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Immune-Mobilizing Monoclonal T Cell Receptors Mediate Specific and Rapid Elimination of Hepatitis B–Infected Cells

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BACKGROUND AND AIMS: Therapies for chronic hepatitis B virus (HBV) infection are urgently needed because of viral integration, persistence of viral antigen expression, inadequate HBV-specific immune responses, and treatment regimens that require lifelong adherence to suppress the virus. Immune mobilizing monoclonal T Cell receptors against virus (ImmTAV) molecules represent a 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 AND 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 toward 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 hybridization of viral RNA assay. Here, we demonstrate that picomolar concentrations of ImmTAV-Env can redirect T cells from healthy and HBV-infected donors toward hepatocellular carcinoma (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 induced cytolysis of antigen-positive HCC cells and cells infected with HBV *in vitro*, causing a reduction of hepatitis B e antigen 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 trials. (HEPATOLOGY 2020;72:1528-1540).

Hepatitis B virus (HBV) is a significant cause of morbidity and mortality, with ~250-340 million chronic carriers of the virus worldwide.[1] Achieving a sterilizing cure is challenging because of the persistence of covalently closed circular DNA (cccDNA) or integrated HBV DNA, the transcriptional templates of HBV antigens, in the

Abbreviations: Ag^+, antigen positive; Ag^-, antigen negative; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; EC_{50}, half-maximal effective concentration; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immunospot; Env, Envelope; GzmB, granzyme B; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; hNTCP, human sodium taurocholate cotransporting polypeptide; IFNγ, interferon-gamma; ImmTAV, immune mobilizing monoclonal T Cell receptors against virus; IL, interleukin; MAIT, mucosal associated invariant T; MSD, Meso Scale Discovery; NA, nucleos(t)ide analogue polymerase inhibitor; PBMCs, peripheral blood mononuclear cells; PHHs, primary human hepatocytes; Pol, Polymerase; scFv, single-chain variable fragment; SPR, surface plasmon resonance; TCR, T Cell receptor.

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nucleus of hepatocytes. The elimination of viral protein production is an important treatment goal given that the continuous secretion of high levels of HBV antigens, including the envelope protein hepatitis B surface antigen (HBsAg), is thought to play a key role in host immunosuppression and immune tolerance of chronic hepatitis B (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; (2) long-term treatment with nucleos(t)ide analogue (NA) polymerase inhibitors, which do not accelerate elimination of the viral reservoir and all have inherent limitations, including the emergence of drug resistance, requirement for lifelong adherence, and related safety concerns associated with long-term use.[6,7] Therefore, there is an urgent need for 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, whereas 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, first, to specifically lyse infected cells and, second, to secrete cytokines that inhibit viral replication and even silence or destabilize cccDNA.[9,10] However, HBV-specific T cells are rare and functionally exhausted in CHB patients, whereas circulating T cells primed against non-HBV antigens are largely unaffected.[3,11] Therefore, strategies that can harness the potential of polyclonal T cells, independently of endogenous HBV-specific T cells, are an attractive approach.

Immune mobilizing monoclonal T cell receptors against virus (ImmTAV) molecules are soluble, bispecific T Cell-engaging fusion proteins comprised of an affinity-enhanced T Cell receptor (TCR), specific for a viral peptide epitope presented in the context of the HLA class I allele molecules on the surface of cells, fused to a humanized anti-CD3 single-chain antibody variable

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fragment (scFv; Fig. 1A). The TCR portion is affinity enhanced to be able to detect very low levels of antigen, whereas the anti-CD3 domain enables the recruitment and activation of endogenous T cells, independently of specificity, to release both cytokines and cytolytic mediators toward antigen-positive cells. This represents an 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 T cell from human immunodeficiency virus (HIV)-infected cells *in vitro* and is currently being used in other therapeutic areas to redirect T cells against cancer (immune mobilizing monoclonal T Cell receptors 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 therapeutic approach for the treatment of CHB.

Materials and Methods

CELL LINES AND CELL CULTURE

T2 cells (174xCEM.T2; CRL-1992) and HepG2 (HB-8065) were purchased from American Type Culture Collection (ATCC; Manassas, VA), and PLC/PRF/5 (85061113) were supplied by Public Health England. T2 were cultured in Roswell Park Memorial Institute (RPMI) media, PLC/PRF/5 A2B2M in Dulbecco's modified Eagle's medium (DMEM), and HepG2 in Eagle's minimum essential medium (all Gibco, Thermo Fisher Scientific, Waltham, MA). All media were supplemented with 10% foetal calf serum and 1% (v/v) penicillin/streptomycin, with RPMI also supplemented with 2 mM of L-glutamine. HLA-A*02:01/β2M (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

