Hepatitis C Virus-Specific T Cell Receptor mRNA-Engineered Human T Cells: Impact of Antigen Specificity on Functional Properties

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ABSTRACT Therapy with genetically modified autologous T cells has shown great promise in cancer therapy. For an efficient control of hepatitis C virus (HCV) infection, cytotoxic T cells (CTL) are pivotal, but persistence of activated T cells may lead to liver toxicity. Here, anti-HCV T cell receptors (TCRs) recognizing the HCV nonstructural (NS) NS3 or NS5 viral peptide target were examined by mRNA transfection of human peripheral blood lymphocytes (PBLs) derived from healthy donors as well as chronically infected HCV patients. Immunological analysis shows that while the CTLs expressing the NS5-specific TCR reduced HCV RNA replication by a noncytotoxic mechanism, the NS3-specific TCR-redirectioned CTLs were polyfunctional and inhibited HCV RNA replication through antigen-specific cytotoxicity. Transcriptome signatures from these two types of CTL responses revealed uniquely expressed gene clusters upon encountering hepatoma target cells presenting endogenously expressed HCV proteins. The NS3 TCR induced a rapid expression of apoptotic signaling pathways and formation of embryonic gene clusters, whereas the NS5A TCR activation induced extended proliferative and metabolic pathways as the HCV target cells survived. Our results provide detailed insights into basic HCV T cell immunology and have clinical relevance for redirecting T cells to target virally infected hepatoma cells.

IMPORTANCE Due to the protective ability of HCV-specific T cells and the hepatotoxic potential that they possess, there is a great need for the understanding of the functional aspects of HCV-specific T cells. To circumvent the low level of precursor frequency in patients, we engineered primary CD8⁺ T cells by mRNA TCR vectors to confer HCV specificity to new T cells. HCV TCRs that differ in antigen specificity and polyfunctionality were examined. mRNA TCR engineering of peripheral blood lymphocytes from healthy donors or chronically infected HCV patients resulted in strikingly high levels of HCV TCR expression and HCV-specific responses. While a cytotoxicity response from a polyfunctional T cell activation caused hepatotoxicity and the rapid induction of apoptotic signaling pathways, the noncytotoxic T cell activation showed extended proliferative, metabolic pathways and persistence of HCV target cells. Our results provide detailed insights into basic HCV T cell immunology and have clinical relevance for immune protection of HCV-associated diseases.

KEYWORDS HCV-specific TCRs, TCR-redirectioned T cell therapy, hepatitis C virus

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Hepatitis C virus (HCV) is a major cause of liver cirrhosis and hepatocellular carcinoma (HCC), and approximately half a million people die each year from HCV-related liver diseases. Although new directly acting antiviral (DAA) drugs are revolutionary and can cure the majority of these infections, problems associated with drug resistance, compliance, medical, or regional political factors restrict the access to these drugs for the majority of infected individuals worldwide (1). In addition, despite DAA treatment, HCV patients previously treated for HCC still have a high risk of liver cancer, and the occurrence of liver cancer is unfortunately not reduced in effectively treated cirrhotic HCV patients (2, 3).

Even though the majority of HCV-infected individuals develop chronic infection, it is estimated that 15 to 45% of infected people spontaneously clear the infection (4). The general consensus is that HCV elimination is associated with strong and sustained T cell responses that target multiple epitopes within the different HCV viral proteins (5–11). While CD4⁺ T cells are central regulators, virus-specific CD8⁺ T cells primarily function as the key effectors. Several lines of evidence support the important role of virus-specific T cells in controlling HCV infection, as there is a clear temporal association between the onset of peripheral and intrahepatic virus-specific T cell responses and HCV clearance (5, 7, 9–13). Elegant *ex vivo* studies of patients' HCV-specific CD8⁺ T cell effector functions revealed that HCV-specific CD8⁺ T cells exert strong antiviral effects primarily by gamma interferon (IFN- γ) but only to a lower extent by cytolytic effector functions (14).

Despite the recruitment of HCV-specific T cells to the infected liver, the failure at the T cell level remains a great challenge for the effective control of HCV infection, as it renders the virus persistent in the majority of infected individuals (15). Several studies illustrated that a protective T cell response has signatures that feature highly polyfunctional HCV-specific CD8⁺ T cells, which contribute to the substantial breadth and height of magnitude of responses to multiple viral determinants, in particular, the viral nonstructural (NS) proteins (16, 17). The impact of polyfunctional T cells on protective immunity is not restricted to HCV but is commonly shared by diseases caused by other infectious pathogens, such as HIV, yellow fever virus, Ebola virus, cytomegaloviruses, and mycobacteria, as well as by cancer (18, 19). However, in spite of its significance, the transcriptional mechanisms underlying antigen-specific T cell polyfunctionality are not completely understood.

We have previously identified HCV-specific T cell receptors (TCRs) in DNA-vaccinated HLA-A2 transgenic mice recognizing two frequently reported HLA-A2-restricted HCV epitopes (NS3₁₀₇₃ and NS5₁₉₉₂) found in HCV patients who resolve their infection (20, 21). Among multiple cloned HCV-reactive TCR candidates generated by this approach, the NS3-H4, NS3-F8, NS5-19, and NS5-69 TCRs were selected for (i) their ability to respond to HCV NS3₁₀₇₃ or NS5₁₉₉₂ peptides in a CD8-independent manner with CD8-negative BW thymoma partners and (ii) their affinity to the respective HCV peptide/MHC pentamers (20, 21). In these previous studies, retroviral TCR gene transfer was used to study these TCRs, in which transduction efficiency varied substantially and was not an optimal approach for global transcriptome studies.

We report here that the synthetic *in vitro*-transcribed TCR mRNA can be used to redirect human T cells to transfer anti-HCV cytotoxic T lymphocyte (CTL) functions to human T cells of peripheral blood lymphocytes (PBLs), as mRNA transfection efficiency was as consistently high, e.g., >90%, in T cells derived from healthy blood donors and HCV-infected individuals. This gave us a prerequisite to examining the global transcriptional signatures of the TCR-redirection CTLs responding to their cognate HCV RNA⁺ hepatoma target cells.

