

Analysis of CD8⁺ T-Cell-Mediated Inhibition of Hepatitis C Virus Replication Using a Novel Immunological Model

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Background & Aims: Virus-specific CD8⁺ T cells are required for the control of hepatitis C virus (HCV) infection. We investigated the extent to which different effector functions of CD8⁺ T cells contribute to the inhibition of viral replication. **Methods:** We developed a novel immunologic model by stably transducing the HLA-A2 gene into the replicon system, matching the epitope sequence of the replicon to the sequence targeted by an HCV-specific CD8⁺ T-cell clone. Luciferase activity was then measured to quantitate HCV RNA replication. **Results:** HCV-specific CD8⁺ T cells strongly inhibited viral replication, through cytolytic and noncytolytic mechanisms, in a dose-dependent manner. HCV replication was almost completely inhibited at an effector-to-target ratio of 1:1 with significant cytotoxicity; however, >95% viral inhibition occurred at ratios as low as 1:100. Importantly, no cytotoxicity was observed at low effector-to-target ratios, indicating a dominant effect of noncytolytic effector functions that was confirmed by Transwell experiments. Neutralization experiments revealed that interferon gamma mediates the noncytolytic inhibition. **Conclusions:** Only a very few HCV-specific CD8⁺ T cells are required to inhibit HCV replication; inhibition occurs primarily by noncytolytic effector functions.

Hepatitis C virus (HCV) is a noncytopathic virus that causes acute and chronic hepatitis and hepatocellular carcinoma.¹ Major histocompatibility complex (MHC) class I-restricted CD8⁺ T cells play a key role in the control of HCV infection.² Indeed, in acutely infected patients and chimpanzees, viral clearance is associated with the appearance of HCV-specific CD8⁺ T cells in peripheral blood and liver.^{3,4} Further, depletion studies of CD8⁺ T cells in chimpanzees revealed that virus-specific CD8⁺ T cells are the main effector cells in controlling HCV replication⁵ and in particular MHC class I alleles were shown to be associated with resolution of HCV infection.^{6,7}

Upon T-cell receptor engagement with peptide/MHC class I complexes, CD8⁺ T cells massively expand and

express multiple effector functions, including cytotoxicity mediated by perforin-granzyme and Fas/FasL pathways and the production of cytokines (eg, interferon [IFN]- γ). Of note, studies in humans and chimpanzees indicated the presence of both effector functions during acute HCV infection. For example, several studies have shown a coincidence between the appearance of virus-specific CD8⁺ T-cell responses in the peripheral blood and liver and the onset of liver disease indicating cytolytic effector functions.^{3,4} The contribution of noncytolytic IFN- γ -induced antiviral effects to the natural course of HCV infection is supported by at least 2 findings. First, during acute HCV infection, virus-specific CD8⁺ T cells switch their antiviral functions from killing to IFN- γ production, coinciding precisely with a 5-log decrease in viremia.⁴ Second, viral clearance in acutely infected chimpanzees can occur in the absence of elevated alanine aminotransferase levels with only minimal histologic evidence of liver cell injury but with detectable IFN- γ messenger RNA in the liver.^{3,8,9} The hypothesis that noncytolytic mechanisms contribute to control of HCV infection has also been supported by studies using replicon systems.^{10,11} For example, IFN- γ has been shown to inhibit replication of subgenomic and genomic HCV RNA.¹² In addition, Liu et al showed that activated HCV-specific CD8⁺ T cells can inhibit viral replication by both cytokine-mediated and direct cytolytic effects.¹³ Indeed, in that study, HCV-specific expansion of CD8⁺ T cells led to a 10-fold decrease in HCV replication and 21% specific lysis. The antiviral effect was quite weak, however, presumably reflecting the low MHC class I expression that is typical for Huh7 hepatoma cells.¹⁴ While the MHC class I expression on these cells can be induced with IFN- γ , this is not feasible for immunologic studies because IFN- α also directly inhibits HCV replication^{10,15} and pro-

Abbreviations used in this paper: anti-IFN- γ , monoclonal anti-human interferon gamma antibody; E/T ratio, effector-to-target ratio; IFN, interferon; MHC, major histocompatibility complex; reIFN- γ , recombinant human interferon gamma.

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fects hepatocytes from CD8⁺ T cell-mediated killing.¹⁶ Currently, little is known about the relative importance of cytolytic versus noncytolytic effector functions and their dose-dependent impact on suppression of HCV replication. Further, the current cellular assays actually do not measure antiviral efficacy, that is, the ability to inhibit viral replication. To overcome this limitation and to determine the relative contribution of cytolytic versus noncytolytic effector functions, we established an in vitro model that allows measurement of the antiviral efficacy of CD8⁺ T cells. Our results show that HCV-specific CD8⁺ T cells strongly inhibit HCV replication in a dose-dependent manner by cytolytic and IFN- γ -mediated noncytolytic effector mechanisms.

Methods

Cell Culture

All Huh7 cells are based on Huh7-Lunet,¹⁷ which were transduced with the blasticidin resistance gene (Huh7_{BLR}), the HLA-A2 gene (Huh7_{A2}), or a JFH1-based selectable subgenomic luciferase replicon (Huh7HCV). Huh7HCV cells were transduced with HLA-A2 gene (Huh7_{A2}HCV) or a mutated epitope (Huh7HCV_{EM}). Further, Huh7_{A2}HCV cells were transduced with a mutated epitope (Huh7_{A2}HCV_{EM}). The mutation in the NS5B₂₅₉₄₋₂₆₀₂ epitope was introduced to match the epitope of HCV genotype 1a that is targeted by an HCV NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T-cell clone. Cells were grown in Dulbecco's modified Eagle medium high glucose (4.5 g/L) with stable glutamine (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal calf serum (Pan-Biotech, Aidenbach, Germany), nonessential amino acids (Biochrom, Berlin, Germany), and 1% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany). For continuous passage, the culture medium was supplemented with 1 mg/mL G418 (PAA Laboratories GmbH) and/or 3 μ g/mL blasticidin S hydrochloride (Carl Roth GmbH + Co, Karlsruhe, Germany).

