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HBsAg-redirected T cells exhibit antiviral activity in HBV-infected human liver chimeric mice

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Abstract

BACKGROUND AIMS—Chronic hepatitis B virus (HBV) infection remains incurable. While HBsAg-specific chimeric antigen receptor (HBsAg-CAR) T-cells have been generated, they have not been tested in animal models with authentic HBV infection.

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Additional methods are provided in the supplementary materials.

Disclosure of Interest:

RLK, TS, SG and KDB have filed a patent application based on this work with Baylor College of Medicine. All other authors declare no conflict of interest.

METHODS—We generated a novel CAR targeting HBsAg, and evaluated its ability to recognize HBV+ cell lines and HBsAg particles *in vitro*. *In vivo*, we tested whether human HBsAg-CAR T-cells would have efficacy against HBV-infected hepatocytes in human liver chimeric mice.

RESULTS—HBsAg-CAR T-cells recognized HBV-positive cell lines and HBsAg particles *in vitro* as judged by cytokine production. However, HBsAg-CAR T-cells did not kill HBV-positive cell lines in cytoxicity assays. Adoptive transfer of HBsAg-CAR T-cells into HBV-infected humanized mice resulted in accumulation within the liver and a significant decrease in plasma HBsAg and HBV-DNA levels in comparison to control mice. Notably, the fraction of HBV core-positive hepatocytes among total human hepatocytes was greatly reduced after HBsAg-CAR T-cell treatment, pointing to non-cytopathic viral clearance. In agreement, changes in surrogate human plasma albumin levels were not significantly different between treatment and control groups.

DISCUSSION—HBsAg-CAR T-cells have anti-HBV activity in an authentic preclinical HBV infection model. Our results warrant further preclinical exploration of HBsAg-CAR T-cells as immunotherapy for HBV.

Keywords

adoptive immunotherapy; CAR T cells; hepatitis B virus

INTRODUCTION

Hepatitis B virus (HBV) is a global pandemic chronically infecting 300 million people across the world today [1]. In these chronic patients, HBV causes a lifelong infection that can lead to liver cirrhosis or cancer in 25% of patients [2]. HBV therapies currently remain limited to reverse transcriptase inhibitors (RTIs) and interferon (IFN)-α. RTIs only suppress HBV-DNA levels without significantly affecting the transcriptional template, covalently closed circular DNA (cccDNA) [3] while IFN-α causes significant side effects with little long-term therapeutic benefit [4]. Thus, new anti-HBV therapies are urgently needed in order to cure the virus.

During the HBV-specific immune response in acutely resolving patients, infiltrating T-cells rapidly purge the liver of HBV [5,6] In chronic HBV patients, however, HBV-specific T-cells are present in only low frequency and/or are anergic [7]. CD8-positive T-cells have been shown to be crucial in resolution of acute HBV infection [6]. They are able to clear HBV in both cytolytic and noncytolytic effector functions [8]. The cytokines, IFN-γ and tumor necrosis factor (TNF)-α, released by T-cells are important in driving noncytolytic suppression of virus [9,10]. Both IFN-γ and TNF-α can induce degradation of intracellular cccDNA [11] explaining part of this mechanism.

The adoptive transfer of T-cells genetically engineered to target hepatitis B surface antigen (HBsAg) on the surface of infected hepatocytes [12] with chimeric antigen receptors (HBsAg-CAR T-cells) is an attractive strategy to reconstitute HBV-specific T-cell immunity. Indeed, HBsAg-CAR T-cells have been shown to eliminate cccDNA from HBV-infected primary hepatocytes *in vitro* [13], and had transient anti-HBV activity in a transgenic HBV mouse model [14]. The HBV transgenic mouse model harbors an integrated copy of the

HBV genome [15], is born with tolerance to viral antigens, and lacks cccDNA formation. Thus, transgenic HBV mice can only model viral suppression and not complete T-cell mediated cure. Given that HBV only naturally infects humans and chimpanzees at high levels [16], finding appropriate models to test cure strategies is challenging. With previous testing only in transgenic mice, it remains an open question whether HBsAg-CAR T-cells can induce a reduction of HBV levels in a model with authentic infection harboring episomal HBV cccDNA. Here we address this question by evaluating human HBsAg-CAR T-cells in HBV-infected human liver chimeric mice. These mice are immunodeficient and repopulated with human hepatocytes [17,18], allowing for spreading infection with HBV entry and cccDNA formation [19]. Thus, this model closely mimics HBV infection, and is ideal to test the ability of HBsAg-CAR T-cell to eradicate HBV genomes and/or infected hepatocytes.

