Research Article
Viral Hepatitis

JOURNAL OF HEPATOLOGY

Functional reconstitution of HBV-specific CD8 T cells by *in vitro* polyphenol treatment in chronic hepatitis B

Greta Acerbi[1,2], Ilaria Montali[1,2], Gennaro Domenico Ferrigno[1,2], Valeria Barili[1,2], Simona Schivazappa[2], Arianna Alfieri[2], Diletta Laccabue[1,2], Alessandro Loglio[3], Marta Borghi[3], Marco Massari[4], Marzia Rossi[1,2], Andrea Vecchi[1,2], Amalia Penna[2], Carolina Boni[2], Gabriele Missale[1,2], Pietro Lampertico[3,5], Daniele Del Rio[6], Carlo Ferrari[1,2,*], Paola Fisicaro[1,2]

[1]*Department of Medicine and Surgery, University of Parma, Parma, Italy;* [2]*Laboratory of Viral Immunopathology, Unit of Infectious Diseases and Hepatology, Azienda Ospedaliero-Universitaria di Parma, Parma, Italy;* [3]*Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Division of Gastroenterology and Hepatology, Milan, Italy;* [4]*Unit of Infectious Diseases, Azienda USL-IRCCS di Reggio Emilia, Reggio Emilia, Italy;* [5]*Department of Pathophysiology and Transplantation, CRC 'A. M. and A. Migliavacca' Center for Liver Disease, University of Milan, Milan, Italy;* [6]*Department of Food and Drug, University of Parma, Parma, Italy*

Background & Aims: In chronic HBV infection, mitochondrial functions and proteostasis are dysregulated in exhausted HBV-specific CD8 T cells. To better characterise the potential involvement of deregulated protein degradation mechanisms in T cell exhaustion, we analysed lysosome-mediated autophagy in HBV-specific CD8 T cells. Bioactive compounds able to simultaneously target both mitochondrial functions and proteostasis were tested to identify optimal combination strategies to reconstitute efficient antiviral CD8 T cell responses in patients with chronic HBV infection.

Methods: Lysosome-mediated degradation pathways were analysed by flow cytometry in virus-specific CD8 T cells from patients with chronic HBV infection. Mitochondrial function, intracellular proteostasis, and cytokine production were evaluated in HBV-peptide-stimulated T cell cultures, in the presence or absence of the polyphenols resveratrol (RSV) and oleuropein (OLE) and their metabolites, either alone or in combination with other bioactive compounds.

Results: HBV-specific CD8 T cells from patients with CHB showed impaired autophagic flux. RSV and OLE elicited a significant improvement in mitochondrial, proteostasis and antiviral functions in CD8 T cells. Cytokine production was also enhanced by synthetic metabolites, which correspond to those generated by RSV and OLE metabolism *in vivo*, suggesting that these polyphenols may also display an effect after transformation *in vivo*. Moreover, polyphenolic compounds improved the T cell revitalising effect of mitochondria-targeted antioxidants and of programmed cell death protein 1/programmed cell death ligand 1 blockade.

Conclusions: Simultaneously targeting multiple altered intracellular pathways with the combination of mitochondria-targeted antioxidants and natural polyphenols may represent a promising immune reconstitution strategy for the treatment of chronic HBV infection.

Lay summary: In chronic hepatitis B, antiviral T lymphocytes are deeply impaired, with many altered intracellular functions. *In vitro* exposure to polyphenols, such as resveratrol and oleuropein, can correct some of the deregulated intracellular pathways and improve antiviral T cell function. This effect can be further strengthened by the association of polyphenols with antioxidant compounds in a significant proportion of patients. Thus, the combination of antioxidants and natural polyphenols represents a promising strategy for chronic hepatitis B therapy.

Introduction

Therapy for chronic hepatitis B (CHB) is principally based on direct acting antiviral drugs that efficiently suppress virus replication, but do not eradicate HBV from the liver, have limited efficacy on serum HBsAg concentration, and require long-lasting treatments to avoid the risk of HBV reactivation at drug suspension.[1] Therefore, there is a pressing need for new therapeutic strategies to induce complete HBV cure in a short definite time of treatment.

Patients with chronic HBV infection show functional defects of HBV-specific T cell responses, which make ineffective antiviral immune control,[2] and reconstitution of an efficient antiviral T cell response is believed to represent a rational strategy to treat patients with chronic HBV infection. In this perspective, a promising possibility is the correction of the severe metabolic alterations that characterise HBV-specific CD8 T cell exhaustion.[3,4] A key metabolic correlate of the impaired antiviral T cell activity is represented by dysfunctional mitochondria, which are abnormally depolarised and produce elevated reactive oxygen species (ROS) levels.[4] An unbalanced ROS production exceeding the neutralisation capacity of the cellular antioxidant systems can be very harmful to the cell function with damage of cellular proteins and organelles, possible proteostasis engulfment, and functional impairment.[5] This is supported by our recent studies

Keywords: Chronic HBV infection; T cell functional reconstitution; Proteostasis; Resveratrol; Oleuropein.
Received 18 February 2020; received in revised form 5 October 2020; accepted 28 October 2020; available online 11 November 2020
* Corresponding author. Address: Department of Medicine and Surgery, University of Parma, Laboratory of Viral Immunopathology, Unit of Infectious Diseases and Hepatology, Azienda Ospedaliero-Universitaria di Parma, Via Gramsci 14, 43126 Parma, Italy. Tel.:+39 521 703622; Fax: +39 521 703857.
E-mail address: carlo.ferrari@unipr.it (C. Ferrari).
https://doi.org/10.1016/j.jhep.2020.10.034

showing accumulation of aggregated proteins in HBV-specific CD8 T cells of patients with CHB.[4]

It is well known that misfolded/damaged protein digestion and recycle are primary tasks of the ubiquitin-proteasome system,[6] which plays important roles in cellular protein quality control.[7] A distinct but mechanistically interconnected component of cellular proteostasis is autophagy, which is a lysosome-mediated degradation pathway involved in cytoplasmic organelle turnover as well as in aggregated protein removal.[6]

Autophagic functions are upregulated when proteasomes are inhibited, thereby acting as an alternative pathway for protein degradation. Instead, autophagy impairment can result either in enhanced proteasome peptidase activity or in proteasome derangement with intracellular accumulation of ubiquitinated proteins.[6] In case of oxidised protein accumulation derived from excess ROS production by dysfunctional mitochondria, proteasome and autophagy functions can be overwhelmed, leading to reduced protein and organelle turnover, which can be ultimately deleterious for the cellular function with possible progression to cell death.[5,8,9] Interestingly, mitochondria are also involved in cytosolic aggregate disassembly by the active import of proteins into the matrix for protease digestion.[10,11]

The interplay between the mitochondria and proteostasis has also been studied in T cells, where proteasome inhibition can induce loss of mitochondrial membrane potential (MMP), translocation of pro-apoptotic mitochondrial proteins to the cytoplasm, and subsequent induction of apoptosis.[12] Moreover, T cell exposure to oxidative stress can decrease proteasome catalytic activities, leading to T cell hypo-responsiveness and mimicking some aspects of T cell ageing.[13] Thus, mitochondrial ROS toxicity provides a link between mitochondrial dysfunction and cellular proteostasis, because proteostasis is needed to regulate and preserve a functional cellular pool of proteins and organelles, which can be damaged and disrupted by ROS excess.

The aim of the present study was to assess the functional implications of the transcriptional deregulation of genes coding for proteins involved in the overall proteostasis machinery, including proteasome as well as phagosome and lysosome effector functions. This can allow a better understanding of the molecular mechanisms that underlie T cell exhaustion and to identify optimal targets for T cell functional restoration strategies. In this perspective, polyphenols and their derivatives are known to express not only an antioxidant effect, but also to stimulate the degradation of misfolded or oxidised proteins.[14] Thus, we asked whether the polyphenolic compounds resveratrol (RSV) and oleuropein (OLE) can further improve the functional T cell restoration effect of mitochondria-targeted (mt) antioxidants, which we reported previously in CHB.[4]

Patients and methods

Study participants

The following groups of patients were enrolled in the study:

- 70 treatment-naive patients with chronic active hepatitis B;
- 6 subjects spontaneously recovered from acute HBV infection; and
- 13 healthy subjects.

Patient characteristics are outlined in the supplementary material and in Table S1.

Autophagy analysis

Autophagy was investigated by LC3B staining in flow cytometry on peripheral blood mononuclear cells (PBMCs) from patients and controls following overnight incubation in the presence or absence of chloroquine (CQ) diphosphate.

The CYTO-ID® autophagy detection kit was also used, upon overnight incubation with CQ and rapamycin.

