T Cells Redirected Against Hepatitis B Virus Surface Proteins Eliminate Infected Hepatocytes

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Background & Aims: **The final goal in hepatitis B therapy is eradication of the hepatitis B virus (HBV) replication template, the so-called covalently closed circular DNA (cccDNA). Current antiviral treatment of chronic hepatitis B depends on interferon α or nucleoside analogues inhibiting the viral reverse transcriptase. Despite treatment, cccDNA mostly persists in the host cell nucleus, continues to produce hepatitis B surface antigen (HBsAg), and causes relapsing disease. We therefore aimed at eliminating persistently infected hepatocytes carrying HBV cccDNA by redirecting cytolytic T cells toward HBsAg-producing cells.** ***Methods:*** **We designed chimeric T-cell receptors directed against HBV surface proteins present on HBV-infected cells and used them to graft primary human T cells with antibody-like specificity. The receptors were composed of a single chain antibody fragment directed against HBV S or L protein fused to intracellular signalling domains of CD3ξ and the costimulatory CD28 molecule.** ***Results:*** **Our results show that these chimeric receptors, when retrovirally delivered and expressed on the cell surface, enable primary human T cells to recognize HBsAg-positive hepatocytes, release interferon γ and interleukin 2, and, most importantly, lyse HBV replicating cells. When coincubated with HBV-infected primary human hepatocytes, these engineered, antigen-specific T cells selectively eliminated HBV-infected and thus cccDNA-positive target cells.** ***Conclusions:*** **Elimination of HBV cccDNA-positive hepatocytes following antiviral therapy is a major therapeutic goal in chronic hepatitis B, and adoptive transfer of grafted T cells provides a promising novel therapeutic approach. However, T-cell therapy may also cause liver damage and therefore needs further preclinical evaluation.**

The human hepatitis B virus (HBV) is a small, enveloped, and noncytopathic virus, with a very narrow host range and strong liver tropism causing acute and chronic liver disease. Worldwide, approximately 350 million patients are infected with HBV. A cytotoxic T-cell response is thought to be responsible for both viral clearance and liver injury during HBV infection.[1] A polyclonal and multispecific T-cell response was observed in patients who had cleared acute infection,[2] whereas a weak and oligoclonal response was described in chronically infected individuals.[3] The ongoing inflammation in chronic infections results in cirrhosis and hepatocellular carcinoma. Current therapeutic options include interferon (IFN) α and nucleos(t)ide analogues inhibiting the viral reverse transcriptase. Nucleos(t)ide analogues impair the viral life cycle but do not target the viral transcription template, the HBV covalently closed circular DNA (cccDNA), in the host cell nucleus. From the episomal cccDNA template, a new HBV replication cycle can be initiated after the end of therapy, often resulting in a hepatitis flare. Therefore, elimination of persistently infected hepatocytes is necessary to cure the disease,[4] which may either be achieved by induction or by adoptive transfer of T cells, which eliminate infected hepatocytes.

Here, we aimed at developing genetically modified cytolytic T cells carrying a chimeric T-cell receptor (cTCR), which targets HBV surface proteins present on HBV-infected cells. HBV-infected cells continuously produce hepatitis B surface antigen (HBsAg) from the cccDNA template even when HBV replication subsides.[5] HBsAg is predominantly composed of the HBV small surface (S) protein with trace amounts of middle and large surface (L) proteins. If patients did not seroconvert from HBsAg to antibodies against hepatitis B surface antigen (anti-HBs), a high number of hepatocytes (5%–30%) remained positive for HBV S protein even after long-term, highly

Abbreviations used in this paper: anti-HBs, antibodies against hepatitis B surface antigen; cccDNA, covalently closed circular DNA; cTCR, chimeric T-cell receptor; GALV, Gibbon ape leukemia virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IFN, interferon; IL, interleukin; L, HBV large surface protein; MHC, major histocompatibility complex; NF-κB, nuclear factor κB; PBL, peripheral blood lymphocytes; PHH, primary human hepatocytes; rcDNA, relaxed circular DNA; S, HBV small surface protein; scFv, single chain antibody fragment.

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potent antiviral therapy.[6] We therefore think that targeting S antigen-positive cells and thus residual infected hepatocytes harboring HBV cccDNA is a promising approach to ultimately eliminate HBV infection following antiviral therapy.

Adoptive T-cell therapy proved safe to reconstitute cellular immunity against cytomegalovirus after allogeneic bone marrow transplantation and effective in the control of Epstein-Barr virus-associated posttransplantation lymphoproliferative disorders.[7,8] These findings provide the rationale to develop adoptive T-cell approaches for malignancies but also for chronic infectious diseases.

Repopulation with receptor modified T cells, transduced to express MART-1-specific TCR, resulted in homing of T cells into the liver and induced profound regression of advanced melanoma.[9] The next generation of adoptive T-cell therapies will likely rely on the ability to endow "fit" cells with elevated cell-surface expression of high-affinity, specific TCRs by gene-transfer technology.[10]

Artificial receptor constructs with antibody-like specificities and a compound signalling domain[11] allow recognition of native, nonprocessed antigen on the cell surface. Thereby, they work independent of classical antigen processing and allow targeting cells with established immune escape mechanisms such as down-regulation of major histocompatibility complex (MHC) molecules or reduced endolysosomal antigen processing. Chimeric receptors were used to specifically target T cells to cancer or human immunodeficiency virus antigens on the cell surface.[12,13]

Here, we use cTCRs composed of single chain antibody fragment (scFv) antibodies against S and L HBV surface protein, respectively, fused to transmembrane and cytoplasmic domains of the costimulatory CD28 molecule and to the CD3ξ signalling domain. We expected the designed cTCRs to recognize HBV S or L protein on the surface of HBV-infected hepatocytes and to licence cytolytic T cells to eliminate these cells.

