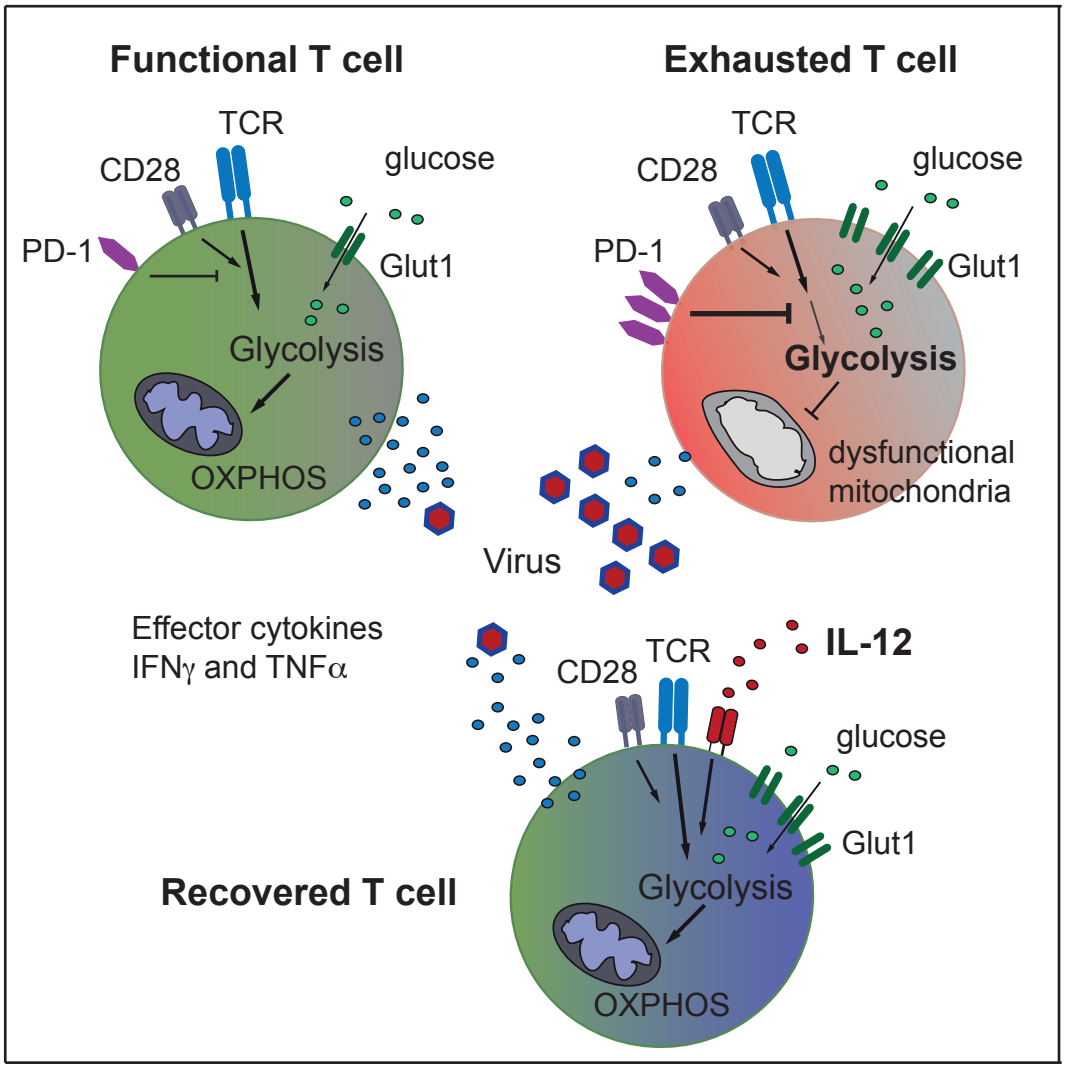
Graphical Abstract



Highlights:

Exhausted CD8 T cells show increased expression of glucose transporter-1

Functional, but not exhausted T cells can use OXPHOS to supplement their energy demand

Exhausted T cells harbour dysfunctional depolarised mitochondria

Interleukin-12 can rescue mitochondrial function and effector responses in exhausted CD8

eTOC

T cells undergo extensive metabolic changes upon activation. Schurich et al find that functional and exhausted human virus-specific CD8 T cells have distinct metabolic phenotypes, shaping their effector capacity.

Distinct metabolic requirements of exhausted and functional virus-specific CD8 T cells in the same host

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Short title: Distinct metabolism of functional and exhausted T cells

Abstract

T cells undergo profound metabolic changes to meet the increased energy demands of maintaining an antiviral response. We postulated that differences in metabolic reprogramming would shape the efficacy of CD8 T cells mounted against persistent viral infections. We found that the poorly functional PD-1^{hi} T cell response against HBV had upregulated the glucose transporter, Glut1, an effect recapitulated by oxygen deprivation to mimic the intrahepatic environment. Glut1^{hi} HBV-specific T cells were dependent on glucose supplies, unlike the more functional CMV-specific T cells, that could utilise oxidative phosphorylation in the absence of glucose. The inability of HBV-specific T cells to switch to oxidative phosphorylation was accompanied by increased mitochondrial size and lower mitochondrial potential, indicative of mitochondrial dysfunction. IL-12, which recovers HBV-specific T cell effector function, increased their mitochondrial potential and reduced their dependence on glycolysis. Our findings suggest that mitochondrial defects limit the metabolic plasticity of exhausted HBV-specific T cells.

Introduction

On average, humans are infected with around 8-12 different persistent viruses during their life-time (Virgin et al., 2009). Most of these infections, like Epstein-Barr Virus (EBV) and cytomegalovirus (CMV) are benign in the vast majority of human hosts and the antiviral T cell response is adapted to keeping the virus at bay whilst limiting organ damage. Other chronic infections such as HIV, hepatitis C virus (HCV) and hepatitis B virus (HBV) cannot be controlled by the T cell response once persistence is established, often resulting in immunopathology and serious sequelae.

An estimated 240 million people worldwide are chronically infected with HBV, which is the leading cause of liver cirrhosis and hepatocellular carcinoma. CD8 T cells are one of the critical mediators of HBV clearance, by IFNγ mediated non-cytopathic mechanisms, possibly supported by direct cytotoxicity. However in chronic HBV infection, the pivotal anti-viral CD8 T cell response is virtually absent. The few HBV-specific T cells detectable are functionally exhausted, with expression of multiple co-inhibitory receptors and poor effector function (Ferrari, 2015), a state that has recently been suggested to allow them to adapt to the onslaught of high-dose antigen (Staron et al., 2014; Utzschneider et al., 2013).

In contrast T cells directed against CMV are a prototype of a functional response able to efficiently contain this highly prevalent, persistent viral infection. CMV-specific T cells can readily be detected in greatly expanded numbers, with conserved clonotypes often dominating the endogenous T cell repertoire (Khan

et al., 2002). They are phenotypically distinct, expressing late differentiation markers such as KLRG-1 rather than the multiple co-inhibitory receptors characteristic of HBV-specific T cells (Schurich and Henson, 2014). CMV-specific T cells produce significant amounts of effector cytokines such as IFNγ and TNF in response to stimulation with their cognate peptide in vitro.

Since HBV and CMV-specific T cells are both directed against persistent viruses but differ markedly in their functionality and phenotype, we were interested to compare their underlying metabolic requirements. It is increasingly recognised that adequate nutrient supply and energy production are key determinants of the capacity of T cells to proliferate and mediate effector function (Pearce and Pearce, 2013). Naïve and resting T cells make use of fatty acid oxidation and the mitochondrial tricarboxylic acid (TCA) cycle, which provides reducing agents for energy production through oxidative phosphorylation (OXPHOS)(Pearce et al., 2009). Recently it has been shown in murine models that mitochondrial activity is also needed for activating and maintaining antigen-specific responses (Okoye et al., 2015; Sena et al., 2013). Upon activation, CD8 T cells have been described to switch their metabolism to become heavily dependent on glycolysis, even in the presence of sufficient oxygen. Despite being less energy efficient, glycolysis provides fast energy and metabolites to support proliferation and effector function(MacIver et al., 2013).

