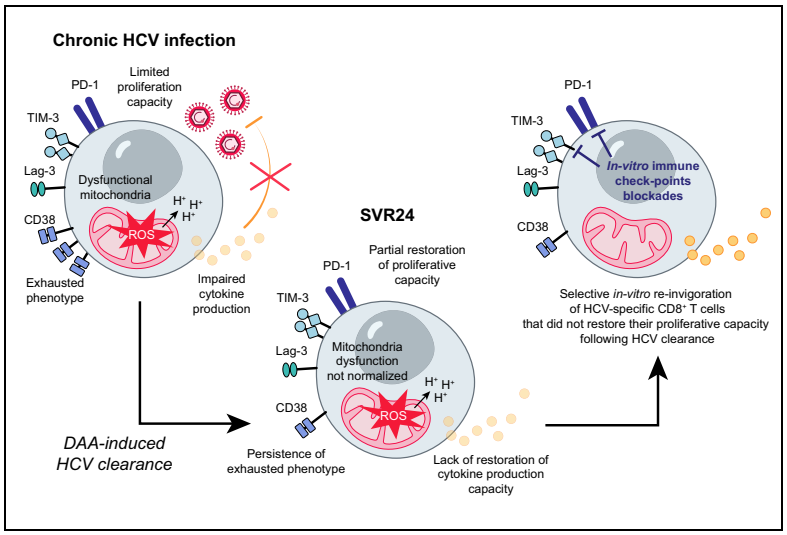
Research Article
Viral Hepatitis

JOURNAL
OF HEPATOLOGY

Elimination of hepatitis C virus has limited impact on the functional and mitochondrial impairment of HCV-specific CD8+ T cell responses

Graphical abstract



Highlights

- HCV-specific CD8+ T cell phenotypes and functional responses are not universally restored during DAA-induced HCV clearance.
- Mitochondrial fitness of virus-specific CD8+ T cells unaltered by cessation of persistent HCV replication.
- *In vitro* immune check-point inhibition mediated selective revival of *in vitro* DAA unresponsive HCV-specific CD8+ T cells.

Authors

Amare Aregay, Solomon Owusu Sekyere, Katja Deterding, ..., Michael Peter Manns, Markus Cornberg, Heiner Wedemeyer

Correspondence

Gastroenterology@uk-essen.de
(H. Wedemeyer)

Lay summary

Direct-acting antiviral therapy results in cure of hepatitis C virus (HCV) in almost all treated patients. However, the impacts of HCV cure on immune responses remain controversial. Whether immune responses to HCV recover is important in cases of re-exposure, or for the resolution of extrahepatic manifestations. The main finding of our study was that HCV-specific T cells remain functionally impaired despite HCV clearance. This finding could explain the fact that HCV cure does not lead to protective immunity and that re-infections have frequently been observed.

http://dx.doi.org/10.1016/j.jhep.2019.06.025
 J. Hepatol. 2019, 71, 889–899

Research Article
Viral Hepatitis

JOURNAL OF HEPATOLOGY

Elimination of hepatitis C virus has limited impact on the functional and mitochondrial impairment of HCV-specific CD8+ T cell responses

Amare Aregay[1], Solomon Owusu Sekyere[1], Katja Deterding[6], Kerstin Port[1], Julia Dietz[3,5], Caterina Berkowski[3,5], Christoph Sarrazin[3,5], Michael Peter Manns[1,2], Markus Cornberg[1,2,4,*,†], Heiner Wedemeyer[1,2,6,7,*,†]

[1]*Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany;* [2]*German Centre for Infection Research, Hannover, Germany (DZIF), Partner-site Hannover-Braunschweig, Germany;* [3]*German Centre for Infection Research (DZIF), External Partner Site Frankfurt, Germany;* [4]*Centre for Individualised Infection Medicine (CIIM), Hannover, Germany;* [5]*Department of Internal Medicine 1, University Hospital Frankfurt, Frankfurt, Germany;* [6]*Department of Gastroenterology and Hepatology, Essen University Hospital, Essen, Germany;* [7]*Integrated Research and Treatment Centre Transplantation (IFB-Tx), Hannover Medical School, Hannover, Germany*

Background & Aims: Hepatitis C virus (HCV)-specific CD8+ T cells are functionally impaired in chronic hepatitis C. Even though HCV can now be rapidly and sustainably cleared from chronically infected patients, the repercussions of HCV clearance on virus-specific CD8+ T cells remain elusive. Here, we aimed to investigate if HCV clearance by direct-acting antivirals (DAAs) could restore the functionality of exhausted HCV-specific CD8+ T cell responses.

Methods: HCV-specific CD8+ T cells in peripheral blood were obtained from 40 patients with chronic HCV infection, during and 6 months following IFN-free DAA therapy. These cells were analyzed for comprehensive phenotypes, proliferation, cytokine production, mitochondrial fitness and response to immune-checkpoint blockade.

Results: We show that, unlike activation markers that decreased, surface expression of multiple co-regulatory receptors on exhausted HCV-specific CD8+ T cells remained unaltered after clearance of HCV. Likewise, cytokine production by HCV-specific CD8+ T cells remained impaired following HCV clearance. The proliferative capacity of HCV multimer-specific CD8 + T cells was not restored in the majority of patients. Enhanced *in vitro* proliferative expansion of HCV-specific CD8+ T cells during HCV clearance was more likely in women, patients with low liver stiffness and low alanine aminotransferase levels in our cohort. Interestingly, HCV-specific CD8+ T cells that did not proliferate following HCV clearance could preferentially re-invigorate their proliferative capacity upon *in vitro* immune-checkpoint inhibition. Moreover, altered mitochondrial dysfunction exhibited by exhausted HCV-specific CD8+ T cells could not be normalized after HCV clearance.

Conclusion: Taken together, our data implies that exhausted HCV-specific CD8+ T cells remain functionally and metabolically impaired at multiple levels following HCV clearance in most patients with chronic hepatitis C. Our results might have implications in cases of re-infection with HCV and for HCV vaccine development.

Lay summary: Direct-acting antiviral therapy results in cure of hepatitis C virus (HCV) in almost all treated patients. However, the impacts of HCV cure on immune responses remain controversial. Whether immune responses to HCV recover is important in cases of re-exposure, or for the resolution of extrahepatic manifestations. The main finding of our study was that HCV-specific T cells remain functionally impaired despite HCV clearance. This finding could explain the fact that HCV cure does not lead to protective immunity and that re-infections have frequently been observed.

Introduction

Hepatitis C virus (HCV) persists and leads to chronicity in the majority of infected patients. Chronic hepatitis C is mainly characterized by functional impairment of virus-specific CD8+ T cells.[1,2] Several mechanisms have been suggested to lead to functional impairment of HCV-specific CD8+ T cells. However, the phenomena that have gained most attention for their contribution to failure of virus-specific CD8+ T cell responses are viral escape variants and CD8+ T cell exhaustion.[3–5] Persistent antigen stimulation during chronic HCV infection is suggested to lead to exhaustion of virus-specific CD8+ T cells. Exhausted virus-specific CD8+ T cells in-turn are characterized by the expression of multiple co-regulatory molecules,[3] limited proliferative capacity,[6] impaired cytokine production[3,6] as well as impaired metabolism.[7–9]

HCV therapy has changed fundamentally since 2013. Interferon (IFN)-free direct-acting antiviral (DAA) therapies resulted in rapid clearance of HCV from infected patients. DAA-mediated rapid HCV clearance is also sustained after the end of treatment; a phenomenon termed sustained virologic response (SVR).

Keywords: DAA; Direct-acting-antivirals; HCV-specific CD8+ T cell exhaustion; Mitochondria; Immune checkpoint blockade.
Received 15 March 2019; received in revised form 12 June 2019; accepted 19 June 2019; available online 8 July 2019

* Corresponding authors. Address: Department of Gastroenterology and Hepatology, Essen University Hospital, Hufelandstraße 44, D-45147 Essen, Germany. Tel.: +49 201 723 3610.
E-mail address: Gastroenterology@uk-essen.de (H. Wedemeyer).
† Contributed equally and share senior authorship.

Importantly, more than 95% SVR rates can now be achieved with IFN-free DAAs, making these therapies a treatment revolution relative to previous IFN-based treatment modalities.[10,11] This makes HCV a unique model to study the effects of clearance of a persistent viral infection on immune responses.

Several groups recently characterized the kinetics of soluble inflammatory mediators, mucosal-associated invariant T cells, gamma delta T cells and natural killer cell diversity during IFN-free DAA therapy.[12–20] These studies revealed partially conflicting data, with some studies showing declines in the activation of immune cells and functional improvements, while others suggested long-term imprints of HCV on immune cells that remain largely unchanged by HCV clearance. Following a first report of improvement of HCV-specific CD8+ T cell proliferative responses after HCV clearance,[21] subsequent studies revealed maintenance of HCV-specific CD8+ T cells with memory potential during DAA therapy.[22] Nevertheless, a detailed understanding of HCV-specific CD8+ T cell functions, metabolism, regulations and associations with underlying clinical patterns during DAA-mediated HCV clearance is still lacking.

Owing to the rapid cessation of HCV replication and the ensuing abrupt clearance of viral antigens mediated by DAAs, we questioned what the overall repercussions would be on exhausted HCV-specific CD8+ T cells, with a particular focus on clinical parameters, functional responses, metabolic fitness and response to immune-checkpoint blockade. As a result, we dissected the functional characteristics of HCV-specific CD8+ T cells upon IFN-free DAA therapy-mediated HCV clearance.

