Human hepatitis D virus-specific T cell epitopes

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Summary
HDV is a small, defective RNA virus that requires the HBsAg of HBV for its assembly, release, and transmission. Chronic HBV/HDV infection often has a severe clinical outcome and is difficult to treat. The important role of a robust virus-specific T cell response for natural viral control has been established for many other chronic viral infections, but the exact role of the T cell response in the control and progression of chronic HDV infection is far less clear. Several recent studies have characterised HDV-specific CD4+ and CD8+ T cell responses on a peptide level. This review comprehensively summarises all HDV-specific T cell epitopes described to date and describes our current knowledge of the role of T cells in HDV infection. While we now have better tools to study the adaptive anti-HDV-specific T cell response, further efforts are needed to define the HLA restriction of additional HDV-specific T cell epitopes, establish additional HDV-specific MHC tetramers, understand the degree of cross HDV genotype reactivity of individual epitopes and understand the correlation of the HBV- and HDV-specific T cell response, as well as the breadth and specificity of the intrahepatic HDV-specific T cell response.

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Introduction
Apart from some initial immunological HDV studies in the 1990s, it was only more recently that several immunological research groups further characterised the HDV-specific T cell response using state of the art methods. In this comprehensive review, we summarise these studies and list all HDV-specific T cell epitopes identified in humans so far, with a focus on their potential significance as well as unresolved knowledge gaps. Detailed knowledge of the HDV-specific T cell epitope repertoire is needed to guide therapeutic vaccine design and to improve immune monitoring in future clinical trials.

Epidemiology
The global prevalence of HDV is estimated at 12 million individuals,1 while others have calculated that it affects 32–61 million,2,3 or even 62–72 million individuals.4 HDV is highly endemic in Africa, the Amazon Basin, Eastern and Mediterranean Europe, the Middle East, and parts of Asia,4 mostly coinciding with high numbers of chronic HBV infections in these areas. HDV genotype 1 has global prevalence, while genotypes 2-8 show distinct regional patterns.5 Genotypes 2 and 4 predominantly cause milder disease, while the South American genotype 3 causes more severe hepatitis.6 Genotypes 5-8 are mostly diagnosed in patients of African origin and have also been linked to milder disease,7 although the latter is a matter of debate.8 Untreated chronic HBV/HDV infection causes severe liver disease in many cases, with 50% of patients developing cirrhosis within 5–10 years.9 Whether HBV/HDV infection is associated with an increased risk of HCC per se,9 or whether this only occurs secondary to cirrhosis,10 is controversial.

HDV virology
HDV is an exceptionally small virus12 and considered defective due to its dependency on the HBV-derived HBsAg to form infectious virions. HDV cell entry is dependent on the interactions between HBsAg/heparan sulfate proteoglycans and sodium taurocholate co-transporting polypeptide (NTCP).13 HDV might also use envelope proteins of other viruses for transmission,14,15 however, the clinical significance is unknown.16 HDV contains a circular single-stranded RNA genome of 1.7 kb, encoding a single 214-amino acid (aa) peptide,1 the hepatitis delta antigen (HDAg), which exists in 2 variants, the small HDAg (S-HDAg) and the large HDAg (L-HDAg), which has 19 additional C-terminal aa.17 Approximately 200 of these molecules are included per virion,18,19 in the form of nucleosome-like ribonucleoproteins consisting of the HDAgs and viral RNA, which have essential roles in viral replication.18,19

Keywords: Hepatitis Delta; HBV; HDV; T cell; CD8+; CD4+; epitope; viral escape

Received 4 January 2021; received in revised form 26 March 2021; accepted 7 April 2021; available online 23 April 2021

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HDV RNA is replicated and transcribed to host-like mRNA in a double rolling circle process by host DNA-dependent RNA polymerases, mainly RNA pol II, but possibly also RNA pol I and III. The small genome and the replication mode are unique among animal RNA viruses and more typical for plant viroids/virusoids. Other than these, HDV hijacks host enzymes and even forces a template shift from host DNA to viral RNA, probably using a histone H3 mimicry strategy.

L-HDAg is translated after ADAR1 (adenosine deaminases acting on RNA 1)-mediated editing of a stop codon at the amber/w site (adenosine 1012) at a portion of the antigenomic RNA, effectively elongating the open reading frame by 19–20 aa. Thus, S-HDAg is the first of the 2 peptides and facilitates replication, while L-HDAg is translated at a later stage and inhibits replication to promote virion assembly.

Post-translational modifications (PTMs) are essential for the function of HDAg. Namely serine-2, -123 and -177 are phosphorylated post-translationally, arginine-13 is methylated, and lysine-72 is acetylated. Cysteine-211, only found in the L-HDAg, is modified by isoprenylation/farnesylation. It has been reported that the acetylation of lysine-72 is required for the subcellular localisation of HDAg and RNA replication. Other important PTMs in this regard include the methylation of arginine-13 and the phosphorylation of serine-177 and -123. The farnesylation of cysteine-211 is required for virus assembly and to inhibit replication.

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Key points

- HDV causes severe hepatitis, often leading to hepatic complications and liver-related death; it is a major public health concern affecting 12 million patients worldwide, with few treatment options.
- The virology and immunology of HDV infection, which is intricately connected with the concomitant HBV infection, is still not completely understood; it is only recently that several studies characterising the T cell response in patients with HDV have been published.
- This review summarises our current knowledge on the virology and immunology of HDV infection, with a focus on the HDV-specific T cell response.
- A comprehensive database of all HDV-specific CD4+ and CD8+ T cell epitopes published to date is presented.
- Detailed functional and phenotypic studies on the peripheral and intrahepatic HDV-specific T cell response during future clinical trials are needed to understand the T cell correlates of HDV control.

