (pool 6, HEV-1116) and the other in RNA-dependent RNA polymerase (RdRp; pool 8, HEV-1527) were chosen for further comparison. Both epitopes were found to be conserved in HEV genotype 3, based on viral genomic data available in the NCBI database. Furthermore, re-analyzing deep sequencing results generated from a separate study with a cohort of 12 patients with cHEV revealed that HEV-1527 was conserved throughout ribavirin treatment[9] (Fig. S3), which reaffirmed that ribavirin-induced mutagenesis is absent in this HEV-1527 epitope.

CD8+ T cells specific for the HEV-1116 epitope were detected by *ex vivo* Dextramer staining in 2 patients with acute hepatitis E. These cells displayed an effector memory phenotype (Fig. 2A). After *in vitro* expansion with the HEV-1116 peptide, we observed HEV-1116-specific T cells in 3 patients with acute hepatitis E. A high proportion of the Dextramer-binding T cells were also cytokine-producing (Fig. 2B). On the contrary, there were fewer HEV-1116-specific T cells in chronic patients compared to acute patients, both *ex vivo* and post-expansion. Only 1 cHEV patient displayed >0.2% Dextramer-positive cells *ex vivo* (Fig. 2A) and *in vitro* expansion with the HEV-1116 peptide did not result in strong responses. Even after PD-L1 blockage, T cell functions were only restored in some but not all of the patients with cHEV (Fig. 2C,D). Similar characteristics of HEV-1527-specific T cell proliferation and function were observed in chronic patients (Fig. S4).

TCR α-chain variable gene (TCRAV) 12-02 was selected in HEV-specific T cells

CD8+ T cells specific for the 2 selected epitopes were sorted using Dextramers from 3 acute patients and 1 donor. Next generation sequencing of TCR repertoires revealed that TCRAV12-02 was the only common V gene found in all sorted samples, ranging from 12–80% of gene usage frequency, albeit targeting 2 different epitopes (Fig. 3A,B). This finding suggests that TCRAV12-02 has an important role in HEV control. The CDR3 sequences are summarized in Table 2; results from patient aHEV6 were excluded from the TCR redirection assay as there was an unresolved β chain, most likely due to its less distinct Dextramer-positive population (Fig. 3A). Seven pairs of TCR to be included in TCR redirection were: 1116.A1 consisting of TCRAV29-01 and TCRBV07-08*01 from patient aHEV5; 1116.B1 consisting of TCRAV12-02 (J31-01) and TCRBV07-09, 1116.B2 consisting of TCRAV12-02 (J21-01) and TCRBV07-09, 1116.B3 consisting of TCRAV12-02 (J31-01) and TCRBV05-01*01, 1116.B4 consisting of TCRAV12-02 (J21-01) and TCRBV05-01*01 from patient aHEV10; 1527.A1 consisting of TCRAV04-01*01 and TCRBV04-02*01, 1527.A2 consisting of TCRAV12-02 and TCRBV04-02*01 from Donor D2 (Fig. S5).

TCR redirection assay using HEV-specific TCRs showed polyfunctional characteristics and cytotoxicity

TCR redirection using synthetic mRNA could bypass T cell clonal expansion to generate effector T cells expressing antiviral TCRs of interest,[22] which suited our approach to characterize the immune potential of the candidate TCRs. Post-redirection, Dextramer staining of the engineered T cells was performed to identify which construct could constitute a functional TCR.

Four TCR constructs were able to recognize respective Dextramers: 1116.A1, 1116.B3 and 1116.B4 for HEV-1116 Dextramer, and 1527.A2 for HEV-1527 Dextramer (Fig. 4A,B). Among the TCRs constructed from patient aHEV10 (1116.B1 to B4), the presence of TRBV07-09 instead of TRBV05-01*01