RESULTS

HCV TCR mRNA redirection of healthy human T cells. *In vitro*-transcribed mRNA encoding the NS3_{1073–1081}-specific TCRs (NS3-H4 and NS3-F8) and NS5A_{1992–2000}-specific TCRs (NS5-19 and NS5-69) were introduced into activated T cells of two HLA-A2⁺ healthy donors by electroporation. Because these are murine TCRs originating

from HLA-A2-transgenic mice, they can be distinguished from human TCRs with antibodies that bind to their mouse V β chain (e.g., for NS3-H4, V β 8.3; for NS3-F8, V β 4; for NS5-19, V β 6; for NS5-69, V β 8.1-8.2) (Fig. 1a). Moreover, these antibodies also gave staining results comparable to those of the respective HCV peptide/major histocompatibility complex (MHC) pentamer for each TCR (20, 21). Here we found that the cell viability after mRNA electroporation was around 95% (Fig. 1a). The surface expression of the introduced TCR was detected in >90% of live CD3⁺ T cells on the day after electroporation (Fig. 1a; Table 1), and the majority of TCR⁺ cells were CD8⁺ T cells with background staining (mock-transfected or TCR-transfected cells stained with isotype controls) at 1 to 3% (Fig. 1a and data not shown). HCV-specific responses, detected following coculture with NS5_{1992–2000} or NS3_{1073–1081} peptide-loaded T2 cells, confirmed that NS3 TCR-redirection effector cells were superior IFN- γ producers to NS5-TCRs (Fig. 1b), which is consistent with the results obtained with retrovirus-modified T cells (21). Cell surface expression of the TCRs lasted for 3 to 4 days (Fig. 1c) and concurred with their target-specific functionality (Fig. 1d). Moreover, NS3-H4-redirection T cells secrete both IFN- γ and interleukin-2 (IL-2), whereas those of NS5-69 produce mainly IFN- γ . Because NS3-H4 and NS3-F8 (NS3_{1073–1081} specific) as well as NS5-19 and NS5-69 (NS5A_{1992–2000} specific) share antigen specificity and similar functional responses, the NS3-H4 and the NS5-69 TCRs were chosen for further comparisons.

HCV TCR mRNA redirection of HCV patients' T cells and comparative functional analysis with healthy human T cells. To examine if PBLs from chronic HCV patients can be redirected with TCR mRNAs with the same efficiency, HLA-A2⁺ PBLs from chronic HCV patients ($n = 10$) and healthy donors ($n = 9$) were tested as described above. Our results showed that irrespective of the liver disease stage, HCV patients' PBLs were as successfully redirected with HCV TCR as in healthy donors (around 90% of CD3⁺ T cells), and the vast majority of CD8⁺ T cells showed surface expression of the respective HCV TCRs (Fig. 2a; Table 1). Moreover, NS3-H4-redirection T cells from HCV-infected patients and healthy donors effectively eliminated T2 target cells loaded with NS3-1073 peptide as early as 5 h after the coincubation (Fig. 2c), while NS5-69-redirection cells did not. Given that T cell polyfunctionality is a hallmark of pathogen control, the two TCRs were compared at the single cell level by intracellular cytokine staining (ICS). We found that a large proportion of NS3-H4-redirection T cells, but not NS5-69-redirection cells, secreted more than one cytokine, e.g., IFN- γ , tumor necrosis factor alpha (TNF- α), or IL-2, and had upregulated the surface expression of CD107 α (Fig. 2b and d). Surface expression of HCV TCR was also found in the CD8-negative cell population, which showed moderate proportions of polyfunctional cells upon stimulation (Fig. 3). In conclusion, the data demonstrate that TCR mRNA delivery is highly effective in producing HCV TCR-redirection T cells that resemble polyfunctional/cytotoxic T cells (NS3-H4) and monofunctional/noncytotoxic T cells (NS5-69).

Antiviral efficacy and hepatotoxic potential of TCR mRNA-redirection T cells. The ability of TCR mRNA-redirection T cells to recognize endogenously processed HCV antigen was examined next. To provide a more biologically relevant condition that allows examination of the hepatotoxic potentials, the redirection T cells were coincubated with hepatoma target cells Huh7^{A2} HCV^{Rep}, human hepatoma cells that persistently replicate the HCV genotype 1 subgenomic RNA and express HLA-A2 (22). As shown in Fig. 3a, both NS3-H4 TCR- and NS5-69 TCR-redirection T cells responded to the Huh7^{A2} HCV^{Rep} cells by secreting IFN- γ , and these results confirmed the observations with T2 cells. Next, the hepatotoxic potential of the TCR-redirection T cells was examined: the Huh7^{A2} HCV^{Rep} target cell number was determined and the aspartate aminotransferase (AST) enzyme release was measured over 5 days of coincubation. We found a steady increase of AST, and the considerable cell detachment in the NS3-H4 T cell and Huh7^{A2} HCV^{Rep} target cell coculture indicated a rapid hepatocellular cell injury (Fig. 4b). On the other hand, the NS5-69 T cells spared the target cells and allowed them to grow to confluence (Fig. 4b). Next, the antiviral properties of TCR mRNA-redirection T cells were tested using PBLs from healthy donors ($n = 9$) and HCV patients ($n = 10$) in overnight cocultures with Huh7^{A2} HCV^{Rep} cells or the control Huh7 HCV^{Rep} cells lacking

