Immunotherapy Advances, 2023, **3**, 1–11
https://doi.org/10.1093/immadv/ltad015
Advance access publication 14 August 2023
Research Article

Lytic efficiency of immunosuppressive drug-resistant armoured T cells against circulating HBV-related HCC in whole blood

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Summary

Recurrence of hepatitis B virus-related hepatocellular carcinoma (HBV-HCC) after liver transplant (LT) is mediated by circulating tumour cells (CTCs) and exacerbated by the immunosuppressants required to prevent graft rejection. To circumvent the effects of immunosuppressants, we developed immunosuppressive drug-resistant armoured HBV-specific T-cell receptor-redirected T cells (IDRA HBV-TCR). However, their ability to eliminate HBV-HCC circulating in the whole blood has never been tested, and whether their lytic efficacy is compatible with the number of adoptively transferred T cells *in vivo* has never been measured. Hence, we developed a microscopy-based assay to quantify CTCs in whole blood. The assay was then used to quantify the efficacy of IDRA HBV-TCRs to lyse free-floating HBV-HCC cells in the presence of Tacrolimus and Mycophenolate Mofetil (MMF). We demonstrated that a panel of antibodies (AFP, GPC3, Vimentin, pan-Cytokeratin, and CD45) specific for HCC tumour antigens and immune cells can effectively differentiate HCC-CTCs in whole blood. Through dose-titration experiments, we observed that in the presence of immunosuppressive drugs, a minimum of 20 000 IDRA HBV-TCR T cells/ml of whole blood is necessary to lyse ~63.5% of free-floating HBV-HCC cells within 16 hours. In conclusion, IDRA HBV-TCR T cells can lyse free-floating HBV-HCC cells in whole blood in the presence of Tacrolimus and MMF. The quantity of IDRA-HBV TCR T cells required can be achieved by the adoptive transfer of 5×10^6 IDRA-HBV TCR-T cells/kg, supporting the utilisation of IDRA HBV-TCR T cells to eliminate CTCs as prophylaxis against recurrence after LT.

Keywords: adoptive T-cell transfer, circulating tumour cells, HBV, hepatocellular carcinoma, TCR T cells

Abbreviations: AFP: Alpha-fetoprotein; Ck: Cytokeratin; CnB: Calcineurin B; CTCs: Circulating tumour cells; GPC3: Glypican-3; HBV: Hepatitis B Virus; HCC: Hepatocellular carcinoma; IDRA: Immunosuppressive drug-resistant armoured; IFNγ: Interferon-γ; IMPDH: Inosine-5′-monophosphate dehydrogenase; LT: Liver transplant; MMF: Mycophenolate Mofetil; PBMCs: Peripheral blood mononuclear cells; TCR: T-cell receptor; TNFα: Tumour necrosis factor.

Introduction

Liver cancer is one of the leading causes of cancer death with more than 800,000 incidents globally in 2015 and Hepatocellular Carcinoma accounts (HCC) for the majority of them [1]. In Asia, Chronic Hepatitis B virus (HBV) infection is the main risk factor for developing this disease (more than 50% of HCC) [2]. Patients with early or intermediate stages of HCC have different treatment options, such as liver resection, liver transplant (LT), checkpoint inhibitors, and anti-angiogenic drug combinations [3, 4]. However, for patients who develop metastasis, particularly after LT, the prospect of treatment is particularly grim. In this clinical setting, conventional molecular therapies have variable but often limited efficacy with a median overall survival rate of approximately 12 months [3, 5]. The low efficacy of systemic therapy in patients with LT is likely caused by the immunosuppressive drugs, utilised to reduce the risk of graft rejection, that reduces immune surveillance of cancer cells. In addition, novel therapies with checkpoint inhibitors are highly controversial in the setting of liver transplant, and several studies have reported allograft rejection and deaths associated with these new therapies [6–8].

For example, a clinical study of recurrent tumours in LT transplant patients under cyclosporine and steroid combinations showed a tumour doubling time of 33.0 days compared to 273.8 days in patients who have undergone

Received: April 12, 2023; Accepted: August 10, 2023

resection and hence, are not under immunosuppressants [9]. Immunosuppressive drugs also increase the rate of HCC relapses. Studies have shown that a greater percentage of patients with high exposure to tacrolimus [10–12] or cyclosporine [10, 12, 13] have a higher recurrence rate compared to those with low exposure. Overall, there is clearly a need for a therapy that will reduce the risk of HCC recurrence in patients after LT, and such therapy should directly target circulating tumour cells (CTCs).

CTCs are cells that have shed from the primary tumour, enter the lymph or blood and can seed metastases in secondary sites. In HCC patients, the appearance of CTCs after resection has been associated with recurrence [14, 15], and an inability of the LT patients to clear CTCs 30 days after surgical HCC removal is associated with higher mortality due to extrahepatic recurrence [16]. Furthermore, CTC numbers also correspond to patient response to therapy. Rau *et al*. demonstrated that those with progressive disease had a median of 50 cells/ml of blood compared to 15 cells/ml in patients with partial response or stable disease [17]. Thus, since interventions to reduce CTCs can benefit patients, we hypothesised that adoptive T-cell transfer of tumour-specific T cells might be used for targeting not only the primary tumour but also CTCs.

Our lab has developed an adoptive CD8 TCR-redirected therapy that targets HBV-associated HCC (HBV-HCC) through the recognition of HBV peptides presented on MHC Class I by the cancer cells [18–23]. We demonstrated that HBV-TCR-redirected T cells can recognise HBV-related HCC *in vitro* and *in vivo*. For example, in a murine model, we showed that injection of HBV-TCR-redirected T cells 4 hours after inoculation with HBV envelope expressing HCC cell line, HepG2-env, can prevent seeding of the circulating HepG2-env, significantly reducing tumour burden, suggesting a potential prophylactic use [18]. We also observed that in some HCC patients, HBV-TCR T-cell therapy can actually cause a reduction of alpha-fetoprotein and a shrinkage of tumour sizes [24, 25]. However, the cytolytic functions of our engineered CD8 T cells against circulating tumour cells are unknown particularly in the setting of immunosuppressive drug treatment.

