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Hepatitis D Virus-specific CD8⁺ T Cells Have a Memory-like Phenotype Associated With Viral Immune Escape in Patients With Chronic Hepatitis D Virus Infection

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Abstract

Background & Aim: Hepatitis D virus (HDV) superinfection of patients with chronic HBV infection results in rapid progression to liver cirrhosis. Little is known about HDV-specific T cells and how they contribute to the anti-virus immune response and liver disease pathogenesis.

Methods: We isolated peripheral blood mononuclear cells from 28 patients with chronic HDV and HBV infection, identified HDV-specific CD8⁺ T-cell epitopes and characterized HDV-specific CD8⁺ T cells. We associated these with HDV sequence variations and clinical features of patients.

Results: We identified 6 CD8⁺ T-cell epitopes; several were restricted by multiple HLA class I alleles. HDV-specific CD8⁺ T cells were as frequent as HBV-specific CD8⁺ T cells, but less frequent than T cells with specificity for cytomegalovirus, Epstein-Barr virus, or influenza virus. The ex vivo frequency of activated HDV-specific CD8⁺ T cells correlated with transaminase

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activity. CD8⁺ T cell production of interferon gamma following stimulation with HDV peptides correlated inversely with HDV titer. HDV-specific CD8⁺ T cells did not express the terminal differentiation marker CD57, and fewer HDV-specific than Epstein-Barr virus-specific CD8⁺ T cells were 2B4⁺CD160⁺PD1⁺, a characteristic of exhausted cells. About half of the HDV-specific CD8⁺ T cells had a memory-like PD1⁺CD127⁺TCF1^{hi}T-bet^{low} phenotype, which associated with HDV sequence variants with reduced HLA binding and reduced T-cell activation.

Conclusions: CD8⁺ T cells isolated from patients with chronic HDV and HBV infection recognize HDV epitopes presented by multiple HLA molecules. The subset of activated DV-specific CD8⁺ T cells targets conserved epitopes and likely contributes to disease progression. The subset of memory-like HDV-specific CD8⁺ T cells is functional, but unable to clear HDV due to the presence of escape variants.

Graphical Abstract

Keywords

mutation; lymphocyte; virus escape; transcription factor

Introduction

Hepatitis delta virus (HDV) infection causes the least frequent but most severe form of viral hepatitis. Acute HDV infection has a fatality rate of at least 5%. Chronic HDV infection leads to rapid development of cirrhosis in 70–80% of infected patients, often presenting as end-stage liver disease in early adulthood.¹ The sole available treatment, interferon alpha (IFNα), rarely achieves long-term viral suppression.²

HDV is a single-stranded circular RNA virus, approximately 1700 nucleotides in length.³ HDV virions have a nucleocapsid-like structure, in which HDV RNA is associated with the hepatitis D antigen (HDAg) and enveloped by hepatitis B virus (HBV) surface antigen (HBsAg).⁴ HDV replication generates a complementary copy of its genome, the antigenome. Unedited genomes have an amber stop codon at position 196, leading to formation of the small HDAg, whereas edited genomes allow translation to proceed to position 215, creating the large HDAg.⁵ Farnesylation of an isoprenylation site at the carboxyterminus of the

HDVAg is required for interaction with HBsAg⁶ and prenylation inhibitors, such as lonafarnib, are currently evaluated in clinical trials.^{7, 8}

Chronic hepatitis D results from either simultaneous infection with HBV and HDV or from HDV superinfection of HBsAg carriers, because HDV requires HBsAg to infect cells. Patients that are already infected with the hepatitis B virus (HBV) are therefore at risk of HDV superinfection and a prophylactic HDV vaccine is not available yet. This is confounded by the fact that the role of adaptive immune responses in HDV clearance and/or disease progression is largely unknown. Two studies described weak proliferative T-cell responses to overlapping HDV peptides in chronically infected patients.^{9, 10} Four CD8⁺ T-cell epitopes were identified in patients who spontaneously recovered from HDV infection,^{11, 12} but all chronically infected patients tested negative. Two CD4 T-cell epitopes were identified in patients with normal alanine aminotransferase (ALT) activity, but HDV RNA levels were not assessed.⁹ To date, no study has characterized HDV-specific CD8⁺ T cells in chronic HDV infection.

Reasoning that CD8⁺ T-cell responses in chronic HDV infection may target subdominant epitopes that differ from those targeted in acute, self-limited infection, we set out to identify CD8⁺ T-cell epitopes and their restricting HLA-alleles in patients with chronic HDV/HBV infection. We then synthesized HLA class I/epitope multimers to characterize differentiation, exhaustion and function of HDV-specific CD8⁺ T cells. These were compared to CD8⁺ T cells that are specific for HBV as the co-infecting virus, cytomegalovirus (CMV) as a readily controlled virus, Epstein-Barr virus (EBV) as a latent virus and influenza virus (Flu) as a cleared virus. The overall goal of the study was to identify immunogenic HDV sequences for vaccination and prevention of HDV infection and to understand the role of HDV-specific CD8⁺ T-cell responses in chronic disease.

Material and Methods

Patients

Immune responses were studied in 28 patients with chronic HDV/HBV infection (Suppl. Table 1). CD8⁺ T-cell epitopes were mapped with fresh peripheral blood mononuclear cells (PBMC) of a group of 17 patients that had completed therapy with the farnesyltransferase inhibitor lonafarnib and the protease inhibitor ritonavir. This was followed by *ex vivo* analysis of phenotype and function of HDV-specific CD8⁺ T cells in patients that had not been treated for HDV at the time point of this study (Suppl. Table 2). All patients tested negative for hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infection and gave written informed consent for research testing under protocols ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02511431) identifier [NCT02511431](https://clinicaltrials.gov/ct2/show/study/NCT02511431), [NCT00023322](https://clinicaltrials.gov/ct2/show/study/NCT00023322), [NCT01495585](https://clinicaltrials.gov/ct2/show/study/NCT01495585) and [NCT00001971](https://clinicaltrials.gov/ct2/show/study/NCT00001971)) approved by the institutional review board of NIDDK/NIAMS.

