T Cells Expressing a Chimeric Antigen Receptor That Binds Hepatitis B Virus Envelope Proteins Control Virus Replication in Mice

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BACKGROUND & AIMS: Antiviral agents suppress hepatitis B virus (HBV) replication but do not clear the infection. A strong effector T-cell response is required to eradicate HBV, but this does not occur in patients with chronic infection. T cells might be directed toward virus-infected cells by expressing HBV-specific receptors and thereby clear HBV and help to prevent development of liver cancer. In mice, we studied whether redirected T cells can engraft after adoptive transfer, without prior T-cell depletion, and whether the large amounts of circulating viral antigens inactivate the transferred T cells or lead to uncontrolled immune-mediated damage. **METHODS:** $CD8^+$ T cells were isolated from mice and stimulated using an optimized protocol. Chimeric antigen receptors (CARs) that bind HBV envelope proteins (S-CAR) and activate T cells were expressed on the surface of cells using retroviral vectors. S-CAR–expressing $CD8^+$ T cells, which carried the marker CD45.1, were injected into $CD45.2^+$ HBV transgenic mice. We compared these mice with mice that received $CD8^+$ T cells induced by vaccination, cells that express a CAR without a proper signaling domain, or cells that express a CAR that does not bind HBV proteins (controls). **RESULTS:** $CD8^+$ T cells that expressed HBV-specific CARs recognized different HBV subtypes and were able to engraft and expand in immune-competent HBV transgenic mice. After adoptive transfer, the S-CAR–expressing T cells localized to and functioned in the liver and rapidly and efficiently controlled HBV replication compared with controls, causing only transient liver damage. The large amount of circulating viral antigen did not impair or overactivate the S-CAR–grafted T cells. **CONCLUSIONS: T cells with a CAR specific for HBV envelope proteins localize to the liver in mice to reduce HBV replication, causing only transient liver damage. This immune cell therapy might be developed for patients with chronic hepatitis B, regardless of their HLA type.**

Keywords: Immunotherapy; Chronic Hepatitis B; Hepatocellular Carcinoma; Adoptive T-Cell Therapy.

BASIC AND TRANSLATIONAL LIVER

Patients chronically infected with hepatitis B virus (HBV) are at high risk for developing cirrhosis and hepatocellular carcinoma (HCC), which lead to more than 0.5 million deaths per year.[1] Antiviral nucleos(t)ide analogues control but do not eradicate the virus because they do not target the nuclear persistence form of the virus, the covalently closed circular DNA (cccDNA).[2] The episomal HBV cccDNA serves as a transcription template and can cause a relapse of hepatitis B when pharmacological treatment ends.[3,4] During acute, self-limited hepatitis B, patients mount a strong T-cell response against multiple viral antigens[5–8] that is required to eliminate cccDNA-positive hepatocytes and to clear the virus.[9] Such a T-cell response is lacking in chronic infection.

The aim of immunotherapy against chronic hepatitis B is to restore efficient antiviral immune responses and complement pharmacological antiviral therapy to eliminate remaining infected cells. A promising immunotherapeutic approach is the adoptive transfer of genetically modified HBV-specific T cells. In infected cells, HBV envelope proteins are incorporated into endoplasmic reticulum membranes, where they either form (sub)viral particles or may reach the cell surface by physiological membrane exchange.[10] These (sub)viral particles can be detected in large amounts in sera of infected patients as hepatitis B surface antigen (HBsAg) and very likely contribute to induction of immune tolerance.[11] Because the expression of HBV surface proteins is not controlled by available antiviral agents and is usually maintained in HCC with integrated viral genomes, HBsAg remains positive under antiviral therapy, even in late stages of chronic hepatitis B in which HCC has developed. Targeting HBV surface proteins therefore seems most promising.

We have previously shown that expression of a chimeric antigen receptor (CAR) directed against the HBV surface proteins enables human T cells to kill HBV-infected human hepatocytes and to eliminate viral cccDNA in vitro.[12] On this basis, we here addressed the question

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Abbreviations used in this paper: ALT, alanine aminotransferase; CAR, chimeric antigen receptor; cccDNA, covalently closed circular DNA; CEA, carcinoembryonic antigen; DC, dendritic cells; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBVtg, hepatitis B virus transgenic; HCC, hepatocellular carcinoma; IFN, interferon; IL, interleukin; S-CAR, hepatitis B virus–specific chimeric antigen receptor; scFv, single-chain fragment variable; TNF, tumor necrosis factor.

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whether CAR-grafted, adoptively transferred T cells would retain their function in vivo and control virus replication without significant T cell–related toxicity in a model of persistent HBV infection in HBV transgenic (HBVtg) mice with a functional immune system.

Materials and Methods

Mice

C57BL/6 (CD45.1$^+$) and HBVtg HBV1.3xfs mice (HBV genotype D, serotype *ayw*[13], CD45.2$^+$) were bred in specific pathogen–free animal facilities. The study was conducted according to the German Law for the Protection of Animals.

Retroviral Transduction

CAR transgenes[12] were cloned into the retroviral vector MP71.[14] Plasmids were amplified using Stbl3 bacteria (Life Technologies, Darmstadt, Germany) and purified with a Midiprep Plasmid DNA Endotoxin-free Kit (Sigma-Aldrich, Taufkirchen, Germany). The packaging cell line Platinum-E[15] was transfected in a 6-well plate with 4 μg of plasmid DNA and 10 μL of Lipofectamine 2000 (Life Technologies). After 16 hours, the medium was replaced with 1.5 mL of T-cell medium. After 24 and 48 hours, the retrovirus supernatant was collected and filtered through a 0.45-μm filter. Splenocytes were isolated from CD45.1$^+$ C57BL/6 mice after lysis of red blood cells.

For in vitro assays, splenocytes were stimulated overnight at a density of 3×10^6 cells/mL with 10 ng/mL interleukin (IL)-2 (R&D Biosystems, Wiesbaden, Germany), 2 μg/mL anti-CD3, and 0.1 μg/mL anti-CD28 antibody (kindly provided by E. Kremmer, Helmholtz Zentrum München) and spinoculated on RetroNectin-coated plates (12.5 μg/mL; TaKaRa Bio Europe SAS, St. Germain en Laye, France) at 850*g* for 90 minutes at 32°C with retrovirus supernatant supplemented with IL-2 and 4 μg/mL protamine sulfate (Sigma-Aldrich).

For in vivo studies, CD8$^+$ T cells were positively selected with magnetic beads (MACS CD8a [Ly2] Microbeads; Miltenyi Biotec, Bergisch-Gladbach, Germany). A total of 1×10^6 CD8$^+$ T cells/well were stimulated overnight with 5 ng/mL IL-12 (see Supplementary Materials and Methods) on 24-well plates pre-coated with anti-CD3 and anti-CD28 antibodies at room temperature overnight (10 μg/mL phosphate-buffered saline [PBS]; eBioscience, Frankfurt, Germany). Fresh retrovirus supernatant was twice spinoculated onto CD8$^+$ T cells supplemented with protamine sulfate.

