TCR-Redirected Human T Cells Inhibit Hepatitis C Virus Replication: Hepatotoxic Potential Is Linked to Antigen Specificity and Functional Avidity

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M ore than 150 million people worldwide are estimated to have persistent infection with the hepatitis C virus (HCV). The propensity of this virus to cause chronic infection has made it one of the leading causes of liver cirrhosis and hepatocellular carcinoma in the world (1). Although current antiviral therapy is effective in achieving sustained HCV clearance in chronically infected patients, the severe side effects and contraindications render it inaccessible to a considerable proportion of the infected patient population. The rate of sustained virological response is still rather low in patients with liver cirrhosis, despite the recently introduced first generation of protease inhibitors. In spite of the fact that HCV is exceptional in establishing chronicity in infected individuals, spontaneous resolution may occur in a minority of these individuals. The immunological mechanisms that mediate the spontaneous viral clearance have been related to the T cell-mediated immune responses. This evidence is supported by facts such as the notion that chronic HCV patients who spontaneously clear their infection have virus-specific T cell responses detectable in the blood just before their elimination of virus infection (2) and the discovery that depletion of CD4 or CD8 T cells from nonhuman primates abrogates protective immunity and results in persistent HCV infection (3, 4).

Recent data have increasingly demonstrated that the T cell response during acute self-limited hepatitis C in HCV-infected patients is characterized by a vigorous, polyclonal, and multispecific cytotoxic and Th cell response. Moreover, patients who spontaneously recover from HCV infection typically mount vigorous HCV-specific CD4+ and CD8+ T cell responses that are directed against multiple viral peptides and are readily detectable in the blood (5–9). By contrast, patients with chronic hepatitis C tend to have late, transient, and/or narrowly focused T cell responses (9, 10). Failure to sustain virus-specific CD8+ lymphocytes is frequently observed in the chronically infected patients, and T cells from these patients are associated with features such as functional exhaustion as well as tolerance induction and developmental arrest (11–15).

Selection of high-avidity CD8 T cells correlates with control of viral infections and polyfunctional profiles as well as potent virus-suppressive activity including HCV (16, 17). Notwithstanding the intrinsic superior antiviral activity of higher avidity TCR CD8 T cells, low-avidity TCR polyfunctional CD8 T cells have been suggested to be more fit in providing disease control during
chronic virus infection (18) and have been reported to reject tumors and can have less problems regarding immune pathology (19). Although high-avidity CTLs are superior for viral and tumor clearance, they also have a greater sensitivity to Ag-induced cell death. As with chronic infections, such as HIV and HCV, as well as in cancer, the host may lose (by clonal exhaustion or other apoptotic mechanisms) the effector cells that are most critical for viral or tumor clearance (18). In the case of HCV, the high-avidity T cells have been suggested to control HCV infection (16, 17); however, what remains unclear is whether low avidity T cells may contribute to antiviral mechanisms against the HCV-infected cells.

Better T cell-based strategies against HCV infection may help patient groups that are prone to failure of standard therapy (2). Infusion with TCR-redirected HCV CTL would be a rational proposal, however, the impact of the TCR avidity on the resulting T cell function and antiviral properties against HCV-infected cells is largely unknown. In this study, we compare HCV TCRs that differ in Ag specificity and functional avidity. Their biological significance in induction of antiviral activities and hepatocellular injury using TCR-transduced human T cells are demonstrated in this study.

Materials and Methods

PBMCs from healthy blood donors and HCV patients

PBMCs from healthy blood donors (n = 10) and patients with persistent HCV infection (n = 6) with serum HCV RNA levels between 2.8 × 10^6 and 5 × 10^7 IU/ml were collected at Karolinska University Hospital under informed consent and isolated using Ficoll-Hypaque density gradient centrifugation. HLA-A2 typing was performed by FACS. Ethical permission was obtained from the Regional Ethical Review Board in Stockholm for use of patient and control samples.

