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Editor's Summary

Turning the Tables on Tolerance

When it comes to transplantation, chronic viral infection may not be so bad after all. Bohne *et al.* demonstrate that chronic infection with hepatitis C virus (HCV) does not prevent the development of tolerance in liver transplant patients. Animal studies have previously indicated that immune responses to infection prevent the establishment of transplantation tolerance, which has resulted in the exclusion of individuals with persistent infection from transplantation tolerance trials. This prospective trial of immunosuppression withdrawal in HCV-infected liver transplant recipients found that the HCV-induced immune response did not influence outcome. Rather, tolerance induction was associated with expression of immunoregulatory genes that were specific for HCV infection. Thus, HCV infection not only does not always prevent the development of transplantation tolerance but also may actually contribute to restraining antitransplant immune responses.

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TRANSPLANTATION

HCV-Induced Immune Responses Influence the Development of Operational Tolerance After Liver Transplantation in Humans

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Pathogen-induced immune responses prevent the establishment of transplantation tolerance in experimental animal models. Whether this occurs in humans as well remains unclear. The development of operational tolerance in liver transplant recipients with chronic hepatitis C virus (HCV) infection allows us to address this question. We conducted a clinical trial of immunosuppression withdrawal in HCV-infected adult liver recipients to elucidate (i) the mechanisms through which allograft tolerance can be established in the presence of an ongoing inflammatory response and (ii) whether anti-HCV heterologous immune responses influence this phenomenon. Of 34 enrolled liver recipients, drug withdrawal was successful in 17 patients (50%). Tolerance was associated with intrahepatic overexpression of type I interferon and immunoregulatory genes and with an expansion of exhausted PD1/CTLA4/2B4-positive HCV-specific circulating $CD8^+$ T cells. These findings were already present before immunosuppression was discontinued and were specific for HCV infection. In contrast, the magnitude of HCV-induced proinflammatory gene expression and the breadth of anti-HCV effector T cell responses did not influence drug withdrawal outcome. Our data suggest that in humans, persistent viral infections exert immunoregulatory effects that could contribute to the restraining of alloimmune responses, and do not necessarily preclude the development of allograft tolerance.

INTRODUCTION

Immune responses to pathogens can have profound effects on alloimmune responses and tolerance induction (*1*). Concurrent infections have been shown to trigger the production of proinflammatory mediators that may overcome the effects of tolerance-promoting therapies (*2*). Furthermore, pathogenic immune exposures, particularly in the form of viral infections, can directly activate T cell clones cross-reacting with alloantigens and generate memory-phenotype cross-reactive allospecific T cell populations (*3*–*7*). Thus, ongoing and/or past infections interfere with the capacity of costimulation blockade to induce transplant tolerance in mice (*5*, *8*, *9*). Because human transplant recipients harbor large memory T cell pools and are exposed to a variety of infective agents, pathogen-induced innate and adaptive immune responses have been proposed as one of the principal barriers to the establishment of transplantation tolerance in the clinic (*3*). The influence of pathogen-host immune interactions on human allograft tolerance, however, has never been investigated because recipients with persistent infections are typically excluded from experimental tolerance-promoting strategies.

Liver allografts have unique tolerogenic properties and a lower susceptibility to rejection than other solid organs (*10*–*12*). Thus, graft survival in the absence of maintenance immunosuppression (IS), commonly referred to as spontaneous operational tolerance, occurs more frequently in liver than in any other clinical transplantation setting. Consequently, clinical trials in which IS drugs are intentionally withdrawn from stable recipients have only been performed in liver transplantation (*10*, *13*–*18*).

We previously reported that IS-free tolerant liver recipients and patients requiring maintenance IS differ in blood transcriptional and cell phenotypic patterns, and that intragraft gene expression signatures accurately predict the outcome of IS weaning (*19*–*21*). The influence of hepatitis C virus (HCV) infection on intragraft molecular patterns was, however, not investigated because HCV-positive recipients were excluded from the study.

HCV chronically infects around 200 million people worldwide and constitutes the leading indication for liver transplantation in the Western hemisphere. HCV immunopathogenesis is far from being completely understood. Both insufficient virus-specific protective immunity and increased nonspecific inflammatory activation are thought to play a key role in HCV persistence and liver injury (*22*). After liver transplantation, recurrence of HCV infection is universal and results in higher viral loads (*23*, *24*) and more rapid HCV-induced liver damage (*25*, *26*) than in nontransplanted individuals. This has been attributed, at least in part, to the inhibition of anti-HCV immunity by pharmacological IS (*25*, *27*, *28*). The development of liver transplant tolerance and the discontinuation of IS, which in HCV-positive recipients occurs as frequently as in HCV-negative patients (*29*), provide a unique setting to understand how viral-induced innate and adaptive immune responses influence the establishment of transplantation tolerance in humans.

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Here, we conducted a prospective IS withdrawal trial in liver recipients with persistent HCV infection to investigate how anti-HCV T cell immunity and HCV-induced inflammatory responses influence the development of operational tolerance. Molecular analyses performed before the initiation of IS weaning revealed that successful IS withdrawal was associated with heightened intrahepatic type I interferon (IFN-I) gene expression. This correlated with overactivation of immunoregulatory pathways in the absence of increased proinflammatory gene expression, and was associated with an expansion of HCV-specific exhausted circulating $CD8^+$ T cells.

RESULTS

A large proportion of carefully selected HCV-infected liver recipients successfully discontinued immunosuppressive therapy

Thirty-four recipients meeting clinical eligibility criteria and exhibiting at least one of two markers previously identified as being highly specific for liver operational tolerance in retrospective cross-sectional studies (high blood Vδ1/Vδ2 γδ T cell ratio and elevated blood *SLAMF7*/*KLRF1* expression) (*19*, *20*) were included in the IS withdrawal protocol (Fig. 1, A and B). The primary endpoint was defined as the successful discontinuation of IS followed by 12 months of stable liver function and a protocol liver biopsy excluding subclinical rejection. IS was discontinued in 17 patients (50%), who were considered operationally tolerant (TOL). Fifteen recipients rejected and were classified as nontolerant (Non-TOL). Baseline clinical and demographic characteristics of TOL and Non-TOL recipients were similar (Table 1). All patients exhibited positive serum HCV RNA and liver inflammatory features suggestive of chronic HCV infection. No improvement in HCV viral load or graft histopathology was noted after IS withdrawal (tables S1 and S2). All episodes of rejection were histologically mild or moderate, and resolved after reinstitution of IS therapy without the need for high-dose steroid boluses. No grafts were lost because of rejection.