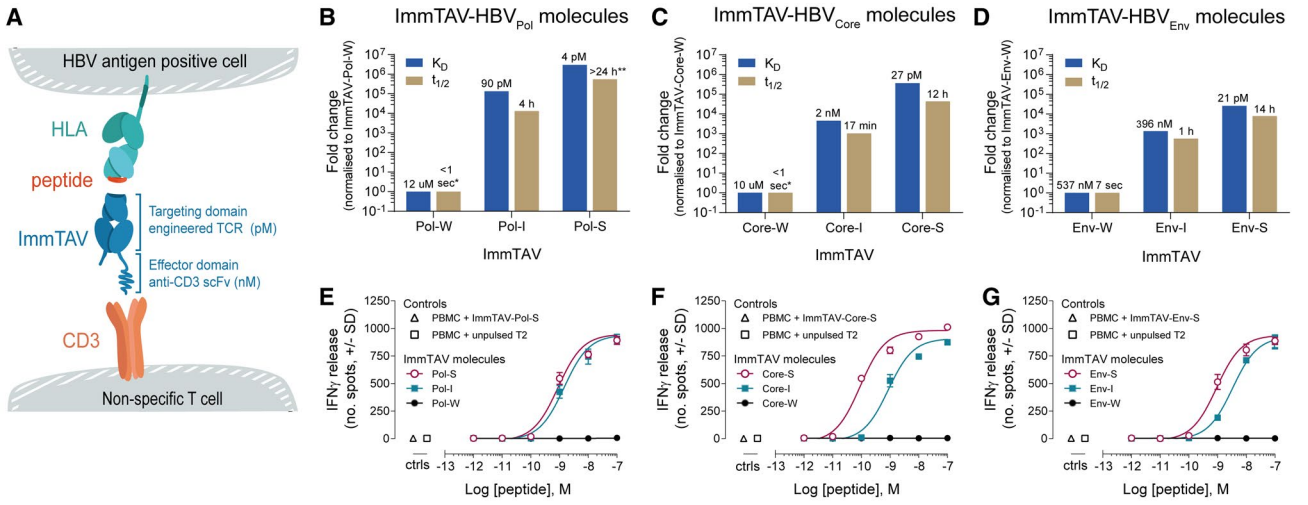

FIG. 1. Affinity enhancement increases the kinetics and potency of ImmTAV molecules. (A) Schematic of T Cell redirection using ImmTAV molecules. (B-D) Affinity (K_D) and half-life ($t_{1/2}$) of (B) ImmTAV-Pol, (C) ImmTAV-Core, and (D) ImmTAV-Env molecules were determined by SPR. Molecules were classified as either weak (-W), intermediate (-I), or strong (-S) affinity according to affinity and half-life measurements. Fold change was determined by normalizing 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 second and ~24 hours, respectively. (E-G) IFNγ ELISpot assays showing activation of PBMCs by 100 pM of 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 PBMCs incubated with T2 without peptide (unpulsed) in the absence of ImmTAV molecules (□) and PBMCs incubated with ImmTAV-x-S alone (Δ). Data points represent mean ± SD. Line of best fit and EC_{50} values were calculated by nonlinear regression log (agonist) versus response (three parameters). Abbreviation: ctrls, controls.

(Teddington, UK) and Mycoplasma Experience Ltd (Redhill, UK), respectively.

PRIMARY CELLS

HLA-A*02:01-positive primary human hepatocytes (PHHs) 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 peripheral blood mononuclear cells (PBMCs) were purchased from Discovery Life Sciences (Huntsville, AL) or StemCell Technologies (Grenoble, France). For assays using purified T cells as effectors, whole blood was obtained from healthy volunteers, PBMCs 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 PBMCs from CHB donors on NAs were obtained from Tissue Solutions and BioIVT (West Sussex, UK).

GENERATION OF ImmTAV MOLECULES

ImmTAV molecules targeting HBV antigens Polymerase (Pol), Core, and Envelope (Env) were prepared as described.[12-15] Briefly, wild-type TCRs specific for an HBV antigen (pol, core, and 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 humanized CD3-specific scFv by a flexible linker. The alpha and beta chains of the resulting ImmTAV were expressed in *Escherichia coli* as inclusion bodies, refolded, and purified as described.[14]

SURFACE PLASMON RESONANCE

Purified ImmTAV molecules were subjected to surface plasmon resonance (SPR) analysis using either a BIAcore T200 (for weak-affinity molecules) or a BIAcore 8K system (for intermediate- to strong-affinity molecules) (GE Healthcare, Chicago, IL). Briefly, biotinylated cognate peptide-HLA complexes were immobilized 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 were analyzed using a 1:1 binding model (GraphPad Prism [v8.3.0] (GraphPad Software, San Diego, CA) for steady-state affinity analysis and Biacore Insight Evaluation [v2.0.15.12933] (Cytiva, Marlborough, CA) for single-cycle kinetics analysis).