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Fig. 1. Immunological course of HBV monoinfection vs. HBV/HDV coinfection. Note that chronic HBV inhibits various pathways of innate immunity and leads to different degrees of T cell exhaustion and deletion. Clearance of HBV is largely dependent on effective CD4+ and CD8+ T cell responses, as well as innate immune response, viral and host factors. Contrary, HDV activates pathways of innate immunity, thereby increasing type-I interferon (β and λ) responses and suppressing HBV replication, HBV epitope presentation and hepatotropic T cell recruitment is enhanced. Which immune responses are primarily required for HDV clearance is not well understood. ISG, interferon-stimulated genes; NKG2D, natural killer group 2 member D. Figure created with Biorender.com.
Other functional domains include a coiled-coil domain that is important for self-dimerisation, a domain that determines the nuclear localisation of HDAG and the unique carboxyterminal region of L-HDAG with the nucleolar export signal. RNA-binding arginine-rich motifs of HDAG have been described, still, oligomerisation seems more important for the activating and inhibitory effects of S- and L-HDAG.

### Heterogeneity and viral evolution
HDV shows great genetic variance, a broad range of viral quasispecies exist within the same infected individual. The intragenotypic genetic variability of HDV genotype 1 is estimated to be 11.3–14.3% and, recently, a subclassification of genotypes was proposed. Like DNA polymerases, DNA-dependent RNA polymerase II is reported to have kinetic proofreading abilities, however, the template switch to RNA might cause a higher error rate. Additionally, strand ADAR1-mediated RNA editing might also contribute to sequence heterogeneity.

The substitution rates range from 3.0·10⁻² to 3.0·10⁻³ for the whole genome and 9.5·10⁻³ to 1.2·10⁻² substitutions per site per year for the HDag open reading frame (determined by next-generation sequencing), decaying over time towards a steady state. High evolution rates correlate with clinical flares, and evolution rates are only higher than other RNA viruses at the beginning of the infection, during adaptation to the host. Non-synonymous mutations happen relatively more often, likely as a result of selection of variants capable of immune escape.

PTM sites and ribozymes seem rather conserved, while 10.6% of codons are under diversifying positive selection. A reduced in vitro sensitivity of HDV to interferon (IFN)-α during treatment has been reported, likely due to the selection of genetic variants that replicate despite IFN. Possibly, HDV is activating the IFN pathways itself to suppress HBV replication and increase RNA editing by the IFN-induced enzymes ADAR1 and apolipoprotein B mRNA editing enzyme (APOBEC). In conclusion, HDV shows higher initial mutation rates than other hepatotropic viruses, while a few conserved genomic regions are described. Mutations and quasispecies may contribute to immune escape and treatment failure.

### HDV therapy
Until recently, the only recommended treatment option for chronically HBV/HDV-infected patients was a long-term (48 weeks) therapy with pegylated-IFN-α (peg-IFN-α). Only few patients respond to treatment and late relapses occur in almost half of responding patients after achieving a ‘sustained’ virological response. Only 11% of patients maintain a virological response after IFN-based regimens and late relapses after therapy discontinuation are not uncommon. Elongation of IFN therapy to 96 weeks is possible with acceptable safety in up to 80% of patients, leading to longer on-treatment suppression of HDV replication and amelioration of fibrosis. However, relapse rates are still high (around one-third of responders) and HBsAg clearance is not improved – even by addition of tenofovir disoproxil fumarate – which is possibly linked to almost undetectably low viraemia. These findings underscore that, while a sustained virological response by negative HCV PCR 12 weeks after therapy indicates cure from HCV, this concept cannot be extrapolated to HDV, where loss of HBsAg and seroconversion remain the best markers for cure of chronic HDV infection. Roche has officially stopped the production of peg-IFN-α which will only be available until the end of 2022. The novel entry inhibitor bulevirtide targets host NTCP and has led to promising results in 2 phase II trials, leading to its conditional marketing authorisation in Europe. Other novel anti-HDV agents are currently being investigated (reviewed in detail). Pegylated-IFN-α showed advantageous tolerability and comparable antiviral activity during 8 weeks of treatment compared to 48 weeks of peg-IFN-α in a randomised, open-label, multicentre study. Tonafarib – an orphan drug for the rare genetic disorder progeria – inhibits the host enzyme farnesyltransferase and is being investigated for HDV therapy in multiple combinations. The HBsAg release inhibitor REP-2139 is another promising candidate for HDV treatment. So far, although paradigm-shifting, none of these novel therapeutic approaches is recommended in international treatment guidelines.

### Immunology of HDV infection
Humans are the only natural host of HDV and only chimpanzees and some non-primates such as Tupaia bengaleri can be infected with (human) HBV and coinfect with HDV. Other mammals used for prospective studies on HDV are woodchucks, wooly monkeys, and bats. HDV-transgenic mice were used to demonstrate that HDV hepatotropism is only due to the entry restriction by HBsAg. HDAG/HBsAg-transgenic mice did not develop liver disease. In vitro, in transient transfection, there was no interference with the cell cycle or apoptosis, whereas in dividing cells a slight growth disadvantage could be observed. Accordingly, HDV may – to some degree – be cytopathic itself and drive histopathologic liver damage together with the immune response. In humanised uPA/SCID/beige mice, HDV mono-infection can persist intrahepatically for at least 6 weeks without HBsAg, while maintaining infectivity and the ability to convert to a productive co-infection after rescue by HBV infection.

HDV and HBV interact in multiple ways. Although HDV is able to replicate without active HBV replication, HBsAg is needed for HDV to form infectious particles. Usually, HDV predominates over HBV and coinfected patients often only show low HBV viral loads, although this pattern might be reversed in some individuals or transiently during early treatment phases.