Cell lines

T2 cells were grown in RPMI 1640 10% FBS with 2 mM l-glutamine, 100 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin. T2 is an HLA A2.1 cell line, a cloned hybrid between the 721.174 (variant of the LCL 721 B lymphoblastic cell line) and CEMR.3 (8-azaguanine and ouabain-resistant clone of the CEM T lymphoblastic cell line). Hepatoma Huh-7-Lunet cells designated Huh-Lia2-1uc-neo/ET (HuH7/HCVrep), Lunet-bifid/neo/ET (HuH7/HCVrep), or Lunet-Huh2-A2 (HuH7/A2) were generated by similar protocol as described previously (20). The Lunet-Huh2-A2-1uc-neo/ET has coexpressed HLA-A2 expression and a selective HCV subgenomic RNA replicon of genotype 1b, harboring replication-enhancing mutations in nonstructural protein (NS) 3 and NS4B (Con1-ET) (21) and expressing the firefly luciferase gene fused to the selectable marker by ubiquitin (22). The control replicon cell line Huh/HCVrep is the same as above but transduced with an empty viral vector without the HLA-A2 gene. Both were maintained in DMEM medium supplemented with 10% FBS, nonessential amino acids, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, with addition of baculovirus S hydrochloride (3 µg/ml) and G418 (1 mg/ml). The other control cell line, HuH-A2, expressing HLA-A2 under baculovirus selection but without the HCV replicon, was maintained in the same DMEM medium as above but with 3 µg/ml baculovirus S hydrochloride and no G418. For the coculture experiments, the hepatoma cells were washed and reseeded 1 d before in fresh medium without the antibiotic selection. Retroviral-transduced PBLs were maintained in AIMV medium 2% human serum and 100 U/ml recombinant human IL-2 (R&D Systems, PeproTech). Phoenix cells were maintained in IMDM. All cells were grown in a humidified incubator at 37˚C and with human IL-2 (R&D Systems, PeproTech). Phoenix cells were maintained above but transduced with an empty viral vector without the HLA-A2 gene. Both were maintained in DMEM medium supplemented with 10% FBS, nonessential amino acids, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, with addition of baculovirus S hydrochloride (3 µg/ml) and G418 (1 mg/ml). The other control cell line, HuH-A2, expressing HLA-A2 under baculovirus selection but without the HCV replicon, was maintained in the same DMEM medium as above but with 3 µg/ml baculovirus S hydrochloride and no G418. For the coculture experiments, the hepatoma cells were washed and reseeded 1 d before in fresh medium without the antibiotic selection. Retroviral-transduced PBLs were maintained in AIMV medium 2% human serum and 100 U/ml recombinant human IL-2 (R&D Systems, PeproTech). Phoenix cells were maintained in IMDM. All cells were grown in a humidified incubator at 37˚C and with 5% CO2. All medium and supplements were purchased from Invitrogen (Carlsbad, CA).

Peptide Ags

HCV peptides: NS3 1073–1081 gT1a (CINGVCWTV), NS5 1922–2060 bT1b (VLTDFTKTLW), NS5 2212–2221 (SPADDLIEANL), NS5 2140–2154 (LLRE-DVTPQV), NS 2352–2360 (ILDSFDPDLR), and HCMV peptide p250265 (NLVPNPMAT) were synthesized (purity >70%) by ChronTech Pharma (Huddinge, Sweden) and EZBiolab (Carmel, IN). T2 target cells were pulsed with the indicated peptide for 12 h before use.

Flow cytometry and multicolor cytokine analysis

All Abs were purchased from BD Biosciences (San Jose, CA) or BioLegend (San Diego, CA), and R-PE–labeled HLA-A*0201 Pro5 pentamers refolded with HCV NS3 1073 or HCV NS5 1922 or hepatitis B virus (HBV) core18–27 were from ProImmune (Oxford, U.K.). For intracellular cytokine staining (ICS) multicolor FACS staining, TCR-transduced PBMCs were incubated overnight with indicated stimuli. GolgiPlugg was added during the final 12 h. PMA 50 ng/ml and ionomycin 500 ng/ml (Sigma-Aldrich) were used as positive controls. Cells were then washed and stained with: Pacific Blue anti-human CD3 (BioLegend), allophyco- cyanin-Cy7 anti-human CD8a (BioLegend), FITC-labeled anti-mouse Vβ 8.3 (BD Biosciences), anti-mouse Vβ8.1-8.2 (BD Biosciences), anti-mouse Vβ4 (BD Biosciences), APC anti-human IL-2 (BioLegend), PE anti-human IFN-γ (BioLegend), and PE-Cy7 anti-human TNF-α (BD Biosciences). BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) was used. CD107a staining was done as described earlier (23). Cells were analyzed using the BD LSRFortessa flow cytometer (BD Biosciences) and FlowJo 9.2 (Tree Star) software.

