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Title: Immune-mobilising monoclonal T cell receptors mediate specific and rapid elimination of Hepatitis B-infected cells

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Abstract

Background & Aims: New therapies for chronic HBV infection are urgently needed due to viral integration, persistence of viral antigen expression, inadequate HBV-specific immune responses and treatment regimens that require life-long adherence to suppress the virus. Immune mobilising monoclonal T cell receptors Against Virus (ImmTAV[®]) molecules represent a novel therapeutic strategy combining an affinity-enhanced T cell receptor with an anti-CD3 T cell-activating moiety. This bispecific fusion protein redirects T cells to specifically lyse infected cells expressing the target virus-derived peptides presented by human leukocyte antigen (HLA).

Approach & Results: ImmTAV molecules specific for HLA-A*02:01-restricted epitopes from HBV envelope, polymerase and core antigens were engineered. The ability of ImmTAV-Env to activate and redirect polyclonal T cells towards cells containing integrated HBV and cells infected with HBV was assessed using cytokine secretion assays and imaging-based killing assays. Elimination of infected cells was further quantified using a modified fluorescent hybridisation of viral RNA assay. Here, we demonstrate that picomolar concentrations of ImmTAV-Env can redirect T cells from healthy and HBV-infected donors towards HCC cells containing integrated HBV DNA, resulting in cytokine release, which could be suppressed by the addition of a corticosteroid *in vitro*. Importantly, ImmTAV-Env redirection of T cells resulted in cytolysis of antigen-positive HCC cells and cells infected with HBV *in vitro*, resulting in a reduction of HBeAg and specific loss of cells expressing viral RNA.

Conclusions: The ImmTAV platform has the potential to enable the elimination of infected cells by redirecting endogenous non-HBV specific T cells, bypassing exhausted HBV-specific T cells. This represents a promising therapeutic option in the treatment of chronic Hepatitis B, with our lead candidate now entering first-in-human trials.

HBV is a significant cause of morbidity and mortality, with ~250-340 million chronic carriers of the virus worldwide (1). Achieving a sterilising cure is challenging due to the persistence of covalently closed circular DNA (cccDNA) or integrated HBV DNA, the transcriptional templates of HBV antigens, in the nucleus of hepatocytes. The elimination of viral protein production is an important treatment goal as the continuous secretion of high levels of HBV antigens, including the envelope protein HBsAg, is thought to play a key role in host immunosuppression and immune tolerance of CHB (2-4). As such, the concept of an immunological or “functional cure”, defined as sustained HBsAg loss and undetectable HBV DNA in serum after completion of a finite course of treatment, is regarded as a more achievable target (5).

The current standard of care for patients with CHB is made up of two treatment strategies: 1) therapies of finite duration using immunomodulators such as pegylated interferon- α , which is the only licensed finite therapy but has significant limitations; and 2) long-term treatment with nucleos(t)ide analogue (NAs) polymerase inhibitors, which do not accelerate elimination of the viral reservoir and all have inherent limitations including the emergence of drug resistance, requirement for life-long adherence and related safety concerns associated with long-term use (6, 7). Therefore, there is an urgent need for novel therapeutic approaches that achieve rapid viral control with sustained off-treatment responses.

The potential for immunotherapeutic strategies to control HBV infection is illustrated in acute-resolving infections, in which the presence of a strong immune response results in natural resolution of infection. Resolution of acute disease is largely driven by CD8⁺ T cells, while the lack of a strong and broad CD8⁺ T cell-mediated immune response is a driving factor in progression to chronic infection (8). The importance of T cells lies in their ability, firstly, to specifically lyse infected cells and, secondly, to secrete cytokines that inhibit viral replication and even silence or destabilise cccDNA (9, 10). However, in CHB patients, HBV-specific T cells are rare and are functionally exhausted, whilst circulating T cells primed against non-HBV antigens are largely unaffected (3, 11). Therefore, new strategies that can harness the potential of polyclonal T cells, independently of endogenous HBV-specific T cells, are an attractive approach.

Immune mobilising monoclonal T cell receptors Against X (ImmTAX™) molecules are soluble, bispecific T cell-engaging fusion proteins comprised of an affinity enhanced T cell receptor (TCR), specific for a peptide epitope presented in the context of the HLA class I allele molecules on the surface of cells, fused to a humanised anti-CD3 single-chain antibody variable fragment (scFv; **Figure 1A**). The TCR portion is

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affinity-enhanced to be able to detect very low levels of antigen, while the anti-CD3 domain enables the recruitment and activation of endogenous T cells, independently of specificity, to release both cytokines and cytolytic mediators towards antigen positive cells. This represents a novel approach that harnesses the power of the immune system yet bypasses the need for rare 'exhausted' antigen-specific T cells by redirecting T cells of any specificity. This technology has been shown to be effective against viral epitopes (ImmTAV[®]) from HIV-infected cells *in vitro* and is currently being used in other therapeutic areas to redirect T cells against cancer (ImmTAC[®]) (12, 13). Here, we have engineered ImmTAV molecules to picomolar affinities against three major HBV antigens and demonstrated the ability of an envelope-specific ImmTAV molecule to potently redirect polyclonal T cells to lyse both cells containing integrated HBV DNA and those newly infected with HBV *in vitro*. This ability to mediate rapid and specific elimination of infected cells demonstrates the promise of ImmTAV molecules as a novel therapeutic approach for the treatment of chronic Hepatitis B.

Experimental Procedures

Cell lines and cell culture

T2 cells (174xCEM.T2; CRL-1992) and HepG2 (HB-8065) were purchased from American Type Culture Collection (ATCC, Manassas, USA) and PLC/PRF/5 (85061113) were supplied by Public Health England. T2 were cultured in RPMI media, PLC/PRF/5 A2B2M in DMEM and HepG2 in EMEM (Gibco). All media were supplemented with 10% foetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin, with RPMI also supplemented with 2 mM L-glutamine. HLA-A*02:01/ β 2M (A2B2M) was ectopically expressed in both PLC/PRF/5 and HepG2 by lentiviral transduction. HepG2-hNTCP (clone A3) were obtained from S. Urban (Ruprecht Karl University of Heidelberg) and maintained in DMEM. Cell line authentication and mycoplasma testing were routinely carried out by the LGC Standards Cell line Authentication Service and Mycoplasma Experience Ltd, respectively.

