Comprehensive Phenotyping of Regulatory T Cells After Liver Transplantation

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Regulatory T cells (Tregs) play an important role in controlling alloreactivity after solid organ transplantation, but they may also impair antiviral immunity. We hypothesized that the Treg frequency and the Treg phenotype are altered in hepatitis C virus (HCV)–infected recipients of liver transplantation (LT) with possible prognostic implications. Tregs from 141 individuals, including healthy individuals, LT recipients with or without persistent HCV infections, and nontransplant patients with chronic HCV, were studied. A comprehensive phenotypic analysis was performed with multicolor flow cytometry, which included standard Treg markers [CD4$^+$, CD25^{hi}, CD127$^-$, and FoxP3$^+$ in addition to HLA DR, CCR7, CD45RA, CD62L, CD49d, CD39, ICOS and LAP-TGFβ stainings. Healthy individuals and LT patients displayed similar Treg frequencies and largely comparable Treg phenotypes, which were stable over time after transplantation. In contrast, Tregs with a CD45RA$^-$CCR7$^-$ effector phenotype were enriched in LT recipients with chronic HCV versus HCV-negative transplant patients. HCV infection, rather than LT, altered the expression of functional markers on Tregs. A principal component analysis revealed distinct Treg phenotypes in HCV-infected LT recipients with rejection and patients with recurrent graft HCV. In conclusion, Treg phenotypes are altered in HCV-infected LT patients. An investigation of Tregs may possibly help to distinguish recurrent HCV from graft rejection. Further functional studies are needed to define the role of Tregs in determining the balance between antiviral and allogenic immunity. *Liver Transpl 21:381-395, 2015*. © 2015 AASLD.

Liver transplantation (LT) is a well-established treatment option for end-stage liver diseases. Since it was first performed in 1963,[1] long-term clinical outcomes have improved significantly; however, no major additional steps forward have been achieved in the most recent decade. Chronic hepatitis C virus (HCV) is the leading cause of LT in the Western world.[2] HCV reinfection of the liver graft is universal.[3] The natural history of HCV after LT is accelerated with higher levels of viremia and more rapid progression to fibrosis and cirrhosis.[4] Graft hepatitis after LT for HCV is, therefore, a clinical challenge, and more in-depth knowledge of the underlying pathogenesis is needed, especially to differentiate between recurrent HCV and rejection of the organ.

Regulatory T cells (Tregs) play a key role in immune homeostasis and are of particular importance after transplantation. Tregs form a subtype of $CD4^+$ cells with a defined immunoregulatory phenotype and are involved in immune tolerance.[5] Tregs are defined as $CD4^+CD25^{hi}FoxP3^+$ cells (where FoxP3 indicates forkhead box P3). In addition, CD127 has been shown to correlate negatively with FoxP3,[6-8] and thus Tregs can also be defined as $CD4^+CD25^{hi}CD127^-$ cells. CD127 has the advantage of being able to identify Tregs with surface staining.

Tregs have been proven to be important in regulating alloreactive immunity after solid organ transplantation in several studies.[9,10] For example, Treg frequencies 1 year after kidney transplantation may be predictive of long-term graft survival.[11] Over time, Tregs tend to decline after transplantation, but their suppressive capacity is maintained or even enhanced against donor antigens.[12] Studies in kidney transplant recipients correlated increased FoxP3 expression in transplanted kidneys with acute rejection episodes,[13] but they described reduced Treg frequencies in peripheral blood during chronic rejection.[14] Similarly, Tregs after bone marrow transplantation have been related to acute and chronic graft-versus-host disease,[15,16] with reduced Treg frequencies possibly being involved in the development of chronic graft-versus-host disease.[17-19]