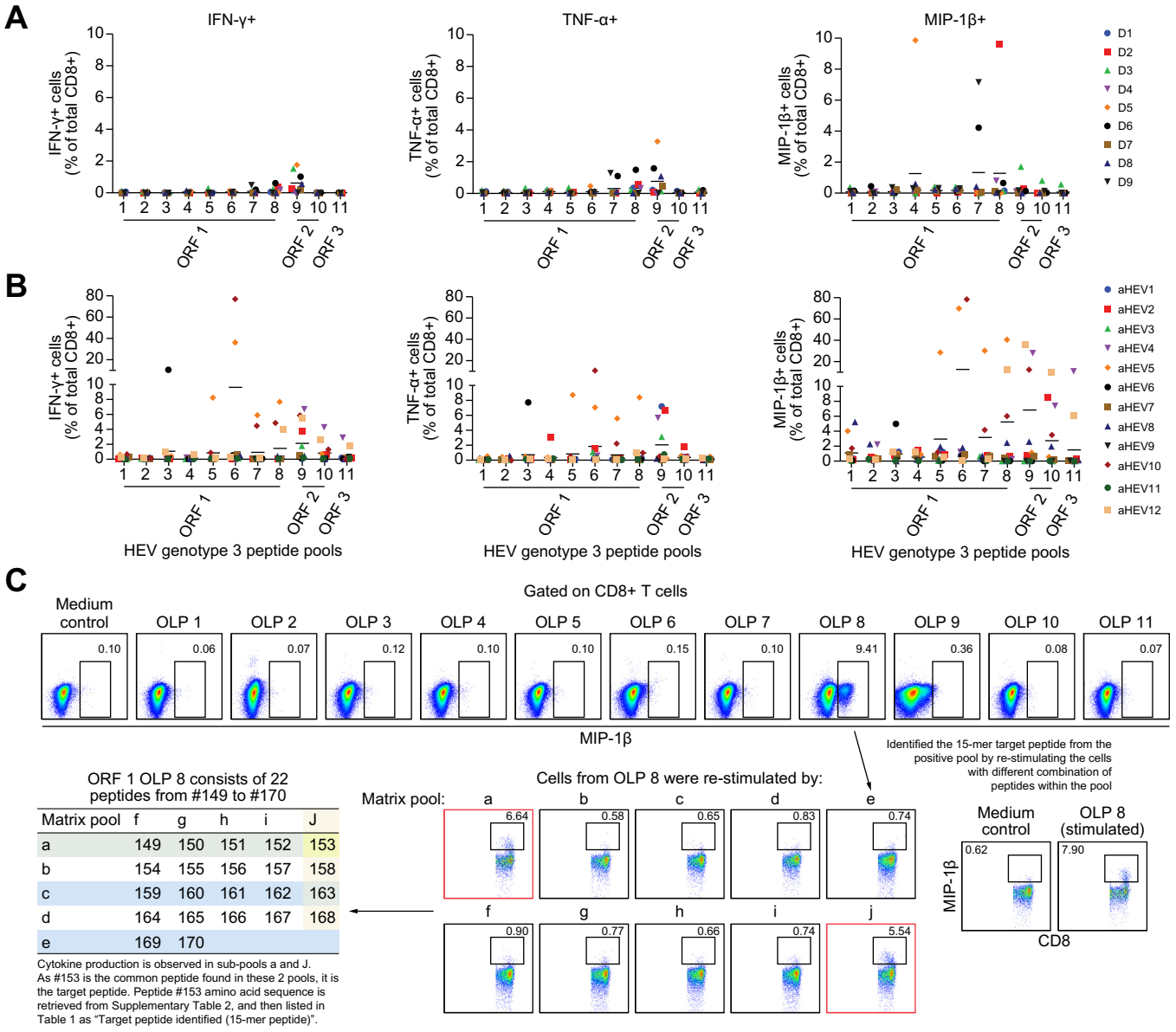

Fig. 1. Identification of HLA-A2-restricted HEV epitopes by ICS and Dextramer staining. CD8+ T cells expanded for 3 weeks were re-stimulated with the respective HEV OLPs for ICS. IFN-γ, TNF-α and MIP-1β were stained in (A) healthy donors, D1 to D9, and (B) patients with acute HEV infection, aHEV1 to aHEV12. Black horizontal lines indicate the mean of the cytokine responses across all donors/patients in each pool. Cytokine responses of ≥2% were considered positive. (C) Schematic illustration of 15-mer target peptides identification from the positive OLPs, by re-stimulating the cells with different combination of peptides within the same pool (matrix sub-pools a to j). Once the 15-mer peptide was known, its amino acid sequence was submitted to the IEDB website to predict the major histocompatibility complex class I 9-mer T cell epitope (http://tools.iedb.org/mhci/), which was subsequently used to synthesize Dextramers. ICS, intracellular cytokine staining; HEV, hepatitis E virus; OLPs, overlapping peptide pools. (This figure appears in colour on the web.)

diminished the functionality of TCR. Intriguingly, having TRAJ31-01 could restore some function in 1116.B1 compared to 1116.B2. This is an interesting observation as J gene, a short linker sequence in the α chain that consists of a few amino acids, could make or break the fate of the TCR.

To ascertain the functionality of TCR-engineered T cells, they were co-cultured with T2 cells loaded with respective target peptides for 6 h, with and without Brefeldin A for intracellular cytokine assays and killing assays, respectively. As expected, TCR-redirected CD8+ T cells that were Dextramer-binding also produced IFN-γ upon encountering their respective target peptides (Fig. 4C). The multiplicity of immune potential, as shown in SPICE analysis on 2 TCR pairs as representatives (1116.B3 and 1527.A2), demonstrated that TCR-engineered T cells were polyfunctional producers of proinflammatory cytokines: IFN-γ, TNF-α, MIP-1β as well as CD107a when stimulated (Fig. 4D). Patient data taken from aHEV10 is shown in Fig. S6, to compare the HEV-1116-engineered T cells against the original clone from this patient. There was a close resemblance in both Dextramer-binding capacity and cytokine production, affirming that the redirection method did not change the functional nature of the TCR.

Using HEV-1527-redirected T cells as an example, cytotoxicity was noted by a reduction of the peptide-loaded T2 cells, a

Table 1. HLA-A2-restricted HEV-specific CD8+ T cell epitopes.

Peptide pool	Encoding region	Total number of responders	HD	aHEV	Responders' codes	Target peptide identified (15-mer peptide)	MHC class I Epitopes (9-mer peptide)*	Unresolved responses ^
ORF1								
1	Methyltransferase	0	0	0	–	–	–	aHEV8 (pool 1)
2	Methyltransferase	0	0	0	–	–	–	
3	Cysteine protease	2	0	2	aHEV6, aHEV12	IAARASRLTATVELA	–	
4	X domain	1	1	0	HD5	HPEGLLGLFPPFSPG	–	
5	RNA helicase	2	0	2	aHEV5, aHEV10	AARVTGGRRVVIDEA VIDEAPSLPPHLLLL	–	
6	RNA helicase	3	0	3	aHEV5, aHEV6, aHEV10	SRVLRSLFWNEPAIG	SLFWNEPAI	
7	RNA helicase	2	0	2	aHEV5, aHEV10	EQGLLYMPQELAVSD	–	D9 (pool 7)
7	RdRp	1	1	0	HD6	SKTFCALFGPWFRAI	–	
8	RdRp	3	1	1	HD2, aHEV10	SGEPGTLLWNTVWNM	LLWNTVWNM	aHEV5 (pool 8)
			0	1	aHEV12	VHNLIGMLQTIADGK	–	
ORF2								
9	Capsid	4	1	2	HD5, aHEV1, aHEV2	AVGGYAISISFWPQT	–	aHEV3 (pool 9)
			0	1	aHEV12	TNLVLYAAPLNPLLP	–	aHEV4 (pool 9)
10	Capsid	4	0	4	aHEV2, aHEV4, aHEV10, aHEV12	PMYVSDTVTFVNVAT	YVSDTVTFV	aHEV8 (pool 9)
11	Capsid	1	0	1	aHEV12	LAPHSALAALEDTID	–	aHEV10 (pool 9)
ORF3								
11	Phosphoprotein	0	0	0	–	–	–	aHEV4 (pool 11)