We, therefore, utilised immunosuppressive drug-resistant armoured (IDRA) HBV-TCR T cells and tested their ability to recognise and lyse circulating HCC cells in the presence of immunosuppressive drugs tacrolimus and mycophenolate mofetil (MMF). Tacrolimus inhibits T-cell activation by binding to the 12-kDA FK506-binding protein (FKBP12) to form a complex that inhibits the function of Calcineurin (CN) subunit B (CnB) [26]. CnB can be mutated at the tacrolimus-FKBP12 binding site to prevent their interactions and allow for downstream signalling even in the presence of the immunosuppressive drugs [27–29]. This is done through the substitution of leucine at position 124 to a tyrosine and insertion of a leucine and an alanine after lysine at position 125 [27–29]. MMF, on the other hand, is first activated by esterase to give mycophenolic acid (MPA), which inhibits inoside-5′-monophosphate (IMP) dehydrogenase isoform II (IMPDH2) found in T cells and is the rate-limiting enzyme for *de novo* synthesis of guanine nucleotides. Consequently, inhibition of IMPDH2 results in the suppression of T-cell proliferation. Similarly, IMPDH2 can be mutated at the MPA-binding site through the substitution of threonine to an isoleucine at position 333 and of serine to tyrosine at position 351 [30, 31]. The IDRA TCR-T cells were engineered by introducing mRNA of a cognate HBV-TCR and of a mutant CnB and IMPDH that confer resistance against the effects of the immunosuppressive drugs [27–30, 32].

To test their ability to lyse CTCs, we first developed an immunofluorescence panel to differentiate HCC cells from peripheral blood mononuclear cells (PBMCs) before validating the protocol to enrich and image CTCs in blood samples from advanced HCC patients. We then used the panel to determine the optimal number of IDRA TCR-T cells that can result in maximum lysis of free-floating HBV-HCC cells. This work is a proof-of-concept for using IDRA HBV-TCR T cells as prophylaxis for HCC patients post-LT.

Material and methods

Cell culture

HepG2.2.15 were cultured in Dulbecco's modified eagle's medium (DMEM) (Gibco) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco), 2% Penicillin-Streptomycin (Gibco), 1% sodium pyruvate (Gibco), 1% non-essential amino acids (Gibco). Hep3B, SNU354, SNU368, SNU387, and SNU475 were cultured in RPMI 1640 (Gibco) supplemented in 10% heat-inactivated FBS, 1% Penicillin-Streptomycin, and 1% Glutamax (Gibco). Immunosuppressive drugs Tacrolimus (Sigma-Aldrich, St. Louis, MO) and MMF (Sigma-Aldrich, St. Louis, MO) were reconstituted in DMSO.

Patient samples

Patients were recruited at the National Cancer Centre, Singapore according to Institutional Review Board (IRB) guidelines (under 2018/3046: Circulating Markers in Upper GI and Hepatopancreatobiliary Cancers) and had provided written informed consent. The study conforms to the ethical guidelines of the Declaration of Helsinki. Healthy donors were recruited according to Institutional Review Board (IRB) guidelines (under NUS-IRB-2022-338: Functional characterisation and modification of virus-specific T-cell receptor-redirected T cells) and had provided written informed consent. The study conforms to the ethical guidelines of the Declaration of Helsinki. Healthy donors were also de-identified. Blood samples were collected using EDTA-coated vacutainer.

Engineering of IDRA HBV-specific TCR T cells

mRNA encoding the HBV-specific TCR (S183-TCR) was produced using the mMESSAGE mMACHINE™ T7 ULTRA Transcription Kit (ThermoFisher #AM1344) as per manufacturer's instructions. T cells were expanded from peripheral blood using a protocol previously described [18]. Briefly, PBMCs were activated with CD3 monoclonal antibody (ThermoFisher #16-0037-81) and expanded with recombinant 600U/ml IL-2 (Miltenyi Biotech). Ten million expanded cells were next electroporated with 20 µg HBV-TCR mRNA, 20 µg mutant IMPDH mRNA and 10 µg mutant CnB mRNA using P3 Primary Cell 4D-Nucleofactor™ X Kit (Lonza) [33] and rested for 24 hours [19]. The expression of S183-TCR on CD8 T cells was determined using HLA-A*0201 FLLTRILTI dextramer (Immudex #WB3290), CD3 PerCP-Cy5.5 (BD Biosciences #340949), CD8 Pe-Cy7 (BD

Biosciences #557746), LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kit for 405 nm excitation (ThermoFisher #L34968).

Incubation of engineered T cells in autologous blood

Five millilitres of fresh blood were obtained from healthy donors. Ten nanograms of tacrolimus and 4 µg of MMF were added per ml of blood before 100,000 HepG2.2.15 cells and autologous engineered T cells were spiked. The blood was incubated in Amersham biosciences hybridisation oven/shaker at 37°C and at eight revolutions per minute for 16 hours using before CTCs were isolated (See below for isolation of CTCs).

Isolation of CTCs/spiked HepG2.2.15 cell and confocal microscopy

Five millilitres of healthy donor blood was incubated with 10 ng/ml of tacrolimus and 4 µg/ml of MMF for 1h before being spiked with 25, 100, or 1000 HepG2.2.15 cells. HepG2.2.15 were then isolated using RosetteSep™ Human CD45 Depletion Cocktail kit as per the manufacturer's instructions. Briefly, tetrameric antibody complexes against CD45, CD66b, and CD235a were incubated with the spiked blood and layered onto Ficoll®-Paque Plus for density centrifugation. HepG2.2.15 were isolated from the interface between serum and Ficoll®-Paque Plus. Isolated cells were fixed and stained using X-Zell Cryoimmunostaining™ Suit and kit as per the manufacturers' instruction (Fig. 2A). CTCs were stained with antibodies against pan-cytokeratin AF594 (Biolegend #628606), vimentin (Vim) AF594 (Abcam #ab154207), alpha-fetoprotein (AFP) AF488 (BD #563016), Glypican-3 (GPC3) AF488 (R&D systems #FAB2119G), CD45 PerCP (Biolegend #304026) and with DNA stain DRAQ5 (ThermoFisher #62251) as detailed in Supplementary Table S1 [34]. Cells were incubated with antibodies in 50 µl of staining buffer at 4°C for 50 minutes as per the manufacturers' instruction. Opera Phenix High Content Screening confocal system (PerkinElmer) with air objective 20× and Harmony High-Content Imaging and Analysis software was used for image acquisition and IMARIS software (Bitplane) was used for image analysis.