In vitro, upon superinfection there is a specific interference between the 2 viruses: HBV DNA, pregenomic RNA and HBsAg decrease while cccDNA and HBsAg stay constant. HDV infection is associated with a type-I IFN response and upregulation of IFN-induced genes. Upregulation of IFN-induced genes not only increases the HDV mutation rate, but also suppresses HBV replication, owing to decreased transcription of covalently closed circular DNA (cccDNA, without a decline in cccDNA abundance) as a result of IFN-α-induced epigenetic changes to the cccDNA. Notably, IFN partially suppresses HDV replication under certain circumstances (reviewed in detail). HDV also enhances HBV epitope presentation, which could be one of the causes of the more severe liver pathology in HBV/HDV coinfected (Fig. 1).

Few studies examined humoral immune responses to HDV. Anti-HDV antibodies are commonly generated, but probably unable to neutralise HDV. Anti-HDV IgM was traditionally used as clinical marker of disease activity before the establishment of standard pan-genotypic PCR assays.
Chromically HDV-superinfected patients have higher serum type 1 to type 2 cytokine ratios, while HBV-monoinfected patients show elevated levels of both type 1 (tumour necrosis factor-α [TNF-α], interleukin [IL]12, C-X-C motif chemokine ligand 9, IFN-γ) and type 2 (IL4, IL13, C-C motif chemokine ligand 26) cytokines. This predominance of type 1 responses, which mainly elicit cellular immune cascades, might explain the more aggressive course of disease in the case of HDV superinfection.

Further, it has been shown that HDV strongly activates an IFN-β/k response mainly through the pattern recognition receptor MDA5 (melanoma differentiation-associated protein 5) and that HDV can replicate in vivo despite this “interferon-activated state”. Further, it has been shown that HDV strongly activates an IFN-β/λ response mainly through the pattern recognition receptor MDA5 (melanoma differentiation-associated protein 5) and that HDV can replicate in vivo despite this “interferon-activated state”.44 Functionally impaired CD56bright natural killer (NK) cells accumulate in viral hepatitis regardless of the virus itself (Fig. 2). The highest total frequencies of NK cells and CD56dim NK cells and the highest amounts of IFN-γ and TNF-α were found for HDV. However, phenotype and functional alterations were attributed primarily to the severity of infection rather than the virus itself. A higher frequency of CD56dim NK cells in HDV-infected patients is associated with better outcome after IFN-α treatment. IFN-α treatment seems to deplete terminally differentiated NK cells and cause functional impairment of NK cells.

Intrahepatic and peripheral frequencies of mucosa-associated invariant T (MAIT) cells are reduced in patients with chronic HDV compared to healthy individuals and patients with HBV monoinfection of similar age. MAIT cells are also functionally impaired and exhibit an activated and exhausted compound phenotype of CD38hiPD-1hiCD28loCD127loPLZFloEomesloHelioslo. Similar to chronic HBV and HCV infection, it is assumed that specific T cell responses are needed for clearance of HDV and, at the same time, are drivers of histopathologic liver damage. There is great variability in the observed frequencies of HDV-specific T cell responses reported in the literature. Grabowski et al. describe HDV-specific cytokine responses after stimulation of peripheral blood mononuclear cells (PBMCs) with a peptide pool spanning the whole HDAg in 94% (16/17) of patients before the start of IFN treatment. Nisini et al. detected HDV-specific CD4+ T cell proliferation in 27% of patients after whole-antigen stimulation (8/30), whereas Landahl et al. detected CD4+ or IFN-α

Fig. 2. Key immunological findings in chronic HDV. CXCR3, CXC-motif chemokine receptor 3; MAIT, mucosal associated invariant T cell; TCR, T cell receptor; TCF-1, T cell factor 1; PD-1, programmed cell death protein 1; Tet, HLA-I tetramer (loaded with HDAg-derived peptide). Based on 82–84,86–89,91,92. Figure created with Biorender.com.
CD8+ T cell responses in 53% of patients (17/32) by intracellular cytokine staining in vitro expansion (ICS). Kefalakes et al. report an HDV-specific T cell response rate of 71% by IFN-γ ICS in a sub-cohort of 17 lonafarnib/ritonavir-treated patients after treatment discontinuation. Ex vivo frequencies of epitope-specific CD8+ T cells were either undetectable, detectable at very low frequencies after enrichment or reported at 0.013% of CD3+CD4- T cells, depending on the epitopes and multimers used.

Even less is known about the phenotype and the clinical correlate of the detection of a broad HDV-specific T cell response. Specific T cell responses have been linked to “inactive disease” – defined as normal alanine aminotransferase (ALT) levels for 1 year and negative anti-HDV IgM. A higher frequency of HDV-specific cytokine responses and a restoration of transiently diminished specific cytokine responses after peg-IFN-α treatment coincided with therapeutic response in the HIDIT-1 trial. However, this correlation between HDV-specific IFN-γ levels, ALT levels and HDV RNA was not significant. Correlations between elevated serum IL2 and IL12 levels and response to IFN treatment suggest that a T helper-1 (Th1)-polarised cellular immune response might be associated with viral clearance.

In contrast, Landahl et al. observed broad low-level HDV-specific T cell responses that did not correlate with HDV viral load, level of transaminases and presence or absence of HDV viraemia. There was also no difference between responses in spontaneous resolvers, treatment-induced PCR-negative patients and chronically viremic patients, but there was a negative correlation between HBV viral load and number of responses. Interestingly, a correlation between activated HDV-specific T cells and aspartate aminotransferase (AST) levels was reported, suggesting that CD8+ T cells may contribute to liver damage in HDV infection. Furthermore, higher frequencies of IFN-γ-producing CD8+ T cells were associated with lower viremia 4 weeks post treatment, emphasising their role in viral clearance. All in all, the exact interplay between specific T cell responses and treatment outcome or disease course remains unclear.

