

Deregulated intracellular pathways define novel molecular targets for HBV-specific CD8 T cell reconstitution in chronic hepatitis B

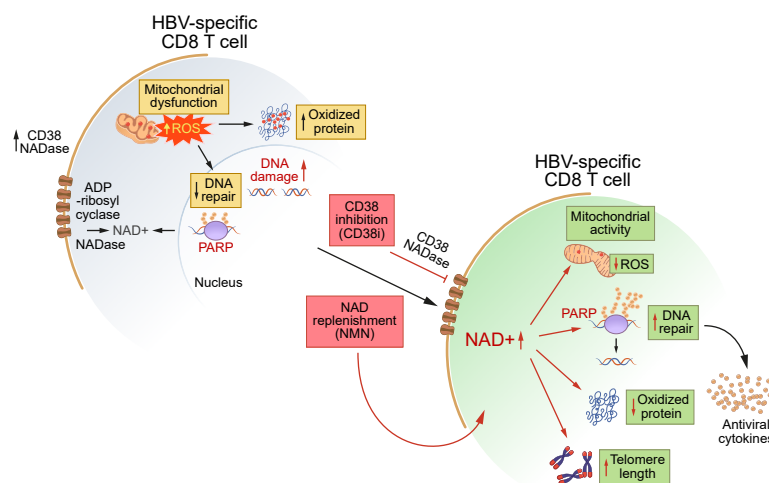
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Graphical abstract



Highlights

- In chronic HBV infection elevated ROS production is associated with high DNA damage in HBV-specific CD8 T cells.
- Response to DNA damage is dysfunctional.
- Upregulation of CD38 and persistent activation of poly-ADP-ribose polymerases contribute to NAD consumption.
- NAD depletion maintains and amplifies cellular dysfunction in exhausted HBV-specific T cells.
- NAD replenishment can restore T-cell function.

Impact and implications

Correction of HBV-specific CD8 T cell dysfunction is believed to represent a rational strategy to cure chronic HBV infection, which however requires a deep understanding of HBV immune pathogenesis to identify the most important targets for functional T cell reconstitution strategies. This study identifies a central role played by NAD depletion in the intracellular vicious circle that maintains CD8 T cell exhaustion, showing that its replenishment can correct impaired intracellular mechanisms and reconstitute efficient antiviral CD8 T cell function, with implications for the design of novel immune anti-HBV therapies. As these intracellular defects are likely shared with other chronic virus infections where CD8 exhaustion can affect virus clearance, these results can likely also be of pathogenetic relevance for other infection models.

Deregulated intracellular pathways define novel molecular targets for HBV-specific CD8 T cell reconstitution in chronic hepatitis B

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Background & Aims: In chronic HBV infection, elevated reactive oxygen species levels derived from dysfunctional mitochondria can cause increased protein oxidation and DNA damage in exhausted virus-specific CD8 T cells. The aim of this study was to understand how these defects are mechanistically interconnected to further elucidate T cell exhaustion pathogenesis and, doing so, to devise novel T cell-based therapies.

Methods: DNA damage and repair mechanisms, including parylation, CD38 expression, and telomere length were studied in HBV-specific CD8 T cells from chronic HBV patients. Correction of intracellular signalling alterations and improvement of antiviral T cell functions by the NAD precursor nicotinamide mononucleotide and by CD38 inhibition was assessed.

Results: Elevated DNA damage was associated with defective DNA repair processes, including NAD-dependent parylation, in HBV-specific CD8 cells of chronic HBV patients. NAD depletion was indicated by the overexpression of CD38, the major NAD consumer, and by the significant improvement of DNA repair mechanisms, and mitochondrial and proteostasis functions by NAD supplementation, which could also improve the HBV-specific antiviral CD8 T cell function.

Conclusions: Our study delineates a model of CD8 T cell exhaustion whereby multiple interconnected intracellular defects, including telomere shortening, are causally related to NAD depletion suggesting similarities between T cell exhaustion and cell senescence. Correction of these deregulated intracellular functions by NAD supplementation can also restore antiviral CD8 T cell activity and thus represents a promising potential therapeutic strategy for chronic HBV infection.

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Introduction

In chronic HBV infection, virus-specific CD8 T cells are dysfunctional with profound metabolic derangements underlying a severe impairment of antiviral activity. Several perturbed intracellular pathways have been identified by transcriptome analysis, among which the most significantly downregulated are involved in mitochondria, proteostasis, and DNA repair functions. These transcriptional deregulations translate into an excess mitochondrial reactive oxygen species (ROS) production and proteostasis engulfment.^{1,2}

Such deregulated processes are partially reminiscent of the functional defects associated with immune-cell senescence. Of note, recent studies of ageing-related cell senescence indicate an association between DNA damage accumulation, poly(ADP-ribose)-polymerase 1 (PARP1) hyperactivation and decreased

NAD levels³ and report that NAD replenishment can improve mitochondrial function and DNA repair.³ Also in exhausted HBV-specific CD8 cells, accumulation of DNA damage, likely induced primarily by elevated ROS levels, should result in persistent activation of DNA repair processes, including parylation, which is catalysed by poly(ADP-ribose)-polymerases (PARPs) that rely on NAD for their activity.⁴

The working hypothesis underlying our study is that chronic liver inflammation and repeated triggering of HBV-specific CD8 cells as a result of their persistent exposure to high antigen concentrations may cause the accumulation of oxidised proteins with proteostasis engulfment, DNA damage with persistent activation of DNA repair mechanisms, including parylation, and CD38 overexpression with subsequent NAD depletion. This can have a negative impact on different effector target molecules, including the activity of NAD-dependent sirtuins,

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with generation of a vicious cycle leading to amplification of the intracellular defects affecting the T cell function. Linking together different impaired metabolic and signalling pathways of HBV-specific CD8 T cells into a functionally interconnected network of deregulated processes underlying T cell exhaustion may shed further light into the mechanistic basis of this condition, allowing key molecular targets for therapeutic functional T cell reconstitution to be identified.

Patients and methods

Patient populations

The following groups of patients were enrolled: 69 treatment-naïve patients with chronic active hepatitis B; 12 individuals who spontaneously recovered from acute HBV infection; and 15 healthy individuals.

Patient characteristics are outlined in the Supplementary material and in [Table S1](#). The number identifying the patients with chronic disease tested in each experiment shown in the figures have been reported in [Table S2](#).

PhosphoH2AX, phosphoATM, 53BP1 staining

For staining of phospho-histone H2AX(ser139), phospho-ATM(ser1981), 53BP1 the BD Phosflow™ kit (BD Biosciences) was used for fixation and permeabilisation.

Poly-ADP-ribosylation (PARylation)

Following fixation and permeabilisation with the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher, Waltham, MA, USA), samples were stained with anti-Poly(ADP-ribose) monoclonal antibody.

Telomere length

To investigate the telomere length in virus-specific CD8 T cells the Prime Flow RNA Assay Kit (Thermo-Fisher) was used, according to the manufacturer's protocol.

Mitochondrial function and aggresome detection

The JC1 and Proteostat probes were used to study the mitochondrial membrane potential and intracellular aggresomes respectively, as previously described.¹

Further materials and methods

Additional information is provided in the Supplementary materials section.

Results

Increased DNA damage and dysfunctional DNA repair in exhausted HBV-specific CD8 T cells

Persistent DNA damage resulting from elevated mitochondrial ROS production should trigger a continuous activation of the DNA repair mechanisms. However, transcriptome analysis¹ of exhausted HBV-specific CD8 T cells highlighted a profound downregulation of transcripts involved in DNA repair, in comparison with influenza (FLU)-specific CD8 cells from the same patients with chronic HBV (CHB) ([Fig. S1](#)).

