Immunotherapy of chronic hepatitis C virus infection with antibodies against programmed cell death-1 (PD-1)

Michael J. Fuller, Nationwide Children’s Hospital
Benoit Callendret, Nationwide Children’s Hospital
Baogong Zhu, Harvard Medical School
Gordon J. Freeman, Harvard Medical School
Dana L. Hasselschwert, University of Louisiana at Lafayette
William Satterfield, M. D. Anderson Cancer Center
Arlene H. Sharpe, Harvard Medical School
Lynn B. Dustin, The Rockefeller University
Charles M. Rice, The Rockefeller University
Arash Grakoui, Emory University

Only first 10 authors above; see publication for full author list.

**Journal Title:** Proceedings of the National Academy of Sciences  
**Volume:** Volume 110, Number 37  
**Publisher:** National Academy of Sciences | 2013-09-10, Pages 15001-15006  
**Type of Work:** Article | Final Publisher PDF  
**Publisher DOI:** 10.1073/pnas.1312772110  
**Permanent URL:** [http://pid.emory.edu/ark:/25593/fsz0x](http://pid.emory.edu/ark:/25593/fsz0x)

Final published version: [http://www.pnas.org/content/110/37/15001](http://www.pnas.org/content/110/37/15001)

**Copyright information:**  
Freely available online through the PNAS open access option.

*Accessed July 15, 2018 7:24 AM EDT*
Immunotherapy of chronic hepatitis C virus infection with antibodies against programmed cell death-1 (PD-1)

Michael J. Fuller,a,1 Benoit Callendret,b Baogong Zhu,b Gordon J. Freemanb, Dana L. Hasselschwert,b William Satterfieldd, Arlene H. Sharpee, Lynn B. Dustinf, Charles M. Ricie, Arash Grakoui,g, Rafi Ahmedg,2, and Christopher M. Walkerah,2

aCenter for Vaccines and Immunity, Nationwide Children’s Hospital, Columbus, OH 43205; bDana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115; cNew Iberia Research Center, University of Louisiana at Lafayette, New Iberia, LA 70560; dDepartment of Veterinary Sciences, Micheal E. Keeling Center for Comparative Medicine and Research, M. D. Anderson Cancer Center, Bastrop, TX 78602; eDepartment of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115; fCenter for the Study of Hepatitis C, Laboratory of Virology and Infectious Disease, The Rockefeller University, New York, NY 10065; gEmory Vaccine Center, Emory University, Atlanta, GA 30329; and hDepartment of Pediatrics, College of Medicine, The Ohio State University, Columbus, OH 43210

Contributed by Rafi Ahmed, July 10, 2013 (sent for review February 26, 2013)

Hepatitis C virus (HCV) persistence is facilitated by exhaustion of CD8+ T cells that express the inhibitory receptor programmed cell death-1 (PD-1). Blockade of PD-1 signaling improves antiviral immune responses by restoring the function of pre-existing virus-specific T cells and decreasing the presence of an exhausted T-cell population. An increased frequency of exhausted T cells is present in HCV patients, and clinical responses to PD-1 blockade have been reported in the setting of HCV infection. In this study, we investigate the effect of PD-1 blockade on chronic HCV infection in chimpanzees.

T cell exhaustion is a defining feature of failed immunity against tumors and persistent viruses. Exhausted CD8+ T cells constitutively express multiple receptors that deliver inhibitory signals, resulting in loss of effector functions and reduced proliferative potential. Blockade of inhibitory signals using antibodies against receptors or their ligand(s) is a promising therapeutic strategy. Blockade of inhibitory signals using antibodies against receptors or their ligand(s) is a promising therapeutic strategy.

To whom correspondence may be addressed. E-mail: christopher.walker@nationwidechildrens.org or rahmed@emory.edu.

This article contains supporting information online at www.pnas.org/cgi/content/doi/10.1073/pnas.1312772110/DCSupplemental.

