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HBV-infected HepG2^{hNTCP} cells serve as a novel immunological tool to analyze the antiviral efficacy of CD8$^+$ T cells *in vitro*

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Abstract

$CD8^+$ T cells are main effector lymphocytes in the control of hepatitis B virus (HBV) infection. However, limitations of model systems such as low infection rates restricted mechanistic studies of HBV-specific $CD8^+$ T cells. Here, we established a novel immunological cell culture model based on HBV-infected $HepG2^{hNTCP}$ cells that endogenously processed and presented viral antigens to HBV-specific $CD8^+$ T cells. This induced cytolytic and non-cytolytic $CD8^+$ T-cell effector functions and reduction of viral loads.

Introduction

The hepatitis B virus (HBV) is a noncytopathic, hepatotropic virus causing acute and chronic necroinflammatory liver disease which may progress to hepatocellular carcinoma (HCC). It is generally assumed that the pathogenesis of HBV infection is determined by virus-host interactions mediated by the immune system, specifically by virus-specific $CD8^+$ T cells (1-5). Analysis of T-cell antiviral efficacy has been constrained by the limitations of available model systems. Furthermore, hepatoma cells used for *in vitro* studies were not susceptible to HBV infection except for low-permissive HepaRG cells (6). Recently, human sodium taurocholate cotransporting polypeptide (hNTCP) was identified as the entry receptor of HBV. Non-permissive HepG2 cells become susceptible to the virus after hNTCP transduction (7, 8) allowing high infection rates. Here, we used HLA-A*02$^+$ HepG2^{hNTCP} cells to establish a novel immunological cell culture model for HBV infection. In coculture assays, we could analyze antiviral effector functions of HLA-A*02-restricted HBV-specific $CD8^+$ T cells and determine immunological mechanisms of HBV control.

Results & Discussion

HBV-infected HepG2^{hNTCP} cells induce effector functions in virus-specific CD8$^+$ T cells

HepG2 cells stably transduced with hNTCP were infected with HBV, genotype D, purified from cell culture supernatant as previously described (7). The efficiency of infection, i.e. HBV protein content on a single cell basis was analyzed by flow cytometry using an anti-HBV core antibody (Figure 1A). Until day 7 post infection the frequency of HBV-infected HepG2^{hNTCP} cells and viral loads detected by qPCR (9) increased (Figure 1B). Subsequently, HBV-infected HepG2^{hNTCP} cells were analyzed for their capacity to induce effector functions in HLA-matched CD8$^+$ T cells from healthy donors retrovirally transduced with a HBV core$_{18-27}$-specific T-cell receptor (TCR) (10). Indeed, coculture of these TCR-redirected T cells with HBV-infected HepG2^{hNTCP} cells overnight led to a strong production of IFNγ and TNF and induced CD107a surface expression/degranulation (Figure 1C) comparable to peptide stimulation. In sum, these results indicate that viral antigens were efficiently synthesized, endogenously processed and presented on MHC class I molecules leading to the induction of effector functions in HBV core$_{18-27}$-specific CD8$^+$ T cells.

HBV core$_{18-27}$-specific CD8$^+$ T cells significantly reduce viral loads in HBV-infected HepG2^{hNTCP} cells

Next, we wanted to quantify the antiviral efficacy of TCR-redirected CD8$^+$ T cells. CD8$^+$ T cells were directly cocultured with HBV-infected HepG2^{hNTCP} cells at an effector to target cell (E:T) ratio of 1:1 (Figure 2A). Viral loads decreased to minimal levels after 72-96h (Figure 2B). Cocultures with CD8$^+$ T cells specific for two HBV epitopes (HBV core$_{18-27}$, HBV env$_{370-379}$ (10)) confirmed that different viral antigens were presented by HepG2^{hNTCP} cells and led to reduction of viral loads. The absence of antiviral efficacy after incubation of HBV-infected HepG2^{hNTCP} with a HCV NS5B$_{2594-2602}$-specific CD8$^+$ T-cell clone (11) revealed the specificity of this effect (Figure 2C). It is well known from cell culture and animal models that viral control requires cytolytic (12, 13) and noncytolytic CD8$^+$ T-cell effector mechanisms (14-16). The antiviral efficacy of both effector functions was assessed by cocultivating HBV core$_{18-27}$-

