Accepted Article

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Article type : Original

Immunosuppressive Drug Resistant Armored TCR T cells for immune-therapy of HCC in liver transplant patients

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/HEP.31662

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Keywords

T cell therapy, T cell receptor, hepatocellular carcinoma, liver transplant, HBV

Word count

5776 words

Number of figures and tables

5 figures; 4 supplementary figures

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

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treatment of virus-related diseases and cancers. A.Chua, Z.Z.H., R.F., L.W. and S.K. are employees of Lion TCR Pte. Ltd. All other authors disclose no conflicts.

Funding

This research is supported by the Singapore Ministry of Health's National Medical Research Council under its Singapore Translational Research Investigator Award (NMRC/STaR/0013/2012).

Author Contributions

M.H., A.T.T., and A.B. designed the study. M.H., A.T.T., and A.B. drafted the manuscript. M.H., M.L., A.Chia., D.T., J.A. and Z.Z.H. performed the experiments. M.H., M.L., A.T.T., Z.Z.H., L.W., S.K. and A.P. analyzed and interpreted the data. L.W., S.K., Z.Z.H. and A.Chua were responsible for manufacturing of trial product. R.F. was responsible for all clinical data and sample collation, as well as management of the clinical trial. W.C., Q.Z., T.L.K., and W.C.C. provided clinical care for the patients, were responsible for the running of the proof-of-concept trials and provided all clinical data/materials.

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List of abbreviations

3D: 3-dimensional

AFP: alpha-fetoprotein

CAR: Chimeric antigen receptor

CD: Cluster of differentiation

CMV: Cytomegalovirus

CnB: Calcineurin B

EBV: Epstein-Barr virus

ENV183-191: Envelope 183-191

FIG: Figure

FKBP1A: Peptidyl-prolyl cis-trans isomerase

HBV: Hepatitis B virus

HCC: Hepatocellular carcinoma

HLA: human leukocyte antigen

IDRA: Immunosuppressive Drug Resistant Armored

IMPDH: inosine-5'-monophosphate dehydrogenase

IS: immunosuppressants

MMF: Mycophenolate Mofetil

MPA: Mycophenolic acid

NFAT: Nuclear factor of activated T-cells

PBMC: Peripheral blood mononuclear cell

S183: HBV envelope epitope 183-191

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SIRNA: Small interfering RNA

TCR: T-cell receptor

TAC: Tacrolimus

mRNA: Messenger RNA

αβ: alpha beta

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Abstract

Background & Aims: HBV-specific T cell receptor (HBV-TCR) engineered T cells have the potential for treating hepatocellular carcinoma (HCC) relapses after liver transplantation, but their efficacy can be hampered by the concomitant immunosuppressive treatment required to prevent graft rejection. Our aim is to molecularly engineer TCR-T cells that could retain their polyfunctionality in such patients while minimising the associated risk of organ rejection.

Approach & Results: We first analysed how immunosuppressive drugs can interfere with the in vivo function of TCR-T cells in liver transplanted patients with HBV-HCC recurrence receiving HBV-TCR T cells, and in vitro in the presence of clinically relevant concentrations of immunosuppressive Tacrolimus (TAC) and Mycophenolate Mofetil (MMF). Immunosuppressive Drug Resistant Armored (IDRA) TCR-T cells of desired specific (HBV or EBV) were then engineered by concomitantly electroporating mRNA encoding specific-TCRs and mutated variants of calcineurin B (CnB) and inosine-5'-monophosphate dehydrogenase (IMPDH), and their function was assessed through intracellular cytokine staining and cytotoxicity assays in the presence of TAC and MMF.

Liver transplanted HBV-HCC patients receiving different immunosuppressant drugs exhibited varying levels of activated (CD39+ Ki67+) PBMCs post HBV-TCR T cell infusions that positively correlates with clinical efficacy. In vitro experiments with TAC and MMF showed a potent inhibition of TCR-T cell polyfunctionality. This inhibition can be effectively negated by the transient overexpression of mutated variants of CnB and IMPDH. Importantly, the resistance only lasted for 3-5 days after which sensitivity was restored.

Conclusions: We engineered TCR-T cells of desired specificities that transiently escape the immunosuppressive effects of TAC and MMF. This finding has important clinical applications for the treatment of HBV-HCC relapses and other pathologies occurring in organ transplanted patients.

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Introduction

An increasing number of severe human pathologies are treated successfully with organ transplantation. Among them, hepatocellular carcinoma (HCC), the sixth most common cancer world-wide(1), represent the best example. Liver transplantation remains the best treatment option for HCC occurring in patients with liver cirrhosis (2) and its efficacy in increasing life expectancy of the HCC patients has led to its progressive adoption world-wide. Unfortunately, with its increased utilization, a parallel increase in the incidence of HCC recurrence after liver transplant has been observed particularly in Asia.

Whether this high incidence is caused by etiological differences (HCC in Asia is mainly HBV related, HBV-HCC) or by differing criteria used for transplant patient selection remains controversial(3-5). What is certain is that the therapeutic options for the treatment of HCC relapses in transplanted patients are minimal and often merely palliative(6,7). In addition, the utilization of new therapeutic combinations of check point inhibitors with anti-angiogenic drugs that have shown increased efficacy in advanced primary HCC(8) should be considered with great care in liver transplanted patients since treatment with check point inhibitors increases the incidence of organ rejections(9).

In the last few years we have first proposed and then developed a therapy for HCC recurrence after liver transplantation that harness the anti-tumor efficacy of CAR/TCR T cell therapy. Since HBV-DNA is integrated in the majority of HBV-HCC and can produce HBV-host chimeric proteins, we engineered HBV-specific TCR-redirected T cells (HBV-TCR T cells) and demonstrated their ability to target HBV derived epitopes presented by HLA-class I molecules on HCC cells in vitro, and in patients with HCC relapses(10). When HBV-specific TCR-redirected T cells are adoptively transferred in liver transplanted patients, the HLA-mismatch between the donor liver and the HCC lesions allows them to exclusively target the tumours and not the HBV infected hepatocytes in the transplanted liver graft (11). A real drawback of implementing this therapy in organ transplant subjects is the continuous administration of immunosuppressive drugs necessary to

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maintain the transplanted organ which can simultaneously interfere with the function of the adoptively transferred T cells. In liver transplant, due to the tolerogenic nature of the liver, immunosuppressive treatment is often maintained with low doses of Tacrolimus or Rapamycin alone or in combination with Mycophenolate Mofetil (MMF)(12), but the combined effect of such immunosuppresants (IS) on the functionality of our TCR-T cells have not been analyzed in detail.

In this work, we first assess the impact of individual immunosuppression regimens on the functionality of adoptively transferred HBV-TCR T cells directly in liver transplanted patients with HBV-HCC relapses. For this purpose, we measured the quantity of activated/proliferating T cells persisting in the circulation of such patients who received HBV-TCR T cell infusions and correlate the findings with clinical parameters of HCC treatment efficacy (Alpha-fetoprotein levels/volumetric alterations of lesions). We then analyzed in vitro the effect of variable quantities of IS (Tacrolimus and MMF) on TCR-T cell function. Finally, we developed a solution that can improve the in vivo functionality of HBV-TCR T cells in organ transplant patients by engineering an inherent resistance to immunosuppressive pharmacological treatments into the T cells. We demonstrated that through the simultaneous electroporation of mRNAs coding for αβ TCR and mutated variants of intracellular molecules specifically utilized by Tacrolimus and MMF signalling, we can produce what we defined as Immunosuppressive Drug Resistant Armored (IDRA) TCR redirected T cells. These cells can be engineered with TCRs of different specificity and are only transiently resistant to the immunosuppressive effect of Tacrolimus and MMF, a built-in safety feature important for implementing the approach in organ transplant patients where immunosuppression has to be carefully maintained.