Cytokine secretion analyses suggest that HDV-specific CD4+ T cell responses that did not correlate with HDV viral load, level of transaminases and presence or absence of HDV viraemia. There was also no difference between responses in spontaneous resolvers, treatment-induced PCR-negative patients and chronically viremic patients, but there was a negative correlation between HBV viral load and number of responses. Interestingly, a correlation between activated HDV-specific T cells and aspartate aminotransferase (AST) levels was reported, suggesting that CD8+ T cells may contribute to liver damage in HDV infection. Furthermore, higher frequencies of IFN-γ-producing CD8+ T cells were associated with lower viremia 4 weeks post treatment, emphasising their role in viral clearance. All in all, the exact interplay between specific T cell responses and treatment outcome or disease course remains unclear.

In HCV infection, the detection of strong and long-lasting epitope-specific CD4+ T cell responses that did not correlate with HDV viral load, level of transaminases and presence or absence of HDV viraemia. There was also no difference between responses in spontaneous resolvers, treatment-induced PCR-negative patients and chronically viremic patients, but there was a negative correlation between HBV viral load and number of responses. Interestingly, a correlation between activated HDV-specific T cells and aspartate aminotransferase (AST) levels was reported, suggesting that CD8+ T cells may contribute to liver damage in HDV infection. Furthermore, higher frequencies of IFN-γ-producing CD8+ T cells were associated with lower viremia 4 weeks post treatment, emphasising their role in viral clearance. All in all, the exact interplay between specific T cell responses and treatment outcome or disease course remains unclear.

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HDV-specific CD8+ T cell epitopes

It is generally thought that HBV-specific CD8+ cells are the main effector cells responsible for viral clearance during acute HBV infection. The role of CD8+ T cells in the different disease courses of patients with chronic HBV is less clear. CD8+ T cells cause liver injury and promote disease pathogenesis. A key event in the persistence of HBV is the exhaustion of virus-specific CD8+ T cells – indeed, diminished frequencies of functionally impaired HBV-specific CD8+ T cells expressing inhibitory receptors have been described. Immune checkpoint inhibition could restore antiviral CD8+ T cells responses.

The role of the HDV-specific CD8+ T cell response in HDV resolution and pathogenesis remains unclear and most of the data are derived from animal models. The definition of the specificities of the anti-HDAg-specific T cells is an essential step towards understanding the heterogeneous disease courses of HDV infection, non-responders, and paving the way for an immunotherapeutic approach.

So far, T cell responses directed against 18 HDV-specific CD8+ T cell epitopes have been identified in 5 different studies, with partial overlap between the studied peptides. Huang et al. described the 2 CD8+ T cell epitopes that were restricted by HLA-A*02:01 as predicted in silico. Out of 4 HLA-A*02:02-positive patients, responses were detected in the 2 PCR-negative patients with normal ALT levels by ELISpot and HLA-A*02:01 tetramer staining. However, these epitopes were not detectable in 4 European HLA-A*02-positive patients by ICS, the overall response rate therefore being 2/8 for each epitope in a total of 2 studies. Possibly, different HLA-A*02 subtypes in Asia and Europe may play a role in these discordant results.

Karimzadeh et al. analysed HLA-B*27-restricted HDV-specific CD8+ T cell responses based on HLA-B*27-associated sequence polymorphisms (indicating viral escape mutations within a putative HDV-specific CD8+ T cell epitope, see below). By IFN-γ ICS, 2 epitopes were confirmed: aa99-108 and aa103/104-112. Three patients with resolved HDV infection responded to these 2 epitopes (1 patient for each epitope plus 1 patient who was only tested for the overlapping peptide containing both epitopes).

In 2019, Karimzadeh et al. expanded their viral sequence-based approach to all HLA class 1 alleles and described 5 additional HDV-specific CD8+ T cell epitopes by IFN-γ ICS of in vitro expanded CD8+ T cells from HLA-matched patients. Out of these HLA-B restricted novel epitopes, aa170-179 was additionally confirmed by direct ex vivo analysis after HLA-B*15:01 tetramer enrichment.

Kefalakes et al. 2019 used overlapping peptides spanning the whole L-HDAg to detect HDV-specific CD8+ T cells. They found responses against a total of 6 HDV-specific CD8+ T cell epitopes, including the aforementioned HLA-B*27 epitope aa104-112 (with an arginine at the C-terminus), the HLA-B*18 epitope aa46-54 (additionally restricted by HLA-B*44:02 and B*44:03), and 4 additional novel epitopes. Most of these
epitopes were further confirmed by direct ex vivo multimer staining. Response rates for these epitopes varied, however, responses clustered against epitopes located at the C-terminus of HDAg, which is unique to L-DHAg, with up to 8/17 patients responding to individual overlapping peptides, irrespective of the individual HLA types.

In addition to the identification and characterisation of HDV-specific CD4+ T cell epitopes (see above), Landahl et al. also found responses against 5 HDV-specific CD8+ T cell epitopes. However, fine-mapping and HLA class I restriction experiments were not performed in this study which focused on HDV-specific CD4+ T cell responses. In silico prediction, as well as HLA typing of responding patients, indicated that these responses were most likely restricted by B*2705 and B*5701, indicating that the optimal epitope(s) may be identical to the 3 epitopes aa191–196, aa192–200, and aa194–202 identified by Kefalakes et al. in this viral region.

Aa101–120 contains the B*27 epitope aa104–112. Interestingly, the responding patient in this study was HLA-B*27 negative. Other epitopes also overlap with those identified by the more CD8+ focused studies by Karimzadeh et al. and Kefalakes et al. (see Table 1 and Fig. 5).

In contrast to CD4+ epitopes that are distributed through the entire HDAg (with hotspots at the N-terminus) and can be presented by multiple HLA types, the known HDV-specific CD8+ T cell epitopes seem to cluster in a few distinct locations (mainly C-terminal) and are restricted mainly by HLA-B subtypes, including relatively infrequent subtypes.