FIG 1 Surface expression and target peptide recognition in human PBLs (from healthy blood donors) transfected with mRNA encoding HCV-specific TCRs. (a) Surface staining of TCRs specific for HCV NS3₁₀₇₃₋₁₀₈₁ (H4 and F8) and NS5₁₉₉₂₋₂₀₀₀ (19 and 69) by their V β antibody (H4, mV β 8.3; F8, mV β 4; 19, mV β 6; 69, mV β 8.1-8.2) 1 day posttransfection. Mock-transfected PBLs served as negative controls. The percentages of indicated TCR mV β -expressing cells were gated on live CD3⁺ T cells that were counterstained with Live/Dead Aqua fixable reagent, anti-CD3, anti-CD8, and the indicated mV β antibody and determined by FACS. (b) HCV-specific IFN- γ secretion following stimulation with T2 cells loaded with their cognate peptide (1 μ g/ml) at the indicated E/T ratios. (c) Transient expression of HCV TCRs (H4, 69) in human PBLs following the mRNA transfection. (d) T cells redirected by the TCR H4 are capable of secreting antigen-specific (NS3₁₀₇₃₋₁₀₈₁) IFN- γ and IL-2, whereas TCR 69-redredirected cells secrete mainly IFN- γ in response to its target peptide (NS5₁₉₉₂₋₂₀₀₀). Transfected T cells from healthy donors were cocultured for 16 to 18 h with T2 target cells with or without peptide from the indicated time points after the transfection, and the harvested supernatant were analyzed with cytokine ELISA. Mock-transfected PBLs were included as negative controls. Coculture experiments were done in duplicate wells and repeated two times with similar results.

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^bpNA, not available.

*Fibroscan scores measure liver stiffness in kilopascals.

FIG 2 TCR mRNA redirection of PBLs from chronically infected HCV patients and healthy donors. (a) HCV TCR cell surface expression on day 1 posttransfection. CD8 and mV β staining for respective TCRs is shown on live CD3-positive lymphocytes as described for Fig. 1. (b) The numbers (Continued on next page)

FIG 3 (a) Proportion of CD8⁻ and CD8⁺ populations of CD3-gated HCV TCR⁺ live T cells. (b) Mean fluorescence intensity (MFI) of HCV TCR V β staining of the CD8⁺ versus CD8⁻ T cells; the individual points indicate the MFI of indicated HCV TCR expression in each donor (healthy donors, $n = 8$; HepC donors, $n = 9$). Bars indicate the mean MFI values of the indicated HCV TCR staining in the patient group. (c) Proportions of antigen-specific cytokine-producing TCR-transfected CD3⁺ CD8⁻ T cells (H4 versus 69) from healthy donors versus hepatitis C patients (Hep C).

HLA-A2, and the HCV replicon-driven luciferase reporter protein was quantitated. The results indicate that regardless of the age of HCV donors, HCV viral load, or liver disease status, the NS3-H4-redirected T cells strongly inhibited HCV replicon activity while the NS5-69-redirected T cells showed mainly a marginal replication inhibition (Fig. 4c; Table 1).

Transcriptome analysis of the TCR-redirected T cell responses against HCV replicon cells. Next, we examined the transcriptional signatures in the TCR-redirected T cells responding to HuhA2 HCV^{Rep} target cells. We chose PBLs from patient K23, as this individual had no detectable endogenous T cell responses against HCV replicon cells, and redirected T cells from this patient showed a consistent response in all experiments and was representative of the responses in the patient cohort tested. The two TCR-redirected (NS3-H4, NS5-69) T cells and undirected T cells (UT) were cocultured with HuhA2 HCV^{Rep} target cells for 6 h, 24 h, and 4 days and enriched for CD8⁺ T cells by

FIG 2 Legend (Continued)

of antigen-specific functions (intracellular IFN- γ , IL-2, TNF- α , or CD107 α upregulation) in TCR mRNA-transfected CD3-gated cytokine-producing CD8⁺ T cells (H4 versus 69) from healthy donors ($n = 2$) versus those from hepatitis C patients ($n = 3$) are summarized in pie charts. Each pie chart represents the mean responses of the different TCRs for total CD3-gated CD8⁺ T cell populations in healthy individuals and chronic HCV disease patients. (c) Effective target-specific elimination by NS3-H4 cocultured with HCV NS3_{1073–1081} peptide-loaded T2 cells (upper panel). Representative forward scatter/side scatter (FSC/SSC) dot plots of cocultured TCR-redirected T cells (H4 in the top panels, 69 in the middle panels) cocultured with T2 cells loaded with or without the respective HCV peptides. TCR-transfected PBLs alone and T2 cells only (bottom panel) served as controls. The experiment was repeated three times using PBLs from three different HCV patient donors, all of which gave similar results. (d) Difference in degranulation capacity in NS3-H4 (H4)- and NS5-69 (69)-redirected cells. Representative dot plots gated on CD3⁺ T cells and the CD107 α surface staining in TCR-transfected T cells (healthy donor and HCV patient). All functional analyses were done 5 h after cocultivation with the target cells. PMA/ionomycin (PMA+ION) and mock undirected T cells were included as controls in all intracellular cytokine staining assays.

FIG 4 Replicon cell recognition, hepatotoxic potential, and antiviral activity of TCR-redirection T cells. (a) HCV-specific IFN- γ secretion in NS3-H4 (H4)- and NS5-69 (69)-redirection T cells from HCV patient donors ($n = 3$) following 24 h of coculture with Huh7^{A2} HCV^{Rep} cells at an E/T ratio of 10:1. (b) Hepatocellular cell injury by T cells redirection with the indicated TCRs. (Right) Percentages (left panel) of the target Huh7^{A2} HCV^{Rep} cells that remain adherent after cocubation with the indicated T cells over 5 h of coculture. After the indicated times of cell cocubation, the suspension cells were removed and the replicon cells remaining adherent were trypsinized and counted by trypan blue exclusion. The percentages of replicon cells were determined against control target replicon cells (RepA2 only). (Right) Aspartate aminotransferase (AST) enzyme release from Huh7^{A2} HCV^{Rep} cells after cocubation with T cells transfected with indicated TCR. Means and standard deviations (SD) are shown. nd, not detected. (c) Antiviral potential of redirection T cells from healthy donors ($n = 9$) and HCV patients ($n = 10$). The HCV replicon luciferase activity is measured in Huh7^{A2} HCV^{Rep} and Huh7 HCV^{Rep} cells after 24 h of coculture with the indicated T cells at an E/T ratio of 0.5:1 in duplicate wells. Each dot represents one individual, and the bar indicates the mean value for the group. Mock undirected T cells (UT) were included as controls. The Mann-Whitney U test was used for group comparisons (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