Results

Ability of HBV-TCR-T cells treatment to modify immunological profile in patients

We reported the utilization of autologous T cells transiently expressing HBV-specific TCR in the treatment of HCC relapses occurring in two liver transplanted patients with recurrence of HBV-

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HCC in Singapore. The treatment induced in one of the two patients, fluctuations of AFP values associated with objective volumetric changes of HBV-HCC tumour lesions(10). However, such objective responses were not detected in the majority of patients recently treated with similar doses of HBV-TCR T cells in a clinical trial performed in Guangzhou (The Third Affiliated Hospital, Sun Yat-sen University; Fig. 1A). Since these liver transplanted patients received individualized treatments with IS at dosages following the Centre's recommendations (Fig. 1A), the autologous HBV-TCR T cells can thus respond differently to the respective IS drugs. We hypothesized that the different pharmacological regimen might exert a variable impact on the adoptively transferred HBV-TCR T cells and therefore we analyzed phenotypic markers of activation that can be indicative of TCR-T cell function in vivo.

We quantified the frequency of activated/proliferating (CD39+ Ki67+) total T cells present in the peripheral blood ~2-5days after adoptive transfer of HBV-TCR T cells. An increased frequency of activated (CD39/Ki67+) total T cells after HBV-TCR T cell transfer was observed only in liver transplanted patients who presented with fluctuations of serum AFP levels and showed clinical signs of tumour regression (Patient S-1 and C-3; Fig. 1B). This was also observed in HBV-TCR T cell treated primary HBV-HCC patients who were not under IS treatment (manuscript in preparation). Note that since the adoptively transferred HBV-TCR T cells were engineered with mRNA electroporation, the transient expression of the HBV-TCR (~3-4 days) on T cells do not allow us to directly monitor the persistence or activation of HBV-TCR T cells in the treated patients.

Differential effects of immunosuppressive drugs on engineered HBV-TCR T cells

As such, we analyzed the impact that individual immunosuppressive drugs can exercise on TCR-T cell function. HBV-TCR T cells were incubated for ~5 hours with clinically relevant concentration of Tacrolimus (2.5-20 ng/ml) and/or MMF (1-3µg/ml). IS-treated HBV envelope TCR-T cells (HBs183-91 TCR) (13)were co-cultured *in vitro* with their specific target (HepG-2215, a hepatoma cell line expressing HBV-specific proteins) and thereafter analyzed for TCR expression, function and viability (Fig. S1). All the IS drugs did not affect the expression of the introduced TCR (Fig. 2A), but short term exposure to all concentrations of Tacrolimus alone or in combination with MMF potently impair T cell polyfunctionality by reducing TNF-α and IFN-γ (Fig. S2) production

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while MMF alone did not show any inhibitory effect on cytokine production (Fig. 2B). We also measured the lytic ability of HBV-TCR T cells in the presence and absence of IS using the xCELLigence real-time killing assay. Tacrolimus alone or in combination with MMF reduced the cytolytic activity of HBV-TCR T cells by up to 50% in comparison with non-treated control (Fig. 2C). In contrast to the potent suppression of T cell cytokine production and cytotoxicity by Tacrolimus, its effect on their viability was negligible (Fig. 2D). MMF on the other hand has a dramatic toxic effect on T cells observable after 48 hours of treatment (Fig. 2D). The capacity of IS drugs to alter HBV-TCR T cell function was also tested in a 3D–system that mimic some aspects of the interaction between T cells and solid tumor in vivo(14). In this 3D microfluidic device, target cells (HepG-2 cells expressing pre-S1 antigen) were embedded in a gel matrix, thereby requiring the T cells to actively migrate within the gel in order to reach and kill the target cells. T cell migration and killing were measured in the presence and absence of Tacrolimus alone or in combination with MMF. Both treatments potently inhibit T cell migratory capacity and lead to reduced target killing (Fig. 2E). Thus both in vivo and in vitro data showed that IS drugs, and in particular the combination between Tacrolimus and MMF has a profound impact on the functionality of TCR-T cells. This encouraged us to design strategies to engineer IS drug-resistant TCR-T cells.

IDRA TCR-T cells engineered through transient overexpression of mutant CnB and IMPDH

First we applied a siRNA approach to develop Tacrolimus resistant T cells. In the cytoplasm, Tacrolimus first binds to its chaperone protein FKBP1A, before interacting with the calcineurin (Cn) heterodimer and blocking the NFAT activation pathway (Fig. 3A). By concomitantly electroporating HBV-TCR mRNA and siRNA to knockdown FKPB1A, HBV-TCR T cells could be engineered to resist the suppressive effects of Tacrolimus. While a 90% knockdown of FKBP1A mRNA was achieved without significant effects on HBV-TCR expression and T cell viability, the immunosuppressive effect of Tacrolimus was only partially abolished (Fig. S3, Fig. 3B). This is likely due to the long half-life of existing FKPB1A protein that could mitigate the siRNA knockdown effects which acts at the mRNA level.

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Thus, to circumvent this drawback, we decided to engineer the TCR T cells by overexpressing a mutant form of calcineurin subunit B (CnB; Fig. 3A). Stable expression of this mutant CnB in EBV-specific T cells expanded in vitro from healthy individuals have been shown to block the suppressive effect of Tacrolimus (15) and resulted in an increased efficacy of these IS resistant T cells in the treatment of viral infections in bone marrow transplanted patients (16). However, due to the potential risk of graft rejection from the long term presence of IS resistant T cells, we engineered IS resistant T cells through mRNA electroporation which will overexpress the mutated form of CnB only transiently, and thus could meet the safety needs for cell therapy in liver transplant patients. HBV-TCR T cells engineered in this way dramatically recovered their functionality (cytokine production and lysis) in the presence of clinically relevant concentrations of Tacrolimus (Fig. 3B), while maintaining equivalent HBV-TCR expression and T cell viability as classical HBV-TCR T cells (Fig. S4). Importantly, by activating the HBV-TCR T cells through CD3/28 bead stimulation which is independent of the HBV-TCR expression, we demonstrate that the resistance to Tacrolimus is transient, and the T cells regain their sensitivity to Tacrolimus ~5 days after electroporation (Fig. 3C).

In addition to Tacrolimus, MMF is frequently used as an adjunct immunosuppressive treatment after liver transplant. MMF is a pro-drug which rapidly hydrolyses to its active form MPA that inhibits inosine 5'-monophosphate dehydrogenase (IMPDH) and subsequently lymphocyte proliferation (Fig. 3A). We have shown that clinically relevant concentrations of MMF do not impact T cell function and cytotoxicity but increased T cell death after 48 hours (Fig. 2A-D). To overcome this effect we engineered TCR-T cells with transient expression of a mutated form of IMPDH (17). Co-electroporation of mutated IMPDH and HBV-TCR mRNA showed minimal impact on TCR expression but maintain T cell viability in the presence of MMF (Fig. 3D). The mRNA electroporation method guarantees the transient presence of mutated IMPDH in the T cells that allows the reversion of MMF resistance after a few days (Fig. 3E).

Finally, we produced dual resistant (Tacrolimus and MMF) IDRA HBV-TCR T cells. Three mRNA coding for mutant CnB, IMPDH and HBV-TCR were concomitantly electroporated into T cells. Fig. 4A shows that concomitant electroporation of s183-TCR, CnB and IMPDH only has a minor impact on T cell viability (up to 15%) and TCR expression (~10-20% reduction and maintenance

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for up to 5 days) 24 hours post electroporation. Again, functional analysis showed that the triple mRNA electroporated T cells retain their ability to produce cytokines and lyse targets in the presence of both IS drugs (Fig. 4B, 4C) for up to 3, 4 days after electroporation and regain IS sensitivity after ~5 days (Fig. 4D). In addition, the transient resistance to the toxic effects of MMF was demonstrated by the viability analysis of the IDRA HBV-TCR T cells after 72 hours of exposure to both IS drugs (Fig. 4E, 4F). The gain of functionality of IDRA HBV-TCR T cells in the presence of both IS drugs was further exemplified in a 3D live imaging experiment. IDRA HBV-TCR T cells exhibited superior killing of HCC targets in the 3D microdevice assay compared to classical HBV-TCR where cytotoxicity was clearly inhibited (Fig. 4F). Importantly, we also analyzed the expression of activation markers CD39 and Ki67 on classical and IDRA HBV-TCR T cells that were co-cultured with TAC and MMF, similar to that performed on the PBMCs isolated from liver transplanted HBV-HCC patients receiving classical HBV-TCR T cells (Fig. 1). We observed a reduction of both markers on classical HBV-TCR T cells, while IDRA HBV-TCR T cells retained their expression, showing how IDRA HBV-TCR T cells can withstand the suppressive effects of TAC and MMF (Fig. 4G).