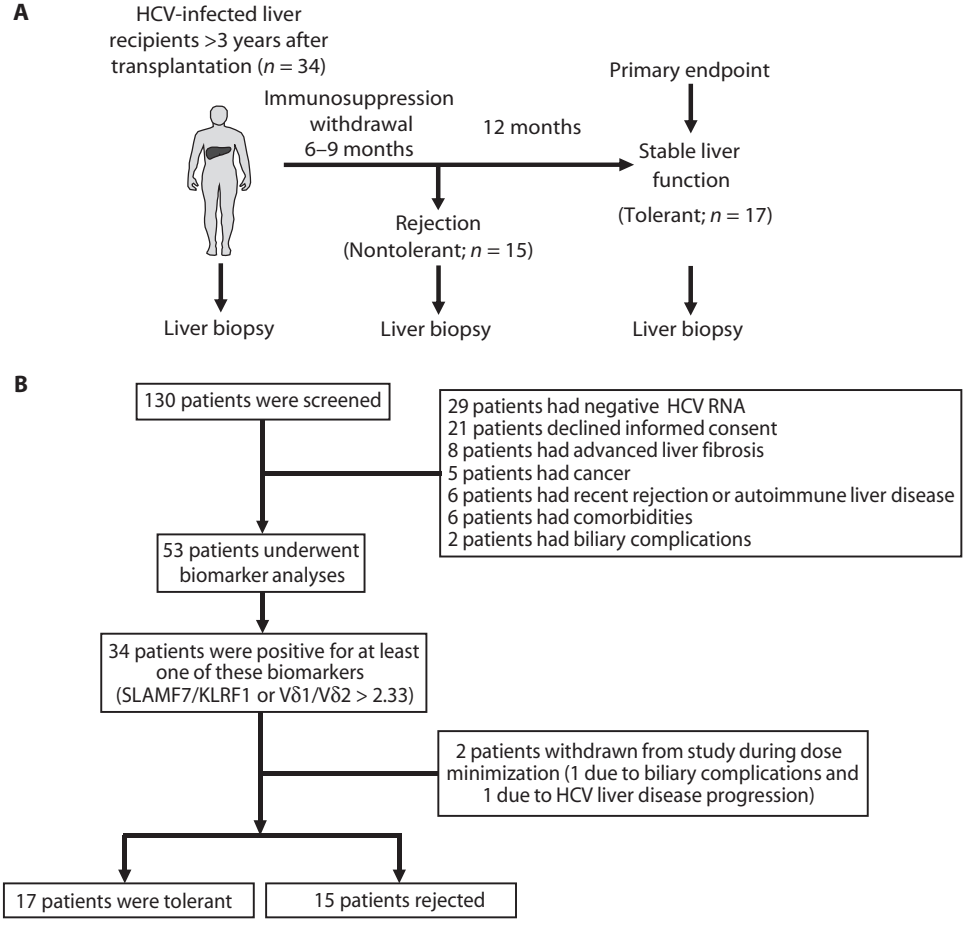

Fig. 1. Study design. (**A**) Study outline: stable HCV-infected liver transplant recipients with >3-year posttransplant follow-up were enrolled in an IS withdrawal clinical trial. Drugs were gradually discontinued over a 6- to 9-month period, and patients were followed up for an additional 12-month period. Protocol liver biopsies were obtained at study onset, at any time rejection was suspected, and 12 and 36 months after complete drug withdrawal in patients who did not reject. Patients who maintained stable graft function during the entire duration of the study and in whom no signs of rejection were noticed in the 12-month post-withdrawal protocol biopsy were considered operationally tolerant (TOL). Patients who underwent rejection at any time during the follow-up were labeled as nontolerant (Non-TOL). (**B**) Screening and enrolment of patients.

Blood Vδ1/Vδ2 T cell ratio was useful in the screening of liver recipients for IS withdrawal

Blood Vδ1/Vδ2 T cell ratio had a good discriminative capacity in predicting the outcome of drug withdrawal, with 82% sensitivity (SN), 53% specificity (SP), 67% positive predictive value (PPV), and 73% negative predictive value (NPV). To further explore the capacity of blood Vδ1/Vδ2 T cell ratio to predict the outcome of IS withdrawal, we reanalyzed the flow cytometric data derived from an independent cohort of 67 liver recipients enrolled in a previously reported IS withdrawal trial (*21*). Vδ1/Vδ2 T cell ratio quantification conducted before the initiation of IS weaning predicted the outcome of the withdrawal protocol with 76% SN, 47% SP, 62% PPV, and 63% NPV. Peripheral blood mononuclear cell (PBMC) *SLAMF7* and *KLRF1* transcript levels, in contrast, exhibited a suboptimal performance (44% SN, 27% SP, 47% PPV, and 25% NPV; table S3), which was worse than originally estimated within retrospective studies (*19*, *20*), and significantly lower than what was reported in the more recent multicenter prospective IS withdrawal trial that mostly enrolled HCV-negative recipients (66% SN, 76% SP, 76% NPV, and 66% PPV) (*21*).

Successful IS withdrawal and operational tolerance occurred despite marked systemic proinflammatory gene expression

To determine whether the magnitude of HCV-mediated inflammatory response in blood could hamper the acquisition of tolerance, we collected PBMCs from TOL and Non-TOL recipients and from healthy

individuals, and quantified the transcript levels of a set of proinflammatory genes whose expression is activated by HCV (*20*). Marked changes were noted between HCV-infected recipients and noninfected healthy individuals, confirming the capacity of HCV to induce substantial systemic inflammatory responses. However, this did not influence the establishment of tolerance, because no differences in the expression of these genes between TOL and Non-TOL recipients were noted before the initiation of IS weaning (Fig. 2 and table S4). The effect of IS withdrawal on HCV-induced inflammation was explored in parallel by replicating the experiments in sequentially collected PBMC samples. In contrast to what we had previously reported in cross-sectional studies (*20*), withdrawal of IS in TOL recipients did not substantially modify the expression of proinflammatory genes (Fig. 2).

The magnitude of HCV-specific effector T cell responses was similar in operationally tolerant and nontolerant recipients

We next investigated whether anti-HCV memory T cell responses negatively correlated with the establishment of tolerance. The frequency of

Table 1. Baseline characteristics of evaluable patients (per protocol).

Characteristic	Total cohort (*N* = 32)	Tolerant group (*n* = 17)	Nontolerant group (*n* = 15)	*P*
Age at weaning start (years)	63 ± 8	63 ± 9	63 ± 8	0.8
Gender (% male)	78	88	67	0.1
Caucasian (%)	100	100	100	
Time from transplant to minimization start (months)*	86 ± 37	92 ± 44	80 ± 28	0.4
Previous episodes of rejection (%)	16	14	23	0.5
Immunosuppressive therapy at weaning start (no. of patients)				0.2
Tacrolimus	18	9	9	
Cyclosporin A	9	7	2	
Mycophenolate	0	0	0	
Sirolimus	2	0	2	
Tacrolimus + mycophenolate	1	1	0	
Cyclosporin A + mycophenolate	2	0	2	
Immunosuppressive drug trough levels at weaning start (ng/ml)*				
Tacrolimus	6.3 ± 3	6.4 ± 3	6.2 ± 4	0.9
Cyclosporin A	148 ± 231	215 ± 286	48 ± 39	0.3
Liver function tests at weaning start*				
Aspartate aminotransferases (U/liter)	54 ± 33	48 ± 27	63 ± 42	0.2
Alanine aminotransferases (U/liter)	69 ± 52	58 ± 57	81 ± 46	0.2
γ-Glutamyl transpeptidase (U/liter)	104 ± 142	87 ± 125	122 ± 160	0.5
Alkaline phosphatase (U/liter)	183 ± 71	179 ± 74	189 ± 71	0.7
Bilirubin (mg/dl)	0.9 ± 0.4	0.9 ± 0.4	1 ± 0.5	0.5
Transient elastography at weaning start (kPa)	8.5 ± 5	10 ± 5.6	7.3 ± 4.2	0.3
HCV genotype (no. of patients)				0.297
1a	10	7	3	
1b	20	9	11	
Others	2	1	1	
HCV RNA at weaning start (log_{10})*	5.9 ± 0.6	5.8 ± 0.6	6.1 ± 0.5	0.2
Receptor IL28B polymorphism (%)				0.6
CC	28	30	25	
CT/TT	72	70	75	
Donor IL28B polymorphism (%)				0.2
CC	36	25	50	
CT/TT	64	75	50	
Antiviral treatment course before weaning (%)	28	29	27	0.6

*Plus-minus values are means ± SD.

HCV-specific memory T cells was quantified in PBMC samples collected before the initiation of IS weaning (baseline), at the time of complete IS discontinuation (or rejection), and 12 months after discontinuation, using an IFNγ enzyme-linked immunospot (ELISpot) assay and a library of overlapping peptides. T cell responses directed against HCV peptides could be detected in most liver recipients, although, as previously described, they were weak and limited in their breadth (*28*). Before the initiation of IS weaning, the number of HCV-specific IFNγ-producing T cells was similar in TOL and Non-TOL recipients (Fig. 3A). Parallel experiments were performed using specific human leukocyte antigen (HLA)–restricted Epstein-Barr virus (EBV) T cell epitopes and a cocktail of immunodominant viral peptides (CEF cocktail). In keeping with the results of the HCV assay, no differences between TOL and Non-TOL recipients in either anti-EBV or anti-CEF T cells were noted at baseline (Fig. 3B). IS discontinuation did not modify the intensity or the width of the repertoire of HCV-specific T cell responses, which remained stable over the entire duration of the study (Fig. 3A). Immune reconstitution was only observed in one case (Fig. 3, C and D), in whom a marked increase in the number of IFNγ-producing anti-HCV and anti-EBV T cells was noted both at the time IS was discontinued and 12 months afterward. This was associated with a decrease in serum aminotransferases and a drop in HCV RNA serum levels. Notably, this patient experienced a rejection episode exactly 12 months after complete IS discontinuation.