Materials and methods

Study participants

Peripheral whole blood samples were collected from a total of 47 HLA-A*02 positive patients at the liver outpatient clinic of Hannover Medical School. This included 40 HCV genotype 1 (1a or 1b) infected patients who received various combinations of IFN-free DAA therapies (namely: sofosbuvir, sofosbuvir/ledipasvir, sofosbuvir+simeprevir, sofosbuvir+daclatasvir, sofosbuvir/velpatasvir/voxilaprevir, ombitasvir/paritaprevir/ritonavir+dasabuvir, glecaprevir/pibrentasvir or grazoprevir/elbasvir) with or without ribavirin and 7 healthy blood donors. All selected patients were HBV surface antigen and anti-HIV negative at the time of blood collection. Of these, a total of 28 patients were non-cirrhotic with only mild fibrosis based on transient elastography and 12 patients had advanced stage liver fibrosis or cirrhosis. Blood samples collected at baseline (therapy start), End of therapy and 24 weeks following the end of DAA therapy were used for further experiments. All patients included in this study successfully cleared the virus at the end of therapy with subsequent attainment of SVR. The serum levels of alanine aminotransferase and aspartate aminotransferase were also normalized in the majority of these patients following HCV clearance. A summary of patient cohorts and characteristics are provided in Table S1 and Fig. S1. For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Ethics statement

This study was conducted in conformity with declaration of Helsinki (1975). Prior approval from local ethics committee of Hannover Medical School was obtained (approval number: 2148-2014) and written informed consent was obtained in all cases.

Results

Limited impact of IFN-free DAA-mediated HCV clearance on the exhausted phenotype of HCV-specific CD8+ T cells during chronic hepatitis C

Attempts at extensively characterizing the phenotypes of HCV-specific CD8+ T cells are often prevented by their low or undetectable frequencies with conventional *ex vivo* detection methods. To circumvent this, we employed the peptide MHCI (pMHCI) multimer-based magnetic-bead enrichment technique[23,24] which enabled us to not only detect virus-specific CD8+ T cells in all patient samples, at the 3 time points analyzed, but also to comprehensively characterize their phenotypes with considerable quantities of detectable cells (Fig. 1A-E). A cocktail of dextramers corresponding to 3 immuno-dominant HLA-A*02-restricted epitopes ($NS3_{1073-1081}$, $NS3_{1406-1415}$ and $Core_{132-140}$) were used for the analyses. In this regard, we analyzed the expression patterns of exhaustion (PD-1, TIM-3, Lag-3 and CD5), activation (CD38 and HLA-DR), terminal exhaustion (CD39 and CD127-$PD1^{high}$) and differentiation (CCR7, CD45RA and CD127) markers on HCV-specific CD8+ T cells. Frequencies and relative expressions of HCV-specific CD8+ T cells expressing the analyzed exhaustion markers were not altered after cessation of HCV replication (Fig. 1B and 1E). Meanwhile, the elevated frequencies of multimer-specific CD8+ T cells expressing CD38 and CD38 + HLA-DR (Fig. 1C and 1E), as well as terminally exhausted cells expressing CD39 (Fig. 1D and 1E), were significantly reduced following HCV clearance. Of note, subset differentiation based on CD127 and PD-1 expression did not show significant differences after HCV clearance (Fig. S2A, 2B and Fig. 1E) despite the trend in reduction of the CD127-$PD\text{-}1^{high}$ subpopulation. These data suggest that despite the observed reduction in activation state of HCV-specific CD8+ T cells, IFN-free DAA therapy-mediated HCV clearance does not fully restore the exhausted phenotype of HCV-specific CD8+ T cells during chronic HCV infection.

Proliferative capacity of HCV-specific CD8+ T cells is restored in a reduced fraction of patients with chronic HCV infection following HCV clearance

The proliferative capacity of virus-specific CD8+ T cells is limited during chronic HCV infection.[6,25] Herein, we assessed whether the proliferative potential of these T cells is restored following DAA therapy-mediated HCV clearance by analyzing the frequencies of multimer-specific CD8+ T cells. Peripheral blood mononuclear cells (PBMCs) were stimulated *in vitro* for 10 days with HLA-A*02 restricted peptides ($NS3_{1073-1081}$, $NS3_{1406-1415}$ or $Core_{132-140}$) that correspond to the well described HCV epitopes. In a combined analysis of *in vitro* peptide stimulated cells obtained from non-cirrhotic patients, the frequency of epitope-specific CD8+ T cells increased from baseline to 24 weeks after HCV clearance (FU24) (Fig. 2A and 2B). At an individual level, however, the increase in proliferation occurred in only 8/18 (44.4%) of patients (Fig. 2C). The T cells isolated from the remaining 10/18 (55.6%) patients did not show increased proliferation in response to any of the analyzed epitopes at FU24 upon *in vitro* stimulation (Fig. 2C). Of note, there was no significant increase in proliferative capacity of

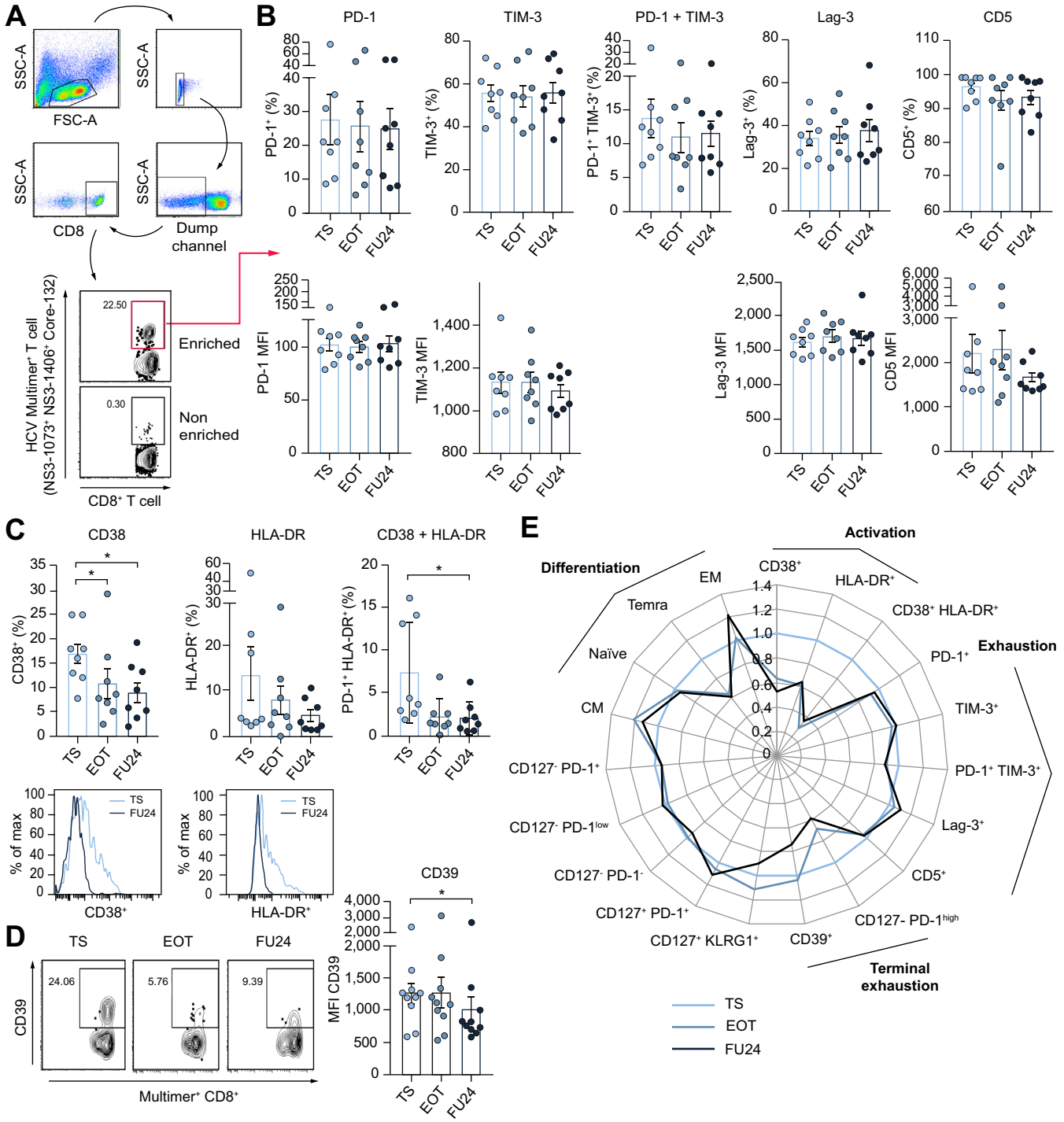


Fig. 1. Limited impact of IFN-free DAA-mediated HCV clearance on the exhausted phenotype of HCV-specific CD8+ T cells during chronic hepatitis C. A) Representative gating strategy used to identify multimer-specific CD8+ T cells before and after pMHC I magnetic-bead multimer-based enrichment in the same patient. PBMCs from patients at TS, EOT and FU24 were analyzed. (B) Summary frequencies and relative expressions of HCV-specific CD8+ T cells expressing the indicated co-regulatory markers at the 3 time points indicated. (C) Frequencies of epitope-specific CD8+ T cells expressing the analyzed activation markers. Representative overlay histograms depicting expressions of the analyzed activation markers before and after HCV clearance are shown below. (D) Exemplary FACS plots showing reduction in frequency of CD39 expressing epitope-specific CD8+ T cells in 1 patient (left) as well as a summary plot showing reduction in relative expression (MFI) of CD39 on multimer-specific CD8+ T cells during clearance of HCV. In all cases horizontal bars represent mean plus standard error of the means. (E) Spider plot summarizing frequencies of the analyzed activation, exhaustion, terminal exhaustion and differentiation markers on multimer-specific CD8+ T cells during DAA-mediated cessation of HCV stimulation. Values indicated in spider plot are means of frequencies of each marker at EOT and FU24 relative to corresponding means at TS. For comparison, baselines (TS) frequencies in spider plot were set at 1. Statistical significance was determined by *t* testing (*p <0.05). DAA, direct-acting antiviral; EOT, end of treatment; FU24, follow-up week 24; HCV, hepatitis C virus; IFN, interferon; MFI, mean fluorescence intensity; PBMCs, peripheral blood mononuclear cells; TS, treatment start. (This figure appears in colour on the web.)

