Enolase represents a metabolic checkpoint controlling the differential exhaustion programmes of hepatitis virus-specific CD8⁺ T cells

Frances Winkler, Anna V Hipp, Carlos Ramirez, Bianca Martin, Matteo Villa, Emilia Neuwirt, Oliver Gorka, Jeroen Aerssens, Susanne E Johansson, Nisha Rana, Sian Llewellyn-Lacey, David A Price, Marcus Panning, Olaf Groß, Erika L Pearce, Carl M Hermann, Kathrin Schumann, Luciana Hannibal, Christoph Neumann-Haefelin, Tobias Boettler, Percy Knolle, Maike Hofmann, Dirk Wohleb, Robert Thimme, Bertram Bengsch

ABSTRACT

Objective Exhausted T cells with limited effector function are enriched in chronic hepatitis B and C virus (HBV and HCV) infection. Metabolic regulation contributes to exhaustion, but it remains unclear how metabolism relates to different exhaustion states, is impacted by antiviral therapy, and if metabolic checkpoints regulate dysfunction.

Design Metabolic state, exhaustion and transcriptome of virus-specific CD8⁺ T cells from chronic HBV-infected (n=31) and HCV-infected patients (n=52) were determined ex vivo and during direct-acting antiviral (DAA) therapy. Metabolic flux and metabolic checkpoints were tested in vitro. Intrahepatic virus-specific CD8⁺ T cells were analysed by scRNA-Seq in a HBV-replicating murine in vivo model of acute and chronic infection.

Results HBV-specific (core, polymerase) and HCV-specific (NS3, NS5B) CD8⁺ T cell responses exhibit heterogeneous metabolic profiles connected to their exhaustion states. The metabolic state was connected to the exhaustion profile rather than the aetiology of infection. Mitochondrial impairment despite intact glucose uptake was prominent in severely exhausted T cells linked to elevated liver inflammation in chronic HCV infection and in HBV polymerase-specific CD8⁺ T cell responses. In contrast, relative metabolic fitness was observed in HBeAg-negative HBV infection in HBV core-specific responses. DAA therapy partially improved mitochondrial programmes in severely exhausted HCV-specific T cells and enriched metabolically fit precursors. We identified enolase as a metabolic checkpoint in exhausted T cells. Metabolic bypassing improved glycolysis and T cell effector function. Similarly, enolase deficiency was observed in intrahepatic HBV-specific CD8⁺ T cells in a murine model of chronic infection.

Conclusion Metabolism of HBV-specific and HCV-specific T cells is strongly connected to their exhaustion severity. Our results highlight enolase as metabolic regulator of severely exhausted T cells. They connect differential bioenergetic fitness with distinct exhaustion subtypes and varying liver disease, with implications for therapeutic strategies.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Accumulation of exhausted CD8⁺ T cells in patients with chronic hepatitis B and C virus (HBV and HCV) infection contributes to the failure to clear viral infection.
⇒ These exhausted T cells lack sufficient antiviral function but the mechanisms behind this dysfunction are unclear.

WHAT THIS STUDY ADDS

⇒ HBV-specific and HCV-specific CD8⁺ T cells exhibit distinct metabolic profiles that correlate with severity of exhaustion.
⇒ Severe mitochondrial depolarisation despite high glucose uptake is present in severely exhausted virus-specific CD8⁺ T cells.
⇒ HBV polymerase-specific CD8⁺ T cells in chHBV infection display more severe exhaustion and mitochondrial dysfunction that correlates with quantitative HB surface antigen levels in contrast to HBV core-specific CD8⁺ T cells.
⇒ Enolase is a metabolic checkpoint limiting glycolytic flux in HBV-specific and HCV-specific CD8⁺ T cells.
⇒ Effector function of enolase-inhibited CD8⁺ T cells is boosted by pyruvate supplementation.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ We identified the glycolytic enzyme enolase as a metabolic checkpoint that can restrict mitochondrial metabolism and effector function of HBV-specific and HCV-specific CD8⁺ T cells.
⇒ This knowledge points to interventions to enhance or bypass enolase activity in order to boost antiviral responses in chronic infection.
⇒ Our data suggest that the exhaustion and metabolic state of HBV polymerase-specific CD8⁺ T cells may act as correlates of differential antigen recognition in cHBV infection which should be further investigated.
INTRODUCTION

Exhausted virus-specific CD8+ T cells (T_{ex}) with limited effector function accumulate in patients with chronic hepatitis B and C virus infection (cHBV/cHCV).1-4 These exhausted T cells are characterised by increased PD-1 expression and other coinhibitory receptors and profound alterations in transcriptional programmes.2,3 Recent reports highlighted substantial heterogeneity among exhausted HBV-specific and HCV-specific CD8+ T cells, including early differentiated PD-1+CD127+ precursors of exhausted T cells with partial memory-like characteristics and more severely exhausted PD-1+CD127- T cells.5-10 Interestingly, many cellular alterations of exhausted virus-specific CD8+ T cells persist even after antiviral therapy.2,7,10-11 However, the precise mechanisms determining these exhausted T cell programmes remain poorly defined.