Engineering of IDRA TCR T cells specific for viral pathologies occurring in IS drug treated patients

Engineered IDRA TCR-T cells could also have the potential for clinical application in other viral pathologies that occurs in situations of obligate immunosuppression. Hence, we tested whether IDRA TCR T cells can be engineered through mRNA electroporation with EBV specific TCRs(18).

In the presence of clinically relevant concentrations of Tacrolimus and MMF, EBV-TCR redirected T cells lost their ability to produce cytokines after antigen-specific recognition (Fig. 5A) and up to 50% reduction of viability could be seen after 3 days of IS drugs exposure (Fig. 5B). Concomitant electroporation of TCR mRNA along with mutant CnB and IMPDH dramatically recovered the function and viability of these T cells in the presence of both immunosuppressant agents (Fig. 5A, 5B). Thus, IDRA T cells can be engineered with different TCR specificity suggesting a possible therapeutic use not restricted only to the treatment of HBV-HCC.

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Discussion

Adoptive T cell therapy emerged as an effective therapeutic approach for certain types of cancer including virally-related cancers. In this approach, patient-derived T cells are modified to express TCR or CAR against tumor-associated antigens (e.g. self or foreign antigens), enabling them to recognize and engage target cancer cells. In the case of HBV-associated HCC, we have previously shown the promising potential of HBV-TCR T cells in treating HBV-HCC recurrence after curative liver transplantation (10). However, the efficacy of the treatment can be hampered due to the presence of IS drugs required to prevent liver rejection. Hence, we first explored how these drugs can impact the in vivo function of TCR-T cells in liver transplant patients with HBV-HCC metastasis. Analysis of the phenotypic markers of activation on PBMCs after adoptive transfer of HBV-TCR T cells showed markedly differing levels of activation (CD39+ Ki67+) that appears to be correlated with clinical efficacy (Fig. 1), showing how IS drugs can have a considerable impact on TCR-T cell functionality in vivo. The data also support the notion that a conservation of TCR T cell function is required for their clinical efficacy, a finding that has important implications in the development of a prognostic biomarker of TCR-T cell treatment efficacy. In vitro experiments of TCR-T cell function in the presence of clinically relevant concentrations of IS drugs further validated the negative impact of commonly used Tacrolimus and MMF (Fig. 2).

Hence, to efficiently implement TCR-T cell therapy in liver transplanted patients with HBV-HCC relapses, HBV-TCR T cells needs to function effectively even in the presence of strong immunosuppression. While previous studies have shown how this can be achieved through permanent genetic modifications using viral vectors, this also gives rise to concerns of organ rejection especially when large numbers of TCR-T cells are usually infused into patients for treatment (100-1000x10^6 T cells). To circumvent this hurdle, we employed the mRNA electroporation strategy to engineer HBV-TCR T cells that can transiently escape immunosuppression while targeting HBV antigens on HCC cells. Primarily, this transient expression of the mutated forms of CnB and IMPDH produced through mRNA electroporation avoids the establishment of a population of potentially alloreactive T cells that is resistant to IS drugs, which minimizes the risk of potential organ rejection. At the same time, it also reduces

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the possible immunogenicity of mutated forms of self-proteins in patients which might cause a progressive loss of efficacy of such strategy.

The ability to engineer HBV-TCR T cells capable of resisting the immunosuppressive effects of two commonly used IS drugs also opens the possibility that the strategy could also be applied to other pathologies occurring in IS treated patients. CMV and EBV–specific T cell therapy has shown clinical benefits in the treatment of HCMV and EBV reactivation that frequently occurs in patients under immunosuppression after bone marrow transplantation (19). Our demonstration of engineering IDRA T cells of different specificity by altering the introduced TCRs exemplifies the therapeutic value of IDRA T cell which is not restricted only to HBV-HCC, but can also be applied to such pathologies that are potentially treatable with TCR-T cells but are not commonly implemented due to the inhibitory effects of IS drugs. Furthermore, the demonstration that IDRA T cells withstand the downregulation of activation markers CD39 and Ki67 upon exposure to the IS drugs (Fig. 4G) supports the use of these markers to monitor the efficacy IDRA TCR-T cells in vivo.

Taken together, in this study we developed an in vitro method to concomitantly modify the specificity and the function of T cells to produce Immunosuppressive Drug Resistant Armored (IDRA) TCR T cells of desired specificity (i.e. HBV and EBV) which can transiently escape the suppressive effect of Tacrolimus and MMF. We hope that the method of engineering IDRA-TCR T cells will soon be adopted for clinical testing in the therapy of pathologies occurring in organ transplanted patients under IS drugs treatment and demonstrate the in vivo gain of function that we have shown here in a laboratory setting.

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MATERIALS AND METHODS

Patients

Liver transplanted patients with HBV-HCC recurrence were recruited and treated with multiple infusions of HBV-TCR T cells in the Singapore General Hospital and in The Third Affiliated Hospital of Sun Yat-sen University in accordance to clinical protocols approved by the respective institutional ethics review board. Informed consent was obtained from all patients and the study was conducted in accordance to the principles expressed in the Helsinki Declaration.

Phenotypic analysis of patient PBMCs

Peripheral blood of the patients were isolated by Ficoll (GE Healthcare, Chicago, IL) density gradient centrifugation and cryopreserved at regular intervals after infusions of HBV-TCR T cells. For the phenotypic analysis, PBMCs were thawed and stained with LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (ThermoFisher Scientific, Waltham, MA) in PBS for 10 minutes at room temperature. Cells were then incubated with a surface antibody cocktail CD3 PerCPCy5.5 (BD, Franklin Lakes, NJ), CD8 V500 (BD, Franklin Lakes, NJ), CD39 PECy7 (ThermoFisher Scientific, Waltham, MA) in staining buffer (PBS/1% BSA/0.1%Azide) for 30 minutes on ice. Subsequently, cells were fixed and permeablized with FOXP3 Buffer Set (BD, Franklin Lakes, NJ) as recommended by manufacturer. For intracellular staining, cells were incubated on ice for 30 minutes with Ki67 FITC (BD, Franklin Lakes, NJ) in perm wash (PBS/1% BSA/0.1% Saponin/ 0.1% Azide). After washing, cells were resuspended in PBS 1% formaldehyde before acquiring using BD LSRII flow cytometer.

Manufacturing of HBV-specific TCR T-cells

Production of HBV-specific TCR T-cells for infusions is similar to that described previously (Tan et al., 2019). In brief, the HBV-TCR mRNA was in vitro transcribed using the mMESSAGE mMACHINE™ T7 ULTRA Transcription Kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. For infusions in patients, peripheral blood mononuclear cells

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from the patient was isolated by Ficoll density gradient centrifugation and activated for 8 days with 600IU/ml of GMP grade IL-2 (Miltenyi, Germany) and 50ng/ml of GMP grade OKT-3 (Miltenyi, Germany) in cell therapy grade AIM-V (Invitrogen) supplemented with 5% CTS Serum Replacement (ThermoFisher Scientific, Waltham, MA). The activated T-cells were then electroporated with the HBV-TCR mRNA using the AgilePulse Waveform Electroporation System (BTX Harvard Apparatus, Holliston, MA) according to the manufacturer's recommended protocol. Quality control experiments were then performed to characterize HBV-specific TCR expression levels of the engineered T-cells before infusion into the patients in a solution containing 5% human albumin. For in vitro experiments, HBV Env183-191-specific TCR (s183-TCR), mutant calcineurin subunit B and mutant IMPDH mRNAs were synthesized using the same kit. PBMCs of healthy subjects were activated similarly as above. Activated T cells electroporated using either the 4DNucleofector™ System (Lonza, Switzerland) or AgilePulse Waveform Electroporation System following the manufacturer's instructions. TCR expression and viability of the cells were analyzed by flow cytometry at the indicated times.

siRNA electroporation

ON-TARGETplus and non-targeting siRNAs for FKBP1A were obtained from Dharmacon (Lafayette, CO). siRNA sequences are listed in Fig. S3. Lyophilized siRNAs were reconstituted in 1X siRNA buffer and stored in -80. Primary T cells were electroporated as described above. Knockdown efficiency was checked by quantitative real-time PCR (qPCR) at indicated times (Fig. S3).