There are fewer data about the role of Tregs after LT. Some studies have reported elevated Treg frequencies in the blood of patients with tolerant grafts not requiring immunosuppressive medications.[20-22] Not only conventional Tregs but specifically $CD45RA^+$ Tregs with a naive phenotype have been shown to be elevated in tolerant patients after LT.[23] In contrast, decreased frequencies of circulating Tregs have been found during acute rejection episodes in LT patients,[24,25] whereas Tregs seem to be enriched in livers with histological evidence of rejection after pediatric[26] and adult LT.[27]

HCV infection has been shown to be associated with an altered Treg phenotype and function. The frequency of Tregs is elevated in patients with chronic HCV versus recovered and healthy controls,[28] and Tregs are able to suppress HCV-specific T cell responses.[29] In HCV infections after transplantation, Tregs have been suggested to play a role in the progression to fibrosis through the determination of the magnitude of the antiviral immune response.[30,31] Nevertheless, previous studies of the role of Tregs in LT have had various limitations, such as rather small sample sizes, a lack of control groups, and limited phenotypic characterization beyond the standard $CD4^+CD25^{hi}FoxP3^+CD127^-$ Treg classification.

The aim of this study was, therefore, to overcome past constraints and perform a comprehensive phenotypical characterization of Tregs in LT recipients with a particular focus on posttransplant HCV. Specifically, we aimed to determine whether specific Treg characteristics are associated with HCV infection and graft rejection. In-depth knowledge of Tregs is fundamental for understanding how Tregs can prevent graft rejection and for determining their role in graft hepatitis.

PATIENTS AND METHODS

Patient Cohort and Study Design

Overall, 141 individuals with available peripheral blood samples were studied; they included liver transplanted patients (LTx, n = 83), nontransplant patients with chronic hepatitis C (HCV chr patients; n = 24), and healthy controls (n = 34).

All patients undergoing transplantation because of HCV-related liver disease (HCV LTx, n = 35) had a graft reinfection, and none of them received antiviral therapy after transplantation until the time of our study. Patients undergoing LT because of hepatitis B virus (HBV) or alcoholic cirrhosis (non-HCV LTx patients; n = 24) had no signs of recurrence of the initial underlying liver disease and were, therefore, used as controls within the transplant cohort for comparison with HCV LTx patients. The remaining patients (n = 24) underwent transplantation because of various causes of irreversible liver dysfunction (primary biliary cirrhosis, primary sclerosing cholangitis, autoimmune hepatitis, Wilson's disease, acute liver failure, cryptogenic cirrhosis, Budd-Chiari syndrome, polycystic liver disease, cystic fibrosis, and primary hepatocellular carcinoma). Patient populations are shown in Supporting Fig. 2.

In all, 121 peripheral blood samples from LT patients were studied (repeat samples were available from 31 patients). Thirty-five samples were collected in the first 6 months after transplantation, 47 samples were collected 7 to 15 months after transplantation, and the rest (n = 39) were collected from patients undergoing transplantation more than 16 months ago. In 87 cases, liver biopsy was performed on the day of the blood drawing; 49 were conducted per protocol, and the rest (n = 38) were indication biopsies. Biopsy samples were evaluated with respect to rejection or graft HCV by 2 independent pathologists (J.S. and T.K.) according to the rejection activity index.[30]

	TABLE 1. Baseline Characteristics of the LT Patients Who Participated in This Study					
	LT Patients (n = 83)		HCV LT Patients (n = 35)		Non-HCV LT Patients (HBV+C2; n = 24)	
Parameter	n/N or Mean ± SD (Median)	% or Range	n/N or Mean ± SD (Median)	% or Range	n/N or Mean ± SD (Median)	% or Range
Recipient age at LT, years	48.9 ± 10.8 (50)	18-68	51.3 ± 7.3 (51.5)	34-64	53.7 ± 9 (56)	31-68
Sex: male	61/83	73	27/35	77	19/24	79
HCC	40/83	48	25/35	71	12/24	50
Genotype 1 HCV			19/23	83	NA	NA
Non–genotype 1 HCV			4/23	17	NA	NA
Immunosuppression						
Tacrolimus	40/83	48	18/35	51	8/24	33
Cyclosporine A	41/83	49	16/35	46	15/24	62
Steroid (as standard)	51/83	61	14/35	40	19/24	79