Primary cells

HLA-A*02:01-positive primary human hepatocytes (PHH) were obtained from Tissue Solutions (Glasgow, UK) or Lonza (Basel, Switzerland) and cultured according to Lonza's 'Plateable Cryopreserved Hepatocyte' instructions. Healthy donor HLA-A*02:01-positive PBMC were purchased from Discovery Life Sciences (Huntsville, AL, USA) or StemCell Technologies (Grenoble, France). For assays using purified T cells as effectors, whole blood was obtained from healthy volunteers, PBMC isolated by density centrifugation and pan T cells isolated by negative selection (Miltenyi Biotec, Germany). The Oxford A REC approved protocol 13/SC/0226 was used to obtain written consent for all blood donations and was fully approved by the National Research Ethics Committee South Central. Cryopreserved PBMC from CHB donors on NAs were obtained from Tissue Solutions (Glasgow, UK) and BioIVT (West Sussex, UK).

Generation of ImmTAV molecules

ImmTAV molecules targeting HBV antigens Polymerase, Core and Envelope were prepared as previously described (12-15). Briefly, wild-type TCRs specific for an HBV antigen (pol, core, env) were obtained from both in-house naïve TCR phage display libraries and through T cell cloning from healthy donors. TCR affinities were significantly enhanced by using directed molecular evolution and phage display selection (15). The beta chains of either the wild-type TCR or resulting strong-affinity TCR were fused to a humanised CD3-specific scFv via a flexible linker. The alpha and beta chains of the resulting ImmTAV were expressed in *E. coli* as inclusion bodies, refolded and purified as previously described (14).

Surface plasmon resonance (SPR)

Purified ImmTAV molecules were subjected to SPR analysis using either a BIAcore™ T200 (for weak affinity molecules) or a BIAcore™ 8K system (for intermediate to strong affinity molecules). Briefly, biotinylated cognate pHLAs were immobilised onto a streptavidin-coupled CM5 sensor chip. Flow cell one was loaded with free biotin alone to act as a control surface. K_D values were calculated assuming Langmuir binding and data was analysed using a 1:1 binding model (GraphPad Prism v8.3.0 for steady state affinity analysis and Biacore Insight Evaluation v2.0.15.12933 for single cycle kinetics analysis).

Enzyme-linked immunospot (ELISpot) assays

IFN γ and Granzyme B (GzmB) ELISpot assays were performed according to the manufacturer's recommendations (BD Biosciences) after culture for 24 or 48 hours, respectively. For peptide-pulsing experiments, T2 target cells were incubated with peptide (Peptide Synthetics, UK) for 2 h before plating. 30,000 PBMC were added with 50,000 target cells per well. This ratio gave the most comparable responses between donors within the optimal window of responses. Spots were quantified using the BD ELISpot reader (Immunospot Series 5 Analyzer, Cellular Technology Ltd).

IncuCyte Killing Assay

In IncuCyte S3 Live-Cell Analysis System (Essen Bioscience Ltd., UK) killing assays target cells were stained with CellTracker Deep Red Dye (Invitrogen, California, USA). PBMC were added at a 10:1 ratio to targets. IncuCyte Caspase-3/7 Green Apoptosis Assay Reagent (Essen Bioscience) was added to all wells. Plates were incubated at 37°C/5% CO $_2$ with images taken every 3 h. In peptide control wells, 10 μ M Env peptide was added for the duration of the assay. The number of apoptotic events/mm 2 was calculated from two-colour images.

Opera Phenix™ Killing Assay

Assays were performed as for IncuCyte Assays, with the exception that Hoechst stain (Thermo Fisher Scientific, USA) was added for nuclear discrimination and apoptosis was imaged using the Opera Phenix High Content Screening System and analysed with the Harmony High-Content Imaging and Analysis software (PerkinElmer, USA). Percent cytolysis was calculated from three-colour images normalised to the total number of cells in each well at each time point.

For assays with mixed target cell populations, PLC/PRF/5-A2B2M were stained with CellTracker Deep Red, HepG2-A2B2M with CellTracker Red and pan T cells with CellTrace Violet (Invitrogen).

Cytokine Analysis by Meso Scale Discovery (MSD)

Supernatants were analysed for the presence of cytokines by MSD's multi-PLEX pro-inflammatory kit (Meso Scale Technologies, USA) following manufacturer's recommendations. In certain cases, increasing concentrations of dexamethasone (Sigma-Aldrich, UK) were added to co-cultures for the duration of the assay. A vehicle control, representing the highest proportion of solvent (H₂O) added, was included as the 0 μ M dexamethasone condition. Healthy PBMC were added at a ratio of 10:1 with either 10,000 PLC/PRF/5-A2B2M or 50,000 PHH. For co-cultures with PHH, IFN γ and GzmB in supernatants was quantified using an MSD U-plex custom assay (Meso Scale Technologies, LLC).

Flow cytometry

To detect degranulation of T cells CD107a-AlexaFluor647 (BioLegend, USA) was added at the start of co-culture and proliferation was detected by labelling PBMC with CellTrace Violet (Invitrogen). T cell subsets were gated using Zombie Live Dead dye (BioLegend) and the antibodies detailed in Supplementary Table 1. Data was acquired on a Sony MA900 (Sony Biotechnology) and analysed by FlowJo v10 (TreeStar Inc., USA).

HBV Infection

HepG2-hNTCP were infected with 500 genome equivalents/cell of HBV (ImQuest Biosciences, USA) overnight in media plus 4% polyethyleneglycol and 2.75% DMSO (v/v). Media without DMSO was changed every other day for 7 days before addition of effectors and ImmTAV-Env in media alone. HBeAg in supernatants was quantified by HBeAg ELISA (Autobio Diagnostics, China) as per manufacturer's instructions.

PrimeFlow

HBV-RNA was stained following the manufacturer's protocol with PrimeFlow probeset VF1-6000704 (Thermo Fisher Scientific). Live cells were distinguished by fixable viability dye eFluorTM 780 (eBioscience) and effector T cells by anti-CD45-PECy7 (BioLegend). Cells were acquired on the MACSQuant X flow cytometer (Miltenyi Biotec) and analysed by FlowJo v10. Percentage reduction in

infected cells was calculated as below, using the average of duplicate wells, where ‘infected only wells’ refer to cultures of infected HepG2-hNTCP alone:

$$\left[\frac{(\% \text{HBV-RNA}^+ \text{ of live HepG2-hNTCP in infected only wells} - \% \text{HBV-RNA}^+ \text{ of live HepG2-hNTCP in sample wells})}{(\% \text{HBV-RNA}^+ \text{ of live HepG2-hNTCP in infected only wells})} \right] \times 100$$

Results:

ImmTAV molecules with picomolar affinity TCRs can be engineered against HBV

Highly conserved and previously characterised HBV-derived peptides presented in the context of HLA-A*02:01 were selected as targets to isolate and engineer enhanced affinity TCRs (3, 16). These epitopes were derived from HBV Envelope (Env), Core (Core) and Polymerase (Pol) and were presented by hepatocellular carcinoma cell lines and antigen-transduced cells, as confirmed by immunoprecipitation of HLA-A*02:01 molecules followed by mass spectrometry (data not shown). Using these epitopes as targets, we generated a series of ImmTAV molecules by fusing HBV antigen-specific TCRs of weak, intermediate or strong affinity to a scFv anti-CD3 domain, designated: ImmTAV-x-W (weak), ImmTAV-x-I (intermediate) and ImmTAV-x-S (strong), where x can be substituted for the Pol, Core or Env-derived epitopes. All ImmTAV-x-W molecules showed binding affinities in a range similar to previously described for naturally occurring viral and cancer-specific TCRs (nM to μ M) (17), as determined by surface plasmon resonance (SPR), with very short detectable half-lives. Iterative affinity enhancement improved the binding affinities and half-lives of all the ImmTAV-x-W molecules by 10^3 - 10^6 -fold with final ImmTAV-x-S molecules reaching picomolar affinity with half-lives extending beyond 12 h (**Figure 1B-D**).