1Present address: Division of Clinical Immunology and Rheumatology, Department of Medicine, The University of Alabama at Birmingham, Birmingham, AL 35294.

2To whom correspondence may be addressed. E-mail: christopher.walker@nationwidechildrens.org or rahmed@emory.edu.


The authors declare no conflict of interest.

Fehlerfrei günstige Online über die PNAS open access option.

www.pnas.org/cgi/doi/10.1073/pnas.1312772110
Results and Discussion

The objective of this study was to reduce or eliminate persistent HCV replication in chimpanzees by antibody-mediated blockade of PD-1 signaling. We have previously demonstrated that the antibody selected for use in this study recognizes PD-1 expressed on chimpanzee T cells (16). Blockade of PD-1 signaling also restored proliferation of HCV-specific CD8+ T cells recovered from the liver of a persistently infected chimpanzee (Fig. S1), validating this animal model for studies of T-cell reconstitution by immunotherapy.

Three chimpanzees with chronic HCV infection were treated with anti–PD-1 antibodies to interrupt virus replication. Features of persistent HCV infection in these chimpanzees are summarized in Table 1. The first treated animal, Ch1535, was persistently infected with a clonal genotype 1a strain of HCV (H77) 12 y before treatment (17). Baseline viremia measured at six time points over a 12-mo period before initiation of anti–PD-1 therapy was stable at about 10^6 international units (IU) of HCV RNA per milliliter of plasma. Anti–PD-1 antibodies were administered i.v. at a dose of 3 mg/kg of body weight at 5 weekly intervals (Fig. 1A). Anti–PD-1 antibody levels peaked at ~0.05 mg/mL of serum after the first two weekly infusions, and despite persisting for 1–2 wk after the fifth and final dose, no concomitant reduction in viremia was observed, and neither was there evidence of hepatocellular damage as assessed by serum alanine aminotransferase (ALT) levels (Fig. 1B and C). An HCV-specific T-cell response, measured by IFN-γ enzyme-linked immunosorbent spot (ELISpot), was not detected in blood at any time before, during, or after treatment. HCV-specific T cells are frequently enriched in the persistently infected liver of humans and chimpanzees. For Ch1535, virus-specific T cells were expanded only intermittently from liver at four of nine sampling points over 2 y of baseline sampling. The pretreatment response, when detected, was comprised of CD8+ T cells (Fig. 1D) that were present at low frequency and narrowly focused on one or two viral proteins (Fig. 1E). HCV-specific T cells were not expanded from liver obtained 1 wk after the start of PD-1 blockade (Fig. 1D). Lack of a virologic response or hepatitis, combined with

<table>
<thead>
<tr>
<th>Chimpanzee</th>
<th>HCV genotype</th>
<th>Duration of infection at treatment, y</th>
<th>Average baseline viral load, IU/mL</th>
<th>T-cell response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch1535</td>
<td>1a</td>
<td>~12</td>
<td>1.98 x 10^6 ± 7.08 x 10^5</td>
<td>Blood: −, Liver: −/−</td>
</tr>
<tr>
<td>Ch5276</td>
<td>3a</td>
<td>~2</td>
<td>5.70 x 10^5 ± 3.36 x 10^4</td>
<td>Blood: −/+, Liver: −/−</td>
</tr>
<tr>
<td>Ch5300</td>
<td>4a</td>
<td>~2</td>
<td>2.01 x 10^4 ± 6.78 x 10^3</td>
<td>Blood: +, Liver: ++</td>
</tr>
</tbody>
</table>

a failure to improve virus-specific T-cell activity in blood and liver indicated that PD-1 therapy was not successful in this animal.