Before IS discontinuation, operationally tolerant and nontolerant patients exhibited significant differences in intrahepatic IFN-I signaling

To understand the mechanisms potentially responsible for the development of operational tolerance in HCV-infected liver recipients, we performed Illumina microarray experiments on liver biopsy specimens collected from TOL and Non-TOL recipients before the initiation of IS weaning, and from a group of healthy HCV-negative individuals. Comparison of HCV-positive and HCV-negative patients revealed that HCV infection had a very substantial impact on intrahepatic transcriptional profiles, with up-regulation of multiple proinflammatory pathways (Fig. 4, A and B, and table S5). Conversely, the gene set differentially expressed between TOL and Non-TOL recipients was not enriched in cytokine and/or proinflammatory gene pathways and was mainly characterized by an overrepresentation of IFN-I–stimulated genes (for example, *OAS1/2, ISG15,* and *IRF1/7/9*). Microarray analyses were validated using a highly sensitive Fluidigm real-time PCR platform. Eleven genes involved in IFN-I signaling were all consistently overexpressed in TOL patients (Fig. 4A). These findings were highly stable and were not influenced by the discontinuation of IS, because IFN-stimulated gene (ISG) transcript levels were similar before and 12 months after complete IS withdrawal (Fig. 4B). Analysis of the microarray data set using Gene Set Enrichment Analysis (GSEA) identified IFN-I as the transcriptional

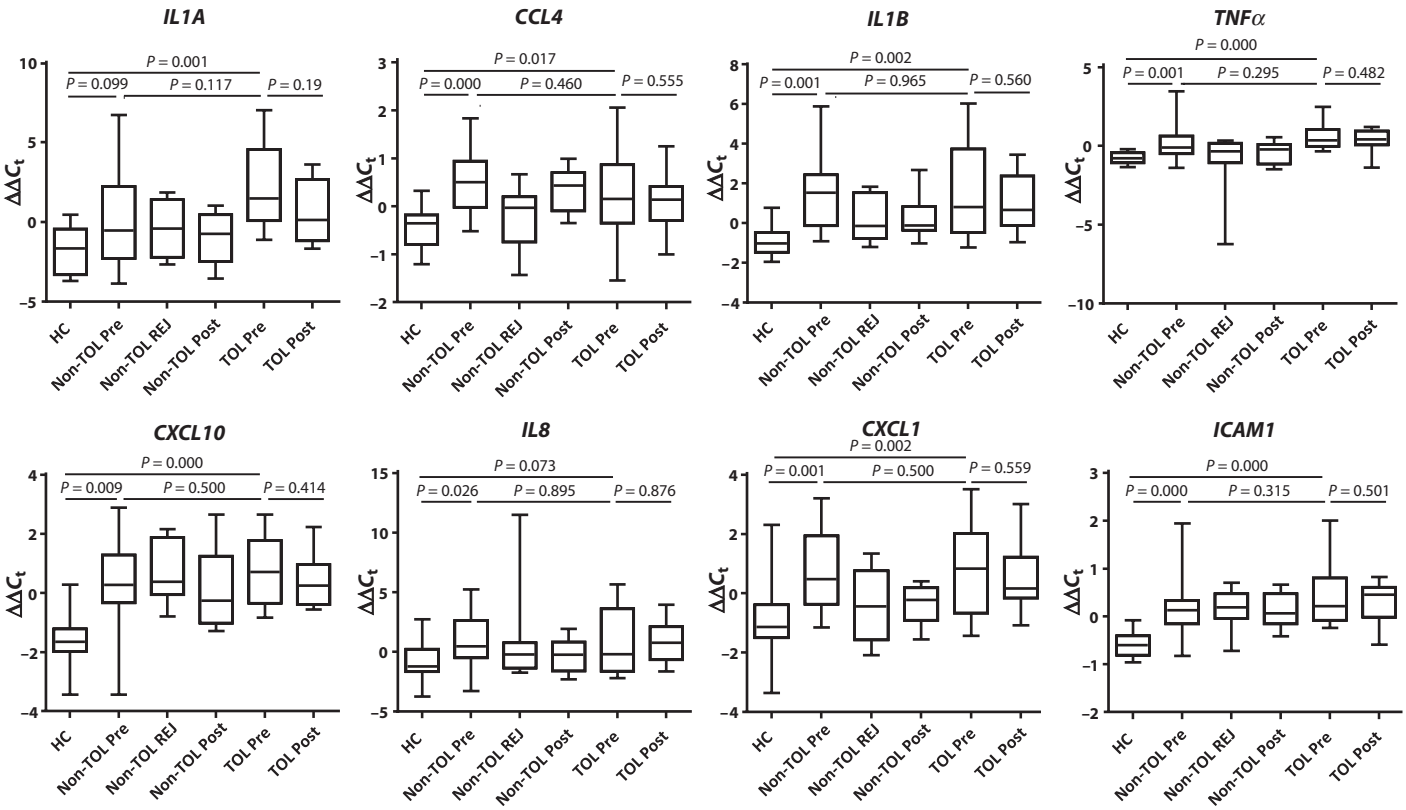

Fig. 2. Blood gene expression differences between operationally tolerant and nontolerant liver recipients. Relative expression of *IL1A, CCL4, IL1B, TNFα, CXCL10, IL8, CXCL1,* and *ICAM1* in PBMCs collected from HCV-negative healthy individuals (HC; $n = 17$) and from HCV-positive TOL ($n = 15$) and Non-TOL ($n = 14$) recipients before the initiation of IS weaning (Pre), at the time of rejection (REJ; $n = 9$), and 12 months after complete IS discontinuation or rejection (Post; $n = 11$ TOL and $n = 8$ Non-TOL). Data were obtained on a Fluidigm real-time polymerase chain reaction (PCR) platform and are displayed as box plots showing $\Delta\Delta C_t$ median (center line), interquartile range (box upper and lower boundaries), and minimum and maximum (whiskers). Student's *t* test was used for all comparisons. All raw data are displayed in table S4.

pathway more significantly associated with tolerance in HCV-infected recipients (Table 2). This is in contrast to what we had previously reported in HCV-negative TOL and Non-TOL recipients, in whom the biological pathway most significantly overrepresented in the operational tolerance-associated expression profile was iron homeostasis and no differences in IFN-I signaling were detected (*21*). Such a discrepancy reflects the fact that HCV-infected patients exhibit substantial intrahepatic lymphocyte infiltration (fig. S1 and table S2), which is absent in HCV-negative patients (*21*). The number of intrahepatic $CD4^+$, $CD8^+$, and $CD4^+Foxp3^+$ lymphocytes was, however, similar in TOL and Non-TOL HCV-infected recipients (fig. S1). Hence, the differences in ISG expression noted between TOL and Non-TOL HCV-infected recipients before the initiation of drug weaning could not be attributed to differences in intragraft lymphocyte infiltration.