Materials and Methods

Cell Culture Conditions and HBV Infection

Human embryonic kidney cells HEK 293T (ATCC CRL-1573), HepG2 hepatoma cells (ATCC HB-8065), and stably HBV transfected HepG2.2.15 cells[14] were maintained in Dulbecco's modified Eagle medium (DMEM)/5% glucose (wt/vol), 10% (vol/vol) fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), nonessential amino acids (0.1 mmol/L), sodium pyruvate (1 mmol/L), and L-glutamine (2 mmol/L) (all from Biochrom AG, Berlin, Germany).

Primary human hepatocytes (PHH) were isolated by a standard 2-step collagenase perfusion and differential centrifugation from liver tissue of patients undergoing partial hepatectomy for metastasis resection after informed consent as approved by the local Ethics Committee. PHH medium was Williams E Medium (Invitrogen GmbH, Karlsruhe, Germany) supplemented with L-glutamine (5 mmol/L), glucose (0.06% [wt/vol]), HEPES (23 mmol/L, pH7.4), gentamycin (50 μg/mL), penicillin (50I U/mL), streptomycin (50 μg/mL), inosine (37 μmol/L), DMSO (1.75%), hydrocortisone (4.8 μg/mL), and insulin (1 μg/mL). Cells were infected with HBV as described before.[15] For cocultures with redirected T cells, we used cortison-free medium.

In cell culture medium of PHH, alanine aminotransferase (ALT) levels were measured using the Reflotron system (Roche Diagnostics, Mannheim, Germany). Live cells were stained with calceine and dead cells with ethidium homodimer-1 (Molecular Probes, Eugene, OR). DNA from cell lysates was prepared by standard phenol-chloroform extraction. HBV cccDNA and total HBV DNA was quantified by Light Cycler PCR (Roche Diagnostic) as described.[16] Hepatitis B e (HBeAg) and surface (HBsAg) antigens were determined by ELISA (Abbott, Dartford, UK). HBV core protein and albumin were detected by Western blot using monoclonal antibody against human albumin (DAKO, Carpinteria, CA) or antiserum raised against truncated HBV core protein (kindly provided by Michael Nassal, University of Freiburg, Germany). Chemiluminescence signals (Super-Signal West Dura, Pierce Biotechnology, Rockford, NJ) were quantified using the Gel Doc 2000 System (Bio-Rad Laboratories, München, Germany).

Construction and Transduction of Chimeric T-Cell Receptors

scFv C8 was selected from an scFv library generated from peripheral blood lymphocytes (PBL) of HBsAg-vaccinated individuals, and scFv 5a19 was derived from a hybridoma expressing the monoclonal antibody 5a19[17] using phage display screening. DNA encoding scFv sequences were cloned 3′ of cytomegalovirus (CMV) I/E promotor and a κ-leader sequence into a cTCR construct[18] and inserted into the retroviral expression plasmid pBullet[19] (Figure 1*A*). Gibbon ape leukaemia virus (GALV) envelope pseudotyped, amphotropic retroviral vectors were produced by cotransfecting the packaging plasmids pHIT60 and pCOLT.

Primary human PBL were freshly prepared from peripheral blood using Ficoll-Hypaque (Biochrom AG) density gradient centrifugation. Non-T cells were depleted by plastic adherence, and T cells were stimulated with anti-CD3 antibody OKT3 and interleukin (IL)-2 before retroviral transduction as described previously.[18,19] To evaluate transduction efficiencies, T cells were stained with PE-labelled murine anti-CD3, -CD4, or -CD8 antibodies (DakoCytomation, Glostrup, Denmark). Transduced cells were identified with FITC-labelled anti-human IgG antibodies recognizing the extracellular Fc-derived spacer of the cTCR, counterstained with propidium iodine, and