A schematic overview of the CD8+ T cell epitopes and their localisation within the HDAg is provided in Fig. 5.

Viral escape from HDV-specific CD8+ T cell responses
Mutational viral escape is a major mechanism of virus-specific T cell failure in persistent viral infections. Viral escape was first described in the lymphocytic choriomeningitis virus mouse model, and has been best characterised in HIV and HCV infection in humans. While there is good evidence that viral escape from virus-specific CD8+ T cell responses impacts the outcome of infection, e.g. viral clearance vs. persistence, there is less evidence for a role of viral escape from virus-specific CD4+ T cell responses. Mutational escape can either occur at HLA binding anchors of epitopes (mostly aa residue 2 and the C-terminal aa residue), at the T cell receptor contact residues of the epitope (mostly the aa residues in the middle of the epitope), or even in the flanking regions of the epitope, interfering with proteasomal processing of the epitope. Since HDV has a relatively high mutation rate (see above), it is reasonable to argue that viral escape from virus-specific CD8+ T cell responses may also take
| Position  | Sequence    | Ref.  | Response | Best assay rate | Assay details | HLA molecule | HLA assay | In silico prediction tool | Comments |
|-----------|-------------|-------|----------|-----------------|---------------|--------------|------------|--------------------------|----------|
|           |             |       |          |                 |               |              |            |                          |          |
| **CD8**   |             |       |          |                 |               |              |            |                          |          |
| 46-54     | DENPWLGNI   | 89,92 | 4/24     | Ex vivo multimer| In vitro ICS (IFNγ) release; ex vivo tetramer ICS | B*18:01; B*44:02, B*44:03 (HLA binding) | In silico, HLA-matched patients in ICS | ANN, netMHCPan (IEDB; SYFPEITHI; BIMAS) |          |
| 81-90     | VDSGPRKRPL  | 92    | 1/1 ICS  | In vitro ICS (IFNγ) | B*37:01 | In silico, HLA-matched patients in ICS | ANN, netMHCPan (IEDB; SYFPEITHI; BIMAS) |          |
| 100-108   | QDHRRRKAL   | 92    | 1/1 ICS  | In vitro ICS (IFNγ) | B*37:01 | In silico, HLA-matched patients in ICS | ANN, netMHCPan (IEDB; SYFPEITHI; BIMAS) |          |
| 140-149   | RERRVAGPPV  | 92    | 1/2 ICS  | In vitro ICS (IFNγ) | B*41:01 | In silico, HLA-matched patients in ICS | ANN, netMHCPan (IEDB; SYFPEITHI; BIMAS) |          |
| 170-179   | SMQGPESPF   | 92    | 10/14 Ex vivo multimer | In vitro ICS (IFNγ), ex vivo tetramer ICS (7/7) | B*15:01 | Ex vivo tetramer ICS, In silico predictions | ANN, netMHCPan (IEDB; SYFPEITHI; BIMAS) |          |
| 192-200   | QGFPWDILF   | 89    | 5/17 Ex vivo multimer | In vitro ICS, ex vivo dextramer ICS | B*35:01; B*52:01 | HLA binding assays with radiolabelled HLA class I, dextramer | n.a. |          |
| 194-202   | FPWDILFPA   | 89    | 5/17 Ex vivo multimer | In vitro ICS, ex vivo dextramer ICS | B*35:01; B*07:02 | HLA binding assays with radiolabelled HLA class I, dextramer | n.a. |          |
| 104-112   | RRKALENK/R  | 89,92 | 2/17 Ex vivo multimer | In vitro ICS, ex vivo pentamer ICS; ICS IFNγ release after restimulation on day 12 | B*27:05 | HLA binding assays with radiolabelled HLA class I, pentamer | ANN, netMHCPan (IEDB; SYFPEITHI; BIMAS) | 103-112 RRKALENK/R is also presented by HLA B*27 and recognised by 1/7; escape mutation K106M |
| 189-196   | RGSQGFPW    | 89    | 6/17 Ex vivo multimer | In vitro ICS, ex vivo tetramer ICS | B*58:01 | HLA binding assays with radiolabelled HLA class I, tetramer | n.a. |          |
| 99-108    | RRDHRRRKAL  | 91    | 1/7 ICS  | In vitro ICS (IFNγ) | B*27:05/02 | UV-mediated peptide exchange assay | IEDB and SYFPEITHI | Escape mutations R105K and K106M; RQDHRRRKAL, REDHRRRKAL, RKDDHRRRKAL are also presented by B*27:05 and recognised by one patient each |
| 98-113    | ERRDHRRRKALE| 91    | 3/8 ICS  | In vitro ICS (IFNγ) | B*27:05 | In silico, HLA typing in responding patients | IEDB and SYFPEITHI |          |

(continued on next page)
| Position Sequence | Sequence | Ref. | Response rate | Assay details | HLA molecule | HLA assay | In silico prediction tool | Comments |
|-------------------|----------|------|---------------|---------------|--------------|-----------|--------------------------|----------|
| 26-34             | KLEDLERDL| 90,91| 2/8 In vitro multimer | Cytotoxicity (mice) and tetramer staining; ELISPOT IFNγ release, tetramer qualitative binding (both after restimulation); In vitro ICS<sup>(1)</sup> | A*02:01 | MHC ligand assay, UV-mediated peptide exchange assay | SYFPEITHI |
| 43-51             | KLEDENPWL| 90,91| 2/8 In vitro multimer | Cytotoxicity (mice) and tetramer staining; ELISPOT IFNγ release, tetramer qualitative binding (both after restimulation); In vitro ICS<sup>(1)</sup> | A*02:01 | MHC ligand assay, UV-mediated peptide exchange assay | SYFPEITHI |

