Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines

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Background & Aims: Virus-specific T cells capable of controlling HBV and eliminating hepatocellular carcinoma (HCC) expressing HBV antigens are deleted or dysfunctional in patients with chronic HBV or HBV-related HCC. The goal of this study was to determine if T cell receptor (TCR) gene transfer can reconstitute HBV-specific T cell immunity in lymphocytes of chronic HBV patients and investigate whether HCC cells with natural HBV–DNA integration can be recognized by genetically modified T cells.

Methods: We used vector-mediated gene transfer to introduce HLA-A2-restricted, HBV-specific TCRs into T cells of chronic HBV as well as HBV-related HCC patients.

Results: The introduced TCRs were expressed on the cell surface, evidenced by Vβ and pentamer staining. TCR transduced T cells produced IFN-γ, TNF-α, IL-2, and lysed HBV infected hepatocyte-like cell lines. Furthermore, HCC cell lines with natural HBV–DNA integration could be recognized by HBV-specific TCR-re-directed T cells.

Conclusions: TCR re-directed HBV-specific T cells generated from PBMC of chronic HBV and HBV-related HCC patients were multi-functional and capable of recognizing HBV-infected cells and HCC tumor cells expressing viral antigens from naturally integrated HBV DNA. These genetically modified T cells could be used to reconstitute virus-specific T cell immunity in chronic HBV patients and target tumors in HBV-related HCC.

Keywords: Immunotherapy; T cell receptor gene transfer; Hepatocellular carcinoma; Hepatitis B virus.

Received 13 May 2010; received in revised form 1 October 2010; accepted 20 October 2010; available online 23 November 2010

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Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; TCR, T cell receptor; c18-TCR, HBc18-27-specific TCR; s370-TCR, HBs370-79-specific TCR.

Introduction

A robust T cell response is associated with control of hepatitis B virus (HBV) infection in both patients and animal models [1]. However, current treatments for chronic HBV infection target the virus reverse transcriptase and rarely establish immunological control over HBV replication, subjecting patients to lifelong antiviral therapy and leaving them at risk of developing progressive complications like hepatocellular carcinoma (HCC) [2]. Eighty percent of HCC cases are associated with chronic hepatitis virus infection [3], making HCC the fifth most common cancer worldwide and the third leading cause of cancer related deaths, due mainly to the limited treatment options [4,5]. Thus, new therapeutic strategies are needed for people chronically infected with HBV.

Data from chronic HBV patients receiving bone marrow transplantation from immune donors or HBV immune recipients receiving a liver from a chronic HBV donor demonstrate that a chronically infected liver can tolerate a fully functional immune response that can control HBV infection without fulminant hepatitis [6–8]. In addition, HBV-specific T cell clones can recognize HCC cells expressing HBV X antigen from chromosomally integrated HBV DNA, demonstrating the anti-tumor potential of virus-specific T cells [9]. However, HBV-specific T cells are deleted or exhausted in chronic hepatitis B and HBV-related HCC patients [10]. As a result, efforts to restore virus-specific T cell immunity in chronic HBV patients using antiviral therapy, immunomodulatory cytokines (IFN-α), or therapeutic vaccination have had little success [11–15].

T cell receptor (TCR) gene transfer has emerged as a method to overcome the obstacles of T cell deletion and dysfunction [16]. TCR gene transfer involves vector-mediated gene transfer of a donor TCR with known specificity into recipient T cells that lack an antigen-specific response. Here, we determined if TCR gene transfer can generate HBV-specific T cells from lymphocytes of chronic HBV patients. We cloned TCRs specific for HLA-A2-restricted core and envelope epitopes and tested the ability of TCR re-directed recipient T cells to recognize HBV infected hepatocytes and HCC cell lines expressing viral antigen from naturally integrated HBV DNA.

Methods

Patients

Peripheral blood mononuclear cells (PBMC) of 20 HLA-A2$^+$ subjects (5 healthy controls and 10 chronic HBV patients, 5 HBV-related HCC patients) were collected under informed consent from the National University Hospital of Singapore. Chronic HBV patients were grouped according to HBeAg status, 5 HBeAg−, and 5 HBeAg$^+$. Basic patient characteristics, transduction efficiency, and functional information are listed in Supplementary Table 1.

Cell lines

T2, HepG2, SNU-475, and PLC-PRF5 cells were cultured in RPMI 1640 supplemented as below whereas Hep3B cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 mM HEPES, 0.5 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, MeM amino acids, Glutamax, MeM nonessential amino acids, (Invitrogen, Carlsbad, CA), and 5 μg/ml Plasmocin (InvivoGen, San Diego, CA). For lentiviral transduced cells, 2 μg/ml puromycin (BD Biosciences, San Jose, CA) was added to maintain selection. Phoenix cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) and HEK-293T cells in DMEM, 10% FBS, 25 mM HEPES, Glutamax, and 5 μg/ml Plasmocin. HBV expressing HepG2.117 cells and the vector control parent line, HepG2TA2-7 were maintained as described [17].

Isolation of T cell receptor alpha and beta chain DNA

Total RNA was isolated from 5×10^6 HBc18-27, HBs183-91, or HBs370-79 specific T cell clones using TRIzol (Invitrogen). The HBc18-27 specific TCR (c18-TCR) Vα3 and Vβ8.2 TCR chain cloning was performed on a contract basis by Primm SRL (Milano, Italy) and supplied in Topo blunt II for downstream cloning. c18-TCR Vα3 and Vβ8.2 chains were cloned individually into the retroviral vector, MP-71, for expression in primary human T cells.

