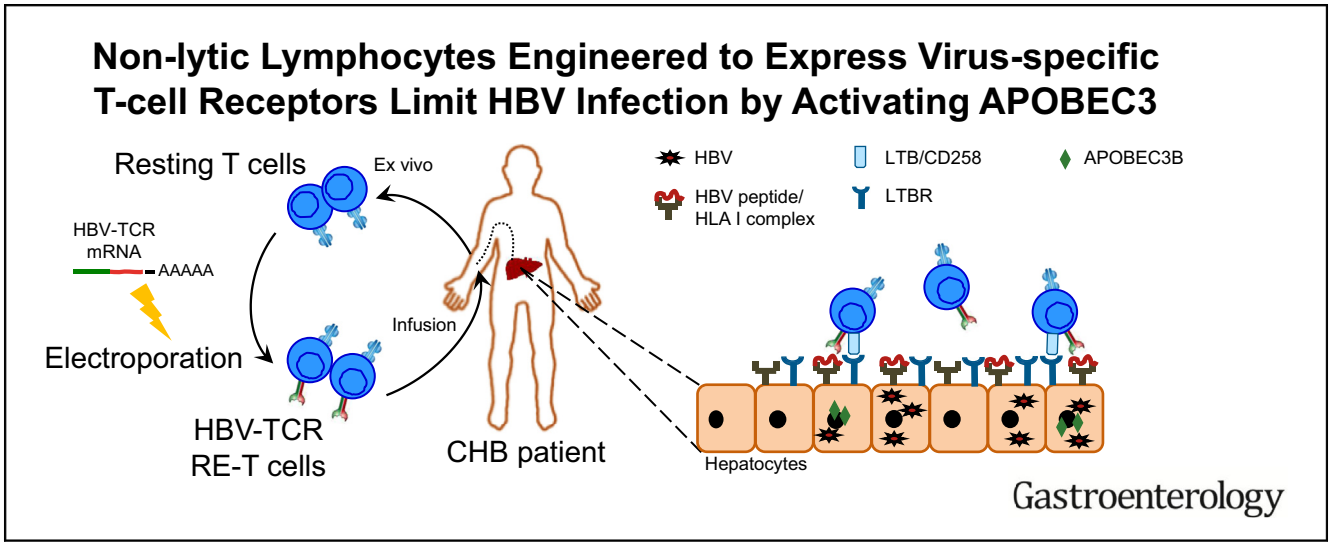
Gastroenterology 2018;155:180–193

BASIC AND TRANSLATIONAL—LIVER

Nonlytic Lymphocytes Engineered to Express Virus-Specific T-Cell Receptors Limit HBV Infection by Activating APOBEC3

Sarene Koh,[1,2] Janine Kah,[3] Christine Y. L. Tham,[1,4] Ninghan Yang,[1] Erica Ceccarello,[5,6] Adeline Chia,[7] Margaret Chen,[8] Atefeh Khakpoor,[7] Andrea Pavesi,[6] Anthony T. Tan,[7] Maura Dandri,[3,9] and Antonio Bertoletti[1,7]

*[1]Singapore Institute for Clinical Sciences, Agency for Science, Technology and Research (A*STAR), Singapore; [2]Lion TCR Private Limited Singapore, Singapore; [3]Department of Internal Medicine, University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [4]NUS Graduate School for Integrative Sciences and Engineering, National University of Singapore, Singapore; [5]Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore; [6]Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), Singapore; [7]Emerging Infectious Diseases (EID) Program, Duke-NUS Medical School, Singapore; [8]Department of Dental Medicine, Karolinska Institutet, Sweden; [9]German Center for Infection Research, Hamburg-Lübeck-Borstel Partner Site, Germany*



See Covering the Cover synopsis on page 6.

BACKGROUND & AIMS: Strategies to develop virus-specific T cells against hepatic viral infections have been hindered by safety concerns. We engineered nonlytic human T cells to suppress replication of hepatitis B virus (HBV) and hepatitis C virus (HCV) without overt hepatotoxicity and investigated their antiviral activity. **METHODS:** We electroporated resting T cells or T cells activated by anti-CD3 with mRNAs encoding HBV or HCV-specific T-cell receptors (TCRs) to create 2 populations of TCR-reprogrammed T cells. We tested their ability to suppress HBV or HCV replication without lysis in 2-dimensional and 3-dimensional cultures of HepG2.2.15 cells and HBV-infected HepG2-hNTCP cells. We also injected TCR-reprogrammed resting and activated T cells into HBV-infected urokinase-type plasminogen activator/severe combined immunodeficiency disease/interleukin 2γ mice with humanized livers and measured levels of intrahepatic and serological viral parameters and serum alanine aminotransferase. Livers were collected for analysis of gene expression patterns to determine effects of the TCR-reprogrammed T cells. **RESULTS:** TCR-reprogrammed resting T cells produced comparable levels of interferon gamma but lower levels of perforin and granzyme than activated T cells and did not lyse HCV- or HBV-infected hepatoma cells. Although T-cell secretion of interferon gamma was required to inhibit HCV replication, the HBV-specific TCR-reprogrammed resting T cells reduced HBV replication also through intracellular activation of apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3 (APOBEC3). The mechanism of APOBEC3 intracellular activation involved temporal expression of lymphotoxin-β receptor ligands on resting T cells after TCR-mediated antigen recognition and activation of lymphotoxin-β receptor in infected cells. **CONCLUSIONS:** We developed TCR-reprogrammed nonlytic T cells capable of activating APOBEC3 in hepatoma cells and in HBV-infected human

hepatocytes in mice, limiting viral infection. These cells with limited hepatotoxicity might be developed for treatment of chronic HBV infection.

Keywords: Antiviral; Immune Response; Immune Therapy; LTB.

Adoptive T-cell therapy using autologous T cells genetically modified to express a classical HLA class I restricted T-cell receptor (TCR) or a chimeric antigen receptor (CAR) targeting tumor cells has generated impressive results in treating some cancers.[1,2] T cells are, however, also essential for the control of chronic viral infections, and virus-specific TCR-redirected T cells have been shown to recognize viral-infected cells in culture and in animal models.[3] Persistent hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are 2 chronic viral diseases for which virus-specific T-cell therapy has a strong rationale.[4–6] Both infections are characterized by defects of antigen-specific T cells, and efficient viral control is associated with T-cell functional recovery. However, because both viruses are not directly cytopathic, the observed hepatitis during natural infection is immune-mediated, and thus T cells can cause liver damage.[7]

A rational approach to restoring the lack of virus-specific T-cell response in patients with chronic hepatic viral infections is to genetically engineer a patient's own (autologous) T cells to express an HBV- or HCV-specific CAR or TCR. Here, we engineered T cells with TCRs recognizing HBV or HCV peptide–HLA complexes. This strategy can offer an advantage over CAR-T cells,[5] particularly in HBV infection. HBV-specific TCR-T cells are not suppressed by the large quantity of circulating antigens (hepatitis B surface antigen [HBsAg] and hepatitis B e antigen HBeAg) that are present in chronic hepatitis B patients' sera,[8] nor could they recognize circulating monocytes and B cells accumulating HBsAg[9] because they are specific for the HLA-class I/HBV epitope complexes generated by the processing of HBV antigens synthesized within the cells. Thus, HBV-specific TCR-T cells only recognize HBV-infected hepatocytes or hepatocytes producing antigens from HBV-DNA integrations.[4]

In addition, to bypass safety concerns related to stable genetic manipulation of T cells, we established a technically simple platform using electroporation of messenger RNA (mRNA) encoding TCR genes to produce TCR-T cells for adoptive immunotherapy of HBV-related hepatocellular carcinoma (HCC).[10] The transient expression of TCR introduced by mRNA electroporation in anti-CD3–activated T cells generates HBV-specific T cells that lyse HCC cells[10] and HBV-infected hepatocytes[11] within a limited time span and induce transient liver inflammation, avoiding the persistence and accumulation of TCR-T cells. However, because a substantial number of hepatocytes could be infected in chronic hepatitis B, T-cell therapy using cytolytic TCR-T cells might still trigger severe liver pathology[12] and hence might be difficult to implement in the clinic.

We hypothesized that because mRNA can also be introduced into nondividing unstimulated T cells,[13] we could engineer virus-specific human T cells with lower granzyme B and perforin contents than activated T cells.[14] These cells should not trigger overt liver damage but maintain antiviral activity when used for immunotherapy. We thus reprogrammed the antigen specificity of resting human T cells to target HBV or HCV, tested their ability to suppress viral replication in vitro and in HBV-infected human liver chimeric urokinase-type plasminogen activator/severe combined immunodeficiency disease/IL2γ mice,[15] and further investigated their mechanism of nonlytic antiviral suppression.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

We engineered non-lytic human T cells to suppress replication of hepatitis B virus (HBV), without overt hepatotoxicity as a safer approach to adoptive T cell therapy for chronic hepatitis B.

NEW FINDINGS

TCR-reprogrammed, non-lytic T cells produced lower levels of perforin and granzyme than activated T cells and activate APOBEC3 in HBV-infected hepatocytes through temporal activation of lymphotoxin-β-receptor.

LIMITATIONS

Multiple treatments of TCR-reprogrammed T cells, in combination with inhibitors of HBV infection might be required to achieve control of HBV infection.

IMPACT

Adoptive T cell therapy using HBV TCR-reprogrammed, non-lytic T cells could be a beneficial therapeutic strategy for chronic hepatitis B without inducing inflammatory pathologies.

Materials and Methods

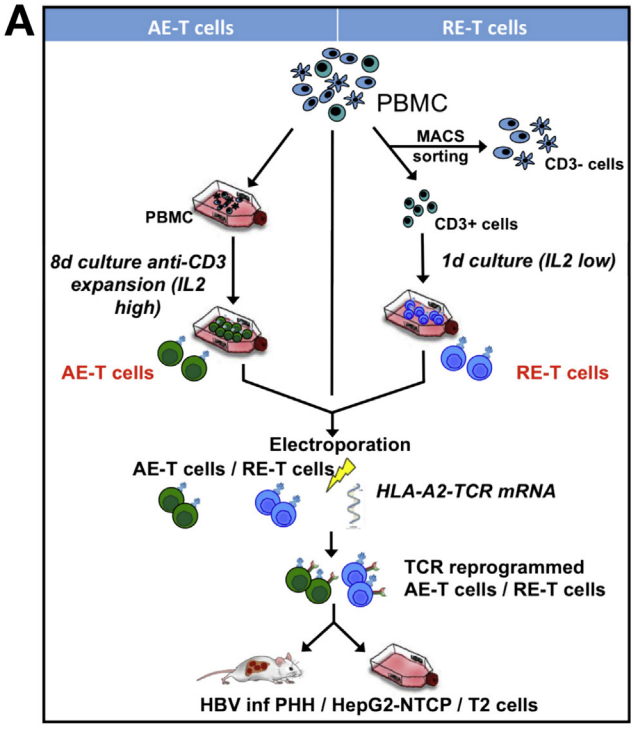
mRNA Electroporation of T Cells

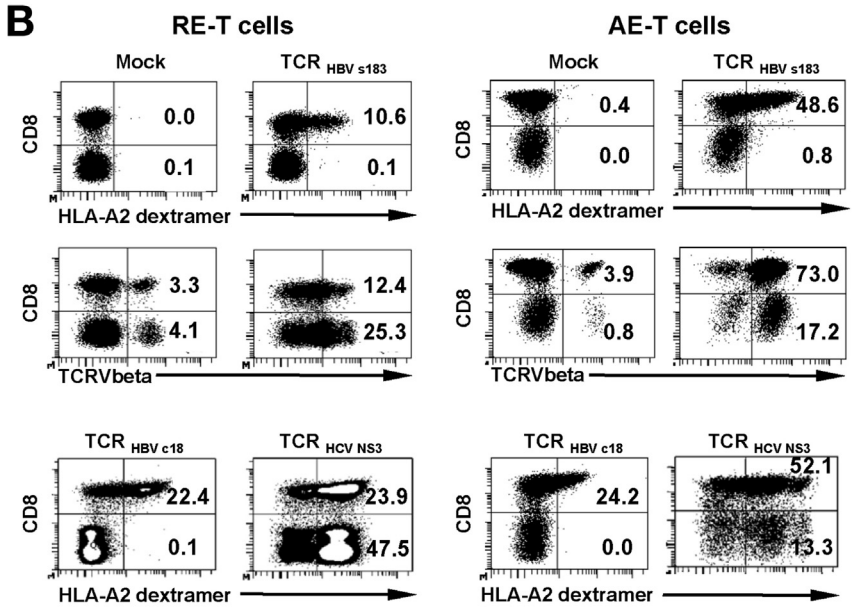
The preparation of activated (AE-T) and resting (RE-T) T cells from peripheral blood mononuclear cells (PBMCs) is described schematically in Figure 1*A* and in the Supplementary Materials. Ten million AE-T or RE-T cells were suspended in 100 μL of supplemented 4D-Nucleofector Solution (Lonza, Cologne, Germany), and TCR mRNA was added at 200 μg/mL. The mixture was electroporated in a Nucleocuvette using preset T-cell programs in the 4D-Nucleofector system (Lonza, Cologne,