ENZYME-LINKED IMMUNOSPOT ASSAYS

Interferon-gamma (IFNγ) and granzyme B (GzmB) enzyme-linked immunospot (ELISpot) assays were performed according to the manufacturer's recommendations (BD Biosciences, Wokingham, UK) after culture for 24 or 48 hours, respectively. For peptide-pulsing experiments, T2 target cells were incubated with peptide (Peptide Synthetics, Fareham, UK) for 2 hours before plating. A total of 30,000 PBMCs 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, Shaker Heights, OH).

IncuCyte KILLING ASSAY

In the IncuCyte S3 Live-Cell Analysis System (Essen Bioscience Ltd., Royston Hertfordshire, UK), target cells were stained with CellTracker Deep Red Dye (Invitrogen, Carlsbad, CA). PBMCs 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 hours. In peptide control wells, 10 μM of Env peptide was added for the duration of the assay. The number of apoptotic events/mm^2 was calculated from two-color images.

Opera Phenix KILLING ASSAY

Assays were performed as for IncuCyte assays, with the exception that Hoechst stain (ThermoFisherScientific) was added for nuclear discrimination and apoptosis was imaged using the Opera Phenix High Content Screening

System and analyzed with the Harmony High-Content Imaging and Analysis software (PerkinElmer, Waltham, MA). Percent cytolysis was calculated from three-color images normalized 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

Supernatants were analyzed for the presence of cytokines using a V-PLEX proinflammatory kit (Meso Scale Technologies, Rockville, MD), following the manufacturer's recommendations. In certain cases, increasing concentrations of dexamethasone (Sigma-Aldrich, now Merck Life Science UK, Gillingham, UK) were added to cocultures for the duration of the assay. A vehicle control, representing the highest proportion of solvent (H_2O) added, was included as the 0-μM dexamethasone condition. Healthy PBMCs were added at a ratio of 10:1 with either 10,000 PLC/PRF/5-A2B2M or 50,000 PHHs. For cocultures with PHHs, IFNγ and GzmB in supernatants were quantified using a custom U-plex assay (Meso Scale Technologies, LLC).

FLOW CYTOMETRY

To detect degranulation of T cells, CD107a-AlexaFluor647 (BioLegend, San Diego, CA) was added at the start of coculture and proliferation was detected by labeling PBMCs with CellTrace Violet (Invitrogen). T Cell subsets were gated using Zombie Live Dead dye (BioLegend) and the antibodies detailed in Supporting Table S1. Data were acquired on a Sony MA900 (Sony Biotechnology, San Jose, CA) and analyzed by FlowJo (v10; TreeStar Inc., Ashland, OR).

HBV INFECTION

HepG2-hNTCP were infected with 500 genome equivalents per cell of HBV (ImQuest Biosciences, Frederick, MD) overnight in media plus 4% polyethylene glycol and 2.75% dimethyl sulfoxide (DMSO; v/v). Media without DMSO was changed every other day for 7 days before addition of effectors and ImmTAV-Env in media alone. Hepatitis B e antigen (HBeAg) in supernatants was quantified by HBeAg enzyme-linked immunosorbent assay (ELISA; Autobio Diagnostics, China) as per the manufacturer's instructions.

PrimeFlow

HBV RNA was stained following the manufacturer's protocol with PrimeFlow probeset VF1-6000704 (ThermoFisherScientific). Live cells were distinguished by fixable viability dye eFluor 780 (eBioscience, San Diego, CA) and effector T cells by anti-CD45-PECy7 (BioLegend). Cells were acquired on the MACSQuant X flow cytometer (Miltenyi Biotec) and analyzed by FlowJo (v10; TreeStar Inc.). 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:

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

Results

ImmTAV MOLECULES WITH PICOMOLAR AFFINITY TCRs CAN BE ENGINEERED AGAINST HBV

Highly conserved and previously characterized 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 Env, Core, and Pol and were presented by hepatocellular carcinoma (HCC) 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 that described for naturally occurring viral and cancer-specific TCRs (nM to μM),[17] as determined by 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- to 10^6-fold with final ImmTAV-x-S molecules reaching picomolar affinity with half-lives extending beyond 12 hours (Fig. 1B-D).

