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Research Article

A rapid method to assess the *in vivo* multi-functionality of adoptively transferred engineered TCR T cells

Anthony T. Tan[1,*], Shou Kit Hang[1], Nicole Tan[1], Thinesh L. Krishnamoorthy[2], Wan Cheng Chow[2], Regina Wanju Wong[3], Lu-En Wai[3,*], Antonio Bertoletti[1]

[1]Emerging Infectious Diseases, Duke-NUS Medical School, Singapore, Singapore
[2]Department of Gastroenterology and Hepatology, Singapore General Hospital, Singapore, Singapore
[3]Lion TCR Pte Ltd., Singapore, Singapore

[*]Corresponding authors. Anthony T. Tan, Programme in Emerging Infectious Diseases, Duke-NUS Medical School, 8 College Road, Singapore 169857, Singapore. E-mail: Anthony.tan@duke-nus.edu.sg; Lu-En Wai, Lion TCR Pte. Ltd., JTC MedTech Hub, 2 Tukang Innovation Grove, #09-03/04, Singapore 618305, Singapore. E-mail: luen.wai@liontcr.com

Abstract

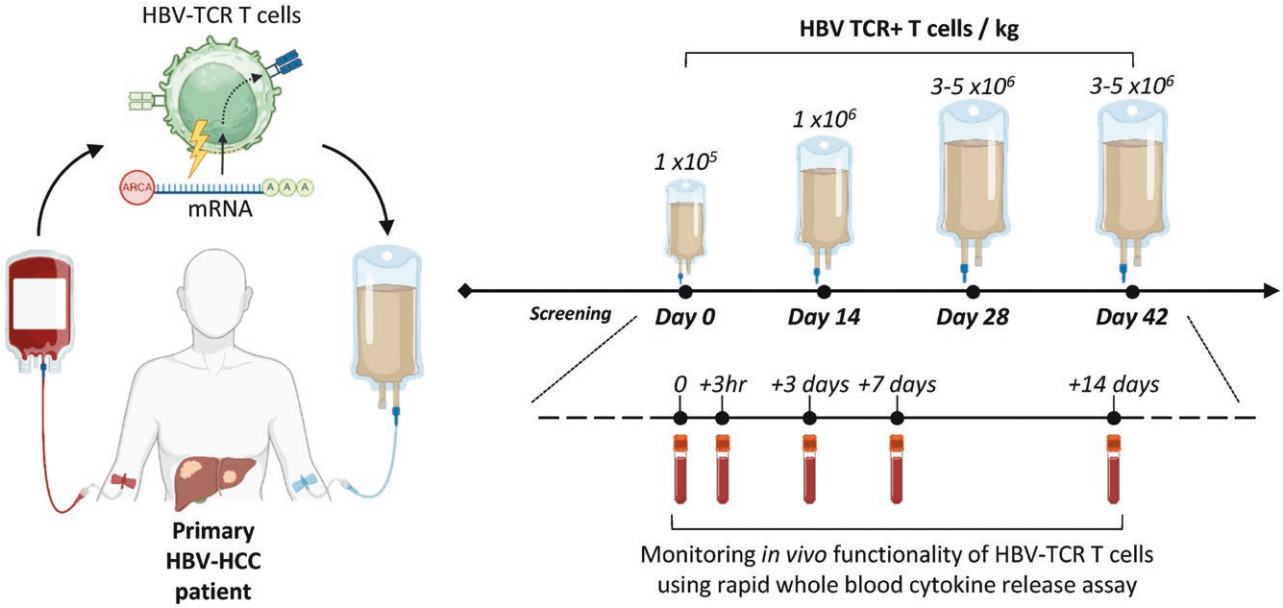
Introduction: The clinical efficacy of chimeric antigen and T cell receptor (TCR) T cell immunotherapies is attributed to their ability to proliferate and persist *in vivo*. Since the interaction of the engineered T cells with the targeted tumour or its environment might suppress their function, their functionality should be characterized not only before but also after adoptive transfer.