Intracellular staining of engineered T cells

10^8/ml of electroporated T cells were stained with 1 µM of CFSE (ThermoFisher #C34554) for 5mins in the dark before being washed in HBSS supplemented in 2% FBS. 2 µg/ml of Brefeldin A, CFSE engineered T cells, and HepG2.2.15 were spiked into whole blood. The spiked blood was next incubated at 37°C and at eight revolutions per minute for 16 hours using the Amersham biosciences hybridisation oven/shaker. Red blood cells were then lysed with Red Blood Cell Lysis solution (Miltenyi Biotec), and remaining cells were stained with LIVE/DEAD™ Fixable Yellow Dead Cell Stain (ThermoFisher #L34968) before being blocked with Human TruStain FcX™ (Biolegend #422301) and stained with CD3 PerCP-Cy5.5 (BD Biosciences #340949), CD8 Pe-Cy7 (BD Biosciences #557746) antibodies. Cells were then fixed and permeabilised using Cytofix/Cytoperm™ Fixation and Permeabilisation Solution (BD) before being stained with IFNγ V450 (BD Biosciences #560371), TNFα APC (BD Biosciences #554514) antibodies.

Quantification of cytokines

Plasma was collected after co-culture of 100,000 or 200,000 IDRA S183-TCR T cells with 100,000 HepG2.2.15 cells in 5 ml of blood. To quantify the IFNγ concentrations, Ella Automated Immunoassay System (ProteinSimple) with microfluidic multiplex cartridge for measurement of IFNγ was used as per the manufacturer's instructions.

Statistical tests

Data were analysed in Prism (GraphPad, San Diego, CA), using the statistical tests stated in the respective figure legends (****$P < 0.0001$, ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

Results

Establishment of a microscopy-based immunofluorescence panel to identify HCC CTCs

A four-colour, six-marker immunofluorescence panel was first set up to identify and differentiate enriched CTCs from contaminating white blood cells in the samples (Table 1) [34, 35]. CTCs are rare and numbers can be can low as less than five in 5 ml of blood [17, 36–39], hence a microscopy-based method that allows visual validation of the analysed cell will reduce false positive rates.

DRAQ5, a cell permeable stain, is used to identify nucleated cells. Two sets of markers were used to stain for HCC: Alpha-fetoprotein (AFP) and Glypican-3 (GPC3), and pan-cytokeratin (Ck) and vimentin (Vim) (Table 1). GPC3 has high sensitivity and specificity of 0.77 and 0.96, respectively, and AFP has a high specificity of 0.97 making them useful markers for HCC cell identification [40–42]. The pan-Ck antibodies stain for epithelial markers: Ck 4, 5, 6, 8, 10, 13, and 18, which are expressed by cells that are of non-hematopoietic origins. In addition, in HCC cells that have undergone epithelial-mesenchymal transition, Vim, a mesenchymal marker, was observed to be expressed [36, 43–46], hence their combination can be used to confirm the presence of HCC CTCs (Table 1). To further allow the differentiation of immune cells from HCC CTCs, antibodies against CD45—a protein expressed only by the former—were used (Table 1). The panel was tested on six different HCC cell lines—HepG2.2.15, Hep3B, SNU354, SNU368, SNU387, and SNU475—mixed with peripheral blood mononuclear cells (PBMCs) at a ratio of 1:1.

We demonstrated that all six HCC cell lines were pan-CK/Vim+ and AFP/GPC3+ but CD45- (white arrows), while PBMCs are CD45+ (pink arrows) (Fig. 1A andB). The HCC cells also have a much larger nucleus with a diffused DRAQ5 staining intensity, while the nucleus of PBMCs appears smaller with higher DRAQ5 intensity (Fig. 1A). This

Table 1. Cell types of interest and their markers

Cell types	Markers	Fluorophore
HCC	Alpha-fetoprotein (AFP) & Glypican-3 (GPC3)	AF488
	Pan-cytokeratin (Pan-Ck) & Vimentin (Vim)	AF594
Peripheral blood mononuclear cells	CD45	PerCP

difference allows for the visual differentiation of HCC cells and immune cells. The mean intensity of fluorophore AF488 (conjugated to anti-AFP and anti-GPC3), AF594 (anti-pan-Ck and anti-Vim), and PerCP (anti-CD45) on each cell was also quantified (Fig. 1B). The mean intensities of these three channels were plotted in a 3D plot and HepG2.2.15 (brown dots) and immune cells (blue dots) formed distinct non-overlapping clusters, hence validating the ability of our panel to effectively differentiate HCC from PBMCs (Fig. 1C).

Detection of HCC CTCs in human whole blood

Having demonstrated that our panel can differentiate HCC cell lines from PBMCs, we next developed a protocol that can enrich CTCs from human whole blood. In the protocol, CTCs