Karimzadeh et al., found no responses in European cohort by ICS (0/4+2/4); only tested in HLA-A*02:01 patients

| 191-210           | GQGFPWDLFPS | 88   | 7/32 ICS | In vitro ICS (IFNγ); ELISPOT IFNγ | B*35:01; B*51:01; B*53:01 | In silico, HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |
| 101-120           | DHRRRKALENKR | 88   | 1/32 ICS | In vitro ICS (IFNγ); ELISPOT IFNγ | A*03:01 | In silico, HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |
| 131-150           | KRLTEEDERRER | 88   | 1/32 ICS | In vitro ICS (IFNγ); ELISPOT IFNγ | A*02:02P/03:01P; B*15:01P/41:01; C*03:04/17:01P | HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |
| 181-200           | RHGECGLVRGG | 88   | 3/32 ICS | In vitro ICS (IFNγ); ELISPOT IFNγ | B*15:01; C*04:01 | In silico, HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |
| 195-214           | PWDLFPSDPFF | 88   | 3/32 ICS | In vitro ICS (IFNγ); ELISPOT IFNγ | A*02:17/02:01; B*35:01 | In silico, HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |

Table 1 (continued)

| Position Sequence | Sequence | Ref. | Response rate | Assay details | HLA molecule | HLA assay | In silico prediction tool | Comments |
|-------------------|----------|------|---------------|---------------|--------------|-----------|--------------------------|----------|
| 26-41             | Data not provided | 87   | 1/3 ³H thymidine proliferation | Epitope-specific ³H thymidine proliferation after cultivation+stim with HDAg and coculture of B-LCL as APCs | DPB1*17:01 | Blocking experiments with MAb; co-culture with B- LCL of known haplotypes | no in silico predictions used |
| 50-65             | Data not provided | 87   | 3/3 ³H thymidine proliferation | Epitope-specific ³H thymidine proliferation after cultivation+stim with HDAg and coculture of B-LCL as APCs | DRB1*11:01; DRB1*10:01 | Blocking experiments with MAb; co-culture with B- LCL of known haplotypes | no in silico predictions used |
| 66-81             | Data not provided | 87   | 1/3 ³H thymidine proliferation | Epitope-specific ³H thymidine proliferation after cultivation+stim with HDAg and coculture of B-LCL as APCs | DQB1*02:01 | Blocking experiments with MAb; co-culture with B- LCL of known haplotypes | no in silico predictions used |

(continued on next page)
| Position Sequence | Ref. | Response rate | Best assay details | HLA molecule | HLA assay | In silico prediction tool | Comments |
|-------------------|------|---------------|--------------------|--------------|-----------|--------------------------|----------|
| 106-121 Data not provided | 87 | 1/3 | ³H thymidine proliferation | DRB1*11:01; DRB1*12:01; DRB1*01:01; DRB1*07:01; DRB1*14:01; DRB5*02:02 | Blocking experiments with MAb; coculture with B-LCL of known haplotypes | no in silico predictions used |
| 11-30 GREEILEQWVN | 88 | 4/32 | In vitro (IFNγ) | DRB1*08:02; DRB1*14:01; DRB1*15:01 | In silico predictions + MHC ligand assay | IEDB Consensus tool (ANN+SMM) |
| 41-60 IKKLEDENPWLG | 88 | 8/32 | Ex vivo ELISpot | DRB1*10:01; DRB1*11:01; DRB1*08:02; DRB1*13:02 | In silico predictions + MHC ligand assay | IEDB Consensus tool (ANN+SMM) | Confirmed by ex vivo ELISpot in acutely superinfected patient |
| 1-20 MSRSESKKNRG | 88 | 1/32 | In vitro ICS (IFNγ) | DRB1*14:04/15:01; DQA1*01:04/01:02; DQB1*05:03/06:02P | HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |
| 21-40 VNGRKKLEEEL | 88 | 1/32 | Ex vivo ELISpot | DRB1*10:01 | In silico, HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) | Confirmed by ex vivo ELISpot in acutely superinfected patient |
| 31-50 ERDLRKIKKKIKK | 88 | 1/32 | In vitro ICS (IFNγ) | DRB1*10:01 | In silico, HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |
| 51-70 LGNIKGLGKDK | 88 | 1/32 | In vitro ICS (IFNγ) | DRB1*15:02 | In silico, HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |
| 61-80 KDXDGECAAPP | 88 | 1/32 | In vitro ICS (IFNγ) | DRB1*11:01P; DQA1*05; DQB1*03:01P | HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |
| 71-90 AKRARTDQMEID | 88 | 2/32 | In vitro ICS (IFNγ) | DRB1*15:01/03:01 | In silico, HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |
| 81-100 IDSGPRKRPLRG | 88 | 1/32 | In vitro ICS (IFNγ) | DRB1*04:05 | In silico, HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |
| 111-130 KRQQLAGGGKSL | 88 | 1/32 | In vitro ICS (IFNγ) | DRB1*15:01 | In silico, HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |
| 141-160 ERRVAGPQVGG | 88 | 1/32 | In vitro ICS (IFNγ) | DRB1*15:01 | In silico, HLA typing in responding patients | IEDB Consensus tool (ANN+SMM) |