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat of healthy donors by Ficoll-Paque (GE Healthcare, Chicago, IL) gradient centrifugation and cryopreserved. HepG2.2.15 cells were cultured in DMEM (ThermoFisher Scientific, Waltham, MA) supplemented with 10% heat-inactivated FBS, MEM non-essential amino acids, 1mM sodium pyruvate, 100IU/ml penicillin, 100µg/ml streptomycin (ThermoFisher Scientific, Waltham, MA) and 200µg/ml G418

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for selection. HepG2 cells transduced with construct containing genotype D HBV envelope protein (HepG2-env) covalently linked to GFP using Lenti-X HTX packaging system (Takara, Japan) were cultured in RPMI 1640 (ThermoFisher Scientific, Waltham, MA) supplemented with 10% heat-inactivated FBS (ThermoFisher Scientific, Waltham, MA), 0.5mM sodium pyruvate, 20mM HEPES, 100IU/ml penicillin, 100µg/ml streptomycin, MEM non-essential amino acids, MEM amino acids with L-glutamine (ThermoFisher Scientific, Waltham, MA), 5µg/ml Plasmocin (InvivoGen, San Diego, CA) and 5µg/ml of puromycin (Takara, Japan) for selection.

Drug preparation and treatment

Tacrolimus and MMF (Sigma, St. Louis, MO) were dissolved in pure sterile DMSO and kept in -20C. Serum trough level of each drug were used to simulate pharmacological interventions in vitro: 2.5-20ng/ml of Tacrolimus, 1-3ug/ml of MMF.

Cytokine and phenotypic analysis

To check T cells functionality, engineered TCR-T cells (CD8+/tetramer+) were co-cultured with HepG2.2.15 target cells for 15 hours at effector to target ratio (E:T) of 1:1 in AIM-V supplemented with 2% human AB serum and 2µg/ml of Brefeldin A (Biolegend, San Diego, CA). TCR-redirected T cells without target cells were used as negative control. T cell cytokine secretion was analyzed by intracellular cytokine staining of TNF-α and IFN-γ (BD Bioscience, Franklin Lakes, NJ). Kinetic of T cell function were analyzed in an antigen-dependent and -independent manner. For antigen-dependent experiment, 24, 48 and 72 hours after electroporation 10^5 T cells were co-cultured with HepG2.2.15 cells at an effector to target ratio of 1:1 in the presence of aforementioned drug concentration and 2µg/ml of Brefeldin A. Intracellular TNF-α and IFN-γ were checked following overnight incubation. For antigen-independent experiment, T cells were activated with Anti CD3 CD28 Dynabeads (ThermoFisher Scientific, Waltham, MA) at a ratio of 2:1 (Bead:cell) for 4-5 hours in the presence of clinically relevant concentration of drugs and 2µg/ml of Brefeldin A followed by cytokine analysis. Phenotypic changes of engineered TCR T cells were analyzed after 24 hours exposure to the drugs using KI67 and CD39 markers as described earlier.

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For EBV redirected TCR-T cells, engineered T cells were co-incubated with HLA-A2+ EBV-specific peptide pulsed (+) or non-pulsed (-) T2 cells overnight. Similar intracellular cytokine staining were performed after the indicated time.

Real-time killing assay

T cells cytotoxicity were measured by xCELLigence Real-Time Cell Analysis (ACEA Biosciences, San Diego, CA) as described previously (10). In brief, 10^5 HepG2.2.15 cells were seeded in E-plates (ACEA Biosciences, San Diego, CA) and TCR-T cells at effector to target ratio (E:T) of 1:1 were added. Target killing were monitored for ~45 hours after TCR-T cell addition.

3D microfluidic assay and live imaging

3D microfluidic assay performed as described previously (Pavesi et al., 2017). In brief, labeled TCR-T cells with CellTracker Violet BMQC (ThermoFisher Scientific, Waltham, MA) and impermeable nuclear dye DRAQ7 (Biolegend, San Diego, CA) were added to the microfluidic device containing dissociated HepG2-Env target cells. Static and time lapse imaging of selected regions were acquired before and after 15 hours of T cell addition. Percentage of dead cells was calculated by analyzing images acquired before and after addition of TCR T cells using Imaris (Bitplane,).

Statistical significance

All data analysed and plotted by Prism (GraphPad, San Diego, CA) using appropriate tests as stated in the Fig. legends. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

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Figure legends

Fig. 1. Alterations of the peripheral blood immunological profile in liver transplanted HBV-HCC patients treated with HBV-TCR T cells. (A) Schematic illustrates the infusion dose and schedule of liver transplanted HBV-HCC patients treated with mRNA electroporated HBV-TCR T cells (n=8). All patients received 4 escalating doses of HBV-TCR T cells before subsequent infusions of 5-10x10^6/Kg HBV-TCR T cells commence. The table details the clinical information and different immunosuppressant regimens that the patients received following liver transplantation. (B) Phenotypic analysis of PBMCs collected from patients before and after receiving HBV-TCR T cells (n=6). Serum AFP levels were monitored before and throughout the treatment (top row). Each dotted line denotes a single infusion of HBV-TCR T cells. Reference range of serum AFP are shown in grey. The frequency of Ki67+ CD39+ CD8 T cells before and after HBV-TCR T cell infusions are shown below. The red circles corresponds to the pre-infusion samples obtained at the time points denoted by the red dotted lines. Patient C-3 received concurrent Lenvatinib treatment for the duration shown (red).

Fig. 2. Functional profile of S183-electroporated T cell treated with clinically relevant concentrations of immunosuppressive drugs. (A) TCR expression of S183 TCR-T cells were evaluated following 24 hours of IS drug treatment. Representative experiment stained for S183-specific TCR (left panel)**.** Percentage of dextramer positive cells out of total live CD8+ T cells were quantified following incubation with the drug (n=3). (B) IS drug treated S183 TCR-T cells were co-cultured with HepG2.215 cells overnight and the frequency of TNF-α-producing CD8+ cells (right panel) out of total live CD8+ T cells were quantified (n=3). Representative experiment stained for TNF-α production (left panel)**.** DMSO-treated T cells cultured without targets served as negative control. (C) T cell cytolysis determined by real-time killing assay in the presence or absence of the drugs. Normalized Cell Index plot (left panel) for HepG2.215 cells with/without drug treated T cell at E: T ratio of 1:1. All samples have been internally normalized for the cell index value measured before T cell addition (indicated by the vertical red dash line). Bar graphs (right panel) demonstrate percentage of T cell cytolysis up to 45 hours after S183-TCR-T cell addition to the

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targets. (D) Drug impact on T cell viability were analysed at different time point using flow cytometry. Representative experiment stained with live/dead discrimination dye (left panel). (E) Representative images of 3D experiment in various conditions. Engineered T cells labelled with BMQC were introduced into a 3D microdevice in the presence of 5ng/ml of Tacrolimus and DRAQ7. Target death was quantified by measuring DRAQ7 positive cells after the indicated time. Scale bar: 80 µM. T cell infiltration into the matrix gel was evaluated at the end of the experiment in each respective condition. Each dot (gray) represents the localization of a single T cell in the gel matrix. In all experiments, each dot represents one individual experiment in the bar graphs. Statistical significance was evaluated by either one-way ANOVA followed by Turkey multiple or 2-tailed t test comparison test (*0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** < 0.0001, n.s., not significant).