Many recent advances in the understanding of T cell metabolism in naïve, effector and memory stages have been made (Pearce and Pearce, 2013). However, the current knowledge of T cell metabolism in chronic viral infections

is essentially limited to a single example, the murine model of LCMV (Schurich and Henson, 2014). Here we examine the metabolic requirements and restrictions of exhausted HBV-specific CD8 T cells to the more functional CMV-specific T cells within the same patients. Our data show that CD8 T cells specific for these two chronic viral infections have distinct metabolic profiles. CMV-specific T cells can fuel their energetic demands by making use of both glycolysis and OXPHOS to exert full effector functions. In contrast, exhausted HBV-specific T cells show an impaired capacity to utilise mitochondrial energy supply (OXPHOS), causing a dependence on glycolysis. Their defect in mitochondrial metabolism is rescued by the pro-inflammatory cytokine IL-12, which can stimulate a recovery in HBV-specific effector function (Schurich et al., 2013). Our data suggest that full effector function in human virus-specific CD8 T cells is dependent on energy supply through both OXPHOS and glycolysis.

Results

Glut-1 expression is higher in CD8 T cells directed against HBV than CMV

Upon TCR stimulation T cells markedly increase the expression of de novo synthesized glucose transporter 1 (Glut1) to facilitate glucose uptake; this correlates with an increase in glycolysis (Pearce and Pearce, 2013). To examine the capacity for glucose uptake by human virus-specific CD8 T cells, we analysed their expression of Glut1 upon activation. First, we stimulated T cells from chronically co-infected patients with HBV or CMV derived peptides for 4hrs directly ex vivo and measured Glut1 expression on dextramer-positive T cells. Glut1 expression was higher in activated virus-specific than in global CD8 T cells (data not shown). Glut1 expression was increased in HBV compared to CMV-specific T cells (Figure 1a), suggesting that a defect in glucose uptake was unlikely to be responsible for their defective cytokine production (Suppl figure 1a).

Since HBV-specific T cells from the circulation can only be detected at very low frequency we next expanded T cells in culture to study their metabolic phenotype in more detail. We confirmed that the differences observed ex vivo were maintained in vitro. HBV or CMV-specific CD8 were stimulated with their cognate peptides and, upon restimulation, identified by their production of IFNγ and co-stained for Glut1. Again, the frequency of Glut1 expression in virus-specific T cells was significantly increased above the amount expressed by the global CD8 cells in the same sample, confirming that Glut1 is upregulated upon antigen-specific activation (Fig1b,c).

We then compared paired HBV and CMV-specific CD8 T cell responses from the same donors, which had been activated to comparable levels as assessed by CD38 expression (Suppl Fig1b). The frequency of Glut1 positive cells was again higher for HBV-specific than CMV-specific CD8 in these paired samples (Fig1b,d). We observed the same increased expression of Glut1 in HBV compared to CMV, when cultured specific T cells were detected by dextramer staining in 3 patients with known responses to both viruses (Suppl Fig1c).

To confirm that increased Glut1 expression was mediating increased glucose uptake, we pulsed virus-specific T cells with the fluorescent glucose analogue 2-NBDG at the end of culture. Increased expression of Glut1 was accompanied by an increase in the uptake of 2-NBDG (Fig1e,f and correlation in Suppl Fig1d).

Glut1 upregulation is associated with T cell exhaustion and can be promoted by hypoxic conditions mimicking the hepatic milieu.

Since the increased Glut1 expression and glucose uptake on poorly functional HBV-specific CD8 was paradoxical, we postulated that it might relate to their site of antigen encounter in vivo. HBV only replicates in the liver, an immune suppressive environment where T cells have restricted supplies of oxygen (Jungermann and Kietzmann, 2000). Glut1 expression was indeed increased in paired patient-derived CD8 T cells from liver biopsies compared to PBMC directly ex vivo. In one HLA-A2+ patient we detected HBV-specific CD8 T cells, confirming increased Glut1 in the liver compared to the periphery (Fig 2a,b). We next recapitulated the hypoxic environment in vitro by stimulating HBV and CMV-specific T cells in normoxic versus hypoxic conditions (5% oxygen to mimic

the concentration in the hepatic circulation; (Jungermann and Kietzmann, 2000)). Glut1 expression was promoted in hypoxic conditions (Fig 2c), an effect which was independent of the amount of T cell proliferation (Suppl Fig2a). It is therefore possible that a phenotype favouring glycolysis might represent a feedback response promoted by the hepatic environment in which T cells encounter their antigen.

A hallmark of exhaustion is the co-inhibitory receptor PD-1 (Wherry, 2011), which is highly expressed on HBV-specific T cells (Ferrari, 2015). We found that even during culture, HBV-specific CD8 maintained higher levels of PD-1, accompanied by lower expansion and reduced production of IFNγ+, than CMV-specific CD8 (Suppl Fig2b,c; (Schurich et al., 2013). This is consistent with recent work showing that the exhausted phenotype is stable (Utzschneider et al., 2013). Antigen-stimulated HBV-responses showed a correlation between their expression of PD-1 and Glut1 (Fig2d), suggesting that exhausted T cells increase Glut1 expression. Furthermore, we found a significant inverse correlation between Glut1 expression and the magnitude of T cell expansion, such that HBV-specific cells from patients with the lowest frequency responses expressed the highest levels of Glut1 (Fig2e). This did not apply to CMV-specific T cells, for which there was no significant correlation between Glut1 and magnitude of response in culture (Fig2f).

To further probe the metabolic phenotype of patient-derived T cells, we measured cellular oxygen consumption rates during a mitochondrial stress test. The extremely low circulating frequency of HBV-specific T cells precluded their

use for this type of biochemical analysis. PD-1 is expressed on recently activated, and maintained on exhausted, CD8 T cells in chronic viral infections (Barber et al., 2005) including HBV (Boni et al., 2007; Fisicaro et al., 2010; Schurich et al., 2013). Studying the impact of PD-1 on global CD8 T cell metabolism in patients with persistent viral infections is of importance since therapeutic blockade of this pathway (currently considered in HBV following recent successes in cancer) will affect all PD-1+ cells. We therefore sorted PD-1+ and PD-1– CD8 T cells and found that both fractions utilised glycolysis and OXPHOS, as measured respectively by their extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), to varying degrees in the 3 patients tested (Suppl Fig2d). However, the spare respiratory capacity (SRC), calculated as percentage change in mean OCR at baseline to maximal OCR upon treatment with FCCP was lower in PD-1+ compared to PD-1– CD8 T cells (Fig2g,h), a feature retained when cultured in vitro (Suppl Fig2e). SRC is a measure of maximal mitochondrial capacity available to a cell (Henson et al., 2014), therefore the low SRC suggests that PD-1+ T cells are less well equipped to function in conditions of increased energy demand. The low SRC in PD-1+ cells was at odds with the higher abundance of memory cells in this population (Suppl Fig2f), described to be high utilisers of OXPHOS (van der Windt et al., 2012). Our findings therefore reinforced the potential relevance of PD-1 expression, rather than T cell differentiation state, as a driver of the metabolic changes observed.

HBV-specific T cells are dependent on glycolysis for immediate effector function

To further examine the paradoxical increase in glucose uptake by exhausted HBV-specific T cells, we tested their dependence on glycolysis to provide energy.

To this end we stimulated T cells in media containing galactose instead of glucose, to prevent them efficiently utilising glycolysis (Chang et al., 2013). After expansion in regular media, cells were split into either glucose or galactose containing media to assess the requirement for glycolysis for immediate effector function upon antigenic restimulation.

CMV-specific cells showed a diverse response to glucose deprivation, with IFNγ production in some patients being completely unaffected or even increased and in others being decreased (Fig3a,b). Overall the frequency of IFNγ+ CMV-specific CD8 in the presence of galactose compared to glucose was slightly reduced, whilst overall the amount of IFNγ produced per cell was unaffected (Suppl Fig3a). In contrast, the IFNγ response of HBV-specific cells declined strikingly upon culture in galactose (Fig3a,b) with IFNγ MFI also decreasing (Suppl Fig3a). Thus HBV-specific CD8 showed significantly more dependence on glycolysis than CMV-specific CD8 when mounting an immediate effector response (Fig3b). Similarly the frequency of T cells able to produce tumour necrosis factor (TNF) upon peptide restimulation in galactose was more affected in HBV- ($p=0.0005$) than CMV-specific ($p=0.0399$) responses (Fig3c).