T cell energy metabolism and effector T cell function are tightly interconnected. Specific roles of glycolytic enzymes in T cell activation and cytokine production have been described, such as glyceraldehyde phosphate dehydrogenase (GAPDH),14 pyruvate dehydrogenase kinase 1 (PDK1)15 or the glycolytic metabolite phosphoenolpyruvate (PEP).17 Conversely, regulation of the metabolic flux may explain limited T cell function. Indeed, bioenergetic regulation downstream of inhibitory receptor signalling was identified as a major hallmark of exhausted T cells in the murine lymphocytic choriomeningitis virus (LCMV) model of chronic viral infection.18 This bioenergetic regulation affected glycolysis and resulted in a significant disorganisation of mitochondrial organelles with alterations in ultrastructure, membrane depolarisation and production of reactive oxygen species (ROS).17,19 Investigations of the metabolic properties of HBV-specific and HCV-specific CD8+ T cells identified related metabolic dysregulation. HBV-specific CD8+ T cells analysed in vitro were highly dependent on glycolysis, and unable to switch efficiently to oxidative phosphorylation (OXPHOS) in settings of glucose restriction.20 Fisicaro et al. identified that significant downregulation of mitochondrial function is connected to ROS production in exhausted HBV-specific CD8+ T cells - comparable to the findings in LCMV infection.21 HBV-specific CD8+ T cells targeting different epitopes, such as HBV core18-20-specific or polymerase463-specific responses differ in their exhaustion phenotype but their metabolic properties are unknown. Mitochondrial function was also investigated in HCV-specific CD8+ T cells and during direct-acting antiviral (DAA) therapy. While one study reported limited changes in mitochondrial function of HCV-specific CD8+ T cells after DAA therapy and HCV clearance,11 another study observed a reduction of cells with depolarised mitochondria.22 However, currently, it remains unclear whether there are differences in the metabolic regulation of HBV-specific and HCV-specific CD8+ T cells, how these relate to the severity of exhaustion programmes, whether they change during antiviral therapy, and if specific glycolytic checkpoints are involved.

To address these important questions, we performed a detailed analysis of the metabolic profiles of HBV-specific and HCV-specific CD8+ T cells and their exhaustion states. We observed major differences in the exhaustion and metabolic programmes of HBV-specific and HCV-specific CD8+ T cells. HCV-specific responses were enriched for more severe exhaustion phenotypes and connected to more pronounced mitochondrial impairment despite high glucose uptake. However, a similar mitochondrial impairment was observed in HBV polymersase451-463-specific CD8+ T cells but not in HBV core18-20-specific CD8+ T cells during HBeAg-negative infection. These differences were connected to more severe exhaustion and in CHCV to a higher level of liver inflammation. In HBV infection, a correlation between HBV polymerase451-463-specific metabolism and quantitative HB surface antigen (qHBsAg) levels was observed. Antigen removal in vivo during DAA therapy partially improved the metabolism of the more severely exhausted T cell subset. Enolase was identified as a metabolic checkpoint. Its reduced expression in severely exhausted CD8+ T cells is involved in regulation of the glycolytic flux, contributing to metabolic dysfunction and reduced antiviral effector function. Bypassing this metabolic bottleneck reinvigorated effector function of virus-specific CD8+ T cells. Enolase deficiency was a conserved feature of severely exhausted murine intrahepatic HBV-specific CD8+ T cells in chronic but not acute infection. Together, these data highlight differential metabolic programming of virus-specific CD8+ T cells in different exhausted T cell subsets in viral hepatitis and highlight a novel metabolic checkpoint.

METHODS

Study cohort

Patient details are summarised in online supplemental tables 1 and 2.

Peptides, tetramers and antibodies

HLA-A*02:01-restricted monomers of immunodominant epitopes for HBV (HLA-A*02:01/core18-27: FLPSDFPPSV; HLA-A*02:01/polymerase451-463: GLSYRVYRI), HCV (HLA-A*02:01/NS3:1073-1081: CINGVCTWT; HLA-A*02:01/NS3:463-471: KLYALGINAV; HLA-A*02:01/NS5B:2194-2202: ALYDVSVKL), EBV (HLA-A*02:01/BMLF1:402-410: GLCTLVALM), CMV (HLA-A*02:01/pp65:260-268: GLLGVFTL) were synthesised and conjugated with allophycoerythrin (APC)-labelled or phycoerythrin (PE)-labelled Streptavidin (Agilent) in a molar 4:1 ratio. Antibodies used are listed in online supplemental table 3.

Statistics

Statistical analyses were performed using GraphPad Prism V9 (GraphPad Prism Software, USA). Normal (Gaussian) distribution of the data was tested using the D’Agostino and Pearson test. Normally distributed data sets (alpha=0.05) were analysed using parametric statistical tests and not normally distributed data sets were analysed using non-parametric statistics. For the comparison of two groups (unpaired t-test, Mann-Whitney U test or Wilcoxon test was used. The comparison of more than two groups was statistically tested using analysis of variance (ANOVA), Kruskal-Wallis test or Friedman test. Tests used in figures 1–7 and online supplemental figures 1–5 were: Unpaired t-test: figure 1C,F and G (centre and right) and online supplemental figure 3F (MTDR, MTDR/MTG) and online supplemental figure 3G (left). Paired t-test: figure 3A (right), figure 4E, figure 6C, online supplemental figure 4H (MTDR/MTG), online supplemental figure 4J, online supplemental figure 5H (left) and I. Wilcoxon test: figure 3A (left), figure 3B,C, figure 4A–C and G–K, figure 6GI and K, online supplemental figure 4H (MTG, MTDR, MTG-MTDR), online supplemental figure 4L, online supplemental figure 5H (right). Mann-Whitney test: figure 1A,B,D,E and G (left), figure 5B–D, figure 7HI, online supplemental figure 1C–E, online supplemental figure 2A, online supplemental figure 3F (MTG, MitoSox), online supplemental figure 3G (right) and online supplemental figure 3H. ANOVA: figure 6L (right), online supplemental figure 5E, online supplemental figure 4J, online supplemental figure 1G,H and online supplemental figure 4B. Friedman test: figure 6HL and L (left) and online supplemental figure 5C,F. Pearson correlation: online supplemental figure 2G (right). Spearman correlation: figure 3D, figure 4DF, figure 5E–F, figure 6DF, online supplemental figure 2C–F, G (left),
H-L, online supplemental figure 3B–D and online supplemental figure 4C–G. Statistical tests used are depicted in the figure legends (p*>0.05; p**>0.01; p***>0.001; p****>0.0001). Please see online supplemental methods for additional methods used.