Plasmids

All genes were cloned into pWPI-BLR, a derivative of the bicistronic lentiviral vector pWPI (a gift from Didier Trono) generated by modification of the MCS and replacing the GFP in the reporter cistron by blasticidin resistant gene (BLR). pWPI-HLA-A2-BLR was generated by transfer of an *Xba*I-*Not*I fragment containing the entire HLA-A2 complementary DNA from pcDNA3.1-HLA-A2¹⁸ into the MCS of pWPI-BLR. pFKI389Luc-ubi-neo/NS3-3'_{dg}JFH was generated by insertion of an *Eco*RI-*Hind*III fragment containing part of the luciferase gene, the entire ubiquitin and neo-coding sequences, and part of the EMCV-IRES from pFI389luc-ubi-neo/NS3-3'/5.1¹² into pFKI389LucNS3-3'_{dg}JFH.¹⁹ This plasmid contains the T7 promoter sequence fused to nucleotides 1-389 of the JFH-1 consensus sequence, followed by a

gene encoding a firefly luciferase-ubiquitin-neomycin phosphotransferase fusion protein, the encephalomyocarditis virus (EMCV) IRES, the NS3-to-NS5B coding sequence, the 3' NTR of JFH-1, the hepatitis delta virus genomic ribozyme (dg), and the T7 terminator sequence and is referred to as HCV wild-type. The epitope-matched mutated sequence, referred to as HCV_{EM}, was generated by replacing 3 amino acids within NS5B (ITQ by VVT) using oligonucleotide A_ITQ2620VVT_JFH (agggaagctttgttacaactgcatagaggccattttc) to create plasmid pFKI389Luc-ubi-neo/NS3-3'_{dg}JFH/B22.

Transduction of Cells With Lentiviral Vectors

Lentiviral particles were prepared in 293T cells exactly as described previously²⁰ using pWPI-HLA-A2-BLR or pWPI-BLR and packaging constructs pCMVR8.91 and pMD.G (provided by Didier Trono).

In Vitro Transcription, Electroporation, and Selection of Replicon Cells

In vitro transcription of replicon RNAs, electroporation of Huh7 cells, and transient replication assays were essentially performed as described.²¹

CD8⁺ T-Cell Lines and Clones

Blood samples were obtained from 2 HLA-A2-positive patients chronically infected with HCV genotype 1 after informed consent was obtained and in agreement with the 1975 Declaration of Helsinki, federal guidelines, and the local ethics committee. Peripheral blood mononuclear cells were isolated from EDTA anticoagulated blood samples through Pancoll density gradient (Pan-Biotech) by centrifugation. One patient displayed a strong response against the HLA-A2-restricted NS5B₂₅₉₄₋₂₆₀₂ epitope and another patient against the HLA-A2-restricted NS3₁₄₀₆₋₁₄₁₅ epitope. HCV-specific CD8⁺ T-cell clones were prepared by selection of allophycocyanin-labeled tetramer-positive cells by anti-allophycocyanin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Selected cells were plated at different cell numbers in 96-well plates together with irradiated autologous feeder cells and human recombinant interleukin-2 (100 U/mL) and expanded for several weeks before testing for HCV specificity. Established clones were supplemented twice a week with 100 U/mL interleukin-2 and were grown in RPMI 1640 and supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, and 1.5% HEPES buffer (1 mol/L) (Biochrom, Berlin, Germany). Fluorescence-activated cell sorter analysis was performed on a BD FACSCanto II flow cytometer using FlowJo software (Tree Star, Ashland, OR).

Antibodies and Cytokines

Mouse immunoglobulin G1 pure, anti-HLA-A2-FITC, anti-CD8-PE, anti-CD8-PerCP, anti-IFN- γ -FITC, and anti-CD107a-PE were obtained from Becton Dick-

inson (Heidelberg, Germany). Recombinant human IFN- γ (recIFN- γ) and monoclonal anti-human IFN- γ antibody (anti-IFN- γ) (clone 25723) were obtained from BIOMOL (Hamburg, Germany) and R&D Systems (Wiesbaden, Germany), respectively.

Degranulation CD107a Marker and Intracellular IFN- γ Staining

CD107a staining was conducted as described,²² and IFN- γ staining was conducted as described.³ Fluorescence-activated cell sorter analysis was performed on a BD FACSCanto II flow cytometer using FlowJo software.

Luciferase Assay

The assay was performed using the Steady-Glo Luciferase Assay System (Promega, Mannheim, Germany) and measured with Lumat LB 9507 (Berthold, Bad Wildbad, Germany).

IFN- γ Enzyme-Linked Immunosorbent Assay

The assay was performed in duplicate according to the technical manual (DuoSet ELISA Development System; R&D Systems). Concentration of each sample was determined through GraphPad Prism version 4 (La Jolla, CA).

⁵¹Cr Release Assay

The cytolytic activity of NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells on Huh7_{A2}HCV_{EM} or Huh7HCV_{EM} cells was determined in a 9-hour ⁵¹Cr release assay as described.²³

Direct Coculture and Transwell Assay

A total of 1×10^5 replicon cells were cocultured with the NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells together in one well or separated through a microporous membrane with 0.4- μ m pore size (Transwell; Corning Schiphol-Rijk, The Netherlands) in 24-well plates at different effector-to-target (E/T) ratios in a total of 600 μ L of medium. After 24 and 48 hours of coculture, media was collected for the measurement of aspartate aminotransferase (AST) and IFN- γ levels and the luciferase assay was performed as described. During transmembrane assays, 1×10^5 Huh7_{A2}HCV_{EM} cells were plated in 24-well plates (outer chamber) and overlaid with the semipermeable membrane. Within the insert (inner chamber), different amounts of virus-specific CD8⁺ T cells were stimulated with 1×10^5 Huh7_{A2}HCV_{EM} cells. After 24 and 48 hours, media was removed from the outer chamber for the respective analyses. Direct coculture was also performed using NS3₁₄₀₆₋₁₄₁₅-specific CD8⁺ T-cell clone, in which 1×10^5 Huh7_{A2}HCV cells were loaded with 10 μ mol/L NS3₁₄₀₆₋₁₄₁₅ peptide for 1 hour before being cocultured at different E/T ratios for 24 and 48 hours.