Materials and methods: We sought to achieve this by adapting a recently developed Severe acute respiratory syndrome *coronavirus 2* (SARS-CoV-2) rapid whole blood T cell assay to stimulate engineered TCR T cells in small volumes of whole blood (<1 ml) without *in vitro* cellular purification. As a proof-of-concept, we used this method to longitudinally study two patients with primary Hepatitis B Virus (HBV)-related hepatocellular carcinoma who received multiple dose-escalating infusions of transiently functional mRNA-engineered HBV-TCR T cells.

Results: We demonstrated that a simple pulsing of whole blood with a peptide corresponding to the epitope recognized by the specific HBV-TCR elicited Th1 cytokine secretion in both patients only after HBV-TCR T cell treatment and not before. The amount of cytokines secreted also showed an infusion-dose-dependent association.

Discussions: These findings support the utility of the whole blood cytokine release assay in monitoring the *in vivo* function and quantity of engineered T cell products following adoptive transfer.

Graphical Abstract



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Introduction

The clinical efficacy of therapies with T cells engineered to express exogenous chimeric antigen (CAR) or T cell receptors (TCR) have been linked with their *in vivo* ability to proliferate and persist [1]. Hence, bioanalytical platforms to measure adoptive T cell therapy efficacy were primarily developed to detect the presence of the engineered T cells in the peripheral blood of treated patients, substantially neglecting an analysis of their *in vivo* effector function after adoptive transfer [2]. Such neglect could have important repercussions as effector T cell functions, like cytokine production, can be modified by repetitive antigen stimulation or by interactions with the immune microenvironment where the adoptive T cell recognizes its antigen [3]. This is particularly evident for T cells that exert their effector functions in organs, like the liver, that possess T cell-tolerant features.

In addition, while majority of CAR- and TCR-T cells have been engineered using viral vector approaches in order to achieve a permanent and stable expression of the introduced transgene, other alternatives that use *in vitro* transcribed mRNA to provide a non-integrating and transient modification of the T cells have also been done [4–10]. In these cases, the labile nature of the mRNA and the transient expression of the CAR or TCR precludes a meaningful monitoring of the *in vivo* persistence of these T cells. Treatment with messenger RNA (mRNA)-engineered T cells would instead benefit from the assessment of the immediate *in vivo* functionality of these functionally short-lived engineered T cells in order to understand its association with treatment efficacy.

As such, we think that it is important to longitudinally assess the functionality of engineered T cells before and after their transfer into patients. While we have previously shown that the cytotoxicity of engineered TCR T cells can be assessed in whole blood *ex vivo* [11], monitoring the functionality of adoptively transferred engineered T cells should ideally be performed using a simple assay that does not require a significant amount of blood or complex procedures.

We sought to achieve this by adapting a recently developed SARS-CoV-2 rapid whole blood T cell assay [12]. In this assay, SARS-CoV-2 peptides were added directly to a small volume of heparinized whole blood (320 µl) to stimulate SARS-CoV-2-specific T cells. The amount of Interferon gamma (IFN-γ) and Interleukin (IL-2) released into the plasma as a consequence of peptide stimulation were proportional to the frequency of the circulating functional SARS-CoV-2-specific T cells. We hypothesized that by adding a peptide, containing the T cell epitope recognized by the specific TCR T cells, directly into the whole blood of patients undergoing adoptive T cell therapy, we could measure the function of the engineered TCR T cells *ex vivo*.

To illustrate the assay utility, here we used this method to longitudinally monitor the *in vivo* functionality of adoptively transferred HBV-specific TCR-engineered T cells in two patients with primary HBV-related hepatocellular carcinoma (HBV-HCC), a treatment approach that we have previously shown to have therapeutic potential against HBV-HCC [8, 9, 13].