Peptides and peptide/HLA multimers

Forty-one 15-mer peptides (Mimotopes, Clayton, Australia), overlapping by 10 amino acids were synthesized (HDV genotype 1, Genbank number [AM902175](https://www.ncbi.nlm.nih.gov/nuccore/AM902175)¹³) and arranged in four mixes, each containing 10 or 11 peptides. Shorter HDV peptides were synthesized at >90%

purity (Genscript, Piscataway, NJ). Immune responses were also assessed with HBV, HCV, CMV, EBV and Flu epitopes and with HLA/peptide multimers presenting epitopes of the respective virus (Suppl. Table 3).

Isolation of peripheral blood mononuclear cells and HLA-typing

PBMC were separated from heparin-anticoagulated blood by Ficoll-Histopaque (Mediatech, Manassas, VA) density gradient centrifugation, washed three times with phosphate-buffered saline (PBS, Mediatech) and used immediately to generate T-cell lines for epitope identification. All other PBMC used in this study had been cryopreserved in 70% fetal bovine serum (FBS, Serum Source International, Charlotte, NC), 20% RPMI1640 (Mediatech) and 10% DMSO (Sigma Aldrich, St. Louis, MO) in liquid nitrogen.

DNA was extracted from PBMC using spin columns (Qiagen, Hilden, Germany). HLA-A and HLA-B typing was performed at two-digit resolution level using sequence-specific primers (One Lambda Inc., Canoga Park, CA).

Generation of HDV-specific T-cell lines and identification of minimal CD8⁺ T-cell epitopes

PBMC were stimulated at 4×10^5 cells/well of a 96-well round-bottom plate in 100 μ l complete medium [AIM-V containing 10% fetal bovine serum, 50 μ g/ml streptomycin sulfate, 10 μ g/ml gentamicin sulfate, 1% L-glutamine and 1% Hepes (all from Cellgro, Herndon, VA)] with pools of 15-mer HDV peptides (5 mg/ml peptide), 10 ng/mL interleukin (IL)-7 (PeproTech, Rocky Hill, NJ), and 300 pg/mL IL-12 (R&D Systems, Minneapolis, MN). On days 3 and 7, 100 μ l complete medium with 20 IU/ml IL-2 (Prometheus, San Diego, CA) were added.

On day 10 cells were pooled, re-stimulated with or without the respective peptide pools for 5 hours, washed and stained with Aqua Viability dye (Molecular Probes, Eugene, OR) and antibodies against surface markers for 20 min at 4°C (Suppl. Table 4). PBMC were fixed, permeabilized and incubated with anti-IFN- γ PE (Becton Dickinson, BD Biosciences, San Jose, CA) for 30 min at 4°C, and analyzed on an LSR II flow cytometer (BD). The remaining cells were tested for IFN- γ production against single peptides either the next day or after additional culture as described below.

To further expand the population of HDV-specific CD8⁺ T cells, day-10 cell lines were subjected to dead cell removal (Miltenyi Biotec, Auburn, CA) and stimulated with individual 15mer HDV peptides for 4 hours in the presence of anti-CD28/anti-CD49d (0.5 μ g/ml, BD Biosciences). IFN- γ -secreting cells were enriched using the IFN- γ secretion assay, labeled with anti-PE UltraPure MicroBeads (all from Miltenyi) for 15 minutes at 4°C and selected using double columns on an AutoMACS Pro Separator (Miltenyi). The positively selected cell fraction was cultured in complete medium containing 10 ng/ml IL-7, 300 pg/ml IL-12 and 20 IU/ml IL-2. The negatively selected cell fraction was irradiated at 3000 rad and added at 2:1 ratio. Fresh medium containing 20 IU/ml IL-2 was added bi-weekly.

After 10 days the cell lines were subjected to a rapid expansion protocol by restimulation with 50 ng/ml anti-CD3 (clone X35, Beckman-Coulter, Atlanta, GA), 3000 IU/ml IL-2, and a ratio of 1:100 irradiated PBMC (6000 rads) from a research blood donor. Fresh medium

containing 3000 IU/ml IL-2 was added bi-weekly, and the cultures were restimulated every 10 days until sufficient cell numbers were reached.

Ex vivo analysis of number, phenotype and function of HDV-specific CD8⁺ T cells in PBMC with peptide/HLA multimers

After thawing of cryopreserved PBMC, CD8⁺ T cells were enriched using the CD8⁺ T-cell enrichment kit (Miltenyi), incubated with 50 nM dasatinib (Axon Medchem, Reston, VA) for 30 minutes at 37°C, stained with the respective HLA/epitope multimer for 10 min at room temperature, and subsequently with fixable viability dye V510 and antibodies against cell surface markers for 15 min at room temperature (Suppl. Table 4).

For transcription factor analysis cells were fixed, permeabilized, stained with antibodies against transcription factors for 30 min at 4°C (Suppl. Table 4) and analyzed on an LSR II flow cytometer (BD Biosciences). A minimum of 20 multimer⁺ cells were analyzed for subset composition of the multimer⁺ population.

For cytokine secretion assays PBMC were stained with PE-conjugated HLA/epitope multimers for 15 minutes at room temperature, washed twice, stimulated with 1 µg/ml HDV, HBV, CMV, EBV, Flu peptide or an irrelevant HCV peptide for 4 hours and processed using the IFN-γ secretion assay and detection kit (Miltenyi) according to the manufacturer's instruction. Multimer staining was repeated, followed by staining with fixable viability dye V510 and antibodies against cell surface markers for 15 minutes at room temperature (Suppl. Table 4) and subsequent analysis on an LSR II flow cytometer (BD Biosciences). Naïve (CD45RO⁻CCR7⁻) cells were excluded in all figures.