We next asked whether there was an association between Glut1 expression and the ability of virus-specific cells to mediate effector function in the absence of glucose. We therefore split our cohort into patients where the response was more severely affected, (reduction in IFNγ frequency below the mean of the cohort, grey shaded area Figure 3b), and those where the response remained above the mean of the cohort. HBV-specific responses most affected by blocking

glycolysis had significantly more Glut1^{+} cells then those that were less affected, while a non-significant difference was observed for CMV-specific cells (Suppl Fig3b). These data suggest that the upregulation of Glut1 reflects a dependence on glycolysis in exhausted T cells.

The finding that HBV-specific T cells were unable to utilise OXPHOS for effector function, prompted us to investigate whether they had a mitochondrial defect. Mitochondrial mass (MM) can be assessed by staining cells with the mitochondrial potential-independent dye mitoTracker green, previously used to assess the presence of enlarged non-functional mitochondria in T cells (Henson et al., 2014). HBV-specific CD8 showed a higher mitochondrial mass compared to CMV-specific CD8 from the same donors (Fig3d). The increased mitochondrial mass in HBV compared to CMV specific CD8 was also apparent ex vivo (Fig3e).

We then used the ratiometric fluorescent dye JC-1 to assess mitochondrial potential, a key readout of mitochondrial function. HBV-specific T cells showed a much lower mitochondrial potential than CMV-specific cells, as indicated by the drop in the red/green fluorescence intensity ratio of the probe, which is independent of mitochondrial size or shape (Fig3f). A decrease in mitochondrial potential could be a sign of apoptosis induction, however we found HBV and CMV-specific T cells to be viable as they were negative for dead cell staining and showed low Annexin V staining, comparable to global T cells in the same culture (Suppl Fig3c). This indicates that mitochondrial dysfunction in HBV-specific T cells limits their capacity to fuel their energy demand for cytokine production by switching to OXPHOS.

The third signal cytokine IL-12 enhances the metabolic function of exhausted HBV-specific T cells

We recently demonstrated that the third signal pro-inflammatory cytokine IL-12 enhanced effector responses in exhausted HBV-specific T cells, while it had little effect on the functional CMV-specific response. When HBV-specific T cells were stimulated with viral peptide in the presence of IL-12, IFNγ production, TNF production and cytotoxicity were significantly increased (Schurich et al., 2013). We postulated that the capacity of IL-12 to reconstitute HBV-specific T cell effector function might be mediated by a change in the efficiency of their glucose metabolism.

To test this, we stimulated and cultured cells in T cell media in the presence or absence of IL-12 and restimulated in the presence of either glucose or galactose. When IL-12 enhanced IFNγ production by HBV-specific T cells, the cytokine did so irrespective of whether the cells were restimulated in glucose or galactose (Fig4a,b). Furthermore, IL-12 stimulated HBV-specific CD8 had a significantly increased ratio of polarised compared to depolarised mitochondria (Fig4c). These findings indicate that IL-12 can stimulate mitochondrial and metabolic changes in exhausted HBV-specific T cells, reversing their dependence on glycolysis for effector function.

Discussion

Glycolysis, accompanied by the upregulation of the glucose transporter Glut1, has been described to be the main metabolic pathway fuelling effector function upon T cell activation (MacIver et al., 2013). Surprisingly, we found that exhausted HBV-specific T cells, which have very limited effector capacity, showed a marked increase in expression of functional Glut1 upon antigenic stimulation, suggesting that their impaired effector function was not due to a lack of glucose uptake. In contrast to HBV-specific CD8, analysis of paired samples revealed that the more functional CMV-specific CD8 expressed less Glut1, despite equivalent levels of antigenic reactivation. This implied that HBV and CMV-specific T cells might utilise different metabolic pathways. We tested this hypothesis by culturing the cells in galactose, which impairs glycolysis, to probe the capacity of cells to use the alternative pathway of generating energy via mitochondrial OXPHOS (Chang et al., 2013). We found that HBV and CMV-specific T cells are differentially affected by this method of favouring OXPHOS over glycolysis. CMV-specific CD8 T cells from many patients produced normal or even elevated amounts of effector cytokines upon culture in galactose, while in others the production declined, but was never completely abrogated. These data do not contradict previous findings regarding the importance of glycolysis for IFNγ production in CD4 T cells (Chang et al., 2013). In HBV-specific cells, effector cytokine production declined or was completely lost. Defects in the glycolytic pathway cannot be formally excluded but our data suggest that exhausted HBV-specific T cells rely on generating their energy through glycolysis and cannot compensate by using other pathways. Our findings suggest that in some virus-

specific effector T cells, mitochondrial respiration is used to supplement glycolysis in order to satisfy the demands of efficient cytokine production; in line with increased proliferation, survival and anti-viral function in CD8 T cells with genetically enhanced OXPHOS (Okoye et al., 2015). The generation of reactive oxygen species (ROS) is vital in the activation of CD4 T cells (Sena et al., 2013) and an inability of HBV-specific T cells to use OXPHOS could partially be due to changes in ROS production. However, we did not find a significant difference in the production of ROS in exhausted compared to functional CD8 (data not shown).

The observed inability of HBV-specific CD8 T cells to use OXPHOS to fuel their effector function pointed to a mitochondrial defect. This was supported by their increased mitochondrial depolarisation compared to CMV-specific T cells, indicative of impaired function. Additionally, an increase in mitochondrial mass in HBV- compared to CMV-specific CD8 could be due to the formation of non-functional giant mitochondria, as have been described to accumulate in terminally differentiated human CD8 T cells (Henson et al., 2014).

We found that the most exhausted T cells, as measured by decrease in cytokine production and increase in co-inhibitory PD-1, showed the highest expression of Glut1 and the lowest ability to maintain effector functions when forced to use OXPHOS. Our data therefore reveal that functional exhaustion is linked to metabolic impairments in human virus-specific CD8 T cells. This link is underscored by the impact of IL-12; its capacity to rescue HBV-specific CD8 from exhaustion and enhance their functionality (Schurich et al., 2013) is paralleled by an increased proportion of cells harbouring polarised mitochondria and a

reduction in their dependence on glycolysis. Of note, we have previously shown that IL-12 can reduce PD-1 expression on CD8 T cells (Schurich et al., 2013) and the contribution of this pathway to the metabolic changes observed is currently under investigation.

Global patient-derived PD-1+ CD8 T cells had a low SRC suggesting that their ability to generate additional energy through oxygen consumption in situations of metabolic stress is reduced. PD-1 signalling has previously been described to reduce glycolysis and promote fatty acid oxidation in global CD4 T cells (Patsoukis et al., 2015) but the impact of PD-1 signalling on the metabolism of exhausted CD8 cells remains to be investigated. It is also important to keep in mind that T cells in patients with chronic HBV express additional inhibitory molecules like Tim-3 and CTLA-4 (Nebbia et al., 2012; Schurich et al., 2011), therefore multiple signals might shape the altered metabolism in these cells.

It will be interesting to establish whether exhausted T cells in other settings (HIV or HCV infection, tumours) have a similar deficiency in their capacity to supplement glycolysis with OXPHOS in order to optimise their energy supply. The mitochondrial defects and adaptation to glycolysis that we documented here may be the result of particular features of chronic HBV, such as high-level antigenic stimulation in the immunosuppressive liver environment. In particular the hypoxic milieu of the liver may be relevant since hypoxia inducible factor (HIF-1α/β) induces the transcription of Glut1 and multiple rate-limiting glycolytic enzymes to sustain glycolysis in activated T cells (Finlay et al., 2012). We found that intrahepatic T cells showed an increased expression of Glut1 ex vivo and that hypoxia during antigenic stimulation in vitro could recapitulate

this upregulation. The liver can produce IL-7 in response to TLR signalling, thereby enhancing T cell survival and function (Sawa et al., 2009); it is plausible this could be partially mediated by the capacity of IL-7 to drive glycolysis through the induction of Glut1 (Loisel-Meyer et al., 2012; Wofford et al., 2008). The metabolic phenotype of HBV-specific T cells could therefore be a consequence of, or an adaptation to, their target environment, allowing the few remaining responses to survive.