Isolation of HCV TCR from HLA-Tg mice and generation of retroviral vector constructs

TCRs designated F8 and H4 (specific for NS3 1073–1081) and 19 and 69 (specific for NS5 1922–2060) were identified from murine CTL clones generated from HLA-A2-transgenic mice that have been immunized with codon-optimized (co)NS3/4A-pVAX1 and coNS5A-pVAX1 DNA vaccines. Thymoma cell (BW5147) fused T cell hybridomas were generated, and the coding sequences were analyzed as previously described (24). Codon-optimized cassettes in which the α and β TCR chains are linked by the F2A autoprotease were synthetically made (GeneArt) and cloned in the retroviral vector pMP71Gpre (25) kindly provided by Wolfgang Uckert, Max-Delbruck-Center for Molecular Medicine). All plasmids, including the control pMP71Gpre-EFGP, were propagated in Mach1 T1 Phage-Resistant (T1ρ) Escherichia coli (Invitrogen) and prepared by Maxi prep (Qiagen) according to the manufacturer’s instructions. Phoenix amphotropic packaging cells (American Type Culture Collection) were transiently transfected with 20 µg each retroviral vector (pMP71-NS3-H4, pMP71-NS3-F8, pMP71-NS5A-19, pMP71-NS5A-69, and pMP71-EFGP) and 12.5 µl 50 µmol chlo-roquine. The supernatant was harvested after 48 h, clarified by centrifugation, and kept at 4˚C until used.

Animals

Six- to 12-wk-old inbred HHD*H-2d/—β2m—/ (HHD) mice transgenic for HLA-A2.1 monochain histocompatibility class I molecule (provided by Dr. F. Lemonnier, Institute Pasteur, Paris, France) were maintained in-house at Karolinska Institutet (Division of Comparative Medicine, Clinical Research Centre, Karolinska University Hospital Huddinge, Stockholm, Sweden). Male and female mice were 6–16 wk of age at the start of experiments. All experimental protocols involving animals were approved by the Ethical Committee for Animal Research at Karolinska Institutet.

TCR gene transfer and functional analysis of transduced T cells

PBMCs were stimulated with 600 or 300 U/ml IL-2 (R&D Systems or PeproTech) and 50 ng/ml anti-CD3 (OKT-3; Biologic Science, San Diego, CA) for 48 h. Untreated 24-well tissue-culture plates were coated with 30 µg/ml Retractin (Takara Bio, Otsu Shiga, Japan) overnight at 4˚C the day prior to transduction. The wells were then washed and blocked with PBS 2% BSA. Lymphocytes were harvested, washed, counted, 5 × 10^6 cells plated into retronectin-coated wells, and mixed with retroviral supernatant and polybrene 1:1000 (Millipore). The plates were then spinoculated for 1 h at 32˚C, 1000 × g. After 4 h, the medium was replaced with AminV 2% human AB serum and 300 U/ml IL-2. The next day, the spinoculation procedure was repeated, and the cells were then maintained in Amin V 2% human AB serum and 100 U/ml IL-2. After 72 h from the first spinoculation, the cells were stained with CD8-PerCP, CD3-allophycocyanin, and Vβ4-8.1-8.2-8.3-FITC (BD Biosciences), anti-mouse Vβ4-FITC, or Vβ8.1-8.1-FITC (BD Biosciences) to monitor TCR expression. For the functional assay, TCR-transduced PBMC were incubated in a V-bottom 96-well plate with 1 × 10^5 peptide-loaded T2 in ratio 1:1 (the amount of TCR-transduced cells was calculated and adjusted on the basis of the CD3+HCTV TCR+ cells). After 24 h coculture, the concentrations of human IL-2 and IFN-γ in the supernatant were measured using the human IL-2 or IFN-γ ELISA (Mabtech, Nacka Strand, Sweden) and calculated against a standard curve generated with the recombinant protein standard.
Bioluminescence cell imaging and transaminase measurement

Huh7\(^{A2}\) HCV\(_{Rep}\) (50,000 or 100,000 cells) or same number of control cells Huh7/HCV\(_{Rep}\) or Huh7\(^{A2}\) HCV were cocultured with transduced (CD3\(^{+}\) HCV\(_{TCR}^{+}\)) or mock-transduced T cells as indicated in each experiment. Following the indicated time of coinoculation, the medium was replaced with luciferin solution prior to imaging with a charge-coupled device camera. Signals from these bioluminescent cells were analyzed with the Living Image Software version 4.2 and an IVIS Spectrum instrument (Caliper Life Sciences, Hopkinton, MA). In some experiments, cell lysates were used for analysis of the luciferase protein by the ONE-Glo Luciferase Assay System (Promega, Madison, WI). Aspartate transaminase (AST) levels in supernatants were quantitated by a validated AST assay at the Clinical Chemistry Laboratory at the Karolinska University Hospital Huddinge using the Modular P apparatus (Roche Diagnostics, Mannheim, Germany). Purification of CD8\(^{+}\) and CD8\(^{+}\) T cell subsets was done with CD8 MicroBeads (Miltenyi Biotec) on day 2 after TCR transduction. Purified T cells were cocultured with target cells at an E:T ratio of 1:0.1.