FIG 5 Transcriptome profiling by RNA-seq in NS3-H4-directed, NS5-69-directed, and undirected T cells after coculture with HCV replicon cells. (a) Schematic flow of cell and RNA isolation of TCR-redirected CD8⁺ T cells; (b) sequence annotation and analysis; (c) Venn diagrams of the gene distribution in NS3-H4 (H4)- and NS5-69 (69)-redirected and undirected (UT) T cells following coculture with HCV replicon cells at the indicated time points. The intersections of circles refer to mRNA transcripts that are shared among the different conditions.

microbeads (Fig. 5a). TCR transfection efficiency for both TCRs was >95%, and 1.5×10^6 CD3⁺ CD8⁺ cells (>98% purity) were obtained and stored from each condition and time point (Table 1 and Fig. 5a). Total RNAs were analyzed by paired-end RNA sequencing as shown in the flowchart in Fig. 5b. We obtained about 346 million mapped “reads” and found a total of 57,780 genes (both protein-coding and noncoding genes) expressed in the isolated CD8⁺ T cells. Before pursuing the analysis of the

sequencing data, a cutoff level of 0.01 for read counts was applied to exclude spurious transcripts.

The uniquely expressed genes in NS3-H4 versus NS5-69 T cell responses. The distributions of the unique and shared expressed genes for each T cell condition and time point are illustrated by the Venn diagrams shown in Fig. 5c. They show that 6 h after the encounter with HuhA² HCV^{REP} target cells, 7,463 genes were uniquely expressed for the mock undirected T cells (UT) whereas only 463 and 1,310 genes were unique for NS3-H4 and NS5-69, respectively. Furthermore, NS3-H4 had the lowest number of unique genes at the three time points. To further examine the protein-coding genes, we applied another threshold of 20 read counts per feature to filter away weakly expressed genes. KEGG enrichment analyses by WebGestalt were performed to study the unique protein-coding genes expressed in each T cell response. As shown in Table 2, the most significant ($P < 0.01$) enriched KEGG signaling pathways among uniquely expressed genes in NS3-H4 T cells were cytokine-cytokine receptor interactions, JAK-Stat signaling, and chemokine signaling pathways at 6 h following HCV replicon cell coculture. While the target cells were eliminated in the 24-h coculture of NS3-H4 (Fig. 4b), the responses shifted to engage steroid hormone biosynthesis, ribosome and chemokine signaling pathways, and then cytokine-cytokine receptor interaction and rheumatoid arthritis-like pathways on day 4 (Table 2). The NS5-69 T cell response, on the other hand, showed a diffuse and variable gene pathway usage: the unique genes expressed at 6 h following target encounter lacked clear enrichment of any signaling pathway, while at 24 h in the presence of confluent HCV replicon targets, the most relevant enriched pathways seen were complement/coagulation cascade, metabolic, and infection pathways, which extended to more than 30 enriched pathways on day 4. The latter include systemic lupus erythematosus (SLE)-like, calcium signaling, adhesion, tight junction, cytoskeleton, cytokine-cytokine receptor interaction, and leukocyte migration as well as transforming growth factor beta (TGF- β) signaling pathways (Table 2). All the uniquely expressed genes regulated within the significantly enriched KEGG signaling pathways for both TCRs are presented in Table S1 in the supplemental material.

DE genes of NS3-H4 and NS5-69 T cell responses—the common genes. Differential gene expression between undirected T cells (UT) and NS3-H4- and NS5-69-redirected T cells was analyzed using read counts for each gene with the DeSeq package, developed for differential expression analysis of high-throughput RNA sequencing (RNA-seq) data. A cutoff level at $\pm 1.0 \log_2$ -fold change was set for the differentially expressed (DE) genes before the downstream analysis. A complete list of all DE genes in each TCR in $\log_2$ -fold change are listed in Tables S2 and S3 in the supplemental material. The upregulated genes in NS3-H4- and NS5-69-redirected T cells relative to the UT control were analyzed with the functional annotation clustering tool in the Database for Annotation, Visualization and Integrated Discovery (DAVID), which clusters related groups and orders these clusters according to their significance as determined by their EASE scores (a modified Fisher's exact P value). Analysis revealed the top three clusters among all identified upregulated genes with the most significant ($P < 0.05$) enriched gene ontology (GO) terms (Fig. 6). Here, the NS3-H4-redirected T cell response included mainly items related to regulation of apoptosis and programmed cell death at 6 h postcoculture with HCV replicon target cells (both positive and negative regulators; $n = 94$ genes). At 24 h, the majority of items involved regulation of ion and membrane transporter activity, cyclic AMP (cAMP) biosynthesis, and metabolic processes ($n = 12$ genes), as well as tyrosine protein kinase activity (EASE score, 2.76 [data not shown]). On day 4, only genes related to embryonic morphogenesis (1 cluster, 5 genes) were found by this analysis. On the other hand, the NS5-69-redirected T cells at 6 h showed significant clusters of items relating to cellular amino acid biosynthetic process, chemotaxis, and positive regulation of activated T cell proliferation ($n = 13$ genes), such as interleukin-2R (IL-2R) as well as chemokine (C-X-C motif) ligands (CXCL) CXCL9, CXCL10, and CXCL11, which are known to attract activated

TABLE 2 Enriched significant KEGG signaling pathways among unique genes in NS3-H4 and NS5-69 T cells responding to Huh7^{A2} HCV^{Rep} target cells at indicated time points^a