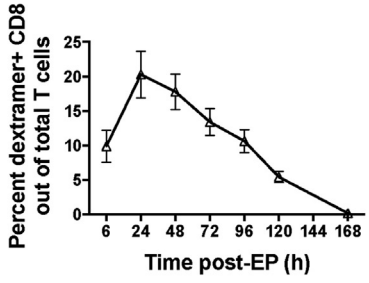
Abbreviations used in this paper: **3-D, 3-dimensional; AE-T, activated T; APOBEC3, apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3; CAR, chimeric antigen receptor; cccDNA, covalently closed circular DNA; E:T, effector cell–to–target cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBcAg, hepatitis B core antigen; HbsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; hNTCP, human sodium taurocholate-cotransporting polypeptide; IFNG, interferon gamma; LT, lymphotoxin; LTB, lymphotoxin β; LTBR, lymphotoxin β receptor; mRNA, messenger RNA; PBMC, peripheral blood mononuclear cell; pgRNA, pregenomic RNA; RE-T, resting T; TCR, T-cell receptor.**

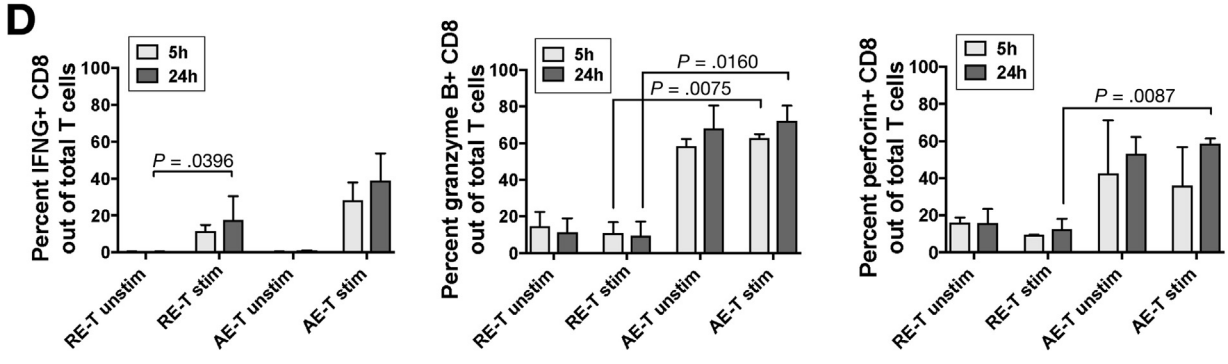
Most current article

0016-5085/$36.00
https://doi.org/10.1053/j.gastro.2018.03.027


A
AE-T cells
RE-T cells
PBMC
MACS sorting
CD3- cells
PBMC
CD3+ cells
8d culture anti-CD3 expansion (IL2 high)
1d culture (IL2 low)
AE-T cells
RE-T cells
Electroporation
AE-T cells / RE-T cells
HLA-A2-TCR mRNA
TCR reprogrammed AE-T cells / RE-T cells
HBV inf PHH / HepG2-NTCP / T2 cells


B
RE-T cells
AE-T cells
Mock
TCR HBV s183
TCR HBV c18
TCR HCV NS3
CD8
HLA-A2 dextramer
TCRVbeta
0.0
0.1
10.6
0.4
48.6
0.8
3.3
4.1
12.4
25.3
3.9
0.8
73.0
17.2
22.4
23.9
47.5
24.2
52.1
13.3


C
Percent dextramer+ CD8 out of total T cells
Time post-EP (h)
0
5
10
15
20
25
6
24
48
72
96
120
144
168


D
Percent IFNG+ CD8 out of total T cells
Percent granzyme B+ CD8 out of total T cells
Percent perforin+ CD8 out of total T cells
5h
24h
P = .0396
P = .0160
P = .0075
P = .0087
RE-T unstim
RE-T stim
AE-T unstim
AE-T stim

Germany). After electroporation, cells were resuspended in AIM-V 10% human AB serum plus 100 IU/mL rIL-2, and cultured at 37°C and 5% CO_2 until analysis.

Function of mRNA Electroporated T Cells

HLA-A2$^+$ T2 cells were pulsed with 1 μg/mL of s183–191 peptide for 1 hour at 10^6 cells/mL and then washed twice. TCR-T cells were cocultured with peptide-loaded T2 cells for 5 hours or 24 hours in the presence of 10 or 2 μg/mL brefeldin A, respectively, and stained for CD8, interferon gamma (IFNG), granzyme, and perforin.

Cytotoxicity Assays

Luciferase-expressing cell lines and TCR-T cells were co-cultured as described in the Supplementary Materials.

Co-culture Experiments of mRNA Electroporated T Cells With Targets

$TCR_{HBVs183}$ T cells were co-cultured with either HepG2.2.15 or HBV-infected HepG2– human sodium taurocholate-cotransporting polypeptide (hNTCP) for 24 hours, and $TCR_{HCV\ NS3}$ T cells were co-cultured with $Huh7_{A2}$HCV cells for 18 hours. To determine target cell lysis, aspartate aminotransferases were measured in supernatants or viability assays were performed using the Cell Proliferation Kit II 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide (XTT) (Roche Applied Science, Mannheim, Germany). For blocking IFNG, 20 μg/mL purified anti-human IFNG or isotype control mouse IgG1 (BioLegend) was added. To block LTα1β2, LTα2β1, and CD258, 1 μg/mL recombinant human lymphotoxin β (LTB) receptor (LTBR)-Fc chimera (R&D Systems, Minneapolis, MN) was used.

Three-Dimensional Microdevice-Based Assay

The seeding of HepG2 targets in microdevices was described previously.[16] TCR-T cells were labelled with 3 μmol/L of CellTracker Violet BMQC (Life Technologies Co., Carlsbad, CA) and resuspended in T-cell media at 3 $\times$ 10^6 cells/mL; 30 μL was added into 1 of the 2 media channels flanking the central gel region of each device and incubated for 15 hours at 37°C before image acquisitions. Details are described in the Supplementary Materials.

HBV Infection of Humanized Mice and T-Cell Administration

Animals were housed under specific pathogen-free conditions according to institutional guidelines under authorized protocols. All animal experiments were conducted in accordance with the European Communities Council Directive (86/EEC) and approved by the City of Hamburg, Germany.

To establish HBV infection, animals received a single intraperitoneal injection of HBV-infectious serum (1 $\times$ 10^7 HBV DNA copies/mouse, genotype D). HBV-infected mice displaying levels of viremia between 1 $\times$ 10^6 to 3 $\times$ 10^8 HBV DNA copies/mL were used for adoptive transfer of TCR-T cells. Then, 0.5 $\times$ 10^6 or 1 $\times$ 10^6 TCR AE-T or RE-T cells (normalized based on frequency of CD8$^+$TCR$^+$ T cells) corresponding to an approximately 1:100 or 1:50 effector cell–to–target cell ratio, respectively, were injected intraperitoneally per mouse. Blood was withdrawn at indicated time points, and liver specimens removed at day 12 were snap-frozen in 2-methylbutane for molecular analyses.

Intrahepatic Virological and Gene Expression Analyses

DNA was extracted from liver specimens using the Master Pure DNA Purification Kit (Epicentre, Madison, WI), covalently closed circular DNA (cccDNA) was isolated by Hirt extraction,[17] and RNA was extracted from liver using the RNeasy RNA Purification Kit (Qiagen, Hilden, Germany). Intrahepatic total viral loads were quantified using primers and probes specific for total HBV DNA and cccDNA.[18] Primers and probes specific for HBV pregenomic RNA (pgRNA) were used for reverse transcription and amplification, and the expression of the human housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. Human apolipoprotein B mRNA editing enzyme (APOBEC3) and LTB gene expression levels were quantified by quantitative polymerase chain reaction using specific primers from the TaqMan Gene Expression Assay System (Thermo Fisher Scientific, Waltham, MA) or custom designed primers (see Supplementary Tables 1 and 2).

Statistical Analysis

Statistical significance was evaluated with 2-tailed *t* test and 1-way analysis of variance with Dunnett's multiple comparisons test using GraphPad Prism 7 software (GraphPad, La Jolla, CA). Only *P* values of less than .05 were considered significant and are displayed in the figures.

Results

TCR mRNA Electroporation in RE-T Cells

HBV-specific TCR-T cells transiently expressing HBV-specific TCRs can be engineered in vitro through direct electroporation of mRNA encoding for specific TCR variable α and β chains in AE-T cells.[10] We previously showed that HBV-specific AE-T cells have antitumor and antiviral

Figure 1. Production of virus-specific TCR AE-T and RE-T cells using mRNA electroporation. (*A*) A schematic of preparation of AE-T and RE-T cells from PBMCs for mRNA TCR electroporation. (*B*) AE-T and RE-T cells from healthy donors (n = 4) were electroporated with $TCR_{HBVs183}$, TCR_{HBVc18}, or $TCR_{HCV\ NS3}$ mRNA. TCR expression was determined 24 hours after electroporation. Mock electroporated T cells served as negative control. Percentages of dextramer$^+$, TCRVβ^+ CD8$^+$, or CD8$^-$ cells were indicated in representative plots. (*C*) The mean frequencies of $TCR_{HBVs183}$ CD8$^+$ cells from 4 healthy donors were measured at indicated time points after electroporation. (*D*) The RE-T or AE-T cells were co-cultured with unpulsed or peptide-pulsed T2 cells for indicated times. Frequencies of IFNG, granzyme B, and perforin-producing CD8$^+$ cells out of total T cells were quantified. EP, electroporation; MACS, magnetic-activated cell sorting.

functions in 2 different animal models of HBV-related HCC and HBV infection.[10,11] In the present study, we investigated whether we could directly express TCR in RE-T cells, which were $CD3^+$ T cells directly purified from PBMCs and left overnight in culture before mRNA TCR electroporation. In contrast, AE-T cells were T cells cultured in vitro for 8 days with high IL-2 concentration after PBMC stimulation with anti-CD3 antibody (Figure 1*A*). The origin, cell culture conditions, and $CD8^+$ and $CD8^-$ T cell compositions in the 2 cell preparations are also summarized in Supplementary Table 3. We electroporated mRNA encoding HLA-A2–restricted TCRs specific for HBV envelope s183–191, HBV core 18–27, or HCV NS3 1073–1081 into RE-T and AE-T cells from different healthy individuals. At 24 hours after electroporation, more than 20% of $CD8^+$ and $CD8^-$ RE-T cells expressed the introduced TCRs (Figure 1*B*). mRNA electroporation is less efficient on RE-T than in AE-T cells (Figure 1*B* and Supplementary Table 3), but the kinetics of TCR expression in both T-cell populations appeared similar[10,11]: they were detectable at 6 hours after electroporation, peaked at 24 hours, and disappeared after 120 hours (Figure 1*C*). More than 70% of $CD8^+$ TCR-expressing RE-T cells were $CD45RA^+CD62L^+$ naïve-like and $CD27^+CD28^+$ (Supplementary Figure 1*A*), and $CD8^+$ TCR-expressing AE-T cells comprised 39% $CD45RA^+CD62L^+$ naïve-like, 45% $CD45RA^-CD62L^+$ central memory-like, and 15% $CD45RA^-CD62L^-$ effector memory-like phenotypes and were $CD27^+CD28^+$ (Supplementary Figure 1*B*). $CD8^-$ TCR-expressing RE-T and AE-T cells comprised a mixture of all 3 phenotypes (Supplementary Figure 1). We have consistently observed bias of $CD8^+$ T cells toward a more naïve-like phenotype in RE-T cells in all healthy donors tested (age range, 25–55 years), as has been reported in a previous study.[19] No difference was detected in the phenotypes of non–TCR-expressing compared with TCR-expressing cells within RE-T or AE-T cells, indicating that mRNA electroporation does not alter T-cell phenotype or preferentially transfect cells of particular differentiation status (data not shown).