The potency of each molecule to redirect T Cell responses against target cells presenting their cognate epitope was tested by measuring IFNγ release upon coculture with peptide-pulsed T2 antigen-presenting cells. No responses were detected with weak-affinity ImmTAV molecules, even at high peptide concentrations (Fig. 1E-G). However, the affinity-enhanced molecules induced robust responses, with half-maximal effective concentration (EC_{50}) 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 it 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 persons supports natural presentation of the epitope.[21] Therefore, ImmTAV-Env-S molecules (hereafter referred to as ImmTAV-Env) were prioritized 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 β2 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 (Supporting Table S2), and the introduction of HLA-A2 and B2M enabled epitope presentation on HLA-A*02:01. In coculture, 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 IFNγ and GzmB release (Fig. 2A,B). Responses toward PLC/PRF/5-A2B2M were induced at low picomolar concentrations of ImmTAV-Env, with an EC_{50} of 36.4 pM for IFNγ release. In contrast, no responses were observed toward HLA-A2-transduced antigen negative (Ag^-) HepG2 cells (HepG2-A2B2M), even at high concentrations of ImmTAV-Env (Fig. 2A,B). In addition to IFNγ release, high levels of interleukin (IL)-2 and proinflammatory cytokines tumor necrosis factor alpha (TNFα) and IL-6 were also detected in supernatants from coculture with PLC/PRF/5-A2B2M (Fig. 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 ~90% for all cytokines tested at ≥50 μM of dexamethasone. IL-6 was the most sensitive to corticosteroid treatment, with effects observed at concentrations as low as 0.1 μM of dexamethasone (Fig. 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, PBMCs were cocultured 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 ≥5 pM (Fig. 3A). Killing was observed from as early as 12 hours of coculture, with maximum cytolysis achieved by 72 hours at concentrations >50 pM of ImmTAV-Env. No cytolysis of Ag^- HepG2-A2B2M was detected at any concentration of ImmTAV-Env, unless cognate peptide was added (data not shown).

Specificity of killing was further demonstrated by coculture 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