Isolation of Liver-Associated Lymphocytes

Livers were perfused with PBS to remove circulating leukocytes. Approximately two-thirds of the liver was mashed with 3 mL medium through a 100-μm cell strainer. Cells that passed were pulled through a 20-gauge needle and collected. The procedure was repeated twice, and then mononuclear cells were separated from other cells using a Ficoll gradient according to the manufacturer's instructions (Lymphoprep; PAA, Pasching, Austria). For cell type analysis, perfused livers were digested with 4500 U collagenase (Worthington, Lakewood, NJ) for 20 minutes at 37°C. Leukocytes were purified in an 80%/40% Percoll gradient (GE Healthcare, Uppsala, Sweden) at 1400*g* for 20 minutes.

Flow Cytometry

Staining was performed for 30 minutes on ice in the dark using primary antibodies (eBioscience) diluted in 0.1% bovine serum albumin/PBS. Transduction efficiency was assessed 1 day after the second transduction by staining the CAR with anti-human immunoglobulin G/fluorescein isothiocyanate antibody (Sigma-Aldrich). To assess cytotoxic degranulation, anti–CD107a-APC was added for 4 hours during incubation of T cells on HBsAg-coated or uncoated plates. For intracellular cytokine staining, Brefeldin A (1 μg/mL; Sigma-Aldrich) was added for 5 hours during antigen stimulation. Before phenotyping, cells were incubated with Fc-Block for 15 minutes (BD Biosciences, Heidelberg, Germany). After staining of dead cells with EMA (Life Technologies) and cell surface molecules, intracellular cytokines were stained using the Cytofix/Cytoperm Kit (BD Biosciences). Cells were analyzed using a FACSCanto II flow cytometer (BD Biosciences), and data were analyzed using FlowJo 9.2 software (Tree Star, Inc, Ashland, OR).

Adoptive Transfer

T cells were isolated, stimulated, and transduced for 3 days before transfer. The cells were then harvested and washed 2 times with ice-cold PBS (180*g*, 4°C, 8 minutes). CAR expression was determined by flow cytometry. The cell number was adjusted to 4×10^6 CAR$^+$ cells per animal dissolved in PBS and injected intraperitoneally. Mice were bled at indicated time points. Recipient mice were 16- to 24-week-old male animals. Groups of mice were matched for age and hepatitis B e antigen titers.

Statistical Analysis

Data are reported as mean values ± SEM. Groups were compared with the nonparametric Kruskal–Wallis test using Prism 5.0 (GraphPad Software, Inc, La Jolla, CA). A *P* value less than .05 was considered statistically significant. Additional methods are described in Supplementary Materials and Methods.

Results

Murine T Cells Redirected by an HBV-Specific CAR Acquire Properties of Fully Activated Effector T Cells

The HBV-specific chimeric antigen receptor (S-CAR) used in this study to redirect T cells contains a single-chain antibody fragment (scFv) that binds to the S domain of all 3 HBV envelope proteins (S, M, and L protein, combined as HBsAg). The scFv is linked to the CD3ζ and costimulatory CD28 signaling domains (Figure 1*A*), providing combined activation signals to T cells when recognizing cell surface–bound HBsAg. The aim of this is to overcome local hepatic coinhibitory signals.[11] A human carcinoembryonic antigen (CEA)-specific CAR served as a control for antigen-independent activation of grafted T cells. After transduction of T cells with CARs using retroviral vectors (Figure 1*B*), only S-CAR–transduced T cells produced high amounts of interferon (IFN)-γ and proliferated in an antigen-specific manner, that is, when cocultured with HBV-replicating human hepatoma cells but not with HBV-negative parental cells (Figure 1*C* and *D*). We observed mobilization of the lysosomal-associated membrane protein 1 on binding of S-CAR–grafted T cells to plate-bound HBsAg (Figure 1*E*), indicating release of cytotoxic granules. Notably, S-CAR–redirected T cells recognized surface