T cell treatments

The tested compounds are:

- trans-RSV, RSV-3-sulphate, dihydroresveratrol, and RSV-3-glucuronide;
- OLE, 3-hydroxytyrosol (HT), 3-HT-3-glucuronide, 3-HT-4-glucuronide, and 3-HT-4-sulphate;
- MitoQ and MitoTEMPO; and
- anti-programmed cell death ligand 1 (PD-L1).

Mitochondrial function and aggresome detection

To analyse the mitochondrial function in T cells, the MMP has been studied by the potentiometric probe JC-1, while the superoxide-sensitive dye dihydroethidium and the superoxide-sensitive probe dichlorodihydrofluorescein diacetate were used for ROS detection. To measure protein aggregate levels, the PROTEOSTAT® Aggresome detection kit was used.

T cell cytokine production assay

T cell cytokine determinations (interferon-γ [IFN-γ], tumour necrosis factor-α [TNF-α], and IL-2) were performed by intracellular cytokine staining, as described previously.[15]

Statistical analysis

Comparisons were done by 2-tailed Mann-Whitney *U* test and Wilcoxon matched-pairs test. Spearman's rank-correlation test was applied for the correlation analysis.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Results

Autophagy is impaired in patients with chronic HBV infection

An impaired proteasome degradation function in exhausted CD8 T cells with abnormal accumulation of intracellular aggresomes was reported previously in patients with CHB.[4] Not only genes related to proteasome, but also transcripts related to autophagosome and lysosome effector functions resulted strongly downregulated.[4] Based on this background, we investigated the lysosome-mediated degradation pathway by looking at the expression of the autophagosome marker LC3B and at the CYTO-ID cationic incorporation into autophagic vesicles (pre-autophagosomes, autophagosomes, and autophagolysosomes).

Changes in LC3B expression were measured upon overnight blockade of the autophagosome turnover by CQ, which inhibits the autophagosome-lysosome fusion, preventing LC3B degradation and leading to its accumulation.[16] As represented in Fig. 1A, CQ-treated HBV-specific CD8 T cells from patients with CHB displayed a significantly lower increase in LC3B expression compared with patients who had been able to resolve spontaneously a previous acute hepatitis B and to influenza (FLU)-specific CD8 T cells from healthy subjects (median increase values 1.079, 1.341, and 1.335 for patients with CHB, resolved, and healthy subjects, respectively), suggesting lower basal

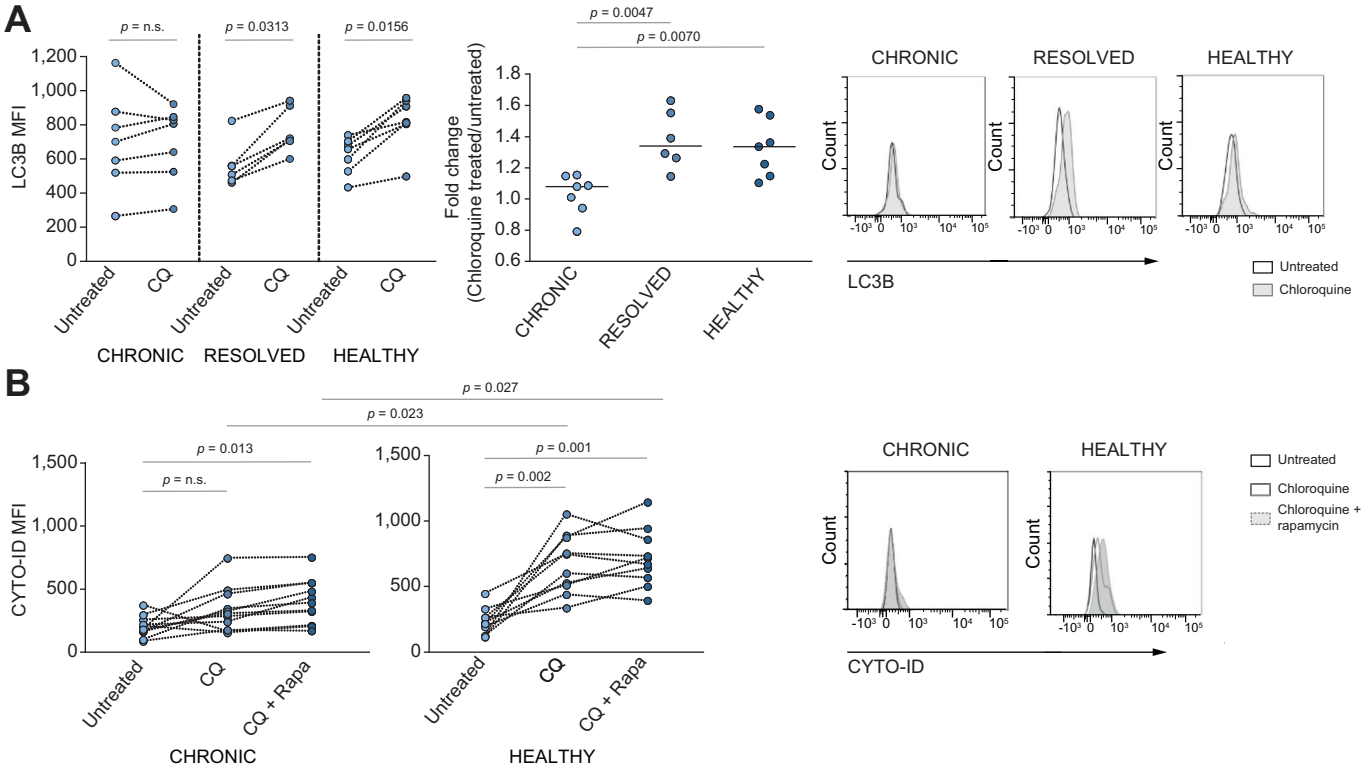

Fig. 1. Autophagic flux in virus-specific CD8 T cells. (A) The left plot shows LC3B MFI values of HBV-specific CD8 cells in CQ-treated or untreated PBMC from patients with chronic HBV infection, subjects who resolved an acute HBV infection (resolved), and FLU-specific CD8 cells from healthy controls (Wilcoxon matched-pairs test). The middle graph represents fold change values (LC3B MFI in CQ-treated/untreated virus-specific CD8 cells; Mann-Whitney test). (B) CYTO-ID MFI values in HBV-specific CD8 cells from patients with CHB and FLU-specific CD8 cells from healthy subjects in untreated samples and treated with CQ or with CQ + rapamycin (CQ + Rapa) (Wilcoxon matched-pairs test). Representative examples are shown on the right of each panel. Plots were generated by gating on HBV-specific CD8 T cells. CQ, chloroquine; FLU, influenza; MFI, median fluorescence intensity; PBMC, peripheral blood mononuclear cell.

autophagosome formation in exhausted CD8 T cells. Data illustrated in Fig. 1A were generated by staining the total LC3B-I cytosolic form, followed by subsequent validation targeting specifically the autophagosome-associated LC3B-II lipidated form, which was stained after LC3B-I removal by selective permeabilisation (see Supplementary Patients and methods). We then used the CYTO-ID cationic tracer dye, which becomes brightly fluorescent upon incorporation into autophagic vesicles in live cells.[16] PBMCs from patients and healthy controls were treated with CQ in the presence or absence of rapamycin, to induce autophagy. As shown in Fig. 1B, lower accumulation of autophagic vesicles was detected in virus-specific CD8 T cells from patients with CHB compared with healthy subjects not only upon CQ-mediated blockade of autophagosome degradation, thus confirming an impairment in the autophagic flux, as observed with LC3B analysis (median increases 1.77 and 2.32 for patients with CHB and healthy controls, respectively), but also in the presence of rapamycin. Indeed, a moderate upregulation of the CYTO-ID probe expression was induced by rapamycin in cells from patients with CHB, but the entity of such increase remained significantly lower compared with what was observed in cells from healthy controls (median increase values 1.91 and 2.79 for patients with CHB and controls, respectively). As the autophagic flux in bulk CD8 T cells is not significantly different in patients with chronic HBV infection and in healthy individuals, our data suggest that the defect in autophagy of patients with chronic HBV involves primarily HBV-specific T cells (data not shown). Thus, our data suggest that exhausted virus-specific CD8 cells display low levels of basal and induced autophagy, further confirming dysfunctional proteostasis.