Responders' codes: HD, healthy donors; aHEV, patients with symptomatic acute HEV infection. *Epitopes of 9 amino acids that were predicted by IEDB (http://tools.iedb.org/mhci/) and confirmed using Dextramer staining. ^Unresolved responses were positive responses found in the peptide pool, but insufficient cells for narrowing down target peptide within the pool.
HEV, hepatitis E virus; MHC, major histocompatibility complex; ORF, open-reading frame.

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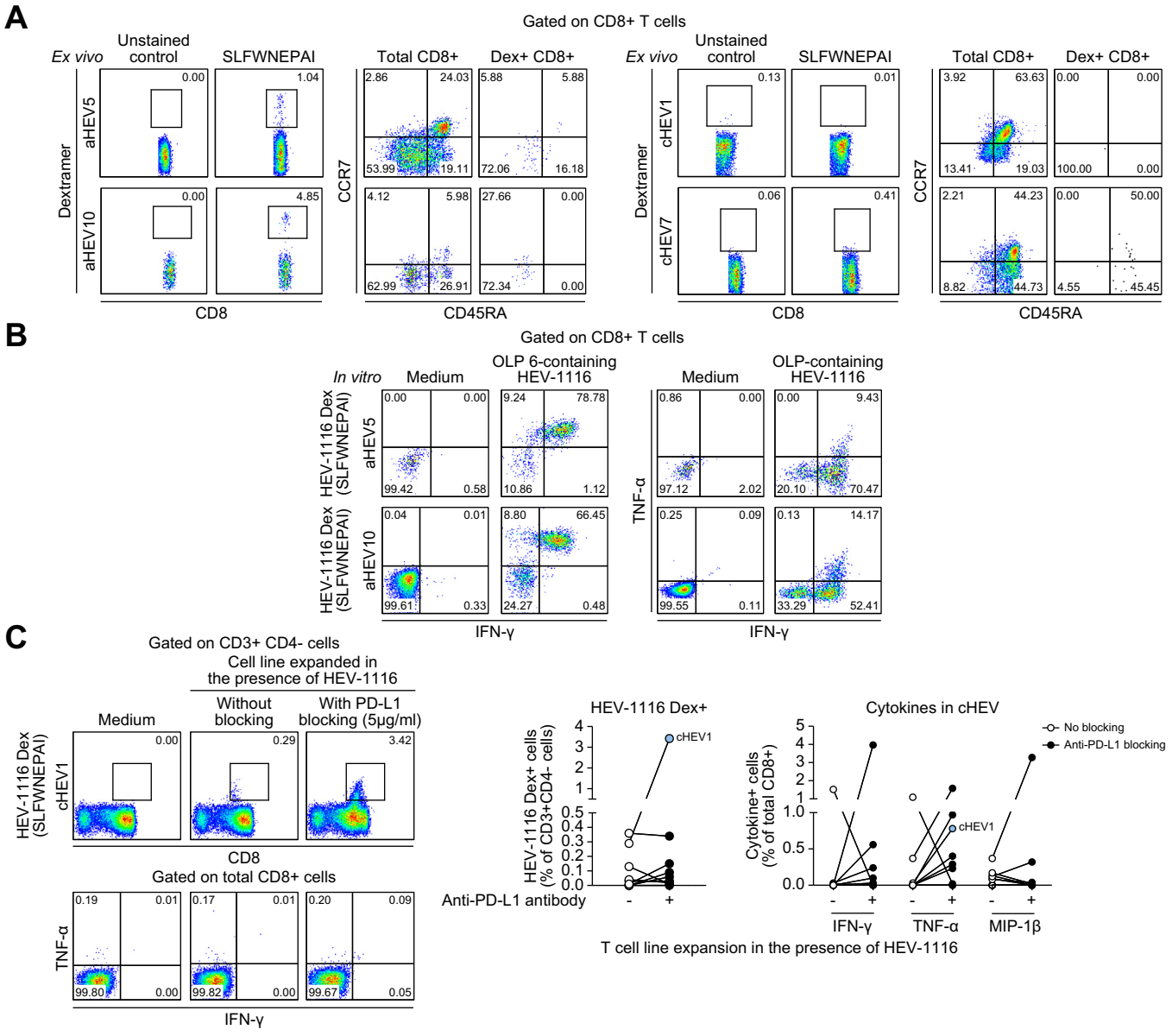

Fig. 2. Characterization of the HEV-1116 epitope in patients with acute HEV and chronic HEV infection. (A) *Ex vivo* HEV-1116 Dextramer staining and co-staining with CCR7 and CD45RA in 2 patients with acute HEV and 2 with chronic HEV. (B) HEV-1116 Dextramer staining and ICS of IFN-γ and TNF-α in *in vitro* culture using HEV-1116 peptide or medium control in 2 patients with acute HEV. (C) HEV-1116 Dextramer staining and ICS after *in vitro* culture with HEV-1116 peptide, with or without PD-L1 blockade, or medium control in 1 patient with chronic HEV. The graphs below summarize the data from 9 patients with chronic HEV. ICS, intracellular cytokine staining; HEV, hepatitis E virus. (This figure appears in colour on the web.)

relative increase of CD8+ T cells within the T2 gate and concurrently an increase in the dead cell population, which was used to calculate net T2 cell death (Fig. S7). Fig. 4E summarizes the net killing effect of the TCR constructs, averaging at about 70–80% after 6 h of co-culturing, with the highest killing observed in constructs 1116.B3 and 1527.A2.