(continued on next page)
place in persistent HDV infection. Indeed, a first study on this issue longitudinally sequenced HDV quasispecies in 4 HLA-A*02+ patients with chronic HBV/HDV infection before and after hepatitis flares. They found evidence for selection pressure in the HLA-A*02-restricted epitope aa43-51 KLEDDNPWL in 3/5 patients and in another predicted HLA-A*02-restricted epitope aa114-122 QLSAGGKSL in 4/5 patients. However, functional T cell assays to confirm that the patients indeed targeted these epitopes were not performed and experimental evidence for the impact of the observed sequence mutations on recognition by epitope-specific CD8+ T cells or at least HLA-A*02 binding was not supplied. Similarly, a more recent study performed a cross-sectional sequence analysis in 34 patients with chronic HBV/HDV infection and identified codons 136-159 to be under positive selection pressure, likely indicating CD8+ or B cell pressure. Unfortunately, HLA typing is not available. The HDAg region under positive selection pressure (aa136-159) overlaps with the HLA-B*41-restricted epitope aa140-149 RERRVAGPPV, however, this CD8+ T cell epitope (restricted by a rather infrequent HLA class I allele) is unlikely to exert selection pressure at a population level. Due to the dominant role of the HLA class I type B*27 in driving viral escape in HIV and HCV infection, the HLA-B*27 background was also used in a pioneer study to functionally demonstrate viral escape from HDV-specific CD8+ T cell responses. Indeed, 2 predicted HLA-B*27-restricted epitopes (aa99-108 RRDHRRRKAL and aa103/4-112 RRKALENKK) were identified. Viral sequence analysis in 8 HLA-B*27+ vs. 96 HLA-B*27- patients demonstrated an enrichment of aa mutations in the epitope region in HLA-B*27+ patients. These HLA-B*27-associated viral sequence polymorphisms (also referred to as HLA-B*27 footprints) indicated that viral escape occurs within these 2 HLA-B*27-restricted HDV-specific CD8+ T cell epitopes. This was functionally confirmed by intracellular IFN-γ staining using wild-type vs. variant peptide, showing little cross-recognition of the variant peptide by wild-type-specific T cell lines. In a following multicentre, multinational study analysing HLA footprints for all HLA class I alleles present in a cohort of 104 patients with chronic HBV/HDV infection, a total of 21 HLA class I footprints were identified. Interestingly, these footprints were restricted by relatively infrequent HLA class I alleles, which might indicate that HDV has already adapted to its host’s HLA class I background at a population level, leading to the extinction of HDV-specific CD8+ T cell epitopes restricted by frequent HLA class I alleles. The most striking example of viral escape affected the HLA-B*15:01-restricted HDV-specific CD8+ T cell epitope aa170-179 SMQGVPESPF: All 8 HLA-B*15:01 patients in the cohort displayed a viral sequence mutation at the N-terminal amino acid residue (S170N) that was detected in a minority of HLA-B*15:01-negative patients only. This mutation impaired cross-recognition by the epitope-specific CD8+ T cell response. The loss of viral control in a patient with acute HDV superinfection coincided with the evolution of this escape mutation, indicating the biological significance of viral escape in HDV persistence. Virus-specific CD8+ T cells targeting this ‘escaped’ epitope displayed a memory-like phenotype (PD-1+CD127+TCF1+) and were thus not terminally exhausted. These data indicate that in parallel to the findings obtained in HCV infection, viral escape and terminal exhaustion are alternative and non-overlapping mechanisms of virus-specific T cell failure. Unfortunately, it was not possible to analyse the phenotype of HDV-specific CD8+ T cells targeting conserved
Non-escaped epitopes in this study. However, Kefalakes et al.\textsuperscript{89} described viral sequence mutations in all 6 HDV-specific CD8+ T cell epitopes identified in 1–4 patients each and could confirm viral escape by HLA binding studies for 6 variant peptides and by functional T cell analysis for 4 variant peptides, respectively. Importantly, HDV-specific CD8+ T cells targeting escaped epitopes displayed a memory-like phenotype (PD-1+CD127+TCF+), without evidence of activation (CD38–), while HDV-specific CD8+ T cells targeting conserved epitopes had a ‘chronically activated’ phenotype (PD-1+CD127 lowTCF1 low, CD38+). These results further underline the complementary, non-overlapping roles of viral escape and terminal exhaustion in HDV persistence and indicate that viral escape needs to be considered in vaccine design.

Discussion

Forty years after the discovery of HDV, its clinical peculiarities remain enigmatic. Clearly, the fate of the HDV infection is intricately intertwined with the replicative cycle and the clinical course of the HBV infection, since HBSAg loss and HBV seroconversion will ultimately terminate HDV propagation. While the current review focuses on the HDV-specific T cell response, the functional interactions between the 2 viruses and their respective effect on HBV- and HDV-specific adaptive and innate immunity must be studied in much more detail. For example, it is not clear why peg-IFN-\alpha leads to relatively infrequent loss of the HBSAg in patients with HDV compared to those with HBV monoinfection. The immune correlates of spontaneous or therapy-induced control of HDV viraemia – apart from the known fact that HBSAg conversion itself can lead to clearance of HDV – and the role HDV-specific T cell responses play are poorly understood.

It is not clear whether immunological interventions in patients with HDV should a) aim at global blockade of co-inhibitory molecules on T cells, or b) be directly aimed at enhancing the HBV-specific immunity to achieve an HBV seroconversion; it is also not clear whether additionally targeting HDV antigens would synergistically help to achieve this aim. We also do not know whether halting HDV replication on its own – by therapeutic HDV vaccination or antiviral therapy\textsuperscript{103} – would be of significant benefit for chronically HBV/HDV-coinfected patients. Lastly, it is not currently clear whether a prophylactic HDV vaccine would be an epidemiologically useful tool to eradicate HDV infection.\textsuperscript{66,103}

Nonetheless, it is essential to conduct further detailed longitudinal studies on the ex vivo phenotype and functionality of the...
HBV- and HDV-specific T cell response during therapeutic trials to understand the immunological correlates of HDV viral control and to achieve sustained virological responses in the majority of patients with HDV by application of antiviral and immunological combination therapies.