To understand this apparent discrepancy, we analysed the DNA damage response in HBV-specific CD8 T cells from patients with CHB. Because H2AX becomes quickly phosphorylated at serine 139 (phosphoH2AX) following DNA-break formation, it is considered a sensitive marker of DNA damage.⁵ Thus, we first analysed phosphoH2AX levels of HBV-specific CD8 cells from patients with CHB and compared them with those of FLU-specific CD8 cells from the same patients and from healthy controls. A significantly higher H2AX-phosphorylation was detected in HBV-specific cells indicating higher levels of DNA damage in HBV core than in control FLU-specific CD8 cells ([Fig. 1A](#), left).

We then analysed the response to exogenously induced DNA damage, by treating samples with the genotoxic agent etoposide.⁶ A greater upregulation of phosphoH2AX was detected in FLU-specific cells from patients with CHB and healthy individuals than in HBV-specific CD8 cells ([Fig. 1A](#), middle). As a result, the ratio of the phosphoH2AX median fluorescence intensity (MFI) in the etoposide-treated/untreated samples was significantly lower in HBV- than in control FLU-specific CD8 cells ([Fig. 1A](#), right).

As T cell proliferation is also known to be associated with spontaneous generation of DNA breaks, we looked at the variation in the phosphoH2AX expression upon T cell activation following anti-CD3/CD28 stimulation. As shown in [Fig. S2A](#), CD8 T cells from controls, but not T cells from patients with CHB, upregulated phosphoH2AX significantly.

The same trend was observed for other mediators of the response to DNA damage, such as phosphorylated ATM (phosphoATM) ([Fig. S2B](#)), which is a key mediator of H2AX phosphorylation in response to DNA damage,⁷ as well as 53BP1, which is recruited by phosphoH2AX to the site of damage⁸ ([Fig. S2C](#)).

Next, we evaluated the formation of ADP-ribose-polymers ('PARylation'), as an indicator of poly-ADP-ribose-polymerase (PARP) activity which, in response to DNA strand break detection, catalyses poly(ADP-ribose) (PAR) synthesis, as a scaffold to mediate early recruitment of repair factors.⁴ Lower parylation levels were detected in HBV- than in FLU-specific T cells by careful comparison of paired HBV- and FLU-specific CD8 cell samples derived from each individual patient with chronic infection ([Fig. 1B](#)), to overcome the potential problem posed by the wide variability in parylation levels among different individuals.⁹ Moreover, following anti-CD3/CD28 stimulation, parylation tended to increase in controls and to decrease in patient samples, resulting in a significant difference in the PAR MFI ratio of stimulated/unstimulated patients' samples ([Fig. 1C](#)).

Altogether, these data demonstrate that HBV-specific CD8 cells from patients with CHB display higher DNA damage, but weaker activation of DNA repair responses.

CD38 is overexpressed in HBV-specific CD8 T cells from patients with CHB

Defective parylation could be caused by a diminished availability of NAD molecules, that represent the ADP-ribose donors for PARP activity.⁴ It is conceivable that persistent ROS generation maintains DNA damage, which may in turn stimulate DNA repair effectors, including PARPs, leading to NAD consumption and progressive DNA repair impairment.¹⁰

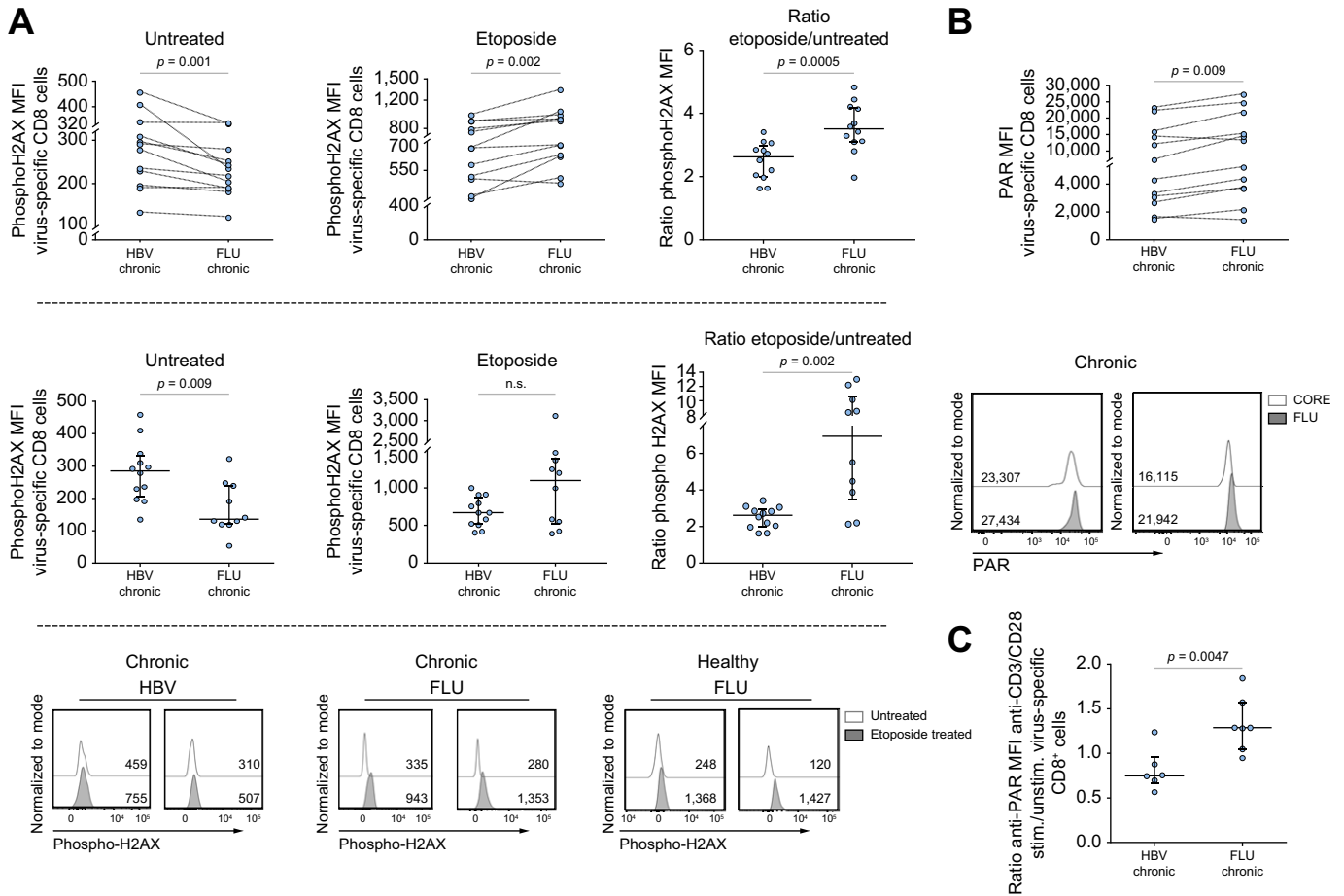


Fig. 1. DNA damage and dysfunctional DNA repair in exhausted HBV-specific CD8 T cells. (A) Left and middle: phosphoH2AX MFI in untreated or etoposide-treated HBV- compared with FLU-specific CD8 cells from the same patients with CHB (top) or from healthy individuals (bottom). The ratio of phosphoH2AX MFI etoposide-treated/untreated is also represented for HBV- and FLU-specific CD8 cells from patients with CHB (top), and for core-specific CD8 cells from patients with CHB and FLU-specific CD8 cells from healthy individuals (bottom). Bottom: representative examples of anti-phosphoH2AX MFI in etoposide-treated and untreated virus-specific CD8 cells. (B) Top: parylation levels in HBV- and FLU-specific CD8 cells from patients with CHB; bottom: representative examples. (C) Ratio between anti-PAR MFI of anti-CD3/CD28 stimulated and unstimulated core- and FLU-specific CD8 cells from patients and controls, respectively. Median and IQR are reported in the graphs. Statistics were determined using the Wilcoxon-matched-paired-test or Mann-Whitney *U* test. CHB, chronic HBV; FLU, influenza; MFI, median fluorescence intensity; phosphoH2AX, phosphorylated H2AX.