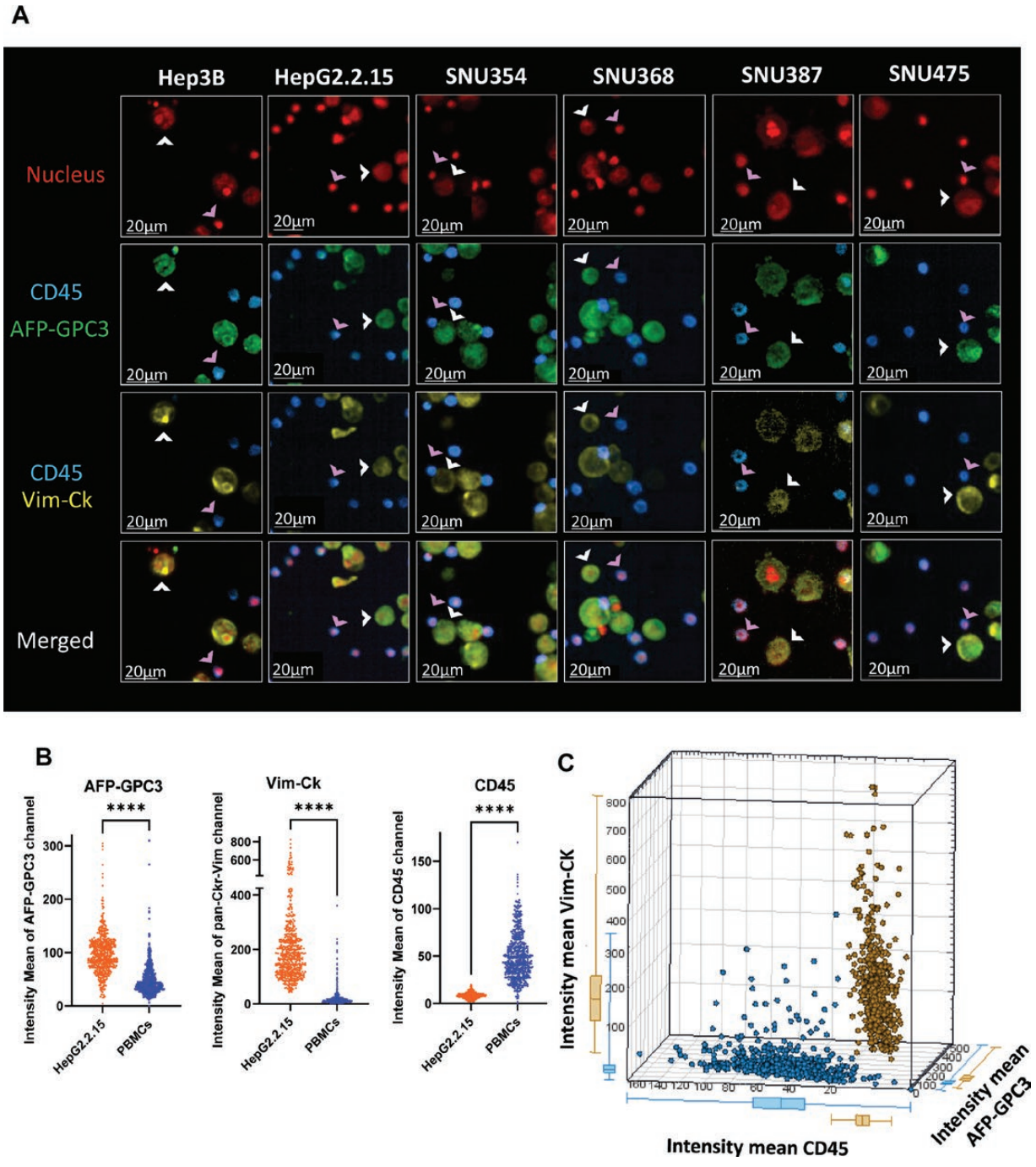

Fig. 1. Establishment of immunofluorescence panel to differentiate HCC cells from PBMCs. (A) Representative images of six HCC cell lines mixed with PBMCs at 1:1 ratio. HCC cells (indicated by white arrows) are positive for cancer markers—AFP and GPC3 (green); and Vim and pan-Ck (yellow) but not immune-marker CD45 (blue), while hematopoietic immune cells (indicated by pink arrows) are positive for CD45. DRAQ5 is used to stain the nucleated cells. (B) Intensity means of AFP-GPC, Vim-CK and CD45 in HepG2.2.15 and PBMCs. Mann–Whitney test was used to determine statistical significance (****$P < 0.0001$). (C) 3D plot with box plots of the intensity means of Vim-Ck, AFP-GPC3 and CD45 expression in HepG2.2.15 cells (brown dots) and PBMCs (blue dots).

are enriched using RosetteSep™ Human CD45 Depletion Cocktail kit as per the manufacturer's instructions and density centrifugation. The enriched CTCs are next isolated, fixed onto microscope slides, and stained with the aforementioned panel using X-Zell Cryoimmunostaining™ Suite, as per the manufacturer's instructions. All fields of views (FOVs) with observable nucleated cells on the slide were counted (maximum of 133 FOVs) (Fig. 2A). To ensure that the low numbers of CTCs in whole blood could be reliably quantified, we first assessed the CTC recovery rate using the protocol. Healthy donor blood was spiked with 25, 100, or 1000 HepG2.2.15 cells in the presence of tacrolimus and MMF. Tacrolimus and MMF are routinely used after liver transplantation, thereby mimicking the *in vivo* scenario and at the same time, inhibiting any potential allogeneic function against HepG2.2.15 cells. We were able to consistently recover ~60% of spiked HepG2.2.15 even at the lowest concentration, where only 25 HepG2.215 cells were spiked in the 5ml of whole blood (Fig. 2B).

The established protocol was then used to enrich and isolate CTCs directly from the blood of HCC patients. Five millilitres of blood from five HCC patients were obtained and processed as described in Fig. 2A. Patients were male and between the age of 64 and 76 (Table 2). Three of five of the patients had HBV-related HCC, one had non-alcoholic liver disease (NASH), while the aetiology of Hep0900 is unknown since a liver biopsy was not performed. Three patients (Hep0679, Hep0702, and Hep900) had evidence of extrahepatic spread and macrovascular invasion and were classified as stage C (or advanced stage) under the Barcelona Clinic Liver Cancer (BCLC) staging system. Patients Hep0788 and Hep0819 did not have signs of extrahepatic spread or macrovascular invasion and were classified as stage B (or intermediate stage). Despite these clinical differences, we were able to isolate CTCs from all five patients (Fig. 2C). This was in contrast to the five healthy controls who were analysed with the same protocol where only one CTC-like cell with poor staining characteristics, likely a false positive, was detected in one of the