The potency of each molecule to redirect T cell responses against target cells presenting their cognate epitope was tested by measuring IFN γ release upon co-culture with peptide-pulsed T2 antigen-presenting cells. No responses were detected with weak affinity ImmTAV molecules, even at high peptide concentrations (**Figure 1E-G**). However, the affinity-enhanced molecules induced robust responses, with EC₅₀ values of 90-900 pM for ImmTAV-x-S molecules.

Epitopes from Env represent an attractive therapeutic target given that HBsAg elimination is essential to achieve resolution of HBV, with high levels associated with cirrhosis and HCC (18) and can be expressed from both cccDNA and integrated DNA (19, 20). Furthermore, the Env target represented a conserved region which is shared across all three envelope proteins (short, medium and long) and CD8⁺ T cell responses observed from chronically infected individuals supports natural presentation of the epitope (21). Therefore, ImmTAV-Env-S molecules (hereafter referred to as ImmTAV-Env) were prioritised for further investigation.

ImmTAV-Env detects antigen positive cells to activate T cells

To confirm that ImmTAV-Env could detect HBsAg-expressing cells, we performed a T cell redirection assay using HLA-A2 and β 2 microglobulin (B2M)-transduced HCC cell line PLC/PRF/5 (PLC/PRF/5-A2B2M) as target cells. In this cell line, epitope expression is driven by transcription of integrated HBV DNA, as confirmed by RNA sequencing (**Supplementary Table 2**), and the introduction of HLA-A2 and B2M enabled epitope presentation on HLA-A*02:01. In co-culture, ImmTAV-Env mediated potent redirection of healthy polyclonal T cells against antigen-positive (Ag⁺) PLC/PRF/5-A2B2M in a dose-dependent manner, as assessed by IFN γ and granzyme B (GzmB) release (**Figure 2A&B**). Responses towards PLC/PRF/5-A2B2M were induced at low picomolar concentrations of ImmTAV-Env, with an EC₅₀ of 36.4 pM. In contrast, no responses were observed towards HLA-A2-transduced antigen negative (Ag⁻) HepG2 cells (HepG2-A2B2M), even at high concentrations of ImmTAV-Env (**Figure 2A&B**). In addition to IFN γ release, high levels of IL-2 and proinflammatory cytokines TNF α and IL-6 were also detected in the supernatants from co-culture with PLC/PRF/5-A2B2M (**Figure 2C**).

In extreme cases, excessive release of proinflammatory cytokines into the circulation *in vivo* can have serious consequences, including cytokine release syndrome (CRS), a condition which may be managed through administration of corticosteroids to inhibit cytokine synthesis (22, 23). ImmTAV-mediated cytokine release to Ag⁺ cells was reduced by approximately 90% for all cytokines tested at \geq 50 μ M dexamethasone. IL-6 was the most sensitive to corticosteroid treatment, with effects observed at concentrations as low as 0.1 μ M dexamethasone (**Figure 2D**).

ImmTAV-Env redirects polyclonal T cells to kill antigen positive hepatocellular cell lines

To verify that activation of T cells by ImmTAV-Env redirection results in killing of Ag⁺ target cells, PBMC were co-cultured with PLC/PRF/5-A2B2M in the presence of ImmTAV-Env for 5 days. Cell death was measured by caspase-3/7 activation. Consistent with the ability to induce cytolytic GzmB release, ImmTAV-Env induced killing of PLC/PRF/5-A2B2M at concentrations \geq 5 pM (**Figure 3A**). Killing was observed from as early as 12 h of co-culture, with maximum cytolysis achieved by 72 h at concentrations \geq 50 pM ImmTAV-Env. No cytolysis of Ag⁻ HepG2-A2B2M was detected at any concentration of ImmTAV-Env, unless cognate peptide was added.

Specificity of killing was further demonstrated by co-culture of T cells with a mixture of both Ag⁺ PLC/PRF/5-A2B2M and Ag⁻ HepG2-A2B2M target cells. Apoptosis of Ag⁺ cells could be observed

during co-culture in the presence of ImmTAV-Env, while Ag⁻ targets remained viable (**Figure 3B & Video 1&2**), demonstrating the ability of ImmTAV-Env to redirect specific lysis of Ag⁺ targets even within a heterogeneous population.

As these assays were performed using PBMC isolated from healthy donors, we next verified the ability of ImmTAV-Env to redirect circulating polyclonal T cells from CHB patients. At concentrations ≥ 10 pM, ImmTAV-Env redirected the lysis of Ag⁺ PLC/PRF/5-A2B2M, but not Ag⁻ HepG2-A2B2M (**Figure 3C&D**). These data demonstrate that non-HBV-specific T cells from chronically infected individuals have lytic capacity when redirected by ImmTAV-Env towards antigen-expressing HCC cells.

ImmTAV-Env redirects activation of different subsets of effector T cells

While CD8⁺ T cells play a major role in killing virus-infected cells, successful antiviral immune responses likely require the engagement of a broad range of effector cells and mechanisms. Therefore, the potential for ImmTAV-Env to activate various T cell subsets from peripheral blood upon exposure to PLC/PRF/5-A2B2M was investigated by flow cytometry (**Figure 4 and Supplementary Figure 1**). Upon co-culture, ImmTAV-Env induced both the degranulation, as measured by CD107a expression (**Figure 4A**), and proliferation (**Figure 4B**) of CD8⁺, CD4⁺, MAIT and $\gamma\delta$ T cells in response to PLC/PRF/5-A2B2M.

ImmTAV-Env was capable of activating all T cell populations tested, illustrating its capacity to activate multiple effector subsets.