Functional Characterization of RE-T Cells

Because the immunological control of HBV and HCV does not necessarily require the direct lysis of infected hepatocytes and can also be achieved through nonlytic mechanisms triggered by antiviral cytokines,[7,20,21] we analyzed the ability of RE-T and AE-T cells to produce cytolytic granules and IFNG. Both $CD8^+$ RE-T and AE-T cells produced IFNG upon 5 hours and 24 hours of stimulation with HBV peptide–pulsed T2 cells, but not in the absence of peptide. A higher frequency of IFNG-producing AE-T cells was determined because of their higher transfection efficiency (Figure 1*C*). Furthermore, 50%–60% of unstimulated $CD8^+$ AE-T cells produced granzyme B and perforin, and these frequencies were slightly increased after 24 hours of stimulation (Figure 1*C*). In contrast, only 10%–15% of unstimulated $CD8^+$ RE-T cells produced granzyme B and perforin, with frequencies similar after 5 or 24 hours of stimulation (Figure 1*C*).

This characterization suggests that RE-T cells would have lower cytolytic capacity than AE-T cells. We tested the killing abilities of both TCR-T cells in a 3-dimensional (3-D) microfluidic device whereby TCR-T cells are interacting with hepatoma cells overexpressing green fluorescent protein covalently linked to HBV envelope protein (HepG2-Env)[16] (Figure 2*A*). AE-T cells (mean ± standard error of the mean, 40.40% ± 1.32%) killed more HepG2-Env cells than RE-T cells (mean ± standard error of the mean, 17.54% ± 1.55%) (Figure 2*B* and Supplementary Figure 2*A*). Although RE-T cells showed reduced ability to migrate toward the HepG2-Env cells (Supplementary Figure 2*A*), the difference in cytotoxicity was not due to lack of target recognition, because there were no significant differences in the proportion of IFNG-secreting cells within the total HBV-specific TCR-expressing RE-T or AE-T cells (Supplementary Figure 2*B*). The reduced lytic ability of RE-T cells was confirmed in 2-dimenional plate-based cytotoxicity assays where RE-T cells, despite being in direct contact with the HepG2-Env cells, did not show substantial killing at various E:T ratios (Figure 2*C*). Co-culture of AE-T cells with the HepG2-Env cells in the microdevices resulted in a distinct elevation of inflammatory factors (RANTES, MIP1A, MIP1B, CCL-22, IL-13, IFNG, sCD40L, and IL-5) (Figure 2*D*). In contrast, co-culture with RE-T cells caused a significant elevation of only IFNG, comparable to that produced by AE-T cells, and most other soluble factors remained unchanged (Figure 2*D*). These data indicate that RE-T cells, after recognition of the specific viral antigen, could concurrently induce significant IFNG production with limited generation of inflammatory chemoattractants and could inhibit hepatotropic viruses without causing overt inflammation and liver damage.

RE-T Cells Inhibit Virus Replication Without Hepatotoxicity

We tested this hypothesis by co-culturing $TCR_{HBVs183}$ RE-T cells with HBV-replicating HepG2.2.15 hepatoma cell line at E:T 1:3 for 24 hours. Intracellular HBV DNA was reduced by 31% within 24 hours (Figure 3*A*), similar to that obtained with AE-T cells. However, unlike AE-T cells, aspartate aminotransferase, a marker of hepatocyte injury, remained unchanged when RE-T cells were used as effectors, confirming their reduced lytic ability (Figure 3*A*). Similar levels of antiviral activity without lysis could also be achieved when HepG2.2.15 cells were co-cultured with either engineered naïve $CD8^+$ or central memory $CD8^+CD62L^+$ resting T cells (Figure 3*B*). The nonlytic antiviral capability of RE-T cells was further confirmed when $TCR_{HCV\ NS3}$ T cells were co-cultured with HLA-A2–expressing Huh7 cells transduced with a subgenomic luciferase replicon ($Huh7_{A2}HCV$).[22] A strong inhibition of HCV replication in $Huh7_{A2}HCV$ cells was observed at all E:T ratios in a dose-dependent manner without target cell lysis, as shown by XTT assay, an alternative method to quantify viable cells based on metabolic activity (Figure 3*C*).

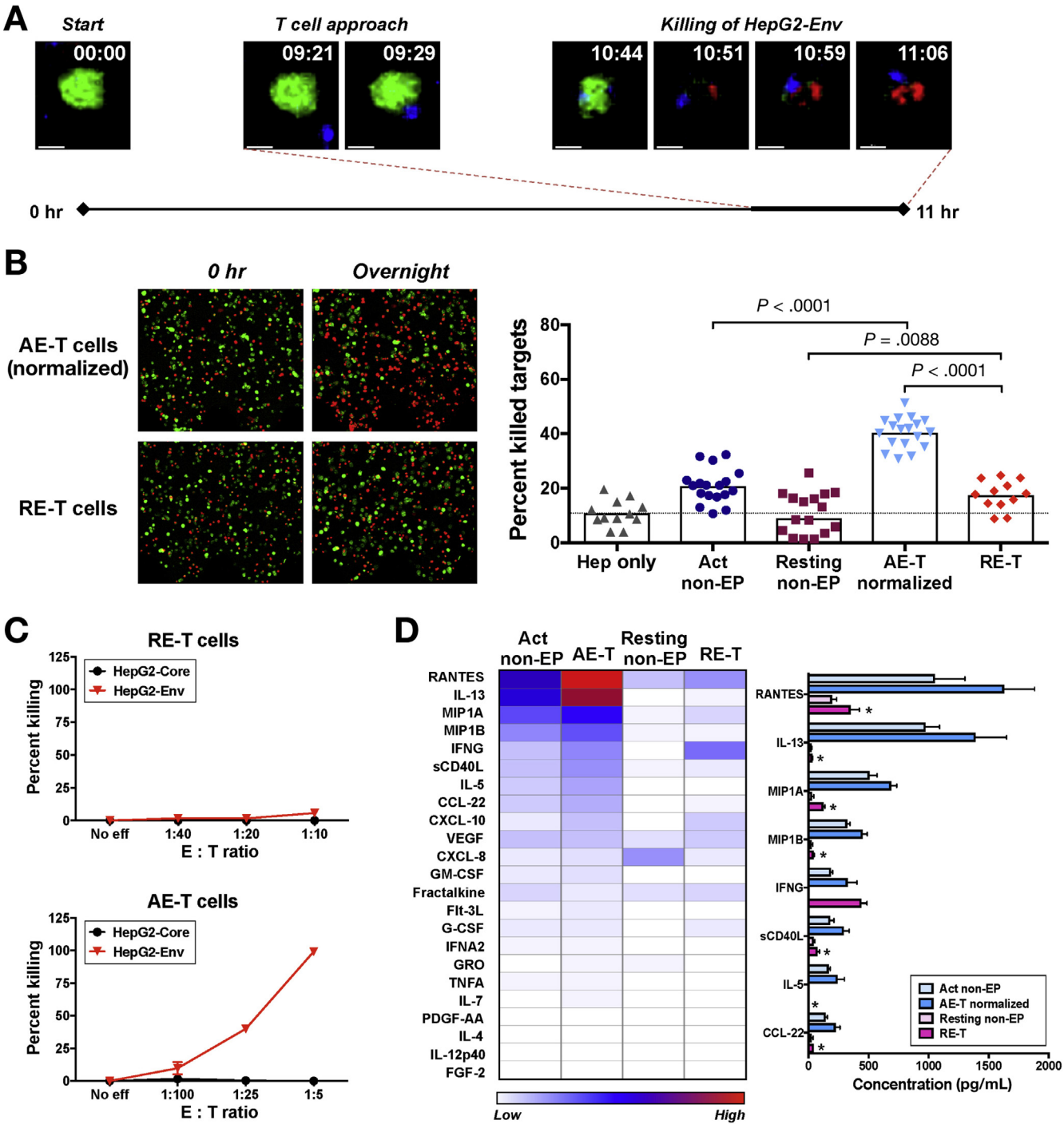
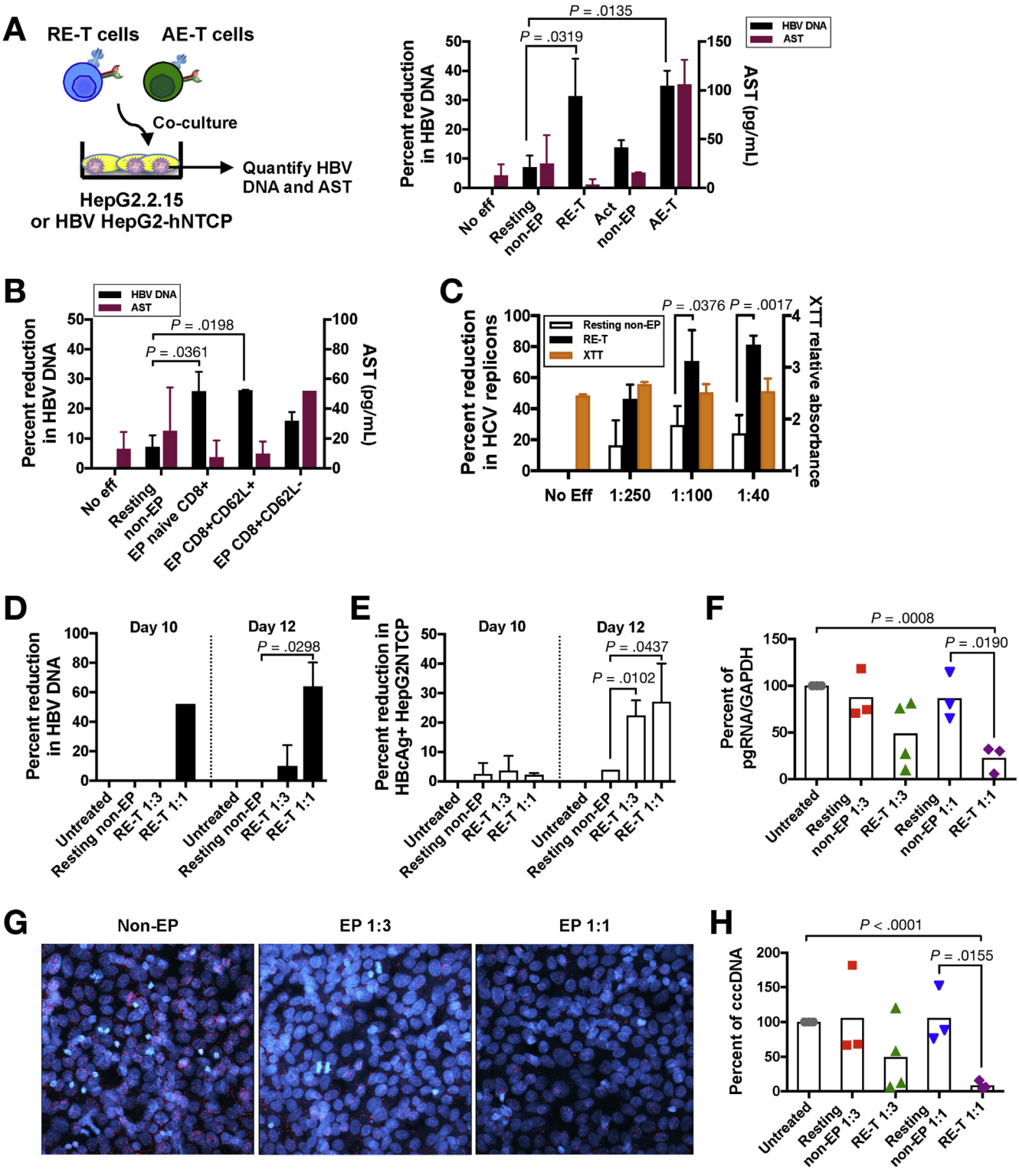


Figure 2. The RE-T cells are nonlytic and noninflammatory. (*A*) A representative 11-hour live-imaging timeline where $TCR_{HBVs183}$ AE-T cells were co-cultured with GFP-expressing HepG2-Env cells in a 3-D microfluidic device. The AE-T cells are displayed as blue cells, and dead cells are labeled red. The magnified maximum-intensity projections of a single HepG2-Env cell are shown at the indicated times. The 10-μm scale bars are included in each image. (*B*) Representative maximum intensity projections of a collagen gel region showing HepG2-Env cells (green) at 0 hours and after overnight co-culture with $TCR_{HBVs183}$ RE-T or AE-T cells. Dead cells are labeled red. The MFIs of GFP (green) and DRAQ7 (red) of each HepG2-Env cell were plotted at 0 hours and after overnight co-culture. Bar chart shows the mean percentage of killed HepG2-Env cells, and each dot represents a single experiment. Devices without T cells or co-cultured with non-electroporated T cells were included as controls. (*C*) Two-dimensional cytotoxicity assay performed using $TCR_{HBVs183}$ RE-T or AE-T cells and HepG2-Env or HepG2-Core cells. The mean percentages of killing obtained with different E:T ratios from 3 independent experiments are shown. (*D*) Heatmap of the relative mean concentration of soluble factors detected in the supernatants collected from microdevices, with indicated engineered T cells (n = 5 each). Factors with concentrations less than 10 pg/mL in all samples were removed from analysis. The bar chart shows the mean concentrations ± standard error of the mean of the top 8 secreted factors, and asterisk indicates statistically significant difference between RE-T and AE-T cells. EP, electroporation; GFP, green fluorescent protein; MFI, mean fluorescence intensity.