Results

Identification of NS3A-specific murine TCRs and gene transfer into human PBL

To identify NS3A-specific TCR, NS5A-specific murine T cell clones were generated from HLA-A2–transgenic mice by DNA immunization using the pVAX1-coNS5A (HCV genotype 1b) plasmid and a protocol we recently described for isolation of HCV-specific murine TCR (24). We obtained eight T cell clones that reacted against the NS3A peptide VLTDFKTLW (NS5A1992–2000), an HLA-A2–restricted CTL epitope that was identified from a patient who succeeded in recovering spontaneously from acute HCV infection (26). All eight clones secreted substantial amounts of IL-2 in response to the NS31073–1081 peptide and not to other HLA-A2–restricted viral peptides (HCV NS52221–2231, HCV NS52145–2154, HCV NS52252–2260, or HCMV pp65596) tested in this study. Two of the clones designated 19 and 69 that demonstrated the highest Ag sensitivity and differed in their TCR V\(^{\beta}\)-chain usage were selected for subsequent TCR gene transfer studies. The recovered full-length TCR genes were codon-optimized to allow optimal expression in human cells, and the V\(^{\alpha}\)- and V\(^{\beta}\)-chains were linked by an F2A autoprotease sequence. The final gene cassettes (Fig. 1A, 1B) were inserted into a retroviral vector using the same conditions as for the previously identified NS3 TCR F8 and H4 that were used for functional comparisons with the newly identified NS5A TCRs. These NS3 TCRs were retrieved with the same approach (24) used in this study and are reactive against the genotype 1a NS3 peptide CINGVCWTV (NS31073–1081), another HLA-A2–restricted CTL epitope that is associated with spontaneous clearance of acute HCV infection (4, 9, 27).

PBL from a healthy donor were transduced with the NS5A-specific TCRs (19 and 69) or NS3 TCRs (F8 and H4) and tested for their peptide sensitivity against peptide-loaded T2 target cells in overnight cocultures (Fig. 1C). In agreement with the observations in their parental NS5A mouse T cell clones that were cocultured with same conditions (Fig. 1D), we found that NS5A TCR-transduced human T cells had a lower functional avidity compared with the high-avidity NS3 TCR F8 and H4 (24). Hence, NS5-TCRs are therefore considered as low-avidity TCRs. The surface expression of these murine TCRs could be analyzed by staining with the respective mouse V\(^{\beta}\)Ab (F8: V\(^{\beta}\) 4; H4: V\(^{\beta}\) 8.3; 69: V\(^{\beta}\) 8.1–8.2; 19: V\(^{\beta}\) 6), which showed that 10–20% of CD3\(^{+}\) T cells expressed the indicated TCR on the cell surface (Fig. 2A). Staining with HLA-A2/NS5 or NS5 pentamer confirmed the presence of heterodimeric form of TCR and that both CD8\(^{+}\) and CD8\(^{+}\) populations were transduced (Fig. 2B).

Next, we investigated if the Ag specificity of the TCRs could be restored in the transduced T cells obtained from healthy blood donors. Moreover, because chronic HCV patients often lack functional HCV-specific T cells, we also included such patients to study the effect of this HCV TCR gene transfer approach with their T cells as recipients. Six HLA-A2\(^{+}\) donors (two HCV\(^{+}\) and four HCV\(^{−}\)) were used for TCR transduction, TCR-transduced T cells were then cocultured overnight with NS51992–2000 or NS31073–1081 peptide-loaded T2 target cells, and the T cell response was determined by IFN-\(\gamma\) ICS. The results shown in Fig. 3A (normalized by total CD8) and Fig. 3B (normalized by the number of TCR\(^{+}\) cells) demonstrate that NS3- and NS5A TCR transduction led to a marked increase of IFN-\(\gamma\)-producing cells that are reactive to their cognate peptide (Supplemental Fig. 1). This was found primarily in the CD8-positive population and to some extent also in the CD8-negative population. None of the donors used showed positive staining for any of these murine TCRs. Interestingly, similar to healthy HCV-negative donors (I and L), T cells from chronically infected HCV patients (KS10, KS11, KS13, and KS16) were successfully transduced and demonstrated substantial numbers of IFN-\(\gamma\)–producing T cells in comparison with ex vivo-derived untransduced T cells (fresh NS3 and NS5A) that have been stimulated with the same peptides.