HLA class I binding assays

Peptide/HLA-binding affinities were determined through inhibition of binding of a high-affinity radiolabeled ligand to purified HLA class I molecules as previously described.¹⁴ The IC₅₀ was the concentration of the HDV peptide yielding 50% inhibition of binding of the radiolabeled high affinity ligand. Utilizing a previously defined threshold,^{15, 16} HDV peptides with an IC₅₀ < 500 nM were defined as having a high HLA-binding affinity and those with an IC₅₀ of 500–5000 nM range as having an intermediate HLA-binding affinity.

HDV sequence analysis

RNA was extracted from plasma or serum using the QIAamp MinElute Virus spin and the QIAamp viral RNA mini kits (Qiagen). Reverse transcription (Superscript IV first-strand synthesis system, Invitrogen, Carlsbad, CA) was followed by a nested polymerase chain reaction to amplify fragments 200–450 bp length using previously published¹⁷ or newly designed HDV-specific primers (Suppl. Table 5) with the following PCR conditions: 1 min at 95°C followed by 35 cycles of 30 sec at 95°C for denaturation, 1 min for primer annealing at different temperatures (Fragment A: PCR1 53°C, PCR2 62°C, Fragment E: PCR1 51°C, PCR2 62°C, Fragment F: PCR1 66°C, PCR2 53°C) and 1 min at 72°C for extension followed by a final extension step of 5 min at 72°C. Polymerase chain reaction products were directly sequenced (GenBank accession numbers [MK333199–333226](#)).

Statistical analyses

Wilcoxon matched-pairs signed rank test, Mann-Whitney U test, Spearman correlation analyses and nonparametric Kruskal-Wallis test with Dunn's multiple comparisons were performed with GraphPad Prism version 7.0 (GraphPad Software Inc, San Diego, CA). $P < .05$ was considered significant. Exhaustion markers were analyzed using SPICE version 5.1 (<http://exon.niaid.nih.gov>).¹⁸ Comparison of distributions was performed using a Student's T test and a partial permutation test.¹⁸ Import of data from FlowJo v10.0.7 into SPICE was conducted using grind (<http://www.github.com/gamanakis/grind>).

Results

Identification of CD8⁺ T-cell epitopes in chronic HDV infection

CD8⁺ T-cell epitopes are viral peptides of 8–11 amino acid length that are presented by HLA class I molecules and recognized by CD8⁺ T cells. Because epitope mapping requires large numbers of virus-specific T cells, we set out to generate HDV-specific CD8⁺ T-cell lines. Reasoning that the effect of lonafarnib/ritonavir therapy on HDV viremia may facilitate the proliferation of HDV-specific T cells we used blood samples from lonafarnib/ritonavir-treated patients to generate these T-cell lines (Suppl Table 2). PBMC were isolated from 17 patients at week 4 and 8 after the end of therapy and stimulated with four pools of overlapping HDV peptides. After 10 days of culture, the resulting T-cell lines were assessed by flow cytometry for IFN- γ production in response to the respective HDV peptide pool (Fig. 1A, left panel). If an HDV peptide pool was recognized, individual peptides of the pool were tested the next day or after additional expansion of the T-cell line. This additional expansion of the T-cell line typically increased the percentage of responding CD8⁺ T cells (Fig. 1A, right panel).

Overall, T-cell lines from 12 of 17 (71%) patients responded to HDV peptides of 15 amino acid length. These T-cell lines were then tested against shorter HDV peptides in dose titration experiments. Epitopes were identified as peptides that yielded half-maximal cytokine responses at the lowest peptide concentration.

As shown in figure 1B, HDV_{192–200} QGFPWDILF and the shorter peptide HDV_{193–200} GFPWDILF were recognized by a T-cell line from an HLA-B*35:01⁺ patient. Interestingly, the longer peptide HDV_{192–200} QGFPWDILF was also recognized by a T-cell line from an HLA-B*52:01⁺ patient (Suppl. Fig. 1). Both peptides were tested in HLA-binding assays and confirmed to have high binding affinity to both HLA-B molecules (IC₅₀ of 118 and 18 nM, respectively, Table 1).

Two additional sequences in that region, HDV_{194–202} FPWDILFPA and HDV_{193–202} GFPWDILFPA, were recognized by an HLA-B*07:05⁺ patient (Fig. 1C). An HLA-B*07:05 expression plasmid was not available, but we confirmed high binding affinity of the shorter peptide FPWDILFPA to HLA-B*07:02 (IC₅₀ of 96 nM, Table 1). IFN- γ ⁺ CD8⁺ T-cell responses to this epitope were also detectable in HLA-B*35:01⁺ patients (not shown) with high binding affinity to that molecule (IC₅₀: 5.7 nM, Table 1).

In addition, HDV_{104–112} RRKALENKR was identified as an HLA-B*27:05-restricted epitope (Fig. 1D), and HDV_{189–196} RGSQGFPW was identified as an HLA-B*58:01 epitope (Suppl. Fig. 2), both with high binding affinity to the respective HLA molecule (IC₅₀ of 109 nM and 14 nM, respectively; Table 1). Finally, HDV_{46–54} DENPWLGNL was identified with T-cell lines from an HLA-B*18:01⁺ patient (Suppl. Fig. 3) and an HLA-B*44:03⁺ patient (not shown) and displayed high binding affinities to HLA-B*18:01 (149 nM), B*44:02 (438 nM) and B*44:03 (99 nM, Table 1).