To further investigate whether other mechanisms can contribute to NAD depletion in patients with CHB, in addition to consumption by parylation, we analysed the expression of the NAD glycohydrolase CD38, which catalyses NAD hydrolysis, thereby representing another major intracellular regulator of NAD levels.¹¹ Confirming at the protein expression level the upregulation previously observed in CD38 gene expression,¹ frequency of CD38 expression and CD38 MFI levels were significantly higher among HBV-specific CD8 cells from patients with CHB than among HBV-specific CD8 cells derived from people who had recovered from an acute HBV infection (resolved) and FLU-specific CD8 cells from individual patients with chronic infection and healthy controls (Fig. 2A).

CD38 and PD-1 expression were directly correlated (Fig. 2B); PD-1 and CD38 were expressed at comparable levels, whereas HLA-DR expression was significantly lower (Fig. 2B). PD-1 was significantly more expressed on virus-specific CD8 cells with high CD38 levels (CD38^{hi}; Fig. 2C).

CD38 expression in exhausted HBV-specific CD8 T cells is inversely correlated with antiviral T cell function

The level of CD38 expression may be correlated with the level of NAD depletion thus suggesting that the extent of CD38 expression in exhausted HBV-specific CD8 T cells should be linked to the severity of T cell dysfunction. As the extremely low HBV-specific CD8 cell frequencies precludes the possibility to reliably detect *ex vivo* cytokine production, we measured IFN- γ production by *in vitro* expanded HBV-specific CD8 cells from patients with CHB upon HBV-peptide stimulation. By this analysis, a sharp segregation of two different populations of patients was observed (Fig. 3A, left) with a significant inverse correlation between IFN- γ production and *ex vivo* CD38 expression ($p = 0.004$, $r = -0.76$; Fig. 3A, right) and cut-off values between the two patient subgroups of 7.87% for IFN- γ + CD8 T cells and 50% for CD38^{hi} HBV-specific CD8 cells, as defined by applying a ROC curve analysis (100% sensitivity and specificity; Fig. 3B).

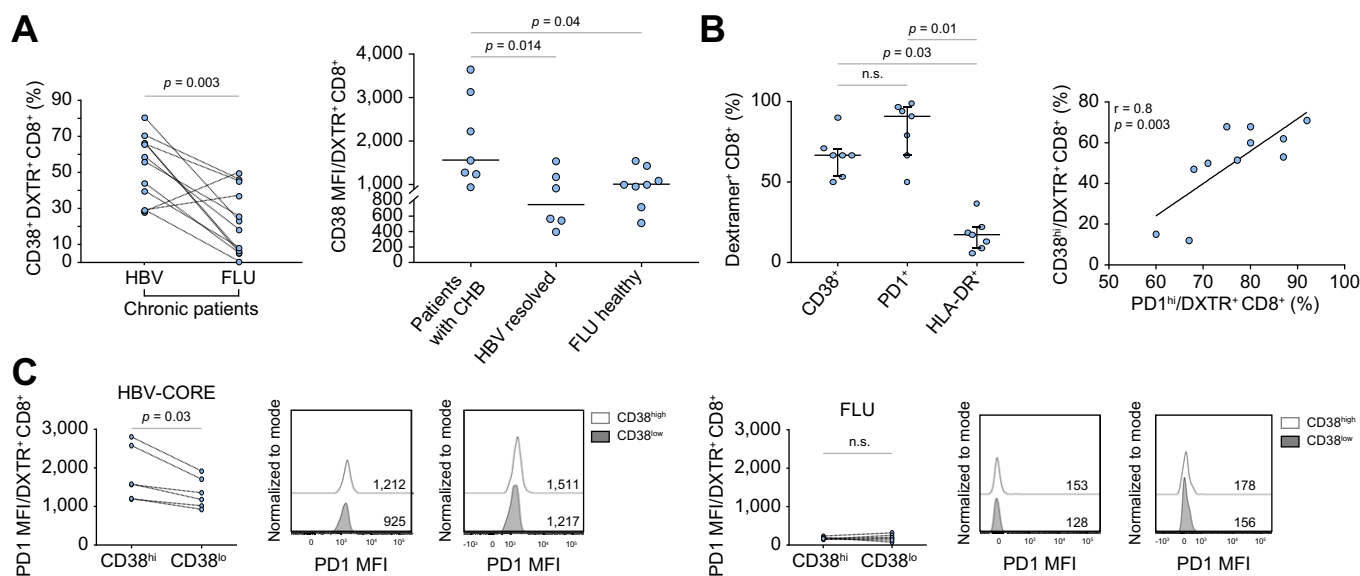


Fig. 2. CD38 expression and telomere length in HBV-specific CD8 T cells from patients with CHB. (A) Left: %CD38+ HBV- and FLU-specific CD8 cells in the same patients with CHB (Wilcoxon matched-paired test); right: CD38 MFI of HBV-specific CD8 cells from patients with CHB, resolved individuals, and FLU-specific CD8 cells from healthy controls (Mann-Whitney *U* test). (B) Left: percentage of CD38, PD1, and HLA-DR-positive HBV-specific CD8 cells from patients with CHB. Median and IQR are shown. Right: correlation between PD1 and CD38 MFI in HBV-specific CD8 cells from patients with CHB (Spearman correlation). (C) Left: PD1 MFI in paired CD38^{hi} and CD38^{low} HBV-specific (top) and FLU-specific (bottom) CD8 cells from patients with CHB (Wilcoxon matched-paired test); right: representative examples. CD38^{hi}, high CD38 levels; CD38^{low}, low CD38 levels; CHB, chronic HBV; FLU, influenza; MFI, median fluorescence intensity.