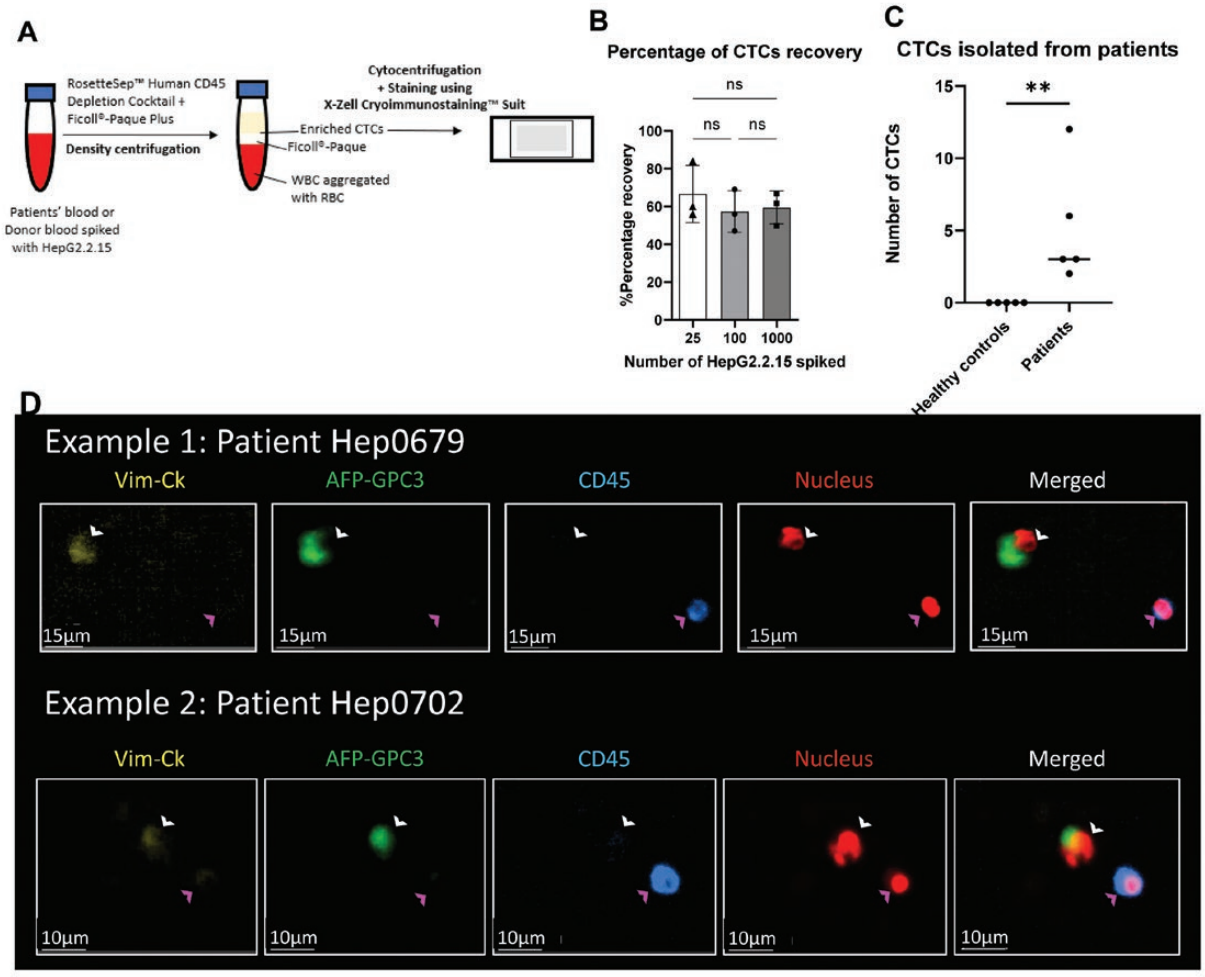

Fig. 2. Protocol to enrich and stain CTCs from patients. (A) Schematic diagram of the isolation and detection of CTCs in patient blood or spiked HepG2.2.15 in healthy donor blood. Whole blood was incubated with RosetteSep™ Human CD45 Depletion Cocktail, layered onto Ficoll®-Paque and centrifuged. CTCs or spiked HepG2.2.15, which were enriched at the serum-Ficoll®-Paque layer, were harvested and concentrated on microscopy slides via cytocentrifugation before being fixed and stained using X-Zell Cryoimmunostaining™ Suit. (B) Percentage of HepG2.2.15 recovered after spiking with 25, 100, or 1000 cells in 5 ml of blood. One-way ANOVA with Tukey's multiple comparisons test was used to determine statistical significance (N.S. > 0.05). (C) Number of CTCs isolated in all patients (n = 5) and healthy donors (n = 5). Two-tailed Mann–Whitney test was used to determine statistical significance (**P < 0.01). (D) Representative images of CTCs isolated from two patients. CTCs are indicated by white arrows while the pink arrows indicate the hematopoietic immune cells.

Table 2. Clinical characteristics of HCC patients and number of CTCs isolated

Patients	Age	Gender	BCLC	Aetiology	Macrovascular invasion	Spread	CTCs isolated
Hep0679	68	Male	C	HBV	No	Lymph node	3
Hep0702	64	Male	C	HBV	Yes	Lymph node	12
Hep0788	76	Male	B	HBV and Alcohol	No	No	6
Hep0819	67	Male	B	NASH	No	No	2
Hep0900	68	Male	C	Unknown	Yes	Portal vein, hepatic vein, and inferior vena cava	3

individual (Fig. 2C and Supplementary Fig. 1). Representative images of HCC CTCs from two patients (Fig. 2D) and the healthy donor are shown (Supplementary Fig. S1). The white arrows of both examples indicate CTCs stained positive for Vim-Ck; cancer markers (AFP-GPC3) and nucleus while immune cells (pink arrows) stain for CD45 and nucleus (Fig. 2D).

Some studies in patients have identified clusters of more than 2 CTCs aggregated together, known as circulating tumour microemboli (CTMs), which are less common than CTCs but have several fold higher metastatic potential [14, 36, 37, 45–48]. We did not detect any CTMs in any of the patients and healthy individuals analysed, likely due to the limited sample size.

Autologous IDRA HBV-TCRT cells can be activated by free-floating HCC cell line in whole blood in the presence of immunosuppressive drugs

Previously, we have demonstrated that our IDRA HBV-TCR T cells can efficiently lyse the HBV-HCC cell line, HepG2.2.15, in the presence of immunosuppressive drugs in 2D and 3D cell cultures [32]. However, it is unknown whether these T cells can directly lyse free-floating HepG2.215 in whole blood in the presence of tacrolimus and MMF, similar to the in vivo scenario of lysing CTCs. To determine this, we measured the production of cytokines after spiking healthy whole blood containing tacrolimus and MMF with 100,000 HepG2.2.15 and 100,000 autologous mock electroporated (MEP) or IDRA HBV-TCR T cells. As previously described, IDRA HBV-TCR-redirected T cells were obtained by expanding CD8 T cells from PBMCs and electroporating them with mRNA that encodes a TCR (S183-TCR), which can recognise cells presenting HBV ENV183-191 peptide on HLA-A*02:01 [18], concomitantly with mutant CnB and mutant IMPDH mRNA [32].