RSV and OLE can improve mitochondrial and proteasomal functions

The effect of 2 polyphenolic compounds, RSV and OLE, which can affect both mitochondrial function and proteostasis, was tested *in vitro* on exhausted HBV-specific CD8 T cells. PBMCs from patients with CHB were cultured for 10 days, and HBV-specific T cells were expanded upon stimulation with a mixture of 15-mer overlapping peptides corresponding to the HBV core protein, or with the core 18–27 HLA-A2-restricted peptide for HLA-A2+ samples, in the presence or absence of polyphenols. Then, to evaluate the effect of RSV and OLE on CD8 cell MMP, the percentage of CD8 T cells with depolarised mitochondria in each lymphocyte culture was analysed by the MMP-sensitive dye JC-1, by gating either on total or on dextramer+ CD8 T cells, respectively, depending on the peptides used to expand the cultures. Treatment with both compounds significantly reduced the fraction of depolarised CD8 T cells (Fig. 2A; Fig. S1A). In the same cultures, intracellular superoxide anion and hydrogen peroxide levels were also significantly reduced in the presence of RSV and OLE, as measured by the specific fluorescent probes dihydroethidium and 2′,7'-

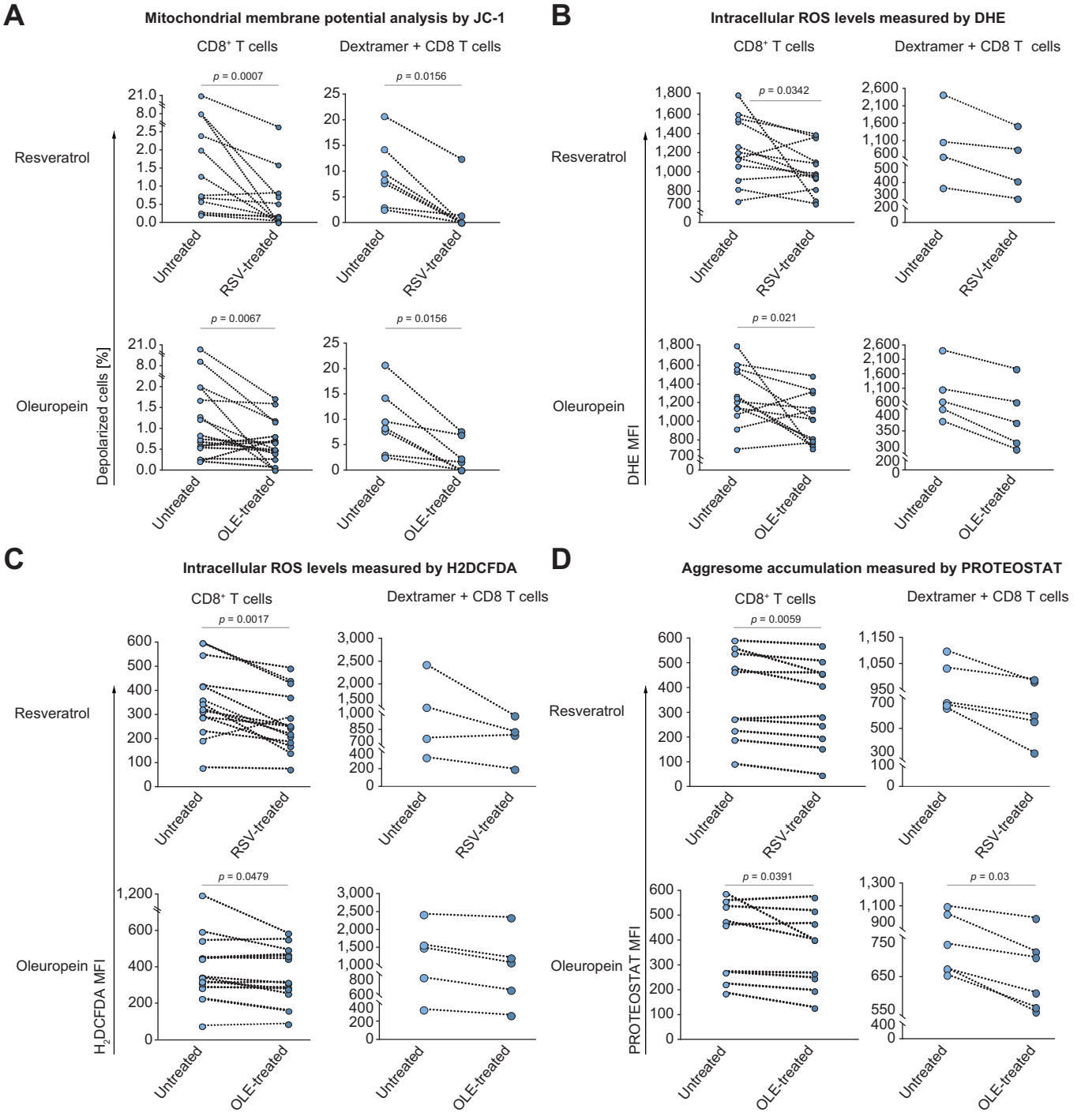

Fig. 2. Effect of resveratrol and oleuropein on mitochondrial function and proteostasis. PBMCs from patients with CHB were stimulated *in vitro* for 10 days either with a mixture of 15-mer core peptides (results derived by gating on CD8+ cells, on the left) or with the core 18–27 HLA-A2-restricted peptide for HLA-A2+ samples (results derived by gating on dextramer+ CD8 cells, on the right), in the presence or absence of RSV or OLE. (A) Percentage of depolarised CD8 cells by JC-1 staining in untreated and polyphenol-treated cultures. (B) Superoxide-anion production by DHE staining. (C) Hydrogen peroxide by H2DCFDA staining (DHE and H2DCFDA MFI are plotted). (D) Intracellular aggresome accumulation as shown by PROTEOSTAT MFI. Statistics by the Wilcoxon matched-pairs test. CHB, chronic hepatitis B; DHE, dihydroethidium; H2DCFDA, 2′,7'-dichlorodihydrofluorescein diacetate; MFI, median fluorescence intensity; OLE, oleuropein; PBMC, peripheral blood mononuclear cell; RSV, resveratrol.

dichlorodihydrofluorescein diacetate, respectively (Fig. 2B and C; Fig. S1B). Finally, the effect of RSV or OLE treatments on cellular proteostasis was evaluated with the PROTEOSTAT Aggresome dye. A significantly lower aggresome accumulation was detected in polyphenol-treated compared with untreated cultures, thus indicating an increased efficiency in damaged protein degradation (Fig. 2D; Fig. S1C).

These results show that RSV and OLE are able to improve mitochondrial function and proteostasis of exhausted CD8 T cells.

Functional restoration of effector CD8 T cell responses by RSV and OLE

We then assessed the effect of RSV and OLE on T cell antiviral functions *in vitro*. To this end, after expansion of PBMC from patients with CHB by HBV core peptide stimulation in the presence/absence of RSV (46 patients) or OLE (34 patients), cultures were tested for cytokine production and CD107a upregulation. RSV induced a significant improvement in cytokine production by stimulated CD8 T cells, with a mean increase of 5.3-, 2.8-, and 3.4-fold for IFN-γ, TNF-α, and IL-2, respectively. IFN-γ, TNF-α, and IL-2 production increased more than 1.5-fold in 63%, 60.8%, and 65.8% of the tested patients, respectively