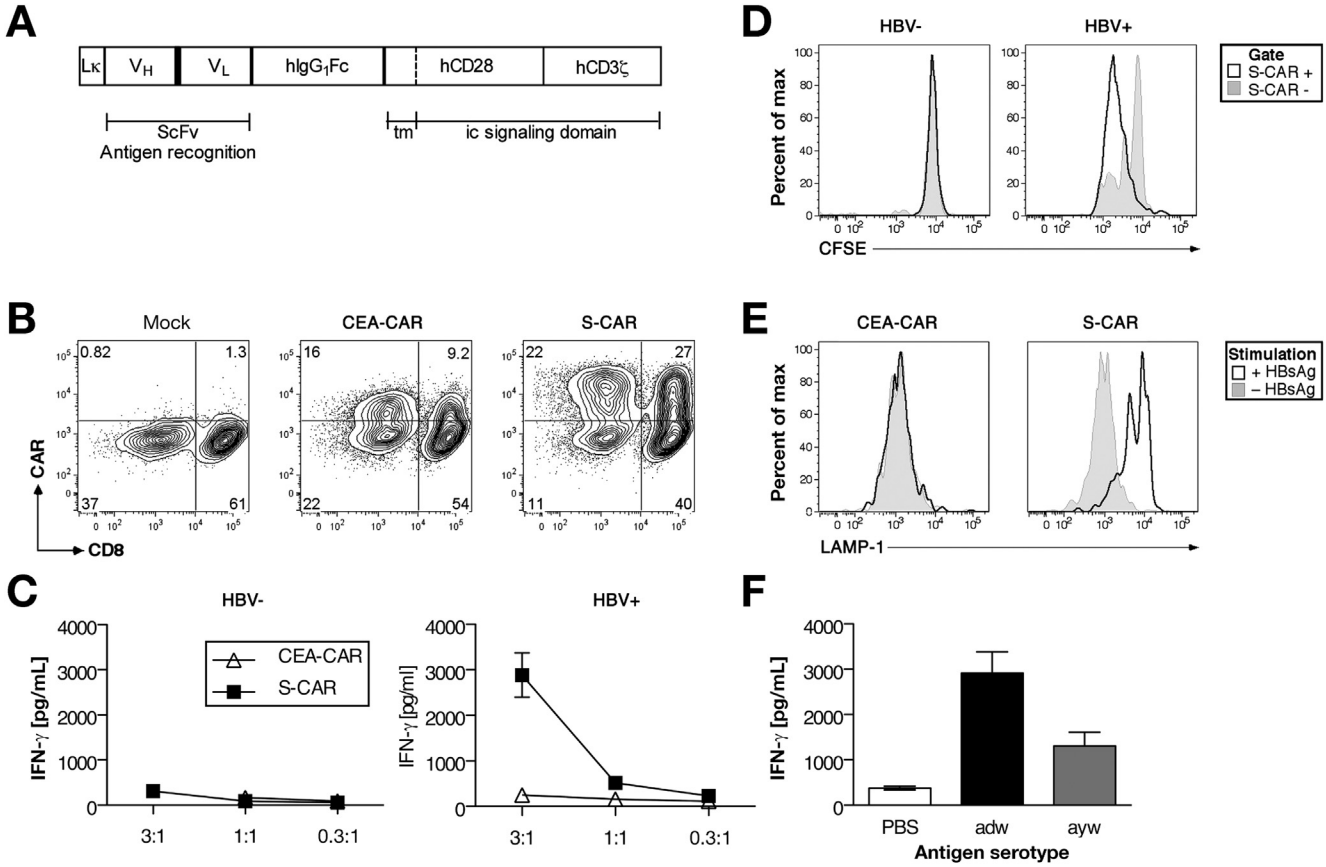

Figure 1. In vitro activation of CAR-grafted primary murine T cells. (*A*) Schematic representation of a CAR construct consisting of a κ leader (Lκ), heavy (V_H) and light (V_L) variable chain of an scFv, the fragment crystallizable (Fc) region of human immunoglobulin G1 ($hIgG_1$), and transmembrane (tm) and intracellular (ic) signaling domains of CD28 and CD3ζ. (*B*) Cell surface expression of CARs on primary murine $CD4^+$ and $CD8^+$ T cells detected by anti-hIgG staining and analyzed by flow cytometry. Splenocytes were gated on $CD4^+$ and $CD8^+$ T cells. Percentages of cells detected in each quadrant are given. CEA-CAR, scFv directed against CEA; S-CAR, scFv directed against the S domain of HBV envelope proteins. (*C*) IFN-γ production (effector to target cell ratio, n = 3) and (*D*) proliferation of CAR-grafted T cells seeded onto HBV-replicating (*right panel*) and HBV-negative (*left panel*) HepG2 hepatoma cells. Proliferation was assayed by flow cytometry of carboxyfluorescein succinimidyl ester (CFSE)-stained S-CAR–grafted T cells. (*E*) Staining for lysosomal-associated membrane protein 1 (LAMP-1) in CAR-grafted $CD8^+$ T cells after incubation with plate-bound recombinant HBsAg. (*F*) Exposure of S-CAR–grafted T cells to recombinant HBsAg of subtype *adw* and *ayw* (n = 3). All data are presented as mean values ± SEM.

antigen of the 2 most prevalent subtypes of HBV: *adw* and *ayw* (Figure 1*F*).

Adoptively Transferred S-CAR–Grafted T Cells Are Attracted to the Site of HBV Replication and Proliferate in the Liver of Immune-Competent Mice

Critical for the success of adoptive cell therapy is the proper functionality of transferred T cells, ensuring that these cells survive and accumulate at the site of antigen expression.[16] We compared classic IL-2 stimulation with IL-12 stimulation of T cells during in vitro expansion and retroviral CAR transduction. IL-12 conditioning led to a 6-fold increase in the frequency of T cells within 6 days after adoptive transfer, which was a significant improvement compared with the established IL-2 protocol (Supplementary Figure 1*A* and *B*). Therefore, in all further experiments, transduction of $CD8^+$ T cells was performed in the presence of IL-12.

To study targeting and antiviral properties in vivo, we transferred CAR^+ $CD8^+$ T cells (4×10^6) carrying the congenic marker CD45.1 into $CD45.2^+$ HBVtg mice. To exclude that a mere capture of virus particles by S-CAR–grafted T cells may contribute to or even initiate antiviral effects, we grafted T cells with an S-decoy(Δ)-CAR that uses the scFv binding site of the S-CAR but lacks functional signaling domains (Supplementary Figure 2*A* and *B*). Whereas numbers of T cells grafted with either CEA-CAR or SΔ-CAR decreased rapidly after adoptive transfer, S-CAR–grafted cells expanded to up to 40% of total circulating $CD8^+$ T cells on day 8 (Figure 2*A*). Because all cells were pretreated with IL-12 in vitro, this indicated antigen-triggered T-cell proliferation in vivo. Quantification of transferred cells on day 12 after transfer revealed preferential T-cell accumulation (Figure 2*B*) and proliferation (Supplementary Figure 2) in the liver of animals that had received S-CAR–grafted T cells. Immunohistochemistry confirmed hepatic infiltration of lymphocytes (Figure 2*C*), which showed cell surface expression of the