Condition and time point	KEGG pathway	No. of genes in pathway	P value
H4 (6 h)	Cytokine-cytokine receptor interaction	6	1.06E−08
	JAK-Stat signaling pathway	3	0.0001
	Chemokine signaling pathway	3	0.0002
	Rheumatoid arthritis	2	0.0014
H4 (24 h)	Steroid hormone biosynthesis	2	0.0026
	Ribosome	2	0.0069
	Chemokine signaling pathway	2	0.0269
H4 (day 4)	Cytokine-cytokine receptor interaction	3	0.0048
	Rheumatoid arthritis	2	0.0061
69 (6 h)	No significantly enriched pathway		
69 (24 h)	Complement and coagulation cascades	5	1.05E−08
	Glycerolipid metabolism	2	0.0013
	<i>Staphylococcus aureus</i> infection	2	0.0015
	Metabolic pathways	5	0.0063
69 (day 4)	Systemic lupus erythematosus	20	3.27E−13
	Neuroactive ligand receptor interaction	27	4.51E−13
	Calcium signaling pathway	17	1.57E−08
	Focal adhesion	17	9.53E−08
	Tight junction	13	5.72E−07
	Cytokine-cytokine receptor interaction	18	1.13E−06
	ECM-receptor interaction	10	2.28E−06
	Pathways in cancer	19	5.46E−06
	Regulation of actin cytoskeleton	15	5.61E−06
	Hypertrophic cardiomyopathy	9	1.43E−05
	Dilated cardiomyopathy	9	2.76E−05
	Leukocyte transendothelial migration	10	3.67E−05
	Axon guidance	10	9.05E−05
	Amoebiasis	9	0.0001
	TGF- β signaling pathway	8	0.0001
	Gap junction	8	0.0002
	Malaria	6	0.0002
	Long-term depression	7	0.0002
	Arrhythmogenic right ventricular cardiomyopathy	7	0.0003
	Long-term potentiation	6	0.0014
	Gastric acid secretion	6	0.0018
	Cell adhesion molecules	8	0.0023
	African trypanosomiasis	4	0.0031
	Arachidonic acid metabolism	5	0.0036
	Vascular smooth muscle contraction	7	0.0042
	Hematopoietic cell lineage	6	0.0044
	Vitamin digestion and absorption	3	0.0080
	Melanoma	5	0.0079
	Viral myocarditis	5	0.007
	Melanogenesis	6	0.0085
	Maturity-onset diabetes of the young	3	0.0090

^aAnalysis of pathways was performed by WebGestalt using the hypergeometric statistical method with significance level set at *P* values of <0.01 and minimum number of regulated genes for each category set at 2.

T cells, while at 24 h, activities attributed to cyclic nucleotide biosynthetic, cAMP, and metabolic processes were well represented along with calcium/metal ion transport genes (*n* = 15 genes), suggesting signal transductions of second messengers. On day 4, multiple items (*n* = 33 genes) resulting in lipid metabolisms (positive regulators of phospholipase and lipase) and cellular metabolic processes, such as arachidonate lipoxygenases, were found. The list of identified DE genes according to their functional clusters is presented together with Ensembl ID, gene name, and log₂-fold change in Table S4 in the supplemental material. Next, GO biological process analyses (performed

FIG 6 Gene ontology (GO) categories enriched among upregulated genes in NS3-H4-redirected T cells and NS5-69-redirected T cells. Biological processes that are upregulated in NS3-H4- or NS5-69 TCR-redirected T cells relative to undirected T cells (UT) at the indicated time points following coculture with Huh7^{A2} HCV_{REP} target cells, identified using the annotation tool DAVID and related processes clustered using the functional annotation clustering tool. The top three clusters with the highest enrichment (EASE) scores and a P value of <0.05 for each GO category for NS3-H4 are presented at 6 h (a), at 24 h (b), and at day 4 (c) and for NS5-69 at 6 h (d), at 24 h (e), and at day 4 (f) after T cell and target cell coculture. The bars represent the respective GO categories, and the x axis is the $-\log_{10} P$ value for enrichment level for each category. pos., positive; neg., negative; reg., regulation; diff, differentiation.

in WebGestalt) of the protein-coding differentially expressed genes between the responses of NS5-69- and NS3-H4-redirected T cells at the different time points (6 h, 24 h, day 4) were performed, with enrichment significance level P values of <0.05 and a minimum level of 10 regulated genes in each category. The enriched GO biological process as well as the parent terms among the DE genes in NS5-69-redirected T cells are illustrated in Fig. 7 and 8, in which the number of genes regulated in each GO category are presented in parentheses. Enriched GO categories among the upregulated genes in NS5-69-redirected T cells compared to the NS3-H4-redirected T cells were indicative of mainly cell-cell signaling, multicellular organismal process, system process, and system development. As for downregulated genes, which were fewer than those that were upregulated, in NS5-69-redirected cells, they were found to be involved in GO categories of signaling, immune system processes, cell communication and activation, chemotaxis, inflammatory response, responses to external stimulus, and locomotion.

DISCUSSION

In this study, we examined a nonviral gene transfer approach to redirect human T cells with antiviral TCRs that recognize two well-characterized human CTL epitopes with clinical relevance in hepatitis C infection. The main reason for choosing these CTL

FIG 7 Significantly enriched gene ontology (GO) biological process among upregulated genes in NS5-69-redredirected T cells compared to NS3-H4-redredirected cells at time points 6 h (a), 24 h (b), and day 4 (c). The regulated pathways are in red, and the numbers of regulated genes in the pathways are in parentheses.