ImmTAV-Env does not redirect T cells towards healthy hepatocytes

Affinity enhancement of the TCR carries the risk of introducing cross-reactivity to peptide mimetics that may be presented on the surface of uninfected cells. To further assess this risk beyond reactivity to an Ag⁻ HCC cell line, we tested the reactivity of polyclonal T cells to healthy primary human hepatocytes (PHH) in the presence of ImmTAV-Env. After 48 h co-culture, ImmTAV-Env did not induce IFN γ or GzmB responses at concentrations below 10,000 pM (**Figure 5A&B**), demonstrating that healthy PHH do not present peptides that could sensitise them to off-target killing by ImmTAV-Env at concentrations shown to induce IFN γ and granzyme B release, and on-target killing (**Figure 2&3**).

ImmTAV-Env mediates rapid cytolysis of HBV-infected cell lines

To test the efficacy of ImmTAV-Env against HBV-infected targets, we utilised a HBV-permissive HepG2 cell line which was transduced with the HBV entry receptor human sodium taurocholate co-transporting polypeptide (hNTCP; HepG2-hNTCP) and expresses natural levels of HLA-A*02:01 (24). Infected HepG2-hNTCP were incubated with PBMC in the presence of ImmTAV-Env and target cell lysis quantified over a 4-day period. At 100 pM and 1,000 pM, ImmTAV-Env redirected polyclonal T cells to induce apoptosis of HBV-infected targets, with cell death detected as early as 6 h (**Figure 6A**). Similar responses were also detected when purified T cells were used as the effector population and correlated with release of adenylate kinase, a marker of cell death, in the supernatant (**Supplementary Figure 2**).

To quantify the specific elimination of HBV-infected cells in this model, we adapted the PrimeFlow assay (25) to distinguish HBV-infected target cells from uninfected cells through the expression of viral RNAs by *in situ* hybridisation (**Figure 6B**). After co-culture with purified T cells, 100 pM ImmTAV-Env induced an 87% reduction in the percentage of HBV-infected cells and up to 97% reduction was observed with 1000 pM, consistent with data obtained from the IncuCyte killing assay in Figure 6A. In parallel, HBeAg released into the supernatant during co-cultures was markedly decreased in the presence of ImmTAV-Env (**Figure 6C**) and reduced production was maintained following a further 48h incubation after co-culture (**Figure 6D**). Together, these data confirm that ImmTAV-Env can detect HBV-infected cells, trigger specific cytotoxicity by polyclonal T cells *in vitro* and reduce viral antigen expression.

Discussion:

In this study, we demonstrate that ImmTAV molecules can be generated to recognise HBV-derived peptides from the Core, Pol and Env proteins, when presented by HLA-A*02:01 on the surface of cells, and that specific targeting of Env resulted in the elimination of HBV+ cells. HLA-A*02 is the most common subgroup of HLA class I alleles and has a high prevalence across ethnicities with CHB infection (1, 26).

HBV envelope proteins include HBsAg which is highly expressed in almost all patients and appears to be well-conserved both between and within patients (27, 28). It can be produced by cells with cccDNA, the episomal source of viral replication, as well as integrated HBV DNA, which may be the source of up to 80% of HBsAg production (19, 20). Achieving a functional cure and elimination of HBsAg and HBV DNA requires targeting both types of transcriptionally active hepatocyte.

Our work demonstrates that ImmTAV molecules mediate the direct elimination of cells containing integrated HBV DNA and cells that are virally infected within hours of co-culture. While HCC cells were transduced for HLA-A*02:01 presentation, infected HepG2-hNTCP presented epitope through natural HLA expression, demonstrating that endogenous epitope presentation levels are sufficient to induce killing. This effect was restricted to cells expressing the cognate antigen, as Ag- HCC cell lines and, importantly, PHH were unaffected in experiments with ImmTAV-Env. These observations demonstrate the potential for a wide therapeutic window owing to the absence of off-target effects with ImmTAV retargeting *in vitro*. Importantly, the activity of ImmTAV-Env is shown to be dose dependent and its induction of cytokines can be downmodulated by the addition of dexamethasone, which may be used in the event of CRS (22, 23). This is supported by previous clinical experience with tebentafusp, our lead ImmTAC molecule in oncology (29, 30).

For CHB, data suggest that T cell exhaustion is largely confined to HBV-antigen specific T cells and that effective responses can be mounted by non-HBV specific T cells (3, 4, 11). In agreement, ImmTAV-Env redirected polyclonal T cells from chronically infected patients to kill an antigen-expressing HCC cell line. These data also agree with responses from HIV-positive donors with HIV-specific ImmTAV molecules, showing that ImmTAV responses bypass antigen-specific T cells to mediate effective antiviral responses (13). Moreover, the ability to activate a range of T cell subsets, including those capable of innate-like

responses to viruses, indicates the possibility of inducing a polyfunctional response. These populations were tested here from the periphery, but have been reported to be resident in and / or recruited to the liver during inflammation (31, 32).

ImmTAV-Env activity also induced a broad cytokine response, which may indirectly suppress viral replication. Specifically, IFN γ and TNF α may limit HBV gene expression and replication through non-cytolytic mechanisms that target cccDNA; TNF α and IL-2 have been shown to reduce HBV mRNA through post-transcriptional mechanisms; and IL-6 has been shown to inhibit HBV entry by up to 90% and reduce cccDNA and HBsAg secretion (33, 34). The low levels of HBsAg produced by infected HepG2-hNTCP (24) prevented measurement of ImmTAV-Env mediated effects on HBsAg release in these assays. However, a decrease in HBeAg levels, together with a reduction in viral RNA, was observed. Whilst non-cytolytic elimination of HBV was not directly measured, there is the potential that these mechanisms contributed to this reduction in viral markers, raising the possibility of achieving additional therapeutic benefit through this mode of action.

Adoptive therapy using T cells expressing chimeric antigen receptors (CAR-T) directed towards HBV proteins such as HBsAg has been demonstrated to eliminate infected hepatocytes in mice through cytolytic and non-cytolytic mechanisms (35, 36). However, results were mixed due to possible interference from the high levels of circulating HBsAg which may sequester the CAR-T cells. Adoptive T cell therapy using TCRs targeting HBsAg-derived epitopes has also been investigated in the treatment of HBV. This approach was shown to prevent HBV-positive HCC tumour seeding in xenograft models and to confer antiviral activity in HBV-infected humanised mice (37, 38). However, success in humans has been limited as this approach offers only transient effects owing to the use of mRNA transduction of T cells due to safety considerations (39). Furthermore, cellular therapies have limitations in terms of scalability and administration (5). ImmTAV molecules offer potential advantages beyond adoptive T cell therapies owing to a rapid plasma clearance, with potential for fine-tuned dosing control, rapid activation of multiple T cell subsets and more scalable production.

In summary, we have demonstrated the ability to produce strong affinity, potent and specific ImmTAV molecules that redirect T cells to lyse both HBV DNA-integrated and virally-infected cells, which is likely to be crucial in achieving a functional cure. These bispecific molecules are first-in-class for CHB and our lead molecule, ImmTAV-Env (IMC-I109V), is now entering first-in-human trials.