HDV epitopes are presented by multiple HLA-B alleles and cluster within the large HDAg

A unique characteristic of the identified CD8⁺ T-cell epitopes was their presentation by multiple HLA alleles, which were almost exclusively B alleles (Table 1). A further unique characteristic was the clustering of epitopes in specific regions of the large HDAg. The B*18:01-, B*44:02- and B*44:03-restricted epitope DENPWLGNL overlaps with the previously published HLA-A*02:01-restricted epitope KLEDENPWL¹¹ at the aminoterminal of the HDAg (Fig. 2A). The HLA-B*27:05-restricted epitope RRKALENKR and the previously described epitope RRKALENKK are identical except for an amino acid difference at the carboxyterminus¹² and they overlap with another HLA-B*27-restricted epitope RRDHRRRKAL¹² (Fig. 2B). Finally, a cluster of several epitopes at the carboxyterminus of the HDAg is presented by HLA-B*02:05, HLA-B*07, HLA-B*35:01, HLA-B*52:01 and HLA-B*58:01 (Fig. 2C). This region was targeted by most of the HDV-specific T-cell lines that were established from 17 patients (Fig. 2D).

Validating our starting hypothesis that treatment-induced reduction in HDV viremia may facilitate the expansion of HDV-specific T cells *in vitro*, we found a negative correlation between the frequency of IFN- γ -producing HDV-specific CD8⁺ T cells in T-cell lines at week 4 post treatment and the level of viremia at that time point ($r=-.6286$, $P=.0141$, Spearman correlation) and at the end of treatment ($r=-.8204$, $P=.0003$). Notably, ALT values increased after the end of lonafarnib/ritonavir treatment (mean ALT $\pm$ SD: 51 $\pm$ 33 U/L at end of treatment, 68 $\pm$ 58 U/L at week 4 post treatment, 118 $\pm$ 98 U/L at week 8 post treatment).

Collectively, these results demonstrate that HDV is very immunogenic despite its small size and hydrophobic amino acid sequence, and suggest that HDV-specific CD8⁺ T cells may contribute to disease pathogenesis.

Frequency and phenotype of HDV-, HBV-, CMV-, EBV- and Flu-specific CD8⁺ T cells in chronic HDV infection

Based on the mapped epitopes we generated six HLA/epitope multimers. The specific binding of the HLA/epitope multimers to the T-cell receptor allows the *ex vivo* detection of low-frequency HDV-specific T cells by flow cytometry irrespective of their ability to expand *in vitro* (Fig. 3A, B).

For this *ex vivo* characterization of HDV-specific CD8⁺ T cells with HLA/epitope multimers in chronic HDV infection we chose patients who had not received prior lonafarnib/ritonavir treatment. The multimers were selected to match the patients HLA haplotype, except for the HLA-B*07:02/HDV_{194–202} multimer, which was used to identify HDV_{194–202}-specific

CD8⁺ T cells in HLA-B*07:02⁺ and HLA-B*07:05⁺ patients, and the HLA-B*27:05/HDV₁₀₄₋₁₁₂ multimer, which was used to identify HDV₁₀₄₋₁₁₂-specific CD8⁺ T cells in HLA-B27:05⁺ and HLA-B*27:02⁺ patients. For comparison, we studied CD8⁺ T cells specific for HBV as the co-infecting virus, CMV as a low level persisting virus, EBV as a latent virus, and Flu as a virus that is completely cleared.

As shown in figure 3C, HDV-specific CD8⁺ T cells were detectable at low frequencies in PBMC of patients with chronic HDV infection (median 0.013% of the CD3⁺CD4⁺ T-cell population). The frequency of HDV-specific CD8⁺ T cells was comparable to that of HBV-specific CD8⁺ T cells in HBV/HDV-coinfected patients, whereas CMV-, EBV- and Flu-specific CD8⁺ T cells were detected at significantly higher frequencies (median 0.08% of EBV- and Flu-specific CD8⁺ T cells, median 0.8% of CMV-specific CD8⁺ T cells, Fig. 3C).

Next, we studied the subset composition of virus-specific CD8⁺ T cells based on cell surface staining for CD45RO, a marker of antigen experience, and CCR7, a lymph node homing marker. About two thirds of virus-specific CD8⁺ T cells were CD45RO⁺CCR7⁺ effector memory cells (Fig. 3D), and most of the remaining virus-specific CD8⁺ T cells were CD45RO⁺CCR7⁺ central memory cells (Fig. 3E). The smallest population consisted of CD45RO⁺CCR7⁺ terminal effector memory RA (TEMRA) cells (Fig. 3F). The size of these cell subsets did not differ among cells with HDV-, HBV-, EBV- or Flu-specific CD8⁺ T-cell populations. CMV-specific CD8⁺ T cells, however, were less frequently CD45RO⁺CCR7⁺ central memory cells and more frequently TEMRA cells in comparison to HDV-specific cells ($P=0.0305$ and $P=0.0028$, respectively, Fig. 3E and 3F).

HDV-specific CD8⁺ T cells are activated, but not terminally differentiated and have a memory-like PD-1⁺CD127⁺ phenotype

The majority of HDV-specific antigen-experienced CD8⁺ T cells expressed the programmed cell death-1 (PD-1) molecule and the IL-7 receptor CD127 (Fig. 4A, left panel). Co-expression of these molecules is typical for a memory-like phenotype^{19, 20} as these cells express PD-1, a marker of T-cell activation and exhaustion but retain their proliferative response to IL-7. The majority of EBV- and Flu-specific CD8⁺ T cells were also PD-1⁺CD127⁺ memory-like, but only the Flu-specific CD8⁺ T-cell population contained a sizable fraction (about 27%) of PD-1⁺CD127⁺ memory cells. The frequency of this Flu-specific CD8⁺ T cell subset was significantly greater than the frequency of HDV- and HBV-specific CD8⁺ T cells ($P=0.0016$ and $P=0.0034$ respectively). Vice versa, an exhausted PD-1⁺CD127⁺ phenotype was found among HDV- and EBV-specific CD8⁺ T cells but was virtually absent among Flu-specific CD8⁺ T cells (Fig. 4A, C, D, left panels).