RESULTS

Heterogeneity of exhaustion profiles indicates bias towards severe exhaustion of HCV-specific CD8+ T cells

We set out to understand how metabolic profiles of HBV-specific and HCV-specific CD8+ T cell responses in chronic infection are connected to their exhaustion profiles. Virus-specific CD8+ T cells were identified by ex vivo tetramer staining which revealed a higher frequency of HBV core18-27-specific compared with HCV NS31073-1081-specific, NS31406-1415-specific and NS5B2594-2602-specific CD8+ T cells (figure 1A). HCV-infected patients had more variable liver inflammation with more severe hepatitis identified by transaminase elevation compared with our cohort of HBeAg-negative cHBV patients, as expected (online supplemental tables 1 and 2). We characterised the expression of exhaustion markers that inform about early differentiated ‘progenitor’ exhausted T cells with homeostatic properties and more severely exhausted T cell populations. PD-1 expression was shared by HBV-specific and HCV-specific CD8+ T cells but expression of the interleukin 7 receptor α-chain (CD127) was significantly reduced in HCV-specific CD8+ T cells, resulting in an enrichment of the PD-1+CD127+ subset in HBV core18-27-specific CD8+ T cells (figure 1B–D). Of note, HCV-specific CD8+ T cells

Figure 1  HCV-specific CD8+ T cells express markers associated with severe exhaustion. (A) Tetramer analysis of PBMCs from therapy-naïve cHBV and cHCV patients. Representative FACS plots of HBV core18-27 and HCV NS31073-1081 specific CD8+ T cells (left). Comparison of tetramer frequencies gated on CD8+ T cells (right). Virus-specific CD8+ T cells from cHBV and cHCV patients were stained for the depicted exhaustion-associated molecules. (B, C) Frequencies of PD-1+, CD127+, (D) PD-1+CD127+, PD-1+CD127−, (E) CD38+, CD39+, (F) TIGIT+, CD57+, (G) CD27+, CD28+ and CD122+ virus-specific CD8+ T cells are indicated. HBV core18-27 (green) and HCV NS31073-1081 epitopes (blue) are represented by circles. HCV NS31406-1415 and HCV NS5B2594-2602 epitopes are visualised by blue squares and triangles, respectively. Mann-Whitney test was performed in (A, B), (D, E) and (G, left). Unpaired t-test was performed in (C), (F, G, centre and right). P values are indicated (p*<0.05, p**<0.01). Error bars indicate mean±SEM. cHBV, chronic hepatitis B virus; cHCV, chronic hepatitis C virus; PBMCs, peripheral blood mononuclear cells.
Hepatitis virus-specific CD8+ T cells exhibit different metabolic programmes

Our exhaustion analysis indicated that the comparison of HBV core18-27-specific CD8+ T cells and HCV NS31073-1081-specific, NS31406-1415-specific and NS5B2594-2602-specific CD8+ T cells also reflected a comparison between mild and severe exhaustion programmes. The comparison of HBV-specific and HCV-specific CD8+ T cell responses thus may not only reflect differences in viral aetiology but also serve as a model to study differences in T cell exhaustion programmes. To understand the metabolic profiles of virus-specific CD8+ T cells in chronic HBV and HCV infection, we first performed transcriptome analysis of sorted virus-specific CD8+ T cells in untreated chronic infection. Interestingly, gene set enrichment analysis (GSEA) of KEGG metabolic pathways indicated significant differences in the metabolic programmes of mildly exhausted HBV-specific compared with severely exhausted HCV-specific CD8+ T cells (figure 2A). In particular, the gene sets for OXPHOS and citric acid cycle (TCA cycle) were significantly enriched in HBV-specific CD8+ T cells, while expression of genes involved in glycolysis had a non-significant trend towards higher expression in HCV-specific CD8+ T cells (figure 2B). These transcriptional analyses suggested differences in energy metabolism of HBV-specific and HCV-specific CD8+ T cells. We thus explored the functional consequences of these altered transcriptomes and functionally interrogated T cell metabolism using metabolism-directed flow cytometry. Glucose uptake was measured using 2-NBDG assay. HBV-specific and HCV-specific CD8+ T cells incorporated more 2-NBDG than naïve T cells, in line with higher bioenergetic requirements of activated antigen-specific CD8+ T cells (figure 2C,D).

We next assessed the mitochondrial properties of virus-specific CD8+ T cells using several mitochondrial dyes that differ in their ability to stain mitochondria depending on their membrane potential. TMRE staining as a direct correlate of ΔΨm electronegativity was significantly higher in HBV-specific compared with HCV-specific CD8+ T cells, indicating elevated mitochondrial membrane potential (figure 2E). In contrast, elevated mitochondrial mass, as indicated by MitoTracker Green (MTG) staining was observed in HCV-specific CD8+ T cells despite similar total cellular uptake of the MitoTracker Deep Red (MTDR) that correlates with mass and potential (figure 2F,G). The MTDR/MTG ratio (informing about relative mitochondrial polarisation as another estimate of mitochondrial membrane potential) was reduced in HCV-specific CD8+ T cells, fitting to the TMRE results (figure 2H). We also observed higher mitochondrial ROS in HBV-specific and HCV-specific CD8+ T cells compared with naïve T cells, however, there was a clear enrichment of ROS in HCV-specific compared with HBV-specific CD8+ T cells (figure 2I). These data informed us that the metabolic differences are connected to the differentiation of exhausted subsets rather than differences inherent to the different hepatitis viruses.