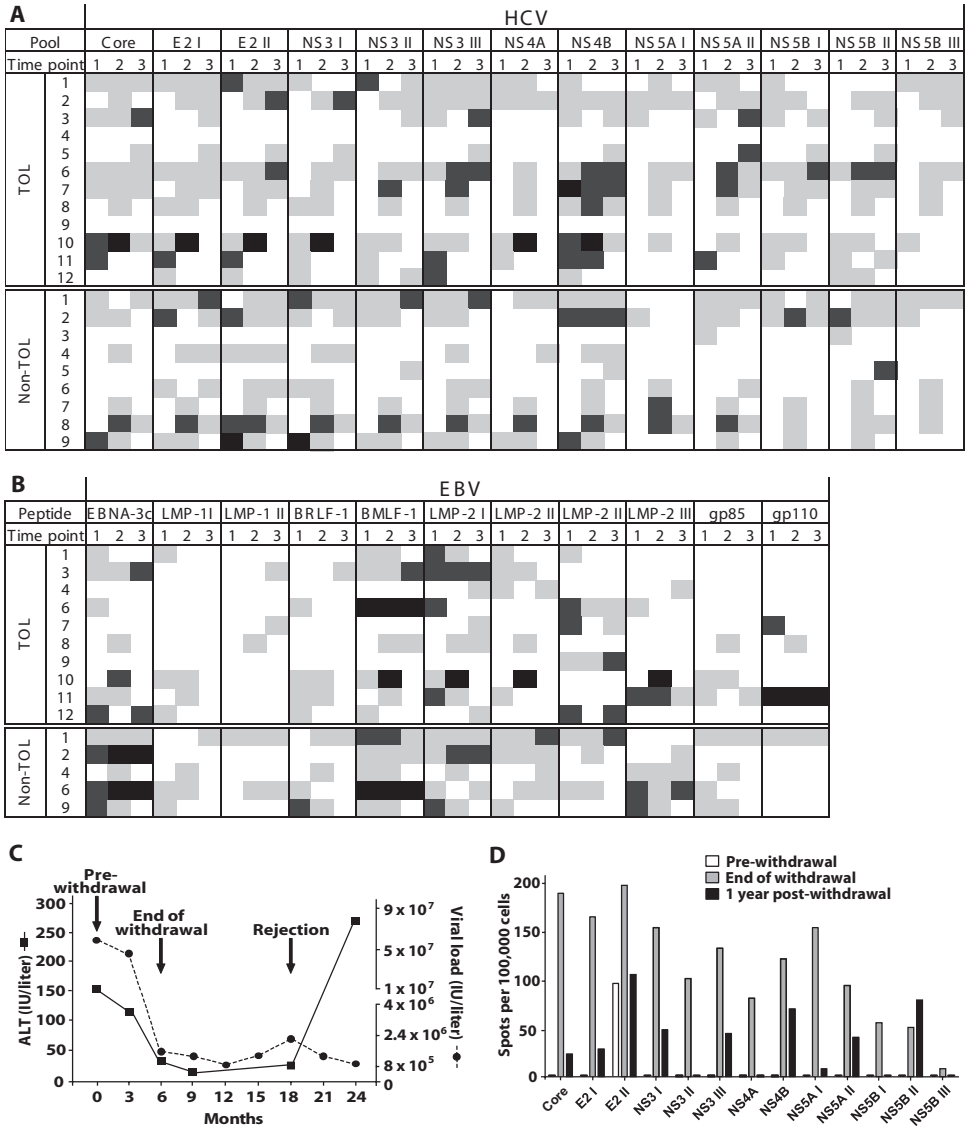

Fig. 3. Sequential changes in HCV-specific T cell responses during IS withdrawal. (**A** and **B**) Checkerboard plots displaying the sequential changes in HCV-specific (A) and EBV-specific (B) T cell responses as assessed by IFNγ ELISpot experiments. Rows represent single patients, and columns represent HCV peptide pools and the specific time points when PBMC samples were obtained (1 = baseline, 2 = end of IS withdrawal or rejection, 3 = 12 months after complete IS withdrawal or rejection). The HIV-negative control plus 2 SDs was considered as the threshold of positivity. Results are shown as a matrix, where white squares correspond to negative results (<threshold), light gray squares to 1- to 1.99-fold threshold, dark gray to 2- to 4.99-fold threshold, and black squares to >5-fold threshold. (**C**) Sequential serum HCV viral load (solid line) and alanine aminotransferase (ALT) levels (dotted line) observed before initiation of IS withdrawal, during withdrawal, and 18 months after discontinuation of IS in Non-TOL patient number 8. (**D**) Frequency of HCV-specific T cell responses as assessed by IFNγ ELISpot in Non-TOL patient number 8 (bars correspond to the number of spots per 100,000 cells).

Transplanted and nontransplanted HCV-infected patients differed in intragraft rejection-associated transcripts but not in HCV-induced IFN-I gene expression

IFN-I can activate signal transducer and activator of transcription 4 (STAT4) and contribute to T helper 1 (T_H1) responses (*30*), which play a central role in orchestrating allograft rejection. To elucidate the extent to which tolerance and rejection overlap in HCV-infected recipients, we undertook additional experiments to assess the expression levels of genes known to be highly specific for liver allograft rejection (*21*). These genes were consistently up-regulated in Non-TOL recipients at the time of rejection (Fig. 4C), but, with the exception of *CD8*, *CENPM*, and *TK1*, they were not differentially expressed between TOL and Non-TOL samples before the initiation of IS weaning. Likewise, most of the IFN-I–related genes maintained stable expression levels during the weaning protocol, and only *STAT1*, *IRF7*, *IRF9*, and *JAK1* were up-regulated at the time of rejection (Fig. 4C). Tolerance and rejection were therefore largely distinct transcriptional entities. To investigate the differences in intrahepatic gene expression between transplanted and nontransplanted HCV-infected patients, we compared preweaning liver tissue samples from all enrolled transplant recipients with age-matched nontransplanted HCV-infected patients. ISG transcript levels were similar in transplanted and nontransplanted individuals (Fig. 4D). Conversely, the expression of rejection-associated genes was significantly inhibited in the liver tissue samples collected from transplanted recipients receiving maintenance IS (Fig. 4D).

HCV-infected operationally tolerant liver recipients exhibit an expansion of immune-exhausted HCV-specific $CD8^+$ T lymphocytes

Chronic HCV infection is known to increase the number of $CD8^+$ T cells expressing inhibitory receptors such as PD1, CTLA4, and TIM3. These cells are typically defined as

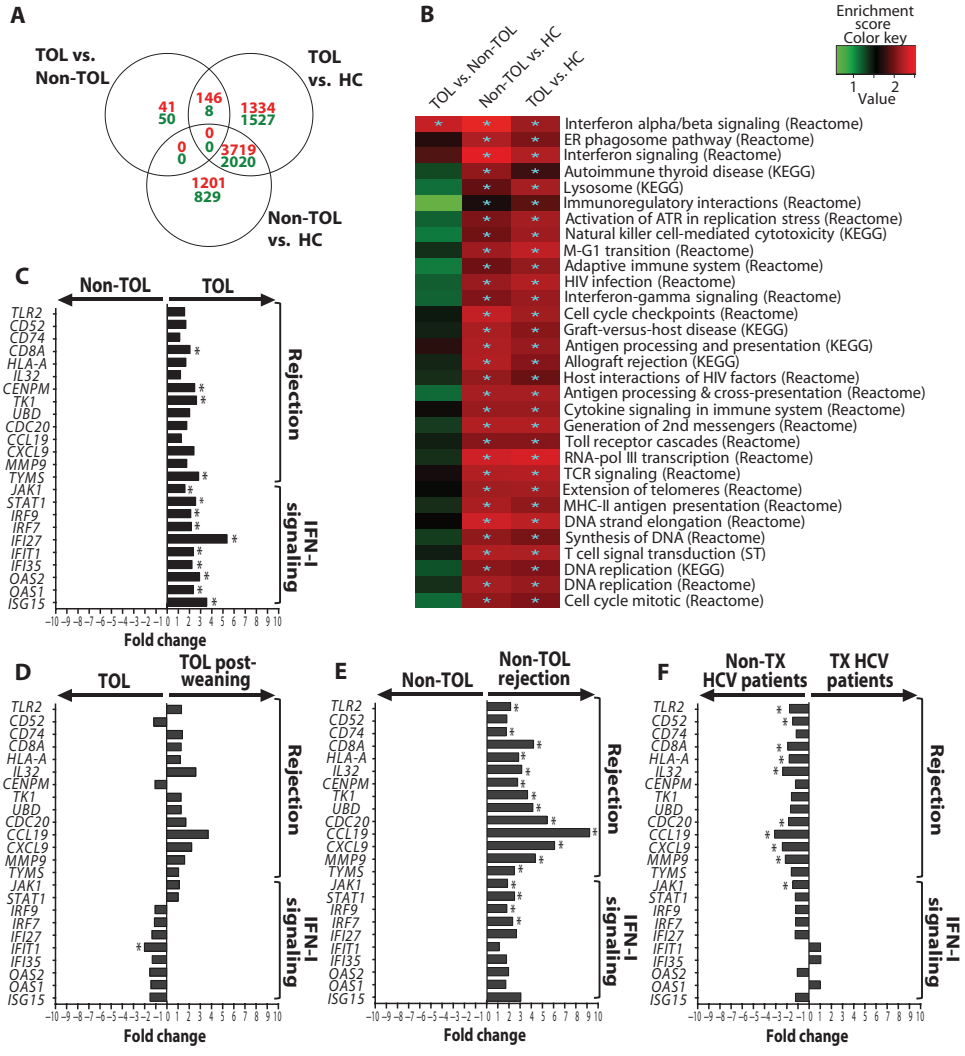

Fig. 4. Liver tissue samples from operationally tolerant and nontolerant recipients differ in the expression of IFN-I–stimulated genes but not in proinflammatory transcriptional markers. (**A**) Venn diagram displaying the number of significantly overexpressed (in red) or underexpressed (in green) genes at FDR <5% when comparing liver tissue samples obtained from TOL (n = 12) and Non-TOL (n = 13) HCV-infected liver recipients before the initiation of IS weaning and from a group of healthy individuals (HC; n = 8). (**B**) Heatmap showing the enrichment score of the 31 transcriptional pathways significantly overrepresented in the list of genes differentially expressed between the liver tissue samples from HCV-negative healthy individuals (HC) and from HCV-positive TOL and Non-TOL recipients. Rows represent transcriptional pathways obtained from MSigDB V.4.0, and columns represent the three different comparisons performed. The intensity of each color is proportional to the magnitude of the enrichment score as computed by GSEA. Asterisks denote statistical significance at FDR <1%. (**C**) Liver tissue expression differences in IFN-I– and rejection-related genes between TOL (n = 13) and Non-TOL (n = 14) recipients at baseline (before initiation of drug withdrawal). (**D**) Liver tissue expression differences in IFN-I– and rejection-related genes between TOL recipients at baseline (n = 13) and 1 year after complete drug withdrawal (n = 16). (**E**) Liver tissue expression differences in IFN-I– and rejection-related genes between Non-TOL recipients at baseline (n = 14) and at the time of rejection (n = 7). (**F**) Liver tissue gene expression differences between TOL and Non-TOL recipients at baseline (TX HCV patients; n = 27) and a group of matched nontransplanted patients with chronic HCV infection (Non-TX HCV patients; n = 16). Bar plots display fold change differences in transcript levels. Individual P values and raw data for (C) to (F) are displayed in table S5. Student's t test was used for all comparisons. *P < 0.05.