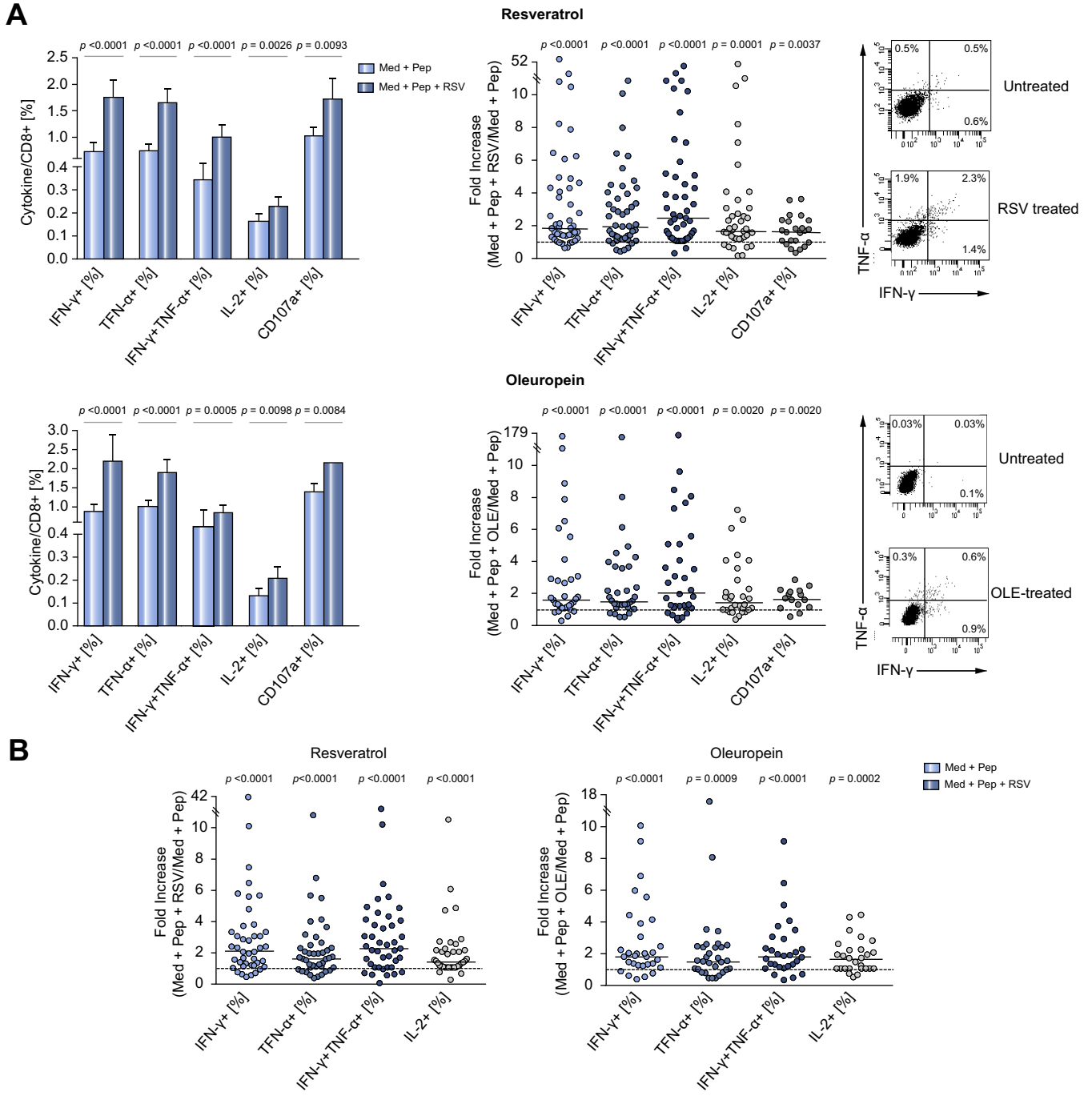

Fig. 3. Functional restoration of exhausted HBV-specific T cells by polyphenols. (A) Percentage of cytokine- and CD107a-positive CD8 cells in T cell lines generated by HBV core peptide stimulation in the presence/absence of RSV (top; N = 46) or OLE (bottom; N = 34). Mean and SE values are represented in the bar plots (Wilcoxon matched-pairs test). Middle graphs show polyphenol-induced variations (fold increase) of cytokine levels in CD8 cells; median fold-increase values are reported (Wilcoxon signed-rank test). Representative examples are shown on the right. Plots were generated by gating on CD8 T cells. (B) Fold increase of cytokine levels upon polyphenol treatment (RSV in the top and OLE in the bottom) in the global CD3+ T cell population. IFN-γ, interferon-γ; OLE, oleuropein; RSV, resveratrol; TNF-α, tumour necrosis factor-α.

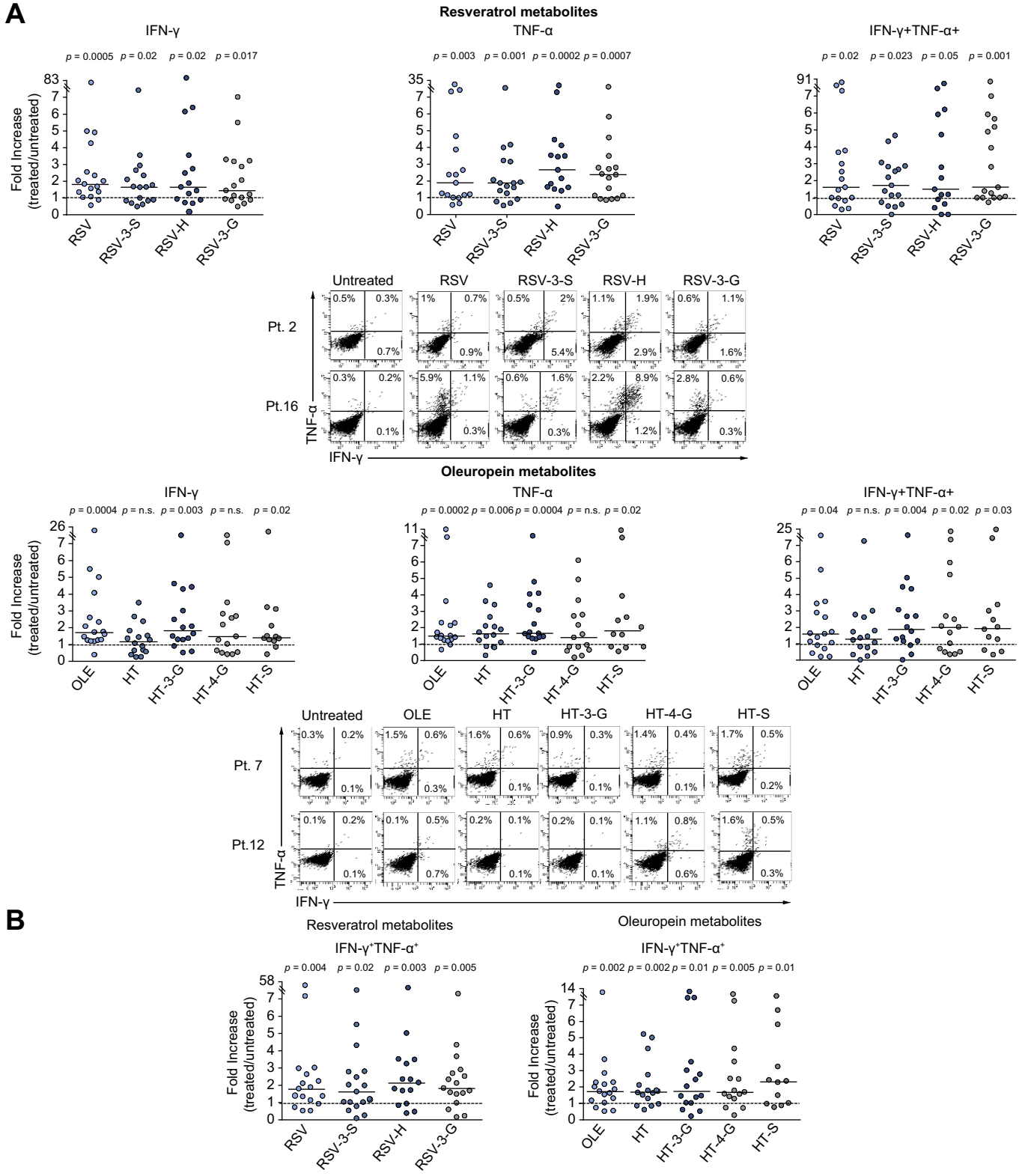

Fig. 4. HBV-specific T cell functional restoration by polyphenol metabolites. (A) Fold increase of IFN-γ-, TNF-α-, and double-IFN-γ/TNF-α-positive CD8 cells in T cell lines generated by HBV core peptide stimulation in the presence/absence of RSV (top; N = 17) and its metabolites RSV-3′-sulphate (RSV-3-S), dihydroresveratrol (RSV-H), and RSV-3′-glucuronate (RSV-3-G). Median fold-increase values are reported (Wilcoxon signed-rank test). Same illustration for OLE (bottom; N = 17) and HT metabolites. Representative examples are illustrated. Plots were generated by gating on CD8 T cells. (B) Fold increase in cytokine levels upon polyphenol and metabolite treatment (RSV in the top and OLE in the bottom) analysed in the global CD3+ T cell population. HT, hydroxytyrosol; IFN-γ, interferon-γ; OLE, oleuropein; Pt, patient; RSV, resveratrol; TNF-α, tumour necrosis factor-α.

(Fig. 3A). With OLE, the mean levels of increase for IFN-γ, TNF-α, and IL-2 were of 8.3-, 5.1-, and 2.1-fold, respectively, and 55.8%, 50%, and 48.3% of the tested patients showed more than 1.5-fold increase in IFN-γ, TNF-α, and IL-2 production (Fig. 3A). Double-positive IFN-γ + TNF-α + CD8 T cells, which have been associated with an enhanced capacity to control viral infections,[17] were also increased significantly by both polyphenolic compound treatments, with mean increases of 4.4- and 7.9-fold for RSV and OLE treatment, respectively (Fig. 3A; Fig. S2). Interestingly, increase in IFNγ + TNFα + CD8 cells in the presence of polyphenols significantly correlated with reduction of depolarised CD8 T cells in the same cultures by JC-1 staining, calculated as the ratio between depolarised CD8 cells in untreated *vs.* RSV- or OLE-treated cultures (Fig. S3A). Also, CD8 cell cytotoxic activity, measured by CD107a upregulation, was significantly improved by RSV and OLE treatments (Fig. 3A). By dextramer staining of HBV-specific CD8 cells it has been possible to evidence that both T cell expansion and cytokine production were increased by RSV or OLE (Fig. S3B). Similarly, a significant enhancement of cytokine production was also detected in total CD3 T cells (Fig. 3B). Direct comparison of different compounds for their capacity to restore the T cell function suggests a hierarchy of efficiency with maximal levels of functional HBV-specific CD8 T cell recovery induced by MitoTEMPO and RSV treatments (Fig. S4).