The mitochondrial profile of HBV-specific and HCV-specific CD8+ T cells is associated with their differential PD-1/CD127 expression

We next examined the extent to which the mitochondrial polarisation depends on the exhausted subpopulations in cHBV and cHCV infection. Analysis of mitochondrial mass, potential and polarisation between PD-1+CD127+ and PD-1+CD127− virus-specific CD8+ T cells in cHBV and cHCV infection revealed significantly reduced mitochondrial polarisation in the more exhausted PD-1+CD127− subset, independent of aetiology of viral infection (figure 3A–C). In line with this observation, linear regression analysis also revealed a direct correlation of mitochondrial polarisation and the PD1/CD127 phenotype. Mitochondrial polarisation (MTDR/MTG) inversely correlated with the PD-1+CD127− subset frequency, in contrast to a positive correlation with the mildly exhausted subset (PD-1+CD127+). These analyses demonstrate a connection of the mitochondrial polarisation with the distribution of different exhaustion subsets of HBV core18-27-specific and HCV NS31073-1081-specific, NS31406-1415-specific and NS5B2594-2602-specific CD8+ T cells and suggest that the metabolic differences are connected to the differentiation of exhausted subsets rather than differences inherent to the different hepatitis viruses.

Antigen clearance improves mitochondrial fitness of HCV-specific CD8+ T cells linked to accumulation of PD-1+CD127+ populations

Since we had identified severely impaired mitochondrial metabolism in exhausted HCV-specific CD8+ T cells associated with a terminally exhausted phenotype (PD-1+CD127+) during chronic infection that is associated with high levels of antigen stimulation and liver inflammation, we sought to understand if direct-acting antiviral (DAA) therapy would reprogramme HCV-specific CD8+ T cell metabolism. Previous studies have shown controversial results on a bulk virus-specific analytic level after overnight culture. Therefore, we performed a longitudinal metabolic analysis of HCV-specific CD8+ T cells ex vivo in cHCV patients treated with DAA.
Figure 2  HBV-specific and HCV-specific CD8+ T cells display different metabolic profiles. (A) Gene-set enrichment analyses (GSEA) of KEGG metabolic pathways were performed on microarray data of sorted therapy-naïve HBV-specific and HCV-specific CD8+ T cells. Significant (p≤0.05) pathways were indicated. (B) GSEA plots for KEGG OXPHOS, TCA cycle and Glycolysis gene sets. Coloured arrows indicate enrichment in HBV-specific or HCV-specific CD8+ T cells. Normalised enrichment scores (NES), false discovery rates (FDR) and p values are shown for GSEA analyses. (C) Naïve CD8+ T cells used as normalisation control for metabolic stainings were gated as CCR7+CD45RA+. (D–I) Virus-specific CD8+ T cells were analysed for metabolic features by staining for glucose uptake (2-NBDG), mitochondrial membrane potential (TMRE), mitochondrial mass (MTG), mitochondrial mass and potential (MTDR), polarisation (MTDR/MTG) and mitochondrial ROS (MitoSox). Metabolic staining intensity was normalised to naïve CD8+ T cells from the same sample. Exemplary histograms of metabolic stainings are shown. HBV core 18-27 (green) and HCV NS3 1073-1081 epitopes (blue) are represented by circles. HCV NS3 1406-1415 and HCV NS5B 2594-2602 epitopes are visualised by blue squares and triangles, respectively. Kruskal-Wallis test was performed in (D–I). P values are indicated (p*<0.05, p**<0.01, p***<0.005, p****<0.001). Error bars indicate mean±SEM. HBV, hepatitis B virus; HCV, hepatitis C virus; ROS, reactive oxygen species.
therapy. Interestingly, ex vivo analysis identified a significant increase of virus-specific cells at week 2 after therapy initiation (figure 4A), suggesting an augmented virus-specific response, that occurred when transaminase levels significantly declined (figure 4B).

Metabolic analysis revealed an increase in the polarisation of mitochondria after 2 weeks of therapy (figure 4C). Interestingly, we observed a negative association between mitochondrial fitness and ALT levels (figure 4D). Thus, our ex vivo analyses indicated a metabolic response to DAA therapy. We next wondered if the improved mitochondrial fitness was associated with a change in the distribution of exhaustion subsets. Indeed, our monitoring showed an enrichment of PD-1+CD127+ cells during DAA therapy (figure 4E). Clearly, the increased frequency of the PD-1+CD127+ subset correlated with higher mitochondrial polarisation (figure 4F).

We, thus, wondered if DAA therapy augmented the metabolism in severely exhausted cells or if the increase in mitochondrial metabolism is due to an accumulation of the metabolically fit subset. We did not observe significant changes in mitochondrial metabolism in the PD-1+CD127+ cells during therapy (figure 4G–I). In contrast, there was an increase in polarisation in the remaining severely exhausted PD-1+CD127− cells during therapy (figure 4G–I). The augmentation of mitochondrial metabolism in the severely exhausted cells compared with the PD-1+CD127+ subset resulted in a similar mitochondrial polarisation of the remaining PD-1−CD127− cells at week 2 (figure 4J,K). In sum, our data link the enhanced metabolic fitness of HCV-specific CD8+ T cells after therapy to an expansion of the PD-1+CD127+ population and improvement of the metabolic fitness of PD-1−CD127− cells.