Target sensitivity, specificity and response rate of engineered T cells

We next examined the characteristics of TCRs in parameters that are of clinical relevance, such as target sensitivity, specificity and time-dependent dynamics in recognizing and responding to target cells.

TCRs with high target affinity are an important determinant in adoptive T cell therapy.[23] To test affinity, T2 cells loaded with various peptide concentrations ranging from 0 to 1 μg/ml were used to stimulate the redirected T cells. It was found that the minimum peptide concentration to elicit cytokine responses was 1 pg/ml (Fig. 5A). Particularly in construct 1116.B3, a gradual reduction of Dextramer-binding intensity was observed when redirected T cells were stimulated with increasing peptide concentrations (Fig. 5B).

Henceforth, we selected TCRs 1116.B3 and 1527.A2 for further characterization, with 1116.B3 representing 1116.A1 and 1116.B4. A validation of TCR specificity was done in a cross-stimulation experiment, whereupon the TCR targeting

Table 2. TCR repertoire of Dextramer-sorted HEV-specific CD8+ T cells.

T cell epitopes	Patient/donor	TCR chain	CDR3 sequence	V gene	D gene	J gene	Frequency %
HEV-1116	aHEV5	α	CAASANDYKLSF	TCRAV29-01		TCRAJ20-01*01	67,3
(SLFWNEPAI)		β	CASSPDRSGQFF	TCRBV07-08*01	TCRBD02-01*02	TCRBJ02-01*01	89,6
	aHEV6	α	CAVPDKLIF	TCRAV12-02		TCRAJ34-01*01	32,4
		α	CAVIQDSNYQLIW	TCRAV08-01		TCRAJ33-01*01	19,7
		β	CASSLGPGSYGYTF	TCRBV05-01*01	TCRBD01-01*01	TCRBJ01-02*01	38,9
		β	CASSRGGMSGELFF	TCRBV07-09	unresolved	TCRBJ02-02*01	21,1 *
	aHEV10	α	CAVHNARLMF	TCRAV12-02		TCRAJ31-01*01	17,1
		α	CAVDKFYF	TCRAV12-02		TCRAJ21-01*01	18,8
		β	CASSFQGNTGELFF	TCRBV07-09	TCRBD01-01*01	TCRBJ02-02*01	14,3
		β	CASSPFQGAIEQYF	TCRBV05-01*01	TCRBD01-01*01	TCRBJ02-07*01	12,0
HEV-1527	HD2	α	CAVANARLMF	TCRAV12-02		TCRAJ31-01*01	55,3
(LLWNTVWNM)		α	CLVTYGGATNKLIF	TCRAV04-01*01		TCRAJ32-01	43,8
		β	CASSSTGGGGELFF	TCRBV04-02*01	TCRBD02-01*01	TCRBJ02-02*01	100 #

*Results from patient aHEV6 were excluded from TCR redirection assay due to an unresolved D gene in one of the TCR β chain.
#Majority of this β chain sequences were non-productive. The remaining productive ones were dominated by this clone.
HEV, hepatitis E virus; TCR, T cell receptor.

HEV-1116 was stimulated by HEV-1527 peptide, and vice versa. As expected, there was no cytokine observed when the mismatch peptide was used as stimulant (Fig. 5C).

Due to the transient nature of this method, the redirected cells will eventually lose the TCR, and consequently, the TCR's functions over time. Fig. 5D shows the Dextramer staining over a period of 5 days post-redirection; 1116.B3 was able to retain Dextramer for a longer duration than 1527.A2. Since Dextramer-binding CD8+ T cells were decreasing exponentially, we expected the cytokine production to follow the same pattern. However, as depicted in Fig. 5E, 1527.A2-specific T cell function remained prominent from day 2 onwards even though Dextramer staining was almost non-existent. As for target cell death, a difference was observed – while the killing effect of 1527.A2 followed the cytokine's diminishing pattern over time, it was a disparate scenario with 1116.B3, as the killing was sustained across 5 days (Fig. 5f). This may be an intrinsic feature of 1116.B3 TCR, whereupon the killing is achieved as long as a minimum cytokine threshold is fulfilled.

Lastly, we investigated the TCRs for their response rate to elicit targeted killing – how fast the TCR could recognize target, produce cytokines and ultimately mediate killing of target cells. Fig. 5G shows that T2 cell death was noticeable (higher than the baseline cell death observed in untransfected controls) starting as soon as 1 h after co-culturing in 1116.B3, and 2 h in 1527.A2.

TCR-redirected T cells isolated from patients with cHEV were polyfunctional and cytotoxic against target cells

We also explored if similar results could be obtained using peripheral CD8+ T cells isolated from 4 patients with cHEV, who were found to be non- or low-responders to PD-L1 blockade (Fig. 2C). Patient-derived T cells were redirected using the same established workflow, with 2 TCR constructs, 1116.B3 and 1527.A2. The redirected T cells were examined for their Dextramer-binding capacity, as well as their functionality and cytotoxicity when stimulated by peptide-sensitized T2 cells. This was done as a proof-of-principle to apply TCR redirection in T cells isolated from patients with persistent infection, to examine its effectiveness. Fig. 6 shows that patient-derived TCR-redirected T cells bind their respective Dextramers, as well as being highly polyfunctional and capable of mediating killing of T2 cells in an HEV-specific manner, to a similar extent as redirected T cells from immunocompetent donors (Fig. 4E).