Fig. 3. mRNA electroporation have superior advantage over siRNA for developing IDRA TCR-T cells. (A) Strategies to engineer IDRA TCR-redirected T cells. Schematic illustrating the methods to develop transient IDRA TCR T cells. 1: Targeting FKBP1A mRNA using smart target pool siRNA. 2: Electroporating mRNA encoding mutant CnB and mutant IMPDH. (B) Frequency of TNF-α-producing CD8+ cells out of total live CD8+ T cells were quantified following overnight incubation with HepG2.215 targets (n=3). Representative experiment stained for TNF-α production (left panel)**.** DMSO-treated T cells without targets served as negative control. T cell cytolysis determined by real-time killing assay in the presence and absence of both drugs (right panel). Bar graphs demonstrate percentage of T cell cytolysis up to 45 hours after TCR T cell addition to the targets. (C) Evaluation of the kinetics of immunosuppressant drug resistance in an antigen independent (CD3 CD28 activation) manner. (D) Viability of IMPDH electroporated T cells evaluated 72 hours after exposure to clinically relevant concentrations of MMF. Representative experiment stained with live/dead discrimination dye (left panel). (E) Longitudinal viability analysis of IMPDH electroporated T cells. In all experiments, each dot represents one individual experiment in bar graphs. Statistical significance was evaluated by 2-tailed t test (*0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** < 0.0001, n.s., not significant).

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Fig. 4. Developing dual-resistant TCR-redirected T cells for liver transplants under MMF and Tacrolimus combination. To develop dual-resistant T cells, all 3 mRNAs including env-s183, mutant CnB and IMPDH were concomitantly electroporated to the T cells. (A) Viability, TCR expression and TCR kinetic of IDRA-T cells evaluated post electroporation. Non-electroporated T cells used as a negative control in the experiments. (B) Frequency of IFN-γ and TNF-α-producing CD8+ cells out of total live CD8 T cells were quantified following overnight incubation with the HepG2.215 targets (n=3). Representative experiment stained for TNF-α and IFN-γ production. DMSO-treated T cells without targets served as negative control. (C) T cell cytolysis determined by real time killing assay in the presence and absence of both drugs. Bar graphs demonstrate percentage of T cell cytolysis up to 45 hours after TCR-T cell addition to the targets. (D) Kinetic analysis of IDRA T cells function in both antigen independent (Anti CD3 CD28 dynabeads) (left panel) and antigen dependent (target: hepG2.2.15) (right panel) manner. (E) Viability of dual resistant TCR-T cells evaluated 72 hours after exposure to clinically relevant concentration of both drugs (left panel). Density plots shows a representative experiment stained with live/dead discrimination dye. Longitudinal viability analysis of dual resistant IDRA TCR-T cells (right panel). In all experiments, each dot represents one individual experiment in bar graphs. (F) Engineered T cells were labelled with BMQC and introduced into 3D microdevice in the presence of 5ng/ml of Tacrolimus and DRAQ7. Target death was quantified by measuring DRAQ7 positive cells at ~30min interval for ~16hours (right panel). Representative images acquired at the indicated times are shown (left panel). Scale bar: 80 μM. Statistical significance was evaluated by either one-way ANOVA followed by Turkey multiple or 2-tailed t test comparison test (*0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** < 0.0001, n.s., not significant). (G) CD39 and Ki67 phenotypic analysis of conventional and IDRA HBV-TCR T cells after 24hr exposure to TAC and MMF. Density plots shows a representative experiment where the frequency of CD39+ Ki67+ T cells out of total live T cells are indicated. Bar graph summarises two independent experiments and the frequency of CD39+ Ki67+ T cells were normalised to the control where cells were cultured without immunosuppressant drugs.

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Fig. 5. Engineering IDRA EBV-specific TCR-redirected T cells. IDRA EBV TCR-T cells were developed by electroporating mRNA encoding EBV-specific TCR, mutant CnB and mutant IMPDH. Engineered T cells were co-incubated with HLA-A2+ EBV-specific peptide pulsed or non-pulsed T2 cells overnight. Intracellular cytokine staining and viability analysis were performed after the indicated time. (A) Representative experiment stained for TNF-α or IFN-γ. Bar graphs demonstrate the percentage of cytokine-positive CD8+ T cells (n = 3). DMSO-treated T cells without targets served as negative control. (B) Viability of T cell were assessed by live-dead staining after 72 hours exposure to the respective drugs. DMSO-treated T cells from same donor served as a control for viability assessment. Representative experiment stained with live/dead discrimination dye (left panel). Each dot represents one individual experiment in bar graphs. Statistical significance was evaluated by 2-tailed t test (*0.01 to 0.05, ** 0.001 to 0.01, *** 0.0001 to 0.001, **** < 0.0001, n.s., not significant).

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Supplementary materials and methods

HBV-TCR T cell infusions in patients

Liver transplanted patients with HBV-HCC recurrence were recruited and treated with multiple infusions of HBV-TCR T cells in the Singapore General Hospital and in The Third Affiliated Hospital of Sun Yat-sen University in accordance to clinical protocols approved by the respective institutional ethics review board. For safety, all patients received the first infusion cycle consisting of 4 infusions of escalating doses ($1x10^4$, $1x10^5$, $1x10^6$, $5\text{-}10x10^6$ / kg) of HBV-TCR T cells given once a week, followed by a month of monitoring. This was then followed by subsequent infusion cycles where $5\text{-}10x10^6$ / kg of HBV-TCR T cells were infused once a week. Informed consent was obtained from all patients and the study was conducted in accordance to the principles expressed in the Helsinki Declaration.

In vitro-transcribed (IVT) mRNA synthesis

HBV Env183-191-specific TCR (s183-TCR), mutant calcineurin subunit B and mutant IMPDH mRNAs were synthesized from purified DNA of aforementioned genes that were subcloned into T7 expression vector (p-VAX1) using the mMESSAGE mMACHINE™ T7 ULTRA Transcription Kit (ThermoFisher Scientific). P-VAX1 vector was linearized by digestion with FastDigest XbaI restriction enzyme (ThermoFisher Scientific) and subsequently transcribed into RNA via addition of T7 RNA polymerase and capped with Anti-Reverse Cap Analog (ThermoFisher Scientific). *E.coli* poly (A) polymerase and ATP were used to add a poly(A)-tail to the synthesized RNA to increase stability. The synthesized mRNA was purified and eluted in nuclease-free water. Quantity of mRNAs were measured by NanoDrop 2000 spectrophotometer (Thermo Scientific).

T cell expansion and mRNA electroporation

PBMCs were cultured in AIM-V (Gibco) supplemented with 2% human AB serum (Gibco) or with 5% CTS Serum Replacement (Invitrogen), 600IU/ml IL-2 (Miltenyi Biotec) and 50ng/ml anti-CD3 (eBioscience) for 7 days. IL-2 concentration was then increased to 1000IU/ml on day 7 and

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expanded T cells were electroporated with indicated mRNA on day 8 using either the 4DNucleofector™ System (Lonza) or AgilePulse Waveform Electroporation System (BTX Harvard Apparatus) following the manufacturer's instructions. Electroporated T cells were maintained overnight in AIM-V media supplemented with 10% human AB serum and 100IU/ml IL-2. T cells expressing s183-specific TCR were detected 24 hours after electroporation using HLA-A201-Env183-191 tetramer (Immudex, Denmark), anti-human CD3 and CD8 m-Abs (BD bioscience). To check the viability, T cells were stained with Live-Dead fixable stain kit (ThermoFisher Scientific) at indicated times and analyzed by flow cytometry.