Results

Two HBV-HCC patients who were not amenable to or have failed conventional treatment were recruited and treated with multiple infusions of autologous mRNA-engineered Hepatitis B Virus-specific T cell receptor-expressing (HBV-TCR) T cells, an approach that we have previously demonstrated to have therapeutic potential against HBV-HCC [8, 9]. The autologous HBV-TCR T cells were engineered by mRNA electroporation using peripheral blood mononuclear cells (PBMCs) obtained through leukopheresis, and cryopreserved at the appropriate quantities for infusion. As the HBV-TCR T cells have the capacity to mediate on-target off-tumour lysis of HBV-infected hepatocytes in the HBV-HCC patients with a background of chronic HBV infection, a dose-escalating infusion protocol was implemented and in this report, only the first four infusions of increasing doses (1×10^5, 1×10^6, $3–5 \times 10^6$, and $3–5 \times 10^6$ TCR+ T cells/kg) given every 14 days are shown (Fig. 1). A small volume of heparinized peripheral whole blood (~ 1 ml) was collected before and after each infusion at the indicated time points (+3 h samples collected starting from 1×10^6 TCR+ T cells/kg dose) and either unstimulated or stimulated with an HBV peptide containing the respective HBV epitope recognized by the engineered HBV-TCR T cells. After overnight incubation, ~100 µl of plasma was collected and analysed for cytokine (IFN-γ, IL-2, Granzyme B [GzB], TNF-α, IL-4, IL-5, IL-13, and IL-10) production.

Cytokines (particularly IFN-γ, IL-2, and GzB) were clearly detectable in both patients only after initiation of mRNA HBV-TCR T cell treatment (3 h) and not before (Fig. 2), indicating that the infusion of HBV-TCR T cells was associated with the increased detection of peptide-specific cytokine response. The profile of secreted cytokines were primarily Th1, while Th2 cytokines and IL-10 were not detected(Fig. 2A and B). This is similar to that observed when healthy donor heparinized whole blood spiked with autologous mRNA HBV-TCR T cells were stimulated with the corresponding peptides and analysed in the same fashion (Fig. 3). The quantity of IFN-γ, IL-2, and GzB in the peptide-pulsed blood samples collected at 3 h post-infusion of mRNA-engineered HBV-TCR T cells was perfectly proportional to the quantity of HBV-TCR T cell infused. All three cytokines were readily detected at lower quantities (<35 pg/ml) upon infusion of 1×10^6 TCR+ T cells/kg and were higher after infusion with 3×10^6 and 5×10^6 TCR+ T cells/kg (Fig. 2A and B). Reflecting the transient TCR expression that characterized mRNA-engineered HBV-TCR T cells (~10% TCR expression after 72 h), IFN-γ and IL-2 quantity waned rapidly at 3 days after infusion and returned to baseline amounts in 7 days (Fig. 2C and D). Comparing the amount of IFN-γ secreted with that from healthy donors (Fig. 3), we estimate that the maximum number of HBV-TCR T cells detected from both patients was between ~2500 and 12 000 T cells per ml of whole blood.

Discussion

In this study, by using mRNA-engineered TCR T cells that are known to lose their TCR-specific functionality over time (~72 h), we were able to implement a dose-escalation infusion protocol and demonstrate with the whole blood assay: (i) T cell-specific cytokine secretion kinetics that was in line with the temporally constrained functionality of the infused mRNA engineered T cells; (ii) cytokine secretion levels correlating with the infused TCR T cell dosage; and (iii) detection of cytokine secretion after infusion of 1×10^6 TCR T cells per kg.