Discussion

There is an unmet need for new treatment alternatives in chronic hepatitis E, as interferon-α is contraindicated in most organ transplant patients. Although ribavirin has been officially endorsed in a recent guideline,[5] side effects restrict its use, along with a risk of increased viral mutagenesis which might enhance replication, leading to therapeutic failure,[9,10] To date, no further treatment regimen is available if patients fail ribavirin or are ineligible. Sofosbuvir, a direct-acting antiviral drug developed to block hepatitis C virus replication by inhibiting the HCV polymerase, was found to be effective against HEV *in vitro*,[24] but effectiveness of sofosbuvir *in vivo* remains elusive as case reports showed conflicting results.[25] Immunotherapy could be a novel approach to treat chronic infectious disease such as cHEV because broad and multispecific T cell responses are known to control acute hepatitis E virus infection and exhausted T cell phenotypes are observed in patients with cHEV.[11] Unlike the active research of immunotherapy in chronic hepatitis B,[13,23] applying this in patients with cHEV has not been proposed, and our work sets in motion the initial groundwork for a new approach towards T cell-based therapy in cHEV.

We i) screened and pursued HEV epitopes that are HLA-A2-restricted in the entire HEV genotype 3 genome, ii) sorted the HEV-specific CD8+ T cells to explore their repertoire diversity, iii) reconstituted the TCRs to characterize their immune affinity, and finally iv) demonstrated, as a proof-of-concept, that TCR-redirected T cells from cHEV patients (who showed no or low HEV-specific CD8+ T cell responses) were conferred with immunogenicity against epitope-loaded target cells.

We used an unbiased method to screen for epitopes using HEV overlapping peptides, and identified 12 potential 15-mer epitopes. However, our method was not intended to identify all possible HEV epitopes. Out of 12 targets identified, there were 3 9-mer epitopes that could be confirmed by using Dextramer staining in multiple individuals. Two of the HLA-A2-restricted HEV epitopes that we reported here, SLFWNEPAI and YVSDTVTFV, coincidently matched those reported by Brown *et al.*[20]

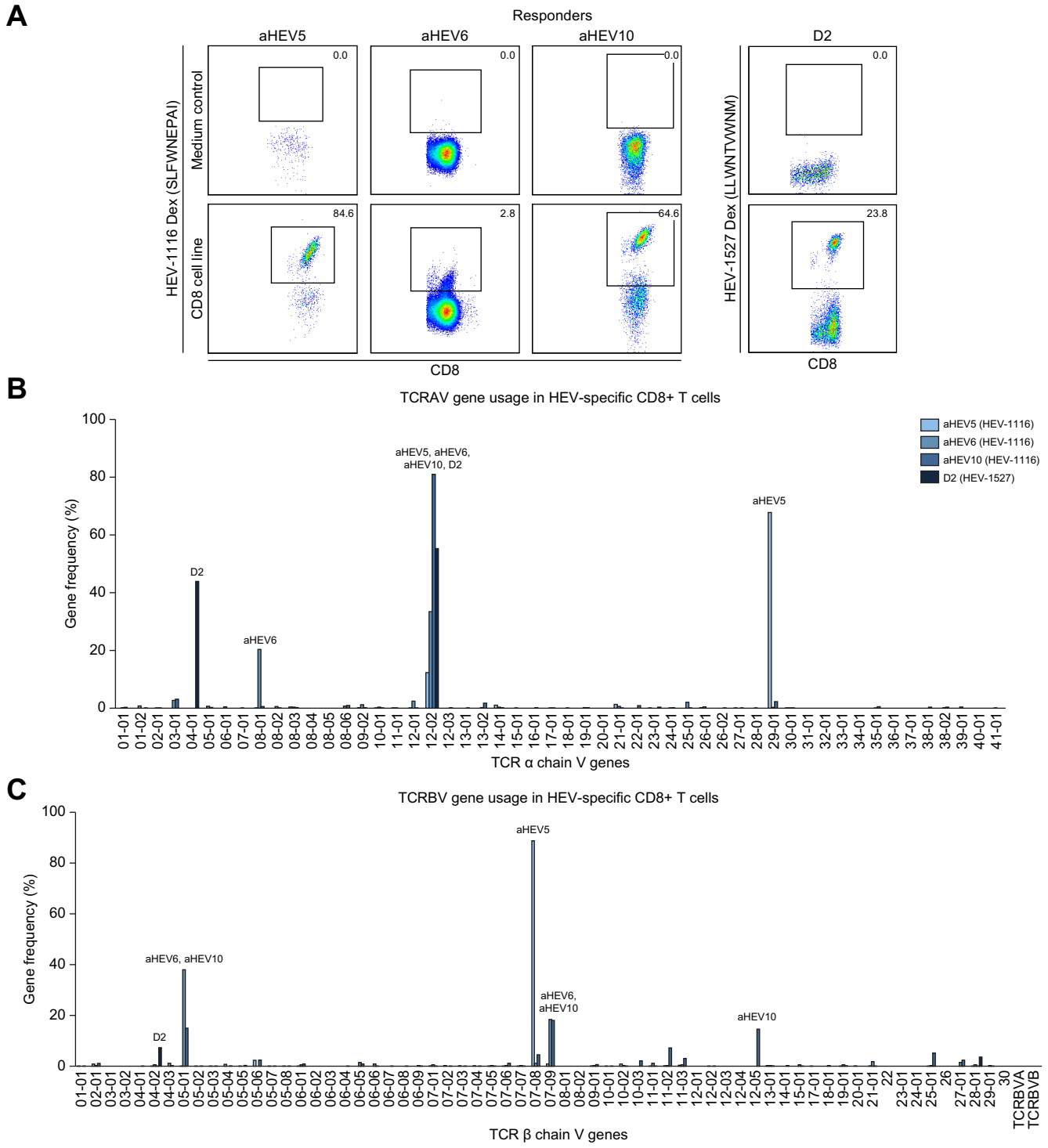

Fig. 3. TCR repertoires of Dextramer-sorted HEV-specific CD8+ T cells determined by next generation sequencing. (A) CD8+ T cells from the selected responders were sorted by Dextramers for TCR sequencing. (B and C) Next generation sequencing results of TCR α and β chains variable gene (TCRAV and TCRBV, respectively) usage in HEV-specific CD8+ T cells. HEV, hepatitis E virus; TCR, T cell receptor. (This figure appears in colour on the web.)