RT-PCR

Total RNA were isolated from $5x10^5$ T cell using RNeasy Plus Micro kit (Qiagen) following the manufacturer's instructions. 200ng of RNA was converted to cDNA using the cDNA iScript synthesis kit (Biorad) following the manufacturer's instructions. Real-time PCR was performed with 20ng cDNA per reaction, 0.5 µM each of forward and reverse primers FKBP1A forward 5'-GGGATGCTTGAAGATGGAAA-3', FKBP1A reverse 5'-TCTGACCCACACTCATCTGG-3', b-actin forward 5'-CCTGGCACCCAGCACAAT-3', b-actin reverse 5'-GCCGATCCACACGGAGTACT-3'and universal Sso Advanced Universal SYBR Green Supermix (Biorad) on CFX96 Touch real-time PCR detection system (Biorad). B-actin was used as internal control.

Real-time killing assay

T cells cytotoxicity were measured by xCELLigence Real-Time Cell Analysis (ACEA Biosciences, Inc). HepG2.2.15 and TCR-redirected T cells were similarly used as target and effector cells in an impedance-based assay. In brief, 10^5 HepG2.2.15 cells were seeded in E-plates (ACEA Biosciences) and incubated overnight. Following 25-30 hours, 150 µl of supernatant removed and replaced with AIM-V 2% AB serum containing TCR-T cell at effector to target ratio (E:T) of 1:1. Killing was evaluated up to 45 hours post T cell addition. Samples have been internally normalized for the Cell Index value measured before T cells addition. The Normalized Cell Index

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plot is converted to an area under the curve to quantify percentage of cytolysis following ~45 hours after TCR-T cell addition.

3D microfluidic assay and live imaging

Freshly trypsinized and dissociated HepG2-Env target cells were mixed in collagen gel solution and injected into the middle region of the microfluidic device and kept for 40 minutes in a humidity box at 37C to permit polymerization. After polymerization, RPMI 1640 supplemented with 10% heat-inactivated FBS (Gibco), 0.5mM sodium pyruvate, 20mM HEPES, 100IU/ml penicillin, 100ug/ml streptomycin, MEM non-essential amino acids, MEM amino acids with L-glutamine (ThermoFisher Scientific), 5µg/ml Plasmocin (InvivoGen) and 5µg/ml of puromycin (Takara) for selection (R10 media) was added to the device to fill both lateral media channels in order to prevent gel dehydration and cell death. 3µM of cell-impermeable nuclear dye DRAQ7 (Biolegend) was added to R10 media. Subsequently, R10 media was replaced with AIM-V supplemented with 2% AB serum, 100IU/ml of rhIL-2 and specific concentrations of drugs listed above if necessary. TCR-T cells were stained in media containing 3µM CellTracker Violet BMQC (ThermoFisher Scientific) prior to addition into the 3D device. Labelled T cells were washed and resuspended in T cell media at $3x10^6$ cells/ml. 30µl of this suspension was added into the microfluidic device. For static imaging, z stack images of selected regions were acquired before and after 15 hours of T cell addition. T cell infiltration were measured by the quantification of labelled T cells inside the matrix gel. Live target cells were quantified via GFP fluorescent intensity. Percentage of dead cells was calculated by determining differences of DRAQ7 fluorescent intensity between time 0 and after overnight incubation with engineered T cells. Time lapse imaging was performed with LSM7800 confocal microscope (Zeiss) equipped with an environmental chamber set at 37°C and 5% CO2. z stack images of selected regions were acquired at the indicated time intervals. All acquired images and videos were analyzed by Imaris software (Bitplane).

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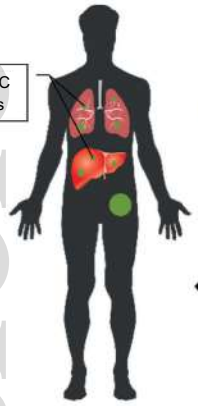
A)



HBV-HCC relapses

Liver transplanted patients (n=8)

Dose-escalation

1x10⁴/Kg TCR-T cells | 1x10⁵/Kg TCR-T cells | 1x10⁶/Kg TCR-T cells | 5-10x10⁶/Kg TCR-T cells

7 days | 7 days | 7 days

≥1 month observation

Subsequent infusions

5-10x10⁶/Kg TCR-T cells

7 days | 7 days | 7 days

	Liver transplanted patients with HCC relapses							
	C-1	C-2	C-3	C-4	C-5	C-6	S-1	S-2
HBV integration	*Not done*	NEG	POS	POS	POS	POS	POS	POS
Total number of HBV-TCR T cell infusions	10	4	9	4	4	8	32	8
Date of liver transplant	23 DEC 2015	20 JULY 2016	09 DEC 2015	18 MAY 2017	30 MAY 2017	21 JUNE 2018	04 DEC 2015	DEC 2013
Date of first HBV-TCR T cell infusion	01 JUNE 2017	20 JULY 2017	21 SEP 2017	26 OCT 2017	15 NOV 2017	19 FEB 2019	11 JAN 2017	07 MAR 2018
*†Immunosuppresant drug administered	TAC/RAPA	TAC/RAPA/MMF	TAV/RAPA	TAC/MMF	RAPA	TAC/RAPA	TAC/MMF	RAPA
‡TAC serum trough levels (5-15 ng/ml)	2.6	2.1	3.5	9.1	-	*Not done*	5.9	-
‡RAPA serum trough levels (4-20 ng/ml)	8.77	9.21	5.12	-	*Not done*	*Not done*	-	*Not done*

*TAC = Tacrolimus; RAPA = Rapamycin; MMF = Mycophenolate Mofetil
†MMF or its active metabolite not measured
‡Serum concentration measured prior to first infusion; reference serum trough levels indicated

B)