To examine the activation and differentiation status of PD-1⁺ CD8⁺ T cells we co-stained for the activation marker CD38 and the terminal differentiation marker CD57. For the HDV- and EBV-specific CD8⁺ T-cell populations, half of the PD-1⁺ cells expressed CD38⁺ and the other half did not (Fig. 4A and C, middle panels). In contrast, HBV-specific CD8⁺ T cells tended to be PD-1⁺CD38⁺ (Fig. 4B, middle panel) and Flu-specific CD8⁺ T cells were significantly more frequently PD-1⁺CD38⁺ ($P=0.0436$, Fig. 4D, middle panel). Overall, non-activated CD38⁺ cells were significantly more prevalent among Flu-specific CD8⁺ T cells than among HDV-specific CD8⁺ T cells ($P<0.01$). This indicates that complete resolution of a

viral infection, such as Flu, results in almost complete loss of the activation marker CD38. About 18.5% of the Flu-specific CD8⁺ T-cell population was negative for both CD38 and PD-1.

In contrast to the activation marker CD38, the terminal differentiation marker CD57 was rarely co-expressed with PD-1 on HDV-specific CD8⁺ T cells (Fig. 4A, right panel). HDV- and EBV-specific CD8⁺ T cells had a small PD-1⁺CD57⁺ T cell subset (median of 7.47% and 21.7% respectively), whereas the frequency of Flu-specific CD8⁺ T cells with PD-1 and CD57 co-expression was significantly lower ($P=0.0137$ and $P=0.0001$ respectively). Flu-specific CD8⁺ T cells were also more frequently PD-1⁻CD57⁻ when compared to HDV- ($P=0.0039$) and HBV-specific ($P=0.0065$) CD8⁺ T cells.

Collectively, these results indicate that HDV-specific CD8⁺ T cells are activated but not terminally differentiated in HDV/HBV co-infected patients. The percentage of activated CD38⁺ T cells in the HDV-specific but not in the HBV-specific CD8⁺ T-cell population correlated with AST activity (Fig. 4E), implying that HDV-specific CD8⁺ T cells contribute to the pathogenesis of hepatitis D.

HDV-specific CD8⁺ T cells are less exhausted than EBV-specific CD8⁺ T cells

Based on these results we set out to more specifically explore the phenotype of HDV-specific CD8⁺ T cells as regards to the two extremes that have been described for virus-specific T cells in other chronic infections: an exhausted phenotype and a memory-like phenotype.

We first determined the expression of cell surface markers associated with exhaustion. This analysis was performed on virus-specific CD45RO⁺CCR7⁻ effector memory CD8⁺ T cells. PD-1 was the single, most frequently expressed marker on these cells (Fig. 5A, turquoise arcs), and HDV-specific effector memory CD8⁺ T cells were more frequently PD-1⁺ than CMV- and EBV-specific effector memory CD8⁺ T cells ($P=0.032$, $P=0.026$, respectively). On the other end of the spectrum, there was a trend for TIM-3 being more frequently expressed on Flu-specific than on HDV-, HBV-, CMV- or EBV-specific effector memory CD8⁺ T cells. These results suggest that activation and exhaustion markers are differentially expressed, depending on the infecting virus and the infection outcome.

As regards to the co-expression of exhaustion markers (indicated by the pies in Fig. 5A and bar graph panels in Fig. 5B), the frequency of 2B4, CD160 and PD-1 triple-positive cells was significantly lower among HDV-specific effector memory CD8⁺ T cells than among EBV-specific effector memory CD8⁺ T cells ($P=0.0001$, Fig. 5A, orange pie slices and Fig. 5B second panel). On the other hand, the frequency of 2B4 and PD-1 double-positive cells (Fig. 5A green slices and Fig. 5B sixth panel) and the frequency of PD-1 single-positive cells (Fig. 5A, dark violet pie slices and Fig. 5B, third last panel) were higher among HDV-specific cells than among CMV- and EBV-specific effector memory CD8⁺ T cells. As expected, exhaustion marker-negative cells were significantly more common among Flu-specific than among HDV-specific effector memory CD8⁺ T cells ($P=0.001$, Fig. 5B last panel).

Collectively, these results indicate that HDV-specific CD8⁺ T cells are chronically activated but less exhausted than EBV-specific CD8⁺ T cells.

Memory like TCF1^{hi} HDV-specific T cells are specific for HDV epitopes with escape mutations

Finally, we explored HDV-specific CD8⁺ T cells for the presence of a memory-like phenotype. To assess whether HDV-specific CD8⁺ T cells were functional in chronic infection we subjected PBMC to a short (4 hour) stimulation with HDV epitopes, stained them with HLA/epitope multimers and assessed multimer⁺ cells for IFN- γ production by flow cytometry. For comparison, PBMC were stimulated with Flu epitope and studied for responses of Flu-specific responses. As shown in Suppl. Fig. 4, HDV-specific CD8⁺ T cells mounted an IFN- γ response upon stimulation ($P=.0078$) albeit the maximal response was less than that of Flu-specific CD8⁺ T cells. This preserved functional response and the PD-1⁺CD127⁺ phenotype of the majority HDV-specific CD8⁺ T cells (Fig. 4A) prompted us to stain for the transcription factor T-cell factor 1 (TCF1), a recently described hallmark of memory-like cells.²¹

Interestingly, TCF1 analysis separated HDV-specific CD8⁺ T cells into two distinct subpopulations: in some cases, HDV-specific CD8⁺ T cells that recognized a specific epitope were mostly TCF1^{hi}, similar to Flu-specific CD8⁺ T cells, whereas in other cases HDV-specific CD8⁺ T-cell populations contained almost no TCF1^{hi} cells (TCF1^{lo}, $P=.0043$, Fig. 6A and Suppl. Fig. 4C).