We then sought to confirm our findings in an infection system. HepG2 cells stably transduced with the HBV entry receptor hNTCP[23] were infected with HBV and, at day 7 after infection, were either left untreated or were treated for 24 hours with $TCR_{HBVs183}$ RE-T cells or non-electroporated RE-T cells. The T cells were removed and, after an additional 48 or 96 hours culture, cytoplasmic HBV DNA, hepatitis B core antigen (HBcAg,



pgRNA, and HBV cccDNA) were measured. Treatment with RE-T cells at E:T 1:1 resulted in 50% reduction in cytoplasmic HBV DNA at day 10 after infection, which increased to 64% by day 12 (Figure 3*D*). The reduction in HBV DNA at day 12 was accompanied by a 27% decrease in the frequency of $HBcAg^+$ HepG2-hNTCP (Figure 3*E*) and a 78% reduction in pgRNA normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) compared with untreated HepG2-hNTCP (Figure 3*F* and *G*). Notably, cccDNA decreased to less than 5% compared with untreated HepG2-hNTCP (Figure 3*H*). Less pronounced antiviral effects were observed at E:T 1:3 (Figure 3*D*–*H*), and similar reductions in viremic parameters were observed using RE-T cells expressing TCR_{HBVc18} (data not shown). Taken together, our data show that RE-T cells mediate a rapid inhibition of HBV and HCV replication in targets with minimal cytolysis.

IFNG Produced by RE-T Cells Does Not Inhibit HBV Replication

Because T cell–derived cytokines IFNG and tumor necrosis factor α can control HBV and HCV replication[20,24,25] and were also shown to degrade HBV cccDNA in infected hepatocytes,[26] we investigated whether RE-T cells might mediate antiviral effects through a similar mechanism. Using IFNG-blocking antibodies, we showed that $TCR_{HCV\ NS3}$ RE-T cell–mediated antiviral effect against $Huh7_{A2}HCV$ cells requires IFNG (Figure 4*A*). Surprisingly, inhibition of HBV replication in transfected (HepG2.2.15) or HBV-infected (HepG2-hNTCP) cells by $TCR_{HBVs183}$ RE-T cells was not reduced by addition of IFNG-blocking antibodies (Figure 4*A*), suggesting that IFNG produced by RE-T cells was not mediating the rapid nonlytic antiviral effect against HBV.

RE-T Cells Activate APOBEC3 Antiviral Pathway for Rapid HBV Inhibition

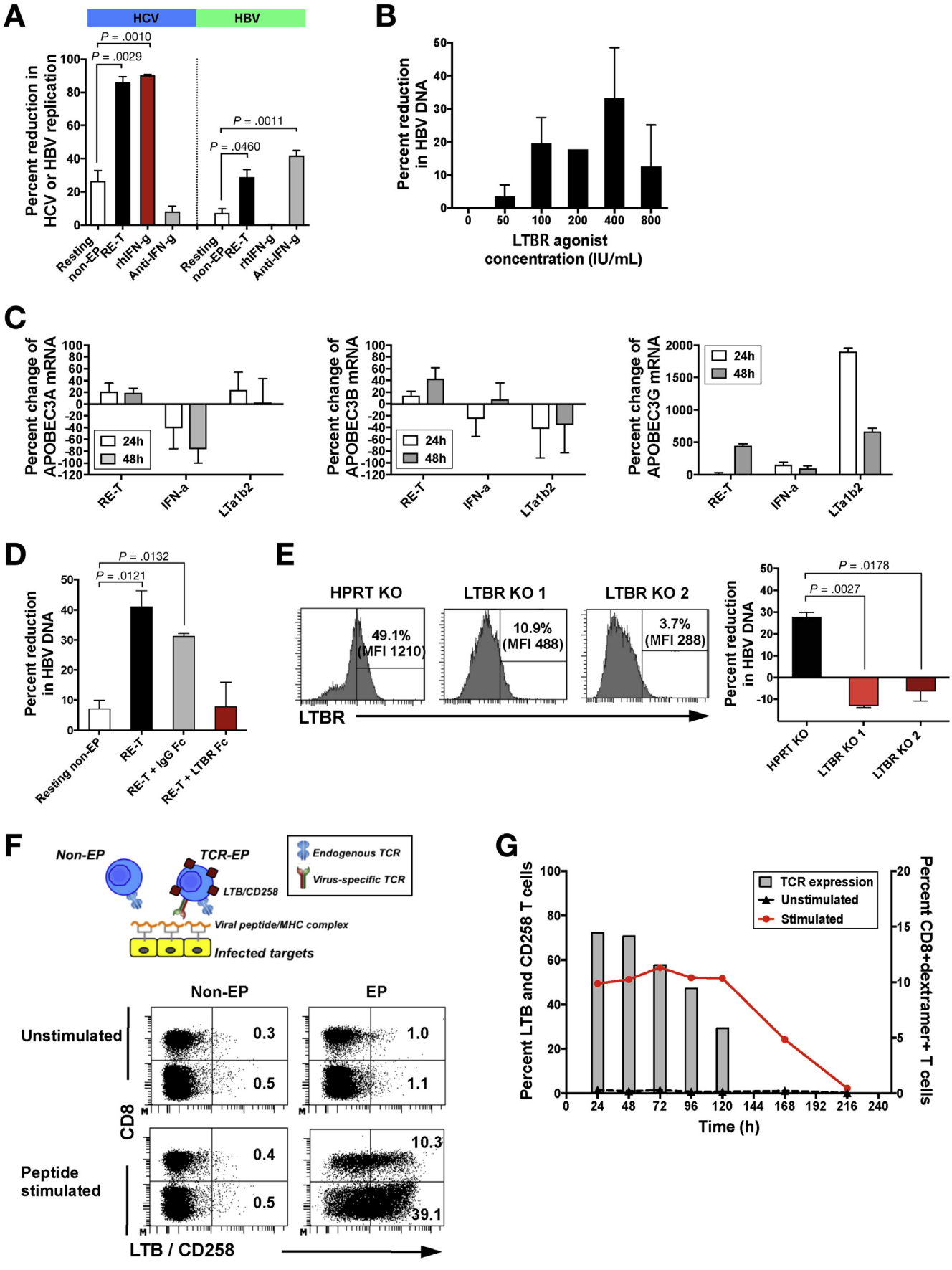
We explored other possible mechanisms of the rapid nonlytic anti-HBV activity. Up-regulation of APOBEC3 cytidine deaminases A3A, A3B, and A3G through interferon alfa[27] and LTBR activation[28] have been shown to inhibit HBV replication by the induction of HBV-DNA hypermutations and might degrade cccDNA without causing hepatotoxicity.[28,29] Thus, we first quantified A3A, A3B, and A3G mRNA expression in HepG2.2.15 after 24 and 48 hours of treatment with RE-T cells, compared with IFN alfa or LTBR agonist (LTα1/β2) treatment (Figure 4*B* and *C*). Treatment with RE-T cells (able to reduce intracellular HBV DNA by 31%) for 24 or 48 hours induced a 20%–40% increase in A3 and A3B relative mRNA, respectively, and a 450% increase in A3G relative mRNA (Figure 4*C*). Similar viral load reductions could be achieved using LTBR agonist (Figure 4*B*), concomitant with significant increase in A3A and A3G relative mRNA (Figure 4*C*). Treatment with IFN-alfa for a short duration of 24 to 48 hours, however, decreased A3A relative mRNA and caused a less robust increase in A3B and A3G relative mRNA. These data suggest that RE-T cells, like LTBR agonist, might be capable of rapidly activating the APOBEC3 innate antiviral pathway by interaction with LTBR on infected targets.

We then investigated if RE-T cells could activate APOBEC3 in other physiological cells. We co-cultured RE-T cells with different immune cell subsets (T cells, B cells, and monocytes) for 24 and 48 hours and quantified A3A, A3B, and A3G mRNA. Significant increases in A3B relative mRNA could be detected in peptide-pulsed T cells, B cells, or monocytes at 48 hours (Supplementary Figure 3), showing that specific activation by RE-T cells can also trigger intracellular the APOBEC3 innate pathway in different primary human cells other than HepG2.2.15.

To confirm the importance of LTBR activation, we blocked the physiological LTBR expressed on HepG2.2.15 with a competing soluble LTBR Fc chimera antibody in the co-culture assay. This blocking resulted in only 8% reduction in intracellular HBV DNA compared with 31% and 41% reduction, respectively, in the co-cultures with or without IgG Fc antibody (Figure 4*D*). In addition, CRISPR/Cas9-mediated knockout of *LTBR,* but not *HPRT* (a housekeeping gene) in HepG2.2.15 followed by co-culture with RE-T cells for 24 hours could not inhibit intracellular HBV DNA (Figure 4*E*), providing the final confirmation that *LTBR* activation is essential for RE-T cell–mediated antiviral activity.

The natural ligands of LTBR are LTB heterotrimers and CD258 (or LIGHT), and they can be expressed on activated

Figure 3. The RE-T cells mediate rapid antiviral effects without cytolysis. (*A*) Mean percentage reductions in intracellular HBV DNA ± standard deviation and means of AST ± standard deviation from 3 independent experiments of $TCR_{HBVs183}$ RE-T (n = 4) or AE-T (n = 3) cells co-cultured with HepG2.2.15 at 1:3 E:T ratio for 24 hours. (*B*) Naïve $CD8^+$, $CD62L^+CD8^+$ and $CD62L^-CD8^+$ memory cells were isolated from RE-T cells and co-cultured with HepG2.2.15 as in (*A*). Results from 2 independent experiments are shown. (*C*) Mean percentage reductions in HCV replication and mean XTT relative absorbance (cell viability) from 3 independent experiments of $TCR_{HCV\ NS3}$ RE-T (n = 3) or non-electroporated T cells co-cultured with $Huh7_{A2}HCV$ at indicated E:T ratios for 24 hours. (*D*) Mean percentage reductions in cytoplasmic HBV DNA ± standard deviation and (*E*) mean percentage reductions in $HBcAg^+$ HepG2-hNTCP ± standard deviation induced by $TCR_{HBVs183}$ RE-T cells co-cultured for 24 hours with HepG2-hNTCP infected for the indicated time (10 and 12 days). (*F*) Mean percentage HBV pgRNA normalized to GAPDH in HepG2-hNTCP at day 12 after treatment with RE-T or non-electroporated T cells. Each symbol represents a single experiment. (*G*) Representative confocal images of HBV pgRNA (pink dots) in HepG2-hNTCP at day 12 after treatment with RE-T or non-electroporated T cells. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). (*H*) Mean percentage HBV cccDNA in HepG2-hNTCP at day 12 after treatment with RE-T or non-electroporated T cells compared with untreated HepG2-hNTCP. Each dot represents a single experiment. Act, Activated; AST, aspartate aminotransferase; Eff, effect; EP, electroporated; XTT, 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide.