The metabolic profile of HBV core18–27-specific and polymerase455–463-specific CD8+ T cell responses significantly differs and correlates with their exhaustion phenotype

In chronic HBV infection, ex vivo HBV-specific CD8+ tetramer responses are preferentially identified in HBeAg-negative infection.6 Comparison of immunodominant epitope responses recently revealed phenotypic and functional differences between HBV core18–27-specific and polymerase455–463-specific CD8+ T cells in patients despite similar low viral loads and inflammation.6 We, thus, wondered if these responses differed in their metabolic profile. Interestingly, HBV polymerase455–463-specific CD8+ T cells had significantly reduced mitochondrial polarisation and increased mitochondrial ROS production compared with HBV core18–27-specific CD8+ T cells (figure 5A), resembling the metabolic programme enriched in severely exhausted T cells and typical for HCV infection. Consistent with our previous analysis, HBV polymerase455–463-specific CD8+ T cells had reduced frequencies of the PD1+CD127+ subset compared with HBV core18–27-specific CD8+ T cells (figure 5B) and higher expression of severe exhaustion-associated markers CD38, CD39, CD57 and reduced expression of CD28 (figure 5C,D). These data indicate that HBV polymerase455–463-specific CD8+ T cells have a more severe mitochondrial dysfunction that is connected to their exhaustion programme. Overall these data are in line with the connection of metabolism to exhaustion state also observed in chronic HCV infection.

Activation and metabolism of HBV polymerase455–463-specific CD8+ T cell responses correlate with qHBsAg levels

Since the differences in metabolism and exhaustion programme of HBV-specific responses could not be explained by the degree of liver inflammation, we wondered if they were connected to antigen levels. We thus determined qHBsAg levels and performed correlation analyses. Linear regression analysis revealed a correlation between CD39 expression, mitochondrial polarisation and quantitative HBsAg (qHBsAg) levels for HBV polymerase455–463-specific responses, while no such correlation was found for HBV core18–27-specific CD8+ T cell responses (figure 5E,F). This suggests that antigen recognition drives the metabolic and exhaustion programme in polymerase455–463-specific CD8+ T cell responses but that this differs in HBV core18–27-specific CD8+ T cell responses.
Increased transaminase levels correlate with mitochondrial depolarisation in PD-1+CD127+ virus-specific CD8+ T cells in chronic HCV infection

The differentiation of exhausted T cells is strongly influenced by viral antigen but is also subject to inflammatory cues. In DAA therapy during HCV infection, we had observed a negative correlation of mitochondrial polarisation with ALT (figure 4D), however, the control of viral replication and resolution of liver inflammation are intertwined. Therefore, we next investigated the connection of the metabolic T cell phenotype with the clinical activity in chronic HBV and HCV infection. Patients with cHCV infection displayed a diverse range of liver inflammation as indicated by aspartate aminotransferase (AST) and alanine aminotransferase (ALT).
enzyme activity and viral loads, while in our cohort, HBV patients had milder hepatic inflammation (online supplemental figure 2A,B, online supplemental tables 1 and 2). We observed an inverse relationship of liver transaminases and mitochondrial polarisation and activation/exhaustion state of HCV-specific CD8+ T cells but not in HBV infection (online supplemental figure 2C–L). There was no clear correlation with viral load as measured by PCR, however, it is unclear if that readout is a good measure of T cell antigen recognition (online supplemental figure 3A–D). There was no direct correlation of metabolic T cell features and transaminase levels in HBeAg-negative chronic infection (ENC1) and HBeAg-negative chronic hepatitis B (ENC2) patients (online supplemental figure 3E–H). These data show a link between the mitochondrial impairment of more severely exhausted virus-specific CD8+ T cells and the degree of hepatic inflammation in cHCV infection.

Enolase expression is reduced in severely exhausted CD8+ T cells and correlates with mitochondrial membrane potential

Our data demonstrate a prominent role for mitochondrial depolarisation in severely exhausted CD8+ T cells. However, this depolarisation occurred despite high glucose uptake by HCV-specific CD8+ T cells (figure 2D) and despite reduced utilisation of glucose for glycolysis in the metabolic flux analysis (online supplemental figure 1). We, therefore, sought to understand whether glycolytic flux was altered due to dysregulation of enzymes required for glycolysis.
Enolase 1 inhibition can drive mitochondrial depolarisation in primary human CD8+ T cells

Since short-term effects of enolase inhibition on respiration in in vitro metabolic flux assays were limited, but the correlations of enolase and mitochondrial respiration were strong in ex vivo analysis, we next asked if prolonged regulation of glycolysis via ENO1 would also impact on mitochondrial polarisation of freshly isolated primary human CD8+ T cells and treated CD8+ T cells from healthy individuals overnight with an enolase-specific inhibitor. We observed that ENO1 inhibition resulted in significantly reduced mitochondrial polarisation, more depolarised mitochondria and trends towards reduced cytokine production (online supplemental figure 4H,I). Thus, ENO1 inhibition resulted in a mitochondrial phenotype typical for severely exhausted virus-specific CD8+ T cells.

Enolase enzymatic activity controls virus-specific CD8+ T cell effector function

Due to the prominent role of glycolysis for T cell effector function, we next wondered if enolase would represent a metabolic regulator of exhausted T cell function in virus-specific CD8+ T cells. We, therefore, examined cytokine production of HBV core18-specific and HCV NS31415-specific and NS5B2594-specific CD8+ T cells after overnight treatment with API-III-a4 hydrochloride or in combination with pyruvate. Treatment did not affect viability (online supplemental figure 4I). After treatment with the ENO1 inhibitor, HBV-specific and HCV CD8+ T cells showed a significant drop in IFN-γ production (figure 6G). In contrast, the addition of pyruvate enhanced cytokine production (figure 6H-I, online supplemental figure 4K-M). The effects of enolase inhibition on IFN-γ production were stronger than on TNF-α production, however, pyruvate supplementation also enhanced the production of this cytokine, in particular in HCV-specific CD8+ T cells (figure 6).