Generation of Ag-specific polyclonotype-producing T cells

The ability of secreting several cytokines is a common feature in polyfunctional effector T cells and it has also been correlated to a superior functional activity (5, 28–30). Because the murine V\(^{\beta}\) is not present on human T cells, we chose it as a marker for transduced T cells and analyzed the cytokine profile of the different TCR-transduced populations. We found, that T cells from healthy donors transduced by the NS3 TCRs (F8 and H4) demonstrated a clear triple-positive (TNF-\(\alpha\), IFN-\(\gamma\), and IL-2) profile within 20 h after stimulation with NS31073–1081 peptide-loaded T2 target cells (Fig. 4, Supplementary Fig. 1). Similar results were found in the transduced T cells from chronic HCV patients. Moreover, in both NS3 TCRs (F8 and H4), it was found that the triple- and double-positive populations account for up to 50% of the V\(^{\beta}\)\(^{+}\)CD8\(^{+}\)CD3\(^{+}\) cytokine-producing effector cells, whereas the other 50% of cells were solely positive for one of the cytokines tested in this study. In contrast, NS5A TCR-transduced CD8\(^{+}\) T cells (19 and 69) were primarily dominated by single-positive populations and demonstrated a preference of IFN-\(\gamma\) production that accounts for up to 60–80% of the cytokine-producing effector cells. A similar pattern (Fig. 4) was found in V\(^{\beta}\)\(^{+}\)CD8\(^{+}\)CD3\(^{+}\) TCR-transduced T cells (e.g., the proportions of double- or triple-positive populations account for >50% of transduced cells), whereas the vast majority of NS5A TCR-positive cells consisted of single cytokine-positive cells following stimulation with the cognate peptide. In addition to HLA-A2–positive donors, we have also tested HLA-A2–negative donors, and a similar functional pattern was observed (data not shown).

Recognition and elimination of HLA-A2\(^{+}\) HCV RNA-replicating hepatoma cells

HCV replicon cells are human hepatoma cells that are characterized by high levels of HCV RNA replication from a subgenomic replicon (31, 32). We therefore used the HLA-A2\(^{+}\) Huh7 HCV-Con1-replicon cells (Huh7\(_{Rep}\) cells, genotype 1b) and investigated in this study if NS5A TCR-transduced T cells could recognize the endogenously processed NS5A1992–2000 peptide, because the NS5A1992–2000 is a conserved CTL epitope shared by HCV genotype 1a and 1b. As previously shown (24), the NS3 TCRs (F8 and H4) demonstrated cross-reactivity to the genotype 1b NS51073–1081 epitope presented by the Huh7\(_{Rep}\) cells by secreting IFN-\(\gamma\) (Fig. 5A) and to some extent TNF-\(\alpha\) (Fig. 5B).
Similar results were obtained with NS5A TCRs (19 and 69), albeit with lower levels of IFN-γ secretion, and this is supported by a small CD3+CD8+ population that had detectable intracellular IFN-γ expression (Fig. 5A, 5B).

The Huh7A2 HCVRep cells are HLA-A2–positive human hepatoma cells persistently harboring a HCV replicon that has been engineered to express the firefly luciferase reporter protein. Because the firefly luciferase reporter protein expression is controlled

**FIGURE 1.** (A) Schematic organization of the four TCR gene constructs used in this study. Codon-optimized (c.o.) α and β TCR genes linked by the F2A autoprotease sequence were synthetically made, and the gene cassettes were then inserted into the retroviral MP71Gpre vector. These TCRs were initially identified from mouse T cell hybridoma clones F8 and H4 reactive to the HLA-A2–restricted HCV NS31073–1081 human CTL peptide and the clones 19 and 69 reactive to the HLA-A2–restricted NS51992–2000 CTL peptide. (B) The junctional sequences and peptide specificity of the TCRs used in this study. Restoration of functional property of the TCRs in transduced human PBL (C), and transduced cells show similar range of functional avidity to their parental mouse hybridomas (D). The experiment was repeated five times with similar results.

**FIGURE 2.** Surface expression of murine TCRs in human PBL. Ab staining for the respective murine TCR Vβ (A), and HLA-A2 HCV pentamer staining with HLA-A2/HCV NS31073–1081 pentamer for clones F8 and H4 and HLA-A2/HCV NS51992–2000 pentamer for clones 19 and 69 on CD3-gated T cells (B). HBV core pentamer is used as negative control. All samples are costained with anti-human CD3 and CD8 Abs, and the populations shown derive from living CD3-positive cells. The Vβ-chain staining was repeated with different donors 26 times with similar results; the pentamer staining was repeated 8 times with different donors.
by the HCV replicase activity, the viral replication activity can be studied by the bioluminescence imaging system (IVIS Spectrum; Caliper Life Sciences) using a high-dimensional charge-coupled device camera. Fig. 5D shows a representative picture of Huh7A2 HCVRep cells after an overnight coculture with decreasing numbers of transduced T cells. It was found that the bioluminescence of the Huh7A2 HCVRep cells was eliminated in a dose-dependent manner according to the ratio of the added T cells, whereas in the control cells without HLA-A2 (Huh7HCVRep), the bioluminescence remained unaffected (Fig. 5C, 5D). In particular, H4 TCR-transduced cells were shown to be very effective (e.g., as few as one transduced T cell was capable of eliminating up to 100 Huh7A2HCVRep cells [ratio 0.01:1] within 16 h of coincubation).