Chimpanzee 1535 had very low levels of HCV-specific T-cell activity in liver before treatment. It is possible that the residual T-cell response was below the threshold for rescue by blockade of PD-1 signaling after more than a decade of persistent HCV replication. We reasoned that PD-1 blockade might be more successful if initiated earlier in the course of chronic infection. Two animals (Ch5276 and Ch5300) were persistently infected with HCV for 2 y when enrolled in this study (Table 1). Ch5276 was infected with a clonal genotype 3a virus (18). Before treatment viremia was stable at ∼2 × 10^7 IU/mL of serum. Administration of anti–PD-1 antibodies at a dose of 3 mg/kg on days 0, 3, 7, 14, and 21 (Fig. 2A) had no impact on serum HCV RNA or ALT levels (Fig. 2B) even though peak antibody titers exceeded those observed in animal 1535. HCV-specific CD4+ or CD8+ T-cell frequencies in liver were very low immediately before and 2 wk after treatment, suggesting that PD-1 blockade failed to improve the weak and narrowly focused response present in the chronically infected liver (Fig. 2D and E). In comparison, T-cell frequencies were substantially higher in this animal during the acute phase of infection (18). Here we further characterized CD4+ and CD8+ T-cell responses at week 14 when cellular immunity is often evident even in those who follow a chronic course, and also at week 22 when infection is transitioning from the acute to chronic phase (Fig. 2D). Acute phase CD4+ and CD8+ T-cell responses were both strong at weeks 14 and 22 compared with the chronic phase of infection (Fig. 2D). Representative data from week 14 show, however, that few HCV proteins were targeted even during the acute phase of infection in this animal (Fig. 2E). Both the CD4+ and CD8+ T cells were focused predominantly on the nonstructural NS3 protein (Fig. 2E). Together these results indicate that HCV-specific T cells surviving in liver after 2 y of persistent infection could neither expand with PD-1 blockade nor broaden beyond the very limited set of HCV proteins recognized much earlier in infection.

The outcome of an identical 3-wk course of anti–PD-1 treatment was quite different for Ch5300. HCV RNA levels were stable at ∼5 × 10^4 IU/mL plasma 2 y after infection with a clonal genotype 4a virus (Fig. 3A) (18). Beginning with the third dose of antibody on day 7, a sharp decline in viremia was observed (Fig. 3B). At day 28, 1 wk after administration of the last antibody dose, HCV RNA decreased to only 64 IU/mL serum, a 100-fold decline from the average baseline value and only slightly above the level of detection for this assay (50 IU/mL serum). HCV replication gradually increased after day 28 and was correlated with a loss of anti–PD-1 antibodies from serum (Fig. 3C). By day 42, HCV RNA titers returned to levels that were approximately equal to those measured before PD-1 blockade (Fig. 3B).

HCV-specific T-cell activity in the liver of Ch5300 was also substantially different after treatment compared with the nonresponders Ch1535 and Ch5276. HCV-specific CD4+ and CD8+ T cells frequencies were low in liver 6 wk before initiation of therapy but increased sharply at study day 7, immediately before viremia began to decline (Fig. 3D). CD4+ T cells increased both in number and diversity after PD-1 blockade. The impact of therapy was greatest on the CD8+ T-cell subset (Fig. 3E). CD8+ T-cell frequencies increased 25-fold and targeted multiple HCV proteins.