TABLE 2. Baseline Characteristics of the HCV-Infected Patients (n = 24)		
Parameter	n/N or Mean ± SD (Median)	% or Range
Age, years	55 ± 14.2 (42)	65-73
Sex: male	16/24	67
Genotype 1 HCV	18/24	75
Non–genotype 1 HCV	4/24	17
Previous antiviral therapy	10/24	42
Albumin, g/L	40 ± 4.5 (42)	30-47
Alanine aminotransferase, IU/L	76 ± 47.4 (65)	11-234
Aspartate aminotransferase, IU/L	63 ± 45 (48)	19-252
Bilirubin, μmol/L	16 ± 26.9 (9)	4-147
Alkaline phosphatase, IU/L	76 ± 20.8 (76)	40-128
Gamma-glutamyltransferase, IU/L	66 ± 53.4 (42)	9-206
Creatinine, μmol/L	75 ± 20.5 (71)	51-157
Platelets, 1000/μL	211 ± 146 (184)	81-887

Standard immunosuppression for the transplant patients consisted of tacrolimus (48%) or cyclosporine A (49%). Sixty-one percent of the patients in all received steroids as part of their standard therapy (40% of HCV LT patients).

Two additional control groups, which consisted of patients with chronic HCV (n = 24) and healthy volunteers (n = 34), were studied. None of the HCV patients were receiving antiviral therapy at the time of the blood drawing.

Baseline characteristics of the LT patients are shown in Table 1, and characteristics of the HCV patients are shown in Table 2. The laboratory values at the time of the blood drawing are shown in Table 3.

Ethics Statement

The study was approved by the ethics committee of Hannover Medical School in Hannover, Germany (record 930-2011), and it conforms with the ethical guidelines of the 1975 Declaration of Helsinki. All patients gave written informed consent to participate in this study. Healthy volunteers were also recruited from Hannover Medical School.

Sample Collection and Cell Isolation

Blood was obtained during the medical examination and was processed on the same day. Peripheral blood mononuclear cells were isolated with a Ficoll density gradient. Peripheral blood mononuclear cells were resuspended in a freezing medium ($15\text{-}20 \times 10^6$/mL) containing 60% fetal bovine serum, 10% dimethyl sulfoxide, and an aliquot in cryotubes. Cryotubes were stored in liquid nitrogen until further analysis.

Flow Cytometry Staining of Tregs

Frozen peripheral blood mononuclear cells were thawed and stained with a master mix of the different antibodies (Table 4) for 15 minute at 4°C in the dark. Afterwards, the wells were washed twice and resuspended in a fluorescence-activated cell sorting (FACS) buffer. For FoxP3 staining, a human FoxP3 staining kit (BD Pharmingen, Franklin Lakes, NJ) was used

TABLE 3. Clinical Parameters at the Time of Blood Samples for the LT Patients

Parameter	LT Patient Samples (n = 121)		HCV LT Patient Samples (n = 51)		Non-HCV LT Patient Samples (HBV+C2; n = 31)		P Value: HCV LT Versus Non-HCV LT
	Mean ± SD (Median)	Range	Mean ± SD (Median)	Range	Mean ± SD (Median)	Range	
Albumin, g/L	38 ± 5 (39)	25-47	38 ± 5 (38)	25-47	38 ± 4 (38)	30-45	0.99
Alanine aminotransferase, IU/L	81 ± 132 (40)	8-1064	81 ± 84 (55)	16-563	82 ± 130 (26)	8-705	0.96
Aspartate aminotransferase, IU/L	74 ± 100 (36)	14-675	89 ± 98 (61)	20-675	54 ± 62 (30)	14-340	0.08
Bilirubin, µmol/L	21 ± 34 (12)	3-223	26 ± 40 (15)	3-223	14 ± 9 (11)	4-37	0.11
Alkaline phosphatase, IU/L	146 ± 132 (106)	47-1028	146 ± 110 (110)	52-570	156 ± 187 (89)	53-1028	0.77
Gamma-glutamyltransferase, IU/L	171 ± 248 (67)	7-1290	210 ± 266 (88)	7-1290	174 ± 282 (49)	14-1272	0.56
Creatinine, µmol/L	101 ± 32 (97)	42-232	103 ± 29 (102)	42-192	99 ± 29 (97)	54-179	0.64
Platelets, 1000/µL	169 ± 88 (158)	32-645	129 ± 67 (112)	32-381	192 ± 87 (177)	88-645	0.001