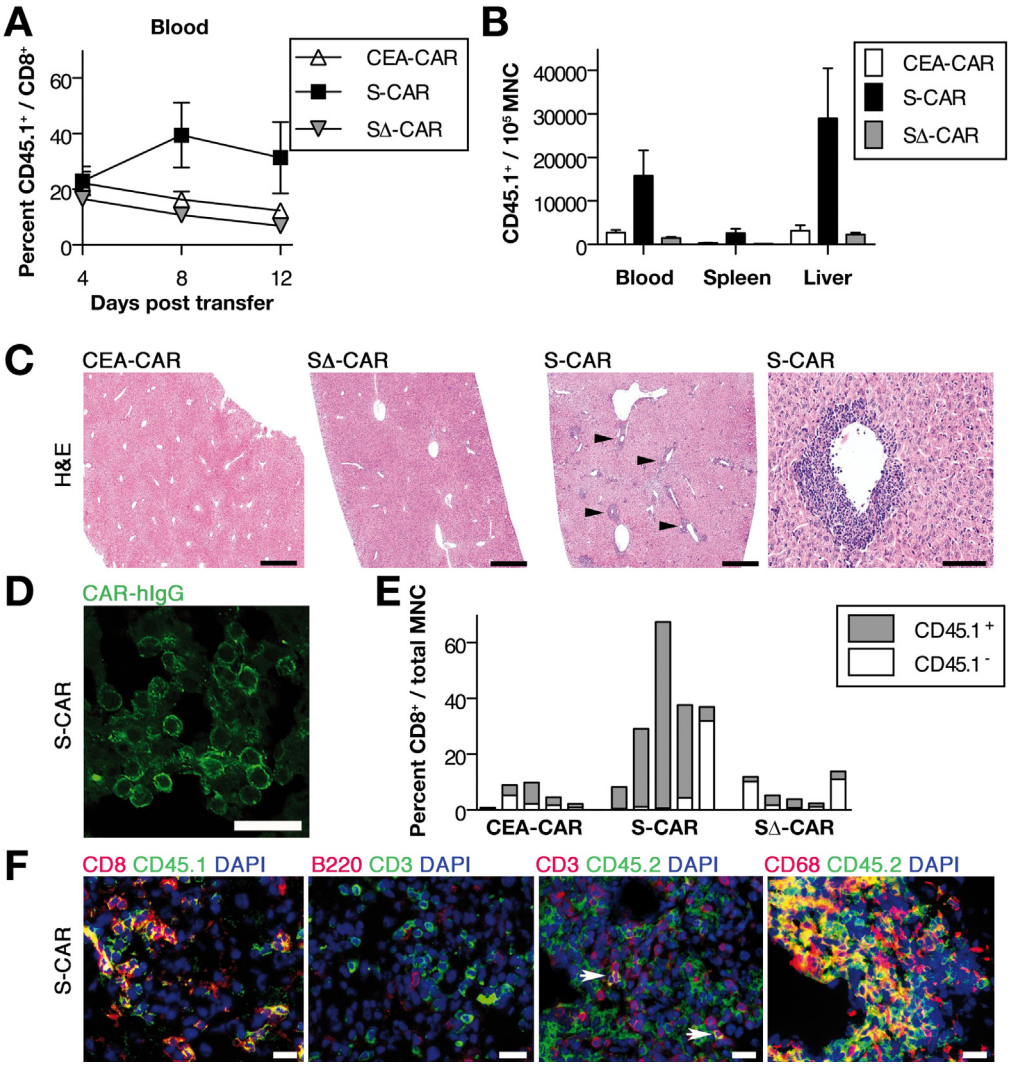

Figure 2. Survival and homing of CAR-grafted, CD45.1$^+$ murine T cells adoptively transferred into CD45.2$^+$ HBVtg mice. CD8$^+$ T cells were grafted with either S-CAR or CEA-CAR or SΔ-CAR for control. (*A*) Proportion of recovered CD45.1$^+$ CD8$^+$ T cells from peripheral blood of recipient mice (mean ± SEM from 5 mice per group). $P = .0324$ comparing all 3 groups on day 8 by Kruskal–Wallis test. (*B*) Numbers of transferred cells per 10^5 mononuclear cells (MNC) in indicated compartments on day 12 after transfer. $P = .0122$ comparing cell numbers in the livers of all 3 groups of mice (n = 5). (*C*) H&E staining of representative liver sections 12 days after transfer. *Scale bar*: *panels 1–3* = 500 μm; *panel 4* = 100 μm. *Arrowheads* indicate lymphocyte infiltrations. (*D*) Visualization of S-CAR$^+$ T cells infiltrating the liver stained with an anti-human immunoglobulin G (hIgG) antibody. *Scale bar* = 20 μm. (*E*) Flow cytometric quantification of endogenous (CD45.1$^-$) and transferred (CD45.1$^+$) CD8$^+$ T cells recovered from the liver of individual mice. (*F*) Representative staining for different cell types in a liver of a mouse after treatment with S-CAR–grafted T cells. *Arrows* indicate endogenous lymphocytes (CD45.2$^+$CD3$^+$). *Scale bar* = 20 μm.

S-CAR (Figure 2*D*). CD3$^+$ lymphocytes accounted for approximately one-half of the infiltrating cells and, with the exception of one mouse, the majority of these were transferred CD45.1$^+$CD8$^+$ T cells (Figure 2*E* and *F* and Supplementary Figure 2*C–E*). Ki67 expression by lymphocytes in intrahepatic infiltrates detected in mice that had received S-CAR–grafted T cells indicated that the adoptively transferred T cells proliferated at the site of HBV replication (Supplementary Figure 2*C* and *H*). Endogenous leukocytes present at the site of inflammation in the liver were mainly macrophages and B cells (Figure 2*F* and Supplementary Figure 2*F* and *G*). These results showed that lymphodepletion before cell transfer is not necessary to allow for engraftment and expansion of chimeric T cells.

Transferred S-CAR–Grafted T Cells Remain Functional Within the Hepatic Microenvironment

The next step was to analyze whether adoptively transferred CAR-engineered T cells executed their effector functions within the hepatic microenvironment. We observed liver damage indicated by serum alanine aminotransferase (ALT) activity peaking on day 8 after transfer and staining of apoptotic hepatocytes only in mice that received S-CAR but not SΔ-CAR or CEA-CAR T cells (Figure 3*A* and *B*). In livers of mice that received S-CAR–grafted T cells, the immunosuppressive cytokine IL-10 (Figure 3*C*) as well as the proinflammatory cytokines IFN-γ and tumor necrosis factor (TNF)-α (Figure 3*D*) were