specific $CD8^+$ T cells in direct contact with their target cells or separated by a semipermeable membrane using Corning Transwell plates (Figure 2A and D). TCR-redirected $CD8^+$ T cells were stimulated with an equal number of irradiated EBV-transformed B cells pulsed with HBV $core_{18-27}$ peptide in transwell cocultures. Importantly, cytoplasmic viral titers were significantly reduced in both coculture conditions. Yet, HBV DNA was more profoundly diminished in $HepG2^{hNTCP}$ cells of direct cocultures (>90%) than of cocultures applying transwell plates (>50%) (Figure 2D). Interestingly, antiviral efficacy of $CD8^+$ T cells is comparably induced in response to HepG2.117 hepatoma cells (17) which were stably transduced with the HBV genome, genotype D (Figure 2E). These data demonstrate that $CD8^+$ T cell-mediated HBV control in hepatoma cells is not altered by HBV infection. Moreover, antiviral efficacy of HBV-specific $CD8^+$ T cells is dominated by cell contact-dependent mechanisms in both cell culture systems. The analysis of transaminase levels further revealed that cell killing is required for efficient eradication of HBV since strong increases in AST (aspartate aminotransferase) levels were observed in direct infectious cocultures (Figure 2F). Of note, AST was only derived from $HepG2^{hNTCP}$ cells. Transaminase concentrations increased over time peaking between 72h and 120h inverse to viral load (Figure 2B and G). Histological signs of cytotoxicity, e.g. a destroyed hepatoma cell monolayer and the appearance of cell debris, could also be monitored microscopically (Figure 2H). Importantly, HBV-infected $HepG2^{hNTCP}$ cells were susceptible to cytokine-mediated antiviral effects (Figure 2I). To elucidate the importance of the antiviral cytokines INFγ and TNF in direct cocultures we performed neutralization assays. Blocking cytokine activity in this setup had no significant impact on the antiviral efficacy of HBV-specific $CD8^+$ T-cell effector functions (Figure 2J). In summary, this $HepG2^{hNTCP}$-based infectious coculture model requires cell killing to eradicate HBV from infected cells which is in line with data obtained from studies in acutely HBV-infected patients and chimpanzees that have reported associations between vigorous HBV-specific $CD8^+$ T-cell responses, ALT elevations and viral clearance (13, 18, 19). However, we could also show significant reductions in viral titers by non-cytolytic effector mechanisms (Figure 2D and E) supporting the hypothesis of several *in vitro* and *in vivo* studies (14-16).

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Patient-derived CD8$^+$ T cells reduce viral loads after peptide-specific expansion

Engineered TCR-redirected CD8$^+$ T cells might respond differently than CD8$^+$ T cells from chronically HBV-infected patients which are detectable only at very low frequencies *ex vivo* (Figure 3A+B, table 1). In a final set of experiments, we therefore analyzed the antiviral capacity of patient-derived CD8$^+$ T cells directly *ex vivo* and after peptide-specific expansion in coculture assays with HBV-infected HepG2^{hNTCP} cells. These results revealed that detection of antiviral efficacy requires an E:T ratio of at least 1:100. We could not obtain these critical numbers of virus-specific CD8$^+$ T cells from chronically HBV-infected patients by *ex vivo* isolation but after peptide-specific expansion (Figure 3C). Indeed, we observed an E:T ratio-dependent reduction of viral loads by expanded HBV-specific CD8$^+$ T cells and TCR-redirected CD8$^+$ T cells, respectively (Figure 3C).

In conclusion, we were able to establish an immunological cell culture model of HBV infection based on HepG2^{hNTCP} cells and thereby provide a new tool to study cytolytic and non-cytolytic antiviral efficacies of HBV-specific CD8$^+$ T cells.

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Figure Legend

Figure 1: Induction of CD8$^+$ T-cell responses through HBV-infected HepG2^{hNTCP} cells.

A) HBV-infected HepG2^{hNTCP} cells were analyzed for endogenous expression of HBV core antigen (clone: 13A9, ThermoFisher) by flow cytometry 7 days post infection (d.p.i.). The frequency of HBV core$^+$ HepG2^{hNTCP} cells is indicated in the representative plot. Dotted line: MOCK-infected HepG2^{hNTCP} cells; tinted line: HBV-infected HepG2^{hNTCP} cells. B) Cytoplasmic viral loads on bulk HepG2^{hNTCP} cells were assessed by qPCR at indicated time points. Frequencies of HBV core$^+$ HepG2^{hNTCP} cells were obtained by flow cytometry. n = 3 C) TCR-redirected CD8$^+$ T cells specific for HBV-core$_{18-27}$/HLA-A*0201 were incubated with MOCK-infected (MOCK infection), HBV-infected HepG2^{hNTCP} cells (HBV infection) (7 d.p.i.) or with 15 µg/ml HBV core$_{18-27}$ peptide (FLPSDFFPSV) overnight. Induction of IFNγ (clone: 25723.11, BD Biosciences) and TNF (clone: Mab11, BD Biosciences) production and degranulation (CD107a, clone: H4A3, BD Biosciences) was analyzed by flow cytometry. Left: representative dot plots are shown. Indicated frequencies were calculated as percentage of responding CD8$^+$ T cells in bulk CD8$^+$ T cells. Indicated MFI refer to the MFI of CD107a on CD8$^+$ T cells. Right: Statistical graphs of 6 independent assays are presented. One way ANOVA with subsequent Tukey post-hoc test was performed for statistical analysis. Means + SD are depicted.