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Figures

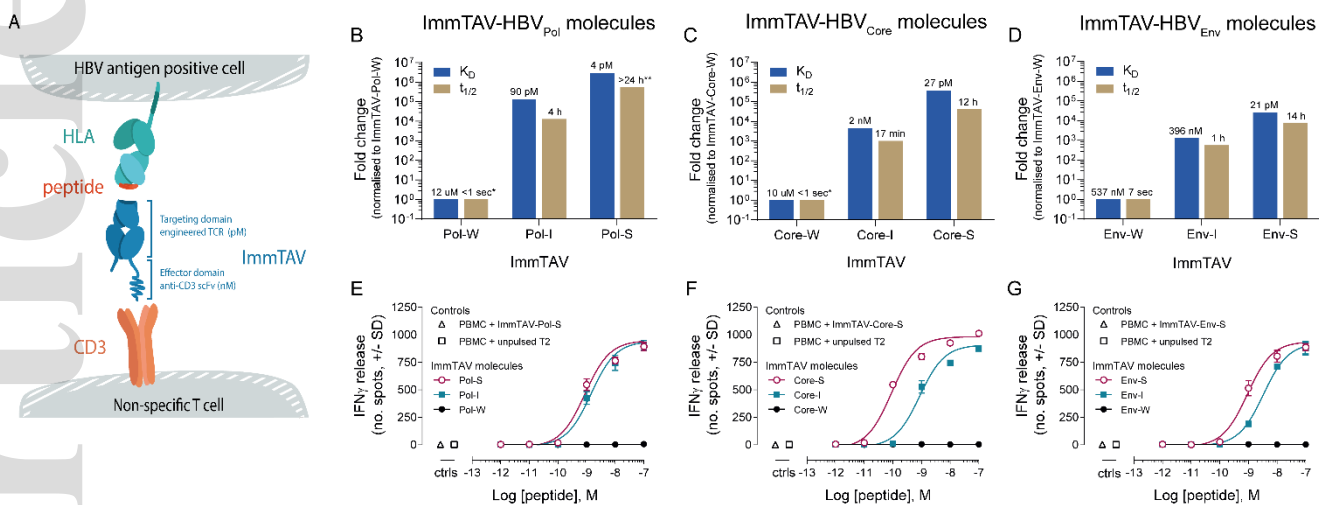


Figure 1 – Affinity enhancement increases the kinetics and potency of ImmTAV molecules

(A) Schematic of T cell redirection using ImmTAV molecules. (B-D) Affinity (K_D) and half-life ($t_{1/2}$) of (B) ImmTAV-Pol, (C) ImmTAV-Core and (D) ImmTAV-Env molecules were determined by SPR. Molecules were classified as either weak (-W), intermediate (-I) or strong (-S) affinity according to affinity and half-life measurements. Fold-change was determined by normalising to K_D and $t_{1/2}$ of ImmTAV-x-W for each target. The *minimum and **maximum detection limits of SPR for $t_{1/2}$ are 1 sec and \sim 24 h, respectively. (E-G) IFN γ ELISpot assays showing activation of PBMC by 100 pM ImmTAV in the presence of T2 cells pulsed with decreasing concentrations of cognate peptide from (E) Pol, (F) Core or (G) Env antigens. Controls (ctrls) represent PBMC incubated with T2 without peptide (unpulsed) in the absence of ImmTAV molecules (\square) and PBMC incubated with ImmTAV-x-S alone (Δ). Data points represent mean \pm SD. Line of best fit and EC_{50} values were calculated by nonlinear regression log (agonist) vs. response (three parameters).

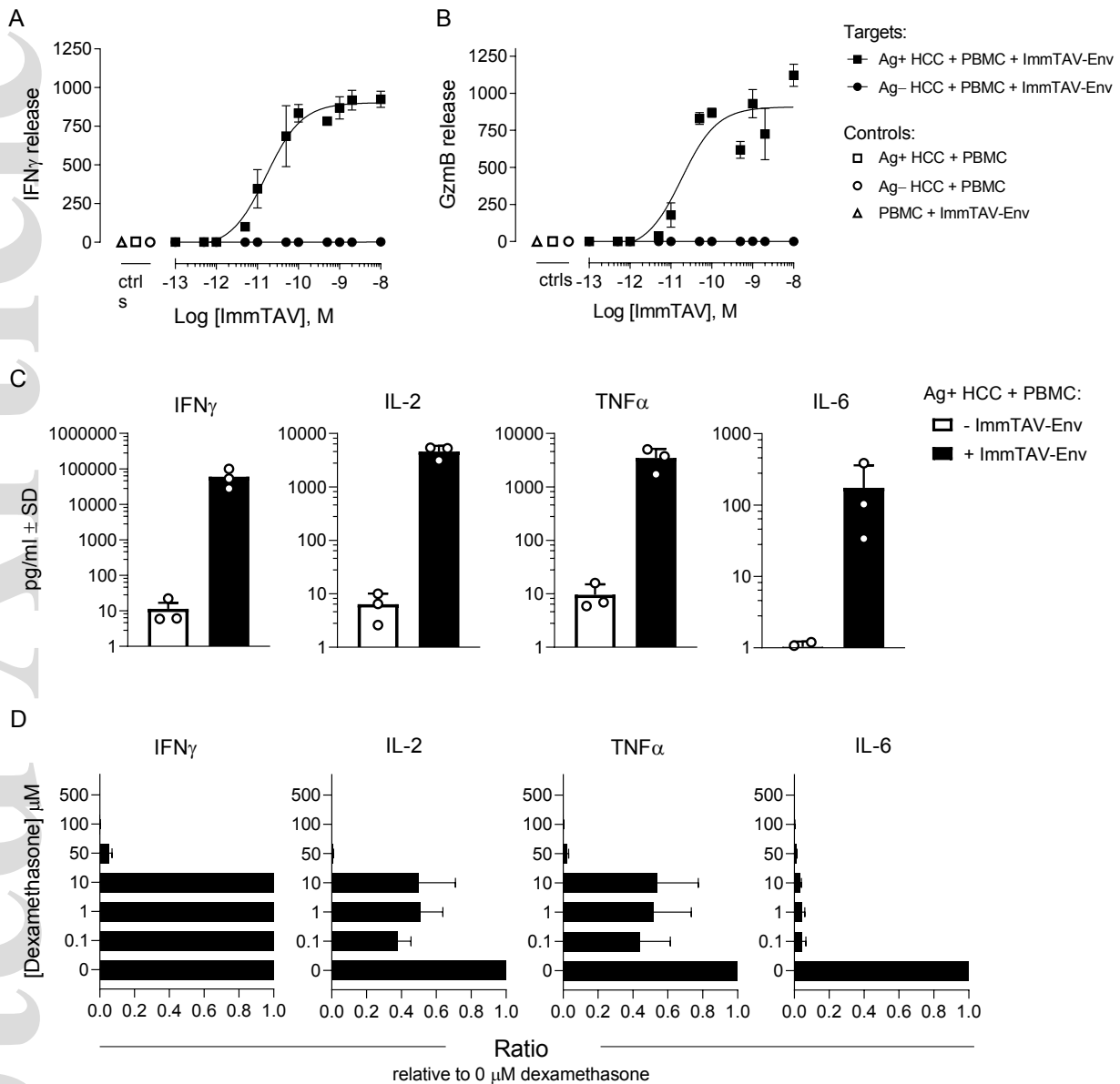


Figure 2 – ImmTAV-Env redirects activation of polyclonal T cells specifically towards antigen-positive cell lines