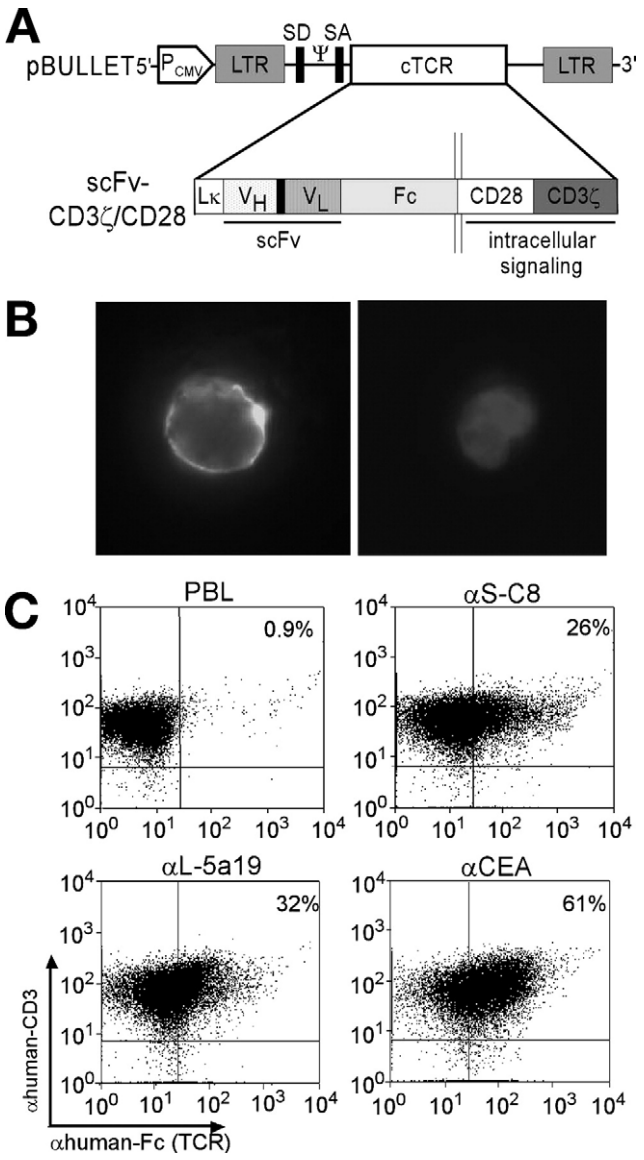

Figure 1. Grafting of primary human T cells with chimeric TCR. (*A*) Schematic representation of the chimeric TCR (*cTCR*) constructs inserted into the retroviral vector plasmid pBullet; long terminal repeat (*LTR*), splice donor (*SD*), splice acceptor (*SA*), packaging signal (ψ).[19] The cTCR is composed of an N-terminal leader sequence ($L\kappa$), heavy (V_H) and light (V_L) chain variable regions of the single chain antibody fragment (*scFv*), the Fc spacer domain (*Fc*) of human IgG1, transmembrane and intracellular regions of the CD28 signalling domain (*CD28*), and the CD3ζ signalling domain (CD3ξ). (*B*) Immunofluorescence microscopy of cells stained with the FITC-conjugated anti-human IgG antibody and DAPI (*left*: αS-C8 transduced; *right*: unmodified T cell). (*C*) Lymphocytes obtained from a healthy donor were retrovirally grafted with cTCR containing scFv recognizing HBV S protein (α*S-C8*), HBV L protein (α*L-5a19*), or carcinoembryonic antigen (α*CEA*) or used as control (*PBL*). Flow cytometric analysis using a PE-conjugated anti-CD3 and an FITC-conjugated anti-human Ig-Fc antibody, which detects the extracellular IgG1 CH2CH3 spacer domain of the receptors, were performed to identify cTCR grafted T cells. Data are presented as *dot blots*, and percentages of double positive cells are given.

analysed using a FACS Canto Flow Cytometer (BD Bioscience, Franklin Lakes, NJ). Alternatively, cells were counterstained with DAPI and analyzed using a fluorescent microscope (IX 81; Olympus, Hamburg, Germany).

T-Cell Activation and Cytotoxicity Assay

Target cells were plated with 5×10^4 cells/well on a collagen type I coated 96-well plate and cultured until confluency was reached. Redirected T cells (effector cells) were added in dilutions to the target cells after removal of IL-2. Grafted T cells were only used if transduction efficacy was $\geq$25%. Dependent on the transduction efficiency, T cells were diluted with unmodified PBL to obtain effector to target ratios (E:T ratio) between 0.02 and 2:1 as indicated. Specific cytotoxicity of receptor grafted T cells against target cells was monitored by an XTT based colorimetric assay (Roche Diagnostics GmbH) as described in detail.[18] To assay proliferation, T cells were stained with CarboxyFluorescein Succinimidyl Ester (CFSE; Molecular Probes, Eugene, OR) before coculture with target cells and analyzed after 48 hours by flow cytometry gating on receptor grafted T cells.

T-cell subpopulations were obtained by magnetic sorting using MACS CD4 and CD8 Microbeads (Miltenyi, Bergisch Gladbach, Germany). Degranulation of activated T cells was stained with LAMP-2 specific antibodies (eBioscience, San Diego, CA). IFN-γ, tumor necrosis factor (TNF) α, and IL-2 were detected by Cytoset-ELISA (Biosource, Camarillo, CA). IFN-γ secretion was analyzed using MACS IFN-γ secretion assay (Miltenyi).

Results

Grafting of Primary Human T Cells With Chimeric TCRs

Figure 1*A* schematically depicts the retroviral vector construct containing the cTCR constructs used in this study. The established cTCR BW431/26scFv-Fc-CD28-ξ,[18] recognizing carcinoembryonic antigen (CEA) was designated αCEA *c*TCR and used as a specificity control because CEA is not expressed on hepatocytes. scFv BW431/26 was replaced by HBV-specific scFv recognizing HBV S (scFv C8) or L protein (scFv 5a19), respectively. scFv C8 binds to a conformational epitope presumably in the "a" determinant of S protein; it recognizes HBV genotypes A and B better than D, and subtype adw better than ayw (data not shown). Antibody 5a19 binds to amino acid (aa) 37 to 43 in the preS1 region of HBV subtype ayw better than adw.[17]

Primary human T cells were engineered to express the cTCR by retroviral transduction with amphotropic retroviruses pseudotyped with a GALV envelope. Up to 60% of CD3+ cells expressed cTCRs following retroviral transduction as determined by flow cytometry (Figure 1*B*). Fluorescence staining using anti-human Fc antibodies visualized surface distribution of the cTCRs on grafted cells (Figure 1*C*).