The HBs183-91-specific TCR (s183-TCR) Vα34 and Vβ28 and HBs370-79-specific TCR (s370-TCR) Vα12 and Vβ7.8 chains were cloned, according to the manufacturer's instructions, by rapid amplification of cDNA ends (RACE) PCR using the Generacer kit (Invitrogen) and ligated into Topo2.1 for sequencing. Following functional confirmation of the TCR, a codon optimized, 2A linked construct was synthesized for the s370-TCR (Genscript) and cloned into MP-71 for expression in primary human T cells.

Retroviral transduction

2×10^6 Phoenix amphotropic packaging cells were seeded into 100 mm tissue culture dishes 24 h prior to transfection. Phoenix cells were transiently co-transfected using calcium phosphate with 9 μg each of MP71-TCR Vα, MP71-TCR Vβ together with 6 μg of amphotropic envelope for 24 h. For the s370-TCR, 18 μg of the 2A-linked TCR construct was co-transfected. IMDM was replaced with Aim-V supplemented with 2% human AB serum and phoenix cells were incubated for an additional 24 h before retroviral supernatants were collected for transduction.

PBMC were stimulated with 600 U/ml interleukin-2 (IL-2; R&D Systems) and 50 ng/ml anti-CD3 (OKT-3; eBioscience, San Diego, CA) for 48 h. Untreated 24-well tissue culture plates were coated with 30 ug/ml Retronectin (Takara Bio, Otsu Shiga, Japan) overnight at 4 °C one day prior to transduction. Wells were then washed with HBSS and blocked with PBS 2% BSA. Lymphocytes were harvested, washed, counted, and 5×10^5 cells plated into retronectin coated wells and mixed with retroviral supernatants collected as described above. Mock transduced cells, included as a negative control, were cultured with the supernatant from untransfected Phoenix cells. Lymphocytes were incubated for 24 h in the retroviral supernatant, medium was replaced and cells were maintained in Aim-V 2% human AB serum plus 100 U/ml IL-2. After 3 d, T cells were stained with CD8-PE-Cy7, CD4-PECy7 (BD Biosciences), Vβ8-PE (c18-TCR; Beckman Coulter), and HLA-A201-HBc18-PE and HLA-A201-HBs183-91 pentamer (Proimmune) or HLA-A201-HBs370-PE tetramers (kindly provided by Gijs Grotenbreg, National University of Singapore) to monitor TCR expression. Flow cytometry was performed using a FACs Canto flow cytometer (BD Biosciences) and data was analyzed with the FACs Diva program (BD Biosciences).

Function and sensitivity of TCR transduced T cells

HLA-A2$^+$ T2 cells were pulsed with various concentrations (1 fg/ml–1 μg/ml) of HBc18-27 (FLPSDFFPSV), HBs183-91, (FLLTRILTI) or HBs370-79 (SIVSPFIPLL) peptides for 1 h at 10^6 cells/ml and then washed. For the functional profile, mock or TCR transduced T cells were incubated with peptide loaded T2 cells (1 μg/ml peptide) overnight in the presence of 2 μg/ml brefeldin A and stained with CD8 PeCy-7 or CD4 PeCy7 and fixed using cytofix/cytoperm (BD Biosciences). Intracellular staining was performed for IFN-γ-APC, TNF-α-Alexa488, and IL-2-PE (BD Biosciences).

For sensitivity of TCR-re-directed T cell activation, T cells from 5 healthy donors, 5 HBeAg$^+$, 5 HBeAg− chronic HBV patients, 5 HBV-HCC patients, and the original T cell clone (C183) were co-cultured with peptide loaded T2 cells for 5 h in the presence of 10 μg/ml brefeldin A. Cells were stained with CD8 PeCy7, fixed, and stained for IFN-γ-PE (R&D systems, Minneapolis, MN). Results are displayed as mean percent of maximum IFN-γ response for all patients within each group. The affinity of TCR transduced CD4 T cells was determined in the same assay by gating on the CD8- cells.

For T cell recognition of HCC lines endogenously expressing HBV antigen (PLC-PRF-5, Hep3B), 7.5×10^4 HCC cells were plated in 96-well flat bottom plates 1 d prior to addition of T cells to permit adherence. TCR transduced T cells were co-cultured with HCC cell lines overnight in the presence of 2 μg/ml brefeldin A and stained for CD8-PE-Cy7 and IFN-γ-APC as previously described.

Cytotoxicity assays

HepG2 and SNU-475 cells were loaded with increasing concentrations of HBc18-27 peptide and HepG2.117 cells expressing HBV, PLC-PRF5, and Hep3B cells were plated overnight to permit adherence. Cytotoxicity was measured using a fluorescent based approach incorporating 75 μM propidium iodide (PI) and FAM conjugated FLICA poly-caspase detection reagent (Immunochemistry Technologies, Bloomington, MN) after co-culture with effector TCR transduced T cells (CD8$^+$/IFN-γ$^+$) at effector:target (E:T) ratio of 1:1. Details can be found in Supplementary methods.

For *in vivo* cytotoxicity assays, 6–9 week old Rag $2^{-/-}$ $\gamma C^{-/-}$ mice were sublethally irradiated (3.5 Gy) one day before 5×10^6 HBV core expressing HepG2 cells (HepG2cAg) were injected subcutaneously into the right flank. Tumors were allowed to form for 15 d. On day 15 and day 18, 1×10^6 or 5×10^6 mock or c18-TCR effector T cells (CD8$^+$/IFN-γ$^+$) were introduced into the tumor site. HepG2cAg tumors were allowed to grow for 15 days before mice were euthanized by CO_2 and tumors removed and weighed.