ELISpot assays of (A) IFN γ and (B) granzyme B (GzmB) release from PBMC in the presence of PLC/PRF/5-A2B2M (Ag+) or HepG2-A2B2M (Ag-) in the absence (ctrls; open symbols) or presence of ImmTAV-Env (closed symbols). PBMC incubated with ImmTAV-Env alone were included as controls (Δ). Graphs shown are representative from one of three donors tested. (C&D) Levels of IFN γ , IL-2, TNF α and IL-6 in supernatants from co-cultures of PBMC and PLC/PRF/5-A2B2M (10:1 ratio) in the presence or absence of 100 pM of ImmTAV-Env were assessed after 24 hrs by Meso Scale Discovery (n=3). (D) Increasing concentrations of dexamethasone were added to co-cultures and the ratio of each cytokine calculated relative to 0 μ M dexamethasone. Data represent mean \pm SEM (n=3).

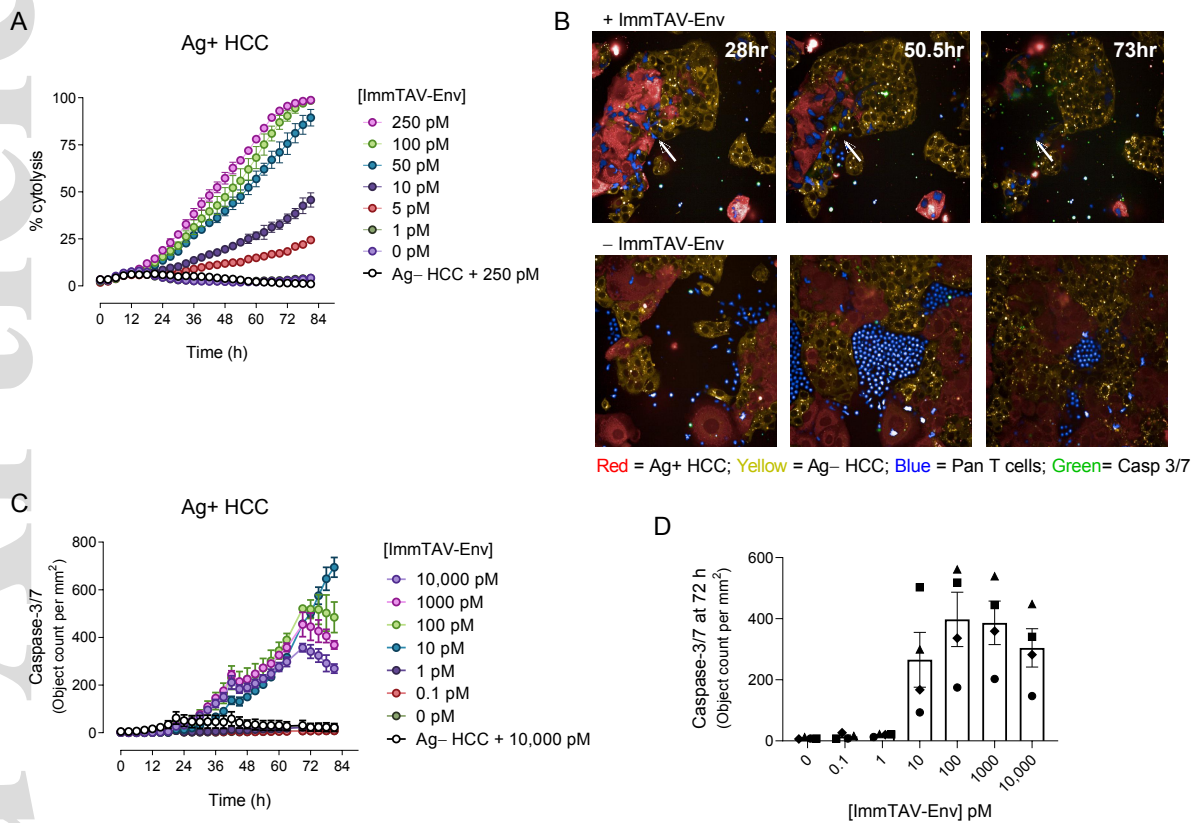


Figure 3. ImmTAV-Env redirects healthy and CHB donor T cells to lyse antigen positive hepatocellular cell lines

(A) Percentage cytolysis of PLC/PRF/5-A2B2M target cells in co-cultures with healthy PBMC at an E:T ratio of 10:1 with various concentrations of ImmTAV-Env was captured by Opera Phenix killing assay. Ag- HepG2-A2B2M with the highest concentration of ImmTAV-Env was included as a control. Data represent mean \pm SD from a representative donor of three donors tested. (B) Confocal images at indicated timepoints after addition of ImmTAV-Env (1000 pM) and pan T cells (blue) at an E:T of 5:1 with both Ag+ PLC/PRF/5-A2B2M (red, indicated with arrow) and Ag- HepG2-A2B2M (yellow) cells, and where activated caspase 3/7 is shown in green. (C&D) Number of apoptotic PLC/PRF/5-A2B2M target cells in co-cultures with PBMC from HBV-infected donors at a 10:1 E:T ratio with ImmTAV-Env was captured by IncuCyte assay. Ag- HepG2-A2B2M with the highest concentration of ImmTAV-Env was included as a control. (C) Data represent mean \pm SD of a representative donor of 4 donors tested, and (D) the number of apoptotic cells per area (mm^2) at 72 h for all 4 donors is plotted as mean \pm SEM of triplicates, where each donor is represented by a unique symbol. The donor shown in C is represented by the square symbols.

Video 1 – Confocal imaging of polyclonal T cells co-cultured with a mixture of Ag⁺ and Ag⁻ target cells in the presence of 1000 pM ImmTAV-Env. Images were taken at 15-minute intervals after addition of ImmTAV-Env.