Enolase 1 expression is reduced in exhausted versus memory-like CD8+ T cells in HBV infection in mice

To understand the role of differential ENO1 expression in hepatitis virus-specific CD8+ T cell exhaustion in the same virus model and intrahepatic environment, and to understand differences between acute and chronic infection, we used a recently developed mouse model of acute or chronic HBV infection.25 In this model, C57Bl/6 mice were infected with different doses of an adenoviral vector encoding for a 1.3-overlength HBV genome (Ad-HBV-Luc) after transfer of naive HBV Core-1-specific CD8+ T cells. Depending on the dose of Ad-HBV-Luc administered, mice develop either acute self-limiting (107 pfu) or chronic HBV infection (108 pfu) (figure 7A). At 15 dpi and 30 dpi, respectively, liver-associated lymphocytes were isolated and Core1-specific CD8+ T cells were analysed by single-cell RNA-seq. UMAP analysis identified different antigen-specific T cell transcriptomes in acute and chronic infection (figure 7B). Clustering analysis revealed four separate clusters with differential expression of exhaustion markers (figure 7C). HBV-specific CD8+ T cells from mice with acute HBV infection were predominantly enriched in cluster 1 (C1). Chronic HBV-specific CD8+ T cells were enriched in C4 (figure 7D). Markers associated with severe exhaustion, such as Pdcd1, Cd38, Cxcr5 and Tmx1 showed increased expression in C4 (figure 7E). In contrast, C1 highly expressed Il7r, Tcf7, Slamf6 and Cxcr5 that indicate homoeostatic and memory-like characteristics. Interestingly, Enol1 expression was significantly higher in acute compared with chronic HBV-specific CD8+ T cells, indicating a loss of enolase expression.
Figure 6  ENO1 lowly expressed in severely exhausted virus-specific CD8+ T cells is a metabolic checkpoint controlling glycolytic flux and T cell function. (A) Schematic overview of the glycolytic pathway. (B) Intracellular ENO1 staining of HBV-specific and HCV-specific CD8+ T cells. ENO1 MFIs are normalised to naïve CD8+ T cells of the respective donor. (C) Ex vivo analysis of PD-1+CD127+ and PD-1+CD127- subsets of HBV- and HCV-specific CD8+ T cells for ENO1 expression. (D) Correlation analyses of ENO1 expression and mitochondrial polarisation (MDTR/MTG) of HBV-specific and HCV-specific CD8+ T cells. (E) Correlation analysis of ENO1 expression and serum ALT level of therapy-naïve cHBV and cHCV patients. (G–L) IFN-γ and TNF-α production of PMA- and ionomycin-stimulated hepatitis virus-specific CD8+ T cells after o/n treatment with DMSO, AP-III-a4 hydrochloride (10 µM) and/or sodium pyruvate (2 mM). Cytokine secretion is shown normalised to DMSO-treated hepatitis virus-specific CD8+ T cells as control samples. HBV core18-27- and polymerase455-463-specific CD8+ T cells are visualised by light green and dark green circles, respectively. HCV NS31073-1081, HCV NS31406-1415 and HCV NS5B2594-2602 epitopes are represented by blue circles, squares and triangles, respectively. Kruskall-Wallis test was performed in (B). Paired t-test was performed in (C). Spearman r correlation analyses were performed in (D–F). Wilcoxon test was performed in (G), (I, K). Friedman test was performed in (H), (J) and (L, left). Two-way ANOVA was performed in (L, right). P values are indicated (p*<0.05, p**<0.01, p***<0.005, p****<0.001). Error bars indicate mean±SEM. ANOVA, analysis of variance; cHBV, chronic hepatitis B virus; cHCV, hepatitis C virus.
during chronic infection (figure 7F). GSEA of exhaustion signatures revealed a significant enrichment of exhaustion genes in HBV-specific CD8⁺ T cells isolated from chronic condition, as expected. Moreover, direct comparison of C1 versus C4 showed a significant enrichment of exhaustion-associated gene expression in C4 and an enrichment of memory-associated features in C1 (figure 7G). Moreover, C1 was significantly enriched for Eno1 expression compared with other clusters (figure 7H). These results are in line with our previous findings on ENO1 expression in less severely exhausted virus-specific CD8⁺ T cells in CHBV and CHCV patients (figure 6C). Moreover, we observed significantly elevated glycolytic gene expression in acute versus chronic conditions, with C1 exhibiting highest expression of glycolytic genes among all clusters, as well as an enrichment of genes responsible for OXPHOS in acute versus chronic HBV infection.

In sum, these data support a role for enolase in metabolically regulating differential exhaustion profiles of intrahepatic virus-specific CD8⁺ T cells (figure 7I).

**DISCUSSION**

T cell exhaustion is a prominent feature of virus-specific CD8⁺ T cells in chronic HBV and HCV infection and linked to metabolic changes. Here, by performing a detailed comparison of the metabolic states of HBV core18⁺-specific and polymerase455-463- specific and HCV NS31071-1081- specific, NS531406-1411- specific and NS5B7594-7602- specific CD8⁺ T cells from patients with chronic infection, we identified major differences in the metabolism of virus-specific CD8⁺ T cell responses with pronounced mitochondrial dysregulation in severely exhausted PD-1⁺CD127⁺ CD8⁺ T cells enriched in chronic HCV infection. In contrast, higher mitochondrial fitness was observed in PD1⁺CD127⁺ CD8⁺ T cells that have also been termed ‘memory-like’ due to their homeostatic features. Interestingly, the link between metabolism and exhaustion subtype was independent of the viral aetiology. In HCV infection, the metabolic state also correlated with the extent of liver inflammation. In HBV infection, we observed different roles for different HBV-specific CD8⁺ T cell responses. In particular, HBV polymerase455-463- specific CD8⁺ T cells showed a more severe mitochondrial dysfunction that correlated with viral antigen levels, while HBV core18⁺-specific CD8⁺ T cell responses were enriched in the PD1⁺CD127⁺ exhaustion phenotype, had relatively intact metabolism but also did not show a clinical correlation. Taken together, these findings suggest a conserved mechanism of metabolic programs involved in exhausted T cells across viral aetiologies and suggested specific metabolic checkpoints governing different exhaustion states. We identified differential enolase expression as a potential metabolic regulator and speculated if it controls the exhaustion states.