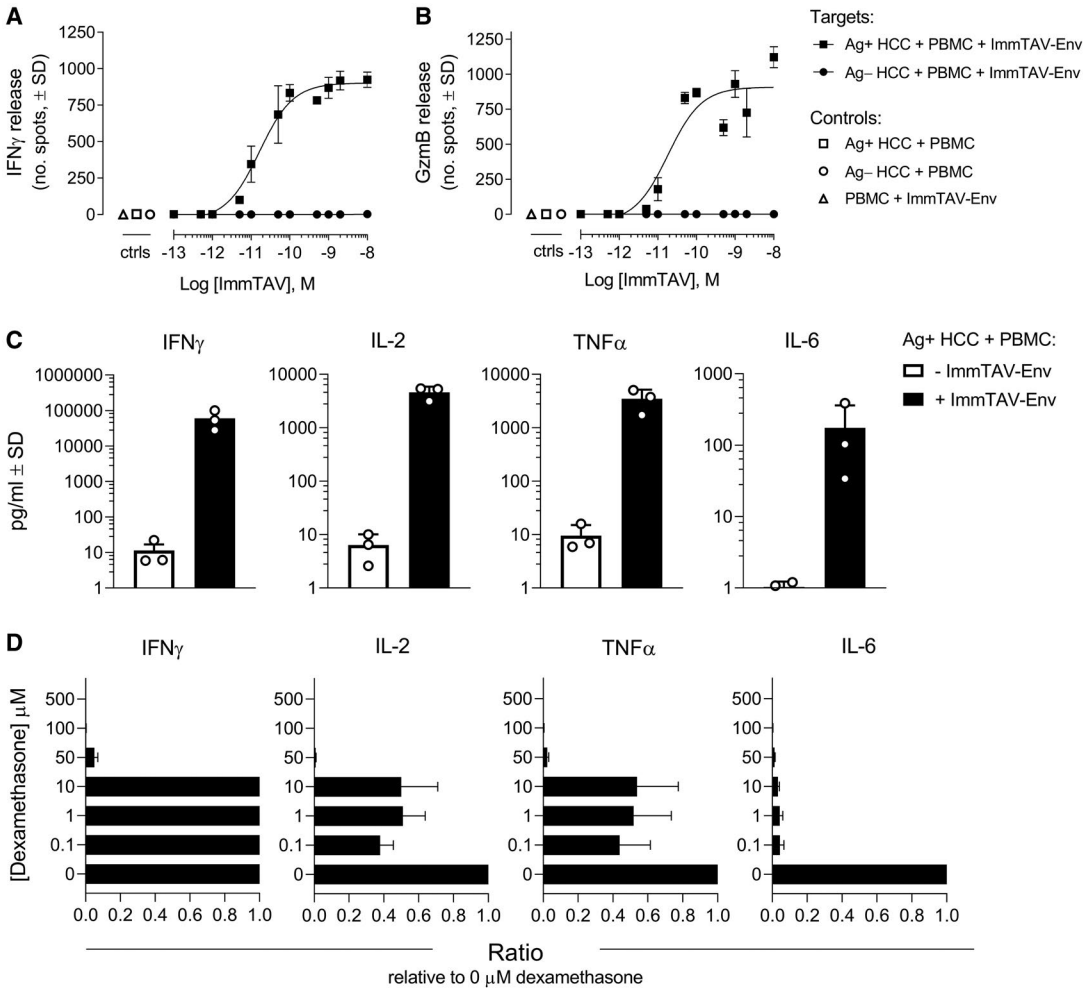

FIG. 2. ImmTAV-Env redirects activation of polyclonal T cells specifically toward antigen-positive cell lines. ELISpot assays of (A) IFNγ and (B) GzmB release from PBMCs in the presence of PLC/PRF/5-A2B2M (Ag^+) or HepG2-A2B2M (Ag^-) in the absence (ctrls; open symbols) or presence of ImmTAV-Env (closed symbols). PBMCs incubated with ImmTAV-Env alone were included as controls (Δ). Graphs shown are representative from 1 of 3 donors tested. (C,D) Levels of IFNγ, IL-2, TNFα, and IL-6 in supernatants from cocultures of PBMCs and PLC/PRF/5-A2B2M (10:1 ratio) in the presence or absence of 100 pM of ImmTAV-Env were assessed after 24 hours by MSD (n = 3). (D) Increasing concentrations of dexamethasone were added to cocultures and the ratio of each cytokine calculated relative to 0 μM of dexamethasone. Data represent mean ± SEM (n = 3). Abbreviation: ctrls, controls.

coculture in the presence of ImmTAV-Env, whereas Ag^- targets remained viable (Fig. 3B; Supporting Videos S1 and S2), demonstrating the ability of ImmTAV-Env to redirect specific lysis of Ag^+ targets even within a heterogeneous population.

Given that these assays were performed using PBMCs 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 (Fig. 3C,D). These data demonstrate that non-HBV-specific T cells from chronically infected persons have lytic capacity when redirected by ImmTAV-Env toward antigen-expressing HCC cells.

ImmTAV-Env REDIRECTS ACTIVATION OF DIFFERENT SUBSETS OF EFFECTOR T CELLS

Although $CD8^+$ T cells play a major role in killing virus-infected cells, successful antiviral immune

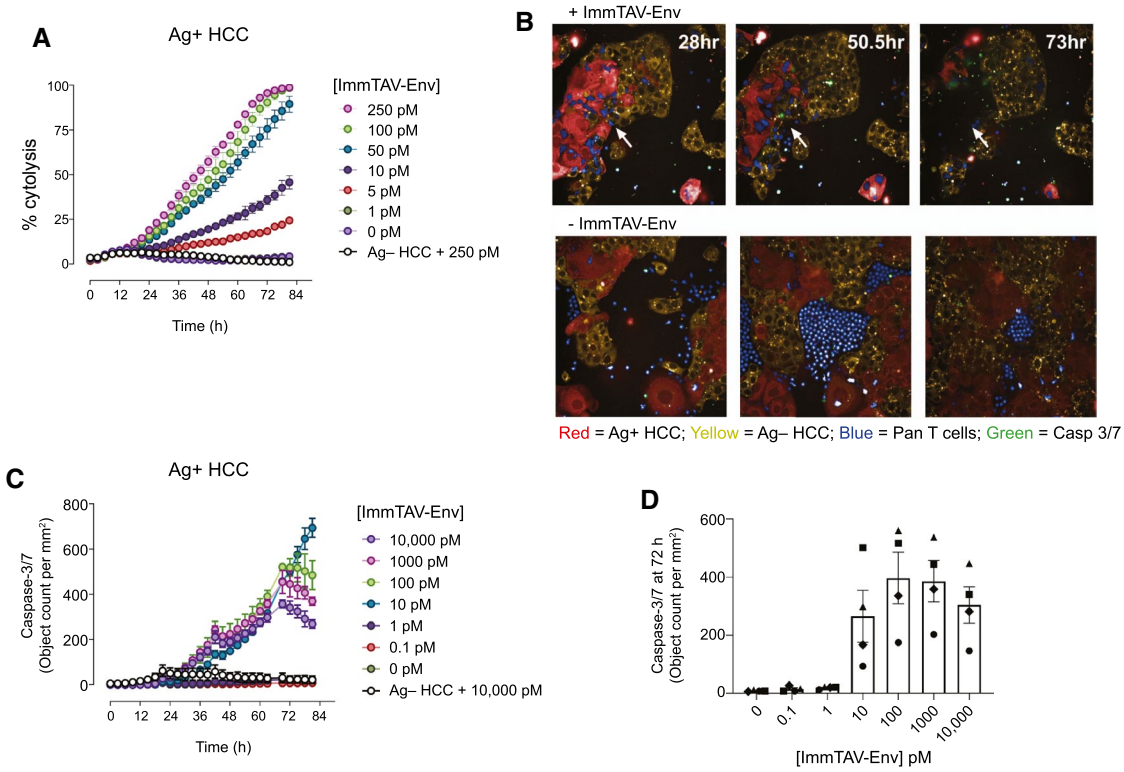

FIG. 3. ImmTAV-Env redirects healthy and CHB donor T cells to lyse antigen-positive HCC cell lines. (A) Percentage cytolysis of PLC/PRF/5-A2B2M target cells in cocultures with healthy PBMCs at an E:T ratio of 10:1 with various concentrations of ImmTAV-Env was captured by Opera Phenix killing assay. Ag^- HepG2-A2B2M with the highest concentration of ImmTAV-Env was included as a control. Data represent mean ± SD from a representative donor of 3 donors tested. (B) Confocal images at indicated time points after addition of ImmTAV-Env (1,000 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 cocultures with PBMCs 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 ± SD of a representative donor of 4 donors tested, and (D) the number of apoptotic cells per area (mm^2) at 72 hours for all 4 donors is plotted as mean ± SEM of triplicates, where each donor is represented by a unique symbol. The donor shown in (C) is represented by the square symbols. Abbreviation: Casp3/7, caspase-3/7.