RESULTS

Generation of a novel CAR targeting HBsAg

We first generated two HBsAg-CARs with a CD28.δ signaling domain and a single chain variable fragment (scFv) derived from the human monoclonal antibody (mAb) 19.79.5, which recognizes HBsAg from different serotypes [20], and has undergone successful Phase 1 testing [21]. Since the length of the spacer region of CARs is critical for their function [22], we first compared long and intermediate spacers. The IgG4 Fc domain with mutated Fc receptor binding sites (HBs-G4m-CAR) served a long [22] and the CH3 domain of IgG1 as an intermediate spacer (HBs-CH3-CAR; Figure 1A). As a control, we constructed a G4m-CAR with an scFv specific for an irrelevant antigen (EGFRvIII [23]; Ctrl-G4m-CAR; Figure 1A). CAR T-cells were generated by retroviral transduction, and the median transduction efficiency was 79.0% (range 60.5-89.9) as judged by FACS analysis with no significant differences between CAR constructs (Figure 1B).

HBs-G4m-CAR T-cells recognize HBV-positive cells *in vitro*

To determine which HBs-CAR recognized HBV-positive cells, we performed 24-hour co-culture assays with HepG2 (HBV-negative) and HepG2.2.15 (HBV-positive) cell lines, washing the cells first before adding CAR-T cells. Only HBs-G4m-CAR T-cells produced significant amounts of IFN-γ in the presence of HepG2.2.15 in contrast to HBs-CH3-CAR and Ctrl-G4m-CAR T-cells (Figure 1C). HepG2 induced only background IFN-γ production confirming specificity. These results demonstrate that a long spacer is needed for CARs with a mAb 19.79.5-derived HBsAg binding domain. In addition to IFN-γ, HBs-G4m-CAR T-cells also produced IL-2 (Figure 1D) and TNF-α (Figure 1E) in the presence of HepG2.2.15 in contrast to Ctrl-G4m-CAR T-cells. Having established that HBs-G4m-CAR T-cells recognize HepG2.2.15 in an HBsAg-restricted fashion, we performed standard cytotoxicity assays with HepG2 and HepG2.2.15 (Figure 1F,G). Only background killing of HepG2.2.15 by HBs-G4m-CAR T-cells was observed.

HBs-G4m-CAR T-cells recognize HBsAg particles *in vitro*

To determine if HBs-G4m-CAR T-cells recognize HBsAg particles, 24-hour co-culture assays were performed with media supernatants derived from HepG2 and HepG2.2.15 cell

lines, the latter containing 80 ng/mL HBsAg. Only HBs-G4m-CAR T-cells secreted significant amounts IFN-γ in the presence of HepG2.2.15-conditioned media in contrast to HBs-CH3-CAR or Ctrl-G4m-CAR T-cells (Supplementary Figure S1A). We confirmed T-cell recognition of HBsAg particles by performing FACS analysis for the T-cell activation markers CD25 and CD69 (Supplementary Figure S2). To acquire more evidence that HBsAg particles bind to HBs-G4m-CAR T-cells, electron microscopy of HepG2.2.15-conditioned media was performed 24 hours after exposure to HBs-G4m-CAR or Ctrl-G4m-CAR T-cells. While HepG2.2.15-conditioned media exposed to Ctrl-G4m-CAR T-cells contained abundant HBsAg particles, there was a significant reduction in the number of viral particles after exposure to HBs-G4m-CAR T-cells (Supplementary Figure S1B,C). Thus, HBsAg particles produced by HepG2.2.15 can bind to HBs-G4m-CAR T-cells and potentially inhibit CAR-T targeting or killing of infected cells.