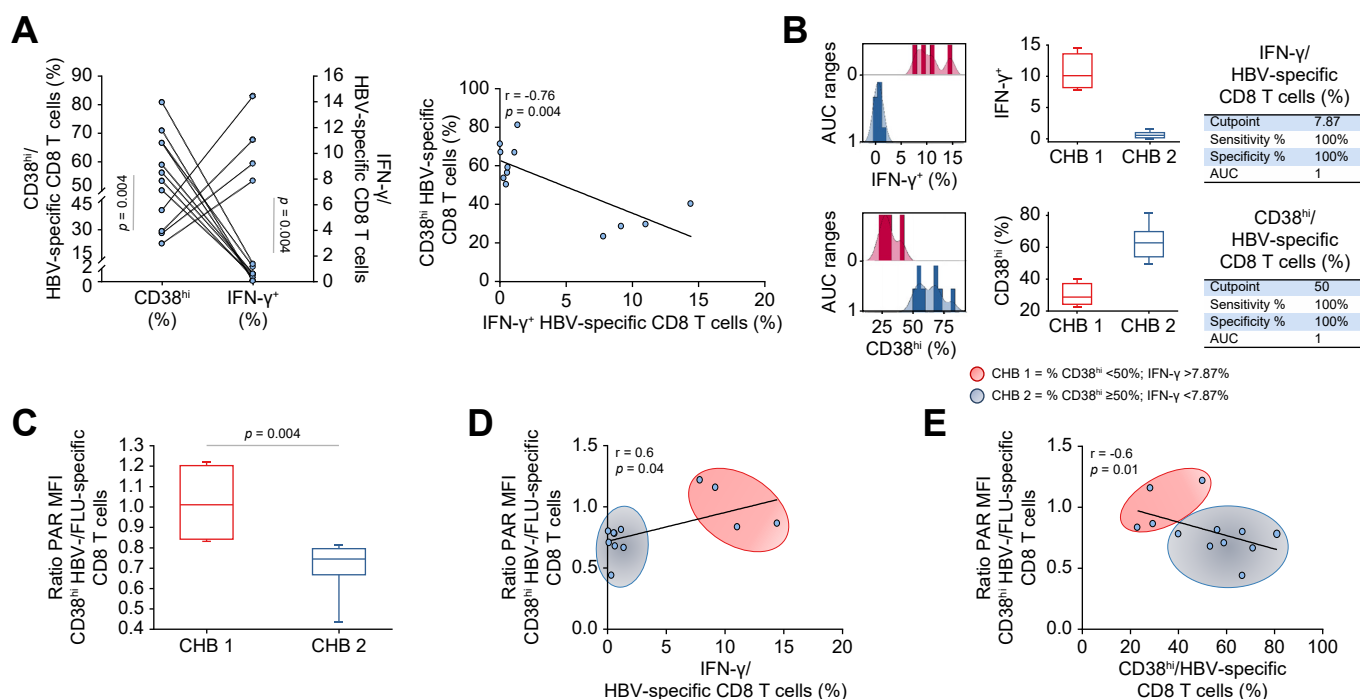


Fig. 3. Correlation between CD38 and T cell dysfunction. (A) Left: paired percentages of CD38^{hi} and of *in vitro* expanded HBV-specific IFN- γ + CD8 lymphocytes for each patient with CHB (Mann-Whitney *U* test). Right: Spearman inverse correlation between %HBV-specific CD38^{hi} and %HBV-specific IFN- γ + lymphocytes upon *in vitro* expansion. (B) Histogram and box plot showing the distribution of %IFN- γ + (top) and %CD38^{hi} (bottom) HBV-specific lymphocytes in patients with CHB, according to the ROC curve analysis. Threshold value, sensitivity, specificity and area under the curve (AUC) are reported on the right. Based on threshold values, patients can be divided into two subgroups (CHB1 and CHB2). (C) Ratio of anti-PAR MFI in CD38^{hi} HBV-specific and FLU-specific lymphocytes derived from individual patients with CHB of the two CHB subgroups distinguished by the parameters described in panel B (Mann-Whitney *U* test). (D,E) Correlation between anti-PAR MFI ratio of CD38^{hi} HBV-specific/FLU-specific cells and %IFN- γ +CD8 cells (D) or %CD38^{hi} (E) among HBV-specific lymphocytes from patients with CHB (Spearman's correlation). CD38^{hi}, high CD38 levels; CHB, chronic HBV; IFN- γ , interferon-gamma; MFI, median fluorescence intensity; PAR, poly(ADP-ribose). (This figure appears in color on the web.)

Interestingly, HBV-specific CD8 cells with higher IFN- γ production and lower CD38 expression displayed also significantly higher parylation compared with CD8 cells of the other patient group (Fig. 3C). Indeed, *ex vivo* parylation levels in HBV-specific CD8 cells normalised to FLU-specific cells from the same patients with CHB, correlated directly with %IFN- γ + HBV-specific CD8 cells ($p = 0.04$, $r = -0.6$, Fig. 3D) and inversely with %CD38^{hi} cells ($p = 0.01$, $r = -0.6$; Fig. 3D, E).

Altogether these results allow to link high CD38 expression to T cell dysfunction, as indicated by reduced parylation and antiviral cytokine production, in line with the known CD38 NAD-hydrolyase activity and its possible role in NAD depletion.

Exhausted HBV-specific CD8 T cells display shorter telomeres than FLU-specific CD8 T cells

DNA repair deficiencies have been reported to result in the accumulation of both non-telomeric and telomeric DNA damage, and ROS can participate in the regulation of telomere length maintenance mechanisms.¹² We thus wondered whether high ROS and DNA damage levels, along with dysfunctional DNA repair mechanisms, could alter telomere length. To investigate this possible link, we compared telomere length by flow cytometry-fluorescence *in-situ* hybridisation (FLOW-FISH)¹³ in HBV- and FLU-specific CD8 cells from the same patients with CHB, to avoid interference of a series of variables (e.g. age, diet, etc.), known to influence the telomere length. By this analysis, telomeres of HBV-specific cells appeared to be significantly shorter than those of FLU-specific CD8 cells (Fig. S3A). This suggests that CHB can lead to telomere attrition in exhausted virus-specific CD8 cells and accelerate T cell senescence.

We then asked whether the oxidative damage related to excess ROS production can represent a cause of telomere shortening. To answer this question, we sought to provide an indirect evidence of ROS involvement, by comparing telomere length in HBV-specific CD8 T cells cultured in the presence or absence of mitochondria-targeted-(mt)antioxidant compounds (MitoQ or MitoTempo), which are known to express mitochondrial ROS-scavenger activity. As shown in Fig. S3B, in mt-antioxidant-treated cultures HBV-specific CD8 cells showed an increased telomere length in comparison with untreated cultures, thus confirming literature data that show a link between mitochondrial excess ROS production and telomere shortening.¹⁴ Interestingly, in antioxidant-treated virus-specific CD8 T cells we observed also PD-1 decrease and a trend to diminished CD38 expression (Fig. S3B).

NAD replenishment can improve intracellular dysfunctions in HBV-specific CD8 T cells

If the level of CD38 expression and the related NAD depletion are causally linked to T cell dysfunction, then NAD replenishment should correct the altered intracellular functions. First, we stained anti-CD3/CD28-stimulated and unstimulated samples with an anti-PAR antibody in the presence/absence of exogenous NAD. Incubation with NAD significantly increased PAR MFI in the stimulated HBV-specific CD8 cells from patients with CHB, but not in the stimulated FLU-specific CD8 cells from healthy individuals (Fig. 4A), supporting the concept that reduced NAD availability can affect parylation in CHB. Then, we treated lymphocyte cultures from patients with chronic

infection with the NAD precursor nicotinamide mononucleotide (NMN), in light of the evidence that its administration can efficiently enhance NAD biosynthesis in different *in vitro* and *in vivo* experimental conditions.¹⁵ Exhausted lymphocytes cultured in the presence of NMN increased phosphoH2AX expression following stimulation with HBV-core 15-mer peptides, as indication of improved DNA repair (Fig. 4B). NMN treatment also reduced the fraction of depolarised CD8 cells, as detected by JC-1 staining (Fig. 4C), and decreased aggresome accumulation (Fig. 4D).

The limited number of the tested HLA-A2+ patients precluded the possibility to assess the prediction power of the combined CD38 and IFN- γ cut-off values on the response to NAD supply.

NMN supplementation can improve the antiviral T cell function

We then assessed whether NMN can also improve T cell antiviral functions. After expansion of PBMC from patients with CHB by HBV-core peptide stimulation in the presence/absence of NMN, a significant improvement in CD8 T cell cytokine production (mean increase 3.4-, 4-, 2.7-fold for interferon-gamma [IFN- γ], tumour necrosis factor-alpha [TNF- α], IL-2, respectively) was observed in the NMN-treated cultures (Fig. 5A). Double IFN- γ /TNF- α -positive CD8 cells increased by 3.8-fold (Fig. 5A) and also CD8 cell cytotoxic activity, measured by CD107a upregulation, was significantly improved by NMN treatment (Fig. 5A). By dextramer staining, expansion of HBV-specific CD8 T cells was also enhanced (Fig. 5B) and a significant increase in cytokine production was detected in total CD3 T cells upon 15-mer HBV peptide stimulation (Fig. S4).