NS5A TCR-transduced T cells, however, were also able to mediate reduction of bioluminescent Huh7A2 HCVRep cells, though in a less effective manner compared with the NS3 TCR H4-transduced T cells. Moreover, because there are some variable levels of the TCR expression in CD8 T cell subsets (Fig. 2), we also carried out an experiment with bead-sorted CD8+ T cells in the functional assay, which showed that the main antiviral function appears to be associated with the CD8+ population (Supplemental Fig. 2).

Because TCR activation induces degranulation, which is a requisite process of perforin-granzyme–mediated killing, we next asked if HCV replicon cells were eliminated through this cytolytic mechanism and analyzed the CD107a surface mobilization because it is a marker of recent lytic activity in Ag-specific CD8+ by the HCV replicase activity, the viral replication activity can be studied by the bioluminescence imaging system (IVIS Spectrum; Caliper Life Sciences) using a high-dimensional charge-coupled device camera. Fig. 5D shows a representative picture of Huh7A2 HCVRep cells after an overnight coculture with decreasing numbers of transduced T cells. It was found that the bioluminescence of the Huh7A2 HCVRep cells was eliminated in a dose-dependent manner according to the ratio of the added T cells, whereas in the control cells without HLA-A2 (Huh7HCVRep), the bioluminescence remained unaffected (Fig. 5C, 5D). In particular, H4 TCR-transduced cells were shown to be very effective (e.g., as few as one transduced T cell was capable of eliminating up to 100 Huh7A2HCVRep cells [ratio 0.01:1] within 16 h of coincubation).

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**FIGURE 3.** Peptide-specific IFN-γ-positive CD8+ T cells in untouched human PBL (fresh) and TCR-transduced T cells (F8, H4, 19, and 69). IFN-γ production normalized by total CD8+ T cells (A) or normalized by the number of CD8+ HCV TCR cells (B) and in gated live CD3+ T cells obtained from healthy blood donors (empty symbols) or chronic HCV patients (filled symbols) determined by ICS assay after 12 h of coculture with peptide-loaded (NS3_1073–1081 or NS5_1992–2000) T2 target cells. nd, Not detected.

**FIGURE 4.** Intracellular cytokine profile of the TCR-transduced T cells obtained from healthy blood donors (n = 2) or chronic HCV patients (n = 4). Each pie chart represents the mean response across the four different TCRs for total CD8+ (A) and CD8+ (B) T cell populations in healthy individuals and chronic patients, respectively.
In this study, our result indicates that the surface CD107a in gated HCV TCR+ cells was upregulated. This was seen both for the mean fluorescence intensity and the percentage of the CD107a expression for all four TCR-transduced cell samples (F8, H4, 19, and 69) that have been stimulated with peptide-loaded T2 target cells (Fig. 6, Supplemental Figs. 3, 4). Similarly, albeit with weaker intensity, the same was found for ionomycin/PHA stimulated TCR-transduced cells. Nevertheless, we found CD107a was only upregulated in NS3 (F8 and H4) but not in NS5A (19 and 69) TCR-transduced T cells that had been stimulated with the Huh7A2 HCVRep or Huh7HCVRep cells after 20 h of coculture with indicated TCR-transduced T cells at E:T ratio 1:1. Average values and SD are given and expressed as percent relative light units for luciferase compared with mock-transduced T cells. Background in medium controls is subtracted. *Indicates under detection level.