IFN-γ was secreted into the plasma only when HepG2.215 was added to the whole blood containing autologous IDRA S183-TCR, but not when mock electroporated T cells were added, showing antigen-specific activation in the presence of tacrolimus and MMF (Fig. 3A). To validate the activation of the spiked IDRA S183-TCR T cells, we performed an intracellular staining of the IDRA S183-TCR T cells for IFNγ and TNFα after co-culturing them with HepG2.2.15 or non-HBV expressing parental HepG2 in autologous healthy whole blood containing tacrolimus and MMF. IDRA S183-TCR were stained with CFSE before being spiked into whole blood to facilitate identification by flow cytometry. Upon addition of HepG2.215, but not HepG2, IDRA S183-TCR T cells were activated and subsequently produced both IFN-γ and TNFα, clearly showing that the IDRA S183-TCR T cells can be stimulated efficiently by free-floating HCC cells in whole blood in the presence of tacrolimus and MMF (Fig. 3B–E).

Minimum number of IDRA S183-TCRT cells required to maximise lysis of free-floating HBV-HCC cells in whole blood in the presence of tacrolimus and MMF

Next, we determined whether the IDRA S183-TCR T cells can also directly lyse and reduce the number of free-floating HepG2.2.15 in autologous immunosuppressant-containing blood. We performed a dose titration of IDRA S183-TCR T cells using concentrations calculated from the infusion schedule of a post-liver transplant patient with extrahepatic HCC recurrence which has undergone similar HBV-TCR T cell therapy [25] (Fig. 4A). We selected the two highest doses of the dose-escalation phase (10^6 and 5×10^6 TCR-T cells/kg) and the dose of the treatment phase (10^7 TCR-T cells/kg) (in red) which is approximately 1000, 5000, and 10,000 IDRA S183-TCR T cells/ml of blood (Fig. 4A). However, this assumes that IDRA S183-TCR T cells will be evenly distributed throughout the body, which is not representative of normal physiology. Here, we assume that the IDRA S183-TCR T cells will be distributed only throughout the extracellular fluid, which makes up 20–30% of total body weight. Therefore, we included concentrations of 20,000 and 40,000 IDRA S183-TCR T cells/ml of blood in the dose-titration. We incubated 100,000 HepG2.2.15 with increasing concentrations of IDRA S183-TCR T cells in whole blood overnight and isolated the remaining HepG2.2.15 cells to determine the rate of lysis (Fig. 4B and C).

By normalising each sample to their respective negative control—mock electroporated (EP) T cells—we demonstrated that the addition of 20,000 IDRA S183-TCR T cells/ml reduces the percentage of remaining HepG2.2.15 after overnight culture to 36.5% (Fig. 4D). We, therefore, show that a maximum lysis of ~63.5% of spiked 100,000 HepG2.215 was achieved in 16 hours using 20,000 IDRA S183-TCR T cells/ml in autologous blood containing tacrolimus and MMF (Fig. 4D). Importantly, ~64% of the spiked HepG2.215 remained when double the amount of conventional S183-TCR T cells were used, showing the superior function of IDRA S183-TCR T cells in the presence of TAC and MMF (Fig. 4D).

Discussion

Immunosuppressants such as tacrolimus and MMF increase the risk of HCC recurrence after LT due to their ability to inhibit immune system function. This functional inhibition also affects HBV-TCR T cells that are adoptively transferred into the liver transplanted patients for treatment of HBV-related