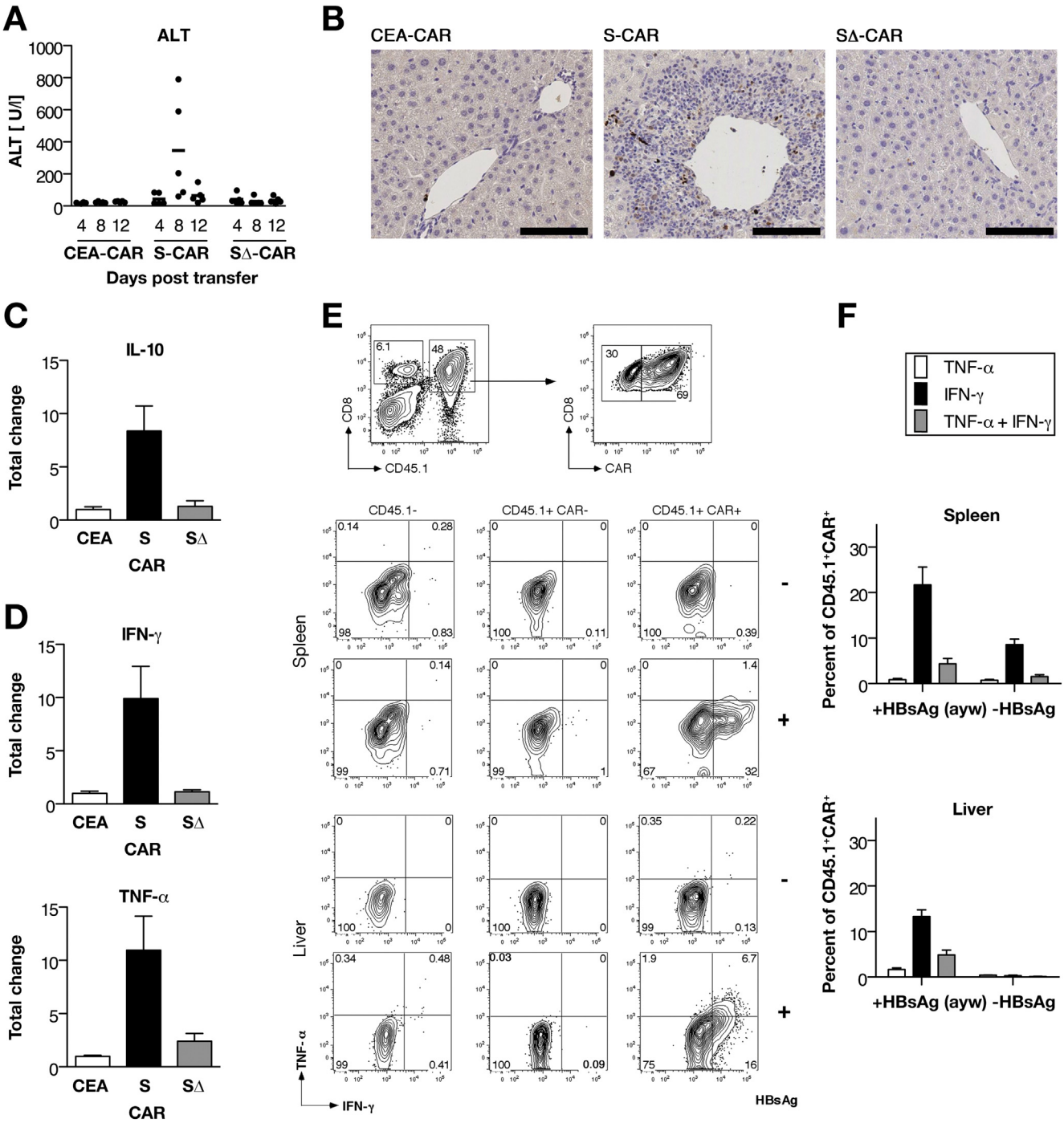

Figure 3. Effector functions exerted by adoptively transferred CAR-grafted T cells. (*A*) ALT activity in mouse sera on days 4, 8, and 12 after transfer. Each dot represents one mouse. $P = .0113$ comparing ALT values of all 3 groups on day 8. (*B*) Representative liver sections stained for cleaved caspase-3 on day 12 after transfer. *Scale bar* = 200 μm. (*C* and *D*) Relative increase in hepatic cytokine expression on day 12 after transfer. Mean values of messenger RNA levels in the CEA-CAR control group were set to 1. $P = .0189$, $P = .0041$, or $P < .0001$ for IL-10, IFN-γ, or TNF-α, respectively (n = 5). (*E* and *F*) Liver-associated lymphocytes (liver) and splenocytes (spleen) were isolated on day 8 after transfer and restimulated on HBsAg-coated plates (+HBsAg) before intracellular cytokine staining. (*E*) Representative flow cytometric analysis gated on endogenous cells ($CD45.1^-$, *column 1*), transferred cells ($CD45.1^+$) without detectable S-CAR expression (*column 2*) or transferred cells expressing the S-CAR (*column 3*). (*F*) Percentage of cytokine-secreting $S\text{-}CAR^+CD8^+CD45.1^+$ T cells in spleen and liver. Data represent mean values ± SEM, n = 3. Splenocytes from each animal were restimulated in duplicate.

strongly up-regulated. Ex vivo restimulation of liver-associated lymphocytes with HBsAg and subsequent intracellular cytokine staining showed that S-CAR–grafted T cells reisolated from spleen or liver produced IFN-γ and/or TNF-α in an antigen-specific fashion (Figure 3*E* and *F*). Additional staining for PD-1 revealed that 20% of CAR^+ T cells were unresponsive to antigen stimulation and expressed the exhaustion marker PD-1. This phenotype

was observed less frequently when S-CAR T cells were transferred into wild-type mice, in which antigen stimulus was missing (Supplementary Figure 3). These results showed that the majority of transferred S-CAR–grafted T cells remain functional even within the immunoregulatory hepatic microenvironment.

Adoptively Transferred T Cells Redirected by the HBV-Specific S-CAR Exhibit a Strong Antiviral Effect In Vivo

A profound reduction of the number of hepatocytes with cytoplasmic expression of HBV core protein (Figure 4*A*) showed the antiviral activity of the adoptively transferred S-CAR–grafted T cells. Moreover, the number of virions circulating in the bloodstream rapidly decreased 100-fold (Figure 4*B*) and replicative forms of HBV DNA almost completely disappeared from the liver within 12 days (Figure 4*C* and *D*). Lacking antiviral activity of CEA-CAR and SΔ-CAR engineered T cells proved that antigen recognition and T-cell activation via the S-CAR were essential to stimulate the antiviral activity of adoptively transferred T cells.

Treatment With S-CAR T Cells Causes Only Minor Side Effects

Animals injected with 4×10^6 S-CAR–grafted T cells did not lose weight over 34 days of treatment (Figure 5*A*) and did not show any obvious signs of distress, although serum TNF-α, IFN-γ, MCP-1, IL-10, and IL-6 levels increased significantly (Figure 5*B*). Levels of immunoglobulin G1 antibodies increased, but levels of other immunoglobulin subtypes were not altered (Figure 5*C*). Twelve days after transfer, the relative amount of $CD4^+$ T cells and B cells decreased in the spleen and liver while B cells and NK cells increased in blood (Figure 5*D*, *left panel*). The relative amount of myeloid immune cells such as inflammatory monocytes, dendritic cells (DC), and neutrophils increased, especially in the liver (Figure 5*D*, *right panel*). Thirty-four days after treatment, the composition of immune cells in all analyzed compartments resembled that of untreated mice again.

S-CAR–Grafted T Cells Exert Stronger Antiviral Activity Than Natural HBV-Specific T Cells

To compare the efficacy of S-CAR T cells with "natural" S-specific T cells, wild-type mice were immunized with recombinant HBsAg and boosted with modified vaccinia Ankara (MVA) virus expressing S-Protein to induce S-specific T cells for adoptive transfer (Supplementary Figure 4). A total of 1×10^6 S-specific $CD8^+$ T cells and 1×10^6 and 4×10^6 S-CAR T cells were injected into HBVtg mice. Most of the vaccine-induced S-specific T cells accumulated in lymph nodes (Figure 6*A*), whereas S-CAR T cells preferentially homed to the liver (Figures 2*B* and 6*A*). ALT levels were not elevated on day 7 in animals that received 1×10^6 S-specific T cells. Transfer