HBs-G4m-CAR T-cells have anti-HBV activity in HBV-infected human liver chimeric mice

We next tested HBs-G4m-CAR T-cell therapy in human liver chimeric FRG mice [17,18], which can replicate HBV (Figure 2A) [19]. Mice received 2×10^7 HBs-G4m-CAR (n=4) or Ctrl-G4m-CAR T-cells (n=3) intraperitoneal, and HBV-DNA and HBsAg levels were measured for five weeks. There was an average 3.0-fold decrease in HBV-DNA levels after 36 days in mice treated with HBs-G4m-CAR T-cells (Figure 2B), whereas HBV-DNA levels increased an average 5.6-fold in mice that received Ctrl-G4m-CAR T-cells. The decrease in HBV-DNA levels was mirrored by an average 4.7-fold decrease in HBsAg levels in HBs-G4m-CAR T-cell-treated mice, and an average 4.6-fold increase in Ctrl-G4m-CAR T-cell-treated mice (Figure 2C, Supplementary Figure S3A,B). There was no significant difference in plasma human albumin levels between groups, indicating that the anti-HBV activity of HBs-G4m-CAR T-cells is noncytolytic toward infected hepatocytes (Figure 2D, Supplementary Figure S3C).

To examine liver sections for the presence of T-cells and HBV directly after T-cell infusion, mice received a second dose of 2×10^7 HBs-G4m-CAR or Ctrl-G4m-CAR T-cells, and were euthanized 10 days post injection. There was a 70.4% decrease in HBV-core staining among human albumin-positive hepatocytes in HBs-G4m-CAR T-cell-treated mice in comparison to control mice (Figure 2E,F), indicating clearance of HBV through noncytopathic mechanisms, similar to studies in chimpanzees [24]. This finding was confirmed by staining for human nuclei (Figure 2E,G). Human T-cells could only be detected in livers of mice treated with HBs-G4m-CAR T-cells in contrast to livers of Ctrl-G4m-CAR T-cell-treated mice (Figure 3), indicating that HBs-G4m-CAR T-cells traffic to livers to exert their anti-HBV activity. At the conclusion of the experiment, we did not detect long-term persistence of CAR T-cells in the liver by immunohistochemistry for CD3 (data not shown).

DISCUSSION

In this study, we demonstrate that HBs-G4m-CAR T-cells are effective in reducing HBV-DNA and HBsAg levels in HBV-infected human liver chimeric mice [19], which possess cccDNA transcriptional templates. After CAR T-cell therapy, a portion of human hepatocytes was also found to have histologically absent HBV core expression,

demonstrating clearance without destruction. However, we did not observe complete elimination of HBV, which is most likely due to the limited persistence of human CAR T-cells in immunodeficient mouse models [25], possibly facilitated in immunodeficient mice on the FRG background by lack of murine SIRPα engagement [26], as opposed to NOD background models [27].

Despite this limitation, HBs-G4m-CAR T cells had superior anti-HBV activity than HBV entry inhibitors [28] that did not reduce the number of HBV-infected hepatocytes in a humanized mouse model of established HBV infection. This finding also extends the work of the previous HBV-CAR publication in transgenic mice, which was unable to assess if HBV could be cleared from hepatocytes, since every cell had integrated virus allowing HBV core expression to quickly return [14]. Our results with HBsAg-CAR T cells were similar to HLA-restricted TCR-redirected T-cells in HBV-infected human liver chimeric mice [29], which observed transient reduction in serum viral markers. In contrast, their study found increases in liver enzymes resembling the administration of PBMCs from HBV-seropositive donors [30]. This suggests that T-cells targeting an extracellular and secreted HBsAg have different effector profiles versus targeting viral peptides on HLA proteins.

Since HBsAg-CAR T-cell therapy employs a different mechanism to eradicate HBV than currently approved therapies, there is an opportunity to explore combinatorial approaches. For example, combining CAR T-cells with RTIs may prevent rebound of viral DNA levels post CAR T-cell therapy observed in our study. Furthermore, hepatitis B immunoglobulin administration prior to CAR T-cell infusion may be useful to reduce serum HBsAg levels. Intriguingly, we found the mouse with the lowest initial HBsAg levels had the greatest HBsAg and DNA knockdown (−1.65 log and −3.67 log, respectively), suggesting some inhibitory role of HBsAg, or that higher disease burden may be too great for CAR T-cells. Future work testing mice with different HBsAg levels will be needed to examine this question. In addition, future studies are needed to characterize the phenotype of HBs-G4m-CAR T cells, and the expression of exhaustion markers (PD-1, LAG3, TIM3) pre-and post-stimulation with an extended panel of HBsAg-positive target cells.