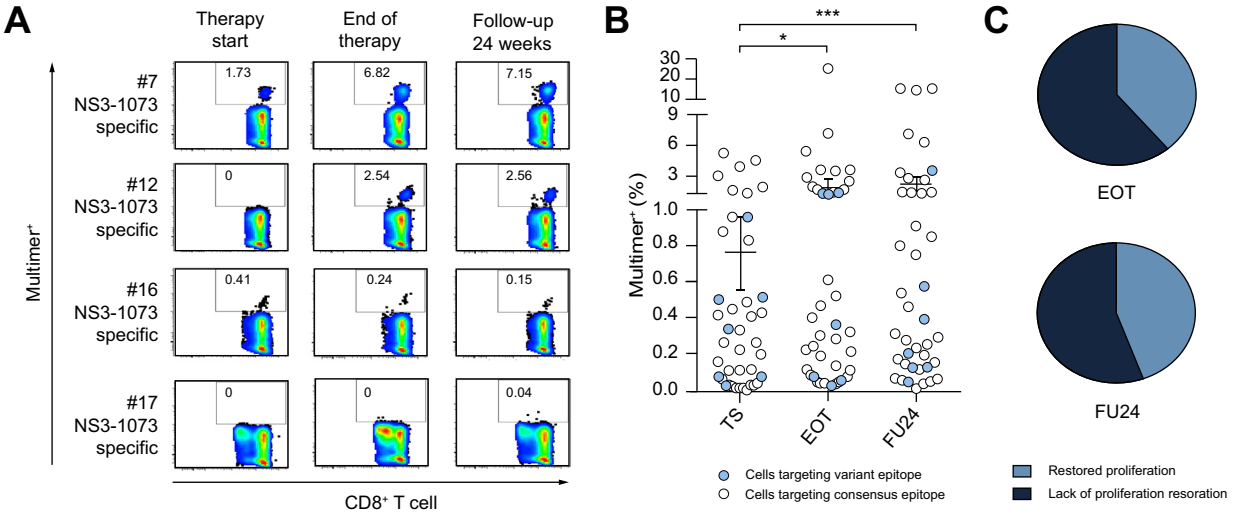


Fig. 2. Proliferative capacity of HCV-specific CD8+ T cells is restored in a reduced fraction of patients with chronic hepatitis C following HCV clearance. (A) Representative FACS plots depicting frequencies of multimer-specific CD8+ T cells for 4 patients during DAA-mediated HCV clearance. (B) Proliferative capacity of HCV multimer-specific CD8+ T cells after 10 days *in vitro* expansion with HLA-A*02 restricted peptides stimulations corresponding to the 3 most dominant HCV epitopes. Eighteen patients with mild stage of liver fibrosis (non-cirrhotic) were included in the analyses. HCV-specific CD8+ T cell response to any of the 3 dominant epitopes are shown. (C) Summary pie chart depicting fraction of multimer-specific CD8+ T cells which restored their proliferative capacity or cells did not restore their proliferative capacity at EOT and FU24 from 18 patient samples analyzed following virus clearance. A minimum of 2-fold increase in multimer-specific CD8+ T cell frequency from baseline to FU24 in any of the epitopes was used to define restored proliferation. Horizontal bars represent mean plus standard error of the mean. Statistical significance was determined by *t* testing (**p* <0.05, ****p* <0.001). DAA, direct-acting antiviral; EOT, end of treatment; FU24, follow-up week 24; HCV, hepatitis C virus. (This figure appears in colour on the web.)

HCV-specific CD8+ T cells among the cirrhotic cohort with advanced stages of liver disease (Fig. S4A).

Continuous antigen stimulation and hence lack of viral sequence variation at the corresponding epitopes were indicated to be associated with limited proliferative capacity of virus-specific CD8+ T cells during chronic hepatitis C.[21] In this study, the majority (87%) of HCV-specific CD8+ T cells recognized the consensus sequence of the epitopes (Fig. 2B, Fig. S3A and Table S2). In sum, our data suggest that successful clearance of HCV is not capable of completely restoring the proliferative capacity of virus-specific CD8+ T cells and that only partial restoration of HCV-specific CD8+ T cells' proliferative capacity is achieved following DAA-mediated HCV clearance.

Impaired cytokine production by HCV-specific CD8+ T cells remains impaired following cessation of HCV replication by IFN-free DAA therapy

Next, we assessed if the impaired functionality of HCV-specific CD8+ T cells manifested by reduced cytokine production during chronic hepatitis C[3,6] is restored upon rapid clearance of HCV. To this end, we analyzed the frequency of HCV-specific CD8+ T cells producing IFN-γ and MIP-1β, following 10 days of *in vitro* expansion. Despite rapid clearance of HCV, IFN-γ and MIP-1β production by HCV-specific CD8+ T cells, as well as polyfunctional IFN-γ + MIP-1β + -producing HCV-specific CD8+ T cells, remained impaired (Fig. 3A-C and Fig. S4B). Indeed, cytokine production by HCV-specific CD8+ T cells targeting wild-type or variant epitopes also remained invariably impaired during HCV clearance (Fig. 3C and Fig. S3B). Taken together, our data indicate that cytokine production by HCV-specific CD8+ T cells remains impaired even after rapid elimination of persisting HCV.

DAA-mediated HCV clearance does not reverse the altered mitochondrial and metabolic dysfunction of exhausted HCV-specific CD8+ T cells during chronic hepatitis C

The preceding results prompted us to further investigate if cessation of continuous antigen stimulation could impact mitochondrial dysfunction and metabolic fitness of exhausted HCV-specific CD8+ T cells. This is important since metabolic regulation of virus-specific CD8+ T cells impacts on multiple functional responses. We first assessed mitochondrial polarization (Fig. 4B and 4C), mass (Fig. 4B and 4D) and reactive oxygen species (ROS) levels (Fig. 4B and 4E) in functional cytomegalovirus (CMV)/Epstein-Barr virus (EBV)-specific CD8+ T cells and exhausted HCV-specific CD8+ T cells from healthy blood donors and patients with chronic HCV infection, respectively. Indeed, we could observe, after overnight anti-CD3 stimulation, that multimer-specific CD8+ T cells from patients with chronic HCV infection exhibit significantly reduced mitochondrial polarization (Fig. 4B) and increased mitochondrial mass (Fig. 4C). In addition, an elevated mitochondrial ROS level was detected in exhausted HCV-specific CD8+ T cells from patients with chronic HCV infection, unlike in CMV/EBV- specific CD8+ T cells from healthy blood donors (Fig. 4D). Interestingly, IFN-free DAA-mediated HCV clearance did not significantly reverse the observed mitochondrial dysfunction in multimer-specific CD8+ T cells from patients with chronic hepatitis C (Fig. 4B-D).

Previous reports indicated that exhausted virus-specific CD8 + T cells have distinct metabolic requirements compared to functional virus-specific CD8+ T cells.[7,8] Virus-specific CD8+ T cells that respond to chronic infections are reported to be characterized by increased expression of glucose transporter 1 (Glut1)[7,8] which leads to a reduction in metabolic plasticity of virus-specific CD8+ T cells.[7,8,26] Therefore, we set out to