In summary, our data show that different virus-specific T cells in the same host have contrasting usage of pathways for glucose metabolism. The exhausted T cell response directed against HBV is characterised by an upregulation of the glucose transporter Glut1, a dependency on glycolysis and mitochondrial defects in OXPHOS and depolarisation. Instead, the highly functional CMV-specific T cell response can utilise OXPHOS for effector function when glycolysis is blocked. Our finding that a more protective T cell response requires mitochondrial respiration is consistent with two recent papers emphasising the importance of OXPHOS for antigen-specific T cell expansion and antiviral immunity in mice (Okoye et al., 2015; Sena et al., 2013). A better understanding of why this pathway fails in exhausted T cells may reveal mitochondrial targets for therapeutic boosting of antiviral immunity.

Experimental Procedures

Patients

Ethics statement: This study was approved by the ethical boards of the Royal Free Hospital and Camden Primary Care and written informed consent was obtained from all participants. All participants were HCV and HIV seronegative and HBV treatment-naïve. HLA-A2 status was determined by specific antibody (AbD Serotec). Patient information is provided in Supplementary Table 1.

Over night and short-term cell culture and stimulation

PBMC were isolated by Ficoll-Hypaque density gradient centrifugation and either analysed directly or cryopreserved. Liver biopsy sections, surplus to diagnostic requirements, were homogenised and filtered to obtain intrahepatic lymphocytes. To examine virus-specific T cell responses, PBMCs from HLA $A2^+$ donors were stimulated with 1μM HBV-derived HLA-A2 restricted peptides (core FLPSDFFPSV, envelope FLLTRILTI, WLSLLVPFV, LLVPFVQWFV, GLSPTVWLSV, polymerase GLSRYVARL, KLHLYSHPI) (Proimmune) or stained with dextramers loaded with the above peptides (Immudex). If derived from HLA-$A2^-$ donors with 1μg overlapping peptides spanning the whole HBV core protein, sequence correlating to HBV genotype D (AYW) (JPT Peptide Technologies). Responses to CMV were measured using 1μM NLVPMVATV peptide (Proimmune) or dextramers (Immudex) for HLA-$A2^+$ donors or overlapping pp65 for HLA-$A2^-$ donors (JPT Peptide Technologies). All cultures were supplemented with 20U/ml rhIL-2 (Miltenyi Biotech) at Day 0 and 4 with addition of rhIL-12 (Miltenyi Biotech) at 10ng/ml at Day 0 where indicated.

PBMCs were restimulated on day 9 for 4hrs by re-adding peptide at the original concentration in the presence of 1μg/ml Brefeldin A (BFA, Sigma-Aldrich). To assess metabolic requirements, samples were split and transferred into media containing either 10mM glucose or 10mM galactose (supplemented with 1mM sodium pyruvate) on day 8 for 24hrs; peptide and BFA was added for the final 4hrs. Virus-specific responses were identified by IFNγ or TNFα production.

Impact of hypoxia

PBMC were cultured and peptide stimulated as above at normoxia (21% 0_2). At the end of culture PBMC were split and peptide restimulated in the presence of BFA over night at 5% O_2 (hypoxia) or 21% O_2 (normoxia). Virus-specific responses were identified by IFNγ.

Flow cytometric analysis

PBMCs were stained for surface markers, CD8 (OKT8), CD3 (UCHT1)(eBiosciences), PD-1 (EH12.2H7) (Biolegend), CD38 (HIT2) (BD Biosciences) Dead cells were always excluded using live/dead fixable dye staining kit (Invitrogen). Cells were fixed and permeabilized for detection of intracellular molecules using anti IFNγ (B27), TNFα (MAB11) (BD Biosciences), Glut1 (202915) (RnD Systems). JC-1 2μM (Molecular Probes), mitotracker green 100nM (Invitrogen) used as per manufacturer's instruction. Samples were acquired on a BD Fortess, analysis was performed using Flowjo (Tree Star).

Metabolic Assay

OCR and ECAR were measured on a XF-24 Extracellular Flux Analyser (Seahorse Bioscience). CD8 T cells were stimulated with 1μg/ml CD3 (OKT3) (eBiosciences) and 20U/ml IL-2 in non-buffered RPMI during the assay. Inhibitors were used at 1μM oligomycin, 1.5μM fluorocarbonyl cyanide phenylhydrazone (FCCP), 1μM antimycinA (Sigma Aldrich) 100nM rotenone (Seahorse Bioscience).

Statistical analysis

Statistical analyses were performed using the non-parametric Mann-Whitney or Wilcoxon matched pairs test as appropriate and significant differences marked on figures ((* = $p<0.05$; ** = $p<0.005$; *** = $p<0.0005$).

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Author Contributions

Conceptualization, AS and MKM; Investigation, AS, LJP, DJ, JW and IO; Resources, USG, NH, PTK, EN, RJG and MKM; Supervision, AS, CF, SMH and MKM, Writing - Original Draft, AS; Writing – Review and Editing, AS and MKM, Funding Acquisition, MKM

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Figure Legends

Figure 1: Increased expression of Glut-1 on HBV-specific compared to CMV-specific CD8 T cells

a) Glut1 expression in HBV and CMV-specific T cells after 4hrs stimulation with cognate peptide directly ex vivo. Example of virus-specific cells detected by staining with HLA-A2+ dextramers loaded with virus-specific or irrelevant control peptides (left panel), overlay of Glut1 MFI in HBV and CMV-specific cells (middle) and summary data (right panel). (b) Glut1 expression in global CD8 T cells (left panel), IFNγ response upon culture with HBV or CMV-specific peptides (middle panel) and Glut1 expression on virus-specific IFNγ CD8 T cells (right panel). Summary data comparing %Glut1^{+} T cells in global and HBV-specific CD8 T cells (c) and HBV and CMV-specific T cells (d) in paired samples. Comparison of Glut1 expression and 2-NBDG uptake in IFNγ+ HBV and CMV-specific CD8 T cells, example (e) and summary data (f).

Figure 2: CD8 T cell Glut1 expression can be induced by hypoxia and correlates with an exhausted phenotype

Glut1 expression in HBV-specific CD8 T cells, detected by HLA-A2 dextramer staining in paired PBMC and intrahepatic lymphocytes (IHL) directly *ex vivo*, gated for CD3+CD8+CD4- T cells (a). Summary data of Glut1 expression on global CD8 T cells from PBMC and IHL (b). PBMC were cultured and restimulated with specific peptides on day 10 for 16hrs in hypoxic (5% O2) or normoxic (21% O2) conditions to analyse Glut1 mean fluorescence intensity (MFI) in HBV and CMV-specific T cells (c). Glut1 MFI was plotted against expression of (d) co-

inhibitory PD-1, (e) %IFNγ+ HBV-specific T cells and (f) %IFNγ+ CMV-specific T cells (d). Oxygen consumption rate (OCR) of purified patient-derived PD-1+ and PD-1– CD8 T cells were measured in real time ex vivo. Cells were stimulated with anti-CD3 and IL-2 at the time of analysis and mitochondrial inhibitors added as indicated (oligomycin blocking ATP synthesis through complex V, FCCP which uncouples ATP synthesis from the electron transport chain by transporting electrons across the inner mitochondrial membrane and rotenone/antimycinA blocking complex I and III) representative example (g). The spare respiratory capacity (SRC) was calculated as percentage change in mean baseline OCR to mean maximal OCR after the addition of FCCP in the three patients tested in two independent experiments (h).

Figure 3: CMV but not HBV-specific CD8 T cells can maintain effector cytokine production when glucose is withdrawn

PBMC were cultured in complete T cell media, before transferring them into media containing either 10mM glucose or 10mM galactose for 24hrs before restimulation. Representative example (a) and summary data showing the magnitude of the virus-specific (b) IFNγ and (c) TNF response in galactose, plotted as fold reduction compared to response in glucose (set to one, indicated as line in the graph). Mean response is shown as grey bars and individual responses as dots. Comparison of mitochondrial mass in cultured HBV and CMV-specific T cells by mitotracker green staining, representative histograms and summary data (d) and in dextramer+ virus-specific T cells ex vivo after 4hrs stimulation with cognate peptide; representative histograms and summary data (e). Determination of mitochondrial polarisation state by staining with the

ratiometric dye JC-1. Red JC-1 staining indicates polarised mitochondria, while loss of red fluorescence shows depolarisation, example FACS plot and comparison of the ratio of polarised/ depolarised mitochondria in HBV and CMV-specific T cells from the same patients (f).

