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

KARIN KREBS,[1,*] NINA BÖTTINGER,[1,*] LI-RUNG HUANG,[2] MARKUS CHMIELEWSKI,[3] SILKE ARZBERGER,[1] GEORG GASTEIGER,[1] CLEMENS JÄGER,[1] EDGAR SCHMITT,[4] FELIX BOHNE,[1] MICHAELA AICHLER,[5] WOLFGANG UCKERT,[6] HINRICH ABKEN,[3] MATHIAS HEIKENWALDER,[1] PERCY KNOLLE,[2] and ULRIKE PROTZER[1]

[1]*Institute of Virology, Technische Universität München/Helmholtz Zentrum München, München;* [2]*Institute of Molecular Medicine, University of Bonn, Bonn;* [3]*Department of Internal Medicine I, University Hospital Cologne, Köln;* [4]*Institute for Immunology, University of Mainz, Mainz;* [5]*Institute of Pathology, Helmholtz Zentrum München, Neuherberg; and* [6]*Max Delbrück Center for Molecular Medicine Berlin-Buch, Berlin, Germany*

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

[*]Authors share co-first authorship.

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.

0016-5085

http://dx.doi.org/10.1053/j.gastro.2013.04.047

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