Results

Expression of introduced TCR in chronic HBV patients

To maximize potential antiviral efficiency and broad applicability for immunotherapy we cloned a T cell receptor specific for the immunodominant, HLA-A2 restricted HBV core 18-27 (HBc18-27) epitope from a HLA-A2$^+$ resolved HBV patient. However, data has suggested that there is global functional skewing of the T cell response in chronic HBV patients due to down-regulation of the CD3 zeta chain, up-regulation of PD-L1 on dendritic cells, increased T regulatory cells, or dysfunctional dendritic cells, which could impact transduction efficiency and TCR expression [18–21]. Therefore, to determine if the introduced TCR was expressed in lymphocytes of chronically infected HBV patients, we transduced PBMC from different cohorts of chronic patients, five HBeAg− (HBV DNA $<10^6$ copies/ml) and five HBeAg$^+$ (HBV DNA $>10^7$ copies/ml), as well as 5 HBV-related HCC (HBV-HCC) patients and compared TCR expression in 5 healthy donors using the HBc18-27 specific TCR (c18-TCR).

Retroviral vectors containing the alpha and beta chain of the c18-TCR were co-transduced into activated T cells from the different patient groups and expression within the CD8 T cell population was measured by staining for Vβ8 (TCR beta chain) and HBc18-27-HLA-A2 pentamer. Mock transduced cells showed that the endogenous expression of Vβ8 was similar in healthy donors and chronic HBV patients (Fig. 1B). Following transduction with the c18-TCR, staining for Vβ8 increased in the CD8$^+$ as well as the CD8$^-$ T cell population (Fig. 1A). Importantly, Vβ8 expression in the CD8 population was similar between healthy donors and chronic HBV/HBV-HCC patients (Fig. 1B).

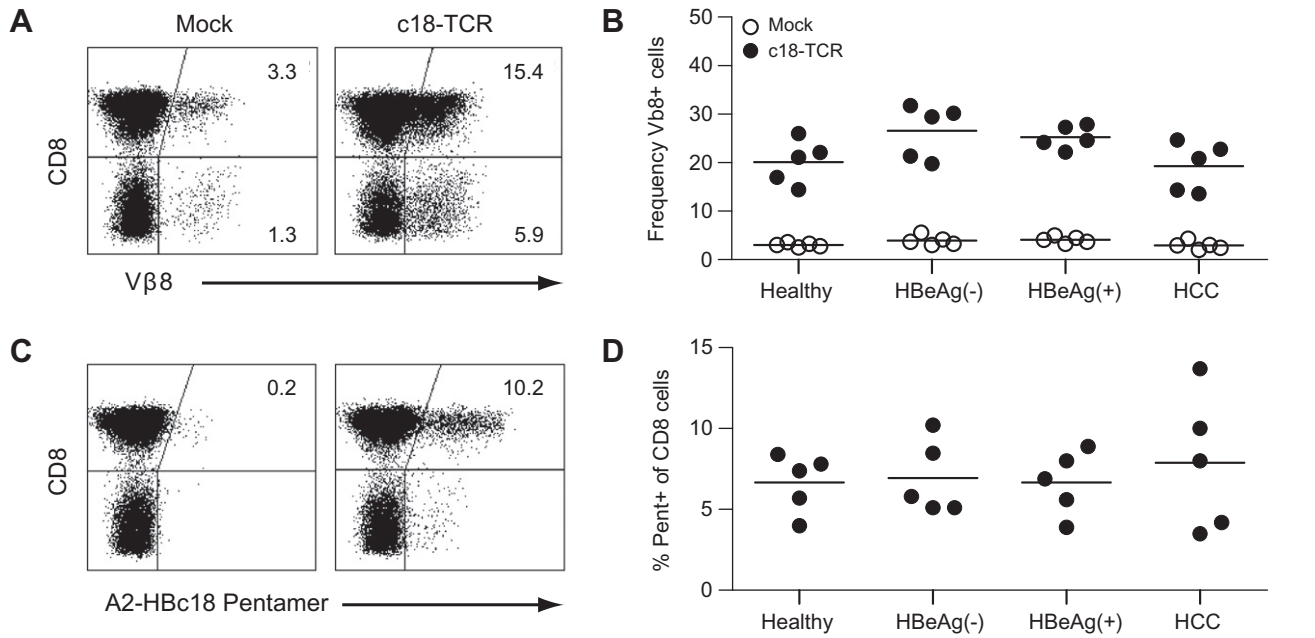

Fig. 1. Expression of introduced TCR. (A) Dot plot of Vβ8.2 expression in mock or c18-TCR transduced T cells from a representative HBeAg− patient. (B) Mean frequency of $CD8^+$ $V\beta8.2^+$ T cells in mock and c18-TCR transduced T cells from five patients in each group. (C) Dot plot of HLA-A2-HBc18-27 pentamer staining in mock or c18-TCR transduced T cells from a representative HBeAg− patient. (D) Mean $CD8^+$ $pentamer^+$ T cells, in mock and c18-TCR transduced T cells from five patients in each group. There was no statistically significant difference between $pentamer^+$ or $V\beta8^+$ cells from each patient group using one-way ANOVA analysis ($p > 0.05$).