Results

Generation of Stably HLA-A2-Transduced Replicon Cells

Human hepatoma cells harboring subgenomic selectable replicons maintain constant levels of HCV replication over years and represent an excellent model of persistent HCV infection.¹¹ Replicon-containing Huh7 cells expressed HLA-A11, -B54, and -B55 (data not shown) at low levels. Therefore, we decided to generate a system on the basis of the common HLA allele HLA-A2. Huh7 cells were transduced with lentiviral vectors expressing a complementary DNA of HLA-A2¹⁸ and a selectable marker conferring blasticidin resistance (BLR) controlled by the constitutive cellular EF1 α promoter. The resulting cell line, designated Huh7_{A2} (Figure 1A), showed a strong and homogeneous HLA-A2 expression. Transient transfection of these cells with a replicon-encoding luciferase (Figure 1B) revealed similar levels of HCV replication, indicating that HLA-A2 expression did not affect viral replication (Figure 1C). Because the amino acid sequence critically involved in recognition of the CD8⁺ T-cell clone differed from our HCV isolates, it was to be expected that the corresponding antigenic peptides might not be recognized efficiently. Therefore, we generated a replicon with a mutated epitope, designated HCV_{EM}, by changing 3 amino acids in the epitope (ITQ \rightarrow VVT). Because these alterations are located close to the active center of the HCV polymerase, their effects on replication fitness of the replicon were analyzed as shown in Figure 1D. HCV_{EM} cells showed similar levels of viral replication as the wild-type replicon cells.

Because HLA-A2 expression and epitope match did not alter the general properties of the Huh7 cells and the replicon, respectively, a panel of Huh7-, Huh7_{A2}- and Huh7_{BLR}-based cell lines harboring persistent replicons with or without epitope match were generated. All cell lines expressed comparable amounts of luciferase (data not shown). The luciferase activity was fully dependent on HCV RNA replication and clearly correlated with intracellular levels of viral RNA and antigens, as shown by a drastic decrease in positive-strand HCV RNA, NS3 protein, and luciferase activity after IFN- α treatment of Huh7_{A2}HCV_{EM} cells (Supplementary Figure 1). Due to the very short half-life of the luciferase protein, measurement of luciferase activity is the most sensitive, quantitative, and reliable way to monitor changes in HCV replication in this system and was therefore used throughout the study.

Specific Recognition by NS5B₂₅₉₄₋₂₆₀₂-Specific CD8⁺ T-Cell Clone

We analyzed the ability of an NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T-cell clone to recognize the newly established replicon cell lines. As shown in Figure 2A, only

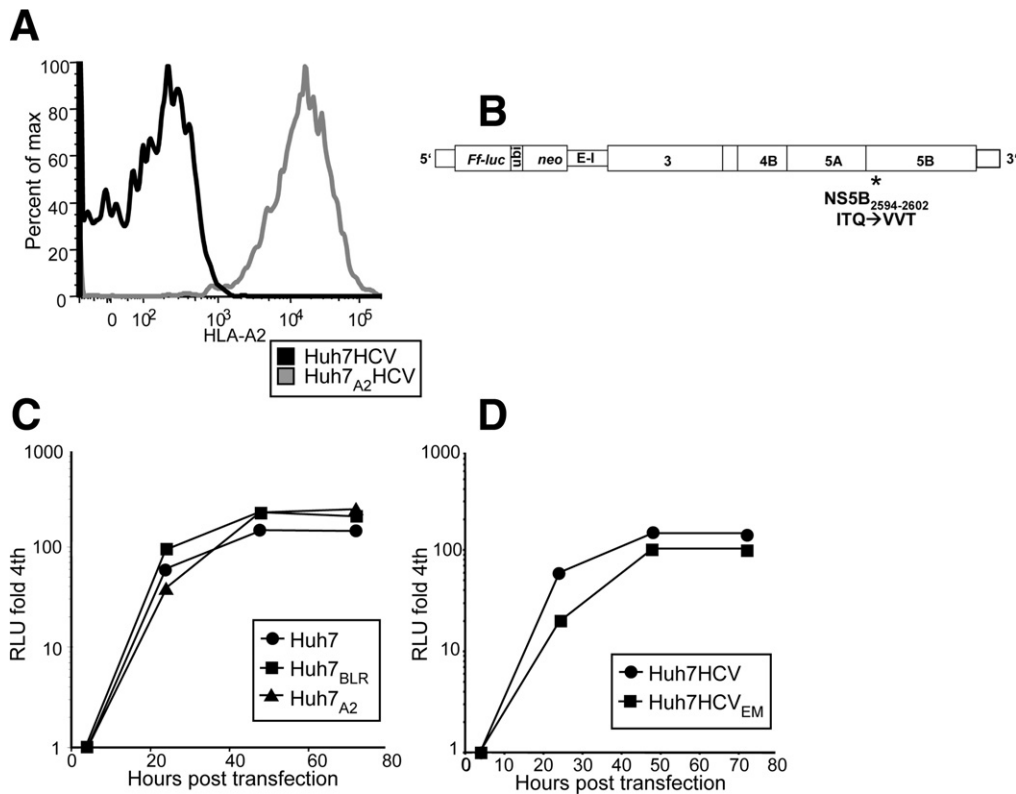


Figure 1. Generation of stably transduced HLA-A2 replicon cells. (A) HLA-A2 expression on Huh7 cells stably transduced with a lentiviral vector. (B) Structure of the subgenomic luc/neo replicon used in this study. The replicon is based on the HCV isolate JFH1 and contains a firefly-luciferase gene (Ff-luc) as well as a selectable marker (neo), conferring resistance to G418, both linked by ubiquitin (ubi). E-1, EMCV-IRES. (C) HCV wild-type replicon RNA was electroporated into Huh7, Huh7_{BLR}, or Huh7_{A2} cells. Luciferase activity was measured in cell lysates 4, 24, 48, and 72 hours after transfection. (D) Huh7 cells were electroporated with the epitope-matched (HCV_{EM}) or parental replicon (HCV). Luciferase activity was determined at the indicated time points. Luciferase activity is given as increase relative to 4 hours after transfection (C and D).

Huh7_{A2}HCV_{EM} cells induced significant IFN- γ production and CD107a mobilization. By comparison, Huh7_{A2}HCV or Huh7HCV_{EM} cells did not induce IFN- γ production and CD107a mobilization. As shown in Figure 2B, antigen-specific induction of CD107a and IFN- γ by NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells was dependent on the ratio of Huh7_{A2}HCV_{EM} cells and was still detectable at an E/T ratio of 100:1. Finally, we tested the ability of NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells to lyse Huh7_{A2}HCV_{EM} cells in a ⁵¹Cr release assay. As shown in Figure 2C, specific killing was dose dependent and still significant at an E/T ratio of 1:1 but not below. Parallel analysis of Huh7_{A2}HCV_{EM} and NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells revealed that AST was released from the replicon cells and not from the T cells (data not shown) and that the number of lysed replicon cells directly correlates with the AST level (Figure 2D). For example, an AST level of 100 U corresponds to the lysis of about 100,000 replicon cells and an AST level of 10 U to 10,000 cells.