FIG 8 Significantly enriched gene ontology (GO) biological process among downregulated genes in NS5-69-redirected T cells compared to NS3-H4-redirected cells at time points 6 h (a), 24 h (b), and day 4 (c). The regulated pathways are in green, and the numbers of regulated genes in the pathways are in parentheses.

epitopes is that they are associated with spontaneous clearance of HCV in acute infection (9, 23–25). Our main findings suggest that the TCR redirection approach examined here is (i) effective in PBLs from both healthy donors and hepatitis C patients that have all four forms of liver fibrosis stages, (ii) functionally preserved and highly reproducible in regard to our previous finding with retrovirally engineered T cells (20, 21), (iii) of transient nature, reducing concerns of vector integration into host genome and long-term expression if administered, and (iv) useful in gaining a systemic insight into HCV-specific CTL responses that differ in their effector functions. These findings add an important translational significance. An immune therapy consisting of efficacious polyfunctional cytolytic HCV-specific T cells would support the immune surveillance in difficult-to-treat hepatitis C patients, presuming that HCV epitopes are still unchanged and are present in the patients. This is particularly relevant given that recent reports suggest that certain HCV patient groups, especially those who are at very high risk of developing hepatocellular carcinoma, may require more-active immune surveillance of infected premalignant cells rather than curative antiviral treatment only (2, 3).

Although much effort and endeavor have been devoted to elucidating the mechanisms that regulate CD8⁺ T cell activation, differentiation, and survival (26–31), a fundamental examination of the genetic pathways in HCV T cell responses to hepatocytes replicating HCV has to our knowledge not been undertaken prior to this study. This has been complicated by the seemingly low precursor frequency of HCV-specific T cells in patients and the requirement for major histocompatibility complex (MHC)-peptide tetramer enrichment, which is difficult to scale up. Single-cell sequencing could eventually surpass this challenge, but our bulk cell analysis here illuminates for the first time the dynamic of an HCV T cell response, allowing an overview of transcriptomic changes in antigen-specific T cells from the encountering to the elimination (or failure of elimination) of their target cells. Although the TCRs examined here originated from HLA-A2 transgenic mice vaccinated with DNA (20, 21), murine TCR expression on human T cells circumvents problems with TCR mispairing, e.g., the formation of nonfunctional TCR “human-human” heterodimers with endogenous host TCRs. Moreover, the functionality of murine TCRs in human T cells has been demonstrated both *in vitro* and *in vivo* in human T cell adoptive therapies (32). In spite of the fact that the ectopic expression of HCV TCR on the human T cell surface lasts for 3 to 4 days, our results indicate that the cells were biologically functional for the entire expression time, i.e., responded to the target cells in an antigen-specific manner.

The HCV RNA replicon cell model is a biologically relevant condition to examine the hepatotoxic potential of the TCRs. The observed polyfunctional profiles in response to processed antigen support the results obtained with peptide-loaded T2 cells: NS3-TCR activation stimulates IFN- γ , tumor necrosis factor alpha (TNF- α), and granzyme B, as confirmed by RNA-seq and injury of HLA-A2-positive HCV RNA⁺ hepatoma cells, while the NS5 TCR's response to processed antigen consists mainly of IFN- γ or TNF- α and no hepatocellular injury. Here, human T cells conferred with NS3-H4 or NS5-69 TCR recognized the endogenously processed peptides in target HCV replicon cells. But contrary to NS5-69, the NS3-H4 TCR was consistently superior in providing polyfunctionality, target cell elimination, and hepatotoxicity, although the two TCRs had similar surface expression and kinetics. It is possible that the differential antiviral activities of the two different peptide-specific transfected T cell lines are due to differential antigen or differential TCR affinity. In this context, peptide titration experiments indicate that although the two TCRs behave in dose-dependent manners, the NS3 TCR is highly sensitive and requires a few log₁₀-fold less peptide than the NS5-TCR to secrete multiple cytokines (22). Hence, the TCR affinity represents an additional important factor accompanying the antigen specificity in an antigen-specific T cell response. Given that T cell responses play a unique part in HCV infection, since CD8⁺ T cells not only contribute to viral clearance and protective immunity in those who recover from infection but also are associated with tissue injury in infected patients, e.g., hepatic flare (33), the two CD8⁺ T cell responses that we report here are thus of clinical relevance

for patients with hepatitis C. Viral clearance during HCV has been thought to reflect the destruction of infected hepatocytes by cytolytic CD8⁺ T lymphocytes, but several elegant studies have suggested that the cytolytic effector pathway is rarely utilized by HCV-specific CD8⁺ T cells in patients with chronic hepatitis C (14, 34, 35), possibly to restrict liver damage *in vivo*.

A typical feature in hepatitis C patients who develop chronic infection is the selection of functionally impaired T cells and upregulation of suppressive cytokines and regulatory cell response such as TGF- β , chemokine ligand 11 (CCL11; eotaxin), and CCL18 and CCL22, known to recruit regulatory T cells (36–38). These were also found to be upregulated here in the NS5-69 T cell response, suggesting that low-avidity CTLs may have anti-inflammatory potentials. In contrast, our data indicate that in a cytolytic CTL response, such as the NS3-H4 CTR having polyfunctional property and increased ability to eliminate HCV target cells, the response has a striking focus on cytokine-cytokine receptor interactions, chemokine signaling, and JAK-Stat signaling, lacking in the noncytolytic NS5-69 CTL response. Interestingly, our functional GO clustering analysis clearly demonstrates that NS3-H4 upregulated apoptosis and programmed cell death gene clusters, including genes encoding TNF/TRAIL, IFN- γ , and CD70, indicative of an activation-induced apoptosis early during the elimination of HCV replicon target cells. Moreover, there were, as well as antiapoptosis genes, clusters of genes encoding BCL-3, IL-2R, and CD27, among which CD27, a costimulatory molecule, is known to be required for the generation of maintenance of T cell immunity (39, 40). The early response seen here could be due to the fact that the PBLs studied here had been preactivated and were already differentiated effector cells when they encountered the HCV target cells. Furthermore, the increased ion and membrane transporter activity, cAMP biosynthesis, and metabolic processes later the following day were not totally unanticipated, since ion channels and transporters in the plasma membrane of T cells are required to modulate the cytoplasmic concentrations of diverse cations; in particular, calcium channels are known to allow second messengers to regulate crucial lymphocyte effector functions, including CD8⁺ T cell survival and control of activation (41, 42). The reason for the appearance of a gene cluster involved in embryonic morphogenesis on day 4 is not clear (perhaps it is a nascent process in T cell rejuvenation and memory cell formation) and deserves further examination.