exhausted because they show decreased proliferative capacity that can be reversed after inhibitory receptor blockade. The number of exhausted CD8$^+$ T cells positively correlates with the degree of intrahepatic IFN-I expression and is associated with failure to respond to IFN-based antiviral therapy (*31*). To elucidate whether these effects could be involved in the establishment of operational tolerance as well, we analyzed the immunophenotype of PBMCs sequentially collected during the performance of the IS withdrawal trial. TOL recipients exhibited a decrease in the proportion of circulating Vδ2-positive T cells (Fig. 5A) and an expansion of terminally differentiated CD8$^+$ T cells (CD8$^+$ CD45RA$^+$ CCR7^{low}; Fig. 5B). In addition, TOL recipients exhibited higher numbers of circulating CD8$^+$ T cells coexpressing CTLA4/PD1 and CTLA4/2B4 than Non-TOL patients (Fig. 5, C and D). No changes in other lymphocyte subsets were noted. More detailed experiments were undertaken next to define the frequency and phenotype of HCV-specific CD8$^+$ T cells. A small but significant increase in tetramer-positive CD8$^+$ T cells was noted in TOL recipients (Fig. 5E). Furthermore, when using HCV peptide stimulation followed by IFNγ intracellular staining, an increase in HCV-specific IFNγ-producing CD8$^+$ T cells coexpressing 2B4, PD1, and CTLA4 was noted in TOL recipients (Fig. 5F).

Before IS discontinuation, HCV-positive, but not HCV-negative, operationally tolerant recipients overexpress immunoregulatory markers in the liver allograft

To determine whether differences in the expression of immune exhaustion and/or negative immunoregulatory receptors were noticeable in the liver as well, we conducted additional real-time PCR experiments in liver tissue samples collected before the initiation of IS weaning from TOL and Non-TOL recipients. The transcript levels of *IL10*, *PD1*, *PDL1*, *BATF*, *TGFB*, and *Foxp3* were significantly higher in TOL than in Non-TOL liver allografts (Table 1). Parallel experiments were performed in pre-weaning liver tissue samples collected from 26 TOL and 35 Non-TOL HCV-negative patients enrolled in a previously reported drug withdrawal trial (*19*–*21*). Direct comparison of liver tissue samples from HCV-positive and HCV-negative recipients revealed that the increased expression of immunoregulatory

genes was only observed in the liver tissue of tolerant recipients with active HCV infection (Table 3). In contrast, differences in the expression of iron-regulatory genes (for example, *HAMP*, *TFRC*, *HFE2*, and *MCOLN1*) were only noted in HCV-negative, and not in HCV-positive, TOL and Non-TOL recipients (Table 3).

DISCUSSION

After liver transplantation, HCV infection universally recurs and induces chronic hepatitis in the new graft. As a result, recipient T cells infiltrate the graft and accumulate in the portal tracts, where they come in close contact with endothelial and biliary cells expressing HLA class I and II molecules. How liver allografts heavily infiltrated by allogeneic T cells avoid rejection and occasionally develop transplantation tolerance remains a key unanswered question in liver immunobiology. The clinical trial and translational research protocol reported here constitute the first attempt to unambiguously address this issue.

In response to HCV, both parenchymal and nonparenchymal liver cells secrete IFN-I (IFNα/β). This induces the expression of hundreds of ISGs, which can exert powerful antiviral effects. In chronically infected patients, however, HCV persists in the face of vigorous ongoing IFN-I signaling. Chronic HCV infection is also associated with dysfunctional HCV-specific T cell responses. Several mechanisms appear to be involved in this phenomenon, such as expression of inhibitory receptors by $CD8^+$ T cells, leading to exhaustion and apoptosis, induction of $Foxp3^+$ regulatory T cells, and inhibition by interleukin-10 (IL-10) and other immunosuppressive molecules (*22*). Dysfunctional HCV-specific T cells are detectable in blood, but they preferentially accumulate in the liver, where their function is more severely impaired (*32*).

The current study has uncovered an association between liver allograft tolerance, heightened intrahepatic ISG and immunoregulatory transcript levels, and increased number of circulating immune-exhausted HCV-specific $CD8^+$ T cells. Although a causal link between high intrahepatic ISG expression and HCV-induced immunoregulation has not been established, the connection is supported by data derived from the murine lymphocytic choriomeningitis virus infection model (*33*, *34*), in which chronic IFN-I signaling directly induces $CD8^+$ T cell immune exhaustion, inhibits antiviral adaptive immune responses, and promotes viral persistence. Further support is derived from the observation that, in humans, failure to clear HCV after IFN-based therapy positively correlates with the magnitude of intrahepatic ISG expression and the pool size of exhausted circulating $CD8^+$ T cells (*31*, *35*, *36*). On the basis of these data, we hypothesize that high intrahepatic IFN-I signaling induced by HCV infection causes T cell exhaustion, inactivates allospecific T cell clones, and promotes a tolerogenic liver microenvironment that facilitates successful discontinuation of IS and operational tolerance. This series of events appears to be elicited by HCV infection, because it is not apparent in the allografts of HCV-negative tolerant liver recipients (*21*). The mechanisms mediating this phenomenon remain undefined, but they may involve cross-reactivity between HCV-specific T cells and allo-HLA molecules [already described for viruses such as cytomegalovirus (CMV) and EBV, but not yet for HCV], linked suppression, or antigen nonspecific mechanisms dependent on cytokines or essential metabolic compounds (*37*). Given that the frequency of tolerant patients among HCV-infected recipients has not been reported to be higher than in HCV-negative patients (*29*), we speculate that the HCV-induced tolerance-promoting mechanisms are only robust enough to ensure successful drug withdrawal in a minority of patients (that is, those with the highest levels of IFN-I signaling). The fact that HCV-specific T cells constitute a very small percentage of the total T cell pool, but typically accumulate in the infected liver (*32*), probably explains why HCV-infected recipients exhibit no signs of general over-immunosuppression and are capable of mounting effective immune responses against extrahepatic pathogens.

Our findings challenge a well-entrenched notion, namely, that inflammatory responses and antiviral heterologous immunity are necessarily detrimental for the establishment of allograft tolerance (*2*, *3*, *5*, *38*). In contrast to what would have been anticipated from experimental animal studies, the development of operational tolerance occurred in the presence of, and independently from, vigorous HCV-induced pro-inflammatory reactions, detectable both in the liver and systemically. Furthermore, the magnitude of anti-HCV T cell responses did not influence the outcome of drug withdrawal. This was not restricted to anti-HCV responses, which are typically very weak after liver transplantation. Similar results were observed for anti-EBV and anti-CEF

Table 2. Molecular pathways overrepresented in the intragraft expression profiles of operationally tolerant recipients. Data correspond to the 10 most significant pathways as assessed by GSEA.