To determine if the alpha and beta chains formed a HBV-specific TCR, transduced T cells were stained with HLA-A2-HBc18-27 pentamers. Following transduction with the c18-TCR we detected a clear pentamer positive CD8 T cell population that was similar between all patient groups (Fig. 1C and D). The lower frequency of pentamer positive cells (≈30% of $V\beta8^+$ cells) is likely due to mispairing of the introduced alpha and beta chains with the endogenous TCR chains. These data demonstrate that transduction and expression of the introduced TCR was similar in all patient groups and not affected by the presence of HBeAg, HCC, the level of HBV replication, or any other potential inhibitory mechanism present in chronic HBV patients.

TCR gene-modified T cells are multi-functional

Having confirmed that the introduced TCR was equally expressed in T cells of healthy and chronic HBV patients we determined if the TCR transduced cells were functional by analyzing their cytokine profile. Mock and c18-TCR transduced T cells from each patient group were stimulated with peptide loaded, $HLA\text{-}A2^+$ T2 cells, and monitored for the production of IFN-γ, TNF-α, and IL-2 by intracellular staining.

In the absence of peptide stimulation there were no cytokines produced by mock or TCR transduced T cells. Co-culture of mock transduced cells with peptide loaded T2 cells did not stimulate cytokine production in healthy donors or chronic HBV patient groups, indicating that there were no detectable endogenous HBc18-27 specific T cells (Fig. 2A and data not shown). In contrast, peptide stimulation of c18-TCR transduced T cells resulted in significant production of IFN-γ and TNF-α, and to a lesser extent IL-2 (Fig. 2B). TCR transduced T cells were multifunctional (Fig. 2C) and cytokine production was observed in both CD8 and CD4 T cell populations, demonstrating that we could reconstitute a cytotoxic and T helper virus-specific T cell response using a single MHC class-I restricted TCR (Fig. 2B; Supplementary Fig. 1). Furthermore, analysis of the frequency of cytokine positive cells from the different patient groups showed that there were no functional differences between TCR transduced T cells from healthy, $HBeAg^-$, $HBeAg^+$, or HBV-HCC patients (Fig. 2D). We also tested whether the presence of HBV antigen in the serum of chronic patients had an impact on transduced T cell function and did not see any difference in the frequency of IFN-γ^+ TCR transduced T cells after 48 h of culture in the presence of 20% $HBeAg^+$ serum (data not shown). Therefore, we could reconstitute a multi-functional T cell response in cells of chronic HBV patients that performs similar to TCR transduced T cells of healthy donors.

Sensitivity of TCR transduced T cells

Due to the potential down-regulation of CD3 zeta in chronic HBV patients [19], we determined if TCR transduced T cells retained sensitivity similar to the HBV-specific CD8 T cell clone derived from a resolved HBV patient, which was the source of the T cell receptor. The activation of TCR transduced CD8 or CD4 T cells from each patient group were compared to the HBc18-27-specific T cell clone, C183. We found that the sensitivity of the c18-TCR transduced CD8 T cells was similar to the original HBV-specific CD8 T cell clone and activation could be observed at 100 fg/ml–1 pg/ml in all patient groups (Fig. 3A). TCR transduced CD4 T cell activation was more variable between the patients due to the relatively low frequency of IFN-γ^+ CD4 T cells and their sensitivity was reduced by approximately 1 $\log_{10}$ compared to C183 (10–100 pg/ml; Fig. 3B). Overall, TCR transduced CD8 T cells generated from T cells of healthy and chronic HBV patients have sensitivity similar to the HBV-specific CD8 T cells selected in HBV infected patients, indicating that a sufficient amount of the