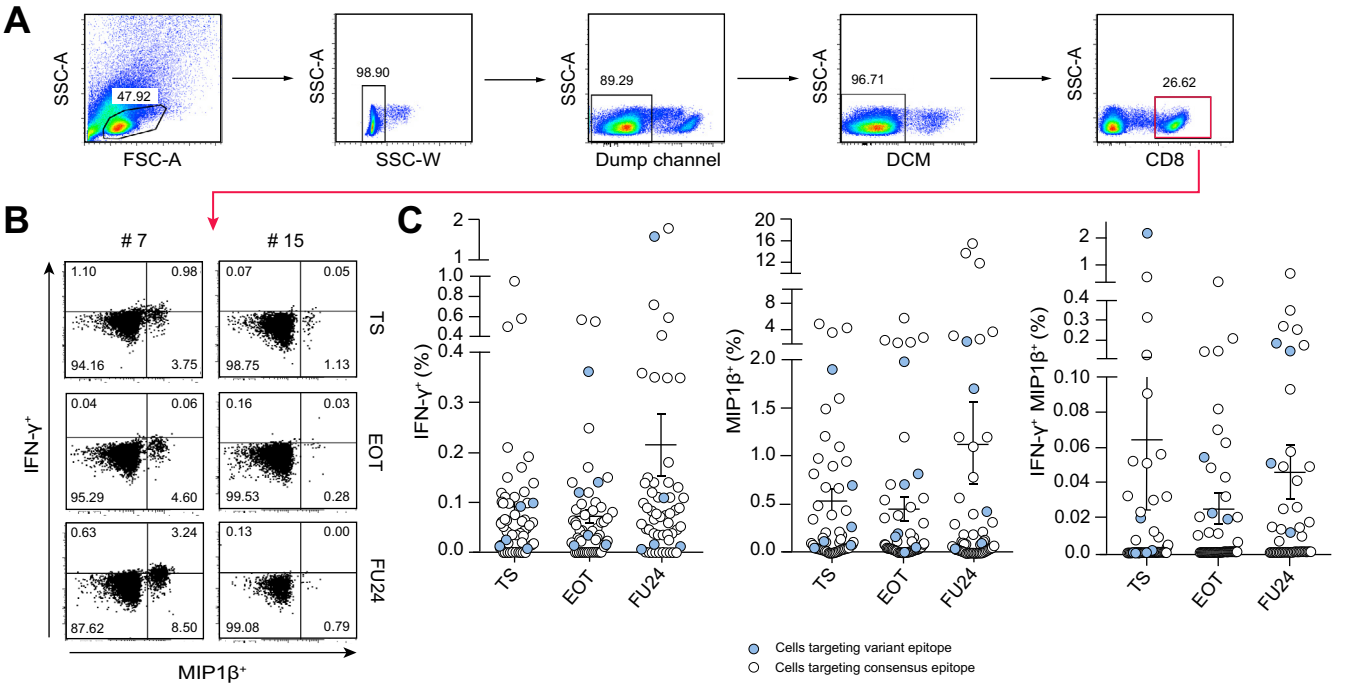


Fig. 3. Cytokine production by HCV-specific CD8+ T cells remain impaired following DAA-induced HCV clearance. PBMCs were expanded *in vitro* for 10 days with the well characterized HLA-A*02 restricted epitope-specific peptides. (A) Gating strategy used to analyze cytokine production by CD8+ T cells after short-term culture with HLA-A*02 restricted peptide stimulations. (B) Representative staining from 2 patients showing production of IFN-γ and MIP-1β by HCV-specific CD8+ T cells following re-stimulation with the respective peptides and intracellular staining during HCV clearance. (C) Frequencies of IFN-γ and MIP-1β producing HCV-specific CD8+ T cells as well as polyfunctional HCV-specific CD8+ T cells producing both IFN-γ and MIP-1β. Co-expression of 2 markers on a single cell was analyzed using Boolean analyses. Horizontal bars represent mean plus standard error of the means. DAA, direct-acting antiviral; EOT, end of treatment; FU24, follow-up week 24; HCV, hepatitis C virus; IFN, interferon; PBMCs, peripheral blood mononuclear cells; TS, treatment start. (This figure appears in colour on the web.)

investigate if Glut1 expression during chronic HCV infection could be normalized upon removal of persistent antigen. Frequencies of Glut1-expressing HCV-specific CD8+ T cells did not show significant alterations 24 weeks following HCV clearance (Fig. S6A, B). In sum, our data indicate that the altered mitochondrial dysfunction and metabolic dysregulation in exhausted multimer-specific CD8+ T cells are not reversed even after the persisting virus is cleared.

DAA-mediated clearance of HCV does not alter memory potential of HCV-specific CD8+ T cells

Immunotherapeutic boosting of virus-specific CD8+ T cell responses could be possible in cells possessing memory potential and self-renewal capability.[27–29] Therefore, it was of interest to assess whether removal of HCV by IFN-free DAAs could alter the memory potential of exhausted HCV-specific CD8+ T cells. Previous reports have demonstrated that in lymphocytic choriomeningitis virus-specific[29] and HCV-specific[30] CD8+ T cells, T cell factor 1 (Tcf1) expression characterizes cells with a memory-like phenotype and sustains CD8+ T cell responses during persistent infections. Remarkably, Tcf1-expressing HCV-specific CD8+ T cells in our cohort remain unaltered after HCV clearance (Fig. 5A and 5B). Previous reports[30] and our data (Fig. S2B) indicate that less-differentiated HCV-specific CD8+ T cells, that co-express CD127 and PD-1, are maintained after HCV clearance. Here, we further demonstrate that the Tcf1+CD127+PD-1+subpopulation is also maintained after HCV clearance (Fig. 5B). Collectively, our data suggests the presence, after the clearance of HCV, of an HCV-specific CD8+ T cell subpopulation with memory potential, corroborating previous findings on the maintenance of memory-like virus-specific CD8 T cells during chronic infections.

Immune-checkpoint inhibition can preferentially increase the proliferative capacity of HCV-specific CD8+ T cells whose proliferative capacity was not restored following HCV clearance

Subsequently, we questioned if functionally impaired HCV-specific CD8+ T cells, which might also harbor memory potential, present after removal of viral antigens could be revived through immune-checkpoint inhibition. This is very important as PD-1/PD-L1 blocking therapy, for example, is being used to treat chronic infections and cancer.[31] In addition to the 10 days of *in vitro* peptide stimulation discussed above, PBMCs were cultured with antibodies targeting PD-L1 and TIM-3 pathways on both day 0 and day 10. Blockade of PD-1/PD-L1 or TIM-3 pathways as well as the simultaneous blockade of the 2 pathways together resulted in enhanced proliferation of some of HCV-specific CD8+ T cells after HCV clearance (Fig. 6A and 6B). However, in line with our previous reports,[3] we could observe heterogeneous responses to immune checkpoint blockade responses at all 3 time points (Fig. 6A and 6B). Interestingly, HCV-specific CD8+ T cells that did not restore their proliferative capacity following cessation of HCV stimulation with IFN-free DAAs were able to preferentially increase their proliferation upon blockade of the PD-1/PD-L1 pathway (Fig. 6B). In contrast, blockade of PD-1/PD-L1 pathway in HCV-specific CD8+ T cells that have already increased their proliferative capacity upon removal of HCV triggering resulted in either no change in proliferation or even

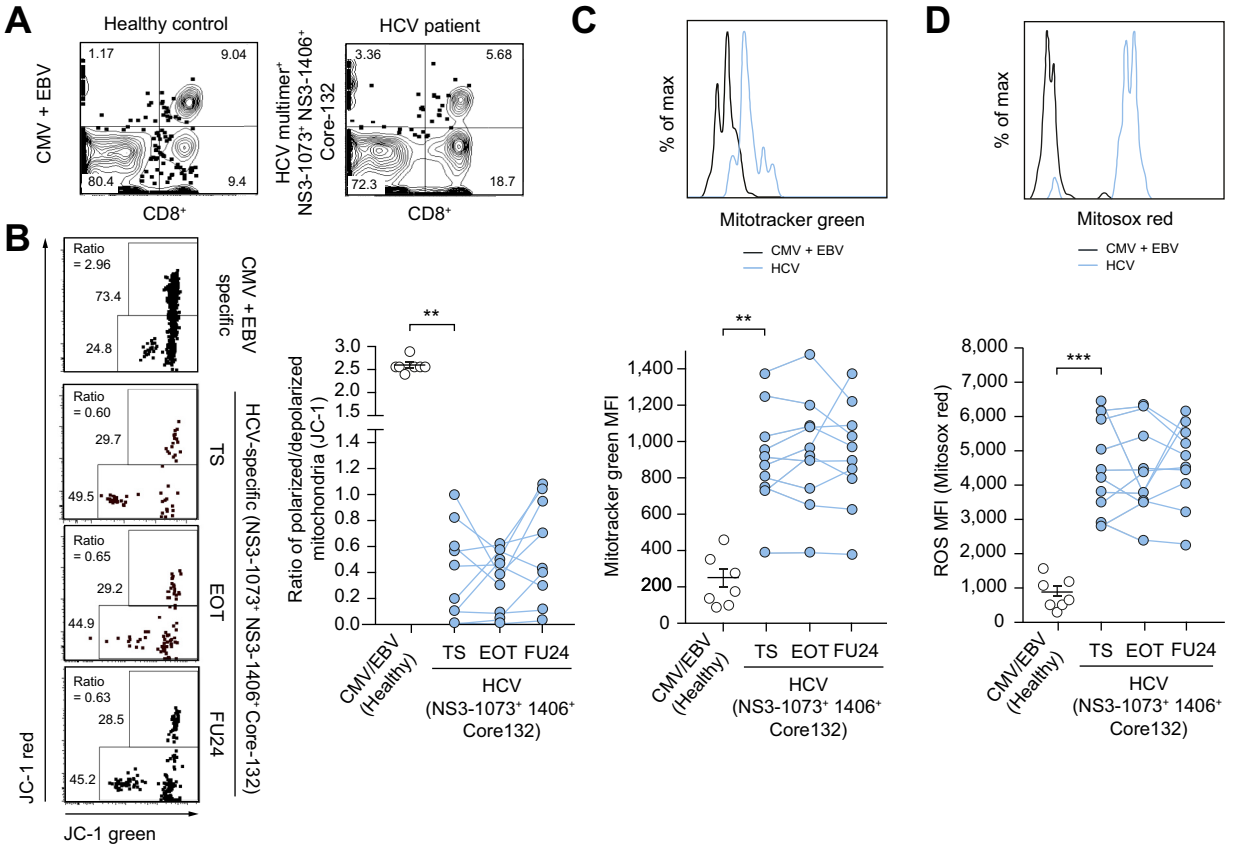


Fig. 4. DAA-induced cessation of chronic HCV stimulation does not reverse the altered mitochondrial dysfunction of exhausted HCV-specific CD8+ T cells during chronic HCV infection. (A) Exemplary stainings that show magnitude of multimer-specific CD8+ T cells after 8h stimulation with anti-CD3 antibody in healthy control and HCV patient. A cocktail of CMV and EBV multimers as well as HCV NS3-1073, NS3-1406 and Core 132 multimers were used to enrich multimer-specific CD8+ T cells in healthy controls and HCV patients, respectively. (B) Representative FACS plots showing mitochondrial polarization by JC-1 staining in a healthy control and HCV patient (during DAA therapy) (left) as well as Summary plots depicting ratio of polarized and depolarized multimer-specific CD8+ T cells by JC-1 staining after 8 h anti-CD3 stimulation (right). (C) Representative overlay histogram and summary plot indicating mitochondrial mass biogenesis as measured by mitotracker green MFI in CMV + EBV- and HCV-specific CD8+ T cells. (D) Representative overlay histogram and summary plot indicating mitochondrial ROS level as measured by mitosox red in CMV + EBV- and HCV-specific CD8+ T cells. PBMCs from 10 HCV patients and 7 healthy blood donors, that were available in sufficient cell quantity, were used for the analysis of mitochondrial functions in virus-specific CD8+ T cells. Horizontal bars represent mean plus standard error of the mean. Statistical significance was determined by t-testing (**p <0.01, ***p <0.001). CMV, cytomegalovirus; DAA, direct-acting antiviral; EBV, Epstein-Barr virus; HCV, hepatitis C virus; PBMCs, peripheral blood mononuclear cells; ROS, reactive oxygen species.