Pathway/function	FDR	Genes with highest enrichment scores
IFNA response	0.001	*RTP4, IFIT1, OAS1, OAS2, MX1, STAT1, EPSTI1, IFIT3, IFITM1, DDX60, IFI44*
IFN-responsive genes	0.001	*XAF1, GMPR, ISG15, TRX1, IFI27, PML, IFI6, IFI35, OAS1, BST2, UBE2L6, OAS2*
Response to tamoxifen	0.001	*ISG15, IFIT1, IFI27, IFI6, IFI35, OAS1, OAS2, MX1, STAT1, IRF7, IFIT3, IFITM1*
Response to extracellular matrix	0.001	*ISG15, IFIT1, IFI6, IFI35, OAS1, MX1, STAT1, PSMB8, IRF7, IFIT3, PSMB10*
Targets of mutated TP53	0.001	*XAF1, ISG15, IFIT1, IFI6, IFI35, DTX3L, OAS1, BST2, MX1, UNG, STAT1, IRF7, TSC22D4*
IFN response	0.001	*ISG15, RTP4, IFI27, OAS1, OAS2, MX1, STAT1, IFITM1, IRF9, IFI44, ISG20, OAS3*
Genes up-regulated in pancreatic cancer decitabine treatment	0.001	*TMEM184B, SLPI, HIST1H2BK, GSN, MAP2K3, WFDC2, RRBP1, CDKN1A, ISG15, IFIT1, LGALS9.*
Response to imiquimod	0.001	*ISG15, IFI6, IFI35, OAS1, OAS2, MX1, STAT1, MICB, IRF7, IFITM1, ISG20, OASL, CCL8*
Response to IFNG	0.001	*RNF213, LGLS3BP, SERPING1, LGALS9, IFI35, DTX3L, OAS1, BST2, UBE2L6, OAS2, MMP25*
Systemic lupus erythematosus	0.001	*XAF1, ISG15, LGALS3BP, SERPING1, AGRN, IFI35, OAS1, OAS2, MX1, STAT1, LY6E*

cellular immune responses. In one patient, however, IS withdrawal elicited immune reconstitution and significantly increased antiviral T cell responses, and ultimately resulted in rejection. Together, these results indicate that the effects of viral-induced immune responses on transplantation tolerance cannot be easily predicted in the clinic, because they are likely to differ among individual patients, and to vary depending on the type of pathogen involved, the magnitude of the immunoregulatory effects exerted by persistent infections, the time between exposure and tolerance induction, and, most important, the type of graft and whether the graft itself is infected by the pathogen. Additional clinical studies will be required to address these questions because there are currently no animal models capable of replicating this specific clinical scenario.

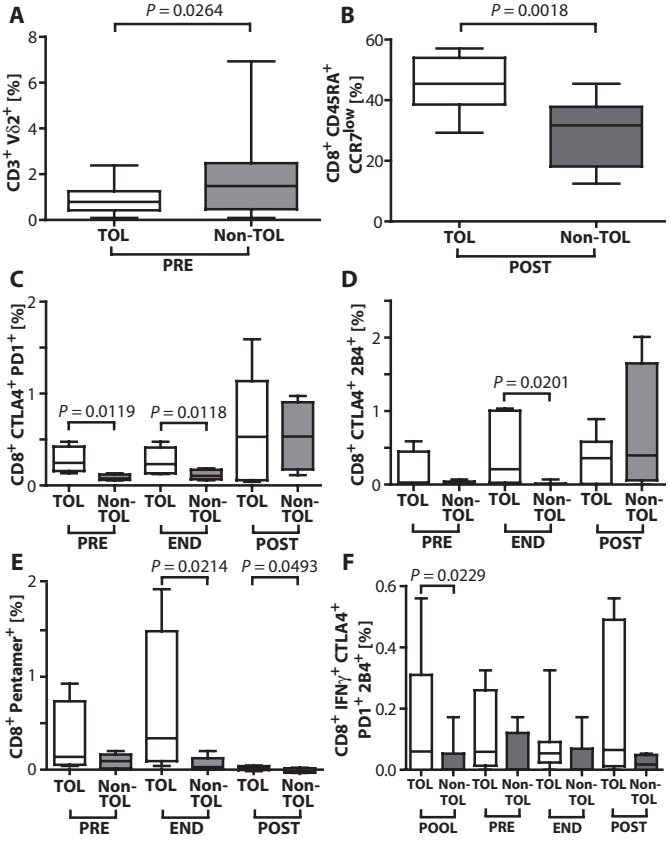

Fig. 5. Operationally tolerant recipients exhibit higher numbers of circulating HCV-specific and immune-exhausted CD8$^+$ T cells than nontolerant recipients. (**A**) Bar plots showing the proportion of blood CDd3$^+$Vδ2$^+$ T cells in TOL (n = 17) and Non-TOL (n = 14) recipients before the initiation of IS weaning (PRE). (**B**) Bar plots showing the proportion of blood CD8$^+$CD45RA$^+$CCR7^{low} T cells in TOL (n = 11) and Non-TOL (n = 9) recipients at the end of the study (POST). (**C** to **F**) Bar plots showing the proportion of blood CD8$^+$CTLA4$^+$PD1$^+$ T cells, CD8$^+$CTLA4$^+$2B4$^+$ T cells, CD8$^+$Pentamer HCV$^+$, and CD8$^+$IFNγCTLA4$^+$PD1$^+$2B4$^+$, respectively, in TOL and Non-TOL before the initiation of IS weaning (PRE; TOL, n = 4; Non-TOL, n = 5), at the time TOL patients completely discontinued IS or Non-TOL rejected (END; TOL, n = 7; Non-TOL, n = 7), and at the end of the study 12 months after complete IS discontinuation or after rejection (POST; TOL, n = 8; Non-TOL, n = 4). Student's t test was used for all comparisons.

From a clinical standpoint, the current study has relevant implications as well. It shows that it is possible to use blood cell phenotypic markers to select liver recipients for drug withdrawal protocols. The association between changes in blood Vδ1/Vδ2 T cell ratio and operational tolerance had been previously described, but ours constitutes the first attempt at using this marker to prospectively select patients for IS withdrawal. The predictive performance of Vδ1/Vδ2 T cell ratio is in agreement with what had been previously described in retrospective case-control studies and in our recently published prospective IS withdrawal trial (*20*, *21*, *39*). Conversely, the performance of *SLAMF7* and *KLRF1* blood transcript levels was clearly suboptimal. Although this appears to be in contrast with the results of our previous IS withdrawal trial, blood transcriptional markers had, in fact, very poor overall reproducibility, and acceptable predictive capacity was only observed in only one of the three participating clinical sites (*21*). Note that in contrast to what we have described for HCV-negative patients (*17*, *21*), iron-related markers were not useful in predicting successful drug withdrawal in HCV-infected recipients. This could reflect the fact that HCV directly inhibits hepcidin expression (*40*), and suggest that different mechanisms can lead to the establishment of allograft tolerance in human liver recipients. Although our results suggest that intrahepatic ISG expression could constitute a biomarker of tolerance in HCV-infected liver recipients, this will need to be validated in future independent cohorts, because our trial was designed as a mechanistic study and was not powered to address this issue. The lack of clinical benefits derived from IS withdrawal is to be expected considering the small number of patients and short follow-up period. Finally, the association between lower ISG levels and rejection explains why HCV-infected liver recipients who reject their grafts during the course of IFN-based treatment are typically those who experience virological response (that is, those with low ISG transcript levels at baseline). Similarly, the observation that maintenance IS modifies the expression profile of HCV-infected grafts by specifically inhibiting genes associated with rejection could account for the fact that these grafts are seldom rejected, despite being heavily infiltrated by recipient T cells, unless IS drugs are discontinued.

Our study has limitations derived from its small sample size and relatively short patient follow-up. Thus, whether IS withdrawal influences HCV-induced liver damage cannot be firmly established. Similarly, the interactions between HCV infection and clinical parameters such as recipient age and time after transplant, known to influence the development of tolerance in HCV-negative recipients (*17*), cannot be elucidated here. Demonstration of a causal link between intrahepatic IFN-I signaling, T cell exhaustion, and allograft tolerance would have required an analysis of the function and allospecificity of intrahepatic T cells, which was not originally envisaged within the current clinical trial. Finally, whether our findings can be extrapolated to other transplantation settings remains to be studied.

Despite these limitations, we provide here the first description of how innate and adaptive antiviral immune responses influence allograft tolerance in humans. The data reported here indicate that in the clinic, liver allograft tolerance can be established even in the presence of an ongoing viral infection. Our results significantly advance our understanding of HCV immunobiology after liver transplantation and suggest for the first time that, within the liver microenvironment, mechanisms used by HCV to evade adaptive immunity and establish a persistent infection may be responsible as well for restraining alloimmune responses during the establishment of operational tolerance.

Table 3. Intrahepatic expression of operational tolerance-related markers in HCV-positive and HCV-negative recipients. ns, not significant.