TCF1^{hi} and TCF1^{lo} CD8⁺ T-cell subsets also segregated as regards to the expression of the transcription factor T-bet. Most TCF1^{hi} HDV-specific CD8⁺ T cells expressed either low levels of T-bet or no T-bet (T-bet^{lo} or T-bet⁻), again resembling Flu-specific CD8⁺ T cells, whereas most TCF1^{lo} HDV-specific T cells expressed high levels of T-bet (Fig. 6B).

Furthermore, TCF1^{hi} HDV-specific cells resembled Flu-specific CD8⁺ T cells in their expression of the activation marker CD38. Specifically, TCF1^{hi} HDV-specific cells and Flu-specific cells were not activated (CD38⁻), whereas TCF1^{lo} HDV-specific cells were activated (CD38⁺, $P=.0303$, Fig. 6C). These *ex vivo* data suggest that TCF1^{hi} HDV-specific CD8⁺ T cells lack *in vivo* stimulation by their cognate antigen, whereas TCF1^{lo} HDV-specific CD8⁺ T cells are continuously stimulated.

To identify the reason for the differential activation status of TCF1^{hi} and TCF1^{lo} HDV-specific CD8⁺ T cells, we determined the sequence of the respective HDV epitope in each patient (autologous epitope sequence) and compared it to the sequence of the prototype HDV genotype 1 peptide that we used in the immunological assays. HDV sequence analysis revealed the presence of viral variants with reduced HLA-binding affinity (increased IC₅₀) only in cases where the epitope-specific CD8 T cells were TCF1^{hi} (Fig. 6D, right panel). In contrast, the HLA-binding affinity of peptides with autologous HDV sequence was preserved when TCF1^{lo} cells were present (Fig. 6D, left panel).

To confirm that reduced HLA-binding affinity of a given peptide affected its ability to stimulate T cells, we tested prototype-specific T-cell lines for recognition of the autologous

viral sequence. In three cases, where the prototype peptide sequence displayed a high and the autologous (variant) peptide sequence a low HLA binding affinity, prototype-specific CD8⁺ T-cell lines produced IFN- γ only in response to the prototype peptide and did not respond to the variant peptide (Suppl. Fig. 5, first three panels). In one additional case, loss of cross-recognition was likely mediated via changes in T-cell receptor rather than HLA contact sites (Suppl. Fig. 5, fourth panel). This was contrasted with two cases where the autologous peptide sequence exhibited only a small or no reduction in HLA binding compared to the prototype sequence. Accordingly, prototype-specific CD8⁺ T-cell lines produced IFN- γ in response to both peptides (Suppl. Fig 5, bottom panels).

Collectively, these results demonstrate that a TCF1^{hi} phenotype of HDV-specific memory CD8⁺ T cells is associated with lack of *in vivo* stimulation with their cognate antigen, implying that HDV developed escape mutations to evade HDV-specific CD8⁺ T-cell recognition in chronic infection.

Discussion

This is the first report that succeeded in identifying and characterizing CD8⁺ T-cell responses *ex vivo* in the blood of patients with chronic HDV/HBV infection. This has been a challenging task, as evidenced by the fact that only four patients, all of them with resolved HDV infection, had been reported to have HDV-specific CD8⁺ T-cell responses against defined epitopes so far.^{11, 12}

The epitopes identified in the current study clustered in specific regions of the large HDAg and several were restricted by multiple HLA alleles. Clustering of epitopes has been described in other viral infections. For example, the HBV core_{18–27} FLPSDFFPSV epitope overlaps with the HLA-B*35:01 and HLA-B*51:01-restricted epitope LPSDFFPSV,²² and EBV-specific CD8⁺ T-cell epitopes cluster in the BZLF1 region of the virus.²³ However, promiscuous binding of CD8⁺ T-cell epitopes to multiple HLA class I molecules is unusual. It is more commonly associated with CD4 T-cell epitopes,²⁴ as the HLA class II binding groove accommodates longer peptides. The very small genome and single open reading frame of HDV may have contributed to both the clustering of epitopes and their promiscuous HLA class I binding by training the immune system on a very short viral sequence. The identified epitopes may be useful to create a prophylactic T-cell vaccine for patients who are HBV-infected and at risk of HDV superinfection. Their restriction by multiple HLA alleles renders them suitable for a genetically diverse patient population.

The *ex vivo* frequency of HDV-specific CD8⁺ T cells was very low in the blood of patients with untreated chronic HDV infection, but comparable to what has been reported for HBV-specific^{25, 26} and HCV-specific CD8⁺ T cells.^{19, 27} A low frequency of virus-specific CD8⁺ T cells is commonly attributed to the fact that they are terminally differentiated and deleted in chronic viral infection.^{10, 12} However, HDV-specific CD8⁺ T cells were activated but not terminally differentiated (Fig. 4), and they expressed less frequently the 2B4⁺CD160⁺PD-1⁺ cell phenotype of exhausted cells than EBV-specific T cells (Fig. 5). They may contribute to liver injury and disease pathogenesis as suggested by the positive correlation between the *ex vivo* frequency of activated (CD38⁺) HDV-specific CD8⁺ T cells in the blood and AST

activity, a serological marker of liver inflammation. Because the current clinical protocol did not include liver biopsies, it will be important to study intrahepatic T cells in future studies to shed more light on the exact role of HDV-specific CD8⁺ T cells in liver disease pathogenesis.