Effect of RSV and OLE metabolites on exhausted HBV-specific CD8 T cells

As RSV and OLE are rapidly degraded after their administration into different metabolites, which can represent *in vivo* the real effectors of their metabolic activity, we then assessed whether the main *in vivo* generated metabolites display *in vitro* the same effects of the parental RSV and OLE molecules on HBV-specific CD8 T cells. RSV-3-sulphate and RSV-3-glucuronide are known as the major circulating forms of RSV because, following oral administration, trans-RSV is rapidly conjugated with glucuronic acid and sulphate at enterocyte and hepatocyte levels.[18] Moreover, in the intestine, the non-absorbed RSV is also metabolised into dihydroresveratrol by the intestinal microflora.[18] Also, OLE is rapidly degraded following oral administration into HT,[19] and its sulphated and glucuronidated forms represent the major metabolites.[20]

To assess the effect of polyphenol metabolites in restoring antiviral functions of exhausted HBV-specific CD8 cells, short-term T cell lines generated by HBV core peptide stimulation of PBMC from 17 patients with CHB were expanded in the presence/absence of RSV, RSV-3-sulphate, dihydroresveratrol, and RSV-3-glucuronide, or OLE, HT, HT-3-glucuronide, HT-4-glucuronide, and HT-3-sulphate. As shown in Fig. 4A and in Table S3, RSV and OLE metabolites significantly enhanced cytokine production and frequency of double IFN-γ + TNF-α + CD8 T cells, with a mean increase comparable with what was induced by the original polyphenol compounds. A similar effect of RSV and OLE metabolites was also observed on the total CD3 T cell population (Fig. 4B).

Combination of polyphenols and mt antioxidants can enhance the effect of individual compounds

We previously showed[4] that the mt antioxidants MitoQ and MitoTEMPO can induce a significant increase in antiviral cytokine production by HBV-specific T cells from patients with CHB. Given the potential complementary effects of mt antioxidants and polyphenols, we asked whether their combination could further improve the effect of individual drugs.

Short-term T cell lines were generated from 13 patients with CHB by PBMC stimulation with HBV core peptides in the presence/absence of mt antioxidants and polyphenols either alone or in combination, and tested for cytokine production and CD107a expression. Despite the good response rate to single compounds, the addition of RSV or OLE to the antioxidants induced a further increase in the percentage of patients responding to treatments, variable from 10% to 60%, depending on individual T cell functions (Fig. 5A). Notably, an increased IFN-γ production of at least 1.5-fold was detected in 100% of the cultures treated with the combination of MitoTEMPO + OLE.

We then evaluated how many of the tested functions (IFN-γ, TNF-α, and IL-2 production and CD107a upregulation) were simultaneously improved in individual patients upon treatment with mt antioxidants and polyphenols alone or in association. When the different compounds were administered alone, a small fraction of patients, variable from 9% to 23%, failed to recover any T cell function (Fig. 5B). Responses were improved by the combination of MitoQ or MitoTEMPO with polyphenols; indeed, all patients showed enhancement of at least 1 antiviral T cell function (Fig. 5B), while a broader simultaneous recovery of 3 or 4 functions was detected in 61.5% and 77% of the patients upon culture with MitoQ + RSV and MitoTEMPO + OLE combinations, respectively (Fig. 5B). The latter combination appeared the most effective treatment for the reconstitution of a broadly polyfunctional antiviral T cell activity. Further analysis of results is reported in Figs. S5 and S6.

Moreover, exhausted HBV-specific CD8 cells resulted more sensitive than CD8 cells of HBV-unrelated specificity to antioxidant and polyphenol modulation (Fig. 6A and B).

Antioxidant and polyphenolic compounds are effective on CD8 T cells of different HBV antigen specificities

As all described analyses were performed on HBV-core-stimulated T cells, we also sought to elucidate whether T cells specific for other HBV antigens were susceptible to the functional restoring effect of MitoTEMPO + OLE treatment. This is important because different levels of T cell exhaustion have been described for HBV-specific CD8 T cells of different HBV antigen specificities.[21,22] To this end, PBMC from patients with chronic HBV infection were stimulated not only with core, but also with HBV envelope and polymerase peptide pools in the presence/absence of MitoTEMPO + OLE. T cell lines generated from 9 patients were tested for IFN-γ and TNF-α production. As shown in Fig. 6C, MitoTEMPO + OLE also significantly enhanced cytokine production induced by envelope and polymerase peptides with an effect comparable with that observed with HBV core peptide stimulation.

Discussion

By a transcriptome analysis of HBV-specific CD8 T cells from patients with chronic HBV infection, we recently described an extensive downregulation of genes coding for different cellular functions, highlighting the central role of mitochondrial metabolic defects, with a high proportion of depolarised mitochondria and elevated ROS production.[4] In addition, deregulation of genes coding for proteins involved in the proteasomal and autophagic functions was also observed.[4] These include genes coding for 26S proteasome subunits; for molecules known to be

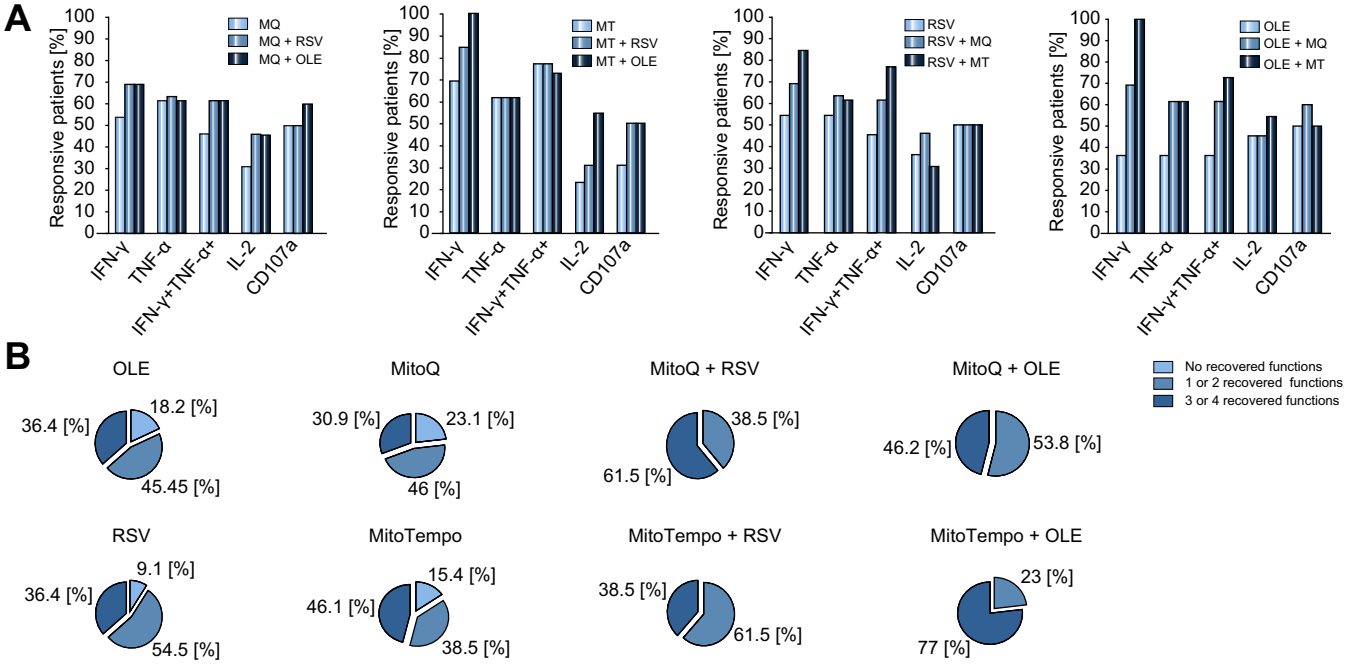

Fig. 5. Effect of the combined mt antioxidant/polyphenol treatments. Combinations of mt antioxidants and polyphenols were tested on lymphocyte cultures from 13 patients with CHB. (A) Percentage of cultures responding to mt antioxidants, polyphenols, or their association. Patients were considered responsive to treatments when cytokine or CD107a expression by CD8 T cells increased at least 1.5-fold in treated *vs.* untreated cultures. (B) Pie graphs represent the increase in polyfunctionality by CD8 cells upon mt antioxidants, polyphenols, or their association. Results are illustrated as percentage of patients showing no recovery of any T cell function, improving 1–2 functions and 3–4 functions simultaneously. CHB, chronic hepatitis B; IFN-γ, interferon-γ; mt, mitochondria targeted; OLE, oleuropein; RSV, resveratrol; TNF-α, tumour necrosis factor-α.