In addition, we found a novel epitope at the RNA-dependent RNA polymerase region, LLWNTVWNM (HEV-1527). To the best of our knowledge, this study is the first to report the repertoire of HEV-specific TCRs. We have identified TCRs targeting 2 conserved HEV epitopes: 3 against HEV-1116 and 1 against HEV-1527 for further TCR redirection.

Because of potential safety concerns, we used a transient mRNA-based TCR redirection method, rather than a retroviral vector transduction.[22,23] The TCR constructs that we tested were able to emulate the original T cell's immunity. Redirected cells were capable of Dextramer binding, highly specific in target stimulation, proinflammatory cytokine

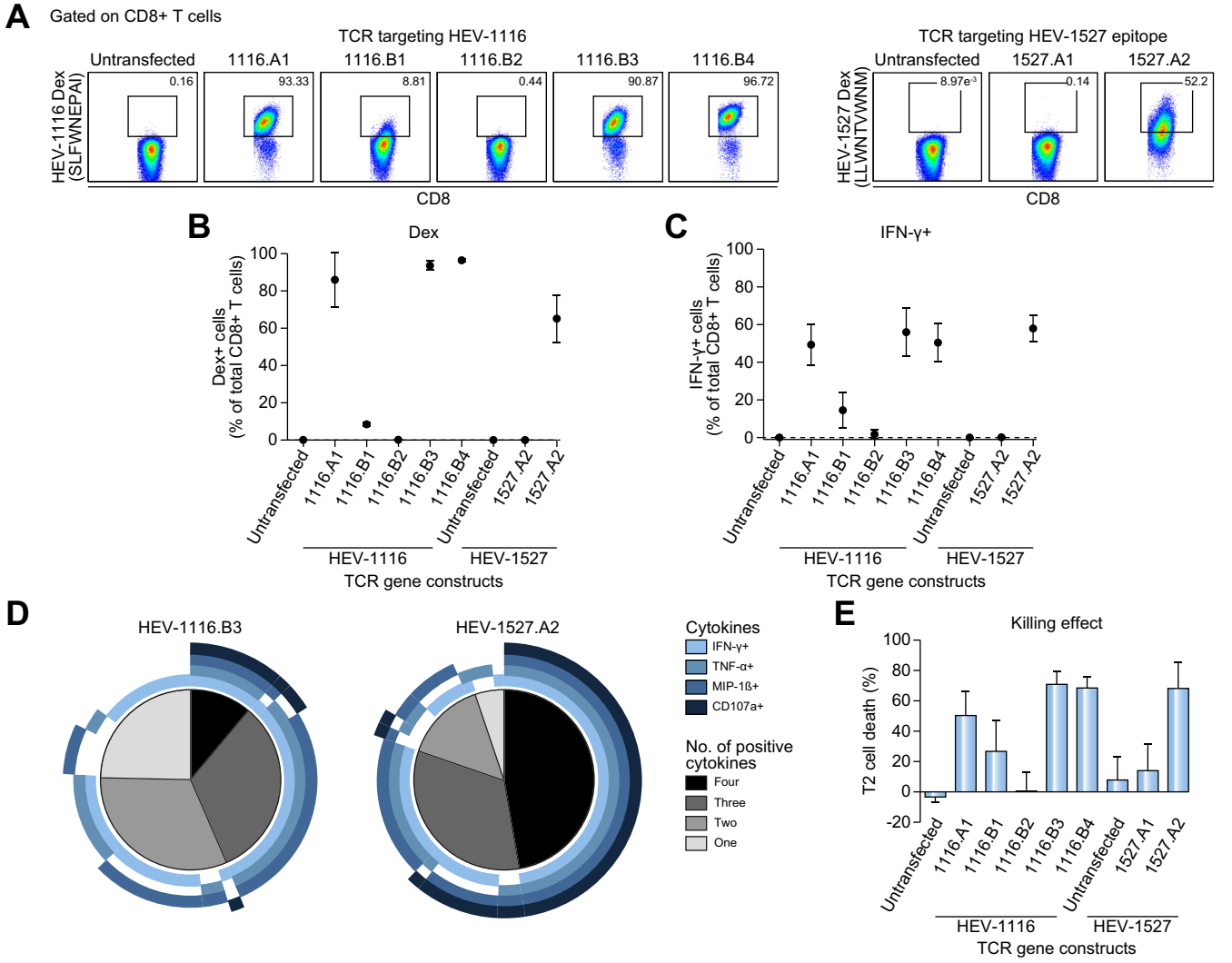

Fig. 4. TCR redirection ICS and killing assay using TCR constructs targeting HEV-1116 and HEV-1527 epitopes. (A) Respective Dextramer staining of T cells isolated from health donors redirected with HEV-1116 and HEV-1527 TCRs. Representative FACS plots were shown. (B) Percentage of Dextramer-binding and (C) IFN-γ-producing CD8+ T cells across all TCR constructs upon peptide-loaded T2 cell stimulation. (D) Polyfunctionality (cytokine production) and cytotoxicity (CD107a) of 1116.B3 and 1527.A2 TCR constructs. Results were mean of 3 experiments. (E) Killing of peptide-loaded T2 cells mediated by T cells redirected with different TCR constructs. Results in B, C and E were expressed as mean ± SD (n = 3). ICS, intracellular cytokine staining; HEV, hepatitis E virus; TCR, T cell receptor. (This figure appears in colour on the web.)

production and cytotoxicity against target cells. Although Dextramer-binding capacity was similar to the original T cell clone, there was a gap between Dextramer-binding and cytokine-producing cells in the TCR-redirected cells. This gap was consistent in all experiments, and was also reported in HBV-specific TCR,[22,26] which may be an intrinsic feature of the assay.