Video 2 – Confocal imaging of polyclonal T cells co-cultured with a mixture of Ag⁺ and Ag⁻ target cells in the absence of ImmTAV-Env. Images were taken at 15-minute intervals.

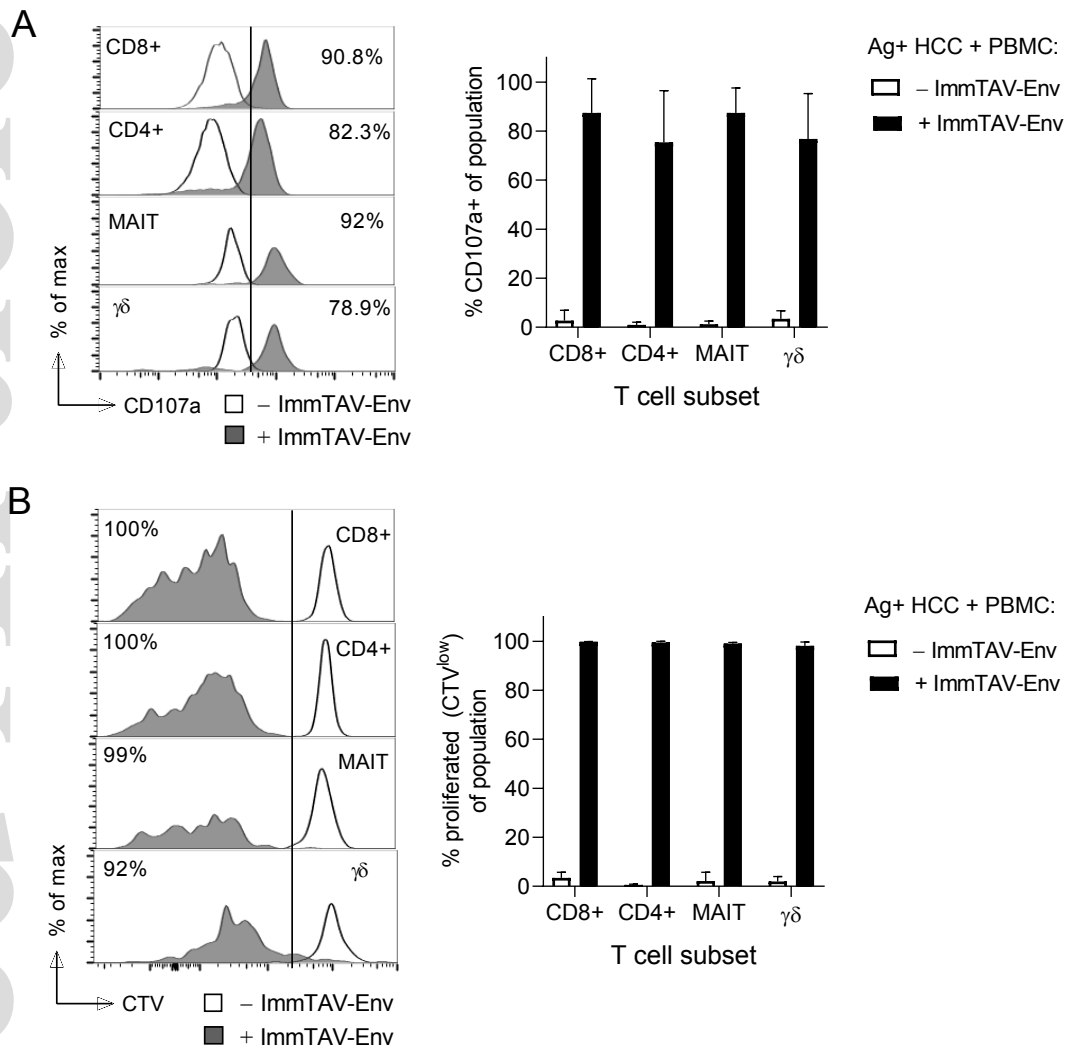


Figure 4. ImmTAV-Env redirects activation of various T cell subsets.

Flow cytometric analysis of (A) degranulation (CD107a⁺) and (B) proliferation (CTV dilution) induced by 100 pM ImmTAV-Env in different T cell subsets from healthy blood in response to PLC/PRF/5-A2B2M after 48 or 120 hrs, respectively. Representative histograms and total percentages are shown for each subset, with gating strategy shown in Supplementary Figure 1. CD107a positive and CTV^{low} gates were set according to no ImmTAV-Env controls (line) and applied to samples with ImmTAV-Env (grey filled).

Data are plotted as mean ± SEM of 4 donors.

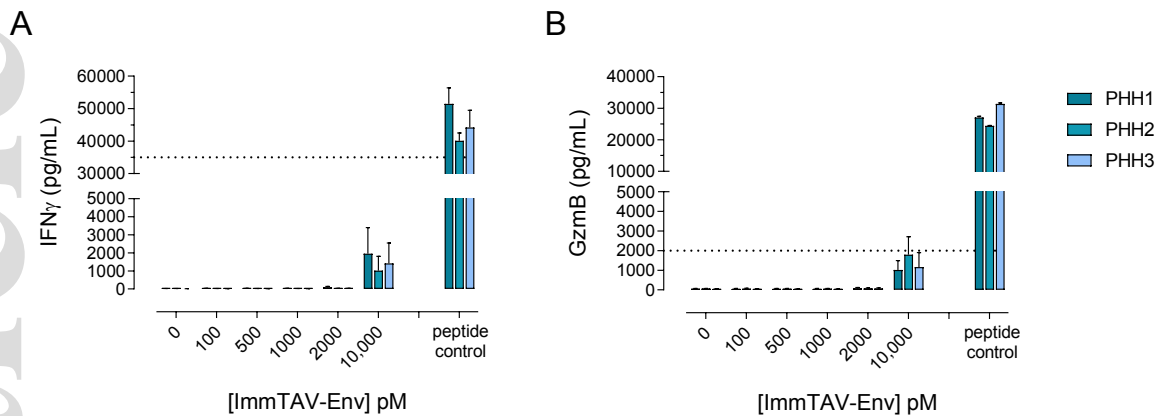


Figure 5 – ImmTAV-Env does not induce cross-reactive responses towards healthy primary human hepatocytes

HLA-A*02:01-positive PHH from 3 healthy donors were incubated with PBMC alone (0 pM) or with PBMC and increasing concentrations of ImmTAV-Env for 48 h before levels of **(A)** IFN γ and **(B)** GzmB were quantified in culture supernatants by MSD. As a positive control, 10 μ M Env peptide was added to PHH with 2000 pM ImmTAV-Env. Data represent the mean \pm SEM of triplicates (n=3) and the dotted line indicates the upper limit of quantification of the MSD; values above this were extrapolated from the standard curve.