Inhibition by NS5B₂₅₉₄₋₂₆₀₂-Specific CD8⁺ T Cells Is HLA Restricted

The inhibitory effect by NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells on HCV replication was analyzed by coculturing lymphocytes and the respective replicon cells at different E/T ratios and was determined by the luciferase assay after 24 and 48 hours of coculture. A strong inhibition of HCV replication in Huh7_{A2}HCV_{EM} cells was observed at both time points (Figure 3A). Indeed, after 48 hours, replication was inhibited by >3 logs at E/T ratios

of 1:1 and 1:10 and still by >2 logs at an E/T ratio of 1:100 and >1 log at an E/T ratio of 1:1000. AST levels were elevated at an E/T ratio of 1:1 after 24 and 48 hours, indicating T-cell-induced killing of replicon cells. Interestingly, AST levels were <10 U/L at E/T ratios of 1:10 and 1:100, respectively, while there was still a strong inhibition of HCV replication of >95%. As shown in Figure 2D, an AST level <10 U corresponds to the killing of <10% of Huh7_{A2}HCV_{EM} cells, indicating that inhibition of HCV replication by >95% is associated with killing of <10% of cells. This observation is in agreement with the results from the ⁵¹Cr release assay (Figure 2C), also showing no specific killing at low E/T ratios. These findings, therefore, suggest that nonkilling mechanisms contribute to the strong CD8⁺ T-cell-mediated inhibition of HCV replication.

An inhibition of HCV replication by about 1 log was also observed when Huh7_{A2}HCV cells were cocultured with the NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells, but only at an E/T ratio of 1:1 (Figure 3B). These results most likely reflect an unspecific activation of CD8⁺ T cells in the presence of a high number of replicon cells. The interpretation is supported by the finding that a minor inhibition of HCV replication was also observed after coculture with Huh7HCV_{EM} or Huh7HCV cells at an E/T ratio of 1:1, but not below (Figure 3C and D), and by the fact that we did not find any cross-recognition of the NS5B₂₅₉₄₋₂₆₀₂(ITQ) peptide by the NS5B₂₅₉₄₋₂₆₀₂(VVT)-specific CD8⁺ T cells (Supplementary Figure 2).

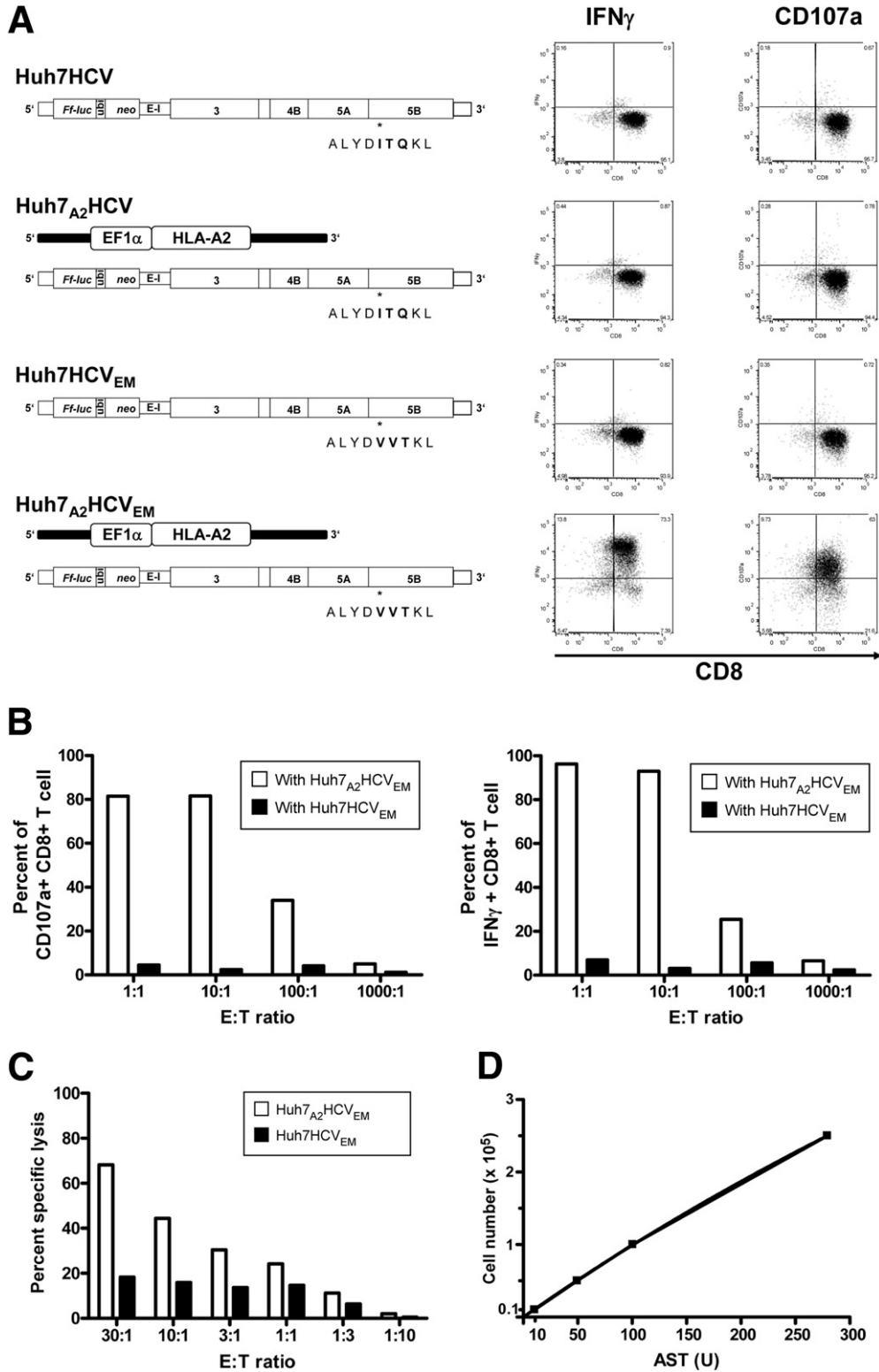


Figure 2. Huh7_{A2}HCV_{EM} cells are specifically recognized by an NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T-cell clone. (A) Comparison of NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T-cell IFN- α production and CD107a mobilization in response to stimulation with various replicon cell lines. The original or mutated epitope is indicated by an asterisk. (B) CD107a mobilization (upper graph) and IFN- α production (lower graph) of NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells after coculture with Huh7_{A2}HCV_{EM} and Huh7HCV_{EM} at different E/T ratios. (C) The cytolytic activity of NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells was determined on Huh7_{A2}HCV_{EM} and Huh7HCV_{EM} cells by ⁵¹Cr release assay. (D) Correlation between AST levels and the number of lysed Huh7_{A2}HCV_{EM} cells.