Despite this, the effectiveness of TCR redirection was proven in patients with cHEV. Patient-originated TCR-engineered T cells were conferred with polyfunctionality and cytotoxicity that was comparable to the redirected T cells isolated from immunocompetent donors. We have previously reported on the lack of HEV-specific T cell responses among patients with cHEV;[11,27] where checkpoint inhibitors could enhance T cell proliferation to some extent. In this study, we included patients with cHEV who were insensitive to PD-L1 blockade in proliferation as well as in T cell function. With the use of TCR redirection, the rarity of HEV-specific T cells is no longer an obstacle to confer viral-specific immunity, because all effector T cells could be redirected with HEV-specific TCR, thereby harnessing the intact signaling machinery in these T cells and turning them into cytotoxic HEV-specific T cells. This also demonstrated that chronic HEV infection has no or limited effect on the overall T cell population. The slightly lower (redirected) T cell function observed in patients with cHEV may be due to immunosuppression. Hence, for chronic patients who are exhausted with current treatment options, immunotherapy with TCR redirection may be more fit-for-purpose.

Moving forward, we hope to test our TCRs *in vivo*, *e.g* in a mouse model, in order to assess their translational potential. Mouse models to study HEV infection have been developed recently,[28–30] and would serve as an excellent avenue for us to examine the redirected TCRs in a chronic setting. Although our work is still in its infancy and restricted to HLA-A2 individuals, it is a milestone for future development into viable immunotherapy for patients with chronic HEV infection.