	TOL HCV$^+$ versus Non-TOL HCV$^+$*	TOL HCV$^-$ versus Non-TOL HCV$^-$*	TOL HCV$^+$ versus TOL HCV$^-$*	Non-TOL HCV$^+$ versus Non-TOL HCV$^-$*
TGFB	0.040 (1.75)	ns	ns	ns
IL10	0.039 (1.62)	ns	0.005 (1.73)	ns
IL10RB	0.036 (1.43)	ns	0.001 (1.59)	ns
PDCD1 (PD1)	0.023 (2.55)	ns	0.001 (3.92)	ns
CD274 (PDL1)	0.047 (1.45)	ns	0.001 (2.01)	ns
FOXP3	0.038 (2.63)	ns	0.003 (2.80)	ns
BATF	0.032 (1.78)	ns	0.001 (2.17)	ns
DDX58 (RIG1)	0.025 (1.87)	ns	0.001 (3.74)	0.03 (1.68)
SOCS1	0.014 (1.82)	ns	0.001 (3.00)	ns
NKG2D	0.048 (1.40)	ns	0.001 (1.71)	ns
NKG2A	ns	ns	ns	0.003 (−6.18)
CDHR2	ns	0.005 (2.03)	0.005 (2.73)	ns
GZMB	ns	<0.001 (2.08)	ns	ns
PRF1	ns	0.021 (1.38)	0.004 (1.66)	ns
MIF	ns	0.009 (−1.47)	ns	ns
TFRC	ns	0.001 (−1.65)	ns	0.026 (1.51)
HAMP	ns	0.006 (2.85)	ns	ns
SH2D1B	ns	0.019 (1.57)	ns	ns
MCOLN1	ns	0.018 (1.41)	ns	ns
HFE2	ns	0.01 (−1.52)	ns	ns
ADORA3	ns	0.012 (1.90)	ns	0.008 (−2.42)
TNFA	ns	0.013 (1.47)	0.007 (1.86)	ns
LAG3	ns	ns	0.001 (2.07)	ns
IFNG	ns	ns	0.005 (2.08)	ns
PDCD1LG2 (PDL2)	ns	ns	0.022 (1.35)	ns
TNFSF10 (TRAIL)	ns	ns	0.001 (1.50)	0.029 (1.34)
TLR7	ns	ns	0.023 (1.71)	ns
TLR3	ns	ns	0.009 (1.61)	ns
TLR9	ns	ns	0.009 (1.66)	ns
CTLA4	ns	ns	0.003 (2.51)	ns
IL28B	ns	ns	0.001 (24.7)	0.004 (11.6)

*Values are *P* values (fold change).

METHODS

Study design

This was a prospective open-label noncontrolled nonrandomized interventional phase 2 trial in which adult HCV-infected stable liver transplant recipients underwent IS drug withdrawal. Inclusion criteria were as follows: liver transplantation for HCV-related liver disease performed at least 3 years before enrolment, positive HCV RNA, no indication for anti-HCV treatment, favorable inclusion biological profile (high blood Vδ1/Vδ2 T cell ratio and/or elevated *SLAMF7*/*KLRF1* transcript levels), no history of autoimmune liver disease, absence of graft rejection during the previous 12 months, and stability of liver function tests. Exclusion criteria were as follows: age <18 years, pregnancy, alcohol or illicit drug abuse, psychiatric or medical illnesses considered incompatible with the safe conduct of the study (for example, kidney failure, cardiac failure, chronic obstructive pulmonary disease, cancer, and major depression), baseline liver biopsy exhibiting signs of acute or chronic rejection according to Banff criteria or fibrosis Metavir stage III to IV, HIV co-infection, and combined liver-kidney transplantation. IS drugs were gradually decreased until complete discontinuation over 6 to 9 months. Patients were then followed up for 12 additional months. The primary endpoint was the successful withdrawal of all IS with maintenance of stable allograft status, as assessed by liver biopsy and liver tests 12 months after complete drug withdrawal. Patients were

followed up for a total of 36 months after the complete discontinuation of IS or rejection. Rejection was diagnosed by the combination of liver function impairment and characteristic liver biopsy findings according to Banff criteria (*41*). Recipients were enrolled from Hospital Clínic (Barcelona) and Hospital Universitario La Fe (Valencia) (ClinicalTrials.gov identifier: NCT00668369).

Patient screening

Measurement of peripheral blood Vδ1/Vδ2 T cell ratio was performed by flow cytometry on whole blood samples. Antibodies were added to the sample and after 15 min at room temperature in the dark erythrocytes were lysed (FACS Lysing Solution, Becton Dickinson) and cells were washed and fixed in 1% paraformaldehyde/phosphate-buffered saline. A receiver operating characteristic analysis of previous retrospective data obtained in a cohort of 16 tolerant and 16 nontolerant recipients (*20*) resulted in an optimal cutoff ratio of 2.33, which, assuming a conservative theoretical prevalence of operational tolerance of 20%, would yield a PPV for tolerance of 46% and an NPV of 90%. Measurement of *SLAMF7* and *KLRF1* transcript levels in PBMCs was performed using a quantitative gene expression real-time PCR platform. Briefly, after RNA extraction (phenol/chloroform, Sigma), DNA was removed from total RNA preparations using TURBO DNA-free DNase treatment (Ambion) and samples were reverse transcribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Real-time PCR was performed using primer/probe combinations for *SLAMF7*, *KLRF1*, and *HPRT1* (TaqMan Gene Expression Assays, Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). *SLAMF7* and *KLRF1* C_t values were normalized to *HPRT1* to generate ΔC_t values. Results were then computed as relative expression between complementary DNA (cDNA) of the target samples and a calibrated sample according to the $\Delta\Delta C_t$ method. A mathematical algorithm derived from preceding studies was used to define a cutoff for increased probability of operational tolerance based on the expression pattern of these genes. When assessed in a retrospective cohort of 28 tolerant and 33 nontolerant recipients (*19*), the combination of HPRT1-normalized *SLAMF7* and *KLRF1* expression discriminated between tolerant and nontolerant recipients with an error rate of 6.45% and an SN, SP, PPV, and NPV of 93.33, 93.79, 93.33, and 93.75%, respectively. Patients were included in the IS withdrawal protocol if they exhibited at least one of the two markers.

Biological samples

Blood specimens were collected at baseline (before initiating the IS withdrawal protocol), at the time of complete discontinuation of IS (in TOL patients), at the time of rejection (in Non-TOL patients), and 12 months after complete IS discontinuation or after the rejection episode. PBMCs were isolated by Ficoll-Hypaque gradient, cryopreserved in freezing medium or lysed in TRIzol (Invitrogen) reagent, and stored at −80°C for RNA extraction. Liver biopsies were obtained from enrolled patients at baseline, at the time of rejection (in Non-TOL patients), and 12 and 36 months after complete IS discontinuation (in TOL patients). A 2- to 3-mm portion of all liver tissue cylinders was stored in RNAlater solution (Ambion) immediately after liver biopsy was performed, and subsequently cryopreserved at −80°C for gene expression experiments.

RNA extraction from liver tissue and PBMC samples

Cryopreserved liver tissue samples were homogenized in TRIzol reagent (Invitrogen) using pestle and nuclease-free 1.5-ml reaction tubes (Ambion). Total RNA was then extracted following the manufacturer's guidelines, and quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies). The same procedure was used to extract RNA from Ficoll-isolated PBMCs.

Liver tissue microarray experiments

Microarray experiments were performed using Illumina HumanHT-12 v4 Expression BeadChip arrays, covering 47,000 probes derived from the National Center for Biotechnology Information (NCBI) RefSeq database, on RNA extracted from the following liver tissue samples: TOL at baseline (n = 12), Non-TOL at baseline (n = 13), Non-TOL at the time of rejection (n = 4), TOL 12 months after IS discontinuation (n = 14). Additionally, eight liver tissue samples obtained from healthy living liver donors undergoing partial hepatectomy were included as nontransplanted controls. RNA samples were processed, hybridized onto BeadChip arrays, and analyzed using a BeadArray Reader (Illumina). Microarray expression data were computed using BeadStudio data analysis software (Illumina) and subsequently processed using quantile normalization using the Lumi Bioconductor package. A conservative probe-filtering step was conducted next to exclude probes with a coefficient of variation >5%, which resulted in a list of 27,955 evaluable probes, corresponding to 20,815 genes. To identify genes differentially expressed between liver tissue samples from TOL and Non-TOL recipients, we used Significant Analysis of Microarray (SAM) (*42*). The complete list of differentially expressed genes with false discovery rate (FDR) <25% and fold change >1.5 can be found at http://bioinfo.ciberehd.org/asf2013. To assess the deregulation of sets of genes associated with specific functional pathways, we computed an enrichment score for each of the gene sets included in the Molecular Signatures Database (MSigDB; www.broadinstitute.org/gsea/msigdb/index.jsp) using the GSEA method (*43*, *44*). The enrichment score reflects the degree to which a gene set is overrepresented at the extremes (top or bottom) of the entire ranked gene list (the entire filtered 20,815 gene list ranked according to SAM). All liver tissue microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO; accession number GSE52420).