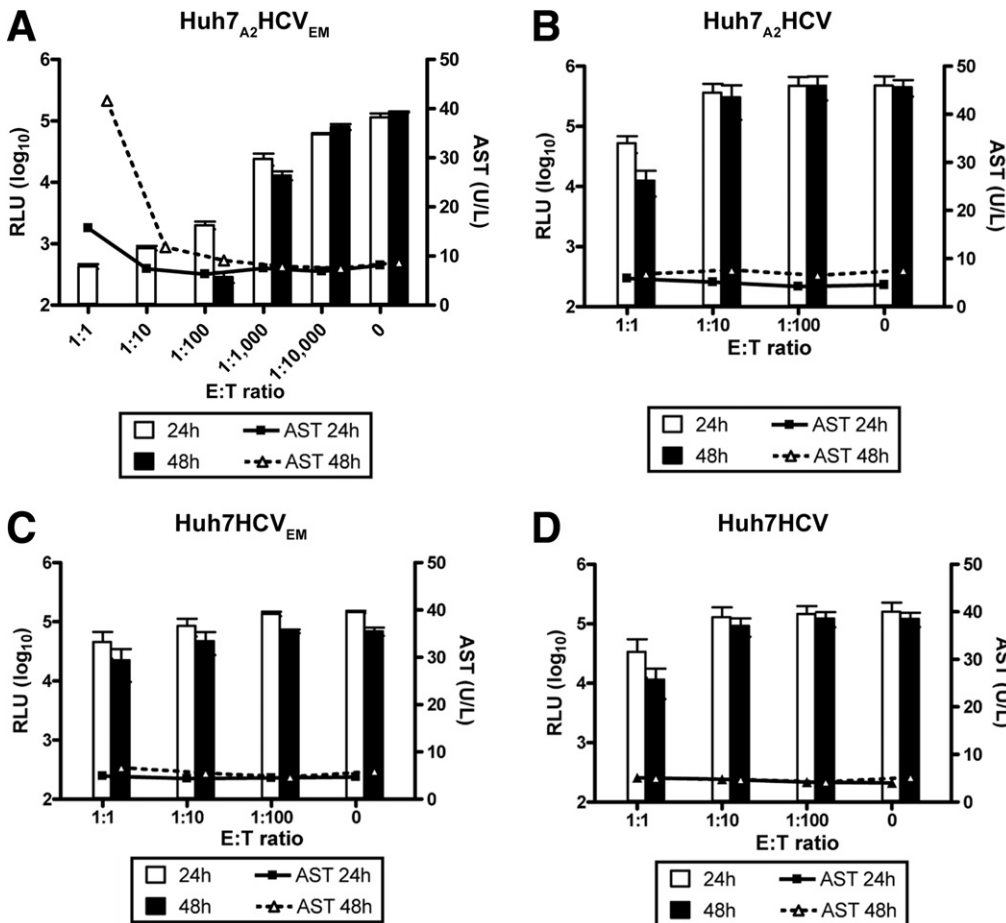


Figure 3. HLA-restricted antiviral effect of NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells. NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells were cocultured for 24 (*white bars*) or 48 hours (*black bars*) with (A) Huh7_{A2}HCV_{EM}, (B) Huh7_{A2}HCV, (C) Huh7HCV_{EM}, or (D) Huh7HCV cells at different E/T ratios. Inhibition of viral replication was measured by luciferase activity. The results are from 3 independent experiments (means ± SEM). The cutoff for detection of HCV replication by the luciferase assay is 10²; therefore, the logarithmic scale starts at 2. AST levels are presented for each coculture (24 hours, *solid line*; 48 hours, *dashed line*).

Inhibition of HCV Replication Without Cell Lysis

Our findings so far indicated that soluble factors secreted by virus-specific CD8⁺ T cells contribute to the strong inhibition of HCV replication, especially at an E/T ratio of 1:10 at which there is no detectable cell lysis. Because killing requires direct cell-cell contact, we addressed this issue by coculturing antigen-specific activated NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells with Huh7_{A2}HCV_{EM} cells either directly or in the Transwell system. As shown in Figure 4, a significant inhibition of HCV replication was observed under both experimental conditions. In the Transwell system, however, at a given E/T ratio, the inhibition of HCV replication was weaker compared with the direct coculture. Taken together, the findings further support the hypothesis that soluble factors secreted by antigen-specific activated NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells significantly contribute to the noncytolytic inhibition.

Noncytolytic Inhibition Is Mediated by IFN-γ

To determine the role of IFN-γ in our system, we incubated Huh7_{A2}HCV_{EM} cells with different concentrations of recIFN-γ for 48 hours. Figure 5A shows that recIFN-γ blocks HCV replication in a dose-dependent

manner with an almost 3-log inhibition at concentrations of 500 and 1000 U/mL, respectively. Even at these high concentrations of recIFN-γ, inhibition of HCV replication was not as strong as that mediated by virus-specific CD8⁺ T cells at E/T ratios of 1:1 and 1:10 in direct coculture (Figure 4). As shown in Figure 5B, the antiviral effect of recIFN-γ can be blocked by anti-IFN-γ up to a concentration of 100 U/mL. To compare the amount of recIFN-γ with the amount secreted by acti-