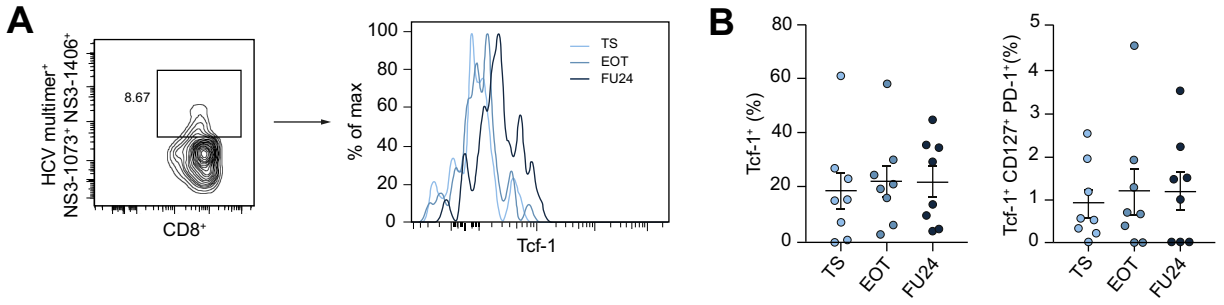


Fig. 5. DAA-mediated Clearance of HCV does not alter memory potential of HCV-specific CD8+ T cells. PBMCs obtained during DAA-mediated HCV clearance, were stimulated with HLA-A*02 restricted peptides overnight and multimer-specific CD8+ T cells were enriched employing magnetic-bead multimer-based enrichment technique. (A) Representative FACS plot depicting magnitude of HCV-specific CD8+ T cells and overlay histogram showing Tcf-1 expressing HCV-specific CD8+ T cells during HCV clearance in one patient. (B) Tcf-1 frequencies of multimer-specific CD8+ T cells as well as CD127 + PD1 + multimer-specific CD8+ T cells expressing Tcf1 during HCV clearance. Horizontal bars represent mean plus standard error of the means. DAA, direct-acting antiviral; EOT, end of treatment; FU24, follow-up week 24; HCV, hepatitis C virus; PBMCs, peripheral blood mononuclear cells; TS, treatment start.

reduced proliferative capacity in some cases (Fig. 6A). Furthermore, there was a positive but not significant correlation between Tcf1 expression and HCV-specific CD8+ T cell responses to PD-1/PD-L1 blockade (data not shown). Taken together, our data suggest that HCV-specific CD8+ T cells that could not restore their proliferative capacity upon clearance of HCV can preferentially revive their proliferative capacity through tailored immune-checkpoint inhibition.

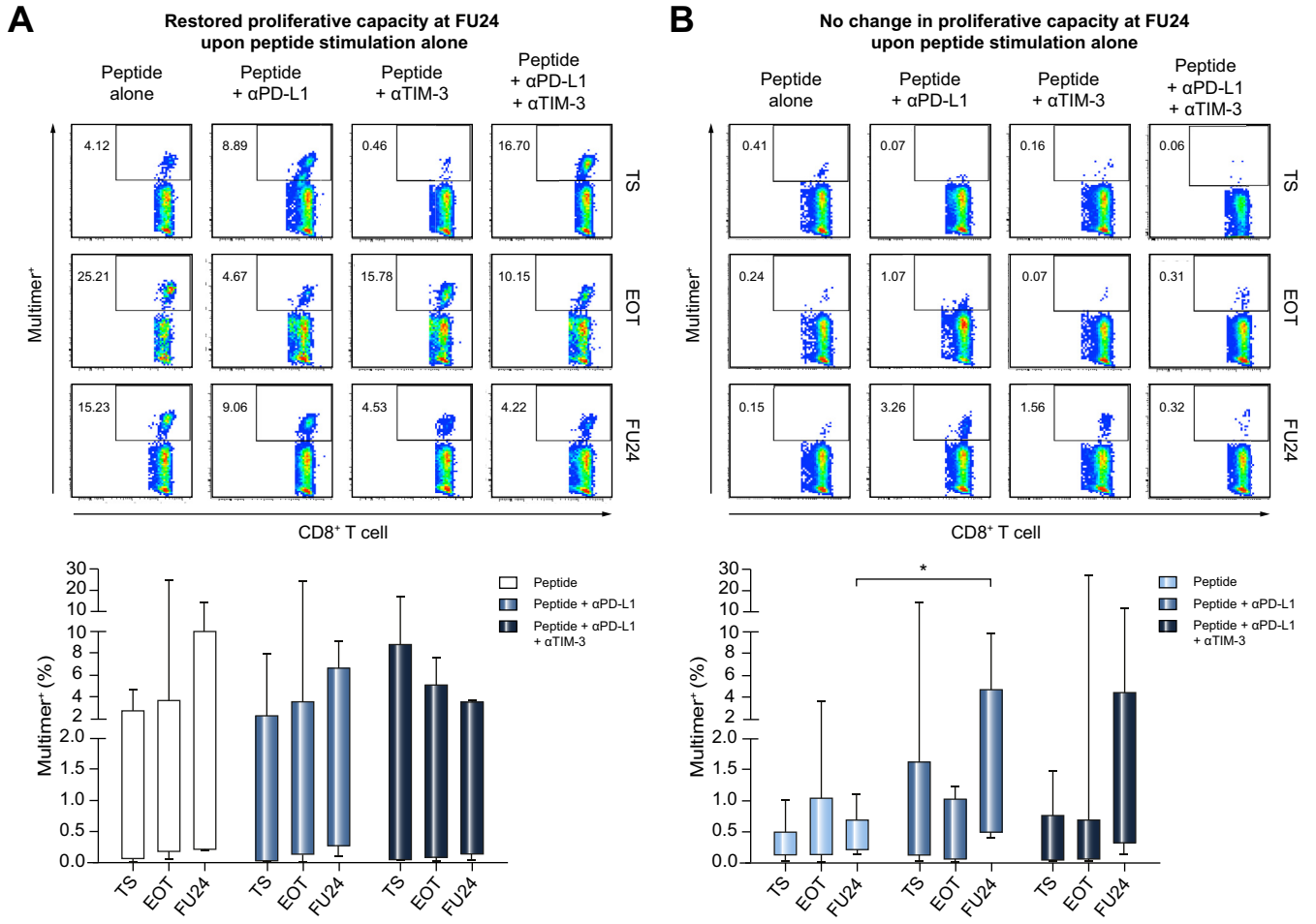


Fig. 6. Immune-checkpoint inhibition can preferentially increase proliferative capacity of HCV-specific CD8+ T cells whose proliferative capacity could not be restored following HCV clearance. PBMCs were expanded for 10 days *in vitro* with the HLA-A*02 restricted epitope-specific peptides alone or in combination with PD-L1 and/ or TIM-3 blocking antibodies. Representative stainings and summary frequencies depicting multimer-specific CD8+ T cell frequencies for 2 patients in whom (A) HCV-specific CD8+ T cells restored their proliferative capacity or (B) did not restore their proliferative capacity, following HCV clearance, upon HLA-A*02 restricted peptide stimulation alone. Corresponding plots for multimer-specific CD8+ T cells upon blockade of PD-L1 and/or TIM-3 pathways during DAA-mediated HCV clearance are also shown. In both cases, the upper panel shows representative staining for 1 patients and lower panel shows summary frequencies of multimer-specific CD8+ T cells, pooled from the respective patients, after *in vitro* peptide stimulation with or without the indicated blocking conditions. Cells available for all the 3 time points during DAA therapy and blocking conditions were included in the analyses. A minimum of 2-fold increase in multimer-specific CD8+ T cell frequency from baseline to FU24 in any of the epitopes was used to define restored proliferation. Horizontal bars represent mean plus standard error of the mean. Statistical significance was determined by multiple *t* testing with a false discovery rate of 10% (*p <0.05). DAA, direct-acting antiviral; FU24, follow-up week 24; HCV, hepatitis C virus; PBMCs, peripheral blood mononuclear cells. (This figure appears in colour on the web.)

Proliferative response of HCV-specific CD8+ T cells following HCV clearance are associated with underlying clinical patterns of patients

We then directed our investigation to see if underlying clinical and pathological characteristics could serve as predictive values for the diverse proliferative responses observed between patients following HCV clearance. In this light, patients were grouped based on baseline age, sex, liver inflammation, liver stiffness, and viral loads immediately before starting therapy. We could observe that female sex was associated with better proliferative response (ratio of HCV multimer-specific CD8+ T cell frequency at FU24 *vs.* treatment start) after HCV clearance (Fig. 7A). In addition, proliferative response of HCV-specific CD8+ T cells upon HCV clearance was significantly higher in patients with lower liver inflammation as measured by lower ALT levels (Fig. 7B). Likewise, HCV-specific CD8+ T cells from patients with lower liver stiffness value (<9.5 KPa) at baseline, and hence lower stage of liver damage, had a significantly improved proliferative response after clearance of HCV (Fig. 7C). Of note, in this cohort, HCV-specific CD8+ T cells proliferative response during HCV clearance did not differ based on patients' age, baseline viral load and previous IFN-based therapies. In conclusion, these data suggest that divergent and individualized HCV-specific CD8+ T cell proliferative responses observed during HCV clearance could partly be associated with underlying clinico-pathological patterns of patients.