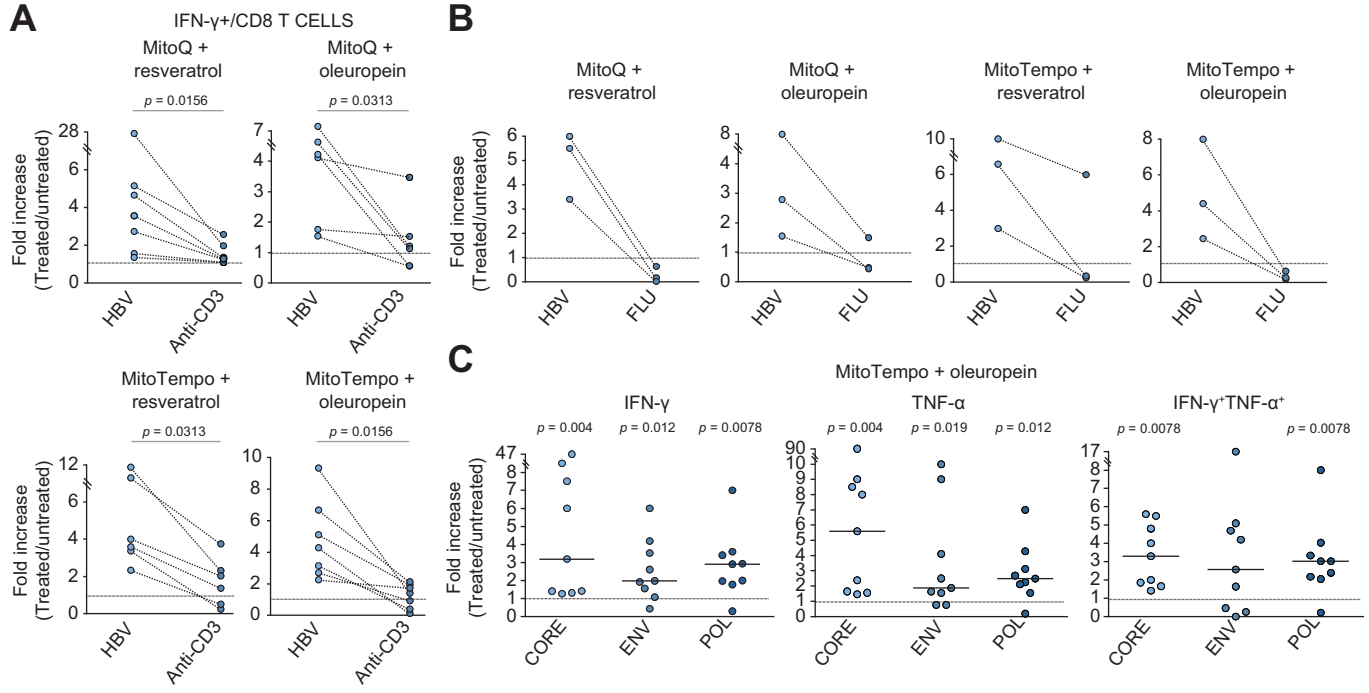

Fig. 6. Effect of combined mt antioxidant/polyphenol treatments on CD8 T cells stimulated with different antigens. (A) Paired T cell cultures generated by HBV core peptides or anti-CD3 stimulation in the presence/absence of mt antioxidants plus polyphenols. Results are expressed as fold increase of IFN-γ-producing CD8 T cells. Statistics by Wilcoxon matched-pairs test. (B) Fold increase of IFN-γ production in paired samples of mt antioxidant/polyphenol-treated HBV-specific and FLU-specific dextramer+ CD8 cells from HLA-A2+ patients with CHB. (C) Fold changes of IFN-γ+, TNF-α+, IFN-γ + TNF-α + CD8 cells stimulated with HBV-core, -envelope, and -polymerase peptide pools, in the presence of MitoTEMPO + oleuropein. Median values are reported. Statistics by Wilcoxon signed-rank test. FLU, influenza; IFN-γ, interferon-γ; mt, mitochondria targeted; TNF-α, tumour necrosis factor-α.

required for autophagosome formation, such as clathrin;[23] for autophagosome maturation, such as *GORASP2*;[24] for autophagosome degradation, such as *TCIRG1* or *ATP6V0C H+-ATPases*;[25] as well as for components of the lysosome degradation machinery, including peptidases and galactosylceramidases. This wide transcriptional deregulation, as well as the abnormal accumulation of intracellular aggresomes,[4] suggests that proteostasis represents a possible target for therapeutic interventions to cure CHB by functional T cell reconstitution.

In this study, we analysed autophagy in HBV-specific CD8 T cells to gain a more complete picture of the overall intracellular protein/organelle degradation machinery. Despite the technical constraint imposed by the low number of antigen-specific CD8 cells detectable in the peripheral blood of patients with CHB, our data strongly support the concept of an impaired autophagic flux in HBV-specific CD8 T cells from patients with chronic HBV. This finding is in line with the higher autophagy rate detected in memory than in terminally differentiated T cells,[26] as well as with the lower autophagy levels reported in less functional T cells with abundant expression of depolarised mitochondria,[26] as samples studied here are from treatment-naive patients with a chronic active hepatitis, who are expected to be deeply exhausted.[27]

Our observations show a wide alteration of the cellular mechanisms responsible for removing misfolded or aggregated proteins and clearing damaged organelles. In combination with the previously described proteasome impairment, they may contribute to the altered HBV-specific CD8 T cell function in CHB, and their correction may represent an additional target for functional T cell reconstitution strategies.

In view of these findings, we asked whether natural polyphenols, which target simultaneously both proteostasis and mitochondria,[14,27] can be beneficial for exhausted T cells from patients with CHB, and whether they can improve the T cell revitalising effect of mt antioxidants, such as MitoQ and Mito-TEMPO.[4] AMPK phosphorylation, with sirtuins upregulation, as well as induction of the nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent antioxidant response can be promoted by both polyphenols, leading to several beneficial effects on cells undergoing oxidative stress.[28–31] Indeed, the Nrf2 pathway has wide cytoprotective functions and represents the master regulator of the cellular redox homeostasis; it has multiple effects on the mitochondrial function and controls several genes involved in the maintenance of the endoplasmic-reticulum physiology, the proteasome activity, and the autophagy function.[32]

Based on this background, we first tested whether OLE and RSV can reconstitute defective mitochondrial function and proteostasis, and thus enhance the antiviral function of primary T cell cultures from patients with CHB. Both polyphenols were able to significantly decrease CD8 T cell mitochondrial depolarisation, ROS levels, and protein accumulation into aggresomes, ultimately leading to an improved cytokine production by HBV-specific T cells. In addition to a direct modulation of intracellular CD8 pathways, polyphenols have also been reported to act on regulatory CD4+ regulatory T cells in cancer models, thereby providing an indirect modulatory effect on the CD8 T cell function,[33] which cannot be excluded in patients with chronic HBV infection.

Such results make the polyphenols attractive therapeutic candidates, but a major limitation to their clinical application is their low bioavailability and rapid metabolism.[34,35] To address this problem, alternative formulations and modes of administration have previously been assessed.[20,35,36] In our study, the problem of the limited bioavailability of the natural compounds was addressed by looking at the effect of some polyphenol metabolites known to be generated *in vivo*, including the glucuronide and sulphate-conjugated forms of RSV and HT, which represent the first molecules generated *in vivo* after RSV and OLE hydrolysis and phase II metabolism.[18–20] Indeed, such molecules have been detected in blood and urine after OLE and RSV administration, even at concentrations significantly higher than those of the parental compounds.[20,37] Importantly, both RSV sulphate and RSV glucuronide have been shown to accumulate into cardiomyocytes *in vivo*, proving their capacity to penetrate mammalian membranes and to maintain their structure within the cell.[38] All tested metabolites induced an increment in cytokine production at a level comparable or even higher than that induced by the original molecules, with the 3′-glucuronide conjugated form of RSV inducing the most significant increment in the percentage of IFN-γ and TNF-α double producing CD8 T cells. These data are in line with a previous study describing ROS-scavenging properties for HT glucuronide higher than those reported for the parental compound.[39] Moreover, the similar efficacy shown by the tested metabolites compared with the parental molecule may be attributable to several reasons. In particular, phenolics and their metabolites have been shown to undergo further phase II transformation steps when put in contact with cells *in vitro*, resulting in deconjugation or further conjugation in relation to the type of molecular scaffold applied and to the type of cells used in culture.[40,41] A possible explanation is that free polyphenols could have been partially or completely formed from deconjugation of their metabolites at cellular level, or *vice versa*, that the tested conjugates have been transformed back, at least partially, to their aglyconic structure during the experiment. Unfortunately, our T cell culture model, where compound concentrations would be too low to be detected by available techniques, did not allow us to investigate such possibilities.