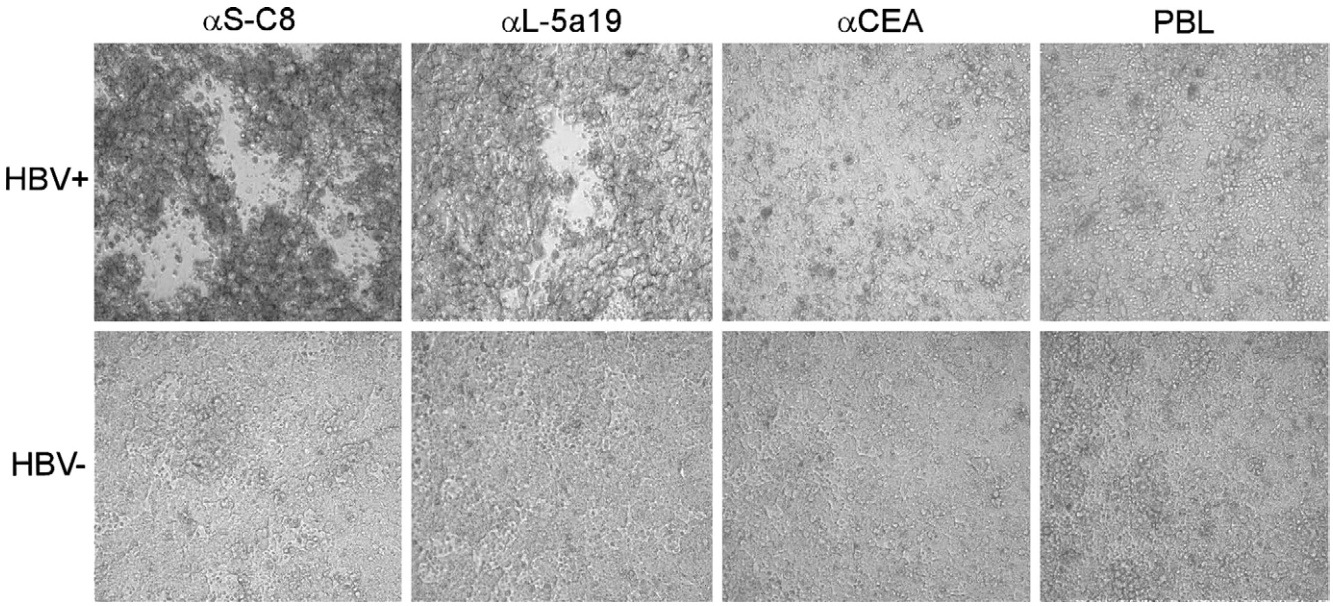

Figure 2. Cytotoxic activity of engineered T cells redirected against HBV surface proteins. Light microscopy of HBV producing (*HBV+*, *upper panel*) HepG2.2.15 and parental HepG2 cells (*HBV−*, *lower panel*), cocultured for 72 hours with redirected T cells (E:T = 2:1) carrying cTCR *αS-C8*, *αL-5a19*, or *αCEA*, respectively, or unmodified control cells (*PBL*).

To monitor the capability of the cTCRs to recognize their target antigens and to induce T-cell activation upon antigen recognition, redirected T cells were challenged with medium containing subviral and viral HBV particles, which carry S and to a low extend middle and L proteins on their surface. Both HBV-specific cTCRs—αS-C8 and αL-5a19—recognized HBV surface proteins on HBV particles leading to T-cell activation (as demonstrated by nuclear factor κB [NF-κB]) and release of IFN-γ (see Supplementary Figure 1 online at www.gastrojournal.org) and TNF-α. cTCR αS-C8, but not αL-5a19, grafted T cells secreted IL-2 (data not shown). Although we observed a low-level NF-κB activation in T cells grafted with the CEA-specific cTCR and in unmodified PBL, this activation did not lead to secretion of IFN-γ, TNF-α, or IL-2. From these data, we concluded that primary human T cells were successfully grafted with our cTCR constructs that recognize native HBV surface proteins resulting in T-cell activation.

Redirected T Cells Are Activated by Lyse HBV-Producing Cell Lines

To test their cytotoxic capabilities, redirected T cells were cocultured with HBV-replicating HepG2.2.15 hepatoma cells or as a control with HBV− parental HepG2 cells. Microscopic evaluation revealed cytotoxic lesions in monolayers of HBV+ target cells when T cells were grafted with αS-C8 or αL-5a19 cTCR but not when T cells were grafted with the αCEA cTCR or when unmodified T cells or HBV− target cells were used (Figure 2).

Next, we performed a time course experiment coculturing grafted and control T cells with HepG2.2.15 cells or with HepG2 cells at E:T ratio 4:1. αS-C8 cTCR grafted cells started to release IFN-γ after 24 hours, accumulating to 22.4 ng/mL at 72 hours. αL-5a19 cTCR grafted T cells showed a delayed response, with IFN-γ secretion starting after 36 hours and reaching levels of 9.9 ng/mL. IFN-γ secretion was neither observed from αCEA cTCR grafted nor unmodified T cells nor on HBV− target cells (data not shown).

To quantify cytotoxicity, redirected T cells were cocultured at different E:T ratios using constant numbers of target cells for 72 hours (Figure 3*A–C*). To reflect a situation, which is likely to be achieved by adoptive transfer of grafted T cells in vivo, we used E:T ratios between 0.2:10 and 1.5:10. αS-C8 cTCR grafted T cells lysed up to 70% and αL-5a19 cTCR grafted T cells up to 58% of HBV+ target cells, whereas only minimal killing activity was observed on HBV− target cells (Figure 3*A*). Using either αCEA cTCR grafted or unmodified T cells on HBV+ cells, background levels of up to 20% target cell lysis were observed.

IFN-γ was released in a dose-dependent fashion upon contact with HBV+ target cells by αS-C8 cTCR grafted and to a lower extent by αL-5a19 cTCR grafted T cells (Figure 3*B*). αS-C8 cTCR grafted T cells secreted IL-2 in a dose-dependent fashion, whereas αL-5a19 cTCR grafted T cells secreted only low levels of IL-2 (Figure 3*C*). Neither αCEA grafted nor unmodified T cells nor T cells in contact with HBV− target cells released IFN-γ or IL-2. αS-C8 cTCR and to a lower extent αL-5a19 cTCR grafted T cells proliferated upon antigen recognition on HBV+ cells as determined by flow cytometric analysis of CFSE dilution (Figure 3*D*).