Discussion

In this study, we comprehensively analyzed the main functional characteristics of exhausted HCV-specific CD8+ T cells during successful IFN-free DAA-mediated cure of HCV. We probed in detail the phenotypes of detectable exhausted HCV-specific CD8+ T cells during therapy mediated clearance of HCV. Importantly, we could show that impaired HCV-specific CD8+ T cell responses during chronic HCV infection, evidenced by limited

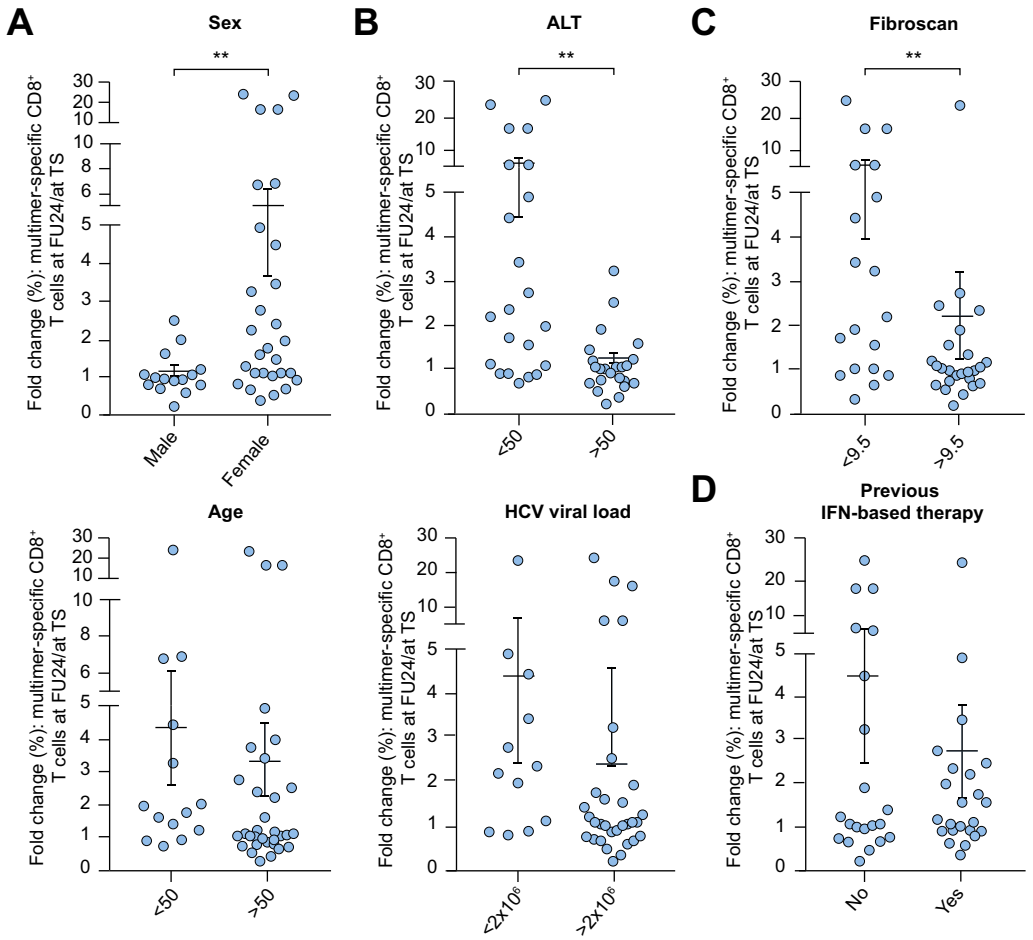


Fig. 7. Proliferative responses of HCV-specific CD8+ T cells following HCV clearance are associated with underlying clinico-pathological characteristics. (A) Associations between age and sex of patients with proliferative response of HCV-specific CD8+ T cells following HCV clearance. (B) Proliferative responses of HCV-specific CD8+ T cells upon HCV clearance based on underlying ALT levels and viral load immediately before starting therapy as well as (C) level of liver stiffness as measure by Fibroscan® immediately before therapy start. (D) Comparison of HCV-specific CD8+ T cell proliferative responses in DAA-treated patients who had previous IFN-based therapy and in patients that did not. Proliferative response was defined as the fold change at FU24 of multimer-specific CD8+ T cells frequencies over TS frequencies. Horizontal bars represent mean plus standard error of the mean. Statistical significance was determined by *t* testing (**p <0.01). ALT, alanine aminotransferase; DAA, direct-acting antiviral; FU24, follow-up week 24; HCV, hepatitis C virus; IFN, interferon; TS, treatment start.

proliferative capacity[6] and defective cytokine production,[3,6] are not restored in the majority of patients following successful HCV clearance. Indeed, HCV-specific CD8+ T cell proliferative responses during HCV clearance could partly be predicted by underlying clinico-pathological patterns of the patients. Most importantly, we hereby provided a firsthand investigation of the impact of completely clearing HCV on mitochondrial and metabolic functions of virus-specific CD8+ T cells. Moreover, we assessed the impact of an *in vitro* immune-checkpoint blockade on HCV-specific CD8+ T cells during HCV clearance. Collectively, our data implies that IFN-free DAA-mediated HCV clearance does not fully re-constitute exhausted HCV-specific CD8+ T cells during chronic hepatitis C.

Compelling data suggest that exhausted HCV-specific CD8+ T cells during chronic HCV infection upregulate expression of multiple co-regulatory molecules including but not limited to PD-1, TIM-3, Lag-3, CD5 and 2B4.[32–34] Our data, however, suggests that clearance of persistent antigens *per se* is not sufficient to reverse the upregulated expression of the analyzed co-regulatory molecules during HCV clearance. Prolonged exposure to persisting antigen leads to the long-lasting presence of activated and ultimately differentiated virus-specific CD8+ T cells[30,35,36] that lack effector functions.[37] Our results showing a reduction in activated HCV multimer-specific CD8+ T cells could be due to the associated loss of CD39-expressing terminally differentiated multimer-specific CD8+ T cells[36] after viral eradication. Indeed, previous studies have also showed that overtly-activated terminally differentiated antigen-specific CD8+ T cells cannot survive following removal of the ongoing antigen.[4,30,35,36]

One major question that needs to be addressed in patients attaining SVR is whether the ensuing virus-specific CD8+ T cells are able to provide protection against re-infection. Indeed, several cases of re-infection with HCV have been reported in patients who already cleared HCV with DAAs.[38,39] Interestingly, studies performed in chimpanzee models indicated that virus-specific CD8+ T cells present after DAA-mediated HCV clearance failed to prevent development of chronicity after re-infection.[40] In the human HCV infection model, specific restoration of the proliferative capacity of HCV-epitope-specific CD8+ T cells was reported in the majority (82%) of patients after HCV cure.[21] In contrast, our data suggest a partial restoration of proliferative

capacity in less than half of patients analyzed. In addition, cytokine production by HCV-specific CD8+ T cells following HCV clearance remained impaired in our cohort, further supporting persistence of functionally impaired HCV-specific CD8+ T cells following cessation of antigen stimulation. Possible reasons for these discrepancies might be the different socio-demographic and clinical backgrounds of patients or the type of DAA drugs and inclusion of ribavirin. Indeed, patients included in our cohort received 8 different types of IFN-free DAAs (5 sofosbuvir-based and 3 without sofosbuvir) (Table S1) unlike patients in the former studies where mainly non-sofosbuvir but protease inhibitor based DAAs were administered to patients.[21] Of note, inhibition of the HCV protease may have additional effects on innate immunity[41] which may also influence adaptive immune responses. Notably, the majority of HCV-specific CD8+ T cells which could significantly restore their proliferative capacity in our study were derived from patients who received DAAs that do not contain sofosbuvir but protease inhibitors (Fig. S5A,B). Additional studies are required to determine if distinct DAAs may display differential effects on cellular immune responses. Preliminary findings from our group indicate that CMV/EBV-specific CD8+ T cells derived from patients who received protease inhibitors may display an increased proliferative capacity at FU24 (data not shown). Furthermore, the epitopes targeted could have also played a role in the discrepancies observed among studies. Unfortunately, HLA B27 epitopes could not be studied in detail here due to limited numbers of patients. It is possible that findings will be different for this immune dominant epitope as shown previously.[21] We were not able to expand $NS5B_{2841\text{-}249}$ specific CD8+ T cells sufficiently to explore this question in detail (data not shown). We also have to highlight that we only studied HCV genotype 1-infected patients to exclude potential impacts of different HCV genotypes. There were no differences between genotype 1a and 1b infected patients in this study.

Notably, the clinical backgrounds of patients included in this study also played a role in the observed heterogeneous proliferative responses following HCV clearance (Fig. 7). Interestingly, female sex and absence of advanced fibrosis were associated with better restoration of HCV-specific T cell proliferation in our cohort. These factors were associated with SVR when treatment regimens with suboptimal antiviral efficacy were used (*e.g.* sofosbuvir+ribavirin)[42]). Collectively, these data suggest that the ensuing HCV-specific CD8+ T cells after DAA-mediated HCV clearance still remain largely impaired in their antiviral responses, with possible implications for re-infection or the use of suboptimal DAA regimens. In fact, the persistence of exhausted HCV-specific CD8+ T cells following HCV clearance might have clinical implications beyond re-infection, affecting the persistence of extrahepatic manifestations,[43] the risk of hepatocellular carcinoma (HCC) recurrence (supported by our recent findings on HCC-specific T cells[44]), as well as possible risk of reactivation of unrelated viruses like HBV and herpes simplex virus.