Figure 2: Antiviral efficacy of TCR-redirected CD8$^+$ T cells.

HBV-infected HepG2^{hNTCP} cells (7 d.p.i.) were cocultured with TCR-redirected CD8$^+$ T cells specific for HBV core$_{18-27}$ for 96h if not indicated differently. Cytoplasmic viral loads were determined by qPCR. Remaining HBV DNA was calculated relative to untreated condition (without CD8$^+$ T cells). All coculture assays were performed using 250,000 HepG2^{hNTCP} cells. E:T ratio = 1:1. A) Schematic experimental setup. B) HBV DNA was determined at indicated time points. n = 2. C) Coculture of CD8$^+$ T cells of indicated specificity with HBV-infected HepG2^{hNTCP} cells. CD8$^+$ T cells from healthy donors were transduced with HBV-specific

TCRs (HBV $core_{18-27}$ and $HBV_{env370-379}$) (10). HCV-specific $CD8^+$ T-cell clones were generated from patients chronically infected with HCV as described before (11). HCV $NS5B_{2594-2602}$-specific $CD8^+$ T-cell clones were tested against HBV-infected $HepG2^{hNTCP}$ cells labeled with the corresponding HCV peptide prior to coculture in order to control functionality. n = 2. D) Coculture of TCR-redirected $CD8^+$ T cells specific for HBV $core_{18-27}$ in direct and indirect (Transwell, TW) conditions (n = 6) applying HBV-infected $HepG2^{hNTCP}$ cells or E) tetracycline-regulated HBV-producing HepG2.117 cells (n=4). F) Comparison of AST levels obtained from 6 infectious coculture assays versus complete lysis using lysis buffer. G) AST levels corresponding to viral loads in B were determined after coculture with HBV $core_{18-27}$-specific TCR-redirected $CD8^+$ T cells at indicated time points. H) Brightfield microscopic analyses of HBV-infected $HepG2^{hNTCP}$ cells and TCR-redirected $CD8^+$ T cells specific for HBV $core_{18-27}$ after 96h coculture. Magnification: 20x; scale bars represent 50 µm. I) Antiviral efficacy of exogenous recombinant cytokines INFγ and TNF (R&D Systems). n = 3. J) Cytokine activity of INFγ and TNF was blocked by adding neutralizing monoclonal antibodies (mAb) against cytokines (10 µg/ml; anti-human INFγ mAb, clone #25723, RnD Systems; anti-human TNF mAb, clone #28401, RnD Systems) in combination with blocking mAb against cytokine receptors (10 µg/ml; anti-human IFNγR1/CD119 mAb, clone #GIR208, RnD Systems; anti-human TNFR1/TNFRSF1A, clone #18605, RnD Systems). n = 3. One way ANOVA with subsequent Tukey post-hoc test was performed for statistical analysis. ns = not significant; TW = transwell. Individual values and means + SD are depicted.

Figure 3: Antiviral efficacy of HBV-specific $CD8^+$ T cells from patients chronically infected with HBV.

A) Frequencies of TCR-redirected $CD8^+$ T cells specific for HBV $core_{18-27}$ and of patient-derived HLA-A*02-restricted HBV-specific $CD8^+$ T cells were determined by multimer staining. Stainings of patient samples were performed directly *ex vivo* and after 14 days of peptide expansion (expanded). Frequencies of multimer positive $CD8^+$ T cells in bulk $CD8^+$ T cells are indicated in representative dot plots. B) Statistical graph of HBV $multimer^+$ $CD8^+$ T

cells of total $CD8^+$ T cells *ex vivo*, expanded $CD8^+$ T cells and HBV $core_{18-27}$-specific TCR-redirected $CD8^+$ T cells. C) MACS-isolated $CD8^+$ T cells of 7 patients with chronic HBV infection (cHB) were cocultivated with 100,000 HBV-infected $HepG2^{hNTCP}$ cells (7 d.p.i.) at indicated E:T ratios for 96h (left). E:T ratios of cocultures with patient-derived $CD8^+$ T cells were recapitulated by titration of TCR-redirected $CD8^+$ T cells specific for HBV $core_{18-27}$ (right). One way ANOVA with subsequent Tukey post-hoc test was performed for statistical analysis. Individual values + means are depicted.