Figure 4: Stimulation with the pro-inflammatory cytokine IL-12 recovers HBV-specific responses even when glycolysis is suppressed

PBMC were stimulated with HBV-derived peptides in the presence or absence of IL-12, cultured for 9 days and transferred into media containing either 10mM glucose or 10mM galactose for 24hrs before peptide restimulation for functional analysis. Representative FACS plots of the percentage of IFNγ+ CD8 when cultured in glucose or galactose and stimulated with HBV peptides +/- IL-12 or IL-12 alone (a). Summary data showing the IFNγ response to HBV peptides in galactose alone, galactose with IL-12 or glucose with IL-12, plotted as the fold change compared to the response of cells stimulated in glucose alone (set to one, indicated as line in the graph). The mean response is shown as grey bars and individual responses as dots (b). Change in mitochondrial polarisation upon stimulation of HBV-specific T cells with IL-12, representative staining with JC-1 (left) and summary data comparing the ratio of polarised to depolarised mitochondria (right) (c).

Figure 1

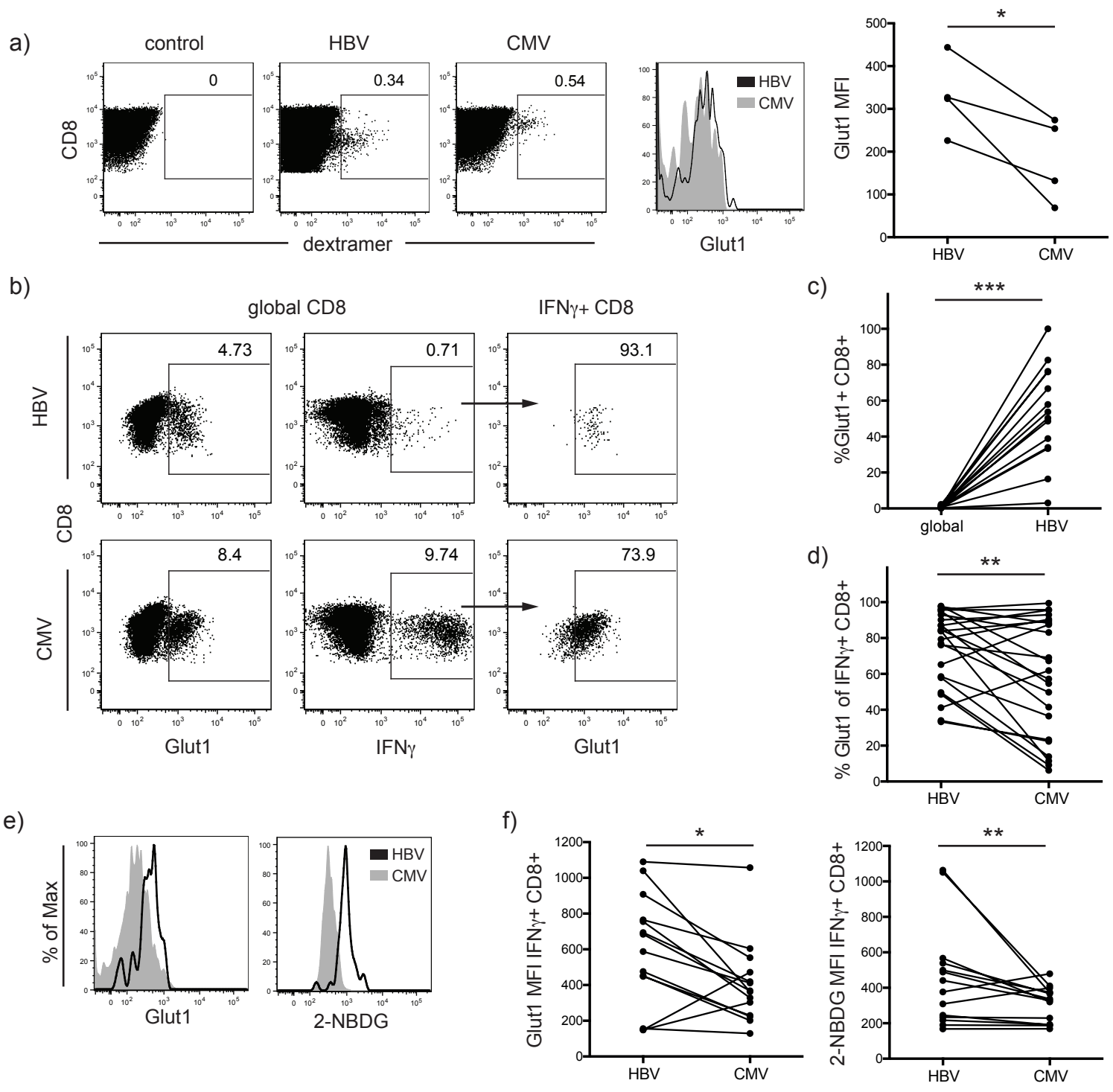

a)
control
HBV
CMV
0
0.34
0.54
CD8
dextramer
HBV
CMV
Glut1
Glut1 MFI
*
HBV
CMV
b)
global CD8
IFNγ+ CD8
4.73
0.71
93.1
8.4
9.74
73.9
HBV
CMV
CD8
Glut1
IFNγ
Glut1
c)
***
%Glut1+ CD8+
global
HBV
d)
**
% Glut1 of IFNγ+ CD8+
HBV
CMV
e)
% of Max
HBV
CMV
Glut1
2-NBDG
f)
*
Glut1 MFI IFNγ+ CD8+
HBV
CMV
**
2-NBDG MFI IFNγ+ CD8+
HBV
CMV