This study also provides new insight into the biology of memory-like T cells, which to date have mostly been studied in the mouse model of lymphocytic choriomeningitis virus (LCMV) infection. This model has been essential in revealing the role of the transcription factor TCF1 in the maintenance of memory CD8⁺ T cells after acute-self-limited infection^{28, 29} and memory-like CD8⁺ T cells in chronic infection.^{20, 30} However, it is limited in its capacity to model virus-host interactions in humans, where chronic infections last for much longer time. Similar to LCMV-specific memory-like CD8⁺ T cells in mice,³⁰ HDV-specific memory-like CD8⁺ T cells in humans are TCF1^{hi} and PD-1⁺CD127⁺. However, whereas TCF1^{hi} cells represented only 6%–10% of CD8⁺ T cells specific for LCMV epitopes in mice,³⁰ they represented 60–100% of CD8⁺ T cells specific for some of the HDV epitopes in our patients (Fig. 6A). Furthermore, LCMV-specific TCF1^{hi} CD8⁺ T cells were highly prevalent in lymphoid organs and completely absent in the peripheral blood, but HDV-specific TCF1^{hi} CD8⁺ T cells were detected in the blood. We attribute this to the dynamics of viral/host interaction and specifically to the emergence of viral variants that represent escape from CD8⁺ T-cell responses.

In chronic HCV infection, it was recently shown that PD-1⁺CD127⁺ TCF1^{hi} memory-like cells persist - presumably for decades - at low frequency in the presence of their cognate antigen and that they undergo expansion after treatment-induced viral clearance.¹⁹ We here propose that viral escape mutations with reduced or completely lost antigen presentation have the same effect: a relative expansion of virus-specific TCF1^{hi} CD8⁺ T cells and a relative loss of chronically stimulated and exhausted effector cells. The following mechanisms may contribute to the expansion of TCF1^{hi} cells in the context of viral escape: first, reduced HLA binding of viral variant sequences results in reduced T-cell activation (Fig. 6) and reduced IFN- γ production (Suppl. Fig. 5). Consistent with this notion, activation of T cells has been shown to downregulate TCF1 expression in T cells of mice and humans.^{31, 32} Second, reduced T-cell receptor/HLA interaction is typically associated with reduced PD-1/PD-L1 interaction, and therapeutic blockade of the PD-1/PD-L1 pathway has been shown to result in a proliferative burst of TCF1^{hi} cells.³⁰ Third, TCF1^{hi} cells can be sustained in the absence of their cognate antigen by low amounts of IL-2 and IL-15,³³ because these cells can undergo homeostatic proliferation in response to IL-7 due to the high expression level of CD127, the IL-7 receptor. Collectively, these mechanisms may explain the presence of TCF1^{hi} memory-like CD8 T cells in chronic HDV infection. A high prevalence of such cells may be useful as a biomarker for viral escape.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BV	brilliant violet
Cy	cyanine
CMV	cytomegalovirus
DNA	deoxyribonucleic acid
DMSO	dimethyl-sulfoxide
EBV	Epstein-Barr virus
FBS	fetal bovine serum
FSC-H/A	forward scatter height/area
FVS	fixable viability stain
HDAg	hepatitis D antigen
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
IFN	interferon
IL	interleukin
LCMV	lymphocytic choriomeningitis virus
NK	natural killer
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
PD-1	programmed cell death protein-1
PD-L1	programmed death ligand-1
SSC-A	side scatter area
T-bet	T-box expressed in T cells
TCF1	T cell factor 1

TEMRA terminal effector memory RA

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Figure 1. CD8⁺ T-cell epitope mapping in chronic HDV infection.

(A) T-cell lines were generated by stimulating PBMC from a chronic HDV patient with pools of overlapping HDV peptides. IFN- γ production was analyzed by flow cytometry after restimulation with or without pool 4 of overlapping 15mer HDV peptides (left panels) or individual peptides from that pool (right panels). (B-D) T-cell lines of an HLA-B*35:01⁺ patient (B), an HLA-B*0701⁺ patient (C) and an HLA-B*2705⁺ patient (D) were tested against shorter peptides to determine the CD8⁺ T-cell epitope. An epitope (marked in red) is defined as the peptide eliciting half-maximal cytokine responses at the lowest peptide concentration.

Figure 2: Characterization of HDV-specific CD8⁺ T-cell epitopes using *in vitro* expanded T-cell lines.

(A-C) The location of HDV-specific CD8⁺ T-cell epitopes in the aminoterminal (A), middle (B) and carboxyterminal HDV amino acid sequence (C) is shown. The HLA molecule presenting the epitope is indicated on the left, an underlined HLA molecule indicates that an HLA/peptide multimer was generated. The previously published epitopes KLEDENPWL,¹¹ RRKALENKK and RRDHRRRKAL¹² are included for reference purposes. (D) Number of patients with T-cell lines responding to the indicated peptides in the HDV peptide pools. Arrows indicate responses that were mapped (after further culture of the T cell lines) to the indicated HLA-restricted minimal optimal epitopes. (E) Inverse correlation (Spearman correlation) between the frequency of IFN γ -producing HDV-specific CD8⁺ T cells in the T-cell lines and the level of viremia in patient serum. The T-cell lines were established from PBMC obtained at week 4 post lonafarnib/ritonavir treatment.

Figure 3: Frequency and phenotype of HDV-, HBV-, CMV-, EBV- and Flu-specific CD8⁺ T-cells in chronic HDV infection.

(A, B) PBMC were depleted of non-CD8 T cells with magnetic beads, antibody-stained and analyzed by flow cytometry following the indicated gating strategy (A) to identify HLA/epitope multimer⁺ cells as HDV-specific CD8⁺ T cells (B). FSC-H: forward scatter height; FSC-A: forward scatter area; SSC-A: side scatter area; FVS: fixable viability stain. (C-F) Frequency of HDV-specific multimer⁺ cells (C) and their subpopulations of CD45RO⁺CCR7⁺ (D), CD45RO⁺CCR7⁻ (E), and CD45RO⁻CCR7⁻ cells (F). Statistics: Kruskal-Wallis test with Dunn's multiple comparisons.