A
HCV
HBV
P = .0010
P = .0029
P = .0011
P = .0460
Percent reduction in HCV or HBV replication
Resting non-EP
RE-T
rhIFN-g
Anti-IFN-g
B
Percent reduction in HBV DNA
LTBR agonist concentration (IU/mL)
C
Percent change of APOBEC3A mRNA
Percent change of APOBEC3B mRNA
Percent change of APOBEC3G mRNA
24h
48h
RE-T
IFN-a
LTa1b2
D
P = .0132
P = .0121
Percent reduction in HBV DNA
Resting non-EP
RE-T
RE-T + IgG Fc
RE-T + LTBR Fc
E
HPRT KO
LTBR KO 1
LTBR KO 2
49.1% (MFI 1210)
10.9% (MFI 488)
3.7% (MFI 288)
LTBR
P = .0178
P = .0027
Percent reduction in HBV DNA
F
Non-EP
TCR-EP
LTB/CD258
Viral peptide/MHC complex
Infected targets
Endogenous TCR
Virus-specific TCR
Non-EP
EP
Unstimulated
Peptide stimulated
CD8
0.3
0.5
1.0
1.1
0.4
0.5
10.3
39.1
LTB / CD258
G
Percent LTB and CD258 T cells
Percent CD8+dextramer+ T cells
TCR expression
Unstimulated
Stimulated
Time (h)

lymphocytes.[30] Therefore, we investigated whether RE-T cells expressed these ligands on their cell surface. Non-electroporated or unstimulated RE-T cells did not express LTB or CD258, but their expression increased only upon antigen-specific TCR recognition (Figure 4*F*). Taken together, these data show that RE-T cells can inhibit HBV replication through activation of the LTBR/APOBEC3 pathway in infected target cells and that this is mediated by up-regulation of LTBR ligands on RE-T cells after TCR-mediated antigen recognition.

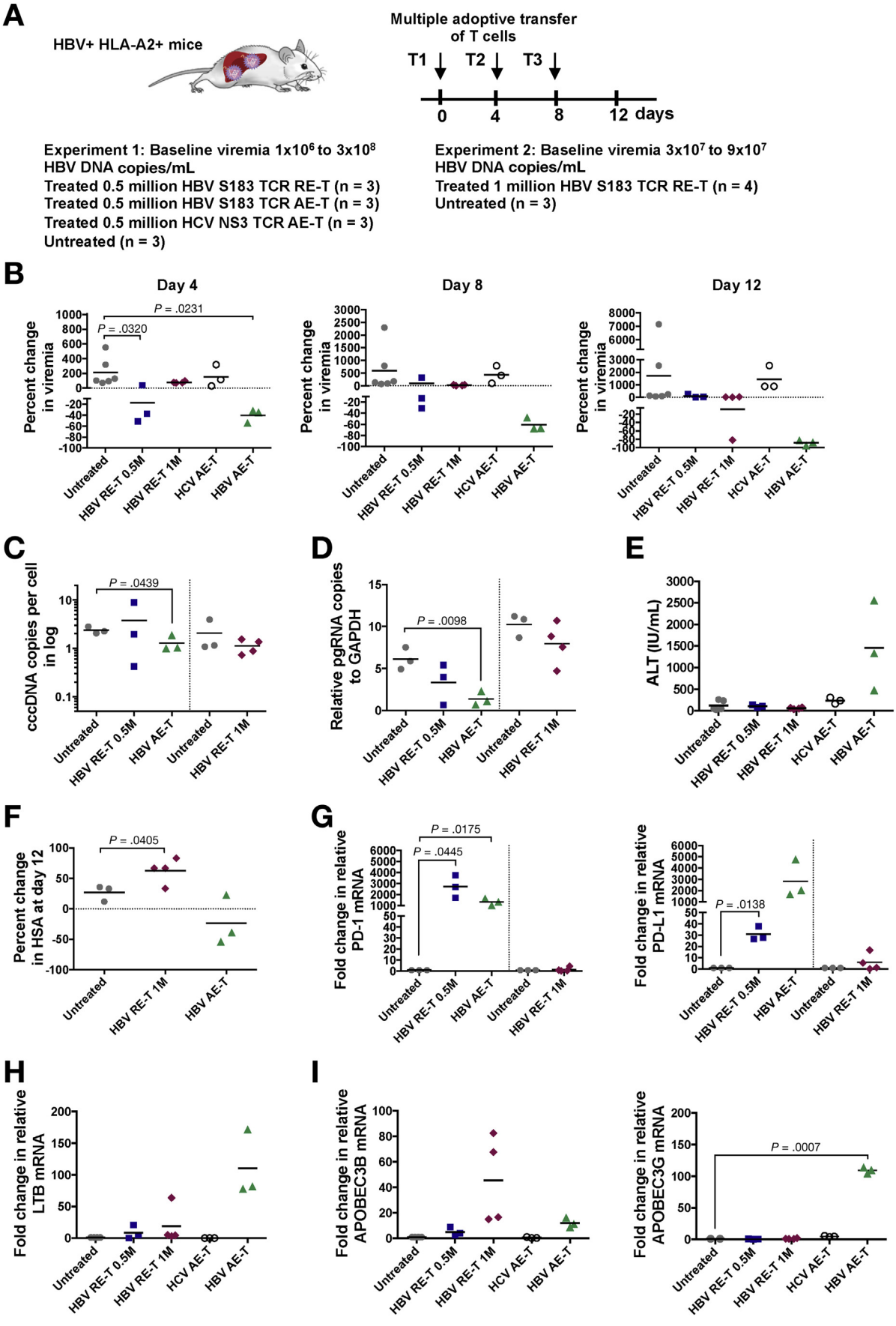
RE-T Cells Transiently Express LTBR Ligands

Despite having the desired antiviral effects, chronic activation of LTBR has also been shown to induce carcinogenesis and inflammation.[31] It is therefore pertinent to know the kinetics of LTB and CD258 expression on RE-T cells. We measured these ligands on RE-T cells longitudinally after antigen-specific activation and found that their expression remained stable from 24 to 120 hours, then decreased progressively with time as TCR expression declined and disappeared after 216 hours (Figure 4*G*). Therefore, in addition to a self-limited expression of virus-specific TCR, RE-T cells can activate the LTBR antiviral pathway in a temporal fashion, minimizing the risk of triggering chronic inflammation or cancer.

RE-T Cells Activate LTB and APOBEC3B in HBV-Infected Primary Human Hepatocytes Without Inducing Liver Inflammation

To assess the antiviral effects of adoptively transferred RE-T cells in vivo, HBV-infected human liver chimeric mice (baseline viremia, 1×10^6 to 3×10^8 HBV DNA copies/ml) previously reconstituted with HLA-A2$^+$ human hepatocytes were used. In the first set of experiments, each HBV-infected human liver chimeric mouse was adoptively transferred with 3 doses of 0.5 million $TCR_{HBVs183}$ RE-T (at 4-day intervals, n = 3), or with $TCR_{HBVs183}$ AE-T cells (n = 3), which were already shown to lyse HBV-infected hepatocytes and cause liver inflammation,[11] or with $TCR_{HCV\ NS3}$ AE-T cells (n = 3). Furthermore, 3 untreated HBV-infected mice served as controls (Figure 5*A*). The antiviral effect of RE-T cells was measured in the serum of treated animals after single (day 4) or multiple T-cell injections (days 8 and 12). A single injection of 0.5 million RE-T cells caused a 20% mean decrease of serum HBV DNA from baseline at day 4 (Figure 5*B*). Although mean HBV DNA viremia further increased to 128% from baseline to day 12 in untreated mice, multiple injections of 0.5 million RE-T cells could inhibit viral replication and so lower viremia or restrain further increase (Figure 5*B*). To determine if we could enhance the antiviral efficacy with a higher T-cell dose, we adoptively transferred 3 doses of 1 million RE-T cells at 4-day intervals into a second group of mice (baseline viremia 3×10^7 to 9×10^7 HBV DNA copies/mL) (Figure 5*A*). This treatment also restrained the increase of viremia from baseline (mean 78% versus 210% increase in untreated mice at day 4) until day 12 (mean 10% reduction versus 1706% increase in untreated), with 1 mouse showing more than 80% reduction (Figure 5*B*). Lower amounts of intrahepatic HBV cccDNA per hepatocyte (mean 50%) were determined in mice treated with 1 million, but not 0.5 million, RE-T cell doses when compared with the matched untreated controls (Figure 5*C*). Such reduced cccDNA amount was also determined in mice treated with AE-T cells. Intrahepatic pgRNA copies relative to GAPDH were also lower in RE-T and AE-T cells treated mice compared with untreated mice (Figure 5*D*). No increase in serum alanine transaminase (Figure 5*E*) or reduction in human serum albumin levels (Figure 5*F*) were determined after multiple injections of RE-T cells. By contrast, multiple injections of AE-T cells increased alanine transaminase values (1500 IU/mL) concomitant with an average 24% reduction in human serum albumin levels (Figure 5*E* and *F*). Despite the lack of induction of inflammatory events, intrahepatic PD-1 and PD-L1 expression levels increased after 3 injections of RE-T cells, showing that RE-T cells were activated in the liver after encountering HBV-infected hepatocytes. High levels of PD-1 and PD-L1 were detected in mice treated with 3 doses of 0.5 million, but not with 1 million, RE-T cells (Figure 5*G*). Circulating hepatitis B e antigen and HBsAg levels remained relatively stable after 2

Figure 4. Increased expression of LTBR ligands on RE-T cells after antigen-specific TCR recognition activates intracellular APOBEC3. (*A*) Mean percentage reductions in HCV or HBV replication ± standard deviation of 2 independent experiments of $Huh7_{A2}$HCV and HepG2.2.15 treated with TCR–RE-T or non-electroporated T cells at a 1:3 E:T ratio in the absence or presence of 10 μg/mL anti-IFNG antibodies or 200 IU/mL rhIFNG alone for 24 hours. (*B*) Mean percentage reductions in intracellular HBV DNA ± standard deviation of 2 independent experiments of HepG2.2.15 treated with indicated doses of LTBR agonist for 48 hours. (*C*) Percent change of A3A, A3B, and A3G mRNA ± standard deviation of triplicates in HepG2.2.15 treated with RE-T or non-electroporated T cells at a 1:3 E:T ratio or 1000 IU/mL IFN-alfa or 400 IU/mL LTBR agonist for the indicated times compared with treatment with non-electroporated T cells. (*D*) Mean percent reductions in intracellular HBV DNA ± standard deviation of 2 independent experiments of HepG2.2.15 treated with RE-T or non-electroporated T cells at a 1:3 E:T ratio for 24 hours in the absence and presence of soluble LTBR Fc chimera antibody or a control IgG Fc antibody. (*E*) Frequency of cells expressing LTBR and the MFI of LTBR expression in LTBR-knockout (clones 1 and 2) and HPRT-knockout HepG2.2.15. Bar chart shows mean percent reductions in intracellular HBV DNA ± standard deviation of 3 independent experiments of knockout clones co-cultured with $TCR_{HBVs183}$ RE-T cells at a 1:3 E:T ratio for 24 hours. (*F*) RE-T or non-electroporated T cells were co-cultured with unpulsed or peptide-pulsed T2 cells for 24 hours, and the frequencies of RE-T cells expressing LTB and CD258 from one representative experiment are shown. (*G*) The frequencies of CD8$^+$dextramer$^+$ T cells (bars) and LTB- and CD258-expressing T cells out of total lymphocytes in unstimulated (dotted black line) and peptide-stimulated (red line) RE-T cells were determined longitudinally at indicated times. EP, electroporated; KO, knockout; MFI, mean fluorescent intensity.


A
HBV+ HLA-A2+ mice
Multiple adoptive transfer of T cells
T1
T2
T3
0
4
8
12 days
Experiment 1: Baseline viremia 1x10^6 to 3x10^8 HBV DNA copies/mL
Treated 0.5 million HBV S183 TCR RE-T (n = 3)
Treated 0.5 million HBV S183 TCR AE-T (n = 3)
Treated 0.5 million HCV NS3 TCR AE-T (n = 3)
Untreated (n = 3)
Experiment 2: Baseline viremia 3x10^7 to 9x10^7 HBV DNA copies/mL
Treated 1 million HBV S183 TCR RE-T (n = 4)
Untreated (n = 3)
B
Day 4
Day 8
Day 12
Percent change in viremia
P = .0231
P = .0320
Untreated
HBV RE-T 0.5M
HBV RE-T 1M
HCV AE-T
HBV AE-T
C
cccDNA copies per cell in log
P = .0439
D
Relative pgRNA copies to GAPDH
P = .0098
E
ALT (IU/mL)
F
Percent change in HSA at day 12
P = .0405
G
Fold change in relative PD-1 mRNA
P = .0175
P = .0445
Fold change in relative PD-L1 mRNA
P = .0138
H
Fold change in relative LTB mRNA
I
Fold change in relative APOBEC3B mRNA
Fold change in relative APOBEC3G mRNA
P = .0007

injections of RE-T cells compared with untreated mice and increased after the third injection (Supplementary Figure 4).