Indeed, enolase inhibition resulted in reduced metabolic flux and mitochondrial depolarisation. Bypassing enolase regulation in HBV-specific and HCV-specific CD8⁺ T cells augmented their glycolysis and T cell effector function. In sum, these findings indicate that hepatitis virus-specific CD8⁺ T cells in CHBV and CHCV infection have different exhaustion states due to distinct metabolic programmes. The identification of enolase 1 as a contributing regulator of these exhaustion programmes may provide opportunities for targeted intervention.

Persistent antigen stimulation is a major driver of T cell exhaustion. DAA therapy in chronic HCV infection causes rapid inhibition of viral replication and served as an *in vivo* model to analyse the effect of tuning viral antigen and associated liver inflammation. Our analysis showed that after 2 weeks of DAA therapy, HCV-specific CD8⁺ T cell metabolism improved with enhanced mitochondrial polarisation. The impact of DAA therapy on T cell metabolism has also been investigated by Arevey et al., who, however, did not find a major effect on mitochondrial HCV-specific CD8⁺ T cell metabolism. In contrast, our DAA therapy results fit to work by Barili et al. who also observed improved mitochondrial polarisation in HCV-specific CD8⁺ T cells by focusing on epitope-matched virus-specific CD8⁺ T cell responses. These studies, different types of exhaustion subsets were not analysed in detail. In our work, the enhanced mitochondrial fitness after DAA therapy was clearly linked to an accumulation of PD1⁺CD127⁺ virus-specific CD8⁺ T cells. However, our data also suggest that more exhausted T cell subsets experience stronger improvement in metabolism by DAA therapy. Thus, different distributions of exhausted T cell subsets at baseline time points may contribute to the different results found in these earlier studies. Interestingly, while we observed a correlation of the metabolic state of HCV-specific CD8⁺ T cells with liver inflammation that is intertwined with viral replication and antigen recognition, we did not observe such a correlation with liver inflammation in chronic HBV infection. However, in CHBV infection we observed clearly distinct roles for HBV core18⁺-specific and polymerase455-463- specific responses, in line with previous results from phenotypic and functional profiling. HCV polymerase455-463- specific CD8⁺ T cell responses displayed more diverse metabolic and exhaustion states. Importantly, we also observed correlations of activation and mitochondrial dysfunction in CHBV polymerase455-463- specific CD8⁺ T cell responses with qHBsAg levels in these patients, suggesting that the exhaustion state and the associated metabolism of HCV polymerase455-463- specific CD8⁺ T cell response is due to the degree of antigen recognition in CHBV infection. These findings also suggest that antigen is a dominant driver of exhausted T cell metabolism in our cohort, although a contribution of the inflammatory microenvironment, in particular in HCV infection, cannot be formally excluded.

Glycolysis is key to effector T cell differentiation and function and subject to regulation by immune checkpoints. Here, in established human chronic infection, we observed high glucose uptake but reduced metabolic activity in severely exhausted CD8⁺ T cells. These cells showed significant mitochondrial disturbances similar to exhausted T cells in LCMV or tumour models. It was puzzling that these exhausted T cells also had high glucose uptake despite limited glycolysis and mitochondrial impairment, which prompted us to dissect the glycolytic pathway. Our analysis revealed reduced ENO1 expression in severely exhausted PD-1⁺CD127⁺ CD8⁺ T cells enriched in chronic HCV infection. Inhibition of ENO1 resulted in decreased glycolytic function that could be reversed by downstream pyruvate supplementation. These results are consistent with work that identified reduced enolase activity associated with insufficient effector function in tumour-infiltrating lymphocytes which was reversed after checkpoint therapy. It also fits to the notion that enolase can act as a metabolic checkpoint, as illustrated by its role in governing the differentiation of regulatory T cells. Enolase is upstream of the production of PEP, a glycolytic intermediate important for sustaining T cell receptor-mediated Ca²⁺-NFAT signalling and effector functions by repressing sarco/ER Ca²⁺-ATPase (SERCA) activity. Reduced enolase activity may therefore limit calcium signalling which may in turn explain the reduced T cell function observed after enolase inhibition. Some HCV-specific CD8⁺ T cell responses with low glycolytic activity were metabolically unresponsive to enolase inhibition, suggesting a lack of enolase enzymatic activity in those cells. However, this was a rather extreme observation in our
Figure 7  Differential enolase 1 expression is linked to different metabolic programmes and exhaustion severities in acute versus chronic HBV infection. (A) Schematic illustration depicting experimental procedure. C57Bl/6 mice were intravenously injected with 10,000 transgenic CD45.1 T cells derived from Cor93 TCR-transgenic mice and after 24 hours infected with 10^7 (acute self-limiting) or 10^8 pfu (chronic) of adenoviral vector (Ad-HBV-Luc). Liver-associated lymphocytes were isolated after 15 (acute) or 30 days (chronic) and subsequently sorted for Cor93-specific CD8+ T cells. Single-cell RNA sequencing was performed on 3000 Cor93-specific cells per mouse. (B) UMAP projection showing Cor93-specific CD8+ T cells coloured by condition (C) and cluster identity. (D) Ratio of the number of cells in each cluster in acute and chronic samples. (E) Densities of gene expression levels are visualised by Nebulosa for selected genes and (F) Eno1. (G) GSEA plots comparing the differentially expressed genes (DEGs) for cluster 1 (C1) versus cluster 4 (C4) with the signatures reported in Utzschneider et al.25 (Exhaustion, Exhaustion memory-like and Memory signatures) and the DEGs of acute versus chronic conditions with the signatures published in Bengsch et al.26 (Upregulated and downregulated epigenomic exhaustion signatures). Coloured arrows indicate the cluster or condition in which the gene set is enriched. (H) Enolase 1 expression in acute versus chronic setting and cluster 1–4 across conditions. Mann-Whitney test was performed in (H–I). P values are indicated (p<0.05, p*<0.01, p**<0.005, p***<0.001). HBV, hepatitis C virus.