Despite the fact that NS5-69-redirected T cells recognize HCV-expressing target cells by secreting a small number of cytokines, they failed to eliminate target cells. The notion that the NS5-69 response had gene clusters indicative of cellular amino acid biosynthesis suggests an active glycolysis associated with activation and effector T cell differentiation (43) at the same time, with increased taxis, and chemokine ligand genes encoding CCL9, CCL10, CCL11, and CCL22 and cell proliferation genes were upregulated. As the incapability to eliminate the target continues, the activities continue to include signal transduction and metabolic processes (cyclic nucleotide biosynthetic, cAMP and metabolic processes, calcium/metal ion transport), which became even more diverse to include gene cluster expressions controlling lipid metabolisms and cellular metabolic processes and pathways sharing features with an SLE-related response.

A further direct comparison of pathways based on enriched GO biological processes used by NS3-H4 and NS5-69 suggests that NS5-69 had upregulated a substantially large number of genes that are associated with cell-cell signaling, multicellular organismal process, system process, and system development. Among the downregulated genes in NS5-69 relative to NS3-H4, albeit much fewer than those that were upregulated, those in pathways involving signaling, immune system process, cell communication and activation, chemotaxis, inflammatory response, response to external stimulus, and locomotion were noted. This supports the idea that NS5-69 activated multiple default pathways as a response to the persistent HCV RNA replicating target cells. The difference in function of the two HCV-specific TCRs might be related to their intrinsic TCR affinity. Alternatively, the HCV replicon cells might process and present more efficiently the NS3 epitope than the NS5 ones. Although there are currently no studies to support

this hypothesis, it is in line with the previously reported hypothesis that activation of NS5-69 TCR requires more target peptide than does that of NS3-H4 (20, 21).

The questions that our study addressed were (i) whether chronic HCV patient PBLs can be effectively redirected by the mRNA approach and (ii) what gave HCV CTL effector cells the ability to eliminate or spare their target cells. The two types of effector T cell responses are considered to have important roles in viral hepatitis cure and progression (44–47). To our knowledge, this is the first study that describes the effector gene profiles distinguishing the cytolytic from the noncytolytic antiviral effector CD8 T cells with specificity to HCV viral targets. Such antiviral effector features may also be of relevance for the biology of antigen-specific polyfunctional T cells in general.

The strength of this study is that the endogenously processed HCV CTL targets and antigen-specific T cells were examined, in which the exposed effector T cells could be isolated without tetramer sorting, and transcriptomically decoded with a powerful pair-end high-throughput RNA sequencing. A limitation of this study is that the transcriptome results are merely descriptive and at the transcript level, and hence further studies are needed at the next level with proteomics and/or metabolomics analyses. Moreover, these TCRs address only HLA-A2-restricted responses and target cells, thus confining their clinical applications. However, a strong antigenic avidity and acquisition of proper effector functions have an impact on the selection of polyfunctional high-avidity CD8 T cells, a quality known to correlate with control of hepatitis C virus infection (48, 49). Our transcriptomic insight into the mechanisms behind a successful versus a less successful HCV CTL response has implications in understanding HCV T cell immunology and T cell biology in general.

MATERIALS AND METHODS

PBMCs from healthy blood donors and HCV-infected patients. Peripheral blood samples were collected under informed consent from healthy blood donors and HCV-infected patients at Karolinska University Hospital. Following HLA typing, HLA-A2-positive donors were included, with subsequent isolation of peripheral blood mononuclear cells (PBMCs) from 9 healthy blood donors and 10 HCV-infected patients (20, 21). Ethical permission was obtained from the Regional Ethical Review Board (EPN) in Stockholm. The Declaration of Helsinki protocols were followed, and all patients gave their written, informed consent.

Cell lines. The Lunet-HLA-A2-luc/neoET (Huh7^{A2} HCV^{Rep}) and Lunet-luc/neoET (Huh7 HCV^{Rep}) cell lines were maintained as described previously (21, 22) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 1 mM nonessential amino acids. Huh7^{A2} HCV^{Rep} cells were selected with puromycin (1 µg/ml) and G418 (0.5 mg/ml) and Huh7 HCV^{Rep} cells with G418 (0.5 mg/ml). Huh7^{A2} HCV^{Rep} cells have ectopic HLA-A2 expression and contain a selectable HCV subgenomic RNA replicon of genotype 1b (Con1-ET). The cells also express the firefly luciferase gene fused to the selectable marker by ubiquitin. Control cell line Huh7 HCV^{Rep} contains only HCV subgenomic RNA replicon of genotype 1b and does not express HLA-A2. The T2 cell line (HLA A2.1⁺) was maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin. Media and supplements were purchased from Sigma-Aldrich, St. Louis, MO.

In vitro synthesis of TCR mRNA and transfection of PBLs. Plasmids encoding NS3 TCRs or NS5 TCRs (pVAX-NS3-H4, pVAX-NS3-F8, pVAX-NS5A-19, pVAX-NS5A-69) were constructed by subcloning TCR genes from retroviral vectors described earlier (20, 21). Qiagen Endo Free Plasmid Maxi kit (Qiagen, Hilden, Germany)-purified and XbaI-linearized DNA was used as the template to synthesize TCR mRNA using the mMESSAGE mMACHINE T7 Ultra kit (Ambion, Austin, TX). T7 RNA polymerase was added to start transcription; RNA was capped with anti-reverse cap analog (ARCA) and poly(A) tailed with poly(A) polymerase and ATP. Lithium chloride-precipitated RNA was stored at -80°C until use. PBMCs were stimulated and prepared as described earlier (50) with 600 U/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 medium (Life Technologies, Carlsbad, CA) supplemented with 2% human AB serum for 7 days. The concentration of rIL-2 was increased to 1,000 IU/ml on day 8. Electroporations were performed with the Nucleofector device (Lonza, Cologne, Germany). Activated T cells were harvested and transfected in Cell Line Nucleofector Solution V (Lonza, Cologne, Germany) at 10 × 10⁶ cells with TCR mRNA (200 µg/ml) in a certified cuvette (Lonza, Cologne, Germany). Immediately after transfection, the cells were resuspended in AIM-V medium (Life Technologies, Carlsbad, CA) supplemented with 2% human AB serum and 100 IU/ml rIL-2 and incubated in a humidified 37°C and 5% CO₂ incubator until further analysis.