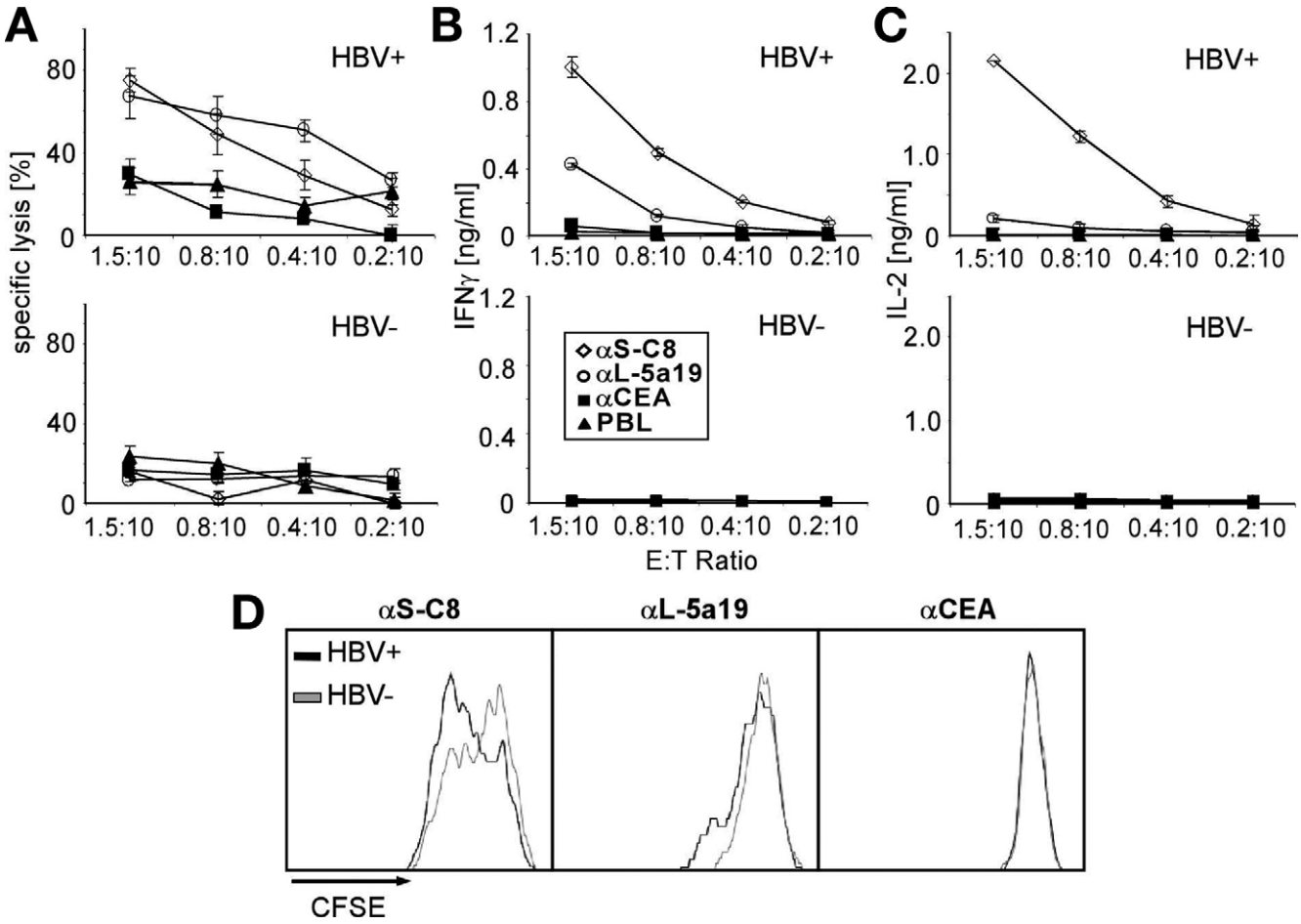

Figure 3. Cytotoxic T-cell response and cytokine secretion. T cells grafted with the cTCR directed either against HBV S (αS-C8, *open diamond*) or L protein (αL-5a19, *open circle*) or against CEA (αCEA, *solid square*) or unmodified cells (PBL, *solid rectangle*) were cultured together with HBV+ (*upper panel*) HepG2.2.15 cells or HBV− (*lower panel*) HepG2 cells. T cells were added in different dilutions to obtain indicated effector to target cell (E:T) ratios and cocultured for 72 hours. (*A*) Specific lysis of target cells in 3 parallel assays is shown. Secretion of IFN-γ (*B*) and IL-2 (*C*) into cell culture media was measured by ELISA. Mean ± SD is given. (*D*) Antigen-specific proliferation was determined by flow cytometry of CFSE stained, cTCR grafted T cells after 48 hours. One representative staining (E:T = 0.8:10) out of 4 stainings is shown.

Next, we determined which T-cell subset contributed to IFN-γ secretion and cytotoxicity (Figure 4). By flow cytometry, we identified IFN-γ secreting cells and cytotoxic activity by cell surface staining of degranulation marker lysosomal membrane protein-2 (LAMP-2).[20] Upon contact with HBsAg, almost all αS-C8 cTCR grafted T cells (35.5% CD4+, 58.8% CD8+) actively secreted IFN-γ but none of the αCEA cTCR grafted control cells (Figure 4*A*). In addition, αS-C8 cTCR but not αCEA cTCR grafted CD4+ as well as CD8+ T cells displayed LAMP-2 on their surface (Figure 4*B*), indicating cytotoxic activity. To verify their cytotoxicity, CD4+ and CD8+ T cells were isolated by magnetic cell sorting following cTCR grafting. When cocultured with target cells, CD4+ as well as CD8+ αS-C8 cTCR grafted T cells specifically lysed HBV replicating HepG2.2.15 cells (Figure 4*C*). Taken together, cTCR binding to HBV surface proteins enable grafted CD4+ and CD8+ T cells to recognize HBV-infected cells, to proliferate, to secrete the cytokines IFN-γ and IL-2, and, last but not least, to lyse HBV-replicating cells.

HBV-Infected Primary Human Hepatocytes Are Specifically Eliminated by Redirected T Cells

Our next aim was to assess the cytotoxicity of redirected T cells toward the natural host cells of HBV, primary human hepatocytes, and to study whether HBV-infected hepatocytes are selectively and efficiently eliminated. We therefore infected primary human hepatocytes with wild-type HBV leading to 5% to 15% of the hepatocytes expressing HBV antigen.[15] After 3 days, we added redirected T cells at effector to target ratio 2:1 and cocultured them for 96 hours. To determine hepatocyte injury, we measured ALT levels in the cell culture media (Figure 5*A*) of a series of independent experiments using hepatocytes from 3 different donors. ALT levels were compared with that obtained when all hepatocytes were killed