**Fig. 2.** PD-1 blockade fails to alter viral load in short-term persistent HCV infection with poor intrahepatic T-cell responses. (A) Dosing regimen for anti–PD-1 antibodies in Ch5276. (B) Serum levels of HCV RNA and ALT were measured in Ch5276 at the indicated time points. Baseline viremia is presented as the mean ± SD of HCV RNA levels over a 4-mo period before initiation of anti–PD-1 therapy. (C) Levels of anti–PD-1 antibodies in serum measured immediately before each dose of anti–PD-1 was administered. Vertical dashed lines in B and C represent time points at which anti–PD-1 antibodies were administered. (D) Magnitude of in vitro–expanded CD4+ (white bars) and CD8+ (black bars) intrahepatic T-cell responses to HCV genotype-matched peptide pools are indicated at various time points. (E) Relative distribution of the intrahepatic CD4+ and CD8+ T-cell responses to each of nine separate pools of genotype-matched HCV peptide pools is depicted to show the breadth of the response to HCV.
proteins by treatment day 7 compared with responses measured before the start of PD-1 blockade (Fig. 3E). Indeed, treatment rescued the frequency and breadth of the posttreatment CD8+ T-cell response to levels that exceeded those measured during the acute phase of infection at weeks 14 and 24 when virus replication was partially controlled (18). The frequency of antiviral T cells also improved in blood by day 21 posttreatment compared with a representative baseline sample taken at day 0 (Fig. 3F). Serum ALT levels did not increase during treatment, indicating that expansion of HCV-specific CD8+ T cells in liver did not cause measurable hepatocellular injury (Fig. 3B). Apparent noncytotoxic control of HCV-infected hepatocytes by CD8+ T cells was described in some humans with acute hepatitis C. It is possible that PD-1 blockade restored production of cytokines like IFN-γ that suppress HCV replication without killing hepatocytes (19–21).

Our primary goal was to restore CD8+ T-cell immunity given the critical role of this subset in control of HCV infection (7, 13, 14). Expansion of CD4+ T cells (as shown in Fig. 3) was not necessarily expected although it is consistent with a recent report of PD-1 expression on HCV-specific helper cells in chronically infected humans (12). It is possible that PD-1 blockade restored production of cytokines like IFN-γ that suppress HCV replication without killing hepatocytes (19–21).

Fig. 3. Transient decline of viral load associated with anti–PD-1 treatment in short-term persistent HCV infection with broad intrahepatic T-cell responses. (A) Dosing regimen for anti–PD-1 antibodies in Ch5300. (B) Serum levels of HCV RNA and ALT were measured in Ch5300 at the indicated time points. Baseline viremia is presented as the mean (±1 SD) of HCV RNA measured over a 4-mo period before initiation of anti–PD-1 therapy. (C) Pharmacokinetic of anti–PD-1 antibodies in serum measured immediately before each dose of anti–PD-1 was administered. Vertical dashed lines in B and C represent time points at which anti–PD-1 antibodies were administered. (D) Magnitude of in vitro–expanded CD4+ (white bars) and CD8+ (black bars) intrahepatic T-cell responses to HCV genotype-matched peptide pools are indicated at various time points. (E) Relative distribution of the intrahepatic CD4+ and CD8+ T-cell responses to each of nine separate pools of genotype-matched HCV peptide pools is depicted to show the breadth of the response to HCV. (F) Combined IFN-γ ELISPOT responses to the nine HCV peptide pools by peripheral blood mononuclear cells are shown for study days 0 and 21.

The virologic response in Ch5300 was most obviously associated with a broad, robust intrahepatic T-cell response absent from the livers of Ch1535 and Ch5276. An additional influence of HCV genotype and/or virus load on the outcome of therapy cannot be excluded. Before initiation of therapy, average baseline viremia was consistently lower in Ch5300 than in Ch5276 and Ch1535. Low-level viremia in Ch5300 may have been a marker of an ongoing, partially effective T-cell response that was more readily rescued by PD-1 blockade. This pattern of immunity may have been established months earlier during the acute phase of infection, when expansion of multispecific T cells was associated with transient control of virus replication in responser Ch5300 but not in nonresponder Ch5276 (18). HCV genotype was probably not a factor in the outcome of PD-1 blockade. The quality of T-cell immunity varies widely in humans and chimpanzees even when infection is established with viruses of the same genotype or subtype (7, 13, 14, 24–29). It is therefore likely that successful PD-1 therapy in Ch5300 was due to priming and/or maintenance of a broad T-cell response that could be achieved by PD-1 blockade.
rescued regardless of HCV genotype. Viremia often oscillates at low levels for a year or more after infection in most humans and chimpanzees, as illustrated by Ch5300 (7, 14, 18). This is thought to reflect antiviral activity by T cells that maintain some residual effector function. Treatment that is delayed until much later in chronic infection, as was the case for Ch1535, may also be less successful.