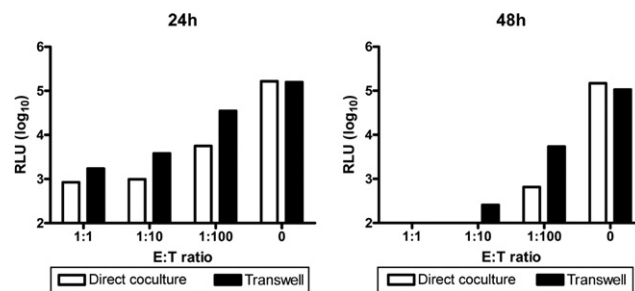
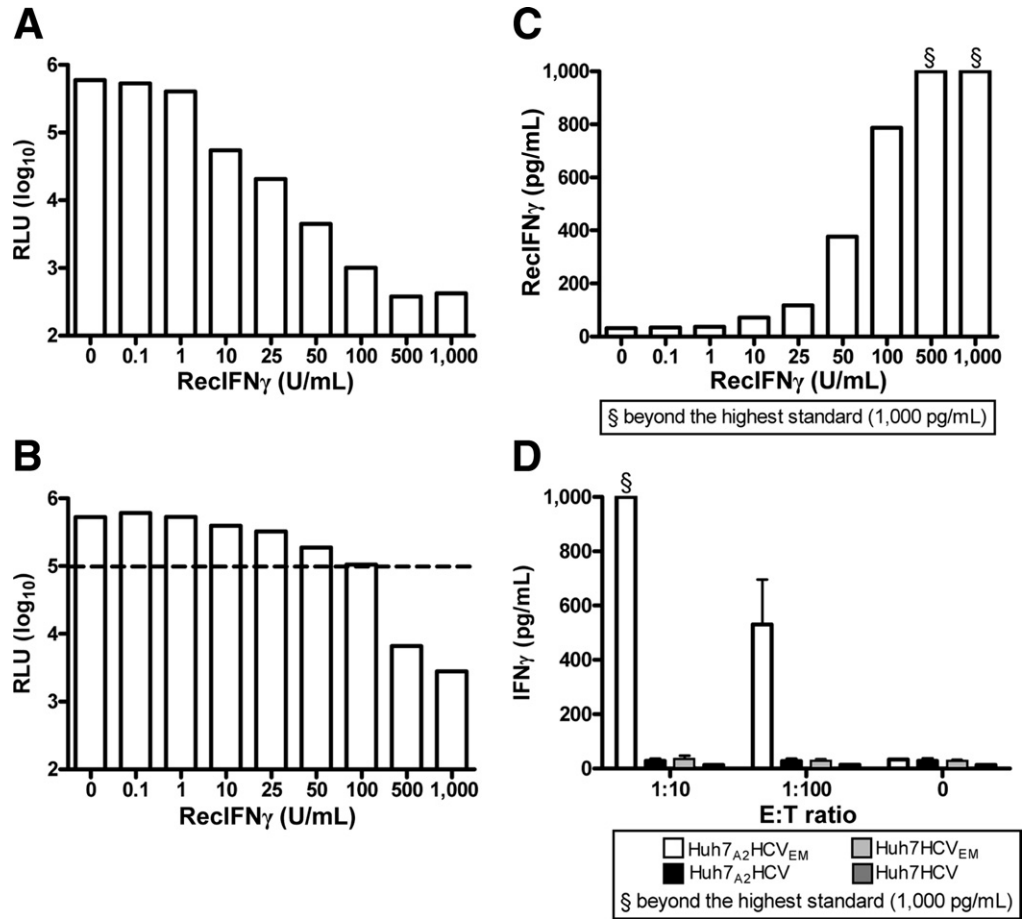


Figure 4. Inhibition of HCV replication by noncytolytic mechanisms. NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells were cocultured with Huh7_{A2}HCV_{EM} cells, either in direct coculture or in the Transwell system, at E/T ratios of 1:1, 1:10, 1:100, and 0 for (*left*) 24 or (*right*) 48 hours. Inhibition of HCV replication was determined by the luciferase assay.

Figure 5. IFN- γ mediates the noncytolytic inhibition on HCV replication. (A) The effect of recIFN- γ on HCV replication in Huh7_{A2}HCV_{EM} cells for 48 hours was measured by the luciferase assay. (B) The effect of 10 μ g/mL anti-IFN- γ and different concentrations of recIFN- γ on HCV replication in Huh7_{A2}HCV_{EM} cells for 48 hours was measured by the luciferase assay. The dashed line indicates the cutoff value of antiviral blockage by anti-IFN- γ . (C) Different amounts of recIFN- γ were added into 1 mL of medium. Subsequently, the concentration of IFN- γ was determined by enzyme-linked immunosorbent assay. (D) Concentration of secreted IFN- γ by NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells after direct coculture with various replicon cell lines at E/T ratios of 1:10, 1:100, and 0 for 48 hours, based on enzyme-linked immunosorbent assay. The results are from 2 independent experiments (means \pm SEM).



vated CD8⁺ T cells, we analyzed the concentration of IFN- γ in media mixed with recIFN- γ (Figure 5C) and in supernatants from NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells cocultured with different replicon cells (Figure 5D). Interestingly, IFN- γ level above the highest standard was observed at E/T ratios of 1:1 and 1:10 and only after cocultured with Huh7_{A2}HCV_{EM} cells (data not shown and Figure 5D, respectively), demonstrating the antigen-specific induction of this cytokine. Based on a titration experiment, the amount of IFN- γ at the E/T ratio of 1:10 was approximately 10,800 pg/mL. At an E/T ratio of 1:100, the amount of IFN- γ was 530 pg/mL and thus equivalent to the concentration of 50–100 U/mL of recIFN- γ . Because anti-IFN- γ is able to block the antiviral efficacy of IFN- γ at these concentrations (Figure 5B), we performed neutralization assays at an E/T ratio of 1:100. First, we analyzed the inhibitory activity of supernatant obtained after antigen-specific stimulation of NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells at an E/T ratio of 1:100. As expected, the supernatant leads to an inhibition of HCV replication by >1 log. Importantly, anti-IFN- γ ablated the CD8⁺ T-cell-mediated inhibitory activity (Figure 6A), indicating that IFN- γ is required by the noncytolytic activity of virus-specific CD8⁺ T cells. Importantly, neutralization experiments in the direct cocul-

ture system demonstrated a strong (>1 log) but not complete ablation of the antiviral effect (Figure 6B). The remaining effect may be due to some degree of cell killing or the inability to neutralize all IFN- γ activity or other antiviral cytokine(s). Of note, we were unable to neutralize the antiviral effect by blocking other cytokines/factors secreted by CD8⁺ T cells, that is, tumor necrosis factor α , transforming growth factor β , or FasL (Supplementary Figure 3).