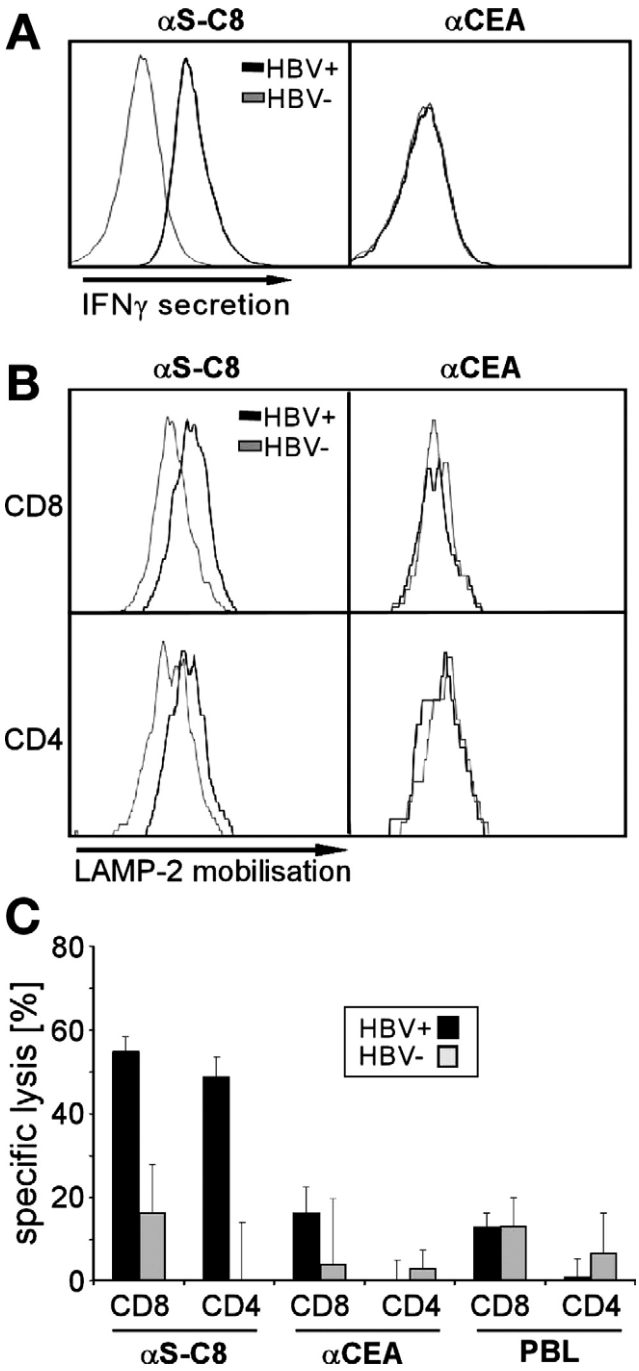

Figure 4. Phenotypic analysis of T-cell subsets. HBV-specific, αS-C8 cTCR-grafted T cells and control T cells grafted with αCEA cTCR were cocultured with HBV+ HepG2.2.15 or HBV− HepG2 cells for 48 hours. (*A*) IFN-γ-secreting cells were PE labelled using the MACS IFN-γ secretion assay and detected by flow cytometry. (*B*) Cytotoxic T cells were identified by staining for CD4, CD8, and LAMP-2 by flow cytometry. (*C*) CD4+ and CD8+ cells were isolated from cTCR-grafted T cells or unmodified cells (PBL) by magnetic cell sorting and incubated for 72 hours with HBV+ HepG2.2.15 or HBV− HepG2 cells. Specific lysis (mean ± SD) of target cells in 2 parallel assays is shown.

by induction of apoptosis using an anti-CD95 antibody.[15] ALT levels significantly increased when αS-C8 or αL-5a19 cTCR grafted T cells were subjected to HBV infected cells ($P < .01$, Student *t* test), whereas ALT levels remained unchanged when noninfected hepatocytes were used as target cells or when αCEA cTCR grafted or unmodified T cells were used as effectors. In addition, αS-C8 or αL-5a19 cTCR grafted T cells secreted 1.3 ± 0.008 ng/mL and 0.8 ± 0.007 ng/mL (mean ± SD) IFN-γ, respectively, if incubated with HBV-infected hepatocytes, indicating antigen-specific activation of cTCR grafted T cells (Figure 5*B*). Again, αS-C8 but not αL-5a19 cTCR grafted T cells secreted IL-2 (Figure 5*C*). HBV-infected hepatocytes released HBsAg and HBeAg at levels of approximately 0.35 μg/mL and 26 ng/mL medium, respectively, indicating that even high amounts of soluble HBsAg do not block activity of αS-C8 TCR grafted T cells. Despite cytotoxic activity of αS-C8 or αL-5a19 cTCR grafted T cells, HBsAg and HBeAg in cell culture media were only slightly reduced (Figure 5*D* and *E*). A possible explanation is the stability of the secreted antigen and that hepatoctyes still released antigen shortly before dying.

To control functional integrity of hepatocytes, we determined intracellular albumin. This also excluded the 10% to 20% of nonhepatocytes present in PHH cultures.[15] In hepatocytes cocultured with αS-C8 cTCR grafted T cells, we observed a 16%–17% reduction of albumin, in hepatocytes cocultured with αL-5a19 cTCR grafted T cells a 10%–14% reduction, respectively, when compared with hepatocytes cocultured with αCEA-grafted or control T cells as quantified by chemiluminescence imaging of Western blots (Figure 5*F*). Together with the ALT activity determined (Figure 5*A*), this indicated that only a minority of hepatocytes was lysed. Regarding that 0.35 μg/mL HBsAg relate to 4.4×10^4 subviral particles per hepatocyte, we concluded that binding of subviral particles did not induce unspecific killing of noninfected hepatocytes.

In contrast to albumin, HBV core protein as well as HBV DNA levels was markedly reduced. αS-C8 cTCR grafted T cells reduced HBV core by 73% and αL-5a19 cTCR grafted T cells by 57% (Figure 5*F*). They reduced intracellular HBV relaxed circular DNA (rcDNA) by 82% and 72%, respectively, as quantified by real-time PCR relative to an external standard (Figure 5*G*).

Most remarkably, the replication template, HBV cccDNA, was eliminated (>99.99% reduction, detection limit 0.0025 copies per cell) by αS-C8 cTCR grafted T cells and reduced to 0.4 copies/cell (>80% reduction) by αL-5a19 cTCR grafted T cells (Figure 5*H*). In control experiments, average levels of 2.5 copies/cell were observed.