Persistent HCV infection silences, and sometimes completely abrogates, HCV-specific CD8+ T cell responses through several mechanisms. In addition to continual antigen triggering-induced virus-specific CD8+ T cell exhaustion, loss of CD4 T cell help,[45] presence of immune modulating cytokines[46] and inability to recognize viral epitopes because of viral escape variants[5] also play a role in functional impairment of virus-specific CD8+ T cells during chronic infections. Importantly, the vast majority (87%) of epitope-specific CD8+ T cells in our cohort recognized wild-type epitope sequences (Fig. S3 and Table S2). Notably, we did not observe a major difference in proliferative capacity or cytokine production capabilities between cells recognizing conserved epitopes and cells targeting escape variants during HCV clearance (Fig. S3). This could be due to the reduced number (17%) of cells that failed to recognize antigens due to variant epitopes in our cohort. Meanwhile, in previous studies, where nearly half of epitope-specific CD8+ T cells recognized variant sequence, a better restoration of proliferative capacity after HCV clearance was reported for CD8+ T cells that target wild-type sequences.[21] Thus, upon inclusion of more patients, it is possible that the repercussions of HCV clearance in virus-specific CD8+ T cells with persistent T cell receptor stimulation might differ from cells that do not recognize viral antigen due to escape variants.

Mitochondrial plasticity and bioenergetics dynamically influence T cell functionality and survival at various levels.[7–9,47,48] Indeed, polarization of mitochondria and ROS levels are shown to be associated with activation[47,49] as well as cell death[50] of T cells. Therefore, we wondered how successful halting of persistent antigen triggering could impact mitochondrial polarization, ROS level and mitochondrial mass. In this study, we showed that HCV-specific CD8+ T cells during chronic HCV display altered mitochondrial dysfunction mainly characterized by depolarized mitochondria, increased ROS level and mass biogenesis compared to CMV/ EBV-specific CD8+ T cells isolated from HCV negative persons. The increased abundance of mitochondrial mass in exhausted HCV-specific CD8+ T cells could be due to defects in mitophagy, a cellular process of removing damaged dysfunctional mitochondria to maintain proper physiological functions of cells.[51] On the other hand, the observed accumulation of mitochondrial ROS in HCV-specific CD8+ T cells can serve a dual role in firing-up activation and cellular degeneration[52] subsequently leading to exhaustion of T cells. Intriguingly, mitochondrial dysfunction in HCV-specific CD8+ T cells was not normalized after HCV clearance. Furthermore, we showed that Glut-1 expression on HCV-specific CD8+ T cells remained unaltered after HCV clearance. To the best of our knowledge, there was no other study that investigated repercussions of persistent antigen clearance on the mitochondrial functionality of virus-specific CD8+ T cells.

Notably, DAA-mediated HCV clearance did not affect the memory potential of HCV-specific CD8+ T cells. Tcf-1-expressing HCV-specific CD8 T cells, which in principle have improved recall response and proliferative capacity independent of antigen,[29,30,27,29] remained stable after HCV elimination in our cohort. Thus, we wondered if further immunotherapeutic re-invigoration of HCV-specific CD8+ T cells could be possible after HCV clearance. Remarkably, HCV-specific CD8+ T cells whose proliferative capacity could not be restored following HCV clearance selectively re-invigorated their proliferative potential upon *in vitro* PD-1/PD-L1 pathway blockade. This is in line with our previous report suggesting individualized heterogeneous responses to targeting of co-regulatory receptor blockades[3] and clinical trials that indicated efficacy of checkpoint blockade in less than half of patients with chronic HCV.[53,54] To the best of our knowledge, no other study has previously described effects of immune-checkpoint blockade in exhausted virus-specific CD8+ T cells from which the persisting antigen has successfully been cleared. One limitation of this study is that we have no complete data on Tcf1 expression for all experiments, *e.g.* when we blocked both PD-L1 and TIM-3.

We suggest that virus-specific CD8+ T cells that restored their proliferative capacity following HCV clearance might have differentiated terminally and further immunotherapeutic interferences, such as checkpoint blockades, have no further effect. In contrast, cells which could not proliferate after HCV clearance might not have been ultimately differentiated and could still have possessed self-differentiation[27,30] capacity upon blockade of immune-checkpoints. In summary, this comprehensive study of functionally and metabolically impaired peripheral HCV-specific CD8+ T cells during DAA-mediated HCV clearance provides compelling evidence that successful cessation of HCV stimulation does not universally restore HCV-specific CD8+ T cell functional responses and metabolism. These results might have implications in case of re-infection with HCV, for persistence of extrahepatic manifestations and for HCV vaccine development, as we also defined subgroups of patients that may mount sufficient immunity to potentially protect from re-infection. Finally, the data on mitochondrial dysfunction and immune-checkpoint blockade open scenarios for alternative treatment options to enhance antiviral immunity. Thus, HCV can serve as an *in vivo* human model to investigate the impact of a complete clearance of a persisting viral infection on immune responses, as well as on the clinical sequelae associated with viral infections in general. Indeed, investigations that look at HCV-specific CD8+ T cell responses beyond 6 months of follow-up are needed to understand the long-term impact of HCV cure. Further studies also need to consider the impact of successful IFN-free DAA-mediated HCV clearance on virus-specific CD8+ T cells residing inside the liver, which is the site of HCV replication.

Financial support

This work was supported by centre for research grants SFB900 “Chronic Infections: Microbial Persistence and its Control” and SFB 738 of the German Research Foundation (DFG), the IFB-Tx (BMBF 01E01302), German Centre for Infection Research (DZIF) and centre for infection Biology of Hannover Biomedical Research School.

Conflict of interest

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

Authors’ contributions

AA, SOS, HW and MC were involved in designing of experiments, drafting and revision of the manuscript. KD, KP and MPM were involved in recruitment of patients. JD, CB and CS sequenced the HCV epitopes. AA acquired and analyzed the data. HW and MC were involved in study concept inception, approval and finalization of the manuscript. All authors read and agreed to the manuscript.

Acknowledgements

We would like to thank all study participants, study nurses, physicians and colleagues who contributed directly or indirectly to this study. Parts of the data included in this manuscript were presented at American Association for Study of Liver Diseases (AASLD) (October, 2017, Washington DC, USA), Joint annual meeting DGI-DZIF (September, 2017, Hamburg Germany), Euro Immunology (June, 2017, Madrid, Spain) and at 25th annual symposium on hepatitis C virus and related viruses (October 2018, Dublin, Ireland).

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.06.025.

References

Author names in bold designate shared co-first authorship

[1] **Heim MH**, **Thimme R**. Innate and adaptive immune responses in HCV infections. J Hepatol 2014;61:S14–25.
[2] **Rehermann B**, **Thimme R**. Insights From antiviral therapy into immune responses to hepatitis B and C virus infection. Gastroenterology 2019;156:369–383.
[3] Owusu Sekyere S, Suneetha PV, Kraft AR, Zhang S, Dietz J, Sarrazin C, et al. A heterogeneous hierarchy of co-regulatory receptors regulates exhaustion of HCV-specific CD8 T cells in patients with chronic hepatitis C. J Hepatol 2015;62:31–40.
[4] Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol 2009;10:29–37.
[5] Kasprowicz V, Kang YH, Lucas M, Schulze zur Wiesch J, Kuntzen T, Fleming V, et al. Hepatitis C virus (HCV) sequence variation induces an HCV-specific T-cell phenotype analogous to spontaneous resolution. J Virol 2010;84:1656–1663.
[6] Wedemeyer H, He XS, Nascimbeni M, Davis AR, Greenberg HB, Hoofnagle JH, et al. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. J Immunol (Baltimore, Md: 1950) 2002;169:3447–3458.
[7] Schurich A, Pallett LJ, Jajbhay D, Wijngaarden J, Otano I, Gill US, et al. Distinct metabolic requirements of exhausted and functional virus-specific CD8 T cells in the same host. Cell Rep 2016;16:1243–1252.
[8] Bengsch B, Johnson AL, Kurachi M, Odorizzi PM, Pauken KE, Attanasio J, et al. Bioenergetic insufficiencies due to metabolic alterations regulated by the inhibitory receptor PD-1 are an early driver of CD8(+) T cell exhaustion. Immunity 2016;45:358–373.
[9] Fisicaro P, Barili V, Montanini B. Targeting mitochondrial dysfunction can restore antiviral activity of exhausted HBV-specific CD8 T cells in chronic hepatitis B. Nat Med 2017;23:327–336.
[10] Wedemeyer H. Towards interferon-free treatment for all HCV genotypes. Lancet 2015;385:2443–2445.
[11] Vermehren J, Park JS, Jacobson IM, Zeuzem S. Challenges and perspectives of direct antivirals for the treatment of hepatitis C virus infection. J Hepatol 2018;69:1178–1187.
[12] **Hengst J**, **Strunz B**, Deterding K, Ljunggren HG, Leeansyah E, Manns MP, et al. Nonreversible MAIT cell-dysfunction in chronic hepatitis C virus infection despite successful interferon-free therapy. Eur J Immunol 2016;46:2204–2210.
[13] **Strunz B**, **Hengst J**, Deterding K, Manns MP, Cornberg M, Ljunggren HG, et al. Chronic hepatitis C virus infection irreversibly impacts human natural killer cell repertoire diversity. Nat Commun 2018;9:2275.
[14] **Hengst J**, **Falk CS**, **Schlaphoff V**, Deterding K, Manns MP, Cornberg M, et al. Direct-acting antiviral-induced hepatitis C virus clearance does not completely restore the altered cytokine and chemokine milieu in patients with chronic hepatitis C. J Infect Dis 2016;214:1965–1974.
[15] Serti E, Chepa-Lotrea X, Kim YJ, Keane M, Fryzek N, Liang TJ, et al. Successful interferon-free therapy of chronic hepatitis C virus infection normalizes natural killer cell function. Gastroenterology 2015;149, 190-200.e192.
[16] Spaan M, van Oord G, Kreefft K, Hou J, Hansen BE, Janssen HL, et al. Immunological analysis during interferon-free therapy for chronic hepatitis C virus infection reveals modulation of the natural killer cell compartment. J Infect Dis 2016;213:216–223.
[17] Golden-Mason L, McMahan RH, Kriss MS, Kilgore AL, Cheng L, Dran RJ, et al. Early and late changes in natural killer cells in response to ledipasvir/sofosbuvir treatment. Hepatol Commun 2018;2:364–375.
[18] Burchill MA, Golden-Mason L, Wind-Rotolo M, Rosen HR. Memory re-differentiation and reduced lymphocyte activation in chronic HCV-infected patients receiving direct-acting antivirals. J Viral Hepatitis 2015;22:983–991.