Figure 2

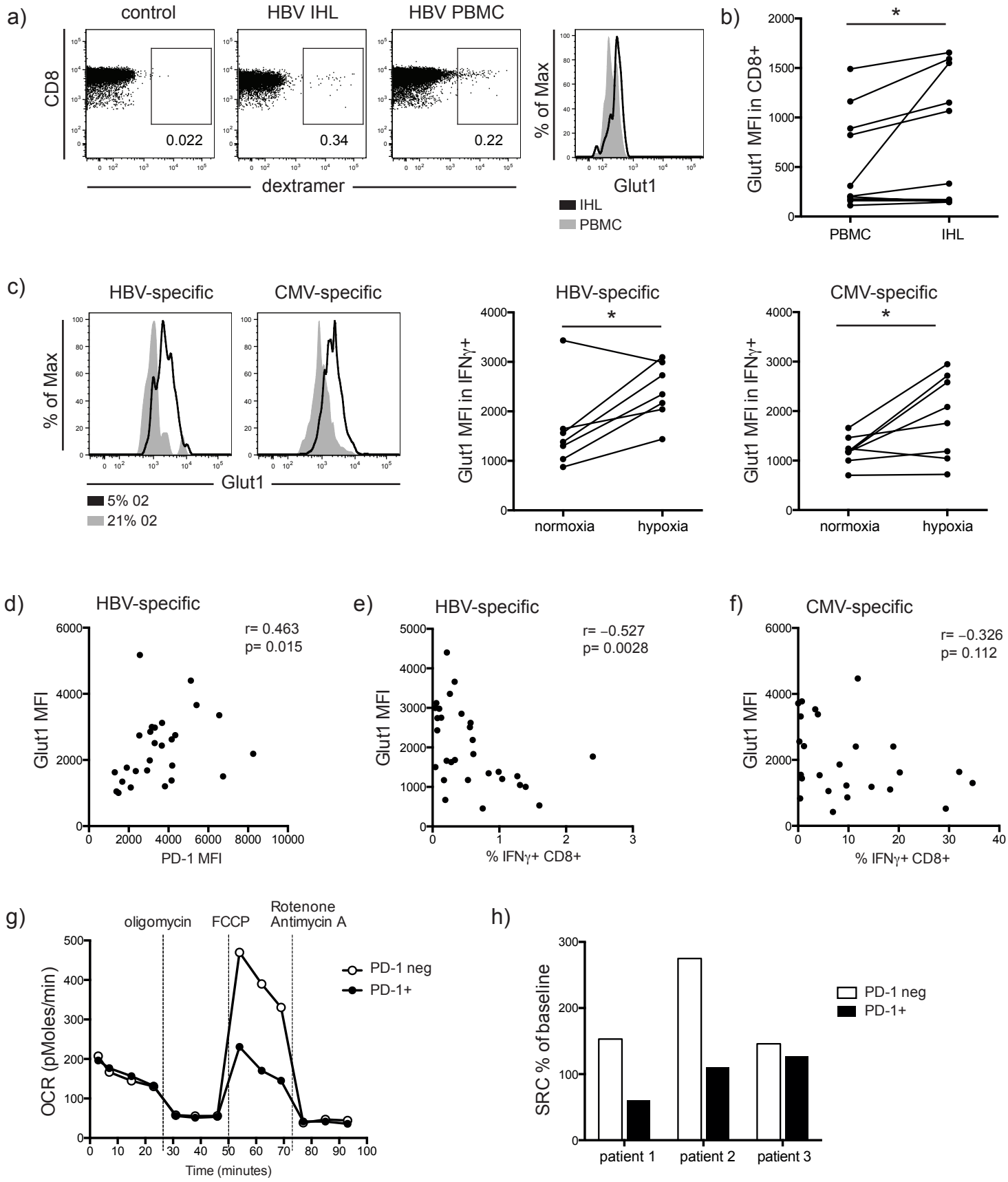

a)
control
HBV IHL
HBV PBMC
CD8
0.022
0.34
0.22
dextramer
% of Max
Glut1
IHL
PBMC
b)
Glut1 MFI in CD8+
PBMC
IHL
c)
HBV-specific
CMV-specific
% of Max
Glut1
5% 02
21% 02
Glut1 MFI in IFNγ+
normoxia
hypoxia
d)
HBV-specific
r= 0.463
p= 0.015
Glut1 MFI
PD-1 MFI
e)
HBV-specific
r= −0.527
p= 0.0028
% IFNγ+ CD8+
f)
CMV-specific
r= −0.326
p= 0.112
g)
oligomycin
FCCP
Rotenone
Antimycin A
OCR (pMoles/min)
PD-1 neg
PD-1+
Time (minutes)
h)
SRC % of baseline
patient 1
patient 2
patient 3