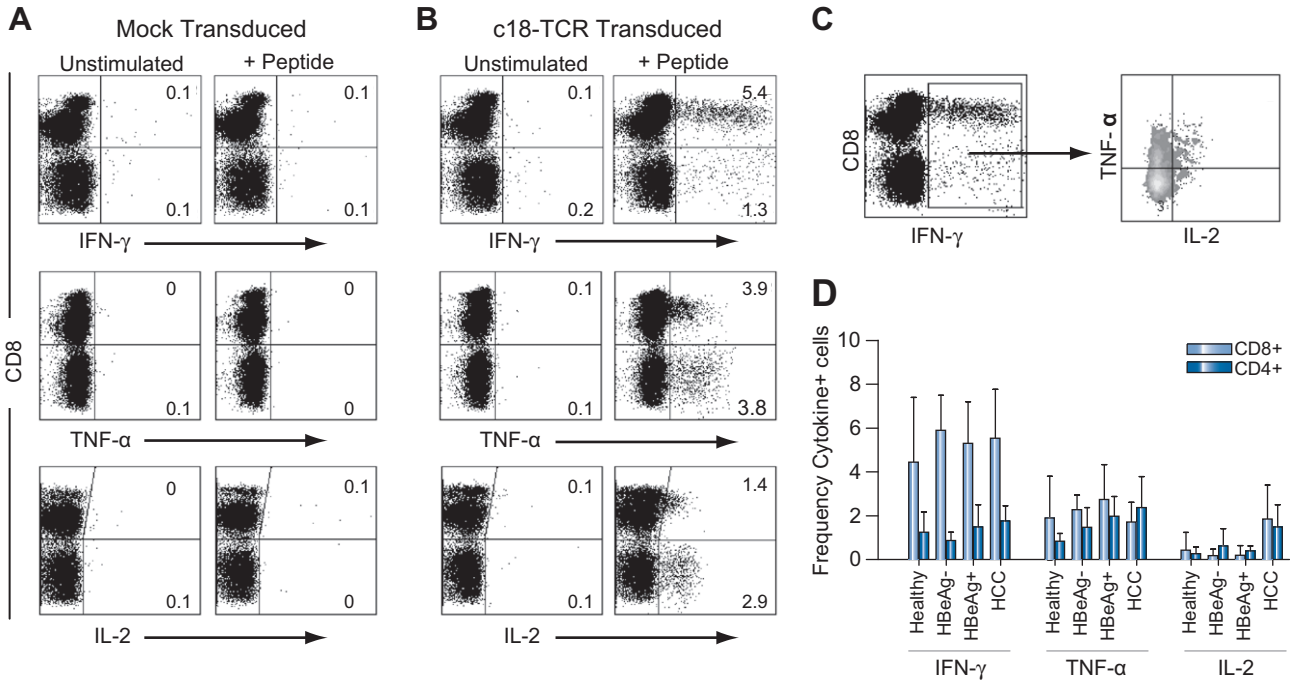

Fig. 2. Functional profile of TCR transduced T cells. Dot plots from a representative HBeAg– (A) mock and (B) c18-TCR transduced T cells, +/– peptide stimulation, stained for CD8 and IFN-γ (top row), TNF-α (middle row) and IL-2 (bottom row). (C) TNF-α and IL-2 production by IFN-γ$^+$ cells to demonstrate multi-functionality of TCR transduced cells. (D) Mean frequency of cytokine positive cells from all patients in each group. Data shown is mean ± standard deviation of the frequency of cytokine positive cells from each patient group.

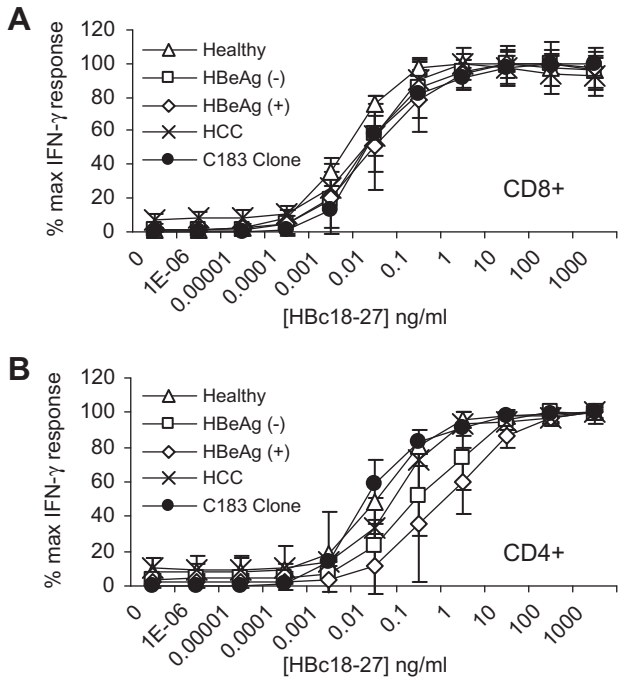

Fig. 3. Sensitivity of TCR transduced T cell activation in (A) CD8 or (B) CD4 c18-TCR transduced T cells from each patient group compared to the original T cell clone, C183. Results are displayed as mean of five patients from each group, +/– standard deviation of percent maximum IFN-γ response obtained from intracellular cytokine staining.

exogenous TCR is present for optimal activation in T cells of chronic HBV patients.

Cytotoxicity of TCR transduced T cells

We then determined the ability of TCR transduced T cells to lyse their specific targets. First, TCR transduced T cells were co-cultured with peptide loaded, HLA-A2$^+$, HepG2, and SNU-475 cells. Incubation of c18-TCR transduced T cells with peptide loaded cell lines resulted in a dose dependent increase in specific lysis (Fig. 4A and B). TCR transduced T cells were then tested against HepG2 cells that stably express the entire HBV genome (HepG2-HBV) and produce infectious virus [17]. As a negative control, TCR transduced T cells were co-cultured with normal HepG2 cells or HepG2 cells stably transfected with the empty expression cassette (HepG2-ctlr), which resulted in minimal background lysis. Co-culture with HBV expressing HepG2 cells resulted in a significant increase in specific lysis by c18-TCR transduced T cells demonstrating that TCR transduced T cells could recognize endogenously processed antigen (Fig. 4C).

The ability of TCR transduced T cells to lyse their cognate target was also demonstrated in a xenograft mouse model. HepG2 cells stably expressing the HBV core antigen were generated by lentiviral transduction and injected subcutaneously into immunodeficient Rag 2$^{-/-}$/γC$^{-/-}$ mice followed 15 days later by adoptive transfer of mock or c18-TCR transduced T cells. Mice receiving mock transduced T cells all exhibited significant tumor growth whereas mice receiving c18-TCR transduced T cells showed a cell number dependent reduction in tumor burden (Fig. 4D).