In conclusion, HBs-G4m-CAR T cells are effective in reducing HBV levels in plasma and tissue in HBV-infected human liver chimeric mice. Thus, further preclinical exploration of our approach to HBV-targeted CAR T-cell therapy is warranted.

METHODS

Generation of retroviral vectors encoding CARs

To generate pSFG-HBs-G4m-28-zeta, a scFv encoding the amino acid sequence VH and VL domains of the XTL-19 antibody (mAb 19.79.5) [20] termed HBs was synthesized (IDTDNA, Coralville, IA), and cloned into an SFG retroviral vector using 5′ NcoI and 3′ BamHI, replacing the antigen binding domain from a second generation CAR vector, IL13Rα2-hIgG1-CD28-zeta CAR [31]. Next, the hIgG1 hinge was replaced with a mini-gene (synthesized by IDTDNA, Coralville, IA) encoding the CH2-CH3 domain from human IgG4 with mutated Fc receptor binding sites [22] (G4m) to generate pSFG-HBs-G4m-28-zeta. To generate pSFG-Ctrl-G4m-28-zeta, the HBs-specific scFv in pSFG-HBs-G4m-28-

zeta was replaced by PCR cloning with an scFv specific for EGFRvIII [23]. pSFG-HBs-CH3-28-zeta was generated by cloning the HBs-specific scFv into the 5′ NcoI and 3′ BamHI sites of a pSFG vector with a CAR.IgG1 CH3. 28-zeta expression cassette (gift of Dr. Maksim Mamonkin, Baylor College of Medicine, Houston, TX). Cloning was verified by sequencing (Lone Star Labs, Houston TX). RD114-pseudotyped retroviral particles were generated by transient transfection of 293T cells as previously described [32].

Generation of CAR-T cells

To generate CAR-T cells, PBMCs were isolated by Lymphoprep (Greiner Bio-One, Monroe, NC) gradient centrifugation and then stimulated on treated non-tissue culture 24-well plates, which were pre-coated with OKT3 (CRL-8001, ATCC) and CD28 (BD Bioscience, Mountain View, CA) antibodies. Recombinant human interleukin-7 and interleukin-15 (IL-7, 10 ng/mL; IL-15, 5ng/mL; PeproTech, Rocky Hill, NJ) were added to cultures on day 2. On day 3, OKT3/CD28 stimulated T-cells (2.5×10^5 cells/well) were transduced on RetroNectin® (Clontech, Mountainview, CA) coated plates in the presence of IL-7 and IL-15. On day 5 or 6, T-cells were transferred into tissue culture plates and subsequently expanded with IL-7 and IL-15. CAR expression was determined 4 to 5 days post transduction by FACS analysis using a human IgG (H+L) antibody (Jackson Immunoresearch, West Grove, PA), and appropriate isotype control.

Cell culture assays

HepG2.2.15 cells (gift of Dr. Betty Slagle, Baylor College of Medicine, Houston, TX) and HepG2 cells (ATCC, Manassas, Virginia) were cultured in Dulbecco's minimal essential media (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Gibco ThermoFisher, Waltham, MA). For co-culture assays, 1 million HepG2 or HepG2.2.15 cells were plated; 24-hours later cells were washed and 2 million T-cells were added in one well of a 24-well plate in duplicate without exogenous cytokines. After 24 hours, supernatant was removed in order to assess cytokine release. For cytokine ELISAs, concentrations of INF-γ were assessed using manufacturer's protocol (R&D Systems, Minneapolis, MN). For IL-2 and TNF-α, cytokine levels were determined using a multiplex assay (Millipore, St. Charles, MO). For cytotoxicity assays, standard chromium release protocols were followed as previously described [31] utilizing the same 2:1 ratio of T-cell to target cell, wherein target cells were plated on the same day as T-cell addition preventing any HBsAg particle accumulation. Cell culture assays were repeated with at least 3 different donors.