CD38 inhibition can complement the NMN T cell functional reconstitution effect

To investigate whether inhibition of the CD38 enzymatic activity can be a complementary strategy to boost cellular NAD levels and modulate T cell functions, we assessed the effect of a small CD38-inhibitor molecule¹⁶ (CD38i) on parylation in HBV-specific CD8 T cells from patients with CHB, following anti-CD3/CD28 stimulation. Similarly to what we observed with NAD supplementation (Fig. 4A), parylation levels increased upon incubation with CD38i (Fig. 6A). Moreover, a trend to telomere length increase and to attenuation of PD1 expression was observed upon combined NMN + CD38i treatment; instead, CD38 expression decreased only in some samples (Fig. 6B).

We then analysed the effect of CD38i addition in the cultures that displayed an increase in IFN- γ production of less than two fold in the presence of NMN. Cytokine production further increased in eight out of 13 (61.5%) of these samples (Fig. 6C). For five of these patients an improvement was observed for all the tested functions, whereas only one patient did not improve T cell activity by the combined treatment with CD38i and NMN. In HLA-A2+ samples, HBV-specific CD8 T cell proliferation was also improved (Fig. 6D). Moreover, exhausted HBV-specific CD8 cells resulted more sensitive than CD8 cells of HBV-unrelated specificity to the combined treatment (Supplementary material and Fig. S5). The prediction power of the combined CD38 and IFN- γ cut-off values on the response to the modulatory effect of NMN alone or in combination with the

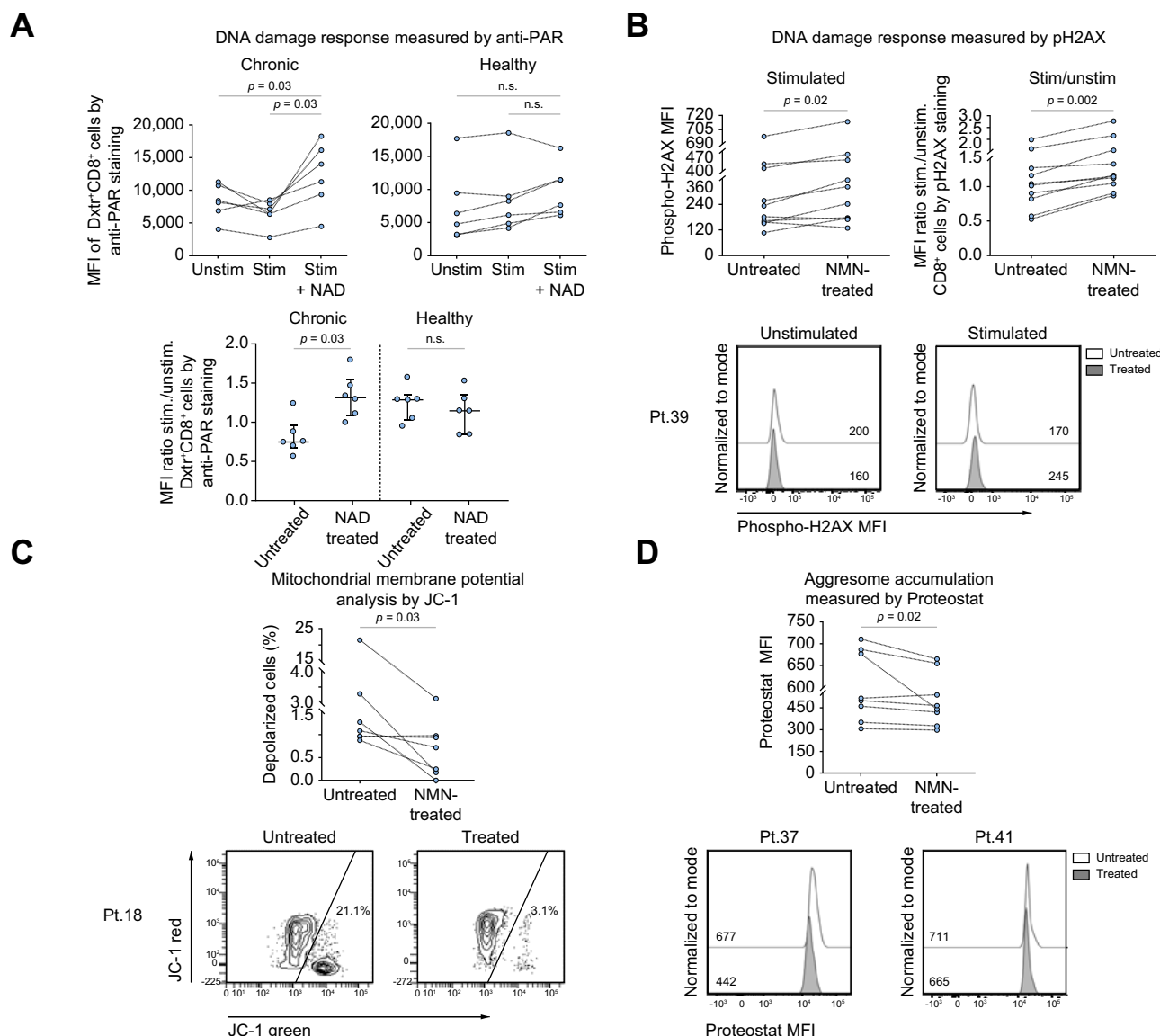


Fig. 4. Effect of NAD supplementation on defective intracellular functions. (A) Top: anti-PAR MFI in unstimulated and anti-CD3/CD28-stimulated virus-specific CD8 cells with/without NAD addition in patients with CHB (left) and healthy individuals (right; Wilcoxon matched-paired test). Bottom: fold-change values of stimulated/unstimulated virus-specific CD8 cells (Mann-Whitney U test). Median and IQR values are reported. (B–D) PBMCs from patients with CHB were stimulated *in vitro* for 10 days with HBV-core peptides, in the presence/absence of NMN and then analysed in paired untreated and NMN-treated samples for (B) phosphoH2AX MFI (left) and MFI ratio of stimulated/unstimulated cells (right) or (C) %depolarised CD8 cells by JC-1 staining or (D) intracellular aggresome accumulation by Proteostat MFI. Representative examples on the bottom of each panel. Statistics by the Wilcoxon matched-paired test. CHB, chronic HBV; NMN, nicotinamide mononucleotide; MFI, median fluorescence intensity; PAR, poly(ADP-ribose); phosphoH2AX, phosphorylated H2AX.

CD38 inhibitor treatment could not be evaluated because most of the tested patients were HLA-A2 negative.

Comparison of HBV-specific CD8 T cells specific for core18–27 or pol455–463 epitopes

Given the low pol-specific CD8 T cell frequency limiting the possibility to compare intracellular defects in CD8 T cells of different HBV specificity, we applied low-input RNA-seq on sorted core18–27 and pol455–463-specific CD8 cells from nine patients with chronic infection and from four individuals who previously had acute hepatitis B (which was now resolved), as controls, to compare their DNA repair pathways. As shown in

Fig. S6A (left), a similar number of DNA repair-related genes (13 and 16 for core and pol, respectively) were significantly deregulated, with prevalence of downregulation. Among these deregulated genes, nine transcripts (corresponding to 56% and 69% of the total deregulated genes) were common to T cells specific for the two epitopes (Fig. S6A, left) and showed a similar positive or negative fold change orientation (Supplementary material and Fig. S6A, centre).