FACS antibody staining and peptides. For fluorescence-activated cell sorting (FACS), APC anti-human CD3, PerCP anti-human CD8α, fluorescein isothiocyanate (FITC)-labeled anti-mouse Vβ 8.3, anti-mouse Vβ8.1-8.2, anti-mouse Vβ6, anti-mouse Vβ4, and the Aqua fluorescent reactive dye (Life Technologies) were used for staining of the TCRs 12 to 16 h after electroporation. Intracellular cytokine staining after antigen stimulation was performed as previously described with Aqua fluorescent reactive

dye, APC Cy7 anti-human CD8, PerCP Cy5.5 anti-human CD107 α ; cells were fixed with Cytofix and Cytoperm (BD Biosciences, CA), stained with Pacific blue anti-human CD3, PE anti-human IFN- γ , APC anti-human IL-2, and PE-Cy7 anti-human TNF- α , and run on the FACS Verse flow cytometer (BD Biosciences, CA). FlowJo version V10.07 software was used for data analysis. All antibodies were purchased from BioLegend, CA, or BD Biosciences, CA. HCV peptides NS31073-1081 gt1a (CINGVCWTV) and NS51992-2000 gt1b (VLTDFTKWL) were synthesized by EZBiolab (Carmel, IN).

Functional analysis of T cells cocultured with T2 cells or HCV replicon cells. On day 1 posttransfection, T cells were cocultured with HCV peptide-loaded T2 cells, Huh7^{A2} HCV^{Rep} cells, or Huh7 HCV^{Rep} cells expressing the endogenous HCV CTL epitopes at the indicated E/T ratios. FACS analysis was done after 5 h of coculture and ICS as described above. Cytokine enzyme-linked immunosorbent assay (ELISA) was done by quantifying IFN- γ and IL-2 in the overnight (16 to 18 h) supernatant (Mabtech, Nacka Strand, Sweden).

HCV antiviral assay and AST measurement. On day 1 posttransfection, T cells were cocultured with Huh7^{A2} HCV^{Rep} cells or Huh7 HCV^{Rep} cells in 96-well tissue culture plates as described earlier (21). After 24 h of coculture, the luciferase activity in target cells was measured using the ONE-Glo luciferase assay system (Promega, Madison, WI). Data are presented as the percentage of HCV replication in Huh7^{A2} HCV^{Rep} cells or Huh7 HCV^{Rep} cells coincubated with T cells by measurement of relative light unit (RLU) values compared to RLU values in Huh7^{A2} HCV^{Rep} cells alone or Huh7 HCV^{Rep} cells alone (without coculture). Background values of T cell control wells were subtracted (10 to 20 RLU). Aspartate aminotransferase (AST) levels in supernatants were quantitated by a validated AST assay at the Clinical Chemistry Laboratory at the Karolinska University Hospital, Huddinge, Sweden, using the Modular P apparatus (Roche Diagnostics, Mannheim, Germany).

Cell purification, RNA isolation, and library preparation for sequencing. TCR-redirected or mock-treated T cells cocultured with Huh7^{A2} HCV^{Rep} cells at an E/T ratio of 10:1 were collected at the indicated times and enriched with anti-CD8 MicroBeads (Miltenyi Biotech) as per the manufacturer's instructions. Enriched CD8⁺ T cells (purity, 98% CD3⁺ CD8⁺) at 1.5 million cells per sample were stored at -80°C . Total RNA was isolated with TRIzol reagent (Thermo Fisher Scientific, MA, USA), and RNase-free reagents were used throughout the isolation. Sample quantity and purity were assessed using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, MA, USA). All RNA samples had RNA integrity number (RIN) values of >9 . The RNA libraries were bar coded and prepared according to the protocol Illumina TruSeq RNA Sample Prep kit v2 (catalog number RS-122-2001) from the manufacturer (Illumina, San Diego, CA, USA).

Paired-end sequencing and sequence alignment. The nine RNA libraries were sequenced in paired ends on an Illumina HiSeq 2000 instrument, where each sample was put in two different lanes, for a total of 18 samples. The runs generated a total of 384 million reads with an average of 43 million paired-end reads per sample, of which 90% mapped to the human genome. The paired-end sequences were aligned to the human genome reference hg19, with TopHat2 version 0.9 using TopHat standard parameters. The numbers of aligned reads per gene were determined with HTSeq 0.6.1. Annotations from Ensembl Homo sapiens grch37.82 and Genome Browser were used to assign features to genomic positions. The R/Bioconductor package DESeq2 was used for differential gene expression on read counts generated by HTSeq 0.6.1.

Functional analysis of gene lists using DAVID and WebGestalt. The default parameters of the Database for Annotation, Visualization and Integrated Discovery (DAVID) were used to achieve GO analysis. *Homo sapiens* was used as background, and the "GOTERM_BP_FAT" option was selected with the highest classification stringency and *P* values of <0.05 . KEGG pathway analysis was performed using the Web-based Gene Set Analysis Toolkit v3 (WebGestalt) with the following parameters: ID type, Ensembl_gene_stable; reference set, *Homo sapiens*; statistical method, hypergeometric; multiple test adjustment, Benjamini & Hochberg (BH); significance level, $P < 0.01$; minimum number of genes for each category, 2.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.00010-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLS file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLS file, 2.6 MB.

SUPPLEMENTAL FILE 4, XLS file, 5.1 MB.

SUPPLEMENTAL FILE 5, XLS file, 0.1 MB.

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We declare that no conflict of interest exists.

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