Figure 4: Most HDV-specific CD8⁺ T cells have a memory-like PD-1⁺CD127⁺ phenotype, and are activated, but not terminally differentiated.

(A) HDV-, (B) HBV-, (C) EBV- and (D) Flu-specific antigen-experienced CD8⁺ T cells were assessed for expression of PD-1 and CD127 (left panels), PD-1 and CD38 (middle panels) and PD-1 and CD57 (right panels). Statistics: Friedman test with Dunn's multiple comparisons. (E) Correlation between serum AST activity and frequency of CD38⁺ cells in the HDV-specific (left panel) or HBV-specific (right panel) CD8⁺ T-cell population using Spearman correlation. *N.s.*, not significant.

Figure 5: HDV-specific CD8⁺ T cells are less exhausted than CMV- and EBV-specific CD8⁺ T cells.

(A) Frequency of HDV-, HBV-, CMV-, EBV- or Flu-specific effector memory (CCR7⁻ CD45RO⁺) CD8⁺ T cells expressing either 2B4, CD160, PD-1 or Tim-3 (pie arcs) or combinations thereof (pie slices, corresponding to individual panels in B). (B-C) Virus-specific effector memory CD8⁺ T cells were identified by flow cytometry and assessed for expression of the surface marker combinations as indicated by bar graphs (B, median with interquartile range) and a heatmap (C). Statistics: Students T test and partial permutation test using SPICE. Only statistically significant differences to HDV-specific CD8⁺ cells are displayed. * $P < .05$, ** $P < .01$, *** $P < .001$.

Figure 6: The memory-like phenotype of HDV-specific CD8⁺ T cells is associated with the presence of viral sequence variants with reduced HLA binding affinity.

(A-C) Frequency of HDV-specific CD8⁺ T cells expressing the transcription factors TCF1(A) or T-bet (B). Statistics: Mann-Whitney test. (C) Expression level (median fluorescence intensity, MFI) of the activation marker CD38 on HDV- and Flu-specific CD8⁺ T cells. (D) The memory-like TCF1^{hi} phenotype of HDV-specific CD8⁺ T cells is associated with the presence of viral sequence variants with reduced HLA binding affinity. HDV-infected patients were divided into two groups with either TCF1^{hi} or TCF1^{lo} HDV-specific CD8⁺ T cells for the respective epitope. For each group, peptides were synthesized according to the prototype genotype 1 HDV sequence and the corresponding autologous sequence present in the patient's serum. Peptides with prototype or autologous sequence were compared in HLA-binding assays. A low IC₅₀ reflects a high HLA-binding affinity. The following autologous sequences were associated with reduced HLA binding (high IC₅₀) and the presence of TCF1^{hi} cells: EENLWLGNI (HLA-B*18:01); QGFPWDMLF,

RGFPWDILF (HLA-B*35:01); **RRKALENKS** (HLA-B*27:01); **FPWDILFPS** (HLA-B*07:02); **TGRQGFPW** (HLA-B*58:01). The following autologous sequences were associated with preserved HLA binding (low IC₅₀) and the presence of TCF1^{lo} cells: **FPWDILFPA**, **QGFPWDLLF** and **FPWDLLFPA**, **FPWDMLFPA** (HLA-B*35:01); The corresponding prototype sequences are shown in Table 1. Statistics: Wilcoxon matched-pairs signed rank test ($P=.0312$) and Mann-Whitney test ($P=.0043$).

Table 1:**Peptide/HLA Binding Capacity**

Epitope	Sequence	HLA binding capacity (IC ₅₀ , nmol/L)			
		A*02:05	B*07:02	B*35:01	B*52:01
HDV ₁₉₁₋₂₀₀	S Q G F P W D I L F	3115	—	—	3657
	D R - - - - -	—	—	—	4682
	N - - I - - - -	7171	—	—	1469
HDV ₁₉₂₋₂₀₀	Q G F P W D I L F	9633	44,263	118^a	18
	- - - - - M - -	9478	—	935	90
	R - - - - -	7951	—	37,953	224
	- - - - - L - -	3405	—	213	135
HDV ₁₉₄₋₂₀₂	F P W D I L F P A	9365	96^a	5.7^a	
	- - - - - T	—	723	26	
	- - - - M - - -	5735	430	1.7	
	- - - - - S	11,715	13,499	27	
	- - - - L - - -	4161	467	18	
HDV ₁₀₄₋₁₁₂	R R K A L E N K R	B*27:05 109^a			
	- - - - - S	13,584			
HDV ₁₈₉₋₁₉₆	R G S Q G F P W	B*58:01 14^a			
	T - R - - - -	1549			
	- - D R E - - -	195			
HDV ₄₆₋₅₄	D E N P W L G N I	B*18:01 149^a	B*44:02 438	B*44:03 99	
	E D - - - - -	17,943	35,054	26,920	
	E - - L - - - -	1991	468	217	
	- D - - - - -	—	45,920	9189	

NOTE. IC₅₀ represents the amount of peptide required for 50% inhibition of binding of the 125-I radiolabeled reference peptide to each HLA molecule. High (IC₅₀ < 500 nmol/L) and intermediate binders (IC₅₀, 500–5000 nmol/L) are highlighted in bold font. A dash represents IC₅₀ > 50,000 nmol/L. The first line indicates the peptide sequence used for immunological analysis; sequence variants found in the patients are listed below.

^aIdentifies IC₅₀ of those peptide/HLA combinations for which we report multimers in this study.