To further define the relative contribution of cytolytic and noncytolytic antiviral activities to the inhibition of HCV replication, we mixed Huh7_{A2}HCV_{EM} and Huh7HCV_{EM} cells before coculture with the NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells at an E/T ratio of 1:10. As shown in Figure 3C, HCV replication in Huh7HCV_{EM} cells was not inhibited at this ratio. By comparison, NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells strongly inhibit HCV replication in mixed cultures of Huh7_{A2}HCV_{EM} and Huh7HCV_{EM} cells (100:0 until 1:99) (Figure 7). This profound effect was completely lost, however, when no Huh7_{A2}HCV_{EM} cells were present in the culture (0:100). These results clearly show that the degree of inhibition of HCV replication is largely independent of the number of HLA-A2-matched replicon cells and therefore is HLA unrestricted and noncytolytic. However, a small fraction of HLA-matched an-

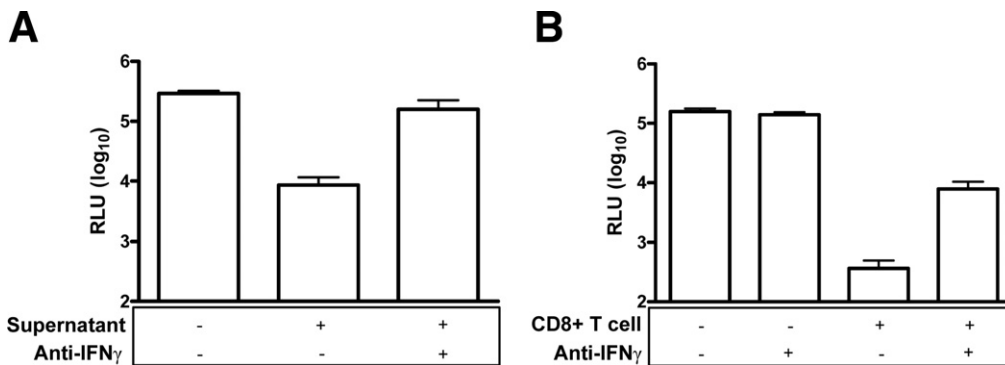


Figure 6. Neutralization of secreted IFN- γ . (A) Supernatant from direct coculture of Huh7_{A2}HCV_{EM} and NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells at an E/T ratio of 1:100 was collected and incubated with 10 μ g/mL anti-IFN- γ for 1 hour. Subsequently, the supernatant was transferred into wells containing only Huh7_{A2}HCV_{EM} cells for 48 hours. Inhibition of HCV replication was determined by the luciferase assay. The results are from 2 independent experiments (means \pm SEM). (B) Huh7_{A2}HCV_{EM} cells were incubated with 10 μ g/mL anti-IFN- γ for 1 hour and then were directly cocultured with NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells at an E/T ratio of 1:100 for 48 hours. The viral inhibition was measured by the luciferase assay. The results are from 2 independent experiments (means \pm SEM).

tigen-presenting cells (1%) is needed to stimulate sufficient IFN- γ production of virus-specific CD8⁺ T cells. These results indicate that the antiviral effect of virus-specific CD8⁺ T cells is class I restricted, leading to the release of IFN- γ that inhibits HCV replication, largely non-class I restricted. The strong induction of IFN- γ confirms our finding that 1% of Huh7_{A2}HCV_{EM} cells still induce IFN- γ production by almost 40% of specific CD8⁺ T cells (Figure 2B). Similarly, enzyme-linked immunosor-

bent assay analysis revealed that the amount of IFN- γ produced by these cultures was higher than the highest standard (data not shown). It is also important to note that no significant AST levels were found in culture supernatants, arguing against a significant direct or bystander cell killing.²⁴

Discussion

The current study was designed to address the mechanisms of CD8⁺ T-cell-mediated inhibition of HCV replication. Based on the replicon system, we developed a novel immunologic model to study CD8⁺ T-cell function by (1) stably transducing the HLA-A2 gene, (2) matching the epitope sequence of the replicon to the sequence targeted by an HCV-specific CD8⁺ T-cell clone, and (3) using luciferase activity to quantitate HCV RNA replication.

One important finding of our study is that HCV-specific CD8⁺ T cells exert strong antiviral effects through 2 distinct mechanisms. The first requires direct cell-cell contact and results in cytolysis, and the second is noncytolytic inhibition mediated by IFN- γ . Our data clearly show that the inhibition of HCV replication by the virus-specific CD8⁺ T cells does not represent the destruction of HCV-replicating hepatocytes only but also involves noncytolytic mechanisms. This conclusion is supported by several findings. First, a strong inhibition of HCV replication by >95% is observed in the presence of mild (E/T ratio of 1:1) or even undetectable AST elevations (E/T ratios of 1:10 and 1:100). Second, inhibition of HCV replication is detectable also in the Transwell system, excluding cell-cell contact-dependent cytolysis. Third, administration of anti-IFN- γ significantly reduces the inhibition of HCV replication by virus-specific CD8⁺ T cells. Fourth, virus-specific CD8⁺ T cells suppress HCV replication equally well in HLA-matched and -mismatched cells as long as the CD8⁺ T cells are antigen-specific activated in an HLA-restricted fashion.

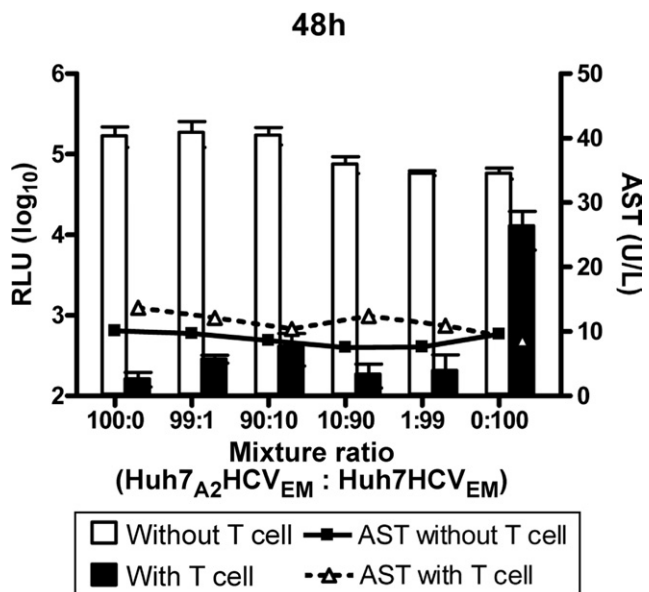


Figure 7. NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells significantly inhibit HCV replication in mixed cultures of Huh7_{A2}HCV_{EM} and Huh7HCV_{EM} cells. Mixed cultures of Huh7_{A2}HCV_{EM} and Huh7HCV_{EM} cells were prepared at different ratios (100:0, 99:1, 90:10, 10:90, 1:99, and 0:100, respectively) and were subsequently cocultured with NS5B₂₅₉₄₋₂₆₀₂-specific CD8⁺ T cells at an E/T ratio of 1:10 for 48 hours. Viral inhibition was measured by the luciferase assay. The results are from 2 independent experiments (means \pm SEM). The AST levels are presented for each coculture (without T cell, solid line; with T cell, dashed line).