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 (Fig. 4 and Supporting Fig. S1). Upon coculture, ImmTAV-Env induced both the degranulation, as measured by CD107a expression (Fig. 4A), and proliferation (Fig. 4B) of $CD8^+$, $CD4^+$, mucosal associated invariant T (MAIT), and γδ 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 TOWARD 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 PHHs in the presence of ImmTAV-Env. After a 48-hour coculture, ImmTAV-Env did not induce IFNγ or GzmB responses at concentrations below 10,000 pM (Fig. 5A,B), demonstrating that healthy PHHs

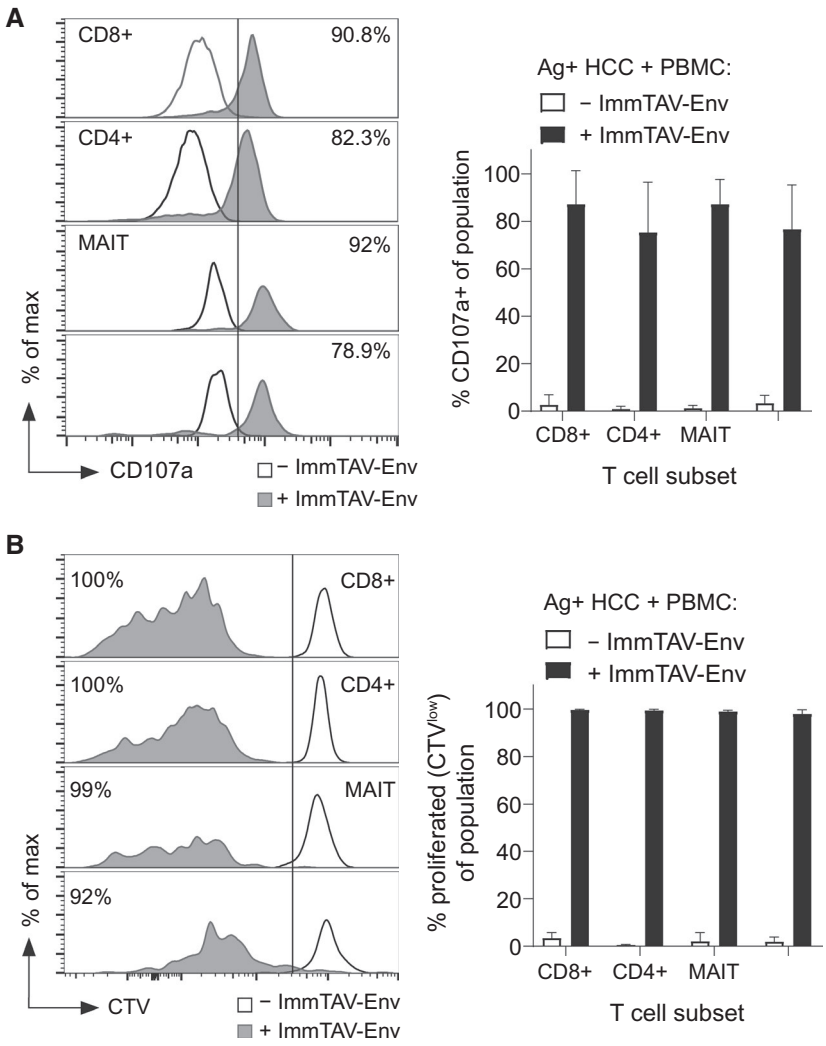

FIG. 4. ImmTAV-Env redirects activation of various T Cell subsets. Flow cytometric analysis of (A) degranulation ($CD107a^+$) and (B) proliferation (CTV dilution) induced by 100 pM of ImmTAV-Env in different T Cell subsets from healthy blood in response to PLC/PRF/5-A2B2M after 48 or 120 hours, respectively. Representative histograms and total percentages are shown for each subset, with gating strategy shown in Supporting Fig. S1. CD107a-positive and CTV^{low} gates were set according to no ImmTAV-Env controls (line) and applied to samples with ImmTAV-Env (gray filled). Data are plotted as mean ± SEM of 4 donors. Abbreviation: CTV, CellTrace Violet.

do not present peptides that could sensitize them to off-target killing by ImmTAV-Env at concentrations shown to induce IFNγ and GzmB release and on-target killing (Figs. 2 and 3).