Figure 3

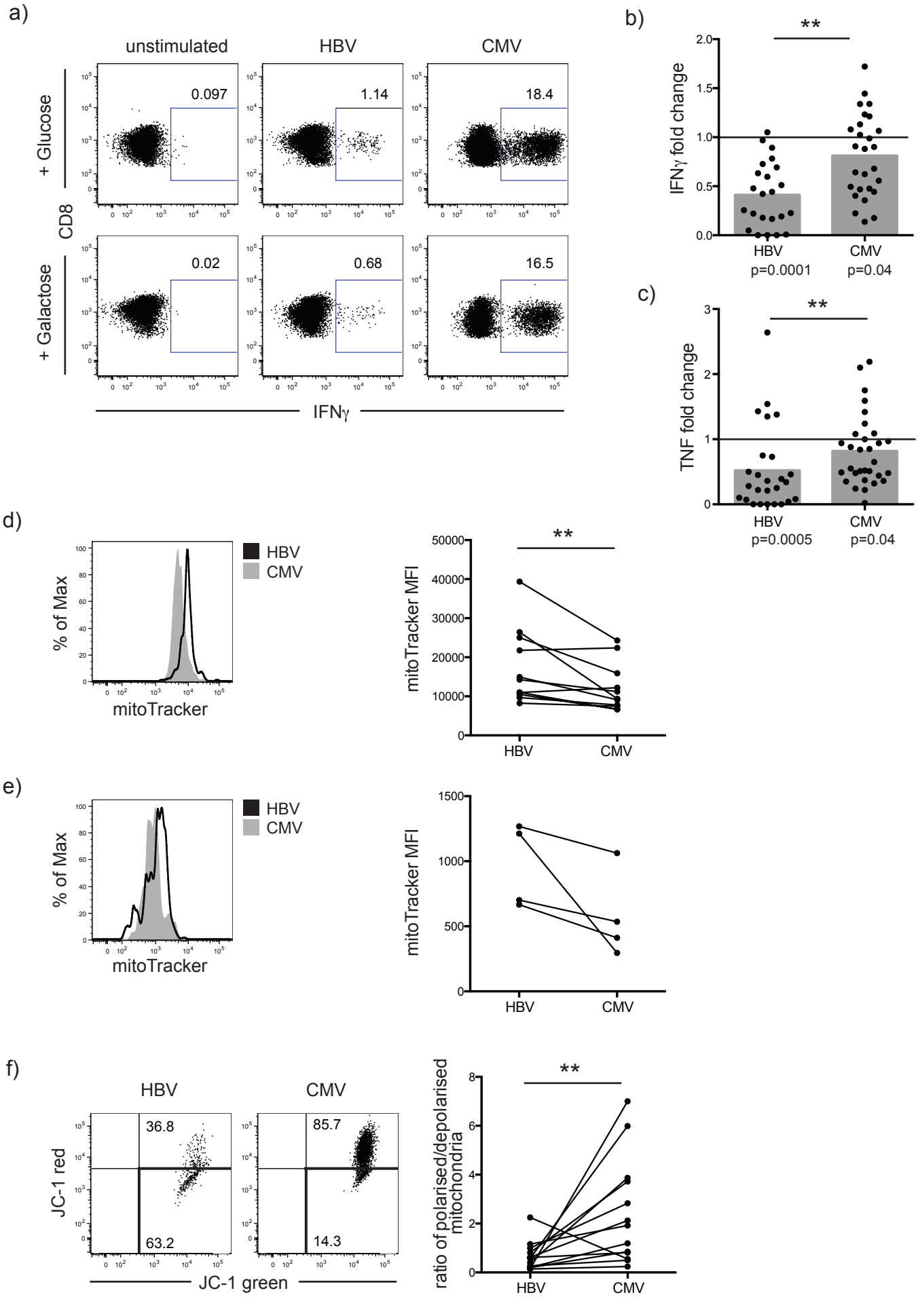
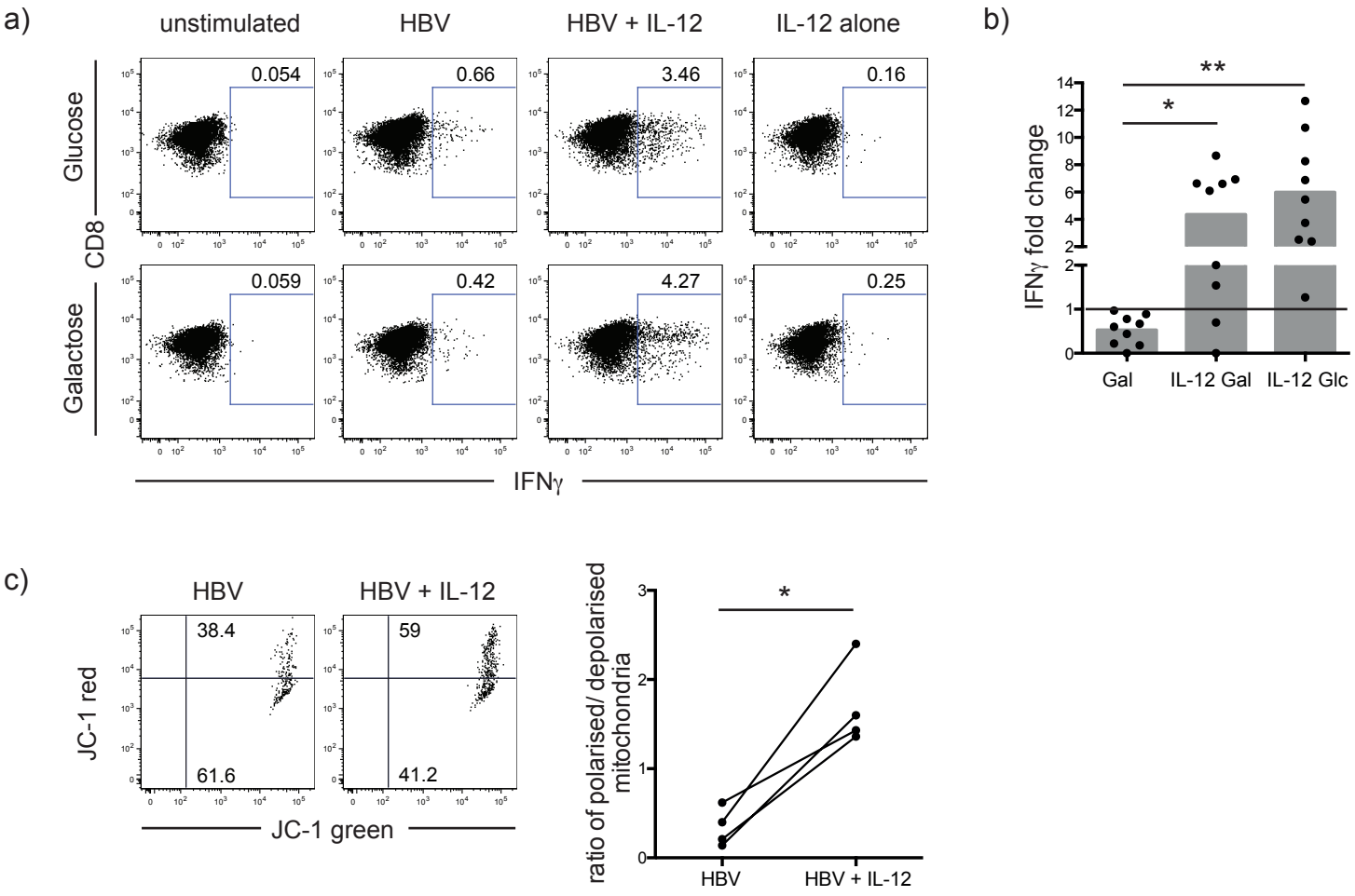
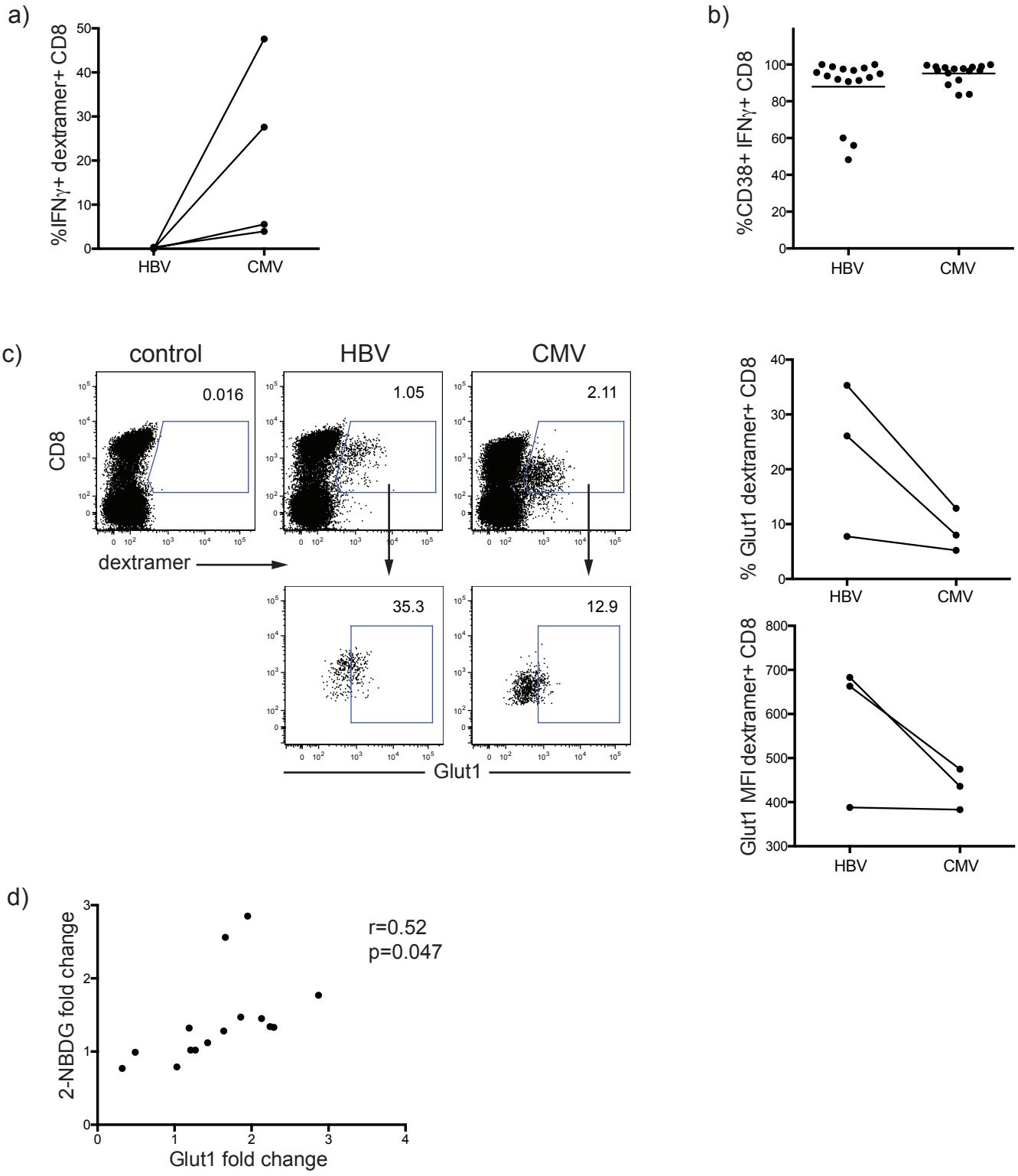

a)
unstimulated
HBV
CMV
+ Glucose
+ Galactose
CD8
IFNγ
0.097
1.14
18.4
0.02
0.68
16.5
b)
IFNγ fold change
HBV
CMV
p=0.0001
p=0.04
c)
TNF fold change
HBV
CMV
p=0.0005
p=0.04
d)
% of Max
mitoTracker
HBV
CMV
mitoTracker MFI
e)
% of Max
mitoTracker
HBV
CMV
mitoTracker MFI
f)
HBV
CMV
JC-1 red
JC-1 green
36.8
63.2
85.7
14.3
ratio of polarised/depolarised mitochondria