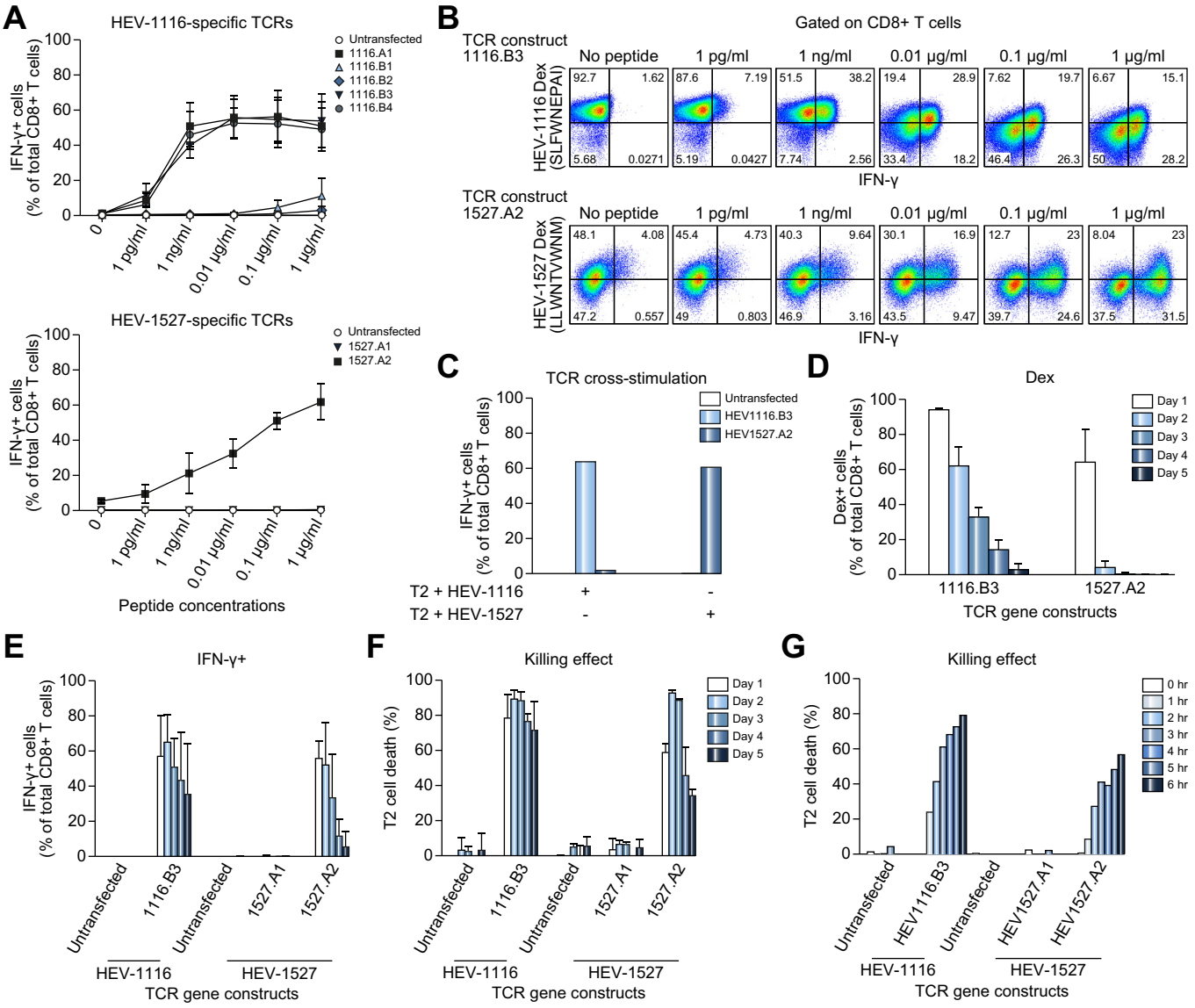

Fig. 5. Characterization of TCR constructs on target sensitivity, specificity and immune response rate. Sensitivity tests on TCR constructs were done by co-culturing TCR-redirected T cells with T2 cells loaded with target peptides at varying concentrations from 0 to 1 μg/ml. (A) IFN-γ production in T cells redirected with different TCR constructs targeting HEV-1116 (upper panel) and HEV-1527 (lower panel) epitopes. Untransfected T cells served as control. (B) Representative FACS plots of Dextramer and intracellular IFN-γ co-staining of CD8+ T cells redirected with TCR constructs 1116.B3 and 1527.A2 upon stimulation with different peptide concentrations. (C) Cross-stimulation of T cells redirected with 1116.B3 TCR with HEV-1527 peptide, and vice versa to validate target specificity. (D) Dextramer-binding capacity, (E) IFN-γ production and (F) killing effect of T cells redirected with different TCRs were analyzed longitudinally from day 1 to 5 post-redirection. Results shown were mean ± SD, n = 2. (G) Targeted killing effect of T cells redirected with different TCRs within 6 h of co-culturing with T2 cells loaded with target peptides. HEV, hepatitis E virus; TCR, T cell receptor. (This figure appears in colour on the web.)

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Conflict of interest

Please refer to the accompanying ICMJE disclosure forms.

Authors' contributions

CFS, MSC, HW and MC contributed to study concept inception and experimental design. PB and MPM recruited patients. CFS acquired and analyzed data. DT analyzed HEV genomic sequences. CFS and MC drafted and revised the manuscript. All authors read and agreed; HW, MSC and MC approved the finalized manuscript.

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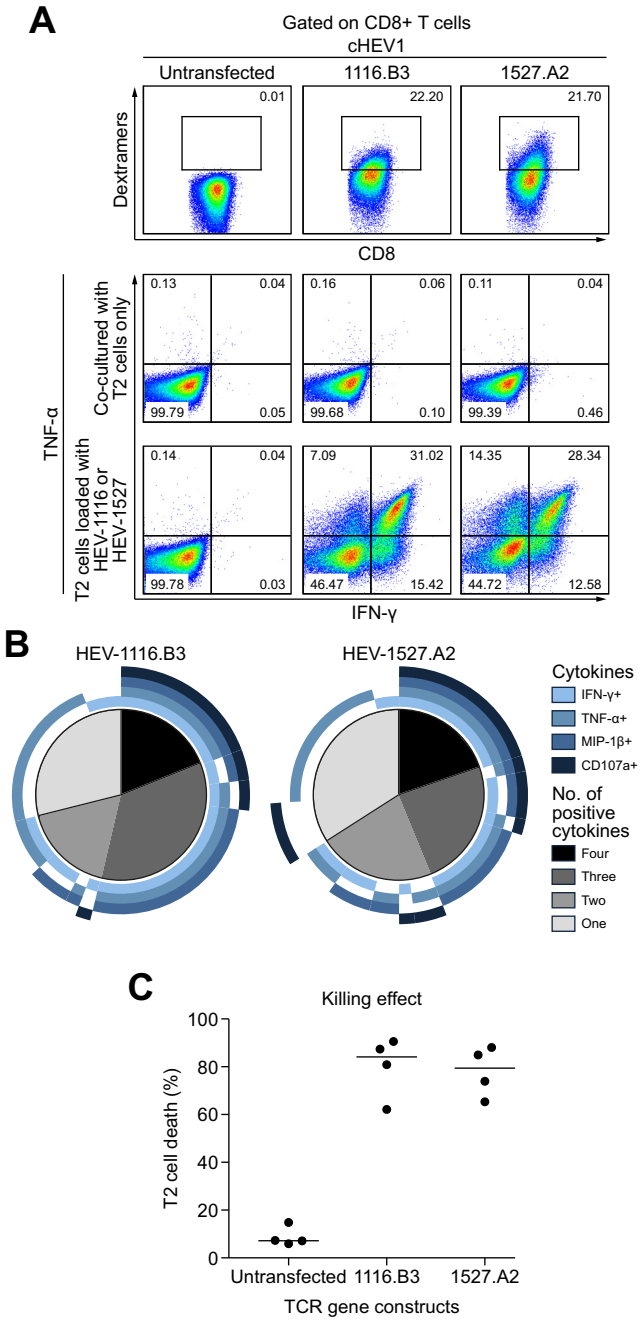

Fig. 6. TCR-redirected T cells isolated from patients with chronic HEV. (A) HEV-1116 and HEV-1527 Dextramer staining and ICS of T cells – isolated from a representative patient with chronic hepatitis E (cHEV1) – redirected with HEV-1116 and HEV-1527 TCRs. Staining was performed 6 h after co-culturing with T2 cells loaded with target peptides. (B) Polyfunctionality (cytokine production) and cytotoxicity (CD107a) of TCR-redirected CD8+ T cells. Results shown were median of 4 patients with chronic HEV. (C) Targeted killing effect of T cells redirected with different TCRs after 6 h of co-culturing with T2 cells loaded with target peptides. Black horizontal lines indicate median values of data from patients with chronic HEV. ICS, intracellular cytokine staining; HEV, hepatitis E virus; TCR, T cell receptor. (This figure appears in colour on the web.)

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.06.005.

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Author names in bold designate shared co-first authorship

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