Given the potential target complementarity of mt antioxidants and polyphenolic compounds, we next asked whether their association could further improve T cell functional reconstitution. Indeed, MitoQ treatment following proteasome inhibition and subsequent cytosolic oxidation has recently been reported to suppress mitochondrial ROS production. MitoQ, however, could not correct the oxidised cytosolic redox state and only partially rescued cell viability, which was conversely better rescued by RSV treatment.[42] In line with these results, the combined treatment with mt antioxidants and polyphenols enhanced the percentage of responding cultures, cytokine production, and polyfunctionality in individual cultures, and maximal effect was induced by the combination of MitoTEMPO and OLE. Whether polyphenols can potentiate the efficacy of anti-PD-L1 remains to be better investigated, also in light of the recent observation that RSV can interfere with PDL1 glycosylation and consequently with its expression and function.[43]

Recent data indicate that T cells able to recognise HBV epitopes within different HBV proteins in chronic HBV infection can express different phenotypic and functional features, suggesting a different degree of dysfunction related to the antigen specificity.[44,45] Although the causative mechanisms are still largely undefined, the different quantities of envelope, core, and polymerase antigens expressed in HBV-infected hepatocytes may at

least partially be involved,[46] in consideration of the evidence that the quantity of antigen presented by hepatocytes can modulate the T cell function.[47] We thus assessed whether the effect of the mt antioxidant and polyphenolic compounds was similar on T cells of different HBV antigen specificities. Results were similar on CD8 cells specific not only for core, but also for envelope and polymerase antigens, which have been reported to be more severely exhausted.[21,22] This differs from previous results of programmed cell death protein 1/PD-L1 blocking experiments, where a functional restoration was primarily detected on core- and polymerase-, but much less on envelope-specific T cells.[15]

The present study was focused on patients with a chronic active hepatitis, as this is the patient population that more urgently requires new therapeutic strategies to shorten the duration of available antiviral therapies and avoid evolution to cirrhosis, hepatocellular carcinoma, and liver decompensation. Instead, we do not have information yet on the degree of metabolic dysfunction and potential T cell restoration efficacy of polyphenols in patient cohorts with no liver inflammation. These patients may be targeted even more efficiently by metabolic modulatory compounds because less exhausted, or *vice versa*, may be minimally affected by these treatments if their antiviral CD8 function may be already close to be optimal.

Finally, a potential limitation of T cell modulatory therapies is the possibility to induce autoimmune pathologies promoted by the undesired stimulation of HBV-unrelated T cell populations of different antigen specificities. To explore whether the effect of these compounds is mostly focused on exhausted rather than functionally preserved T cells, we compared their effect on T cell lines obtained either by HBV- or unrelated antigen-specific stimulation (*i.e.* influenza peptides or CD3). Importantly, although some cytokine increase was observed also in control cultures, a significantly higher effect was detected in HBV-specific cells, suggesting a preferential effect on exhausted T cells. This may limit, but cannot totally exclude, the likelihood of undesired autoimmune reactions. In addition, increase in cytokine production by polyphenols was selectively detected in HBV-peptide-stimulated T cell lines, but not in T cell lines expanded only with IL-2 without any HBV-specific stimulation, further supporting the conclusion that polyphenols are more active on dysfunctional, but poorly on functional T cells (data not shown).

In conclusion, our study provides novel information about the functional impairment of the intracellular degradation processes responsible for removing misfolded/aggregated proteins and damaged organelles in exhausted HBV-specific CD8 T cells and characterise additional mechanisms potentially underlying T cell exhaustion. In addition, our data indicate that the combined modulation of mitochondrial functions and proteostasis can induce a significant improvement of antiviral CD8 T cell activity, which is only weakly detectable on non-exhausted T cells of different specificities. This effect was expressed on CD8 T cells of all HBV antigen specificities (envelope, core, and polymerase), which are expected to display different levels of exhaustion. Targeting simultaneously multiple altered intracellular pathways with the combination of mt antioxidants and natural polyphenols thus represents a promising strategy in the perspective of novel immune reconstitution therapies for CHB. Studies of homogeneous groups of patients with CHB with more or less active HBV infection and with different severity of T cell exhaustion and metabolic impairment, ranging from highly viraemic patients to low viraemic and inactive carriers harbouring functionally more preserved HBV-specific CD8 T cells, are now needed to predict the patient populations that may be more likely to benefit from this type of immune metabolic modulation.

Abbreviations

CHB, chronic hepatitis B; CQ, chloroquine; FLU, influenza; HT, hydroxytyrosol; IFN-γ, interferon-γ; MMP, mitochondrial membrane potential; mt, mitochondria targeted; Nrf2, nuclear factor erythroid 2-related factor 2; OLE, oleuropein; PBMC, peripheral blood mononuclear cell; PD-L1, programmed cell death ligand 1; ROS, reactive oxygen species; RSV, resveratrol; TNF-α, tumour necrosis factor-α.

Financial support

This work was supported by a grant from Emilia-Romagna Region, Italy (Programma di Ricerca Regione-Università 2010-2012; PRUa1RI-2012-006), and by a grant from the Italian Ministry of Health, Italy (Ricerca Finalizzata RF 2013-02359333).

Conflicts of interest

CF received grants from Gilead and AbbVie. He is a consultant for Gilead, AbbVie, Vir Biotechnology, Arrowhead, Transgene, and Bristol Myers Squibb. PL is an advisor and speaker bureau for Gilead, Roche, Bristol Myers Squibb, GlaxoSmithKline, Merck Sharp & Dohme, Arrowhead, Alnylam, Spring Bank, Janssen, and EIGER. AL is consultant for MYR Pharmaceuticals and Gilead. MM is an advisory board for AbbVie. The other authors declare no conflicts of interest that pertain to this work.

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

Authors' contributions

Study conception and design: P.F. Funds acquisition: C.F. Patient recruitment and characterisation: S.S., A.A., A.L., M.B., M.M., C.B. Reagent provision: D.D.R. Experiment execution: G.A., I.M., G.D.F., V.B., M.R., A.V., A.P., P.F. Study supervision: C.F. Administrative support: D.L. Data acquisition: G.A., I.M., G.D.F., V.B., M.R., A.V., A.P., P.F. Data analysis: G.A., P.F. Data interpretation: G.A., I.M., G.D.F., V.B., M.R., A.V., A.P., G.M., P.L., P.F. Statistical analysis: G.A., I.M., G.D.F., V.B., M.R., A.V., A.P. Drafting of paper: G.A. Writing of paper: P.F. Critical revision of paper: G.M., P.L., D.D.R., C.F.

Data availability statement

The authors declare that all data included in this article will be made available upon request.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2020.10.034.

References

Author names in bold designate shared co-first authorship

[1] Zoulim F, Lebossé F, Levrero M. Current treatments for chronic hepatitis B virus infections. Curr Opin Virol 2016;18:109–116.
[2] Bertoletti A, Ferrari C. Adaptive immunity in HBV infection. J Hepatol 2016;64:S71–S83.
[3] 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.