The important role of the noncytolytic IFN- γ -mediated inhibition of HCV replication is supported by studies in humans and chimpanzees acutely and chronically infected with HCV. Indeed, during acute HCV infection, the first decline of HCV RNA correlates precisely with the initiation of IFN- γ production of virus-specific CD8⁺ T cells in humans,⁴ the up-regulation of MHC class I alleles, and the appearance of IFN- γ -producing virus-specific CD8⁺ T cells in the liver.^{3,5} Of note, the latter scenario has also been observed in the absence of significant liver disease, further supporting the noncytotoxic nature of this pathway.³

Importantly, in chronically HCV-infected patients, HCV-specific CD8⁺ T cells in the peripheral blood²⁵ and liver²⁶⁻²⁸ have an impaired ability to produce IFN- γ . Thus, it is tempting to speculate that the persisting inability to produce IFN- γ may be one mechanism that contributes to viral persistence. Clearly, several other important mechanisms are involved in the development of viral persistence, for example, emergence of viral escape mutation or lack of T-cell priming. The mechanisms responsible for the failure of virus-specific CD8⁺ T cells to produce IFN- γ are currently not completely understood. As possible causes, a lack of CD4⁺ T-cell help,²⁹ the action of suppressive regulatory T cells,³⁰ an unresponsiveness due to high antigen levels, and immunomodulatory functions by the PD-1/PD-L1 pathway or interleukin-10 have been discussed.

The highest E/T ratio used in our assays was 1:1 because we observed unspecific viral inhibition of virus-specific CD8⁺ T cells at higher E/T ratios, similar to what has been described for the inhibition of simian immunodeficiency virus.³¹ Importantly, E/T ratios of 1:1 or less may best reflect the situation in vivo.³² Indeed, based on an assumed total of 10¹¹ human hepatocytes with an estimated frequency of 10%–30% HCV-infected cells and a total of 2 \times 10¹¹ CD8⁺ T lymphocytes in the body with HCV-specific CD8⁺ T cells in an average range of 0.01% to 1%,³³ the E/T ratios in vivo would range between 1:10 and 1:1000.

In the absence of an appropriate small animal model for HCV infection, the CD8⁺ T-cell/hepatocyte interactions cannot be analyzed in vivo. However, the important role of the noncytotoxic pathway has previously been elegantly shown in the hepatitis B virus (HBV) transgenic mouse model. Indeed, after adoptive transfer of HBV-specific CD8⁺ T cells, antigen recognition in the liver triggers the release of antiviral cytokines that inhibit viral replication noncytotoxicity and activates the cytolytic potential of these T cells, resulting in necroinflammatory liver disease that temporally follows the inhibition of replication.³⁴ Indeed, both effector functions are regulated nonsynchronously and oscillate with a rapid induction and subsequent loss of IFN- γ and a delayed cytolytic activity.³⁵ In keeping with these observations, the same group has shown that the onset of viral clearance in

HBV-infected chimpanzees is closely correlated with the appearance of IFN- γ -producing virus-specific CD8⁺ T cells in the liver that precedes the peak of liver disease.³⁶⁻³⁸

As a strength of our immunological model, it allows us to analyze the antiviral action of virus-specific CD8⁺ T cells. While enzyme-linked immunosorbent spot, MHC multimers, and intracellular cytokine staining assays are widely accepted to track CD8⁺ T-cell frequencies, these assays fail to differentiate T-cell populations with respect to their antiviral efficacy. Thus, the dependence of CD8⁺ T cell-mediated HCV suppression on epitope specificity, T-cell receptor diversity, T-cell phenotype, or HLA restriction could not be analyzed to date, even though a possible role of some of these factors has been shown for human immunodeficiency virus.³⁹ In this context, it is interesting that we observed a significant inhibition of HCV replication also by an HLA-A2-restricted NS3₁₄₀₆₋₁₄₁₅-specific CD8⁺ T-cell clone (Supplementary Figure 4) as well as by an HLA-B27-restricted CD8⁺ T-cell clone (data not shown).

Importantly, the immunologic assay system is not only flexible with respect to the different HLA alleles but also is applicable to other HCV isolates. In our study, we used a replicon based on the HCV genotype 2a isolate JFH1 for 2 reasons. First, this isolate replicates with very high efficiency in cell culture.²¹ Further, the sequence of a second available HCV isolate (Con1, genotype 1b¹¹) also did not match to our particular T-cell clone, hence we used JFH1 to provide the most robust and reproducible levels of replication. Second, JFH1 is the only HCV isolate capable of efficiently producing virus in cell culture,⁴⁰ opening the possibility of analyzing virus-infected cells. While the findings will probably not differ between persistent replicon cell lines and virus-infected cells in terms of HCV replication and antigen presentation, the production of infectious virions might be important for other questions, such as the selection of escape variants or the importance of epitopes of interest located in genes encoding structural proteins. There is, in principle, no limitation to expand the system to other HCV isolates replicating in cell culture.⁴¹

Based on this system, our study clearly shows that virus-specific CD8⁺ T cells have the ability to inhibit HCV replication by both cytotoxic and IFN- γ -mediated noncytotoxic effector functions. Thus, successful prophylactic or therapeutic T-cell vaccines need to elicit HCV-specific CD8⁺ T-cell responses with a strong ability to produce IFN- γ .

Supplementary Data

Note: To access the supplementary material accompanying this article visit the online version of *Gastroenterology* at www.gastrojournal.org and at doi: 10.1053/j.gastro.2008.12.034.

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Conflict of interest

The authors disclose no conflicts.

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