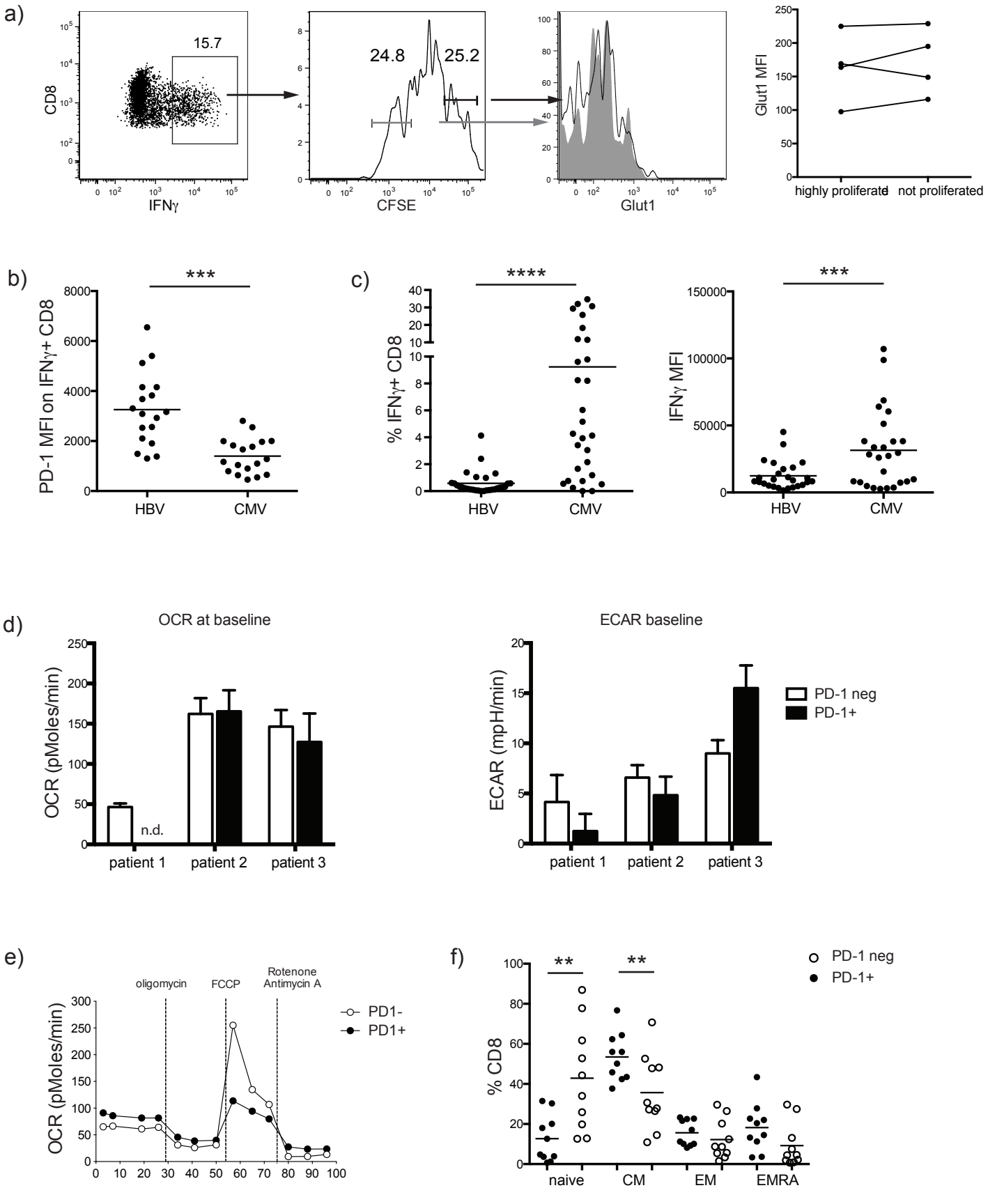
Figure 4


a)
unstimulated
HBV
HBV + IL-12
IL-12 alone
Glucose
Galactose
CD8
0.054
0.66
3.46
0.16
0.059
0.42
4.27
0.25
IFNγ
b)
IFNγ fold change
**
*
Gal
IL-12 Gal
IL-12 Glc
c)
HBV
HBV + IL-12
JC-1 red
38.4
61.6
59
41.2
JC-1 green
ratio of polarised/ depolarised mitochondria
*
HBV
HBV + IL-12

Supplementary Figure 1

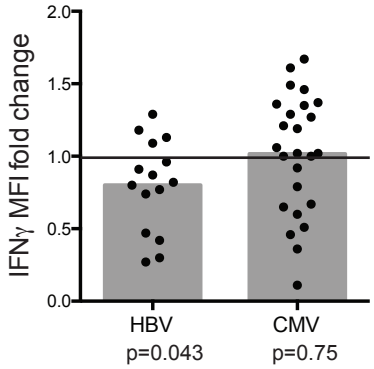


Supplementary Figure 2

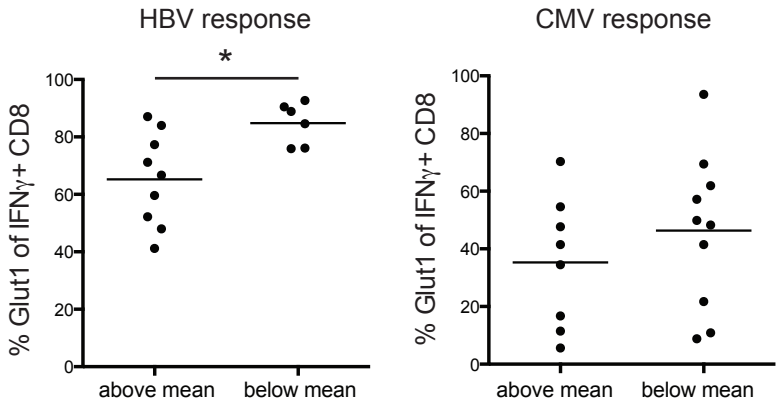


Supplementary Figure 3

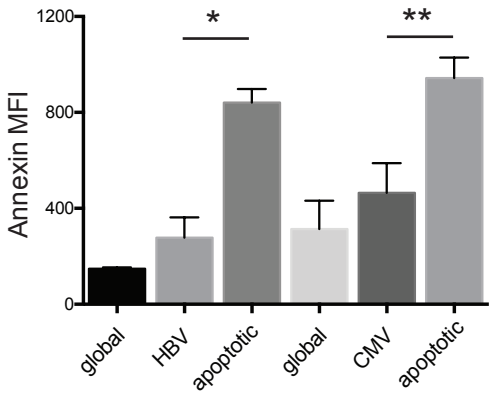
a)



b)



c)



Supplementary Figure 1, related to Figure 1: HBV and CMV-specific T cells retain distinct phenotypes in culture

Summary data of paired HBV-and CMV-specific T cells from patients with CHB detected ex vivo through HLA-A2 dextramer staining and then stimulated for 4hrs with cognate peptide to induce IFNγ production (a) and after 10day culture: activation status assessed by %CD38 expression of IFNγ$^+$ CD8 (b). Glut1 expression in virus-specific CD8 T cells, detected through HLA-A2 dextramer staining (c). Correlation of the fold change in HBV compared to CMV mediated glucose uptake with the fold change in Glut1 expression between the two subsets (d).

Supplementary Figure 2, related to Figure 2: Metabolic profile of patient-derived PD-1$^+$ and PD-1 negative CD8 T cells ex vivo and after culture

Glut1 expression is independent of proliferation, representative CMV-response (left) and CFSE profile (cells divided over night in 5% oxygen) (middle), Glut1 expression is compared in the 25% most highly divided (grey shaded) and 25% least divided (black line) cells and summary (far right) (a). Expression of the co-inhibitory receptor PD-1 on IFNγ$^+$ CD8 (b) and frequency (%) and amount of IFNγ produced (mean fluorescence intensity MFI) by CD8 (c). Comparison of PD-1$^+$ and PD-1$^-$ CD8 T cells stimulated with anti-CD3 and IL-2 *ex vivo.* Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured in real time (mean of 4 base line readings shown) (d). Example of metabolic profile of PD-1$^+$ and PD-1 negative CD8 T cells after 10day culture *in vitro*. Cells were stimulated with anti-CD3 and IL-2 during measurement of OCR and mitochondrial inhibitors added as indicated (e). Differentiation status of

global PD-1+ and PD-1 negative CD8 T cells in chronic HBV defined as naïve: CD45RA+, CD27+, central memory (CM) CD45RA-, CD27+, effector memory (EM) CD45RA-, CD27- and terminally differentiated (EMRA) CD45RA+, CD27- (f).

Supplementary Figure 3, related to Figure 3: CD8 expressing high Glut1 are the most dependent on glycolysis.

(a) Summary data showing the IFNγ MFI of the virus-specific response upon restimulation in galactose, plotted as fold change compared to response in glucose (set to one as indicated by line in the graph). The mean response is shown as grey bars and individual responses as dots. (b) Glut1 expression in $IFN\gamma^+$ HBV-specific T cells (left panel) or CMV-specific T cells (right panel), divided according to whether the response to culture galactose is above or below the mean (all samples falling in grey shaded area in Fig3b left panel). (c) Staining with the apoptosis marker Annexin V in global, virus-specific and apoptotic dead cells within the same wells (n=6). (d) Model of metabolism in functional CMV-specific compared to exhausted HBV-specific CD8 T cells.

Supplementary table 1, related to materials and methods

	Number	Gender (m)	Age	HBV DNA (IU/ml)	ALT (U/L)	eAg+
CHB patients	132	77	35 (18-64)	825 (1-300,000,000)	31 (10-250)	16

Patient characteristics. Data shown is the median value and spread from lowest to highest values in brackets. In cases where viral load was below quantification the value was set to 1.