ImmTAV-Env Mediates Rapid Cytolysis of HBV-Infected Cell Lines

To test the efficacy of ImmTAV-Env against HBV-infected targets, we utilized an HBV-permissive HepG2 cell line, which was transduced with the HBV entry receptor human sodium taurocholate cotransporting polypeptide (hNTCP; HepG2-hNTCP) and expresses natural levels of HLA-A*02:01.[24] Infected HepG2-hNTCP were incubated with PBMCs in the presence of ImmTAV-Env and target cell lysis quantified over a 4-day period. At 100 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 hours (Fig. 6A). Similar responses were also detected when purified T cells were used as

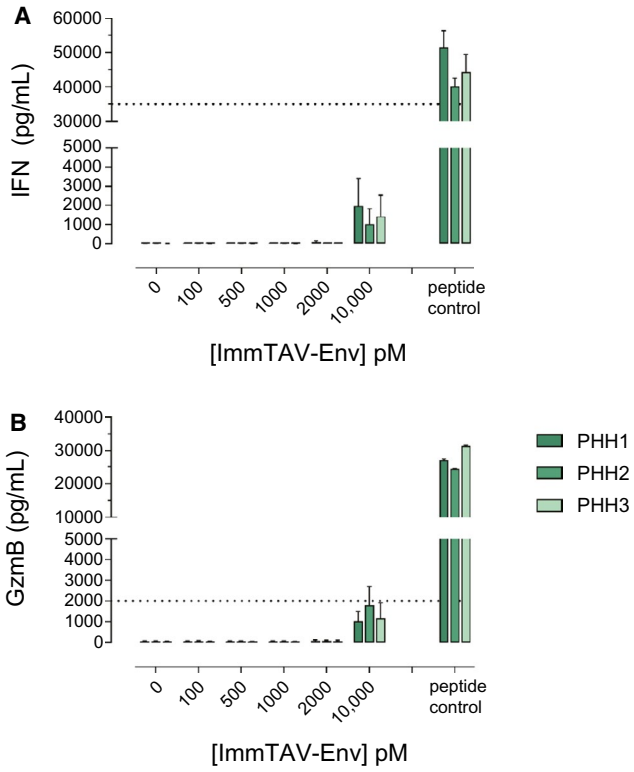

FIG. 5. ImmTAV-Env does not induce cross-reactive responses toward healthy PHHs. HLA-A*02:01-positive PHH from 3 healthy donors were incubated with PBMCs alone (0 pM) or with PBMCs and increasing concentrations of ImmTAV-Env for 48 hours before levels of (A) IFNγ and (B) GzmB were quantified in culture supernatants. As a positive control, 10 μM of Env peptide was added to PHHs with 2,000 pM of ImmTAV-Env. Data represent the mean ± SEM of triplicates (n = 3), and the dotted line indicates the upper limit of quantification; values above this were extrapolated from the standard curve.

the effector population and correlated with release of adenylate kinase, a marker of cell death, in the supernatant (Supporting Fig. S2).

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* hybridization (Fig. 6B). After coculture with purified T cells, 100 pM of ImmTAV-Env induced an 87% reduction in the percentage of HBV-infected cells and up to 97% reduction was observed with 1,000 pM, consistent with data obtained from the IncuCyte killing assay in Fig. 6A. In parallel, HBeAg released into the supernatant during cocultures was markedly decreased in the presence of ImmTAV-Env (Fig. 6C), and reduced production was maintained following a further 48-hour incubation after coculture (Fig. 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 recognize 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 virally infected cells, within hours of coculture. While HCC cells were transduced for HLA-A*02:01 presentation, infected HepG2-hNTCP presented an 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, given that Ag^- HCC cell lines and, importantly, PHHs 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