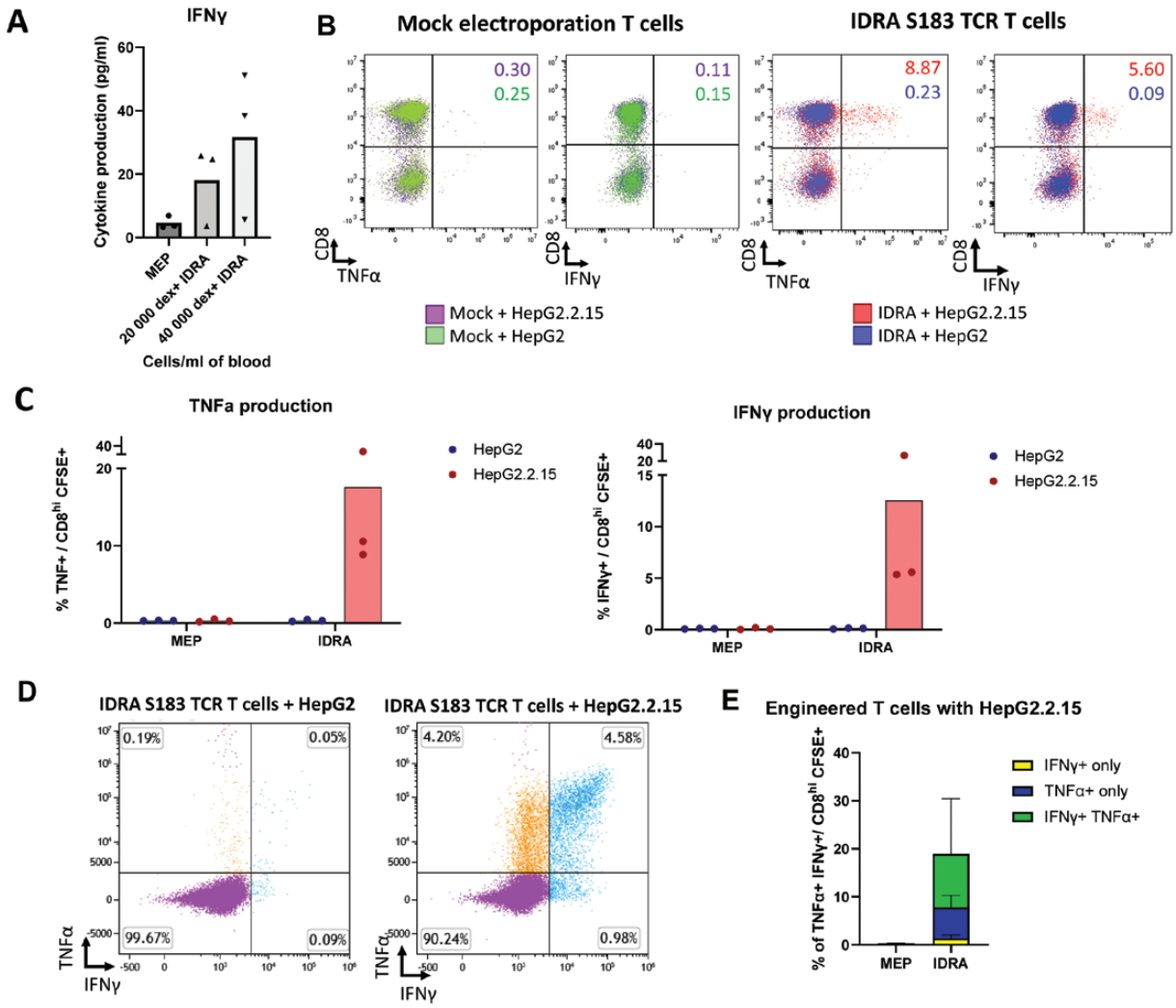

Fig. 3. IDRA HBV-TCR-redirected T cells are activated by free-floating HepG2.2.15 in whole blood in presence of tacrolimus and MMF. (A) IFNγ concentrations in plasma were analysed after mock electroporated T cells (MEP), 20,000 or 40,000 IDRA HBV-TCR T cells are added to each ml of whole blood spiked with HepG2.2.15 (n = 3). (B) Representative overlaid plots of TNFα or IFNγ production by IDRA S183-TCR (antigen-specific) or MEP T cells from a single donor, in the presence of HepG2 (control) or HepG2.2.15 (antigen). The percentage of TNFα and/or IFNγ producing CD8+ CFSE+ IDRA S183-TCR and mock electroporated T cells (out of total CFSE+ CD8+ T cells) are indicated in the top right corner of each graph. (C) Summary of percentage of TNFα and IFNγ producing CFSE+ CD8+ IDRA S183-TCR and MEP T cells (n = 3). (D) Representative flow plots of IFNγ and TNFα production by IDRA S183-TCR or MEP T cells from a single donor, in the presence of HepG2.2.15. The percentage of cytokine producing CD8+ CFSE+ T cells are shown in each quadrant. (E) Summary of IFNγ and TNFα production by IDRA S183-TCR and MEP T cells when co-cultured with free-floating HepG2.2.15 in whole blood (n = 3).

HCC metastasis. To bypass such effect, we engineered IDRA HBV-TCR T cells that preserved their function in the presence of immunosuppressants in 2D and 3D *in vitro* cell culture [32]. Here, we showed that our IDRA HBV-TCR-redirected T cells can also efficiently carry out their effector functions against free-floating HCC cells in whole blood containing tacrolimus and MMF at quantities present in patients.

Since CTCs are involved in the metastasis of tumour cells, it is important to reduce their numbers. However, the rarity of CTCs in the blood (less than five cells/ml of blood) makes their quantification challenging. For this reason, we first developed a protocol to isolate and identify CTCs in patients. We utilised an immunofluorescence microscopy-based approach in order to have high confidence in positively identifying the CTCs in the blood through the overexpression of HCC (AFP and GPC3) and epithelial markers (Vim and Ck), nuclear morphology (size and DRAQ5 staining), and the lack of hematopoietic marker expression (CD45). Using our developed protocol, we were able to detect CTCs in the blood of patients with advanced stages of HCC where we expect CTCs to be present, while it was absent in healthy individuals, except for one CTC-like cell with diffuse/poor staining (likely to be a false positive) detected in a single individual (Fig. 3C and Supplementary Fig 1). The rates of such false positives will have to be further explored in larger future studies. Nonetheless, this ability to isolate and quantify CTCs has several applications. It can be used as a less invasive means to predict recurrence after therapy or as an estimate of treatment efficacy as the number of CTCs in HCC patients is associated with recurrence, tumour burden, and stage [44, 49–51]. An increase in the number of CTCs in the blood in HCC patients during the follow-up after resection was also observed to precede detection of recurrence [44, 52].