Upregulation of the CD38 coding gene was also detected in polymerase-specific CD8 T cells (Fig. S6B) and a comparable increase in cytokine production was detected upon NMN + CD38i treatment of core18–27- and pol455–463-specific CD8 T cell stimulated with either core18–27 or pol455–463 epitopes or

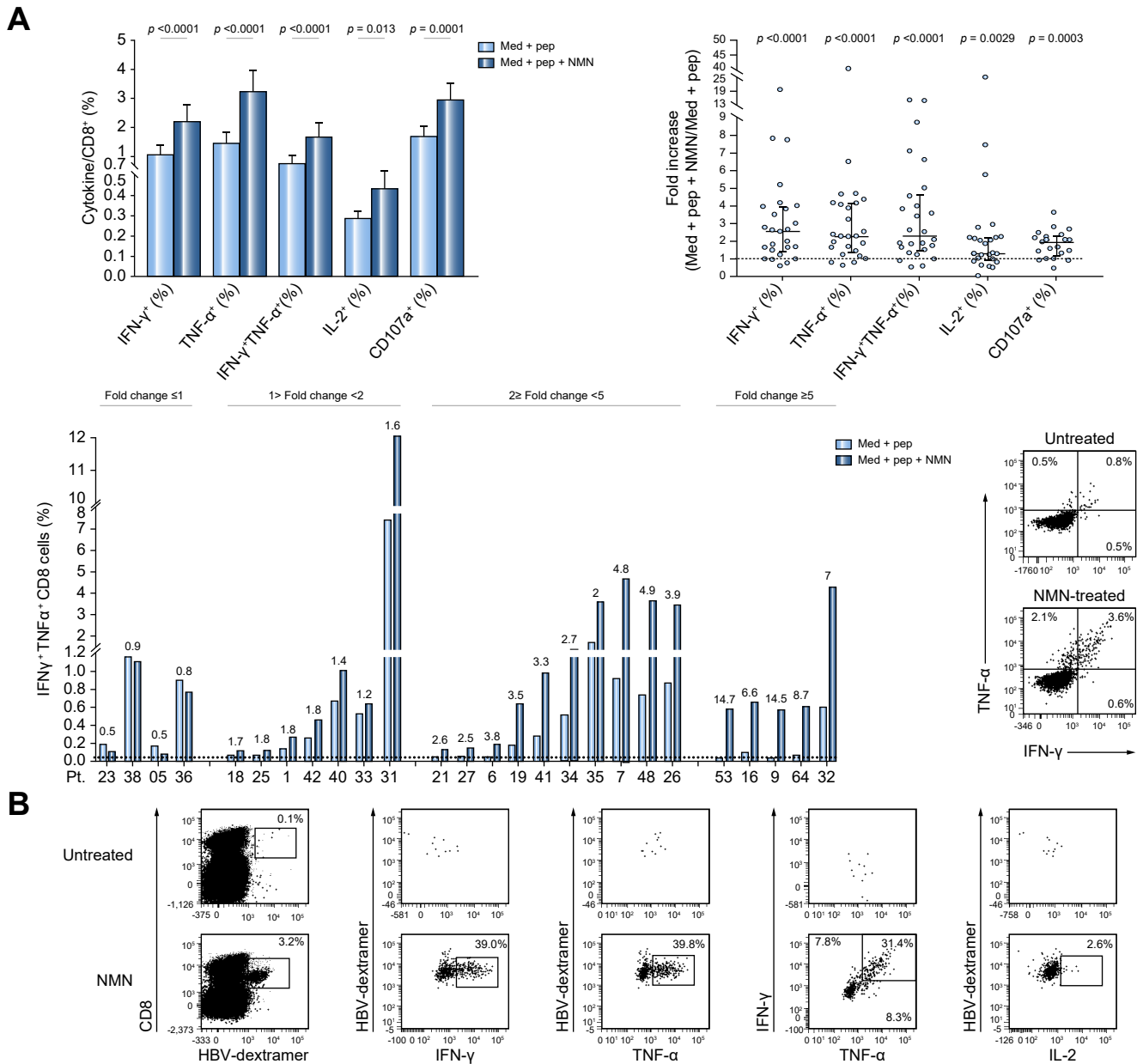


Fig. 5. Functional restoration of exhausted HBV-specific CD8 T cells by NMN. (A) Top-left: mean and standard-error values of %cytokine+ and %CD107a+ CD8 cells in T cell lines generated by HBV-core peptide stimulation for 10 days in the presence/absence of NMN (N = 26) (Wilcoxon matched-paired test). Top-right: NMN-induced increase in cytokine levels in CD8 cells of each patient (ratio NMN-treated/untreated samples); median and IQR values are reported (Wilcoxon signed-rank test). Bottom-left: %IFN γ +TNF α + T cells of each patient in the presence/absence of NMN; numbers on the top of each couple of bars indicate the NMN-induced fold-increase. (B) NMN effect on %IFN γ +, TNF α +, IL2+, and IFN γ +TNF α + HBV-specific CD8 cells in a representative patient. IFN- γ , interferon-gamma; NMN, nicotinamide mononucleotide; TNF- α , tumour necrosis factor-alpha.

with 15-mer overlapping peptides corresponding to the whole core and polymerase protein sequences (Fig. S6C).

Thus, the DNA repair function is similarly impaired in core- and polymerase-specific CD8 T cells and NAD supplementation represents an effective strategy for HBV-specific CD8 T cell restoration regardless of T cell antigenic specificity.

Discussion

Persistent T cell exposure to high antigen loads in patients with CHB causes DNA replicative stress with DNA damage accumulation and likely plays a key role in the maintenance of CD8 T cell exhaustion. This effect may be further amplified if DNA

repair mechanisms are defective. Persistent T cell stimulation may also lead to overexpression of CD38, which is involved in T cell activation. Our data show that CD38 expression in CD8 T cells of chronic HBV patients correlates directly with PD1 levels and inversely with cytokine production, suggesting its involvement in T cell dysfunction. This finding is in line with literature data showing increased CD38 expression associated with T cell exhaustion and with metabolic and epigenetic alterations in different models of chronic infection and cancer,¹⁷⁻¹⁹ thereby identifying CD38 as an additional marker of T cell exhaustion. Moreover, in aged mice CD38 overexpression was associated with low mitochondrial membrane potential