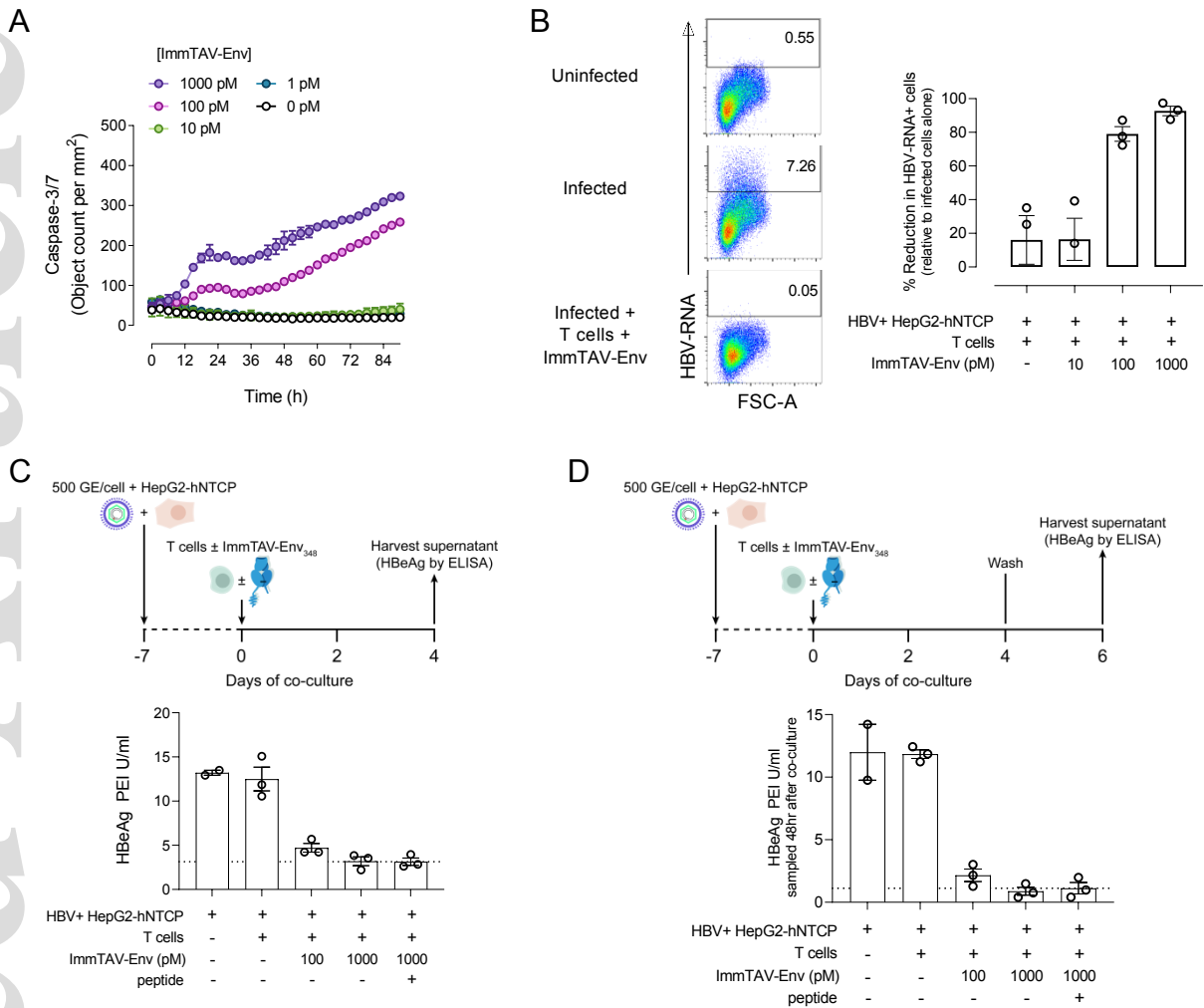


Figure 6 – ImmTAV-Env redirects T cells to eliminate HBV-infected HepG2-hNTCP cells

HepG2-hNTCP cells infected with 500 genome equivalents (GE) per cell HBV for 7 days before addition of PBMC (10:1 ratio) and various concentrations of ImmTAV-Env. **(A)** The number of apoptotic cells per area (mm^2) was determined every 3 h for 4 days by IncuCyte assay. Data represent mean \pm SD of a representative donor of 3 donors tested. **(B)** The percentage of HBV-RNA expressing targets cells after 4 days of co-culture with pan T cells was quantified by PrimeFlow and shown as representative dot plots. From this, the percentage reduction in infected cells was calculated and plotted as mean \pm SEM. **(C & D)** Levels of HBeAg in the supernatants were quantified by ELISA after a 4-day co-culture with pan T cells and ImmTAV-Env. Supernatants were sampled either at the end of co-culture **(C)** or following a further 48 h culture after washing **(D)** according to the schematics shown. Data represent mean \pm SEM ($n=3$). Dashed lines indicate HBeAg level in peptide controls where 10 μM of Env peptide was added to co-cultures with 1000 pM ImmTAV-Env.

Footnote Page

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List of abbreviations: TCR, T cell receptor; SPR, surface plasmon resonance; scFv, single chain variable fragment; HLA, human leukocyte antigen; Ag⁺, antigen-positive; Ag⁻, antigen-negative; NA, nucleos(t)ide analogue polymerase inhibitor

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Disclosure of potential conflicts of interest

JRF, ZW, MMC, APW, RJS, DWH, CB, WB, BSC, SC, MD, ND, JD, LFG, TG, DH, MLM, CP, AS, FUS, PKS, BT, AV, AW, SL, KW, LD, RS and AK are or were employees of Immunocore Ltd. MKM's lab has received unrestricted funding from Gilead, Roche and Immunocore. MKM has sat on advisory boards/provided consultancy for Gilead, Hoffmann La Roche, Immunocore, VIR, Galapagos NV, GSK, Abbvie, Freeline.

A patent has been filed on the sequence and utility of the ImmTAV-Env molecules described in this study.

Author contributions

Study design or conception: JRF, ZW, MMC, APW, DWH, CB, BSC, KS, BT, SL, MKM, KW, LD, RS and AK; Performed experiments and / or data analysis: JRF, ZW, MMC, APW, RJS, DWH, CB, BSC, WB, SC, MD, JD, LFG, TG, DH, MLM, CP, AS, FUS, PKS, KAS, AV, AW, SL, MKM, KW, LD, RS and AK; Methodology: JRF, ZW, DWH, BSC, ND, KAS, AW, SL, MKM, KW, LD, RS and AK Manuscript writing and preparation: JRF, APW, MLM, LD, RS and AK; Project coordination: SL, MKM, KW, LD, RS and AK