For cytokine assays based on co-culture with HBV particles, the HepG2.2.15 supernatant was collected (measured to be 80 ng/mL using HBsAg ELISA protocol below). Culture media contained 50% HepG2.2.15 supernatant with 1 million CAR-T cells. At 24 hours, supernatant was collected for use in cytokine ELISA assays and electron microscopy particle counts. T-cells were also collected at this time point for analysis by flow cytometry using established protocols [31] and staining cells with anti-CD25-PE (BD Biosciences, San Jose, CA) and anti-CD69-APC (BD Biosciences, San Jose, CA). Flow cytometric data were acquired by Gallios (Beckman Coulter, Brea, CA) and analyzed using FlowJo ver.10 (FlowJo, Ashland, Oregon).

Animal experiments

Fah−/−Rag2−/−Il2-rγ−/−(FRG) mice were repopulated with human cadaveric hepatocytes, as described previously [17,19]. For the current experiment, all human liver chimeric mice were repopulated with hepatocytes from the same hepatocyte donor and lot in order to minimize differences. Furthermore, at the initiation of cell injection, mice were kept on 100% NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione) in order to stabilize the levels of human hepatocytes, and prevent ongoing destruction of murine hepatocytes and proliferation of human hepatocytes, that otherwise might contribute to either increasing HBV levels, or the loss of cccDNA after hepatocyte mitosis.

One million genome equivalents of HBV genotype D (serotype ayw) were inoculated intraperitoneal into humanized FRG mice as previously described [19]. The infection was allowed to spread over 1-2 months until mice reached high levels of HBV infection, monitored by qPCR and HBsAg ELISA. HBsAg-CAR or Ctrl-CAR T-cells were injected intraperitoneal into humanized mice. Mice were monitored with retro-orbital bleeds collected into EDTA containing tubes, and plasma collected after centrifugation for 30 minutes at 2.3G. Collected plasma was frozen until further use for HBV-DNA, HBsAg, and human albumin analysis.

Plasma analysis

Plasma HBsAg levels was quantified using commercially available ELISA reagents (International Immuno Diagnostics, Foster City, CA) and HBsAg standards (Alpha Diagnostic International, San Antonio, TX). Plasma HBV DNA levels were determined by quantitative PCR as previously described [33]. Human plasma albumin levels were assessed by ELISA (Bethyl laboratories, Montgomery, TX) and was performed according to manufacturer’s protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

HBV	Hepatitis B virus
RTIs	reverse transcriptase inhibitors
IFN	interferon
TNF	tumor necrosis factor

cccDNA	covalently closed circular DNA
HBsAg	hepatitis B surface antigen
HBsAg-CAR T-cells	T-cells genetically engineered to target HBsAg with chimeric antigen receptors
HBs-G4m-CAR	CAR-T cell targeted HBsAg with an IgG4 Fc domain with mutated Fc receptor binding sites as the spacer domain
HBs-CH3-CAR	CAR-T cell targeted HBsAg with the CH3 domain of IgG1 as an spacer domain
Ctrl-G4m-CAR	CAR-T cell target to EGFRvIII with an IgG4 Fc domain with mutated Fc receptor binding sites as the spacer domain
FRG	*Fah−/−Rag2−/−Il2-rγ−/−*mouse strain used for human liver repopulation
NOD	non-obese diabetic mouse background
TCR	T-cell receptor
HLA	human leukocyte antigen, presenting antigens on the major histocompatibility complex in human cells
VL	variable light domain of antibody
VH	variable heavy domain of antibody
DMEM	Dulbecco’s minimal essential media
NTBC	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione

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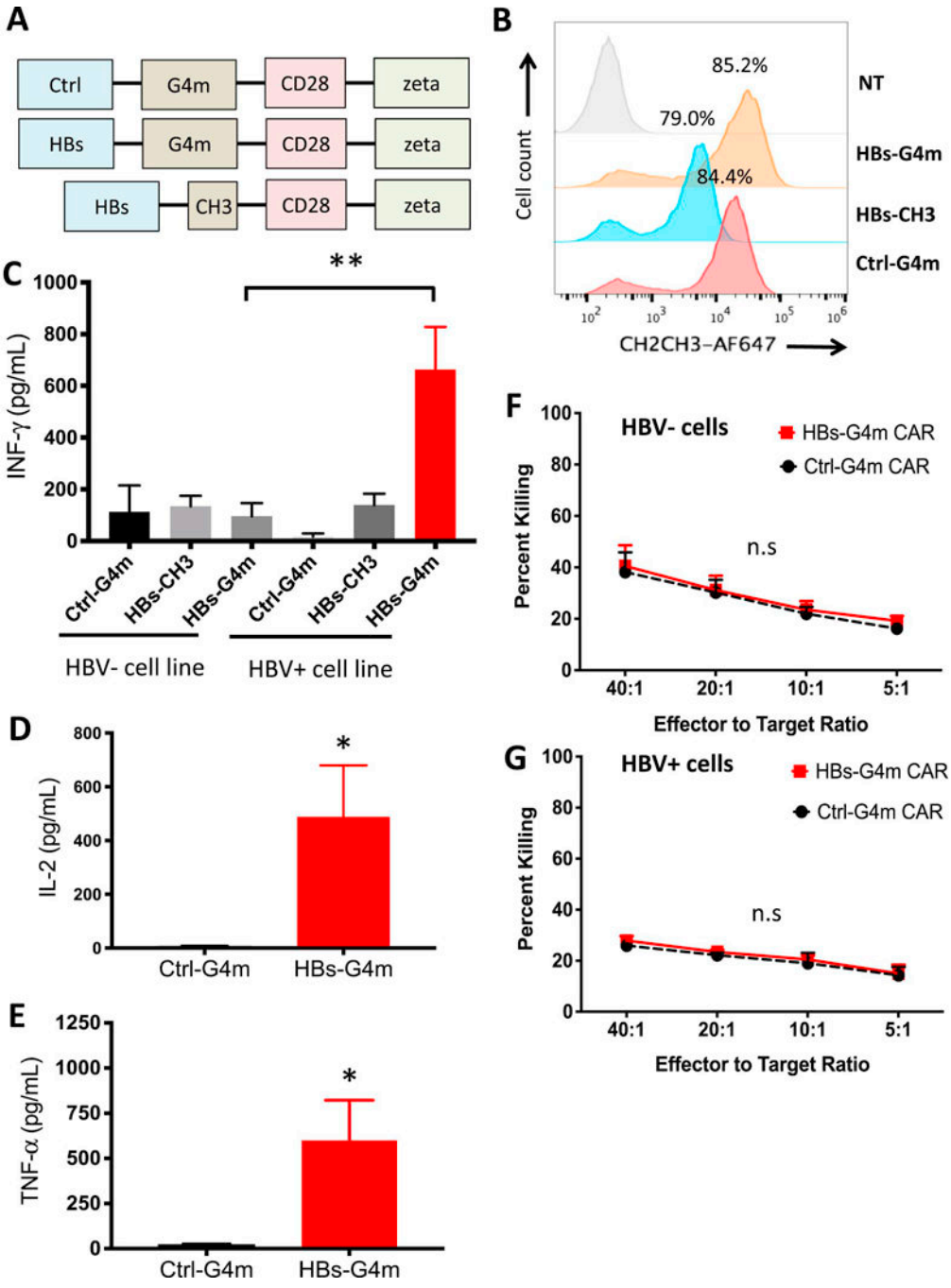

Figure 1. Generation and functional characterization of HBsAg-CAR T-cells

(**A**) Scheme of HBs-G4m, HBs-CH3, and Ctrl-G4m CAR constructs. (**B**) Representative FACS analysis of HBs-G4m-CAR (orange), HBs-CH3-CAR (blue), and Ctrl-G4m-CAR T-cells (red) confirming CAR expression (gray: non-transduced T-cells, NT). CAR-T cells were co-cultured with HBV+ or HBV-cell lines. Cytokine production, (**C**) IFN-γ, (**D**) IL-2, and (**E**) TNF-α, was measured by ELISA after 24 hours (for IFN-γ: **p<0.01, n=4; for IL-2, and TNF-α: *p<0.05, n=3). CAR-T cells were tested in a 5-hour chromium release

assay against (**F**) HBV-or (**G**) HBV+ cell lines (n.s.: not significant, n=3). Error bars represent S.E.M. and significance is determined by unpaired, one-tailed t-tests.