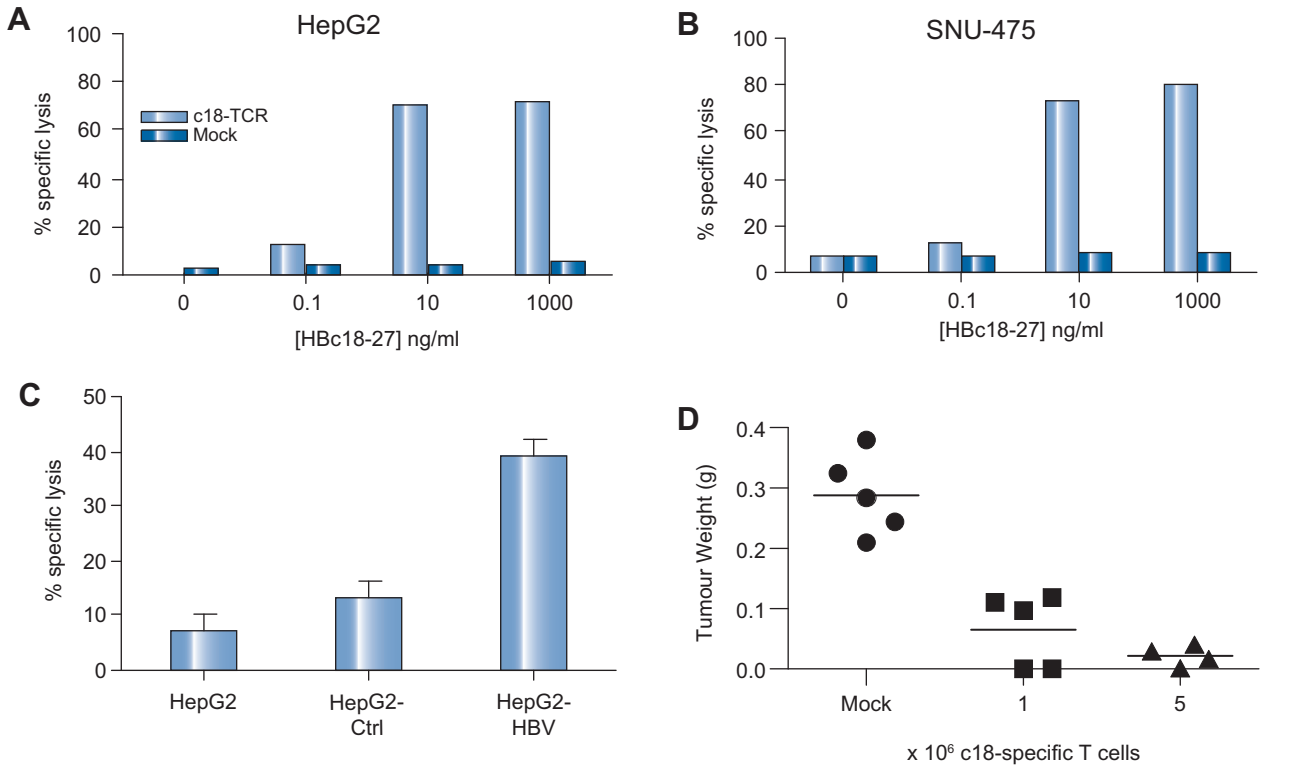

Fig. 4. Lysis of hepatocyte cell lines. Dose dependent lysis of peptide loaded (A) HepG2 and (B) SNU-475 HCC by mock or c18-TCR transduced T cells. (C) Lysis of HBV expressing HepG2 cells (HepG2-HBV) by c18-TCR transduced T cells. HepG2 cells or HepG2 with empty expression cassette (HepG2-ctrl) served as negative control. Each panel is representative of at least three separate experiments with T cells from different patients. (D) *In vivo* cytotoxicity assay. Size of subcutaneous tumors 15 d after adoptive transfer of 1 or 5 × 10^6 c18-TCR T cells.

Recognition of HCC lines naturally expressing HBV antigen

We then analyzed whether the TCR-re-directed T cell approach could be extended to a HBV-related HCC system, where tumor cells can express HBV proteins from integrated portions of HBV. To test this, we used two well characterized HCC cell lines, PLC-PRF-5 and Hep3B. These cell lines have multiple, random integrations of HBV DNA but are HLA-A2 negative, making them invisible to the HLA-A2-restricted TCRs [22–24]. To overcome this issue we used a lentiviral vector to stably express HLA-A2 in both cell lines (Supplementary Fig. 2). C18-TCR T cells were incubated with the HLA-A2 transfected PLC-PRF5 and Hep3B or the parental HLA-A2 negative cell lines as negative controls. C18-TCR T cells were not activated following co-culture with either the HLA-A2- (PLC-puro & Hep3B-puro) or the HLA-A2$^+$ (PLC-A2 or Hep3B-A2) HCC cells (Fig. 5A and B; top row), consistent with these cells being negative for HBV core antigen by western blot (data not shown).

Based on this data, HBV core or even a fragment containing the HBc18-27 epitope was not present within these cells or not processed and presented. However, the HBV surface antigen is known to be constitutively expressed by both PLC-PRF5 and Hep3B. This prompted us to clone two additional HLA-A2-restricted, HBV surface antigen specific TCRs (HBsAg183-91, (s183-TCR) and HBsAg370-79, (s370-TCR), which were successfully expressed on T cells and generated a multi-functional HBV-specific T cell response (Fig. 6 and Supplementary Table 1). Incubation of HBsAg-specific TCR transduced T cells with the HLA-A2 negative PLC-PRF5 and Hep3B cell lines did not result in T cell activation, but different profiles of T cell activation were observed when s183- and s370-TCR T cells were tested against the HLA-A2 expressing cell lines. Significant s183-TCR T cell activation was only observed after co-culture with PLC-PRF5-A2 while s370-TCR T cells were activated by both PLC-PRF5-A2 and Hep3B-A2 (Fig. 5, rows 2 and 3). Cytotoxicity assays performed against both HCC lines using s370-TCR transduced cells confirmed the ability of TCR transduced T cells to recognize HCC tumor cells with naturally integrated portions of HBV–DNA (Supplementary Fig. 3).