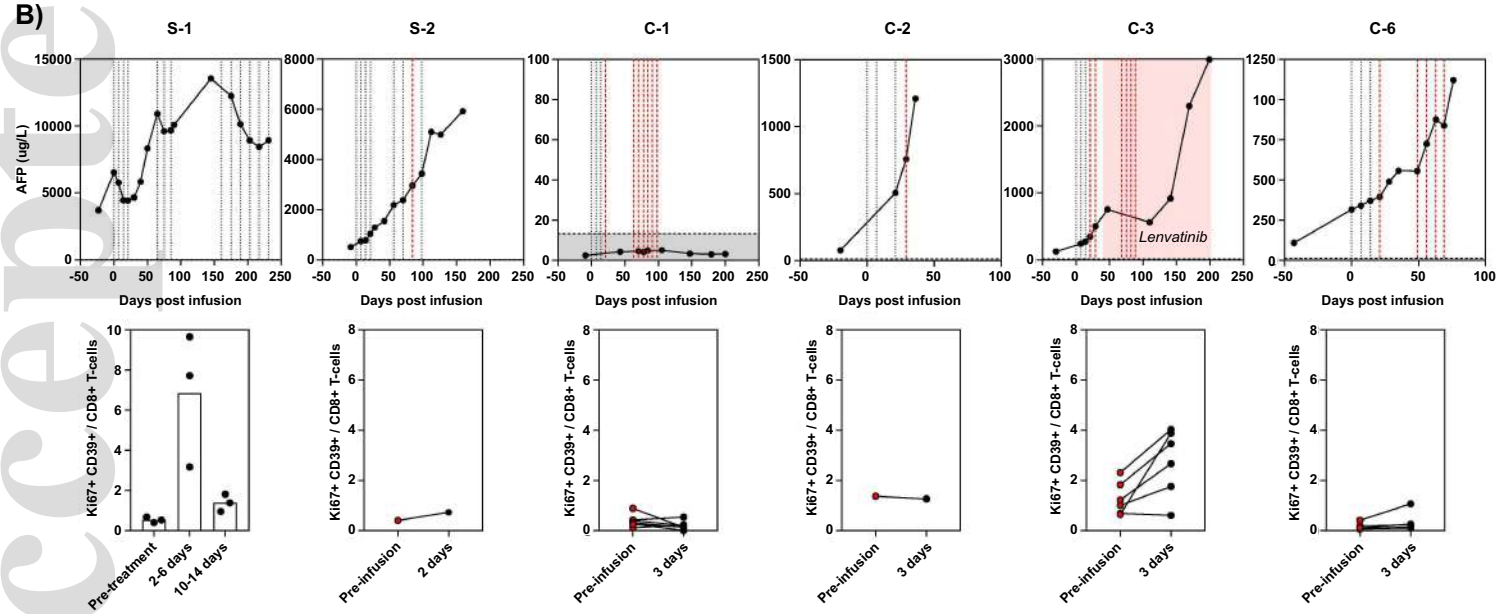


Figure 2

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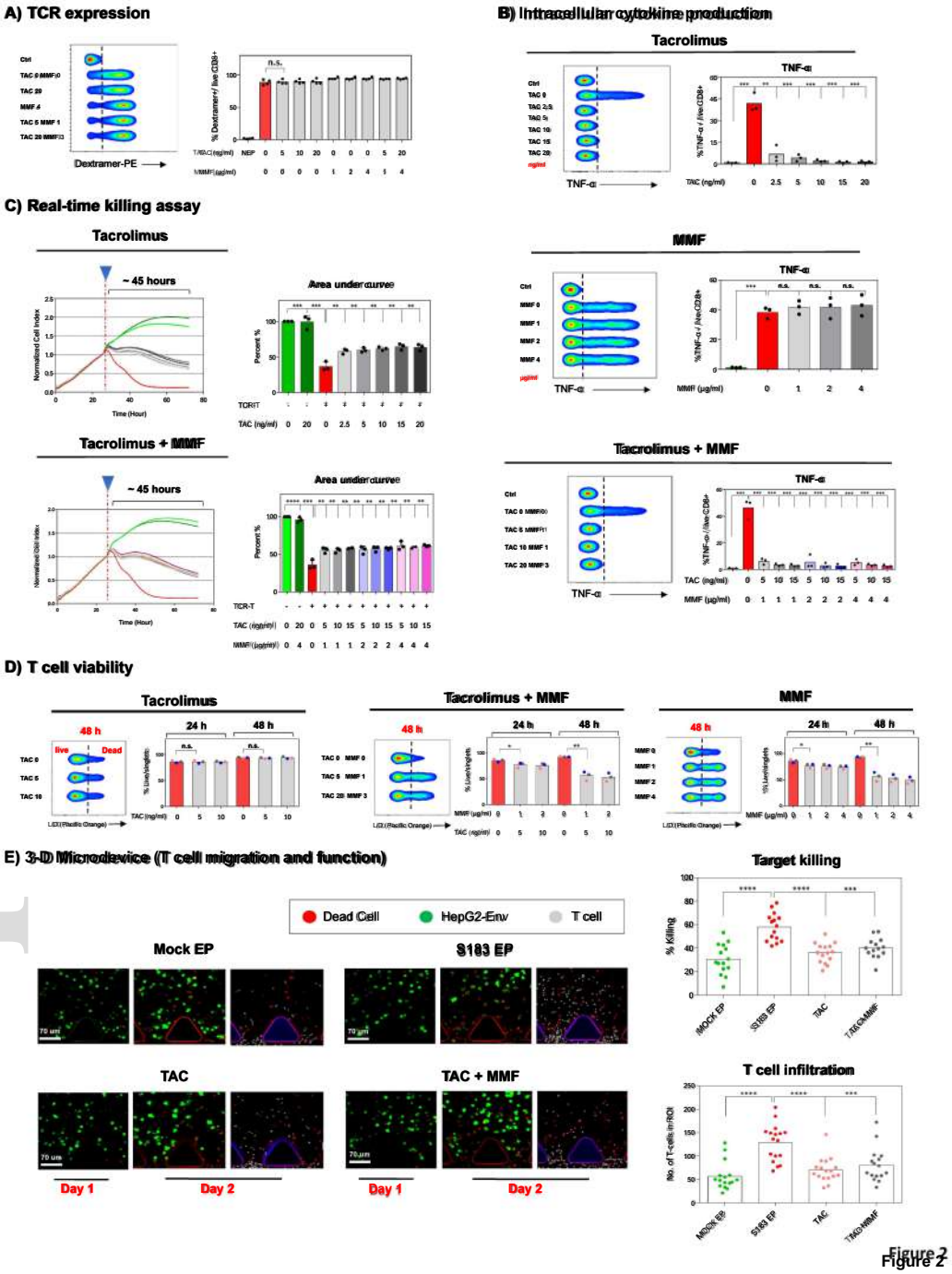

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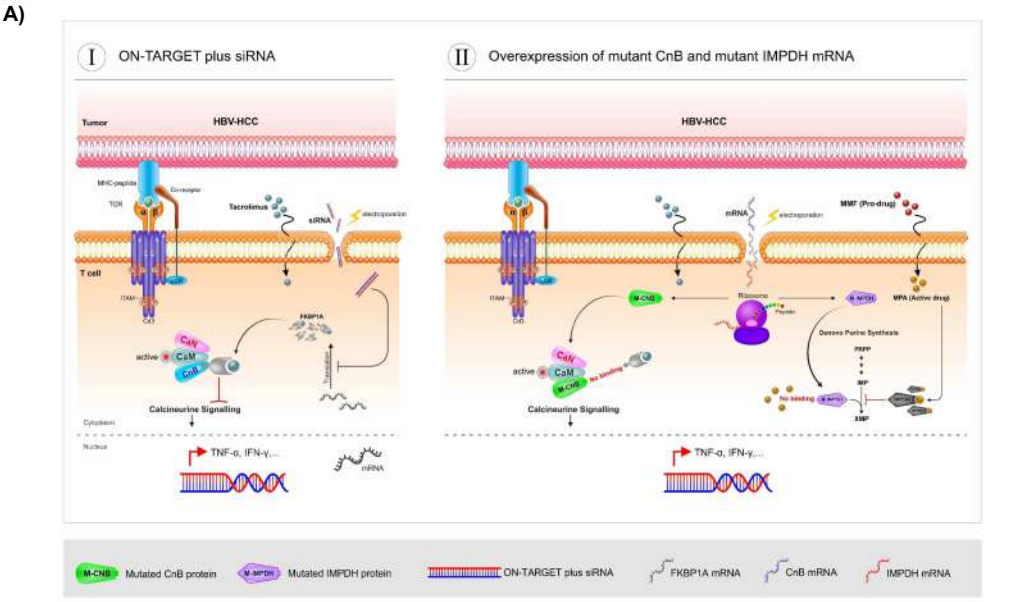