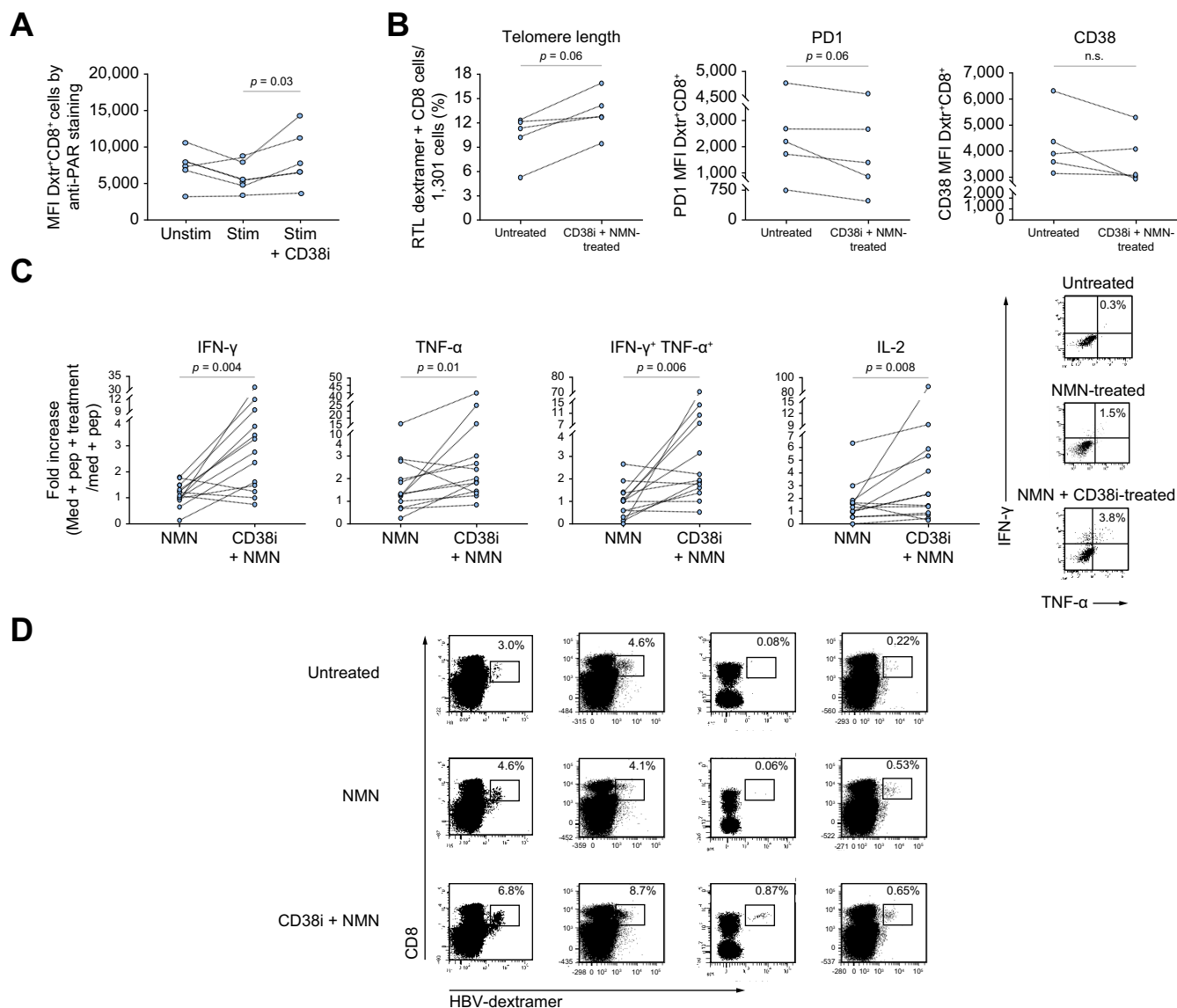


Fig. 6. Effect of CD38 inhibition on the HBV-specific CD8 T cell function. (A) Anti-PAR MFI in unstimulated and anti-CD3/CD28 stimulated HBV-specific CD8 cells from patients with CHB in the presence/absence of CD38i (Wilcoxon matched-paired test). (B) RTL, PD-1 and CD38 MFI in paired untreated and NMN + CD38i-treated HBV-specific T cells from patients with CHB expanded *in vitro* by HBV-core18-27 peptide stimulation (Wilcoxon matched-paired test). (C) Cytokine fold-increase in paired NMN- or NMN + CD38i-treated samples stimulated with HBV-core peptides (Wilcoxon matched-paired test). A representative example is shown on the right. (D) Effect of NMN or NMN + CD38i on HBV-specific CD8 cell proliferation stimulated with the HBV-core18-27 peptide. CD38i, CD38-inhibitor; CHB, chronic HBV; MFI, median fluorescence intensity; NMN, nicotinamide mononucleotide; PAR, poly(ADP-ribose), RTL, relative telomere length.

and increased dependence on glycolysis,²⁰ metabolic features common to HBV-specific CD8 T cells in CHB.²¹

CD38 has been recognised as the major NAD consumer.^{11,20} In aged mice CD38 overexpression has been associated with high NAD consumption and subsequent depletion, and with altered mitochondrial function.²⁰ Therefore, CD38 upregulation may be causally involved in NAD shortage also in exhausted HBV-specific CD8 T cells.

In our study NAD depletion is also suggested by the inverse correlation of CD38 expression with parylation, that we observed in HBV-specific CD8 cells from patients with CHB. Indeed, parylation is well known to be catalysed by PARP enzymes in response to DNA damage sensing and to be dependent on NAD molecules, as donors of ADP-ribose units.⁴ Although a direct quantification of intracellular NAD levels

was precluded by the extremely low frequency of circulating HBV-specific CD8 cells in patients with CHB, the improvement of parylation in response to exogenous NAD supplementation in exhausted CD8 T cells further supports the possibility of intracellular NAD shortage.

In addition to parylation impairment, poorly efficient DNA repair is indicated also by the altered activation of other key mediators of the DNA damage response. These include defective phosphorylation of histone H2AX and of the serine/threonine kinase ATM, that promotes H2AX phosphorylation.⁷ As detection of phospho-H2AX is typically considered a marker of DNA damage,⁵ abnormally elevated levels of phospho-H2AX in the absence of exogenous stimuli indicate not only reduced DNA damage sensing, but also accumulation of DNA damage in exhausted HBV-specific CD8 cells.

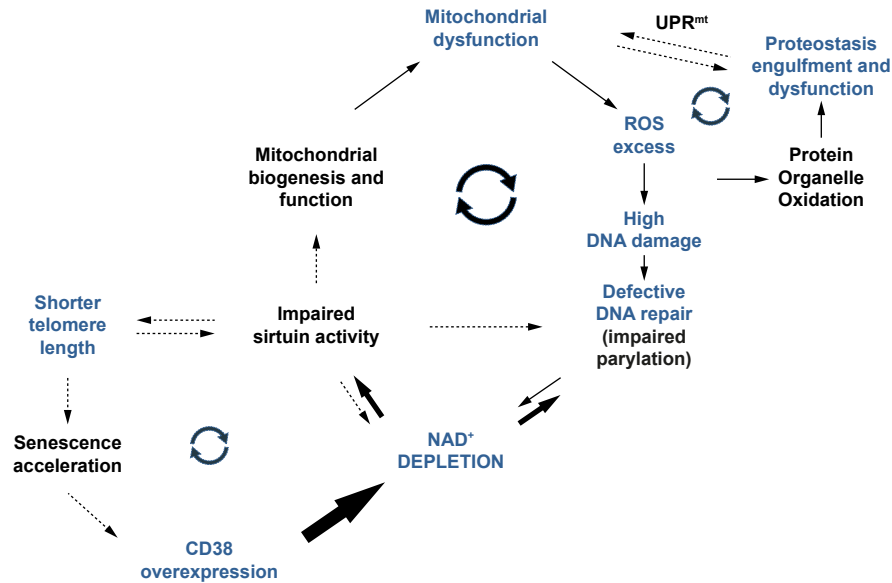


Fig. 7. Intracellular network of deregulated functions underlying HBV-specific CD8 T cell exhaustion. High mitochondrial ROS levels can increase protein and DNA oxidation with subsequent accumulation of intracellular aggregates, affecting proteostasis. Mitochondria can contribute to re-establish protein homeostasis via the ‘mitochondrial-unfolded-protein-response (UPR^{mt})’. DNA damage stimulates persistent activation of DNA repair, including NAD-dependent parylation, that consumes NAD. The upregulation of CD38, the major NAD⁺ hydrolysing enzyme, contributes to NAD depletion. This can in turn affect the activation of sirtuins and reduce the NAD/NADH ratio, with a potential impact also on the availability of acetyl-coenzyme A for histone acetylation. Reduced sirtuin activity can contribute to mitochondrial dysfunction, affect telomere length and several DNA repair pathways generating a vicious cycle which would maintain persistent DNA damage and NAD consumption. All experimentally validated steps are highlighted in blue. ROS overproduction, proteostasis engulfment, NAD depletion, CD38 upregulation, and epigenetic deregulation represent potential targets for immune-modulatory therapies. ROS, reactive oxygen species.

Dampened ATM activation has been reported to be a cause of DNA damage accumulation also in T cells derived from patients with chronic HCV or HIV.^{22,23} Interestingly, parylation has been demonstrated to be required for optimal ATM activation,²⁴ suggesting that also the observed reduction of ATM function and the consequently impaired phosphorylation of its downstream targets, could be causally linked to diminished NAD availability.