Only some of the recent studies included detailed T cell analysis and only 3 studies utilised ex vivo assays like MHC class I multimer stainings. Indeed, due to the generally low ex vivo frequencies of circulating HDV-specific T cells, many researchers use peptide pool stimulation, measuring HDV-specific responses after in vitro expansion and re-stimulation. Epitope mapping by stimulating T cells with different pools of peptides spanning the whole antigen rather than testing each peptide individually greatly increases efficiency, while assay sensitivity may be slightly reduced, especially when using larger pool sizes. In vitro expansion and subsequent re-stimulation help to identify responses at low frequencies but carry the risk of altered T cell phenotypes and functionalities, limiting the comparability to the in vivo situation. Only 3 studies could detect T cell responses directed against 8 different peptide epitopes by direct ex vivo staining. Landahl et al. detected up to 800 HDV-specific T cells/10⁶ PBMCs by ex vivo ELISPOT in patients with acute HDV and Kefalakes et al. performed ex vivo multimer stainings in 17 chronically HDV-infected patients after discontinuation of lona-farnib/ritonavir therapy. Karimzadeh et al. managed to detect responses against 2 epitopes ex vivo by bead-based CD8+T cell enrichment, thereby increasing the assay sensitivity in HLA-matched patients. It is conceivable that a broad and strong specific response is induced in acutely infected patients, which diminishes as the infection persists. Analogously, suppression of viral replication by therapy could lead to partial recovery of exhausted specific memory cells, enabling them to initiate stronger, multi-specific responses upon re-stimulation ex vivo, similar to chronic HBV and HCV.

HLA restriction is an additional insufficiently characterised aspect of HDV epitopes. None of the studies describing CD4+ T cell epitopes confirmed the HLA restriction experimentally, e.g. using multimer stainings, but rather relied on in silico predictions followed by antibody blocking and HLA-haplotype specific co-culturing of CD4+ T cells, or in vitro binding assays or HLA fine typing of responding patients. Regarding CD8+ T cell epitopes, 3 studies included multimer assays to confirm HLA restrictions, with 6 epitopes confirmed by direct ex vivo multimer staining.

Another aspect which limits the current evidence base is the fact that only 4 studies mapped the whole HDAg for T cell epitopes. Consequently, only a limited number of patients (8 in Nisini et al. 1997, 32 in Landahl et al. 2019, 4 in Karimzadeh et al. 2018, and 17 in Kefalakes et al. 2019) were included in complete mappings. Two studies – Kefalakes et al. 2019 and Karimzadeh et al. 2018 – focused on CD8+ T cell responses (including 21 patients in total). Landahl et al. 2019 aimed to measure both CD8+ and CD4+ T cell responses, although the peptide length of 20 aa is suboptimal for MHC class I presentation, which favour peptides of 8 to 10 aa, and Nisini et al. only measured CD4+ responses. Karimzadeh et al. mapped HDAg for CD8+ T cell responses by IFN-γ ICS, a high-quality method for epitope mapping, although limited by the small number of patients. In this sense, a particular strength of the study conducted by Kefalakes et al. 2019 is the relatively large number of individual patients mapped by a high-quality method (namely ICS). Additionally, the authors were able to confirm epitopes and HLA binding by ex vivo multimer stainings of untreated HBV/HDV patients, and even to further characterise HDV-specific CD8+ T cells as discussed earlier in this review. Other studies performed in silico predictions of most probable epitopes and their HLA restrictions, which were subsequently experimentally confirmed. This approach carries the inherent risk of missing responses to epitopes with low binding affinities or restriction by uncommon HLA types. Usage of alternative binding pockets in MHC class I molecules and generally shallower (and thus more variable) binding pockets in MHC class II molecules further complicates this approach. Additionally, CD8+ T cell epitopes may span longer aa sequences than classically assumed, and thus be overlooked in silico predictions presuming lengths of 8 to 10 aa. Of note, most HDV peptide sets used to experimentally screen for HDV-specific T cell responses are based on single genotype 1-based sequences and there is neither a consensus sequence available nor is there an understanding about the degree of cross-genotype reactivity of these epitopes. Ideally, these peptides should be based on, or compared with, autologous circulating HDV sequences to rule out T cell responses against suboptimal heterologous sequence variants.

Furthermore, studies analysing the breadth, specificity, and functionality of the intrahepatic HDV-specific CD8+ T cell response have not yet been performed. In summary, we have provided a detailed review of the current knowledge on HDV-specific T cells and a database of all human T cell epitopes of the hepatitis delta virus characterised to date. This evidence base will help to further elucidate the complicated immunology of this enigmatic viral infection that still has grave clinical implications for too many patients.

Abbreviations
aa, amino acid(s); ADAR1, adenosine deaminases acting on RNA; ALT, alanine aminotransferase; AST, aspartate aminotransferase; cccDNA, covalently closed circular DNA; ELISPOT, enzyme-linked immune spot assay; HDAg, hepatitis delta antigen; ICS, intracellular cytokine staining; IFN-α, interferon-α; L-HDAg, large hepatitis delta antigen; MAIT, mucosa-associated invariant T cells; NK cells, natural killer cells; NTCP, sodium taurocholate co-transporting polypeptide; PBMCs, peripheral blood mononuclear cells; PD-1, programmed cell death protein 1; Peg-IFN-α, pegylated interferon alpha; PTT, post-translational modification; S-HDAg, small hepatitis delta antigen; TCF, T cell-specific transcription factor; Th1, T helper 1; TNFα, tumour necrosis factor-α.

Financial support
DFG (German Research Foundation) grants to Julian Schulze zur Wiesch (SBF841 and SFB1328) and Christoph Neumann-Haefelin (TRR-179 “Determinants and dynamics of elimination versus persistence of hepatitis virus infection”, Project 02). German Center for Infection Research (DZIF) grants to Julian Schulze zur Wiesch and Christoph Neumann-Haefelin.

Conflict of Interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.
Authors’ contributions
Conception MK, JL, and JSzW. Draft MK, JSzW, and CNH. Proofread all authors, important contributions to all authors.

Acknowledgments
The authors thank Janna Heide for helpful advice on visualisation and tabular summary of epitopes.

Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2021.100294.

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Author names in bold designate shared co-first authorship

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