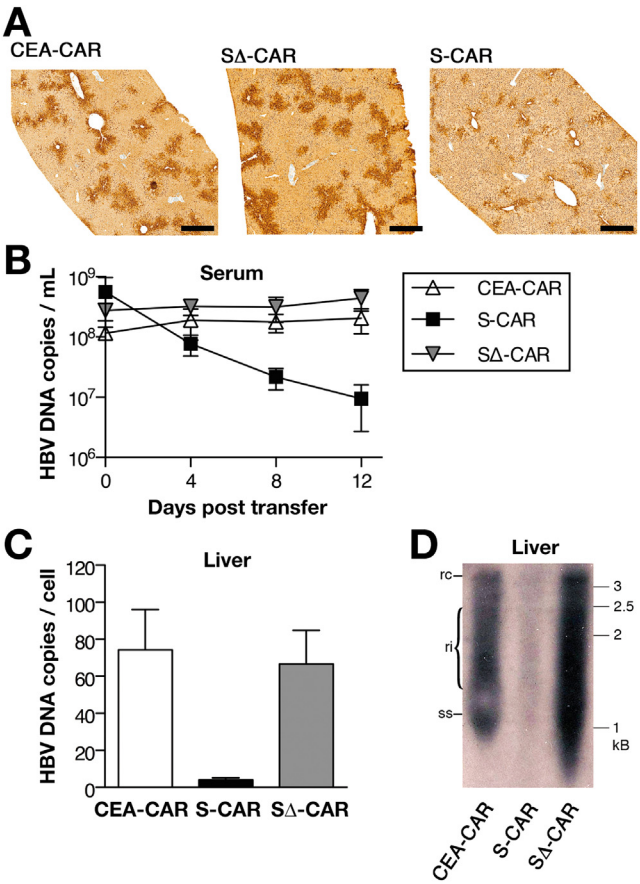

Figure 4. Antiviral effect of adoptively transferred T cells grafted with S-CAR, CEA-CAR, or SΔ-CAR. (*A*) Staining of HBV core–positive hepatocytes in liver sections from HBVtg mice 12 days after T-cell transfer. *Scale bar* = 500 μm. (*B*) HBV viremia determined by quantitative polymerase chain reaction. $P = .0242$ or $P = .0122$ comparing HBV DNA copies of all 3 groups on day 8 or 12, respectively. (*C* and *D*) HBV replication in the liver on day 12 after transfer. (*C*) HBV DNA copy numbers determined by quantitative polymerase chain reaction were normalized to the single copy gene *Nid2*. $P = .009$ comparing S-CAR with CEA-CAR and SΔ-CAR. All data are presented as mean values ± SEM (n = 5). (*D*) Southern blot analysis of pooled total liver DNA. Equal amounts of liver DNA from each animal were pooled per group and probed for HBV DNA. Relaxed circular (rc), single-stranded (ss), and other replicative intermediate (ri) forms of HBV DNA are indicated.

of the same amount of S-CAR T cells led to an increase in ALT activity to approximately 150 U/L. Transfer of 4×10^6 S-CAR T cells led to an ALT activity of approximately 800 U/L (Figure 6*B*). Accordingly, S-CAR T cells reduced cytoplasmic hepatitis B core antigen expression more profoundly than vaccine-induced T cells (Figure 6*C*). However, the effect on HBV viremia (Figure 6*D*) and HBV replication in the liver (Figure 6*E*) was not significantly different among the 3 treatment groups, indicating a dominant role of noncytopathic antiviral mediators.[17,18] Cytoplasmic hepatitis B core antigen and virus DNA in the serum increased to levels before treatment, when S-CAR T cells had almost vanished from the liver on day 34 (Figure 6*C*–*E*) and HBV replication was driven again by the stable transgene, which cannot be eliminated. Taken together, vaccine-induced T cells elicited their effect

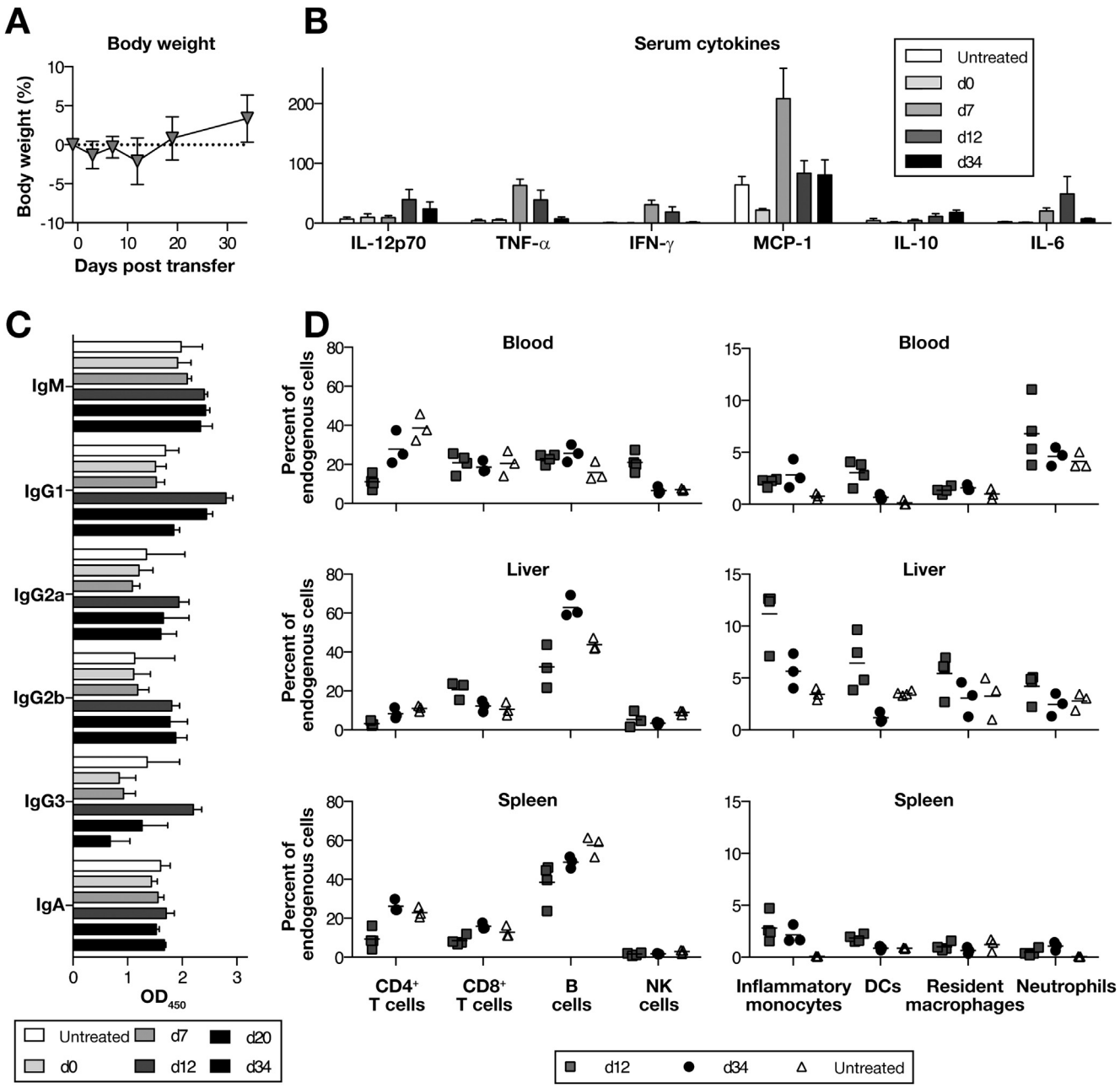

Figure 5. Potential side effects of 4×10^6 adoptively transferred S-CAR–grafted T cells. (*A*) Body weight of mice relative to their individual weight before transfer. (*B*) Cytokines measured in sera on different days after T-cell transfer, compared over all days (n = 3–7): IL-12 ($P = .1163$), TNF-α ($P = .0049$), IFN-γ ($P = .0032$), MCP-1 ($P = .0061$), IL-10 ($P = .0238$), or IL-6 ($P = .0047$). (*C*) Immunoglobulin clonotypes in sera on different days after T-cell transfer. $P = .0041$ for IgG1, comparing all days (n = 3–7). Data represent mean values ± SEM. (*D*) Relative amounts of different cell types in blood, liver, and spleen on days 12 and 34 after T-cell transfer and in untreated mice. Each symbol represents one mouse.