HBV, hepatitis C virus.
cohort, since most exhausted CD8+ T cell responses analysed had residual enolase activity. We also observed reduced mitochondrial polarisation and OXPHOS after overnight ENO1 inhibition, suggesting that enolase provides metabolic substrates for the TCA cycle and its regulation could be involved in throttling metabolic flux upstream of the mitochondrial changes in exhausted T cells. In sum, enolase serves as a metabolic checkpoint of exhausted CD8+ T cells in viral hepatitis.

Our data demonstrate that HBV-specific and HCV-specific CD8+ T cells exhibit distinct metabolic profiles during chronic infection associated with differences in their exhaustion programmes that are linked to antigen recognition and chCV infection, with liver inflammation. We identified the glycolytic enzyme enolase as an upstream metabolic checkpoint contributing to the regulation of the metabolic and functional programmes. Boosting enolase function could represent a novel strategy to counteract reduced effector function of virus-specific CD8+ T cells in chronic viral hepatitis.

Author affiliations
1 Clinic for Internal Medicine II, Freiburg University Medical Center, Faculty of Medicine, University of Freiburg, Freiburg im Breisgau, Germany
2 Faculty of Biology, University of Freiburg, Freiburg im Breisgau, Germany
3 Health Data Science Unit, Medical Faculty, University of Heidelberg, Heidelberg, Germany
4 Max Planck Institute of Immunobiology and Epigenetics, Freiburg im Breisgau, Germany
5 Institute of Neuropathology, Freiburg University Medical Center, Faculty of Medicine, University of Freiburg, Freiburg im Breisgau, Germany
6 Signalling Research Centres BIOUS and CIBSS, University of Freiburg, Freiburg im Breisgau, Germany
7 Translational Biomarkers, Infectious Diseases Therapeutic Area, Janssen Pharmaceutica, Beerse, Belgium
8 Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff University, Cardiff, UK
9 Systems Immunity Research Institute, Cardiff University School of Medicine, Cardiff, UK
10 Institute of Virology, Freiburg University Medical Center, Faculty of Medicine, University Hospital Freiburg, Freiburg im Breisgau, Germany
11 The Bloomberg-Kimmel Institute for Cancer Immunotherapy, Johns Hopkins Medicine Sidney Kimmel Comprehensive Cancer Center, Baltimore, Maryland, USA
12 Institute for Medical Microbiology, Immunology and Hygiene, Technical University of Munich (TUM), Munich, Germany
13 Department of General Pediatrics, Laboratory of Clinical Biochemistry and Metabolism, Medical Center-University of Freiburg, Adolescent Medicine and Neonatology, Faculty of Medicine, University of Freiburg, Freiburg, Germany
14 German Center for Infection Research (DZIF), Munich Partner Site, Munich, Germany
15 Institute of Molecular Immunology, Klinikum Rechts der Isar, Technical University of Munich, Munich, Germany
16 German Cancer Consortium (DKTK), Partner Site Freiburg, Heidelberg, Germany

Acknowledgements We would like to thank all participants who contributed to this study. We thank J. Wersing and the Lighthouse Core Facility for help with cell sorting. The graphical abstract was created using BioRender.com.

Contributors FW performed the experiments and analysed the data with the help of AHV, CR, EN, OGórkA, LH and NR. BM performed sorting of hepatitis virus-specific CD8+ T cells and analysed transcrptonic data with the help of JA and SEJ. SL-L and DAP provided the peptide-MHC class I monomers. MP collected clinical data. FW and BB wrote the manuscript. MV, EL, Ogórkó, CMH, PK, DW, KS, CN-H, TB, RT and MH proofread the manuscript and revised it for important intellectual content. BB designed and supervised the study, accepts full responsibility for the work and the conduct of the study, had access to the data and controlled the decision to publish.

Funding This study was funded by grants from the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation, TRR179 project no. 272983813, project no. 502099232, SFB1160 project no. 250673931, SFB1479 project no. 44189347 and CIBSS-EXC-2189 project no. 390939984). DAP was supported by a Wellcome Trust Senior Investigator Award (100326/Z/12/Z).

Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involves samples from human participants recruited through the HBV/Freeze biobank and was approved by the Ethics Committee of the Albert-Ludwigs University, Freiburg 242/18, 474/14. Participants gave informed consent to participate in the study prior to enrolment.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request.

Supplemental material This content has been supplied by the author(s). It has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution 4.0 Unported (CC BY 4.0) license, which permits others to copy, redistribute, remix, transform and build upon this work for any purpose, provided the original work is properly cited, a link to the licence is given, and indication of whether changes were made. See: https://creativecommons.org/licenses/by/4.0/.

ORCID iDs
David A Price http://orcid.org/0000-0001-9416-2737
Tobias Boettker http://orcid.org/0000-0002-1195-055X
Maike Hofmann http://orcid.org/0000-0001-8410-8833
Robert Thimme http://orcid.org/0000-0003-1417-4135
Bertram Bengsch http://orcid.org/0000-0003-2552-740X

REFERENCES


Hepatology

Gut: first published as 10.1136/gutjnl-2022-328734 on 4 August 2023. Downloaded from http://gut.bmj.com/ on November 1, 2022 by guest. Protected by copyright.
Hepatology