The rapid whole blood T cell assay has several features that makes it more amenable than conventional methods to monitor the presence and functionality of engineered T cells after

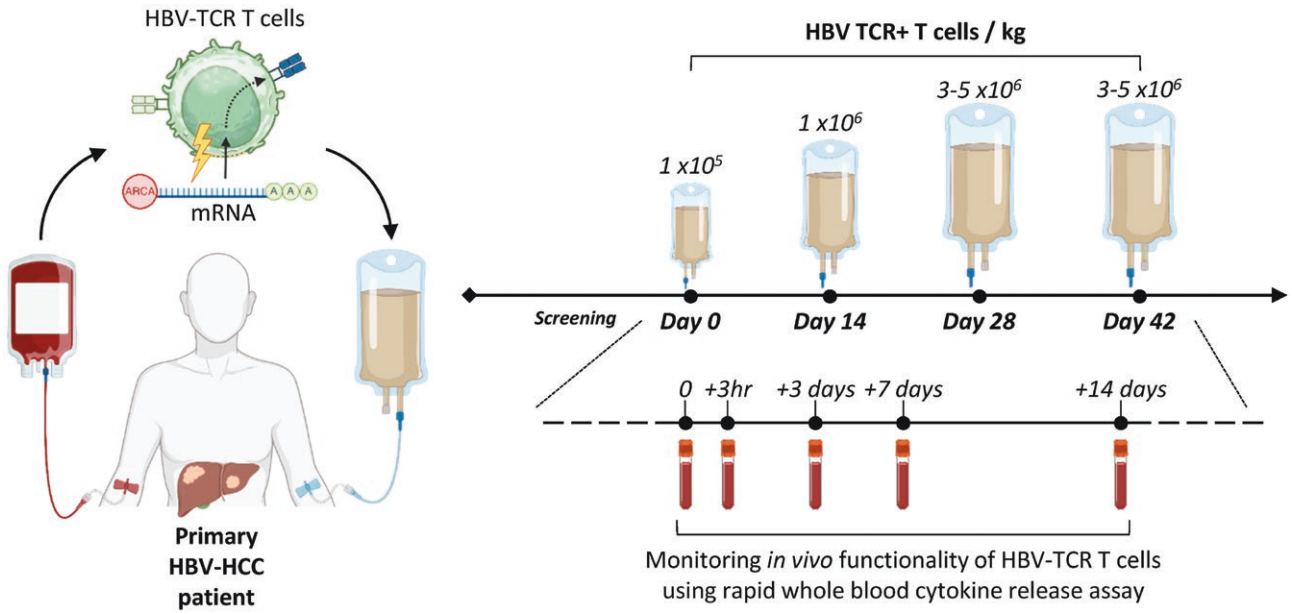

Figure 1. Infusion and whole blood cytokine release assay analysis schedule. Autologous PBMCs from the primary HBV-HCC patients was isolated, expanded, and engineered to transiently express a specific HBV-TCR through mRNA electroporation. HBV-TCR T cells were infused every 14 days and whole blood was collected after each infusion at the indicated time points and analysed with the whole blood cytokine release assay. Created with BioRender.com.

adoptive transfer. Firstly, it does not require any prior isolation of PBMCs from the blood unlike conventional techniques like Enzyme-linked immunosorbent spot assay (ELISPOT) or flow cytometry where PBMCs are used as a starting material. The assay also has the added advantage of being able to assess the functionality of the engineered T cells in its native environment (whole peripheral blood), without the addition of protein transport inhibitors that will modify the milieu of soluble factors, a step that is necessary for the successful detection of intracellular cytokines by flow cytometry. Most importantly, we have demonstrated that the assay can be easily adapted to detect T cells of other specificity by modifying the stimulatory peptide used. While it is plausible to use the assay to monitor CAR-T cells or even tumour infiltrating lymphocytes, as long as information on the specificity of the T cells are known, it remains to be seen whether the performance of the assay will be affected if whole antigens rather than short peptides are used.

In all, we think that this simple assay is ideally suited to monitor the functionality of different TCR T cell products in patient's peripheral blood, including commonly used stable TCR/CAR-expressing T cells where dosage of infused T cells are much higher, ranging between 10^6 and 10^8 per kg [14]. Functional tracking of these stable T cell products could shed some light on the exhaustion/suppression induced by the tumour targets or by its microenvironments over time, providing important information on their functional kinetics and its association with treatment efficacy, an assessment which at the moment remains largely unexplored.