mainly in a noncytopathic fashion, whereas S-CAR T cells killed HBV-replicating hepatocytes in addition.

Discussion

Currently there is no cure for chronic hepatitis B. Novel antiviral agents very efficiently control HBV but cannot eliminate it. Immunotherapy using T cells that are genetically modified to express an HBsAg-specific receptor seems a promising addition to current antiviral therapy. This therapy could cure chronic hepatitis B, which is a premalignant condition, but may also be applied to treat HBsAg-positive HCC.

Our study showed that T cells redirected by an HBV-specific CAR, when transferred into immunocompetent mice, (1) recognize HBV envelope proteins on the surface of HBV-replicating hepatocytes, (2) engraft and (3) expand in vivo, (4) infiltrate the liver, and (5) effectively control HBV replication. This new immunotherapy approach proved safe and did not lead to excessive liver damage after contact of T cells with circulating viral antigen or to functional exhaustion of the adoptively transferred T cells. Our results strongly suggest that S-CAR–engineered T cells will be able to cure HBV infection. However, this cannot be proven in the HBVtg

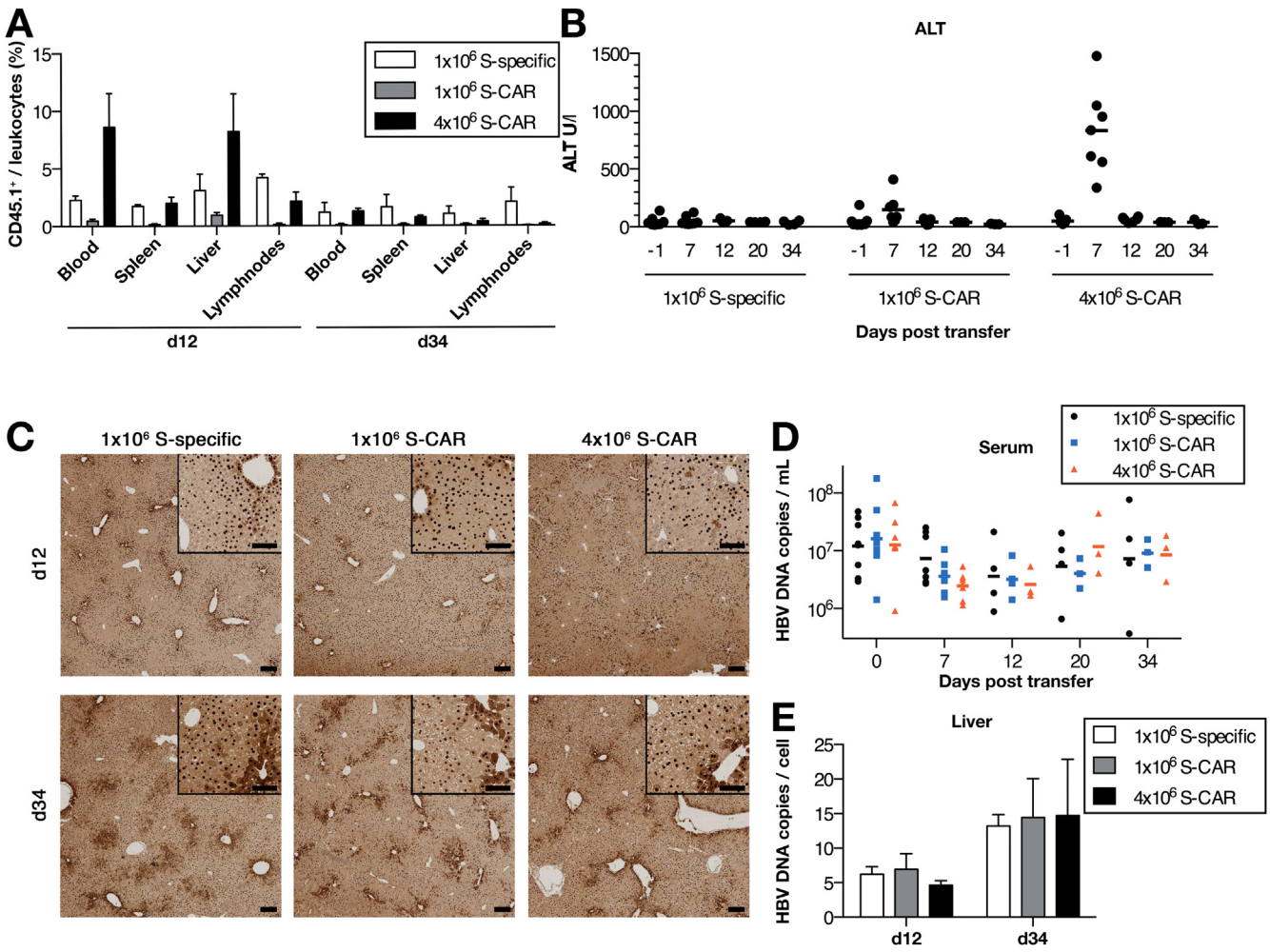

Figure 6. Antiviral activity of adoptively transferred S-CAR–grafted or natural S-specific T cells. $CD8^+$ T cells were isolated and 1×10^6 S-specific (2.5×10^7 total $CD45.1^+$) as well as 1×10^6 and 4×10^6 S-CAR (1.4×10^6 and 5.5×10^6 total $CD45.1^+$) T cells were injected into HBVtg mice. (*A*) Relative amount of transferred T cells per total leukocytes in different compartments on days 12 and 34 after transfer. Data are presented as mean values ± SEM (n = 3–4). (*B*) ALT activity in mouse sera before and 7, 12, 20, and 34 days after transfer. Each dot represents one mouse. (*C*) Staining of HBV core–positive hepatocytes in liver sections from HBVtg mice 12 and 34 days after T-cell transfer. *Scale bar* = 200 μm, *inset* = 100 μm. (*D* and *E*) HBV viremia determined by quantitative polymerase chain reaction in (*D*) serum and (*E*) liver.

mouse model used in this study, because HBV is not transcribed from cccDNA but from a transgene, which cannot be eliminated.