NS3- and NS5A TCR-redirected T cells differ in their hepatotoxic potentials

Because our NS5A TCRs did not show a clear sign of CD107a mobilization as a response to the Huh7A2 HCVRep cells, we therefore investigated the degree of hepatocellular injury in Huh7A2 HCVRep replicon cells that have been cultured with the TCR-transduced T cells. We performed a kinetic analysis by using an E:T ratio of 1:1, and at indicated time points, culture supernatants were harvested for measurement of the AST enzyme release. The remaining adherent cells were lysed and subjected to quantification of the luciferase protein. As shown in Fig. 7B, it was found that NS3- but not NS5A- or mock-transduced TCR (Mock) had induced an elevation of AST level after the overnight coculture with Huh7A2 HCVRep cells. The control cells Huh7HCVRep showed no significant increase in the AST levels, indicating that the cell injury is HLA-A2 restricted. Hepatocyte injury leading to cell detachment and cell loss was significantly increased after the overnight coculture with NS3-TCR–transduced T cells (Fig. 7C). The loss of bioluminescence in Huh7A2HCVRep was found to be an effective and rapid process that begins already at 8 h after addition of the T cells (Fig. 7A). This decrease in bioluminescence was not found in Huh7A2 HCVRep cultured with mock-transduced T cells or in the control cells, Huh7HCVRep. Taken together, our results indicate that although the NS3 TCRs function through cytotoxicity-mediated killing, leading to significant target cell injury and cell death, the NS5A TCR-transduced T cells function primarily through a non-cytotoxic CTL mechanism.

Discussion

In this study, we compared different TCRs directed against two different viral CTL epitopes of HCV, NS37073–1081 and NS51992–2000, with regard to their ability to arm primary T cells against hepatoma cells that harbor persistent HCV RNA infection. HCV NS5A
and NS3 are essential components of the viral replicase complex and are constantly present in the HCV-replicating cells because their enzymatic activities are required in the life cycle of the HCV (33). We aimed at targeting HCV NS5A and NS3 protein-positive hepatocytes to eliminate residual HCV-replicating cells by inducing a pre-engineered cytotoxic T cell response mediated by Ag-specific TCRs. Using NS5A- and NS3-specific TCR as Ag receptors to HCV-replicating cells, we demonstrated Ag-specific activation of engineered T cells resulting in cytokine secretion and cytotoxic as well as noncytotoxic elimination of HCV-replicating hepatoma cells.

For our study, we selected four murine TCRs, F8, H4, 19, and 69, for grafting HCV specificity to human T cells. They were selected because they are directed against CTL determinants of HCV that have been consecutively found in patients who spontaneously recover from their HCV infection (34). Among all TCRs tested, H4 showed the highest functional avidity and specificity and proved to be the most effective and specific in targeting and T cell activation. Ag recognition by T cells bearing H4 and F8 TCR, respectively, resulted in polycytokine responses consisting of the proinflammatory cytokines IFN-γ, TNF-α, and IL-2. Because IFN-γ has a direct antiviral effect on HCV replication in hepatocytes (20), it is anticipated that IFN-γ secretion by activated, redirected T cells contributes to virus control. This is supported by the fact that TCR 19 and 69, which primarily produce a monocytokine response consisting of IFN-γ, could have an antiviral effect on HCV replicon hepatoma cells and yet spare the target cells destructive cell injury. Though this is a less effective effector mechanism, as E:T ratio dilution experiments accordingly showed in this study that NS5A-TCR–transduced effector cells also led to a less effective elimination of HCV replicon hepatoma cells in comparison with the cytotoxic H4 and F8 TCRs.

Viral clearance during HBV and HCV infection has been thought to reflect the destruction of infected hepatocytes by cytolytic CD8+ T lymphocytes. However, noncytolytic T cells with antiviral mechanisms may contribute to viral clearance during viral hepatitis by purging viral replicative intermediates from the cytoplasm (35). Direct suppression of viral replication in HBV- or HCV-infected hepatocytes can be mediated by IFN-γ and TNF-α, independent of cell-to-cell contact with virus-specific CD8+ T cells (20, 35). Because IFN-γ induces several key antiviral enzymes, most notably protein kinase R, ADAR adenosine deaminases, and guanylate binding proteins GBP1 and GBP2, which inhibit viral protein synthesis by phosphorylating the eukaryotic initiation factor 2, and catalyzes the generation of nonfunctional viral proteins to inhibit viral replication (36), this antiviral mechanism can thus act directly on the replicon activity. This activity is reflected in a rapid reduction of luciferase activity, as luciferase has a very short t_1/2 (2 h) (37).

Whether the TCRs identified by this approach have an effect in vivo is currently under investigation in another study using a mouse model engrafted by xenogeneic tumors. It would also be important to study if NS3 and NS5A TCRs complement each other to provide a more effective elimination of HCV-infected hepatocytes.