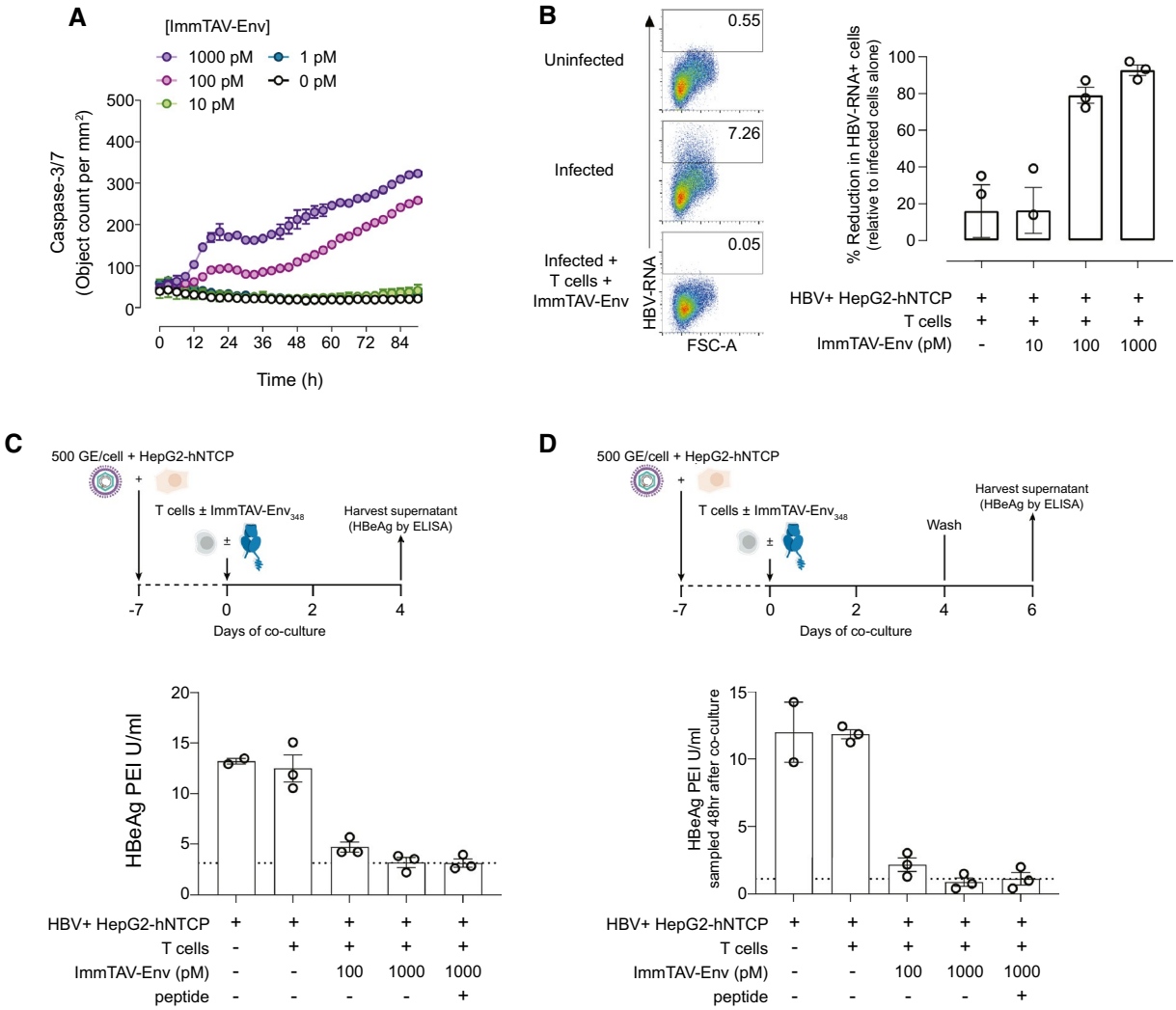

FIG. 6. ImmTAV-Env redirects T cells to eliminate HBV-infected HepG2-hNTCP cells. HepG2-hNTCP cells infected with 500 genome equivalents (GE) per cell of HBV for 7 days before addition of PBMCs (10:1 ratio) and various concentrations of ImmTAV-Env. (A) The number of apoptotic cells per area (mm^2) was determined every 3 hours for 4 days by IncuCyte assay. Data represent mean ± SD of a representative donor of 3 donors tested. (B) The percentage of HBV-RNA expressing targets cells after 4 days of coculture 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 ± SEM. (C,D) Levels of HBeAg in supernatants were quantified by ELISA after a 4-day coculture with pan T cells and ImmTAV-Env. Supernatants were sampled either at the end of coculture (C) or following a further 48-hour culture after washing (D) according to the schematics shown. Data represent mean ± SEM (n = 3). Dashed lines indicate HBeAg level in peptide controls where 10 μM of Env peptide was added to cocultures with 1,000 pM of ImmTAV-Env. Abbreviations: FSC-A, forward scatter area; PEI, Paul Ehrlich Institute.

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. The populations tested here were 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, IFNγ and TNFα may limit HBV gene expression and replication through noncytolytic mechanisms that target cccDNA; TNFα and IL-2 have been shown to reduce HBV mRNA through posttranscriptional 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. Although noncytolytic 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 toward HBV proteins such as HBsAg has been demonstrated to eliminate infected hepatocytes in mice through cytolytic and noncytolytic mechanisms.[35,36] However, results were mixed because of possible interference from the high levels of circulating HBsAg, which may sequester 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 tumor seeding in xenograft models and confer antiviral activity in HBV-infected humanized mice.[37,38] However, success in humans has been limited given that this approach offers only transient effects owing to the use of mRNA transduction of T cells related 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. The data presented here supports the entry of our lead ImmTAV-Env molecule, IMC-I109V, into clinical trials.

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