Discussion

Using TCR gene transfer we expressed exogenous HBV-specific TCRs on T cells from chronic hepatitis B and HBV-HCC patients and reconstituted multifunctional HBV-specific T cells capable of lysing hepatocyte-like cells expressing cognate HBV antigens. In addition, TCR-gene-modified T cells could recognize HCC tumor cells expressing viral antigens from naturally integrated HBV DNA. This study represents a step forward in the development of potential immune based therapies to limit antiviral therapy and treat patients with chronic HBV infection and HBV-related HCC, which are characterized by profound defects in their HBV-specific T cell response [10].

Antibody based approaches, proposed both in the past [25] and recently [26], are unlikely to have an effect in chronic hepatitis B

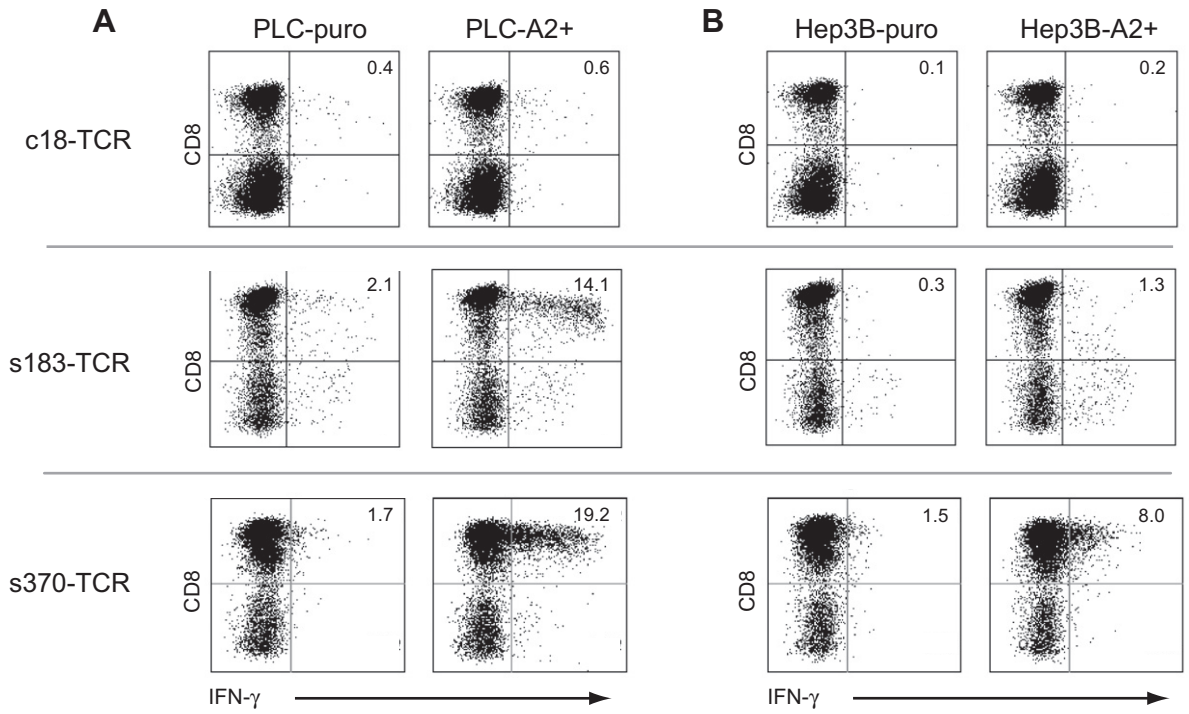

Fig. 5. Recognition of HCC cell lines naturally expressing HBV proteins. C18-TCR (top row), s183-TCR (middle row), and s370-TCR (bottom row) transduced T cell IFN-γ production following overnight co-culture with (A) HLA-A2 negative PLC-puro and HLA-A2$^+$ PLC-A2 or (B) Hep3B-puro and Hep3B-A2 cells. T cells used for this experiment were derived from healthy donors.

patients because the large quantity of circulating antigens (HBsAg and HBeAg) present in these patients will likely interfere with the direct recognition of HBV-infected cells. In contrast, HLA class-I restricted TCR-re-directed T cells do not recognize soluble antigen and thus should be able to engage HBV-infected cells and HCC cells with naturally integrated HBV DNA when used in patients.

Notably, the tolerogenic mechanisms implicated in global T cell exhaustion/deletion in chronic HBV patients did not carry over to T cells expressing exogenous TCRs *in vitro* [18–21]. CD3 zeta down-regulation and arginine depletion are likely reversed due to *in vitro* culture required for TCR transduction and regulatory cells or altered dendritic cell function do not impair T cell proliferation