[19] Owusu Sekyere S, Suneetha PV, Hardtke S, Falk CS, Hengst J, Manns MP, et al. Type I interferon elevates co-regulatory receptor expression on CMV- and EBV-specific CD8 T cells in chronic hepatitis C. Front Immunol 2015;6:270.
[20] **Ravens S**, **Hengst J**, Schlapphoff V, Deterding K, Dhingra A, Schultze-Florey C, et al. Human gammadelta T cell receptor repertoires in peripheral blood remain stable despite clearance of persistent hepatitis C virus infection by direct-acting antiviral drug therapy. Front Immunol 2018;9:510.
[21] Martin B, Hennecke N, Lohmann V, Kayser A, Neumann-Haefelin C, Kukolj G, et al. Restoration of HCV-specific CD8+ T cell function by interferon-free therapy. J Hepatol 2014;61:538–543.
[22] Wieland D, Kemming J, Schuch A, Emmerich F, Knolle P, Neumann-Haefelin C, et al. TCF1(+) hepatitis C virus-specific CD8(+) T cells are maintained after cessation of chronic antigen stimulation. Nat Commun 2017;8:15050.
[23] Schmidt J, Neumann-Haefelin C, Altay T, Gostick E, Price DA, Lohmann V, et al. Immunodominance of HLA-A2-restricted hepatitis C virus-specific CD8+ T cell responses is linked to naive-precursor frequency. J Virol 2011;85:5232–5236.
[24] Nitschke K, Flecken T, Schmidt J, Gostick E, Marget M, Neumann-Haefelin C, et al. Tetramer enrichment reveals the presence of phenotypically diverse hepatitis C virus-specific CD8+ T cells in chronic infection. J Virol 2015;89:25–34.
[25] Klenerman P, Thimme R. T cell responses in hepatitis C: the good, the bad and the unconventional. Gut 2012;61:1226–1234.
[26] Pearce EL, Pearce EJ. Metabolic pathways in immune cell activation and quiescence. Immunity 2013;38:633–643.
[27] Im SJ, Hashimoto M, Gerner MY, Lee J, Kissick HT, Burger MC, et al. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. Nature 2016;537:417–421.
[28] He R, Hou S, Liu C, Zhang A, Bai Q, Han M, et al. Follicular CXCR5-expressing CD8(+) T cells curtail chronic viral infection. Nature 2016;537:412–428.
[29] **Utzschneider DT**, **Charmoy M**, **Chennupati V**, **Pousse L**, Ferreira DP, Calderon-Copete S, et al. T cell factor 1-expressing memory-like CD8(+) T cells sustain the immune response to chronic viral infections. Immunity 2016;45:415–427.
[30] Wieland D, Kemming J, Schuch A, Emmerich F, Knolle P, Neumann-Haefelin C, et al. TCF1(+) hepatitis C virus-specific CD8(+) T cells are maintained after cessation of chronic antigen stimulation. Nat Commun 2017;8:15050.
[31] Zou W, Wolchok JD, Chen L. PD-L1 (B7–H1) and PD-1 pathway blockade for cancer therapy: Mechanisms, response biomarkers, and combinations. Sci Transl Med 2016;8:328rv324.
[32] **Bengsch B**, **Seigel B**, Ruhl M, Timm J, Kuntz M, Blum HE, et al. Coexpression of PD-1, 2B4, CD160 and KLRG1 on exhausted HCV-specific CD8+ T cells is linked to antigen recognition and T cell differentiation. PLoS Pathog 2010;6 e1000947.
[33] Nakamoto N, Kaplan DE, Coleclough J, Li Y, Valiga ME, Kaminski M, et al. Functional restoration of HCV-specific CD8 T cells by PD-1 blockade is defined by PD-1 expression and compartmentalization. Gastroenterology 2008;134:1927–1937, 1937.e1921-1922.
[34] Alanio C, Nicoli F, Sultanik P, Flecken T, Perot B, Duffy D, et al. Bystander hyperactivation of preimmune CD8+ T cells in chronic HCV patients. eLife 2015;4 e07916.
[35] Paley MA, Kroy DC, Odorizzi PM, Johnnidis JB, Dolfi DV, Barnett BE, et al. Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. Science (New York, NY) 2012;338:1220–1225.
[36] **Gupta PK**, **Godec J**, **Wolski D**, Adland E, Yates K, Pauken KE, et al. CD39 expression identifies terminally exhausted CD8+ T cells. PLoS Pathog 2015;11 e1005177.
[37] Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, et al. Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med 1998;188:2205–2213.
[38] **Ingiliz P**, **Martin TC**, Rodger A, Stellbrink HJ, Mauss S, Boesecke C, et al. HCV reinfection incidence and spontaneous clearance rates in HIV-positive men who have sex with men in Western Europe. J Hepatol 2017;66:282–287.
[39] Grebely J, Dore GJ, Greenwald R, Swan T, Barua S, Taylor LE. Hepatitis C virus treatment and persons who inject drugs. Ann Intern Med 2016;164:203.
[40] Callendret B, Eccleston HB, Satterfield W, Capone S, Folgori A, Cortese R, et al. Persistent hepatitis C viral replication despite priming of functional CD8+ T cells by combined therapy with a vaccine and a direct-acting antiviral. Hepatology 2016;63:1442–1454.
[41] Welsch C, Haselow K, Gouttenoire J, Schneider M, Morikawa K, Martinez Y, et al. Hepatitis C virus variants resistant to macrocyclic NS3-4A inhibitors subvert IFN-beta induction by efficient MAVS cleavage. J Hepatol 2015;62:779–784.
[42] Albillos A, Lario M, Alvarez-Mon M. Cirrhosis-associated immune dysfunction: distinctive features and clinical relevance. J Hepatol 2014;61:1385–1396.
[43] Aregay A, Dirks M, Schlaphoff V, Owusu Sekyere S, Haag K, Falk CS, et al. Systemic inflammation and immune cell phenotypes are associated with neuro-psychiatric symptoms in patients with chronic inflammatory liver diseases. Liver Int 2018;38:2317–2328.
[44] Owusu Sekyere S, Schlevogt B, Mettke F, Kabbani M, Deterding K, Wirth TC, et al. HCC immune surveillance and antiviral therapy of hepatitis C virus infection. Liver Cancer 2019;8:41–65.
[45] Battegay M, Moskophidis D, Rahemtulla A, Hengartner H, Mak TW, Zinkernagel RM. Enhanced establishment of a virus carrier state in adult CD4+ T-cell-deficient mice. J Virol 1994;68:4700–4704.
[46] Ng CT, Oldstone MB. IL-10: achieving balance during persistent viral infection. Curr Top Microbiol Immunol 2014;380:129–144.
[47] Sena LA, Li S, Jairaman A, Prakriya M, Ezponda T, Hildeman DA, et al. Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. Immunity 2013;38:225–236.
[48] Buck MD, O'Sullivan D, Pearce EL. T cell metabolism drives immunity. J Exp Med 2015;212:1345–1360.
[49] D'Souza AD, Parikh N, Kaech SM, Shadel GS. Convergence of multiple signaling pathways is required to coordinately up-regulate mtDNA and mitochondrial biogenesis during T cell activation. Mitochondrion 2007;7:374–385.
[50] Devadas S, Zaritskaya L, Rhee SG, Oberley L, Williams MS. Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and fas ligand expression. J Exp Med 2002;195:59–70.
[51] Pickles S, Vigie P, Youle RJ. Mitophagy and quality control mechanisms in mitochondrial maintenance. Curr Biol: CB 2018;28:R170–r185.
[52] Murphy MP, Siegel RM. Mitochondrial ROS fire up T cell activation. Immunity 2013;38:201–202.
[53] Gardiner D, Lalezari J, Lawitz E, DiMicco M, Ghalib R, Reddy KR, et al. A randomized, double-blind, placebo-controlled assessment of BMS-936558, a fully human monoclonal antibody to programmed death-1 (PD-1), in patients with chronic hepatitis C virus infection. PLoS ONE 2013;8 e63818.
[54] Sangro B, Gomez-Martin C, de la Mata M, Inarrairaegui M, Garralda E, Barrera P, et al. A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C. J Hepatol 2013;59:81–88.