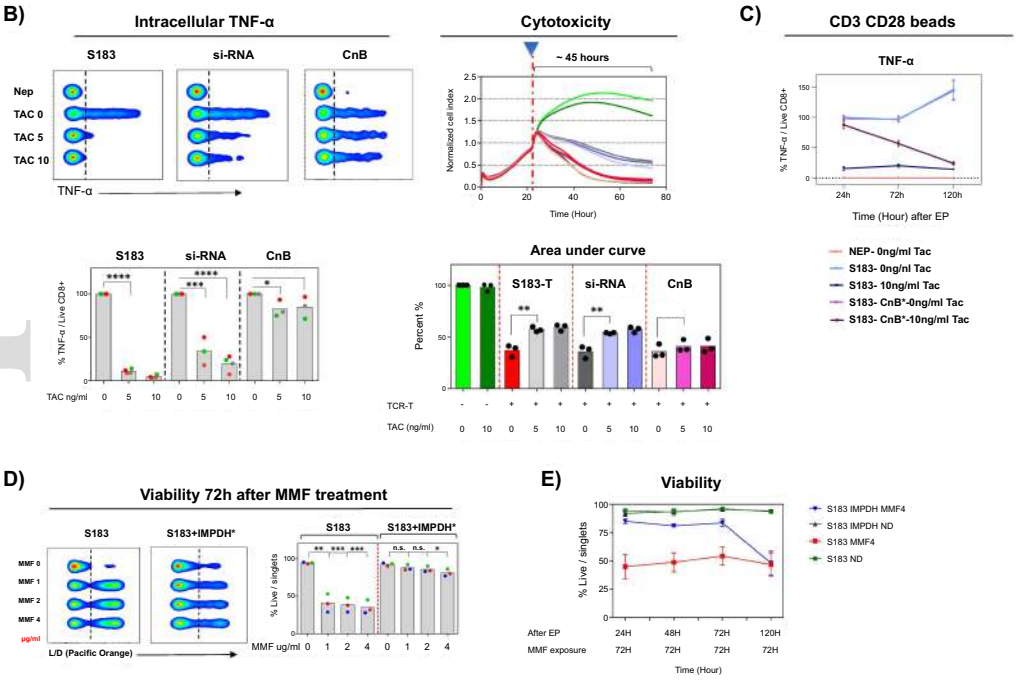

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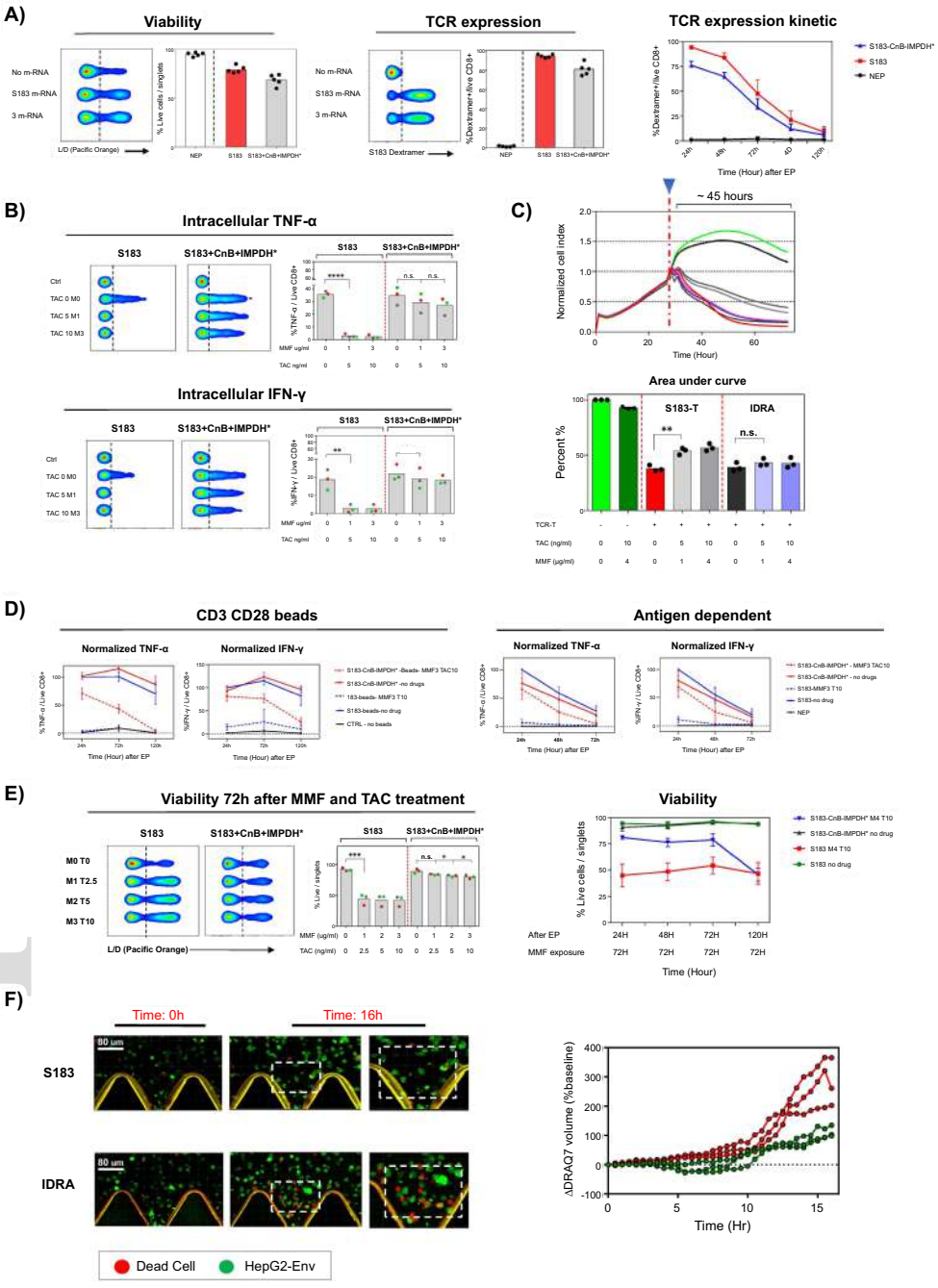

Figure 4

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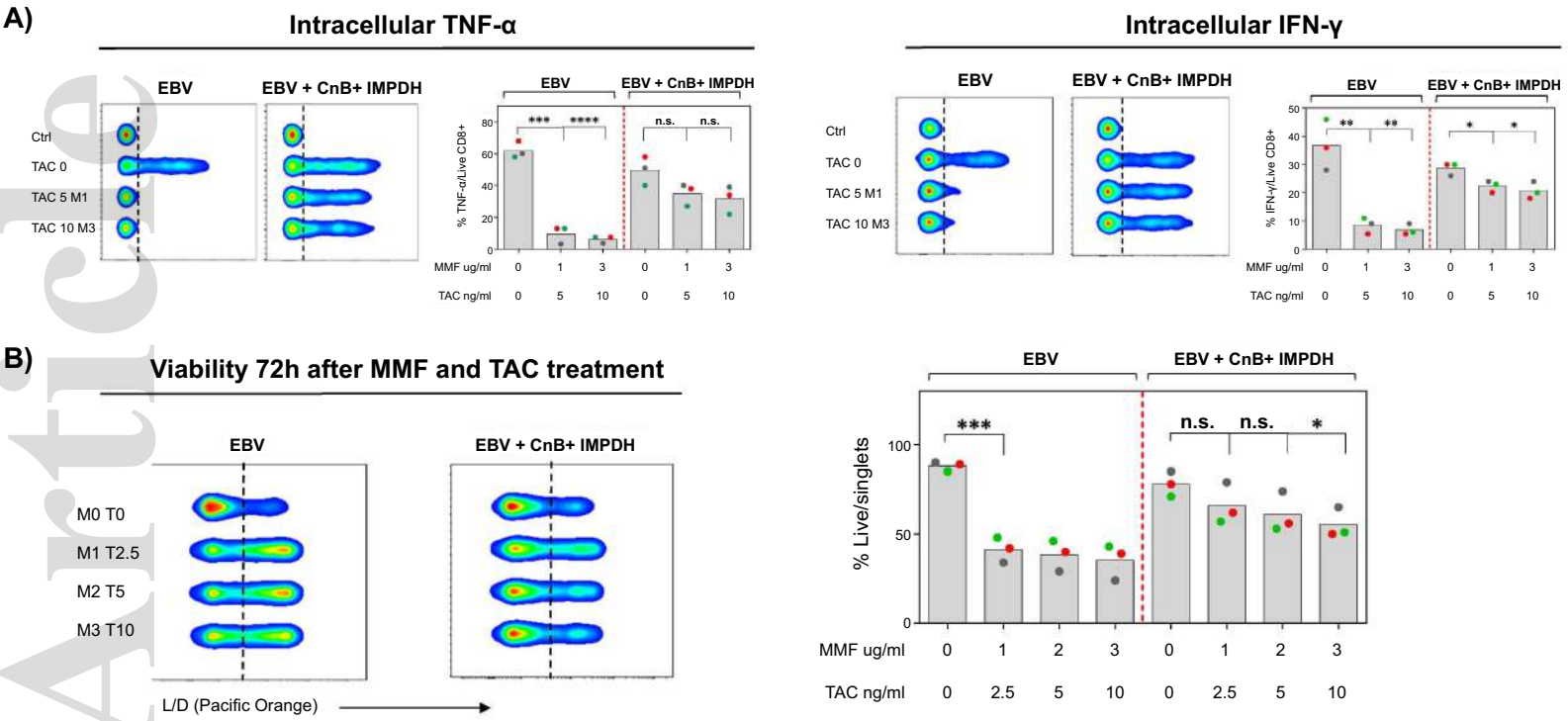

Figure 5

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