A prerequisite for successful adoptive T-cell therapy is that transferred cells engraft in the recipient. Clinical trials using adoptive T-cell therapy for malignant diseases showed that persistence of infused cells 4 weeks after transfer was associated with complete response to treatment.[11,16] In these studies, depleting lymphocytes by chemotherapy or irradiation facilitated the engraftment of T cells. T-cell depletion or immunosuppression, however, is obsolete in patients with chronic hepatitis B. For tumor therapy, it would be advantageous not to suppress the patient's immune system. Our study shows that successful adoptive T-cell therapy without prior T-cell depletion or immunosuppression is feasible. This may enable the few endogenous antigen-specific T and B cells to restore their antiviral or antitumoral capacity when assisted by transferred T cells.

Using IL-12 instead of IL-2 for stimulation during expansion and transduction of $CD8^+$ T cells, we were able to improve survival and engraftment of antigen-specific T cells. Lower numbers of transferred T cells in control groups suggested that engraftment was not merely an effect of in vitro IL-12 treatment but also required triggering of the S-CAR by HBsAg. Mechanistically, IL-12 induces upregulation of antiapoptotic molecules such as B-cell lymphoma 3-encoded protein (Bcl-3)[19] and cellular caspase-8 (FLICE)-like inhibitory protein (c-FLIP)[20] while reducing proapoptotic caspase activation.[20,21] Moreover, IL-12 improves memory cell differentiation.[21,22] With IL-12 pretreatment, lymphodepletion before cell transfer was not necessary to allow for engraftment and expansion of chimeric T cells. Whether pretreatment with IL-12 will be applicable in a clinical trial setting remains an open question. IL-12 has been used in several clinical settings[23] but currently cannot be purchased although clinical-grade production is urgently needed.[24]

An advantage of not administering immunosuppressive therapy before adoptive T-cell therapy is that the regulatory function of immune cells in the liver and other organs is preserved. In our experiments, the increasing ALT activity in the serum selectively after transfer of S-CAR–engineered T cells suggested that the S-CAR mediated the killing of HBV-positive hepatocytes in vivo and thus induced liver damage. Liver damage, however, was transient. This may be explained by either increased levels of the immunosuppressive cytokine IL-10 in the liver, inducing an exhausted phenotype, or contraction of the effector T-cell population after massive clonal expansion,[25,26] resulting in low-level cytotoxicity.[27,28] Restriction of liver damage by IL-10 was observed in several models of immune-mediated liver damage.[29,30] The cellular source of IL-10 may be liver-resident T-helper 2 or regulatory T cells,[31] Kupffer cells,[32,33] or even transferred, IL-12–primed $CD8^+$ T cells.[34] Self-limitation of immune-mediated damage in the liver by any of these means will ensure organ integrity but may limit the efficiency of immunotherapy.[11] The rapid decrease of HBV replication without severe liver disease is very likely due to the fact that S-CAR–grafted T cells, like natural HBV-specific T cells,[18,35] control HBV in transgenic mice in a noncytopathic fashion via antiviral cytokines in addition to directly killing HBV-replicating hepatocytes. This idea is supported by the fact that ALT levels in mice treated with 1×10^6 T cells were much lower but the antiviral activity was comparable to animals that received 4 times more cells.

Development of T-cell therapy for hepatitis B has been encouraged by several observations. Control of HBV replication is obtained after transfer of splenocytes from immunized wild-type mice into HBVtg mice.[18,27] More importantly, cure of HBV infection in patients has been reported after transfer of specific immunity against HBV through allogeneic bone marrow transplantation.[36,37] Bone marrow transplantation, however, is complex and cannot be broadly applied for treatment of an infectious disease because it requires an exact major histocompatibility complex match of cells derived from an immunized donor as well as profound immunosuppression to allow engraftment. Using the patient's own T cells and redirecting them with an HBV-specific receptor seems a more feasible approach to treat chronic hepatitis B or HBsAg-positive HCC. CAR-grafted T cells, which function independently of the patient's HLA haplotype and recognize different HBsAg subtypes, seem to be particularly suited because they will in principle be applicable to almost all HBV-infected patients.[38]

Our preclinical model has similar levels of circulating HBsAg (approximately 1000–1200 IU/mL) as detected in the low-replicative phase of chronic hepatitis B.[39] In this model, we observed elevation of cytokines but no severe side effects during T-cell therapy. However, in a patient with high replication, preexisting liver inflammation, and tissue damage, the situation may be different. Pronounced elevation of ALT levels was observed in transplant recipients with cleared HBV infection,[37] indicating that hepatocyte killing was needed for elimination. S-CAR T cells and T cells induced by immunization of donor mice showed comparable antiviral efficacy in our model, but elevation of ALT levels and clearance of hepatitis B core–positive hepatocytes indicating elimination of HBV-positive hepatocytes was only observed after S-CAR T-cell transfer. To avoid or reduce potential hepatotoxicity in a clinical setting, patients will be pretreated with antiviral agents before T-cell transfer to reduce the amount of HBsAg-positive hepatocytes and the grade of inflammation and increase selection pressure on the virus to minimize the risk for emergence of viral variants, which could escape CAR recognition.[40] In addition, redirected T cells can be specifically eliminated by a safeguard mechanism. For clinical use, we have added a truncated version of the epidermal growth factor receptor to the CAR construct, which allows for depletion of CAR transduced cells with the clinically approved antibody cetuximab.[41]

We have previously reported that human T cells that are engrafted with the S-CAR can eliminate the nuclear persistence form of HBV, the cccDNA, from HBV-infected hepatocytes.[12] In an alternative approach, Gehring et al[42] generated 2 HBV-specific, HLA-A2–restricted T-cell receptors for grafting and showed that HBV-specific T cells generated from peripheral blood mononuclear cells of patients with chronic HBV and HBV-related HCC became multifunctional and capable of recognizing HBV-replicating hepatoma cells and HCC tumor cells expressing viral antigens from naturally integrated HBV DNA. We also have established a series of such recombinant T-cell receptors of diverse receptor avidity (unpublished data; October 2011) and are currently comparing these with respect to optimal functionality.

The in vivo study presented here showed that S-CAR–grafted T cells (although vast amounts of subviral particles are present in the blood of HBVtg mice) infiltrate the liver, remain functional, and lead to a profound reduction of viral load. From the data obtained, we conclude that immunotherapy with S-CAR–grafted T cells is a feasible and promising approach to treat chronic HBV infection, providing proof of concept for further translation of adoptive T-cell therapy for chronic hepatitis B into the clinic.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.04.047.

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