Materials and methods

Study approval

The recruited patients are part of the 'Phase 1b safety and tolerability study of redirected HBV-specific T-cells in patients with Hepatitis B Virus (HBV)—related hepatocellular carcinoma (SAFE-T-HBV)' study (ClinicalTrials.gov: NCT04745403). The study has been approved by the Singhealth Centralised Institutional Review Board and written informed consent was received prior to study participation.

Study protocol

Upon successful recruitment into the study, autologous PBMCs from the respective primary HBV-HCC patient was isolated, expanded, and engineered to transiently express a specific HBV-TCR through mRNA electroporation as described previously [8]. The engineered HBV-TCR T cells were formulated at different quantities to correspond to an escalating dose infusion protocol (three escalating doses: 1×10^5, 1×10^6, $3–5 \times 10^6$ TCR+ T cells/kg; five high doses: $3–5 \times 10^6$ TCR+ T cells/kg) and cryopreserved. The appropriate quantity of engineered HBV-TCR T cells were then thawed and infused into the patient on the same day according to the infusion schedule. Each patient was given a total of eight infusions every 14 days and only data from the first four infusions were analysed here. Heparinized whole blood was collected after each infusion at the indicated time points (+3 h timepoint was only collected for the second to fourth infusion) and analysed with the rapid whole blood cytokine release assay described below.

Rapid whole blood cytokine release assay

The whole blood cytokine release assay was performed similar to what was described previously [12]. Three hundred and twenty microlitres of freshly drawn heparinized blood or heparinized blood spiked with autologous mRNA-engineered HBV-TCR T cells were mixed with 80 µl of RPMI and stimulated with 2 µg/ml of peptide containing the epitope (Pt. 1: 10-mer from HBV Envelope, HLA-A24 restricted; Pt. 2: 9-mer from HBV Envelope, HLA-A2 restricted) recognized by the respective HBV-TCR T cells or with dimethyl sulfoxide (DMSO) as a control. After 16 h of incubation, plasma