Because the noncytolytic mechanism would spare the host from unwanted liver injury, noncytolytic antiviral T cells may be a sensible alternative to include in T cell therapy to treat chronic viral hepatitis to minimize the degree of liver injury. Adding NS5 T cells
might be an advantage when reconstituting a multispecific antiviral T cell response in vivo to avoid the potential overkilling of hepatocytes; however, the ultimate answer to which of the T cells are more beneficial remained to be evaluated in vivo. With regard to the tolerability of mouse TCR, it was recently reported that mouse TCR are well tolerated in vivo (38, 39). In the melanoma trial (39), it was shown that patient lymphocytes transduced with highly reactive mouse or human antitumor TCRs mediated cancer regression in 19 and 30%, respectively, of melanoma patients. In that study, both mouse- and human TCR-transduced cells traffic to and destroy melanoma tumors in the patients. Because melanoma TCRs are often selected to be highly reactive to melanoma/melanocyte Ags, they also mediated in vivo destruction of tissues that express the target Ag (39, 40). In this regard, though the in vitro performance of redirected T cells is very promising, several safety precautions have to be considered before translation of HCV TCR gene therapy into clinical testing. In particular, one needs to explore how strictly redirected T cells differentiate between infected hepatocytes and neighboring noninfected cells. In addition, hepatocytes in the inflamed liver may be more sensitive to cytolysis. We think it is reasonable to consider low-avidity, noncytotoxic, mouse TCR-transduced CTL in T cell therapy in chronic HCV infection, as they might contribute a minor pathogenic potential. However, it remains open how efficiently redirected T cells will invade the liver parenchyma and whether they will escape silencing in a tolerogenic organ that modulates immune responses. Although our sample size is not sufficiently large to make conclusions about potential differences between normal controls and HCV-infected patients, this is the first documentation, to our knowledge, that HCV-positive patients can be used as autologous donors to generate functional HCV TCR-redirected effector cells. This also supports that most of their cells are not HCV specific, and thus one would not expect any issues with the functionality of transduced cells.

NS3- and NS5A-specific T cells are detected in persistent HCV infection, and the frequencies of these T cells during persistent HCV infection can be rather high. However, not all patients develop this T cell response, and issues with HCV-specific T cells that are dysfunctional and incapable of secreting IFN-γ effector cytokines upon HCV Ag stimulation have also been described (41), which differ from the TCR-redirected T cells shown in this study. It is true that a single response will not be able to mediate viral clearance, but as the research in this field is advancing, more HCV TCRs recognizing other viral targets can be identified with this approach.

FIGURE 7. The ability to induce hepatocellular injury and antiviral activity by T cells that are transduced with indicated TCR. (A) Inhibition of viral replication in Huh7A2HCVRep and Huh7HCVRep cells measured by luciferase activity at the indicated time points after incubation with indicated TCR-transduced T cells. *Under detection limit. (B) AST enzyme release from Huh7A2HCVRep and Huh7HCVRep after 24 h of coincubation with T cells transduced with indicated TCR. The experiment was repeated four times with similar results. (C) Percentage of the target hepatoma cells that remain adherent 20 h after coincubation with indicated TCR-transduced cells. *p < 0.01, **p < 0.0001 compared with hepatoma cells alone without T cells (No T cells), calculated by paired t test with two-tailed p values and 99% confidence intervals. TD, Transduction.
which could potentially be combined with the different HCV TCRs that have been identified so far (24, 42, 43). Reconstituting a multispecific antiviral T cell response would thus not be impossible in the near future and is worth consideration as an option for patients who are difficult to treat with antiviral drugs.

Given the attractiveness of high response rates achieved with new direct-acting antivirals, there are great hopes that HCV will end with its eradication. In light of this, however, there is also a uniform opinion that although the introduction of direct antiviral agents is a major breakthrough in hepatitis C therapeutics, it adds significant complexity and introduces important challenges (44). It has been noted recently that treatment failure is not rare, and drug-resistant viruses have been found in patients who receive new triple combination therapy, in particular in patients with advanced liver fibrosis and those with prior IFN-null response (44). This suggests that some patients seem more difficult to treat with antiviral drugs. Strategies such as adoptive T cell therapy therefore might have a role in the future, including new combinations with, for instance, antiviral drugs to meet the potential challenges from resistant virus strains.

Taken together, we have generated and qualitatively compared TCRs with specificities for two different HCV viral proteins. After these TCRs were retrovirally delivered to primary human T cells, these redirected effector cells were activated to secrete cytokines and enabled to eliminate human hepatoma cells with persistent HCV RNA replication. The antiviral response was indirectly monitored with the decrease of bioluminescence in HCV replicon cells in which the luciferase gene was under the control of the HCV replicative system. This reporter system allowed us to see that the cytotoxic activity was effective, rapid, and specific toward the HCV replicon cells. Additionally, Ag recognition resulted in polyfunctional redirected T cells, which is a key requisite for effective protection against viral infections.

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Disclosures
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