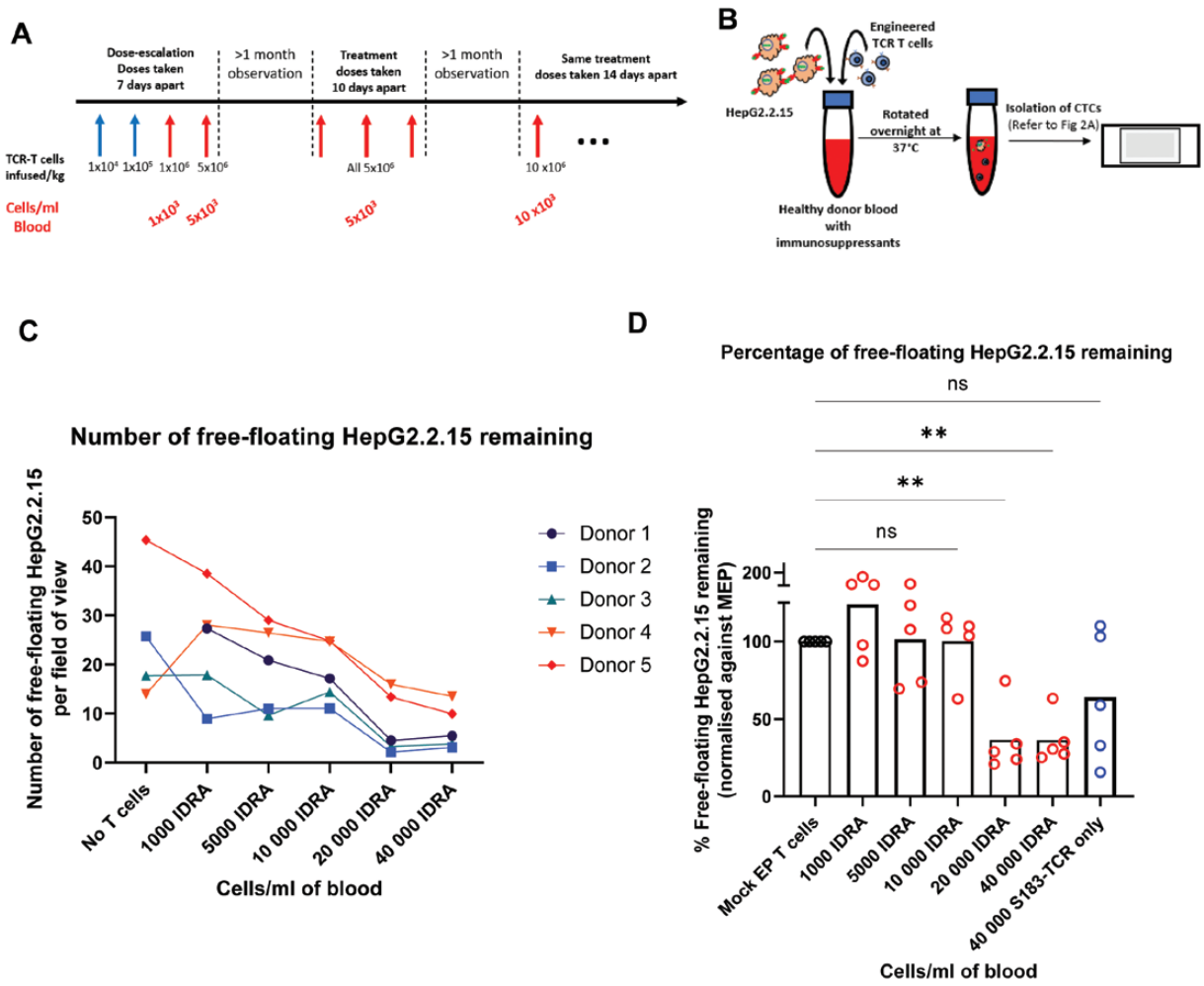

Fig. 4. Cytotoxic function of IDRA HBV-TCR-redirected autologous CD8 T cells against free-floating HepG2.2.15 cells in autologous whole blood. (A) Schematic diagram showing number of S183 TCR-T cells infused (arrows) into a previous patient (Tan *et al.* [24]). Indicated in black font are the infusion doses given to the patient while in red font are the corresponding calculated doses used in the dose-titration experiment. (B) Schematic diagram depicting the experimental design where HepG2.2.15 and increasing doses of engineered T cells were spiked into autologous whole blood with immunosuppressants. Blood was incubated in Amersham biosciences hybridisation oven/shaker at 37°C and rotated at eight revolutions per minute for 16 hours. Free-floating HepG2.2.15 were then isolated as described in Fig. 2A. Forty-nine fields of view were imaged and the number of HepG2.2.15 in each field of view was counted. (C) Average number of free-floating HepG2.2.15 in one field of view after overnight incubation with increasing concentrations of IDRA S183-TCR T cells ($n = 5$). (D) Percentage of free-floating HepG2.2.15 remaining after co-culturing with different quantities of IDRA S183-TCR T cells. Data were normalised to negative control –mock electroporated (MEP) T cells ($n = 5$). One-way ANOVA with Tukey's multiple comparisons test was performed to determine statistical significance (****$P < 0.0001$, ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; N.S. > 0.05).

We also developed a model to predict the ability of our engineered T cells to lyse CTCs. To mimic CTCs in an *in vitro* model, we spiked a predetermined number of HepG2.215 cells, a HBV-HCC cell line that can seed distal HBV-HCC in murine xenograft models [18], into healthy donor whole blood. We demonstrated that IDRA HBV-TCR T cells can be efficiently activated by these free-floating HCC cells in immunosuppressant containing autologous whole blood, resulting in both cytokine secretion and lysis of the tumour cells (Figs 3 and 4). Importantly, by combining the CTC quantification assay with a dose titration of IDRA HBV-TCR T cells, we estimated that 20,000 IDRA HBV-TCR T cells/ml provided maximal lysis of 100,000 circulating CTCs in the presence of tacrolimus and MMF. Assuming that the IDRA S183-TCR T cells will be distributed only throughout the extracellular fluid, this translates to an infusion dose of ~5,000,000 HBV-TCR T cells/kg. The adoptive transfer of this quantity and up to 10×10^6 HBV-TCR T cells/kg have been reliably performed multiple times in several HCC patients with or without LT [24, 25], indicating the feasibility of the approach. Furthermore, given the low numbers of CTCs present in the whole blood of liver transplant patients with HCC recurrence, the quantity of IDRA HBV-TCR T cells required to provide maximal lysis of CTCs *in vivo* is likely to be lower.

In summary, the data presented herein is a proof-of-concept that demonstrates the ability of the immunofluorescence-based assay to detect the low quantities of CTCs present in the whole blood and the capacity of IDRA HBV-TCR T cells to effectively lyse CTCs in blood containing immunosuppressants. These data support the concept of using IDRA HBV-TCR T cells as a prophylaxis against HCC recurrence in patients who have undergone liver transplant. Clearly, it has to be evaluated in the future in patients before and after infusion of IDRA HBV-TCR T cells, or other treatments, to determine whether the dynamic changes in the number of CTCs could still be enumerated by the assay and whether the IDRA HBV-TCR

T cells could effectively reduce CTC frequencies in the blood resulting in a reduction of HCC recurrence. Nevertheless, we hope that this work can spur both the development of HBV-TCR T cells as a prophylactic treatment for HCC recurrence and the quantification of CTCs using the immunofluorescence-based assay as a means of determining HCC treatment efficacy.

Supplementary material

Supplementary data are available at *Immunotherapy Advances* online.

Acknowledgements

We would like to thank Hang Shou Kit for his help with the Ella Automated Immunoassay System and; Maricel Felipe Ang and Tan Yen Leng for coordinating the collection of patients' blood. The Editor-in-Chief, Tim Elliott, and handling editor, Staphanie Dougan, would like to thank the following reviewers, Yi Li and Malcolm Sim, for their contribution to the publication of this article.

Funding

This work was supported by the National Medical Research Council (MOH-STaR17nov-0001).

Authors contributions

M.L., A.T.T., A.B., and S.C.B. designed the study. M.L., A.T.T., and A.B. drafted the manuscript. L.W. and T.W. manufactured the IDRA HBV-TCR T cells. M.L. and D.T. performed the experiments. M.L., A.T.T., and A.P. analysed and interpreted the data. D.W.M.T and J.J.X.L. recruited the patients.

Ethical approval

Patients were recruited at the National Cancer Centre, Singapore according to Institutional Review Board (IRB) guidelines (under 2018/3046: Circulating Markers in Upper GI and Hepatopancreatobiliary Cancers). The study conforms to the ethical guidelines of the Declaration of Helsinki.

Patient consent statement

Patients were recruited at the National Cancer Centre, Singapore and had provided written informed consent.

Conflict of interest

S.C.B. owns stock in and receives compensation from X-Zell, a company owning patents and commercialising technologies covering rare-cell detection and characterisation. A.P. is a consultant and shareholder of AIM Biotech Pte. Ltd. A.B. and A.T.T. are the Scientific Founder and the Scientific Consultant of Lion TCR Pte. Ltd, respectively, a biotech company developing T-cell receptors for the treatment of virus-related diseases and cancers. All other authors disclose no conflicts.

Data availability

The data used to support the findings of this study are included in the article.

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