Table 1: Study cohort.

Characteristics of patients included in the study. 7 HLA-A*02^+ patients with chronic HBV infection presenting at the outpatient hepatology clinic of the University Hospital Freiburg were included in the study after obtaining written informed consent from the patients and approval by the ethics committee of the Albert-Ludwigs-Universitaet, Freiburg. All investigations have been conducted according to the principles expressed in the Declaration of Helsinki. n.s. = HBV genotype could not be sequenced; n.d. = not determined.

Figure 1

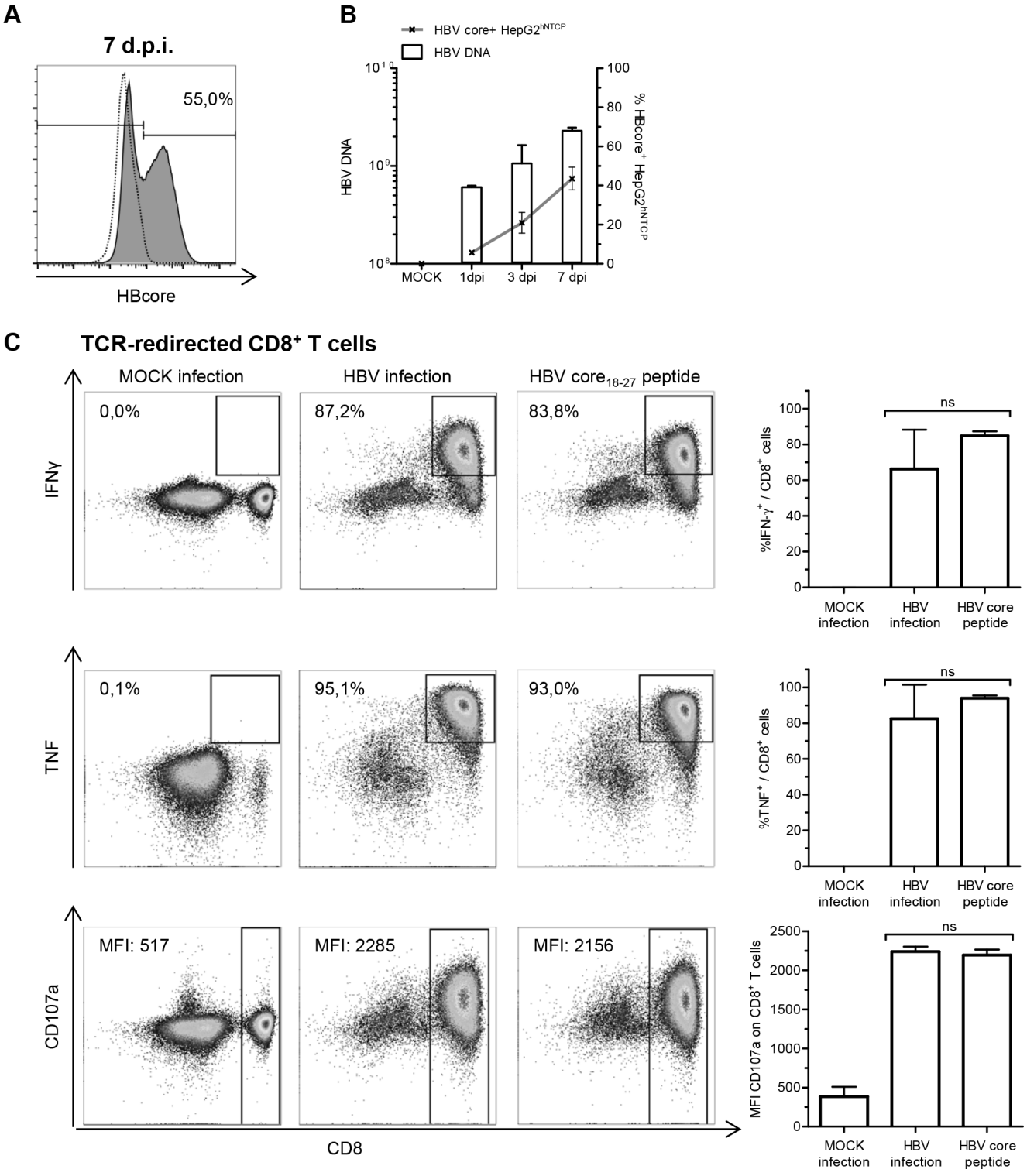

Figure 2

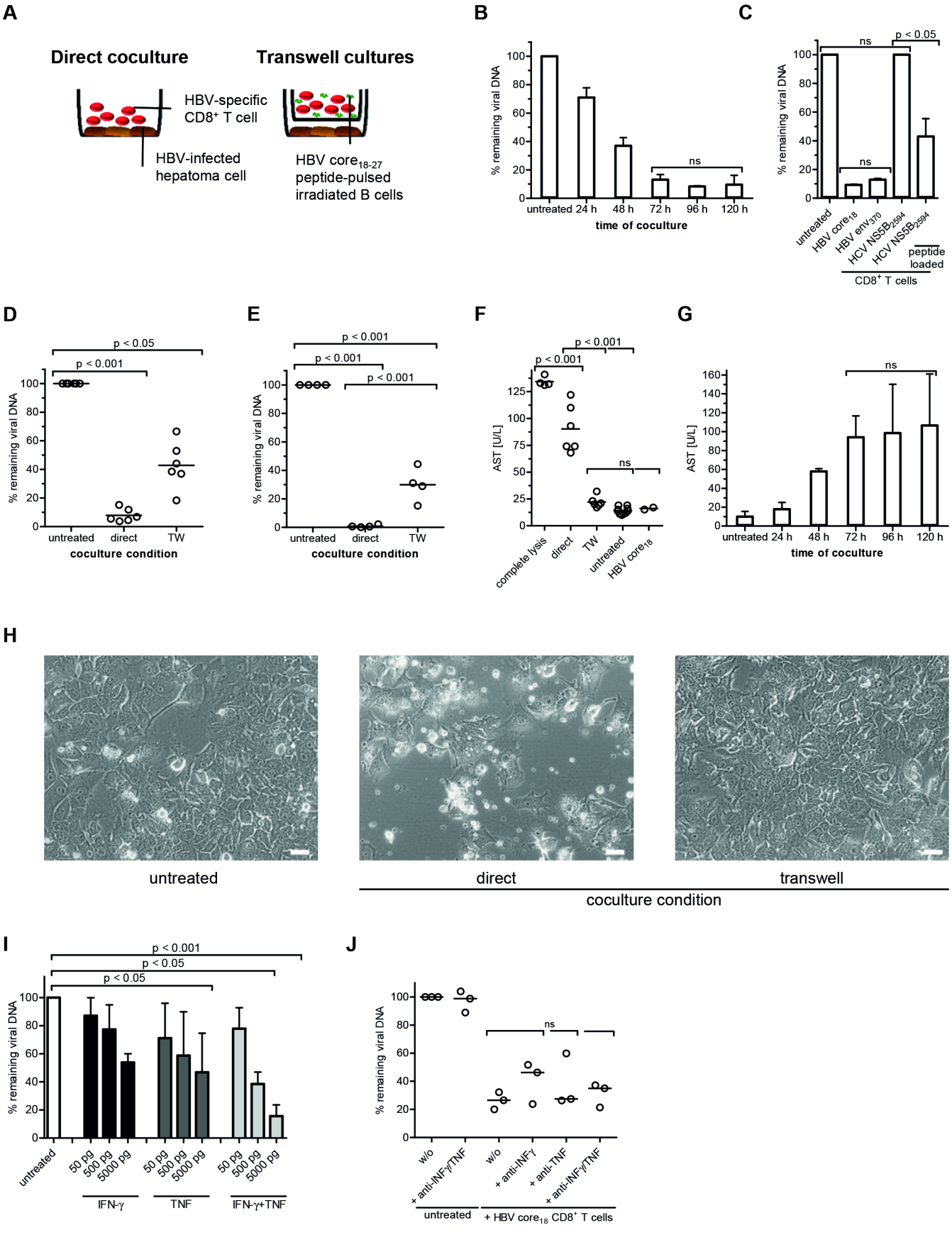

Figure 3

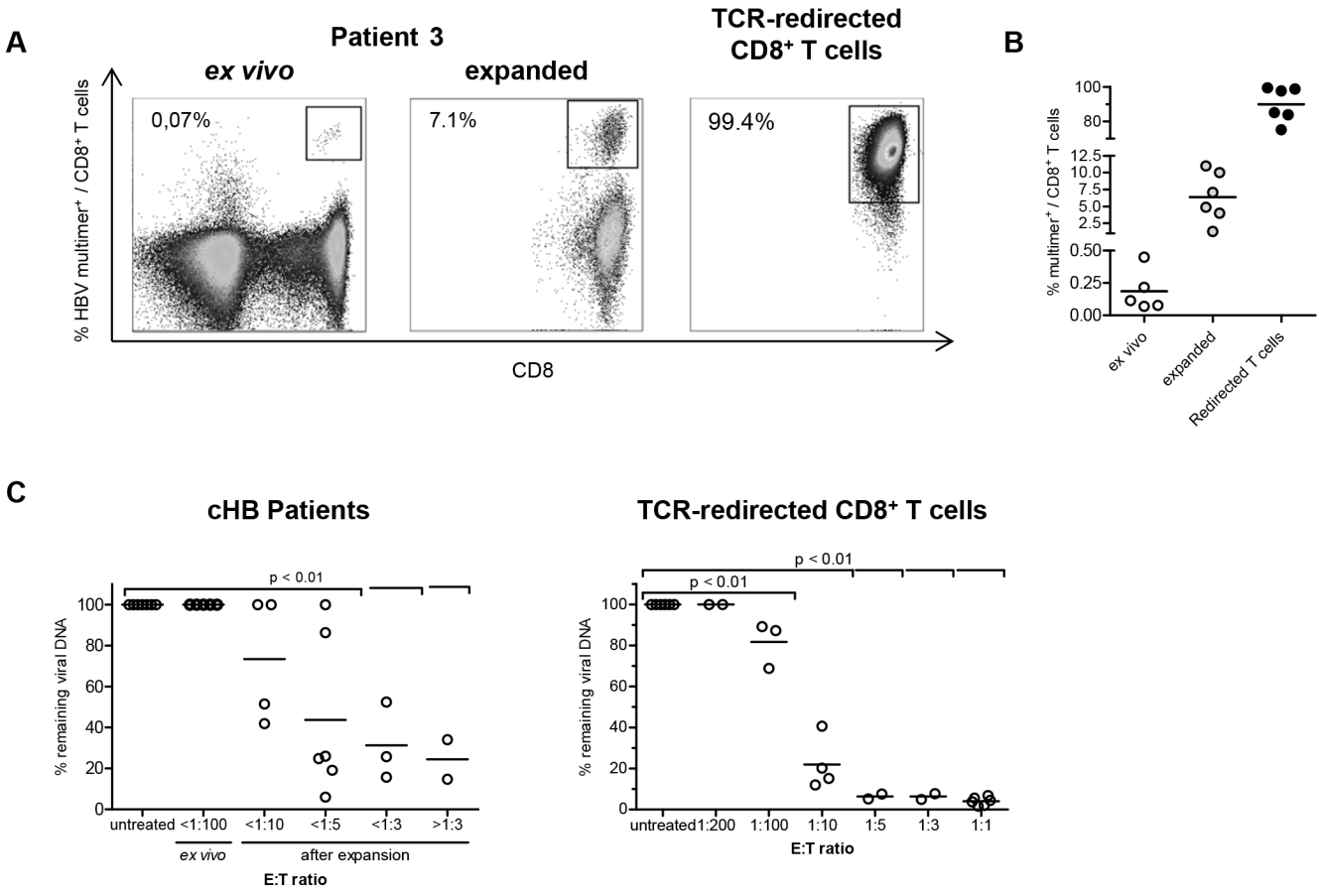

Table 1: Study cohort.

Patient-ID	Sex	Age (yr)	HBV genotype	HBV epitope	Multimer+ *ex vivo*	HBeAg	Viral load (IU/ml)	ALT (U/L)
Pat-1	F	63	D	Env183 Env335	0.45% 0.20%	Pos	19,729	1,601
Pat-2	M	57	A	Core18	0.22%	n.d.	1,711	40
Pat-3	F	46	n.s.	Core18	0.07%	Neg	<34	8
Pat-4	F	26	D	Core18	0.08%	Neg	1,822	18
Pat-5	F	25	n.s.	Core18	0.11%	Neg	117	20
Pat-6	M	68	A	Pol455	/	Pos	<34	24
Pat-7	M	25	n.s.	Pol455	/	Pos	<34	24