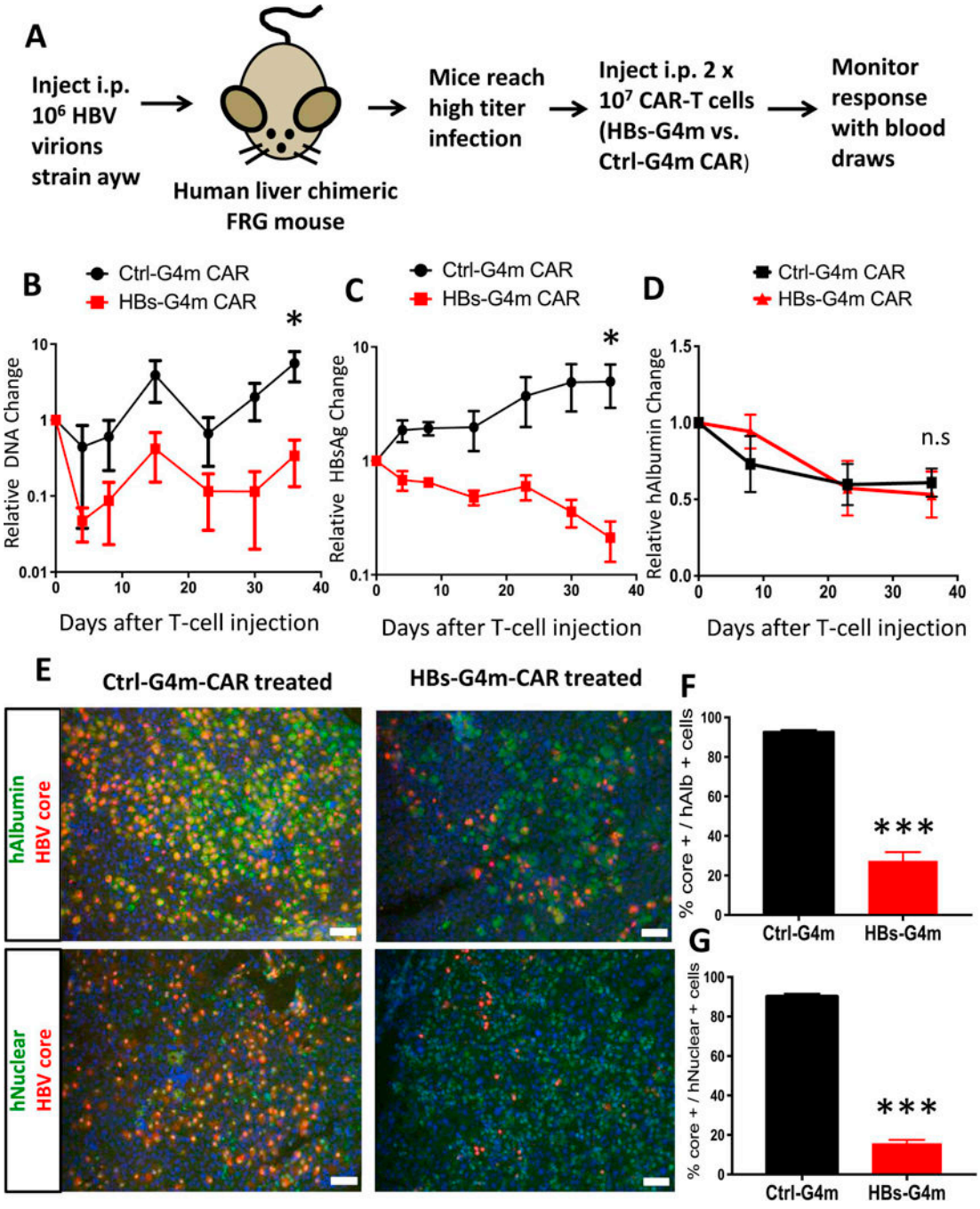

Figure 2. HBs-G4m-CAR T-cells have anti-HBV activity *in vivo*

(**A**) Scheme of animal experiment. Median pre-treatment values were 17,221 ng/mL HBsAg, and 6.04×10^7 copies/ml HBV DNA. Serial monitoring of (**B**) HBsAg, (**C**) HBV-DNA, and (**D**) human albumin (hAlbumin) in plasma of infused mice. Data points were normalized to values pre T-cell injection in individual mice and averaged together. Average value +/− SEM is depicted; n=4 for HBs-G4m-CAR T-cell group; n=3 for Ctrl-G4m-CAR T-cell group; *p<0.05, n.s.: not significant). (**E**) Immunofluorescence of liver sections for hAlbumin (green), HBV-Core (red), and DAPI (blue) or human nucleus (green), HBV-Core (red), and