To determine which intracellular antiviral pathway was activated, we performed a transcriptomic analysis of immune genes in the mice livers. We detected up-regulation of genes related to the interferon response pathway (*MX1, STAT1, STAT2, STAT6, JAK2, GBP1, TLR3, IRF1, IRF8, IFITM1)* in mice treated with multiple injections of 1 million RE-T cells (Supplementary Figure 5*A*). Interestingly, we measured increased expression of apoptosis-related genes (*BAX, BCL10, BCL2L11*) and of protein kinase genes (*MAP4K4, CHUK, MAPK1, MAPKAPK2*) involved in LTBR signaling leading to downstream nuclear factor κB activation pathways (Supplementary Figure 5*A*). A slight increase (3-fold) of human ki67 and caspase 3 transcripts were determined in the livers of mice treated with RE-T cells (Supplementary Figure 5*B*), suggesting that apoptosis and some compensatory cell proliferation may have occurred.

To confirm the activation of the LTBR/APOBEC3 antiviral pathway, we measured LTB and A3B and A3G mRNA in the mice livers at day 12. Multiple injections led to a 9-fold and 19-fold increase in LTB mRNA in mice treated with 0.5 million and 1 million RE-T cells, respectively, but not in untreated or HCV AE-T cell–treated mice (Figure 5*H*). A3B but not A3G mRNA increased 45-fold in mice treated with 1 million RE-T cells (Figure 5*I*), confirming that activation of APOBEC3B by RE-T cells in HBV-infected hepatocytes can inhibit HBV replication.

Discussion

In this study, we show that antiviral nonlytic T cells can be engineered through the transfer of virus-specific TCR mRNA on resting primary human T cells without the requirement for extensive pre-activation and culture in vitro or selection of particular T-cell subsets. These TCR-reprogrammed RE-T cells specifically direct their antiviral effect on infected cells through the activation of the intracellular LTBR/APOBEC3 antiviral pathway. This modality is masked on classical effector CD8 T cells that preferentially lyse their targets but is clearly detectable on RE-T cells that have low lytic contents. We propose that this feature, combined with the inability to activate liver inflammatory events, can be exploited for new therapeutic modalities for the treatment of persistent hepatic viral infections, in particular because induction of APOBEC3B activity in hepatocytes has been proposed as a strategy to inhibit HBV replication[29] and destabilize HBV cccDNA.[28]

The direct antiviral efficacy of our TCR-reprogrammed RE-T cells in limiting HBV infection is quite remarkable. Robust HBV DNA suppression (64%) accompanied by more than 80% reduction in pgRNA and cccDNA could be detected in HBV-infected HepG2-hNTCP after 24 hours of treatment with RE-T cells at a 1:1 effector cell–to–target cell (E:T) ratio. Less pronounced antiviral effects were induced at a 1:3 E:T ratio, but nonetheless, this treatment resulted in 50% reduction in pgRNA and cccDNA. In high-viremic humanized mice, 3 injections of 1 million RE-T cells each resulted in HBV viremia reduction and up to 50% reduction in intrahepatic HBV cccDNA without triggering unspecific liver damage. A lower dose of RE-T cells (0.5 million/injection) reduced HBV viremia initially at day 4, but the antiviral effect was transient and could not be incremented with the subsequent 2 injections. An explanation is that RE-T cells have lower cell motility than AE-T cells, as has been observed in the 3-D microfluidic model, and in addition, a significant number of RE-T cells were trapped in the spleens of the chimeric mice, and thus fewer RE-T cells than AE-T cells could traffic to the livers of these mice. Consequently, a higher T-cell dose is required to achieve similar antiviral effects, at least in this chimeric model. The observed increased expression of programmed death-ligand 1 on HBV-infected hepatocytes could also limit subsequent activation of RE-T cells and incremental inhibition of HBV replication with repetitive injections. The human liver chimeric mouse model is immune deficient. Thus, the potential suppressive effect on the adoptively transferred T cells exerted by immune cells present in a chronically infected liver might not be measured here. Nonetheless, compared with antiviral treatment with nucleoside analogues that requires in the same mouse system approximately 1 month to achieve a 2-log decrease of viremia,[32] mice receiving RE-T cells displayed substantially lower levels of viremia and of HBV RNA amounts, and pathways with the potential to lower cccDNA had been activated. We also showed that APOBEC3B activation in HBV-infected hepatocytes, a mechanism that was already been shown to promote partial cccDNA degradation,[28] is associated with lower intrahepatic cccDNA loads. However, a slightly increased rate of apoptosis and human proliferation markers (3-fold increase of caspase 3 and hKi67 expression) was measured in RE-T cell–treated mice compared with untreated mice, suggesting that hepatocyte

Figure 5. RE-T cells activate APOBEC3B in HBV-infected primary human hepatocytes in mice. (*A*) Schematic of 2 independent experiments performed in HBV-infected human liver chimeric mice treated with 3 doses of 0.5 or 1 million $TCR_{HBVs183}$ RE-T cells or AE-T cells on days 0, 4, and 8. HBV-infected human liver chimeric mice were left untreated or treated with $TCR_{HCV\ NS3}$ AE-T cells as controls. The total number of cells injected for each TCR-T cells was normalized to the frequency of $CD8^+TCR^+$ T cells. (*B*) Viremia changes in the blood expressed as a percentage relative to baseline levels at day 0 were determined at the indicated times upon multiple injections of TCR-T cells. (*C*) Intrahepatic levels of cccDNA copies expressed per human hepatocyte (β-globin) at day 12. (*D*) Intrahepatic HBV pgRNA amounts relative to GAPDH at day 12. (*E*) Serum ALT levels (IU/mL) were analyzed at day 12. (*F*) Percentage change in human serum albumin at day 12 relative to day 8 after treatment with multiple injections of TCR-T cells or untreated. Transcript levels of (*G*) PD-1 and PD-L1 mRNA, (*H*) LTB mRNA, and (*I*) APOBEC3B and APOBEC3G mRNA in treated mice at day 12 normalized to GAPDH and ribosomal protein L30 and expressed as fold change compared with untreated mice. ALT, alanine aminotransferase; M, mol/L.

division could also contribute to cccDNA reduction.[17] Even though RE-T cells will not be able to provide a virus-specific memory T-cell response, infusions of RE-T cells, in combination with inhibitors of HBV infection, like antibodies or Myrcludex-B[33,34] (a lipopeptide that blocks HBV entry) can possibly purge HBV cccDNA from the large number of infected hepatocytes present in chronic hepatitis B patients and progressively lead to a control of the chronic viral infection.

In addition, the antiviral effect detected in HBV-infected cells at high E:T ratio is rapid and specific, occurring 1–3 days after RE-T cells contact their specific targets. The combined rapid down-regulation of the introduced TCR, LTB, and CD258 molecules on the membrane of antigen-stimulated RE-T cells at 5– days after TCR-mRNA electroporation temporally restrict the antiviral action of RE-T cells but at the same time limit their potential off-target effects. We clearly showed here the complete absence of inflammatory phenomena observed in HBV-infected cells and, most importantly, in human liver chimeric mice using RE-T cells, despite the distinct up-regulation of APOBEC3 proteins in liver cells. The absence of inflammatory phenomena contrasts with the clear induction of liver inflammation observed in different experimental systems in which LTBR activation was not temporally or anatomically restricted, for example, by constitutive overexpression of LTB on hepatocytes[35] or by adenovirus-mediated *LIGHT* expression[36] and concavalin A-induced expression of LIGHT[37] in the liver. Because sustained LTB/LIGHT-LTBR signaling is critically involved in the pathogenesis of chronic liver inflammation,[31,38] modulating LTB/LIGHT-LTBR signaling in infected hepatocytes to control viral infection and limit excessive inflammation could be beneficial to the host.

The restricted temporal activation of RE-T cells is also the likely cause for the inability to detect, in our experimental system, a HBV antiviral effect of IFNG. Inhibition of HBV replication in an HBV transgenic mouse model by secretion of antiviral cytokines (IFNG and tumor necrosis factor α) was achieved with classical effector $CD8^+$ cytotoxic T cells that persisted in mice[25,39] and were sequentially activated in the HBV-expressing liver.[40] Similarly, loss of HBV cccDNA mediated by IFNG was shown in vitro after prolonged stimulation of up to a week with high-dose IFNG.[26] In contrast, even though our RE-T cells were able to produce IFNG and they inhibited HCV replication with high efficiency in the IFNG-sensitive HCV replicon system, the temporal limited production of IFNG appeared unable not only to inhibit HBV replication but also to stimulate high levels of inflammatory cytokine/chemokine production from the hepatic cells.

In conclusion, our work highlights an approach to reprogramming resting primary human T cells through TCR mRNA transfer to specifically recognize viral-infected cells and suppress viral replication without lysis. Their ability to selectively activate LTBR signaling and APOBEC3 upon antigen recognition in a temporal fashion and without triggering inflammatory events could be a beneficial therapeutic strategy for chronic hepatitis B.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2018.03.027.

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Author names in bold designate shared co-first authorship.

Received May 17, 2017. Accepted March 8, 2018.

Reprint requests
Address requests for reprints to: Antonio Bertoletti, MD, Emerging Infectious Diseases, Duke-NUS Medical School, 8 College Road, Singapore 169857. e-mail: antonio@duke-nus.edu.sg; or Sarene Koh, PhD, Lion TCR Private Limited Singapore, 61 Biopolis Drive, #08-02B Proteos, Singapore 138673. e-mail: sarene.koh@liontcr.com.

Acknowledgments
The authors thank Professor Mala Maini (University College London, London, United Kingdom) for critical reading of the manuscript.

Author Contributions: Sarene Koh, Antonio Bertoletti, and Maura Dandri designed the research. Sarene Koh and Antonio Bertoletti wrote the manuscript. Sarene Koh, Janine Kah, Christine Y.L. Tham, Erica Ceccarello, Adeline Chia, Ninghan Yang, Atefeh Khakpoor, Andrea Pavesi, and Anthony T. Tan performed the experiments. Sarene Koh, Antonio Bertoletti, Janine Kah, Maura Dandri, and Anthony T. Tan analyzed the data. Margaret Chen provided the HCV NS3 TCR material for the experiments.

Conflicts of interest
Antonio Bertoletti participates in advisory boards on hepatitis B virus immune therapy for Gilead, Janssen, and Medimmune and is a co-founder of Lion TCR Private Limited, a biotech company developing T-cell receptors for treatment of virus-related cancers and chronic viral diseases. Sarene Koh is the Director of Research and Anthony T. Tan is a consultant of Lion TCR Private Limited. Sarene Koh and Antonio Bertoletti have applied for a patent on the subject matter of this paper. Andrea Pavesi is a consultant of AIM Biotech Private Limited. The remaining authors disclose no conflicts.

Funding
The study was supported by a Singapore Translational Research (STaR) Investigator Award (NMRC/STaR/013/2012) to Antonio Bertoletti, by the German Research Foundation (DFG) grant to Maura Dandri (Collaborative Research Centre SFB 841 A5), and by a Heisenberg Professorship to Maura Dandri (DA1063/3-2).

Supplementary Materials and Methods

Preparation of Activated and Resting T Cells

The present study using human peripheral blood mononuclear cells (PBMCs) was reviewed and approved by the institutional review board of the National University of Singapore. For activated (AE) T cells, PBMCs were collected after we received informed consent from healthy donors and were stimulated with 600 IU/mL IL-2 (rIL-2; R&D Systems, Minneapolis, MN) and 50 ng/mL anti-CD3 (OKT-3; eBioscience, San Diego, CA) in AIM-V + 2% human AB serum and expanded in vitro for 8 days, and rIL-2 was increased to 1000 IU/mL 1 day before electroporation. Resting (RE) T cells were isolated from PBMCs using the pan T cell isolation kit (Miltenyi Biotec, GmbH, Germany) and cultured overnight in 100 IU/mL rIL-2 before electroporation.