A delay in the downward trajectory of viremia in Ch5300 until day 14 posttreatment (EBV) is consistent with a process that requires expansion of T cells targeting existing epitopes. It is not clear from our study if continued PD-1 blockade would have terminated infection in Ch5300. The rebound in HCV replication after discontinuation of antibody treatment suggests that T cells remained susceptible to inactivation even after expansion in liver and a reduction in viremia to just above the threshold of detection. Premature termination of immunotherapy may therefore carry the same risk of relapse as current modes of therapy that directly target virus replication.

In summary, our study documents that anti–PD-1 antibodies can be administered safely at doses that can lead to control of viremia. Although PD-1 blockade has shown efficacy in other chronic viral infections, including LCMV infection of mice and SIV infection of rhesus macaques (4, 5), they do not address the particular concern of hepatotropic viruses that tend to persist in the uniquely tolerogenic environment of the liver. In this study we demonstrate that therapeutic strategies focused on blocking inhibitory signaling through PD-1 can potentially overcome that tolerogenic environment and lead to viral control. The chimpanzees selected for this study approximated the wide variability in the breadth and perhaps also the quality of intrahepatic T-cell immunity in the persistently infected human liver. Based on findings presented here, success in treating human chronic hepatitis C with anti–PD-1 antibodies might also be mixed. Effective reconstitution of immunity in nonresponders like Ch5276 and Ch1535 may require simultaneous blockade of signaling by other inhibitory receptors like CTLA-4 and TIM-3 that are coexpressed by exhausted T cells (10, 11). Narrow intrahepatic T-cell responses like those observed in Ch5276 and Ch1535 might also be improved by combining anti–PD-1 therapy with therapeutic vaccination, a concept that has been successfully tested in mice persistently infected with LCMV (30). Expression of PD-1 on exhausted T cells is a feature of chronic HIV and hepatitis B infections. So blockade of this signaling pathway may be generally useful for control of persistent virus replication (8, 9, 31). Our results may be particularly relevant to chronic infection of the liver with HBV. Reversal of immune tolerance is considered important to control of chronic hepatitis B because the virus can be suppressed, but not eliminated, by direct acting antiviral drugs. There is precedent for breaking immune tolerance against HBV with type I IFN, but it is successful in only about 30% of treated patients (9). Interruption of T-cell tolerance in this infection by PD-1 signaling blockade may provide another approach to long-term control of persistent HBV infection.

Materials and Methods

Animals and Treatment. Chimpanzees (Pan troglodytes) Ch1535, Ch5276, and Ch5300 were maintained under standard conditions for humane care and in compliance with National Institutes of Health guidelines. Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Louisiana at Lafayette New Iberia Research Center and the M.D. Anderson Michale E. Keeling Center for Comparative Medicine and Research. Ch1535 was housed at the University of Louisiana at Lafayette New Iberia Research Center, and Ch5276 and Ch5300 were housed at the Michale E. Keeling Center for Comparative Medicine and Research, M.D. Anderson Cancer Center. The inoculations of these animals with infectious clones of HCV corresponding to the genotype 1a H77 strain (Ch1535), the genotype 3a strain S52 (Ch5276), and the genotype 4a strain ED43 (Ch5300) have previously been described (17, 18), and at the time the animals were enrolled in this study, they all had developed persistent viremia. All animals received a humanized anti–PD-1 monoclonal antibody by i.v. injection at a dose of 3 mg antibody per kilogram of body weight. Plasma was analyzed for viral load by HCV RNA 3.0 bDNA assay (Siemens) and serum levels of ALT were also monitored in each animal over the course of treatment.