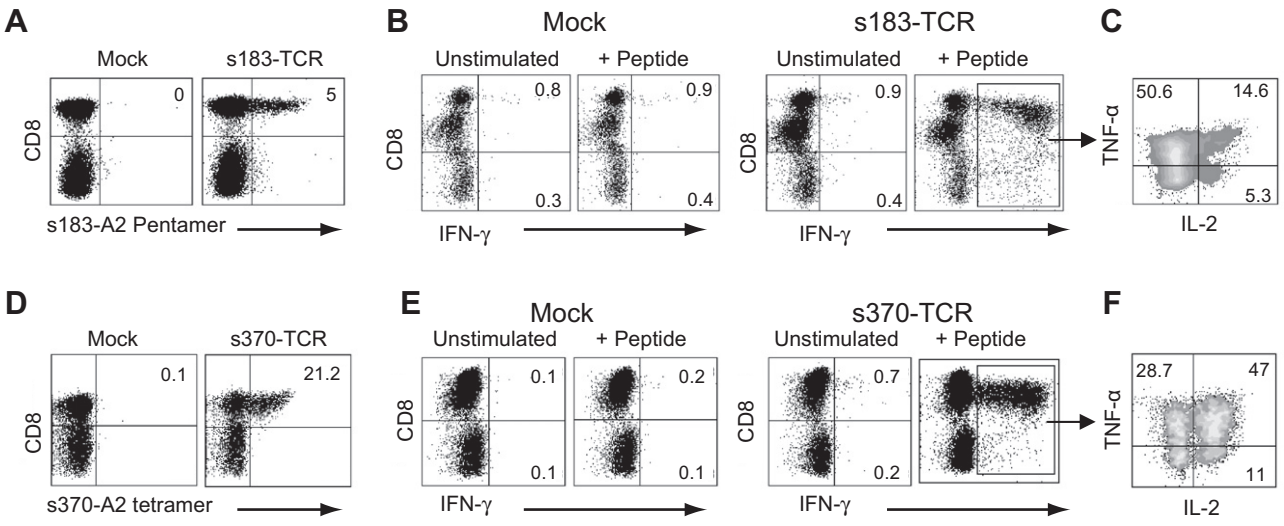

Fig. 6. Characterization of s183- and s370-TCR expression and transduced T cell function. HLA-A2 pentamer staining of mock and (A) s183-TCR or (D) s370-TCR transduced T cells. IFN-γ production in mock and (B) s183-TCR or (E) s370-TCR transduced T cells. Transduction with (C) s183-TCR and (F) s370-TCR generates multi-functional T cells. Panel shows TNF-α and IL-2 production by IFN-γ$^+$ T cells.

needed for efficient retroviral transduction. Whether these mechanisms would have an effect in chronic HBV patients will require clinical studies but, at least *in vitro*, TCR-re-directed T cells from chronic patients expressed similar levels of introduced TCRs and had a functional profile similar to genetically modified T cells from healthy donors.

We did observe differences in the expression and functionality between cells re-directed with different HBV-specific TCRs. Such variability is in accordance with the notion that the variable regions of the alpha and beta TCR chains play a significant role in the pairing of a TCR [27,28]. It is also important to consider that mispairing of the introduced TCR can affect expression and potentially contribute to autoimmune reactions by giving rise to TCRs of unknown specificities [29]. Since modifications to the TCR constant region can help direct proper pairing, reduce autoimmunity, and increase T cell functionality [29–31], we are currently working to develop optimized constructs for the TCRs described in this report.

The functionality of TCR gene modified CD8 T cells is in line with what is observed for *in vitro* expanded HBV-specific T cells from resolved HBV patients; they produce IFN-γ, TNF-α, and to a lesser extent IL-2 (unpublished data). We could possibly improve the multifunctionality of the TCR transduced T cells using the optimized TCR constructs mentioned above and further refining culture conditions to efficiently generate multi-functional T cells. Furthermore, functionality of the TCRs within the CD4 T cell population indicates that we can generate a cytotoxic and helper T cell response using the TCRs. However, because CD4 T cells lack the CD8 co-receptor to stabilize the TCR-HLA interaction, CD4 T cell activation is dependent on the affinity of the HLA-A2-restricted exogenous TCR and will differ depending on the TCR. As such, signaling may not be optimal in CD4 T cells but we were still able to see the production of significant IL-2 that could provide help for adoptively transferred T cells.

We also demonstrated that TCR transduced T cells could recognize HCC cell lines expressing HBV antigens from naturally integrated HBV DNA. Thus, our data, as well as the previous demonstration that HBV X-specific T cell clones can kill HCC tumor cells naturally expressing HBV X protein [9], shows that at least two HBV proteins can act as non-self tumor antigens. How many HCC express HBV antigens in a form recognizable to CD8 T cells, which HBV antigens are most frequently expressed by HCC and what is the level of antigen expression compared to HBV infected hepatocytes is unclear. HBV–DNA integration is common in HBV-related HCC tumors [32,33] but expression of intact HBV antigens can be variable [24] and HBV sequences are often fragmented [34]. Nevertheless, CD8 T cells recognize fragments of limited length (9–10 amino acids) and expression of intact HBV antigens is not necessary; even short integrated HBV DNA fragments can theoretically produce HBV epitopes. This variability in antigen expression potentially explains the different profiles of HBs-specific T cell activation that we observed. We are in the process of sequencing the HBsAg within these HCC cells to determine if the HBs183-91 epitope is not present in Hep3B or is present in a mutant form. In any case, the expression of HBV antigens and epitopes will differ between tumors, so developing TCRs specific for multiple HBV epitopes will be necessary to broaden the applicability of TCR gene therapy for the treatment of HBV-related HCC. While such cell based therapy may seem distant, adoptive transfer of TCR-re-directed T cells is already being safely performed and achieving results in other human tumors [35].

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Acknowledgments

We thank Michael Nassal at University Hospital Freiburg for providing the HBV expressing HepG2 cells, Gijs Grotenbreg at the National University of Singapore for synthesizing our HBs370-79 tetramer, Francis Chisari at the Scripps Research Institute for providing the HBcAg plasmid and Paul McCary at the National University of Singapore for providing the HLA-A2 plasmid. This work was supported by the Agency for Science Technology and Research (A*Star), Singapore.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2010.10.025.

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