Flow Cytometry

Antibodies for cell surface staining were obtained from BD Biosciences (Franklin Lakes, NJ) (anti-human CD8-PE-Cy7, CD8-V500, CD45RA-APC, and CD62L-PECy7), eBioscience (San Diego, CA) (anti-human CD45RO-eFluor650), Immudex (Copenhagen, Denmark) and Proimmune (Oxford, UK) (HLA-A201-HBs183-191-PE, HLA-A201-HBc18-27-PE, and HLA-A201-NS3 1073-1081-PE dextramer or pentamer), and R&D Systems (human LTβ receptor-Fc chimera). Antibodies for intracellular cytokine staining were obtained from BD Biosciences (anti-human IFNG-APC, TNFA-Alexa488, IL-2-PE, and Granzyme-APC) and Diaclone (Besancon, France) (anti-human perforin-FITC). Intracellular cytokine staining was performed by fixing and permeabilizing cells with Cytofix/Cytoperm (BD Biosciences). Flow cytometry was performed using a FACS Canto flow cytometer or LSRII (BD Biosciences) and data was analyzed with FACS Diva program (BD Biosciences).

Production of TCR mRNA

The HBV envelope s183–191 TCR (s183-TCR) and HBV core 18–27 (c18-TCR) cloned in pVAX1 were made in house. The HCV NS3 1073–1081 TCR cloned in pVAX1 was a gift from Margaret Sällberg Chen (Karolinska Institutet, Solna, Sweden). The plasmids were propagated purified from *Escherichia coli* using the One Shot Top10 *E. coli* kit (Invitrogen), purified using Qiagen Endo Free Plasmid Maxi Kit (Qiagen, Valencia, CA) and linearized using the XbaI restriction enzyme. TCR mRNA was produced the TCR using the mMESSAGE mMACHINE T7 Ultra kit (Ambion, Austin, TX).

Cytotoxicity Assays

HepG2-Core–, HepG2-Env–, and $Huh7_{A2}$HCV–expressing luciferase cell lines were plated overnight in 96-well flat bottom plates to permit adherence. Luciferase-expressing cell lines were cocultured with TCR–AE-T or RE-T cells (calculated based on frequency of $CD8^+$dextramer$^+$ cells) at indicated E:T ratios in triplicate in AIM-V + 2% human AB serum for 24 hours. Cytotoxicity was measured by quantifying luciferase expression in remaining cell lines. Briefly, culture medium was discarded and 100 μL of Steady-Glo reagent (Promega, Madison, WI) was added to each well and incubated for 5 minutes to allow cell lysis. Luminescence was measured with a microplate reader (Tecan, Männedorf, Switzerland). Cell lines without effectors were used as a reference for maximum luminescence. Results were expressed as percent reduction in viral replication = 100% – (luminescence remaining/maximum luminescence)% and calculated as the mean of triplicate measurements ± standard deviation.

3-D Microdevice Assay and Imaging

Microdevices with empty gel regions (control) were prepared by adding collagen gel solution without HepG2 targets. The cell impermeable nuclear dye DRAQ7 (Biolegend, San Diego, CA) was added in R10 media at a concentration of 3 μmol/L to discriminate between live and dead cells. The devices were incubated for 24 hours to permit the interaction of the HepG2 cells with the collagen matrix. Before injecting TCR-T cells in the devices, R10 media was replaced with AIM-V 2% human AB serum + 100 IU/mL rIL-2 + DRAQ7. The frequency of TCR-expressing AE-T cells was normalized to that of RE-T cells using activated nonelectroporated T cells such that the same total number of T cells were injected.

Live imaging (time-lapse) experiments were performed using either the LSM7800 confocal microscope (Zeiss, Oberkochen, Germany) or FV1200 confocal microscope (Olympus, Tokyo, Japan) equipped with an environmental chamber set at 37°C and 5% CO_2. The microscope was programmed to acquire Z stacks of the selected regions at the stated time intervals. For static imaging experiments, confocal images of the same region of interest were acquired before T-cell addition and after 15 hours of incubation. All images acquired from the confocal microscope systems (live and static imaging) were visualized and analyzed using Imaris (Bitplane, Zurich, Switzerland).

HBV Infection of HepG2-hNTCP

Approximately 80- to 100-fold concentrated supernatant of HepAD38 cells was used as HBV inoculum. HepG2-hNTCP cells seeded overnight in 24-well plates were inoculated for 24 hours with approximately multiplicities of genome equivalents of 3000/well HBV in medium containing 4% polyethylene glycol (Sigma Aldrich). After infection, cells were washed with phosphate buffered saline (PBS) 3 times, and culture medium with DMSO was added and changed every 2 days.

Quantigene View RNA In Situ Hybridization Cell Assay for HBV pregenomic RNA

Cells were fixed with 4% formaldehyde, washed with 1× PBS and rehydrated with ethanol at decreasing concentrations (100%, 70%, and 50%). In situ hybridization (ISH) was performed using Quantigene View RNA ISH Cell Assay (Affymetrix, San Diego, CA). Cells were permeabilized

with working Detergent QC (Affymetrix), digested with Protease (Affymetrix) 1:4000 dilution in PBS, and then hybridized with custom-designed Quantigene View RNA probes against conserved regions of HBV pregenomic RNA among genotypes, for 3 hours at 40°C. The unbound probes were washed away, and bound probes were amplified through PreAmp (Affymetrix) hybridization for 1 hour at 40°C, followed by Amp (Affymetrix) hybridization at 40°C for 1 hour. Label probes (Affymetrix) targeting pgRNA probe type was added for 1 hour at 40°C. Cell nuclei were stained for 4′,6-diamidino-2-phenylindole, and images were acquired at 40× magnification using Carl Zeiss confocal laser scanning upright microscope. Images were analyzed using Imaris (Bitplane).

Quantification of HBV Genome Equivalent Copies

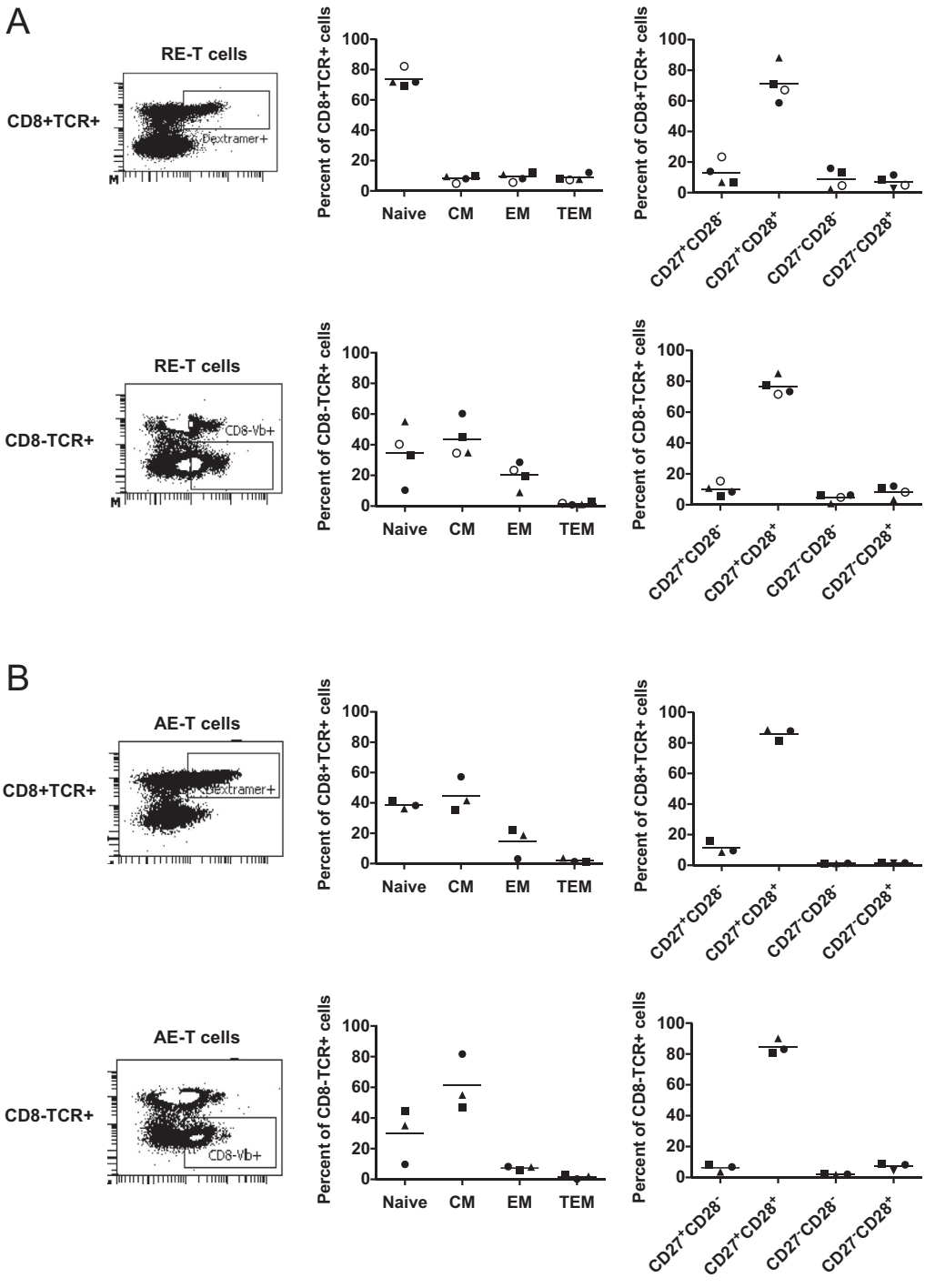
RLT buffer with β-mercaptoethanol or lysis buffer containing 50 mmol/L Tris, 140 mmol/L NaCl, 1.5 mmol/L MgCl, and 0.5% NP-40 at pH 8 was added to lyse HepG2.2.15 or HepG2-hNTCP cells for isolation of intracellular viral nucleic acids using QIAamp MinElute Virus Spin kit (Qiagen, Valencia, CA). Viral DNA were extracted from mouse serum samples (5 μL) using the QiAmp MinElute Virus Spin kit and quantified against a HBV DNA standard. HBV DNA was quantified by quantitative polymerase chain reaction (qPCR) using the artus HBV RG PCR kit in a Rotor-Gene Q 2-plex instrument (Qiagen).

APOBEC3 and LTB Gene Expression Analyses

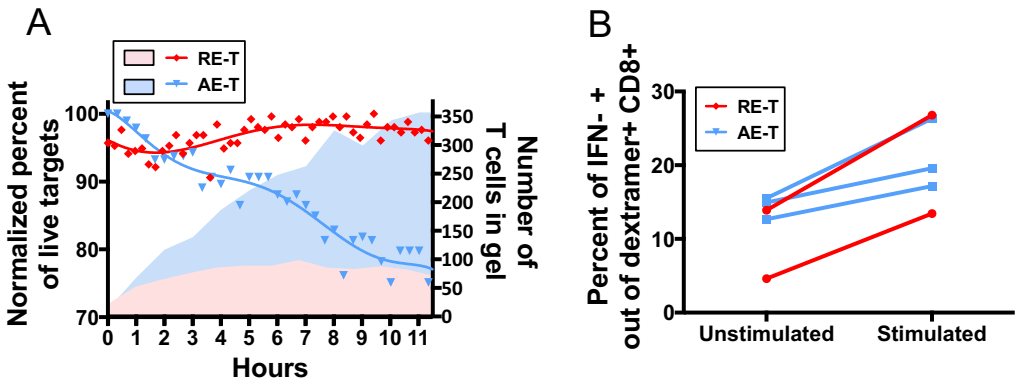
Intrahepatic RNA was extracted from liver specimens or HepG2.2.15 cells using the RNeasy RNA purification kit (Qiagen), transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and used as template for real-time qPCR. Human APOBEC3- and LTB-specific primers from the TaqMan Gene Expression Assay System or custom-designed primers (see Supplementary Tables 1 and 2) were used, and expression levels were quantified by qPCR performed using the ViiA 7 Real-Time PCR system (Life Technologies, Carlsbad, CA) or LightCycler system (Roche Diagnostics, Risch-Rotkreuz, Switzerland) and analyzed using the second derivative maximum method that includes both normalization to the reference genes and primer efficiency. Relative mRNA expression levels of all target genes were normalized to the mean of at least 2 housekeeping genes (actin, GAPDH, or ribosomal protein L30). Negligible mRNA was extracted from the same number of T cells alone, indicating that changes in mRNA expression were measured in hepatic cells.