DAPI (blue), scale bar=50 μm. (**F,G**) HBV-Core+, hAlbumin+, and human nuclear+ hepatocytes were quantified. Ratio of HBV-Core+ to hAlbumin+, and HBV-Core+ to human nuclear+ cells is shown (***$p<0.0001$, n=4 fields). Unpaired, two-tailed t-tests determined significance.

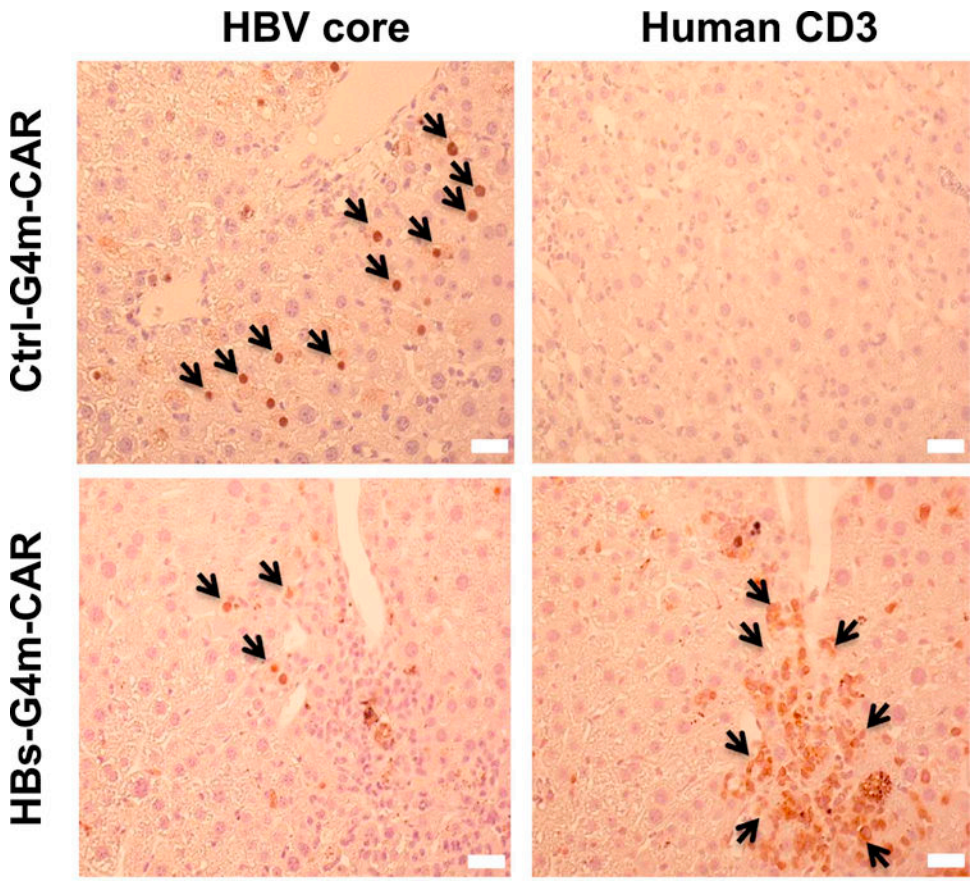

Figure 3. HBs-G4m-CAR T-cells redirected to HBsAg accumulate in the liver of HBV-infected humanized mice

Localization of Ctrl-G4m-CAR and HBs-G4m-CAR T-cells in human liver chimeric mice 10 days post injection was studied using serial sections of paraformaldehyde fixed, paraffin embedded liver tissue, stained for core protein to detect HBV-infected cells (left panels; brown, nuclear stain delineates HBV core protein) and human CD3 to detect human T-cells (right panels; brown, membranous stain delineates CD3 protein; scale bar=20 μm).