These data indicate that T cells grafted with HBV S protein-specific cTCR αS-C8 are able to eliminate HBV-infected primary human hepatocytes. A lesser, but also

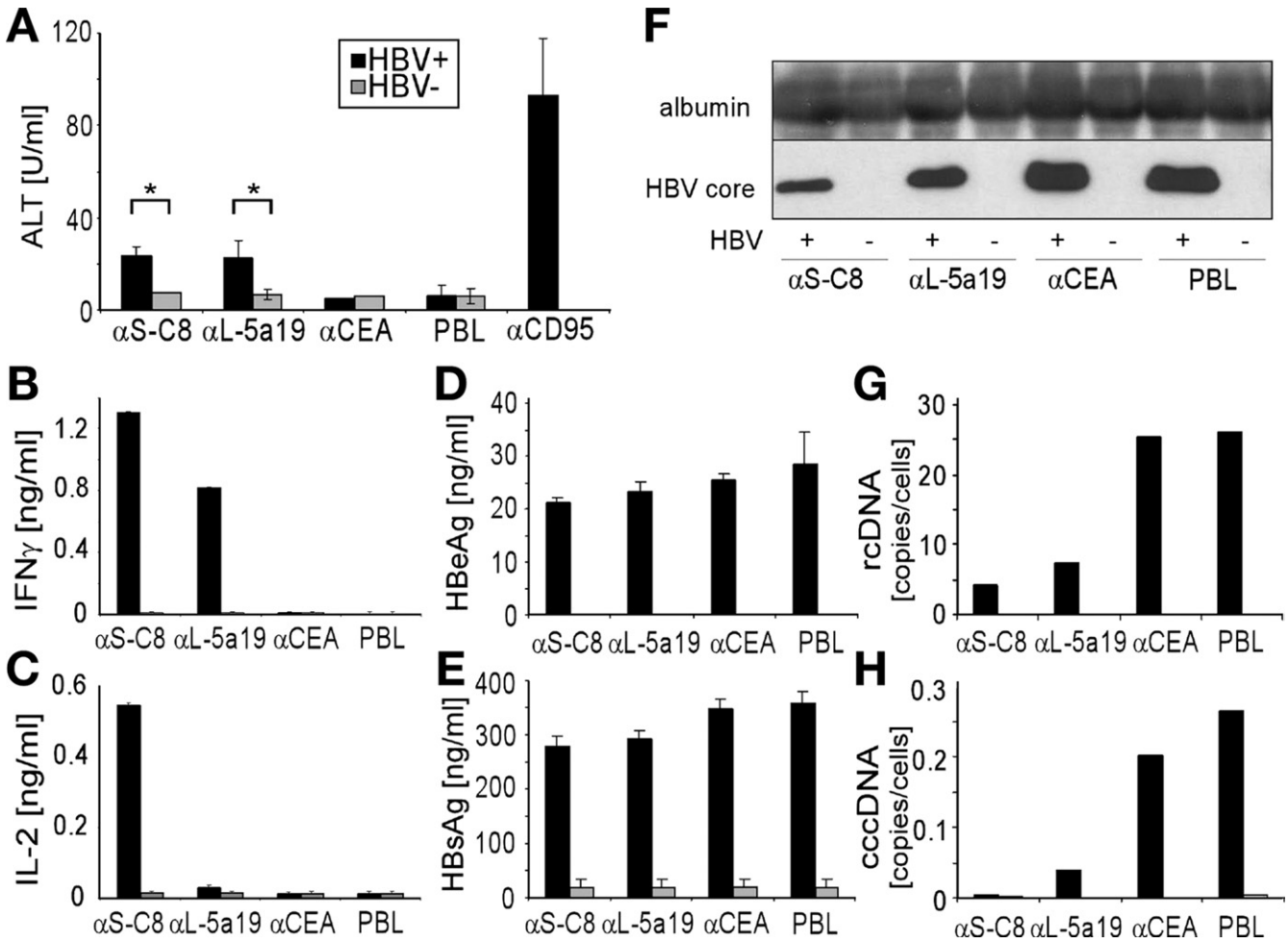

Figure 5. HBV-infected primary human hepatocytes are eliminated by antigen-specific, engineered T cells. Primary human hepatocytes were infected with HBV and cultured for 3 days prior to the addition of redirected T cells (HBV+ target cells: *black columns*; HBV− control cells: *grey columns*). T cells grafted with cTCR αS-C8, αL-5a19, or αCEA, respectively, or unmodified cells (PBL) were added as an effector to target cell ratio of 2:1 and cocultured for 96 hours. (*A*) Liver transaminase (*ALT*) levels were determined in cell culture media as a marker for hepatocyte lysis. Mean values and standard deviations obtained from 3 independent infection experiments are given. For comparison, ALT levels of αCD95-treated cells undergoing apoptosis are shown. (*B*) IFN-γ and (*C*) IL-2 were measured in the culture supernatant by ELISA. (*D*) HBeAg and (*E*) HBsAg were determined in cell culture supernatants by ELISA. (*F*) Western blot analysis of HBV-infected PHH cells for intracellular albumin and HBV core proteins. (*G*) HBV rcDNA and (*H*) HBV cccDNA were quantified by real-time PCR in cellular DNA preparations from infected hepatocyte cultures upon coculture with redirected T cells.

specific, effect was observed for T cells equipped with the HBV L protein specific cTCR αL-5a19.

Discussion

In this study, we compared cTCRs directed against 2 different surface antigens of HBV, the large L and the small S protein, with respect to their ability to arm primary T cells against HBV-infected hepatocytes. HBV surface antigens S and L are constantly present on the surface of HBV replicating cells[21] because of the targeting of HBV surface proteins into the endoplasmic reticulum (ER) membrane and steady exchange of ER membranes with the plasma membrane.[22] We aimed at targeting HBV S and L protein positive hepatocytes to eliminate residual HBV-replicating cells by inducing an artificial cytotoxic T-cell response mediated by antigen-specific cTCRs. Using S- and L-specific scFv as antigen recognition domains of the cTCRs, we demonstrated antigen-specific activation of engineered T cells resulting in cytokine secretion, expansion of this T-cell population and, most importantly, cytotoxic elimination of HBV-replicating hepatoma cells as well as HBV-infected primary human hepatocytes.