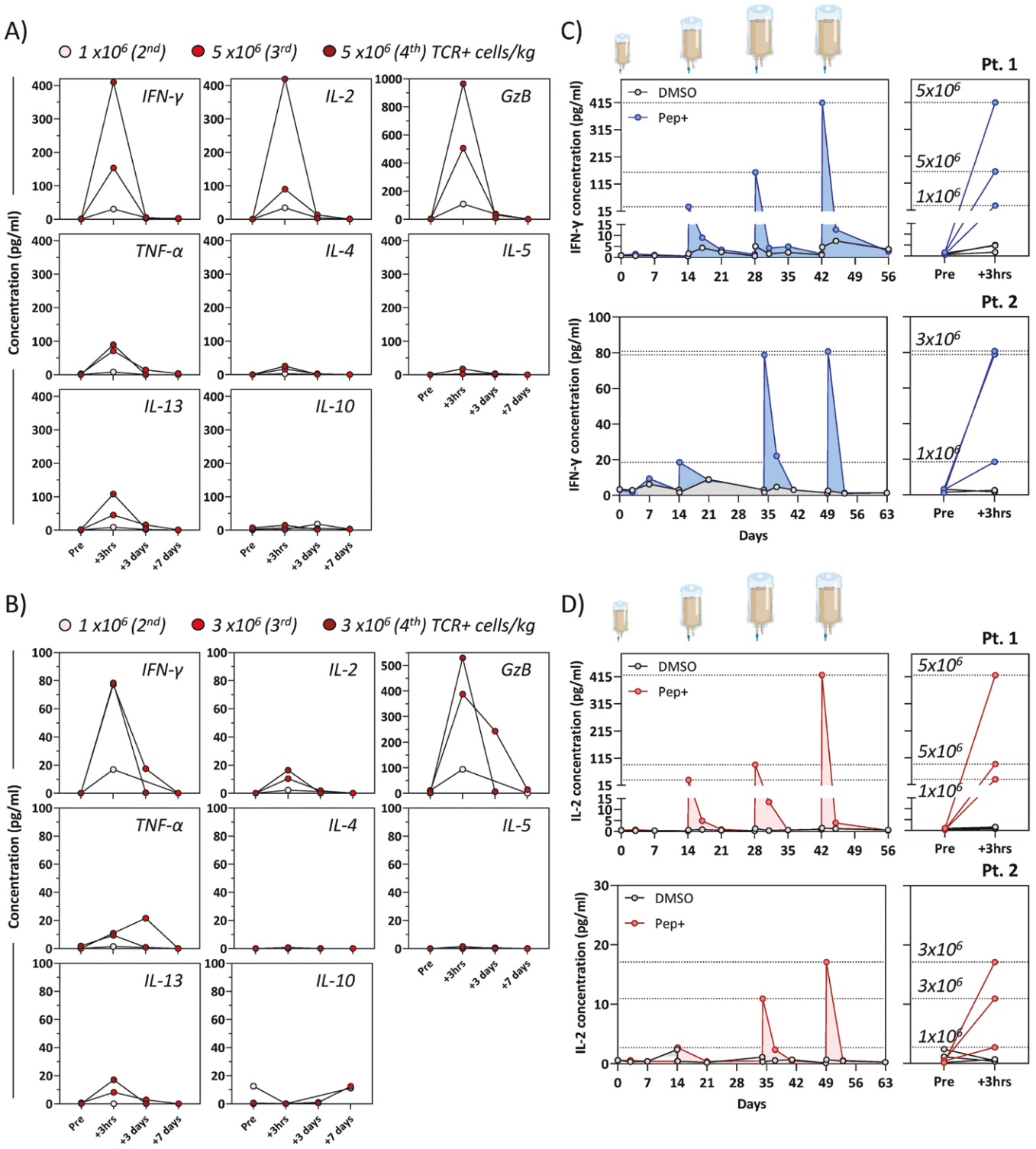

Figure 2. Assessment of the *in vivo* functionality of adoptively transferred HBV-TCR T cells. Concentration of cytokines in the plasma after overnight peptide stimulation of whole blood collected from patient 1 (A) and patient 2 (B) at different time points after HBV-TCR T cell transfer (second, third, and fourth infusion). Longitudinal monitoring of the peptide-induced IFN-γ (C) and IL-2 (D) secretion in the two primary HBV-HCC patients. The concentration of cytokines in the plasma after peptide stimulation (data from 3 h post-infusion are shown on the right for clarity) as well as the background from the unstimulated DMSO containing negative control wells (grey) are shown.

separation of the heparinized whole blood will occur. To ensure maximum recovery of the plasma, the samples were centrifuged at 700 rcf (relative centrifugal force) for 10 min at room temperature. Approximately 100 µl of plasma was collected and stored at –80°C. To quantify the amount of cytokines present in the collected plasma, frozen plasma was first thawed and cytokine concentrations were analysed using the microfluidic Enzyme-linked immunosorbent assay