As observed in pathologies characterised by an impaired DNA damage response, where NAD depletion is caused by PARP hyperactivation,²⁵ when DNA breaks fail to be repaired, the persisting stimulus to PARP activation can lead to a profound consumption and depletion of the intracellular NAD pool.¹⁰ Similarly, the increased DNA damage, likely triggered in early exhausted HBV-specific CD8 cells by the persistent replication stress, may result in PARP hyperactivation, with NAD consumption and depletion, which, in turn, may negatively affect the parylation activity of late exhausted cells.

NAD consumption by CD38 and PARPs can also negatively affect mitochondrial homeostasis by limiting NAD availability to sirtuins. In particular, sirtuin-3 (SIRT3) transcription is known to be induced through sirtuin-1 (SIRT1)-controlled peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α) activation. A reduced NAD availability within the nucleus is expected to interfere with the NAD–SIRT1–PGC1 α axis,²⁶ which in turn can affect SIRT3 transcription, as shown by SIRT3 gene downregulation in our RNAseq analysis on sorted HBV-specific CD8 cells from patients with chronic infection (data not shown). Sirtuins are also critical regulators of DNA repair.²⁷ Therefore, reduced sirtuin function, as a result of diminished NAD availability may, in turn, exacerbate DNA damage and mitochondrial dysfunction, with possible generation of a self-maintaining negative loop.³

A defective DNA repair function is not only a characteristic of core18–27-specific CD8 T cells, but seems to be common also to CD8 T cells of different HBV specificity, as indicated by RNAseq analysis of sorted pol455–463-specific CD8 T cells.

Mitochondrial dysfunction with ROS elevation, DNA damage accumulation with defective DNA repair, and decreased NAD levels sustained by CD38 overexpression and PARP hyperactivation have also been described in cell senescence,^{3,20,25} which is typically associated with telomere erosion.²⁸ This phenomenon has been observed in HIV infection where it has been linked to ATM kinase deficiency.²³ Also HBV-specific CD8 cells, that show a defective ATM activation, display a reduced telomere length. This is in line with findings both from the literature and from our experiments with antioxidant-treated T cell cultures indicating an accelerated telomere attrition caused by ROS-induced oxidative stress.¹⁴ To telomere shortening may also contribute the marked telomere sensitivity to mitochondrial ROS,²⁹ as a result of the susceptibility of the telomere GGG site to ROS-induced 8-oxoguanine formation.^{26,30} Notably, CD28 that we previously found to be transcriptionally downregulated in HBV-specific CD8 cells,¹ has been shown to play a role in the induction of telomerase activity.³¹ Thus, ROS may drive senescence by directly causing telomere damage and by negatively regulating telomere length maintenance mechanisms.

Taken together, our results delineate a functional network of intracellular deregulated pathways related to mitochondria, proteostasis, and DNA repair processes that can lead to NAD depletion, which, in turn, can further fuel the overall dysfunctional network. This creates a vicious cycle whereby ROS overproduction contributes to DNA damage accumulation and persistent activation of DNA repair mechanisms (Fig. 7). PARP hyper-activation and CD38 overexpression would lead to

progressive NAD depletion with consequent impairment of the DNA repair machinery, further amplification of mitochondrial dysfunction and gene silencing. NAD shortage can epigenetically affect gene expression both by limiting sirtuin activity³² and through metabolic modifications.³³

All this information is crucial for an in-depth understanding of the intracellular mechanisms involved in T cell exhaustion and for the identification of key deregulated intracellular processes to be deployed as targets for the improvement of antiviral T cell control. Some of these mechanisms represent a link between T cell exhaustion and cellular ageing. In animal models of premature ageing, targeting NAD metabolism has been proposed as a potential therapeutic approach to improve health span by attenuating mitochondrial alterations, DNA damage response defects, and oxidative stress.¹⁵ NAD replenishment has been effective in eliciting mitochondrial biogenesis and respiratory chain activity also in human mitochondrial myopathies.³⁴

Based on indirect evidence pointing to NAD consumption and subsequent depletion in patients with CHB, we tested *in vitro* the effect of NAD replenishment with the NAD precursor NMN.¹⁵ The treatment efficiently restored mitochondrial membrane potential, aggregates digestion and DNA repair activation. As a likely consequence of the broad correction of different interconnected pathways, the antiviral T cell function was found to be significantly improved in NMN-treated cultures.

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Abbreviations

CD38^{hi}, high CD38 levels; CD38i, CD38-inhibitor; CHB, chronic HBV; FLOW-FISH, flow cytometry-fluorescence *in-situ* hybridisation; FLU, influenza: IFN- γ , interferon-gamma; MFI, median fluorescence intensity; mt, mitochondria-targeted; NMN, nicotinamide mononucleotide; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose)-polymerase; PARP1, poly(ADP-ribose)-polymerase 1; PARYlation, poly-ADP-ribosylation; PGC1 α , peroxisome proliferator-activated receptor gamma coactivator 1-alpha; phosphoH2AX, phosphorylated H2AX; ROS, reactive oxygen species; SIRT1, sirtuin-1; SIRT3, sirtuin-3; TNF- α , tumour necrosis factor-alpha.

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Conflicts of interest

CF received a grant from Gilead and Abbvie outside the submitted work; is a consultant for Gilead, Abbvie, Vir Biotechnology Inc, Arrowhead, Transgene, and BMS. PL is an advisor and speaker bureau for BMS, Roche, Gilead Sciences, GSK, MSD, Abbvie, Janssen, Arrowhead, Alnylam, Eisai, MYR Pharma, Antios, and Aligos. AL is a speaker bureau of MYR Pharma and Gilead Sciences. MM is an advisory board for Abbvie. The remaining authors declare no conflicts of interest that pertain to this work.

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

To further improve the effect of NMN supplementation, we also tested a combination of NMN and a CD38 inhibitor (CD38i).³⁵ For these experiments we used a small CD38 NADase activity inhibitor molecule,¹⁶ that was shown to reverse age-related NAD decline in mice.³⁵ Interestingly, CD38i enhanced parylation, counteracted telomere shortening in HBV-specific CD8 cells and improved significantly cytokine production, especially in samples that responded more weakly to NMN supplementation. This effect was more evident for T cells stimulated with HBV peptides compared to total anti-CD3 or FLU-peptide stimulation, thus suggesting a preferential effect on NAD-deficient T cells. Remarkably, NAD supplementation and CD38 inhibition were effective not only on core-specific, but also on polymerase-specific CD8 T cells. Thus, this strategy may be effective in HBV-specific CD8 T cell restoration regardless of T cell antigenic specificity.

In conclusion, our data show increased DNA damage with limited activation of the DNA repair machinery in HBV-specific CD8 T cells from patients with CHB. This strongly suggests that NAD-consuming enzymes, particularly overexpressed CD38, may play a pivotal role in NAD depletion. Reconstitution of many interconnected intracellular functions by NMN supplementation indicates that NAD depletion likely represents an important determinant of T cell exhaustion. Based on our results, NAD boosting could be worthy of consideration as a novel strategy for T cell functional reconstitution in CHB.

Authors' contributions

Execution of experiments: IM, CCB, GA, VB, MR, AV, AP, CT, VR, PF. Acquisition of data: IM, CCB, GA, VB, MR, AV, AP, CT, VR, PF. Statistical analysis: IM, CCB, GA, VB, MR, AV, AP, CT, VR, GP. Analysis and interpretation of data: IM, CCB, GA, PF. Manuscript drafting: IM. RNA-Seq data analysis: MM. GSEA analysis: BM. Recruitment and characterisation of the patients: CB, AA, MP, AL, ED, MB, RP, GA, CB, MM. Administrative support: DL. Interpretation of data: GM, PL. Critical revision of the manuscript: GM, PL, CF. Obtained funding: CF. Study supervision: CF. Study concept and design: PF. Writing of the manuscript: PF.

Data availability statement

RNA-Seq data are available at NCBI GEO: GSE217838.

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Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2023.02.035>.

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