Quantitative real-time PCR experiments

Quantitative real-time PCR gene expression experiments were performed using a Fluidigm BioMark HD system. DNA was removed from total RNA preparations using TURBO DNA-free DNase treatment (Ambion), and RNA was then reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). A preamplification step was performed as per Fluidigm BioMark HD protocol. To quantify transcript levels, target gene C_t values were normalized to the housekeeping genes to generate ΔC_t values. The results were then computed as relative expression between cDNA of the target samples and a calibrated sample according to the $\Delta\Delta C_t$ method. A total of 91 target genes (plus 3 housekeeping genes) was quantified in 148 liver tissue samples, which included specimens obtained before the initiation of IS weaning (13 TOL and 14 Non-TOL), at the time of rejection (7 Non-TOL), and 12 months after complete IS discontinuation (16 TOL). In addition, we included the following comparison groups: (i) liver tissue samples collected from nontransplanted HCV-infected patients matched for age, gender, and histological HCV activity/staging (n = 16), and (ii) liver tissue samples collected before the initiation of IS weaning in HCV-negative liver transplant recipients (26 Non-TOL and 35 TOL). HCV-negative TOL and Non-TOL recipients were patients who had been enrolled in a previously conducted trial of

IS withdrawal (*17*) and who had followed an identical IS withdrawal clinical protocol as the one described here. In addition to the liver tissue experiments, we quantified the expression of 45 target genes (and 3 housekeeping genes) in PBMC samples collected before the initiation of IS weaning (15 TOL and 14 Non-TOL), at the time of completely discontinuing IS (12 TOL), at the time of rejection (9 Non-TOL), and 12 months after the complete discontinuation of IS or rejection (11 TOL and 8 Non-TOL). PBMC samples from a group of 17 HCV-negative age- and gender-matched healthy individuals were also analyzed.

IFNγ ELISpot assays

Cryopreserved sequential PBMC samples collected at baseline, end of withdrawal/rejection, and after completion of the follow-up period were thawed at 37°C and diluted stepwise (1:1 every 2 min) in RPMI medium. Cells were incubated for 2 hours to rest and subsequently washed in fresh medium, counted, and diluted to a final concentration of 2×10^5 cells per well in an ELISpot 96-well microtiter plate precoated overnight with anti-IFNγ monoclonal antibody. Cells were challenged with HCV peptide pools containing 7 to 33 13- to 18-mer peptides overlapping by 11 to 12 amino acids at a final concentration of 8 μg/ml. Overlapping peptides covered the most immunogenic proteins of HCV (core, NS3, NS4a, NS4b, NS5a, and NS5b) for genotypes 1 and 3, subtypes 1a (H77), 1b (HC-J4), and 3a (K3a/650). HCV peptides were provided by the National Institute of Allergy and Infectious Diseases, Biodefense and Emerging Infections Research Resources Repository (BEI Resources). To detect anti-EBV responses, we used a library of HLA-restricted EBV epitopes selected on the basis of patients' HLA genotype and used at final concentrations of 12 μg/ml. These included 11 epitopes restricted by HLA-A*02, 1 for HLA-A*03, 3 for HLA-A*11, 3 for HLA-A*24, 3 for HLA-B*08, 1 for HLA-B*18, 3 for HLA-B*35, 3 for HLA-B*44, and 2 for HLA-Cw*6 (*45*). As positive controls, we included the CEF-antigen pool (containing highly immunogenic peptides derived from CMV, EBV, and influenza virus; National Institutes of Health Repository) and phytohemagglutinin. A peptide pool partially covering the GAG protein of HIV was used as a negative control. The threshold of positive signals was calculated as the value of the negative control plus two times the SD as described (*46*).

Flow cytometry immunophenotyping

For immunophenotyping studies, fresh whole blood samples sequentially collected before initiation of IS withdrawal (baseline), at the end of IS withdrawal or at the time of rejection, and after completion of the 12-month follow-up period were stained with the following fluorochrome-conjugated antibody combinations: CD4/CD8/CD45RA/CD62L/CCR7 (naïve/memory), CD3/CD16/CD56 [natural killer (NK)/NKT cells], CD4/CD25/CD45RO/CD127/FOXP3 (regulatory T cells), αβTCR/γδTCR/Vδ1/Vδ2 (αβ and γδ lymphocyte subsets), and CD19/HLA-DR (B cells). All antibodies were purchased from Becton Dickinson, except for antibodies against Vδ1 (Thermo Scientific) and against Vδ2 (Beckman Coulter). Samples were analyzed using a FACSCanto II cytometer, and fluorescence-activated cell sorting (FACS) data were analyzed using FlowJo software (Tree Star). Statistical significance was assessed using GraphPad Prism software.

Quantification of HCV-specific exhausted T cells

Cryopreserved PBMC samples sequentially collected before initiation of IS withdrawal (baseline), at the end of IS withdrawal or at the time of rejection, and after completion of the 12-month follow-up period were gradually thawed and rested overnight for 16 hours. Viable cells (10^6) were either stimulated with HCV peptide pools containing overlapping NS3 and NS5a peptides or left unstimulated. PBMCs were first stained with Fixable Near Infrared LIVE/DEAD Cell Stain Kit to discriminate dead cells (Invitrogen), and subsequently incubated with the exhaustion antibody panel containing the following fluorochrome-conjugated antibodies: CD3 (Beckton Dickinson), CD4 (Beckman Coulter), CD8 (eBioscience), 2B4 (eBioscience), CTLA4 (Biozol), PD1 (eBioscience), and Tim3 (eBioscience). Cells stimulated with HCV peptides were fixed and permeabilized using the Cytofix/Cytoperm solution, washed, and stained for intracellular IFNγ using an IFNγ-Al700 antibody (Becton Dickinson). The percentage of marker-positive cells was computed after subtracting the results obtained with the unstimulated controls, assigning "0" to samples with negative results. For each experiment containing two to seven samples, a full set of compensation controls was applied. Additional experiments were conducted by directly staining viable cells with fluorochrome-conjugated HCV-specific pentamers (HLA-A*02:01–restricted NS3-Pentamer CVNGVCWTV; ProImmune). All experiments were performed on an LSRFortessa cytometer (Becton Dickinson).

Immunostaining of liver sections

For quantification of intrahepatic lymphocytes, up to three formalin-fixed, paraffin-embedded sequential sections were analyzed per patient. After deparaffinization and antigen retrieval, the following antibodies and reagents were used: mouse anti-human CD4 (clone BC/1F6), rabbit anti-human CD8 (clone SP16) (both from Abcam), goat Cy5-conjugated anti-mouse (115-176-071, Jackson ImmunoResearch), goat Alexa Fluor 488–conjugated anti-rabbit (A11034, Invitrogen), rat biotin-conjugated anti-human Foxp3 (PCH101, eBioscience), Cy3-conjugated streptavidin (016-160-084, Jackson ImmunoResearch), and 4′,6-diamidino-2-phenylindole. Blocking, staining, and washing steps were performed with tris-buffered saline with 0.05% Tween 20 at room temperature. To avoid unspecific binding, we blocked first with goat serum (5%) and before the biotinylated antibody and streptavidin separately with avidin (0.01%), biotin (0.005%), and mouse serum (5%). Fluorescence microscopy was carried out with an Axio Imager.M1 at a magnification of ×200 (Carl Zeiss). Evaluation of portal infiltration and quantification of $CD4^+$, $CD8^+$, and $Foxp3^+$ cells were performed with AxioVision 4.6 software (Carl Zeiss MicroImaging) in a blinded fashion.

Statistical analyses

Statistical significance was analyzed using GraphPad (GraphPad Software Inc.), and $P < 0.05$ was considered statistically significant. Normal distribution of samples was determined using Kolmogorov-Smirnov, D'Agostino-Pearson omnibus, and Shapiro-Wilk normality test. Upon normal distribution, an unpaired Student's *t* test was used; in other cases, a nonparametric Mann-Whitney test was used. If not stated differently, data are presented as means ± SEM.

Study approval

The study was approved by the Institutional Review Boards of the two participating institutions, and written informed consent was obtained from all study patients.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/6/242/242ra81/DC1
Fig. S1. Intrahepatic T cell subset infiltration.

Table S1. Biochemical and virological parameters of evaluable patients (per protocol).
Table S2. Central review of liver histopathology findings obtained during the course of the study (per protocol).
Table S3. Results of screening marker analyses.
Table S4. Blood gene expression differences between operationally tolerant and nontolerant liver recipients.
Table S5. Differences in IFN-I–related and rejection-associated transcript levels.

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