Anti–PD-1 and PD-L1 Antibodies. The binding domain of the EH12 anti–PD-1 mAb (32) was humanized by Antitope, Ltd., by grafting the complementarity determining regions (CDR) into human heavy- and light-chain framework sequences. DNAs encoding humanized heavy-chain variable region and the CH1-hinge-CH2-CH3 region of human IgG4 were cloned into an expression plasmid containing the elongation factor-1A promoter constructed by Gordon Freeman (designated pEFGP) by In-fusion (33, 34). Ser228 of the human IgG4 core hinge region was mutated to Pro, identical to human IgG1, to ensure efficient dimerization. Similarly, the humanized light-chain variable region and the Kappa-constant region were cloned into the pEFGP expression vector by In-fusion. DHFR-deficient CHO cells (DG44) were electroporated with MIU-linearized plasmids and selected for metotrexate resistance as described (34). Antibody was purified from CHO cell culture supernatants by affinity chromatography on protein A. Endotoxin levels were determined to be less than 2 endotoxin units/ml protein. The 29EZA3 anti–PD-L1 mAb has been described (35).

Anti–PD-1 ELISA. Serum levels of the humanized anti–PD-1 mAb were monitored by a standard sandwich ELISA. Briefly, titrated serum samples were incubated on ELISA plates coated with a human PD-1–Mouse IgG2a fusion protein (36). Captured antibody was detected with an HRP-conjugated goat Fab2 anti-human IgG antibody (Jackson Immunoresearch), and plates were developed with SureBlue TMB Microwell Peroxidase Substrate (KPL). The colorimetric reaction was stopped with TMB Stop Solution (KPL), and absorbance at 450 nm was measured on a tunable microparticle reader. Actual concentrations were calculated by comparing results to a standard curve.

Direct ex Vivo IFN-γ ELISPOT. Whole blood was collected in EDTA at various time points, peripheral blood mononuclear cells (PBMC) were separated by centrifugation over a Ficoll gradient, and HCV-specific responses were quantified by IFN-γ ELISPOT assay (U-CyTech) following in vitro restimulation with genotype-matched pools of peptides corresponding to the HCV polymerase as previously described (18).

Intrahepatic HCV Immune Responses. Chimpanzee liver needle biopsies were gently disrupted in PBS containing 2% (vol/vol) FBS, CD4+ and CD8+ lymphocytes were positively selected by magnetic bead (Invitrogen) purification, and cultured in a 24-well plate together with peptides spanning the entire HCV polymerase and 7 × 10^6 irradiated (5,000 rad) autologous PBMC to serve as feeder cells. Cells were cultured for 2 wk in Roswell Park Memorial Institute (RPMI)-1640 media containing γ-glutamyl, antibiotics, 10% FBS, and 50 U/ml rHuG-CSF (clone media), with half the volume replaced every 3 to 4 d. Cells were then nonspecifically stimulated for an additional 2 wk by the addition of 3 × 10^5 irradiated (5,000 rad) human PBMC feeder cells and anti-human CD3 antibodies, with half the volume of clone media being replaced every 3 or 4 d. Cells were then screened for HCV-specific IFN-γ production by ELISPOT assay following stimulation with one of nine peptide pools spanning the appropriate genotype HCV polymerase.

Online Supplemental Material. Fig. S1 shows the in vitro restoration of proliferative capacity for HCV-specific intrahepatic CD8+ T cells following PD-1 blockade.

Acknowledgments. This work was supported by grants from the Gates Foundation (to R.A.) and the US Public Health Service (USPHS) and National Institutes of Health [R37 AI47367 and U19 AI48231 (to C.M.W.) and P01 AI080192, U19 AI056299, and R01 AI074417 (to G.J.F.)]. This work was also supported by USPHS Grants AI070101 and DK083356 and Yerkes Research Center Base Grant RR-00165 (to A.G.) and the New Iberia Research Center (9U42 OD014838). M.J.F. was funded in part by a postdoctoral fellowship from the American Cancer Society.