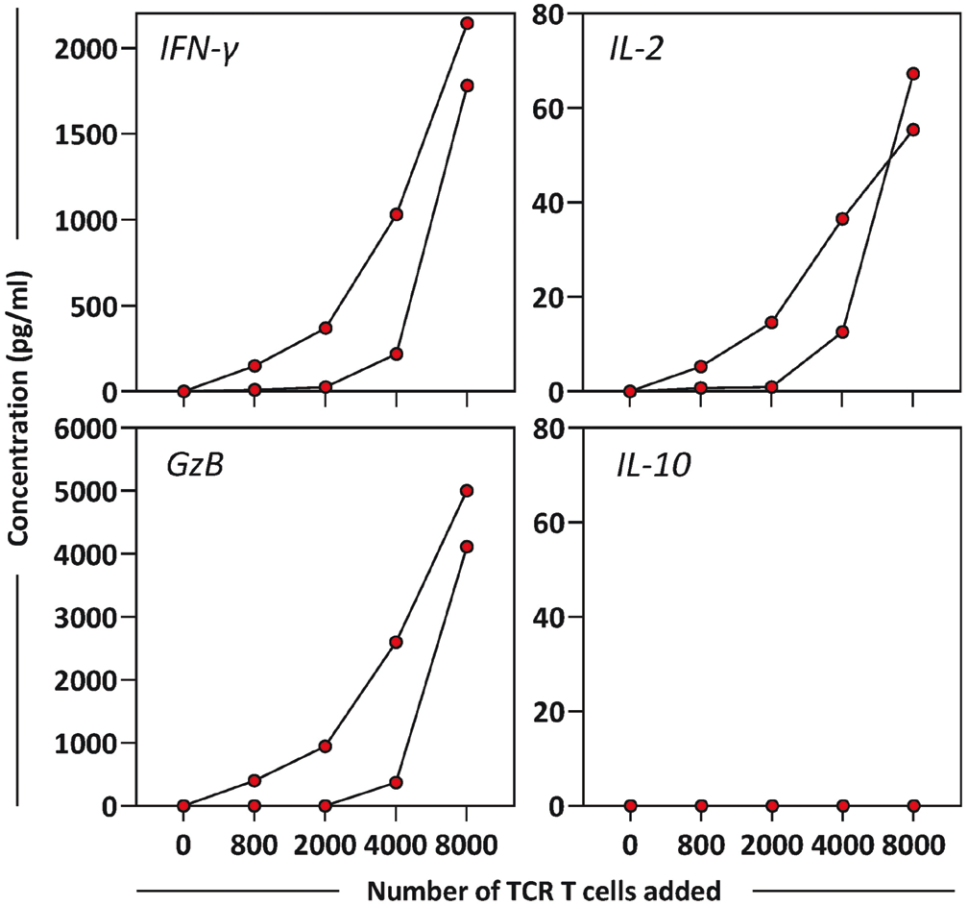

Figure 3. *In vitro* cytokine secretion profile of mRNA-engineered HBV-TCR T cells. Indicated quantities of HBV-TCR T cells engineered from PBMCs obtained from two healthy donors were added into 320 µl of autologous whole blood. The concentration of cytokines released in the plasma after overnight peptide stimulation of the whole blood were analysed using the Protein Simple ELLA platform.

(ELISA)-based ELLA system according to the manufacturer's protocol (Protein Simple). Unless specified, background cytokine values from the unstimulated DMSO containing negative control wells were subtracted from the data shown.

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Author contributions

Anthony Tan (Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing—original draft, Writing—review & editing), Shou Kit Hang (Data curation, Formal analysis), Nicole Tan (Data curation, Formal analysis), Thinesh Krishnamoorthy (Investigation, Project administration, Resources, Writing—review & editing), Wan Cheng Chow (Investigation, Project administration, Resources, Writing—review & editing), Regina Wong (Project administration, Writing—review & editing), Lu-En Wai (Investigation, Project administration, Resources, Writing—review & editing), and Antonio Bertoletti (Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing—original draft, Writing—review & editing)

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Conflict of interest

The authors disclose the following: 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 treatment of virus-related diseases and cancers. R.W.W and L-E.W. are employees of Lion TCR Pte. Ltd. All other authors disclose no conflicts. Lion TCR Pte. Ltd. has a patent application related to this work (WO2021148110A1).

Ethical approval

The study has been approved by the Singhealth Centralised Institutional Review Board and written informed consent was received prior to study participation.

Clinical trial registration

The recruited patients are part of the 'Phase 1b safety and tolerability study of redirected HBV-specific T-cells in patients with

Hepatitis B Virus (HBV)—related hepatocellular carcinoma (SAFE-T-HBV)' study (ClinicalTrials.gov: NCT04745403).

Data availability

Values for all data points shown in the figure are available upon request.

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