For our study, we selected 2 scFv designated C8 and 5a19 for grafting specificity to the recombinant TCR. scFv C8 was selected because it is directed against a superficial epitope in the "a" determinant of HBV S protein, which is expressed at very high amounts by infected cells. Among all scFv tested, scFv C8 showed highest binding efficiency and specificity and proved most effective and specific in targeting and T-cell activation. To obtain a suitable scFv against HBV L protein,

which is expressed in markedly lower amounts by infected cells, we used hybridoma cells producing well-established monoclonal antibody 5a19.[17]

Antigen recognition by T cells bearing αS-C8 and αL-5a19 cTCR, respectively, resulted in high-level secretion of the proinflammatory cytokine IFN-γ. Because IFN-γ has a direct antiviral effect on HBV replication in hepatocytes,[23] we assume that IFN-γ secretion by activated, redirected T cells contributes to virus control. Activation of the CD28 signalling domain in the cTCR construct[24] resulted in secretion of IL-2. Cytokine levels were markedly higher if T cells grafted with αS-C8 cTCR were used in comparison with αL-5a19 cTCR grafted T cells. The minor activation potential of the αL-5a19 TCR might be due to the lower abundance of L protein on the surface of viral and subviral particles, which contain 83%–94% S protein, but only 1%–2% L, and 5%–15% middle protein.[25] Time course experiments accordingly showed a delayed activation kinetic of T cells with the L protein-specific TCR αL-5a19 in comparison with the HBV S protein-specific cTCR.

cTCR grafted T cells bound subviral HBV particles leading to activation of NF-κB and secretion of IFN-γ. This is in contrast to observations that soluble, unlike membrane-bound, CEA does not lead to activation of αCEA cTCR[26] and that soluble monomeric HIV-1 envelope protein gp120 bound less efficiently to a specific cTCR than the respective transmembrane protein.[13] Because TCR-complex clustering is required for T-cell activation via genuine TCRs[27] as well as via recombinant cTCRs,[28] our data imply that the surface area of HBV viral and subviral particles is sufficient to induce TCR clustering.

To address the question of whether activation of redirected T cells results in nonspecific lysis of uninfected hepatocytes in the neighborhood of infected cells, we used primary human hepatocytes as target cells, which can be infected with HBV and support all steps of the HBV replication cycle. In cell culture, only a minority (5% to 15%) of hepatocytes is productively infected, although hepatocytes are incubated with HBV at high multiplicity of infection.[15] When cocultured with HBV-infected primary human hepatocytes, αS-C8 as well as αL-5a19 cTCR grafted, but not αCEA cTCR grafted, T cells or control PBL, secreted IFN-γ. However, they lysed only a proportion of the hepatocytes. Mild ALT activity in cell culture media and reduction of albumin production by hepatocytes, as well as microscopic evaluation, indicated that the majority of hepatocytes remained intact. In contrast, αS-C8 cTCR grafted T cells reduced HBV cccDNA representing the nuclear persistence form of HBV by at least 99.99% to undetectable levels. αL-5a19 cTCR grafted T cells reduced HBV cccDNA by more than 80%. This strongly argues for a specific elimination of HBV-infected hepatocytes by redirected T cells.

Although we propose that HBV-infected hepatocytes were eliminated by αS-C8 grafted T cells, HBV core protein and HBV rcDNA remained detectable. A possible explanation may be the mode by which redirected T cells eliminate their target cell. We found activation of effector caspases 3 and 7 in target hepatocytes and were able to block cytotoxicity by a pancaspase inhibitor (data not shown). We therefore expect an induction of caspase activated DNAses,[29] which will preferentially degrade histone-bound nuclear cccDNA but not HBV rcDNA, which is contained in viral capsids and therefore protected from DNAse activity.

Because αL-5a19 and αS-C8 cTCR grafted T cells bound subviral particles, we asked whether this binding might either block T-cell effector functions or induce nonspecific killing of noninfected cells. High levels of HBsAg were present in the supernatants of cocultures with primary hepatocytes. These amounts are comparable with levels achieved after antiviral therapy with pegylated interferon α and nucleoside analogues[6] and correspond to approximately 40,000 subviral HBV particles per hepatocyte or 20,000 per T cell. This vast excess of HBV particles did not impair the cytotoxic activity of redirected T cells, indicating that they did not block T-cell effector functions. Furthermore, HBV cccDNA-positive cells were selectively lysed, although all hepatocytes were subjected to high amounts of HBsAg. This indicates that soluble HBsAg itself does not mediate the cytotoxic effect.

Although the in vitro performance of redirected T cells is very promising, several safety precautions have to be considered before translation into the clinics. In particular, one needs to explore how strictly redirected T cells differentiate between infected hepatocytes and neighboring noninfected cells, which may bind HBV particles on their surface. In addition, hepatocytes in the inflamed liver may be more sensitive to cytolysis. We think it is reasonable to consider T-cell therapy after suppression of HBV replication by antiviral therapy. However, it remains open as to how efficiently redirected T cells will invade the liver parenchyma and whether they will escape silencing in an organ, which modulates immune responses.

Taken together, we have generated and qualitatively compared cTCRs with specificities for 2 different HBV surface proteins. These TCRs were retrovirally delivered to primary human T cells, activated them to secrete cytokines, and enabled them to kill stably HBV transfected as well as HBV infected primary hepatocytes. Soluble HBV particles bound to cTCR but did not block them. The cytotoxic response was specific toward HBV-replicating cells and was sufficient to eliminate HBV-infected cells from a primary human hepatocyte culture. Additionally, antigen recognition resulted in expansion of redirected T cells, a key requisite for effective repopulation when used for an adoptive transfer.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi:10.1053/j.gastro.2007.11.002.

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