Generation of Humanized Mice

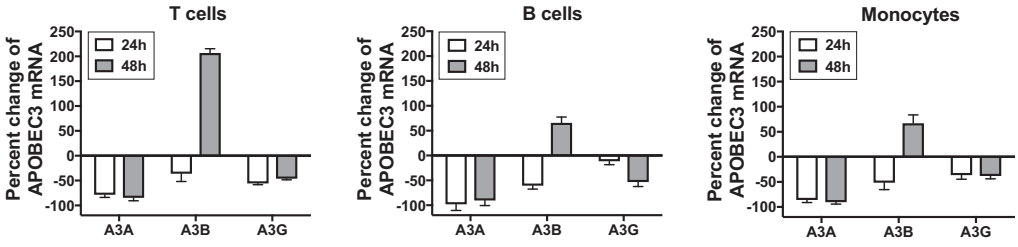
Primary human hepatocytes were isolated from rejected explant livers using protocols approved by the Ethical Committee of the City and State of Hamburg (OB-042/06) and according to the principles of the Declaration of Helsinki. Human liver chimeric uPA/SCID mice were generated by transplanting 1 million thawed human hepatocytes in 3-week-old mice anesthetized with isofluorane. Generation of stably HBV-infected human liver chimeric mice was performed as previously described. Levels of human liver chimerism were determined by measuring HSA levels in mouse serum using the human albumin ELISA quantitation kit (Bethyl Laboratories, Biomol GmbH, Hamburg, Germany).



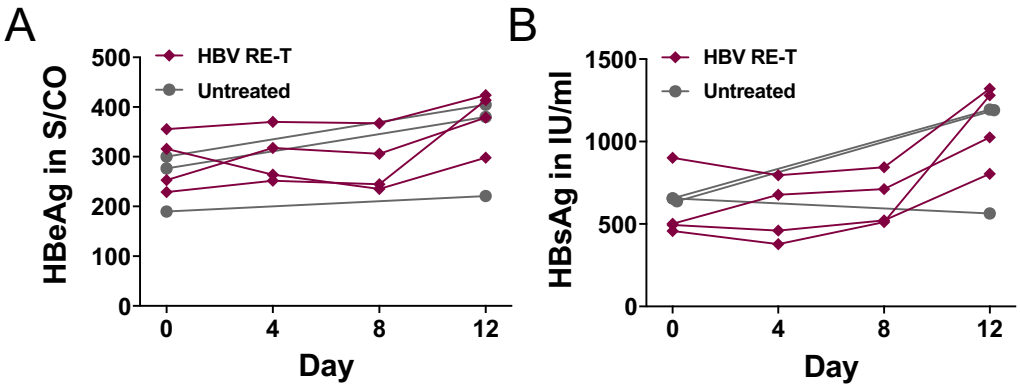
Supplementary Figure 1. $CD8^+$ TCR-expressing RE-T cells are $CD45RA^+CD62L^+$ naïve-like and $CD27^+CD28^+$, whereas $CD8^+$ TCR-expressing AE-T cells comprise $CD45RA^+$ $CD62L^+$ naïve-like, $CD45RA^-CD62L^+$ central memory-like and $CD45RA^-CD62L^-$ effector memory-like phenotypes and are $CD27^+CD28^+$. The percentages of $CD45RA^{+/-}$, $CD62L^{+/-}$, $CD27^{+/-}$, and $CD28^{+/-}$ T cells within the gated $CD8^+$ dextramer$^+$ or CD8-TCRVbeta$^+$ populations in (*A*) RE-T ($n = 4$) and (*B*) AE-T ($n = 3$) cells were determined by flow cytometry. T cells were classified into different subsets: naïve-like ($CD45RA^+CD62L^+$), central memory (CM)-like ($CD45RA^-CD62L^+$), effector memory (TM)-like ($CD45RA^-CD62L^-$) and terminally differentiated effector memory (TEM)-like ($CD45RA^+CD62L^-$).



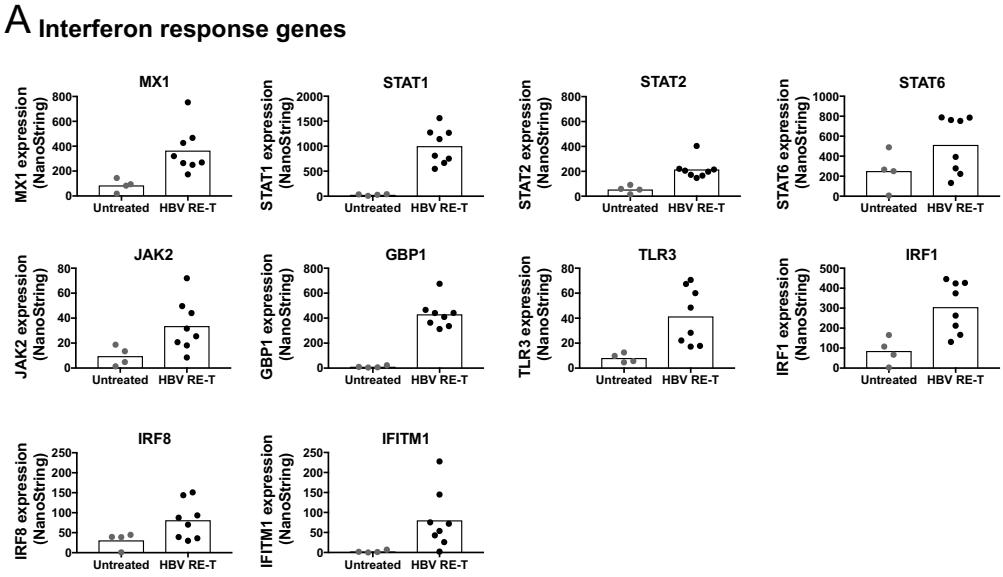
Supplementary Figure 2. AE-T cells kill HepG2-Env cells, but RE-T cells are non-cytolytic. (*A*) $TCR_{HBVs183}$ AE-T (blue line) or RE-T cells (red line) were seeded into separate microfluidic devices, and the percentage of live HepG2-Env was quantified over time by live imaging. The percentage of live HepG2-Env was normalized to the highest quantity of live HepG2-Env measured for the duration of the live imaging. Shaded areas of the corresponding colors show the total number of AE-T or RE-T cells invading the gel region. (*B*) $TCR_{HBVs183}$ AE-T or RE-T cells were co-cultured with peptide-pulsed T2 cells in a 2-D 96-well based assay. The percentages of interferon gamma–producing $CD8^+$ T cells out of dextramer$^+$ $CD8^+$ T cells are shown. The TCR-T cells were concurrently used for 3-D microdevice assays shown in (*A*).

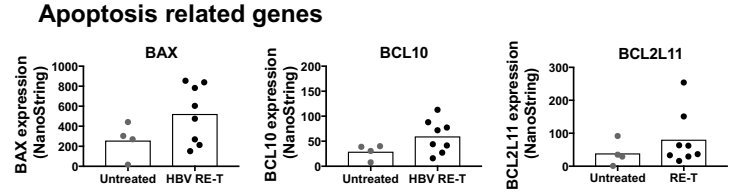


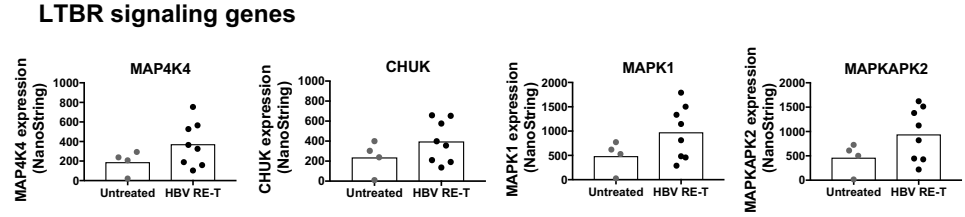
Supplementary Figure 3. APOBEC3 proteins can be activated in other immune cells. $TCR_{HBVs183}$ RE-T cells were co-cultured with HBV envelope peptide-pulsed primary human T cells, monocytes, or B cells for indicated times at an E:T ratio of 1:3. Expression of APOBEC3 family members were normalized to GAPDH and actin, and results were expressed as percent change of APOBEC3 relative mRNA expression compared with untreated targets ± standard deviation of triplicates. Negligible mRNA was extracted from the same number of RE-T cells alone, indicating that changes in mRNA expression were measured in peptide-pulsed immune cells.

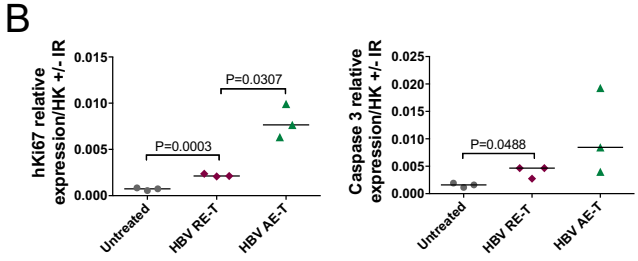


Supplementary Figure 4. Circulating HBeAg and HBsAg levels remained relatively stable in RE-T cells treated mice. (*A*) Longitudinal changes in levels of circulating HBeAg and (*B*) HBsAg were determined by ELISA in HBV-infected untreated mice and mice treated with 3 doses of 1 million $TCR_{HBVs183}$ RE-T cells.









Supplementary Figure 5. Intracellular antiviral pathways are activated in RE-T cell–treated mice. (*A*) NanoString nCounter Human Immunology Profiling (NanoString Technologies, Inc., Seattle, WA) of immune genes in livers of HBV-infected untreated mice and mice treated with 3 doses of $TCR_{HBVs183}$ RE-T cells. (*B*) Expression of human Ki67 and caspase 3 transcripts normalized to GAPDH in livers of HBV-infected untreated mice and mice treated with 3 doses of $TCR_{HBVs183}$ RE-T or AE-T cells.

Supplementary Table 1. Genes Analyzed Using TaqMan Gene Expression Assay

Gene	Array number
GAPDH	Hs03929097_g1
RPL30	Hs00265497_m1
LTB	Hs00242739_m1
APOBEC3B	Hs00242340_m1
APOBEC3G	Hs00222415_m1

Supplementary Table 2. Sequences of custom-designed primers

Oligonucleotide name	Sequence (5′ → 3′)
APOBEC3A fwd	GAGAAGGGACAAGCACATGG
APOBEC3A rev	TGGATCCATCAAGTGTCTGG
APOBEC3B fwd	GACCCTTTGGTCCTTCGAC
APOBEC3B rev	GCACAGCCCCAGGAGAAG
APOBEC3G fwd	CCGAGGACCCGAAGGTTAC
APOBEC3G rev	TCCAACAGTGCTGAAATTCG
GAPDH fwd	AGGGCTGCTTTTAACTCTGGT
GAPDH rev	CCCCACTTGATTTTGGAGGGA
Actin fwd	CACCATTGGCAATGAGCGGTTC
Actin rev	AGGTCTTTGCGGATGTCCACGT

fwd, forward; rev, reverse.

Supplementary Table 3. Distinct features of mRNA TCR electroporated AE-T and RE-T cells

Features	AE-T cells	RE-T cells
Origin	PBMCs	T cells purified from PBMCs
In vitro culture	10 days	Overnight rest
Culture conditions	Stimulation with anti-CD3 and IL-2	No stimulation
T-cell composition, mean % ± SD of $CD8^+$ and $CD8^-$ cells[a]	$CD8^+ = 74.8 \pm 9.7$ $CD8^- = 25.2 \pm 9.7$	$CD8^+ = 32.2 \pm 6.4$ $CD8^- = 67.8 \pm 6.4$
TCR expression, mean MFI ± SD[a]	2295 ± 193	2401 ± 1179
Mean % ± SD of $CD8^+TCR^+$ cells[a]	33.4 ± 20.1	17.5 ± 10.4
Mean % ± SD of $CD8^-TCR^+$ cells[a]	17.4 ± 8.7	23.4 ± 7.6

MFI, mean fluorescent intensity; SD, standard deviation.
[a]Values expressed are the mean ± SD of 4 healthy donors.