[4] Fisicaro P, Barili V, Montanini B, Acerbi G, Ferracin M, Guerrieri F, et al. Targeting mitochondrial dysfunction can restore antiviral activity of exhausted HBV-specific CD8 T cells in chronic hepatitis B. Nat Med 2017;23:327–336.
[5] Korovila I, Hugo M, Castro JP, Weber D, Höhn A, Grune T, et al. Proteostasis, oxidative stress and aging. Redox Biol 2017;13:550–567.
[6] Kocaturk NM, Gozuacik D. Crosstalk between mammalian autophagy and the ubiquitin-proteasome system. Front Cell Dev Biol 2018;6:128.
[7] Chin L, Olzmann J, Li L. Aggresome formation and neurodegenerative diseases: therapeutic implications. Curr Med Chem 2008;15:47–60.
[8] Gumeni S, Trougakos IP. Cross talk of proteostasis and mitostasis in cellular homeodynamics, ageing, and disease. Oxid Med Cell Longev 2016;2016:1–24.
[9] Sorrentino V, Romani M, Mouchiroud L, Beck JS, Zhang H, D'Amico D, et al. Enhancing mitochondrial proteostasis reduces amyloid-β proteotoxicity. Nature 2017;552:187–193.
[10] **D'Amico D**, **Sorrentino V**, Auwerx J. Cytosolic proteostasis networks of the mitochondrial stress response. Trends Biochem Sci 2017;42:712–725.
[11] **Ruan L**, **Zhou C**, Jin E, Kucharavy A, Zhang Y, Wen Z, et al. Cytosolic proteostasis through importing of misfolded proteins into mitochondria. Nature 2017;543:443–446.
[12] **Berges C**, **Haberstock H**, Fuchs D, Sadeghi M, Opelz G, Daniel V, et al. Proteasome inhibition activates the mitochondrial pathway of apoptosis in human $CD4^+$ T cells. J Cell Biochem 2009;108:935–946.
[13] Das R, Ponnappan S, Ponnappan U. Redox regulation of the proteasome in T lymphocytes during aging. Free Radic Biol Med 2007;42:541–551.
[14] Dhouafli Z, Cuanalo-Contreras K, Hayouni EA, Mays CE, Soto C, Moreno-Gonzalez I. Inhibition of protein misfolding and aggregation by natural phenolic compounds. Cell Mol Life Sci 2018;75:3521–3538.
[15] **Boni C**, **Fisicaro P**, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, et al. Characterization of hepatitis B virus (HBV)-specific T cell dysfunction in chronic HBV infection. J Virol 2007;81:4215–4225.
[16] Chan LL-Y, Shen D, Wilkinson AR, Patton W, Lai N, Chan E, et al. A novel image-based cytometry method for autophagy detection in living cells. Autophagy 2012;8:1371–1382.
[17] Harari A, Dutoit V, Cellerai C, Bart P-A, Du Pasquier RA, Pantaleo G. Functional signatures of protective antiviral T cell immunity in human virus infections. Immunol Rev 2006;211:236–254.
[18] Nunes S, Danesi F, Del Rio D, Silva P. Resveratrol and inflammatory bowel disease: the evidence so far. Nutr Res Rev 2018;31:85–97.
[19] Vissers MN, Zock PL, Roodenburg AJC, Leenen R, Katan MB. Olive oil phenols are absorbed in humans. J Nutr 2002;132:409–417.
[20] de Bock M, Thorstensen EB, Derraik JGB, Henderson HV, Hofman PL, Cutfield WS. Human absorption and metabolism of oleuropein and hydroxytyrosol ingested as olive (*Olea europaea* L.) leaf extract. Mol Nutr Food Res 2013;57:2079–2085.
[21] Schuch A, Salimi Alizei E, Heim K, Wieland D, Kiraithe MM, Kemming J, et al. Phenotypic and functional differences of HBV core-specific versus HBV polymerase-specific CD8+ T cells in chronically HBV-infected patients with low viral load. Gut 2019;68:905–915.
[22] Hoogeveen RC, Robidoux MP, Schwarz T, Heydmann L, Cheney JA, Kvistad D, et al. Phenotype and function of HBV-specific T cells is determined by the targeted epitope in addition to the stage of infection. Gut 2019;68:893–904.
[23] **Ravikumar B**, **Moreau K**, Jahreiss L, Puri C, Rubinsztein DC. Plasma membrane contributes to the formation of pre-autophagosomal structures. Nat Cell Biol 2010;12:747–757.
[24] Zhang X, Wang Y. The Golgi stacking protein GORASP2/GRASP55 serves as an energy sensor to promote autophagosome maturation under glucose starvation. Autophagy 2018;14:1649–1651.
[25] Sun-Wada G-H, Tabata H, Kawamura N, Aoyama M, Wada Y. Direct recruitment of H^+-ATPase from lysosomes for phagosomal acidification. J Cell Sci 2009;122:2504–2513.
[26] Swadling L, Pallett LJ, Diniz MO, Baker JM, Amin OE, Stegmann KA, et al. Human liver memory $CD8^+$ T cells use autophagy for tissue residence. Cell Rep 2020;30:687–698. e6.
[27] Boni C, Laccabue D, Lampertico P, Giuberti T, Viganò M, Schivazappa S, et al. Restored function of HBV-specific T cells after long-term effective therapy with nucleos(t)ide analogues. Gastroenterology 2012;143:963–973. e9.
[28] **Rigacci S**, **Miceli C**, Nediani C, Berti A, Cascella R, Pantano D, et al. Oleuropein aglycone induces autophagy *via* the AMPK/mTOR signalling pathway: a mechanistic insight. Oncotarget 2015;6:35344–35357.
[29] **Price NL**, **Gomes AP**, Ling AJY, Duarte FV, Martin-Montalvo A, North BJ, et al. SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. Cell Metab 2012;15:675–690.
[30] **Ajami M**, **Pazoki-Toroudi H**, **Amani H**, Nabavi SF, Braidy N, Vacca RA, et al. Therapeutic role of sirtuins in neurodegenerative disease and their modulation by polyphenols. Neurosci Biobehav Rev 2017;73:39–47.
[31] Martínez-Huélamo M, Rodríguez-Morató J, Boronat A, de la Torre R. Modulation of Nrf2 by olive oil and wine polyphenols and neuroprotection. Antioxidants 2017;6:73.
[32] Dinkova-Kostova AT, Kostov RV, Kazantsev AG. The role of Nrf2 signaling in counteracting neurodegenerative diseases. FEBS J 2018;285:3576–3590.
[33] Bahrami A, Fereidouni M, Pirro M, Bianconi V, Sahebkar A. Modulation of regulatory T cells by natural products in cancer. Cancer Lett 2019;459:72–85.
[34] Walle T. Bioavailability of resveratrol. Ann N Y Acad Sci 2011;1215:9–15.
[35] Rigacci S, Stefani M. Nutraceutical properties of olive oil polyphenols. An itinerary from cultured cells through animal models to humans. Int J Mol Sci 2016;17:843.
[36] Pannu N, Bhatnagar A. Resveratrol: from enhanced biosynthesis and bioavailability to multitargeting chronic diseases. Biomed Pharmacother 2019;109:2237–2251.
[37] Boocock DJ, Faust GES, Patel KR, Schinas AM, Brown VA, Ducharme MP, et al. Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. Cancer Epidemiol Biomarkers Prev 2007;16:1246–1252.
[38] **Bresciani L**, **Calani L**, **Bocchi L**, Delucchi F, Savi M, Ray S, et al. Bioaccumulation of resveratrol metabolites in myocardial tissue is dose-time dependent and related to cardiac hemodynamics in diabetic rats. Nutr Metab Cardiovasc Dis 2014;24:408–415.
[39] Tuck KL, Hayball PJ, Stupans I. Structural characterization of the metabolites of hydroxytyrosol, the principal phenolic component in olive oil, in rats. J Agric Food Chem 2002;50:2404–2409.
[40] Aragonès G, Danesi F, Del Rio D, Mena P. The importance of studying cell metabolism when testing the bioactivity of phenolic compounds. Trends Food Sci Technol 2017;69:230–242.
[41] Ardid-Ruiz A, Ibars M, Mena P, Del Rio D, Muguerza B, Arola L, et al. Resveratrol treatment enhances the cellular response to leptin by increasing OBRb content in palmitate-induced steatotic HepG2 cells. Int J Mol Sci 2019;20:6282.
[42] Maharjan S, Oku M, Tsuda M, Hoseki J, Sakai Y. Mitochondrial impairment triggers cytosolic oxidative stress and cell death following proteasome inhibition. Sci Rep 2015;4:5896.
[43] **Verdura S**, **Cuyàs E**, Cortada E, Brunet J, Lopez-Bonet E, Martin-Castillo B, et al. Resveratrol targets PD-L1 glycosylation and dimerization to enhance antitumor T cell immunity. Aging (Albany NY) 2020;12:8–34.
[44] Kakimi K, Isogawa M, Chung J, Sette A, Chisari FV. Immunogenicity and tolerogenicity of hepatitis B virus structural and nonstructural proteins: implications for immunotherapy of persistent viral infections. J Virol 2002;76:8609–8620.
[45] Webster GJM, Reignat S, Brown D, Ogg GS, Jones L, Seneviratne SL, et al. Longitudinal analysis of $CD8^+$ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. J Virol 2004;78:5707–5719.
[46] Hirsch RC, Lavine JE, Chang L, Varmus HE, Ganem D. Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for revemsrse transcription. Nature 1990;344:552–555.
[47] Gehring AJ, Sun D, Kennedy PTF, Nolte-'t Hoen E, Lim SG, Wasser S, et al. The level of viral antigen presented by hepatocytes influences CD8 T cell function. J Virol 2007;81:2940–2949.