according to the manufacturer's instructions. Briefly, cells were first stained with surface antibodies, and then cells were fixed and permeabilized with 2 different buffers. A FoxP3 antibody was then added to the cells, and they were incubated for 30 minutes at room temperature in the dark. When it was necessary, cytotoxic T lymphocyte antigen 4 (CTLA4) and latency-associated peptide–transforming growth factor β (LAP-TGFβ) were added to the intracellular antibody master mix to also intracellularly stain those markers. After they were washed twice and transferred to FACS tubes, the cells were placed in a BD LSRII. The acquiring software was FACSDiva (BD Biosciences, Franklin Lakes, NJ). To set the gating for each population, fluorescence minus 1 controls were additionally acquired for each fluorochrome. The analysis was performed with FlowJo (TreeStar, Inc., Ashland, OR). A representative staining and gating strategy is shown in Fig. 1A.

Antibodies

The following fluorochrome-conjugated monoclonal antibodies were used for flow cytometry (clones): anti-CD4 APC-H7 (RPA-T4), anti-CD25 PE and PE-Cy7 (M-A251), anti-CD45RA V450 (HI100), anti-CD49d PE-Cy5 (9F10), anti-CD62L FITC (DREG-56), anti-CD127 AF647 and PerCP-Cy5.5 (hIL-7R-M21), anti-CTLA-4 PE and APC (BNI3), anti-FoxP3 PE and AF647 (259D/C7), anti-HLA-DR FITC (TU36), anti-ICOS FITC (ISA-3), LAP-TGFβ APC (27232).

Additionally, the following biotin-conjugated monoclonal antibodies were used: anti-CD39 biotin (A1) and anti–chemokine (C-C motif) receptor 7 (anti-CCR7) biotin (3D12). They were conjugated with SA Texas Red and SA AF405.

Statistical Analysis

The statistical analysis was performed with Prism for Macintosh (GraphPad, San Diego, CA). Data sets were tested for normal distributions with the D'Agostino and Pearson omnibus test or with the Shapiro-Wilk test when the sample size was smaller than 20.

The homogeneity of variances (homoscedasticity) was analyzed with the F test or the Bartlett test. A t test and an analysis of variance were used whenever parametrical testing applied (normal distribution and homoscedasticity), and the Mann-Whitney test and the Kruskal-Wallis test were used when the data set had to be analyzed with a nonparametric test. $P \leq 0.05$ was considered significant. Statistically significant differences are marked as follows: $*P \leq 0.05$, $**P < 0.01$, and $***P < 0.001$.

A principal component analysis (PCA) of the clinical and experimental parameters of this study was conducted with Qlucore Omics Explorer 2.3 software (Qlucore AB, Lund, Sweden).

PCA is a well-established method that transforms a large set of parameters into 3 summary variables (main components), which are illustrated as 3 axes. PCA visualizes multidimensional data because it creates a 3-dimensional image that can instantly be

TABLE 4.   Staining Panels Applied for the Phenotyping of Tregs

Panel						Markers
A	CD4	CD25	FoxP3	ICOS	CD49d	LAP-TGFβ	CD39
B	CD4	CD25	FoxP3	CD127	HLA-DR		
C	CD4	CD25		CD127	CD62L	CCR7	CD45RA
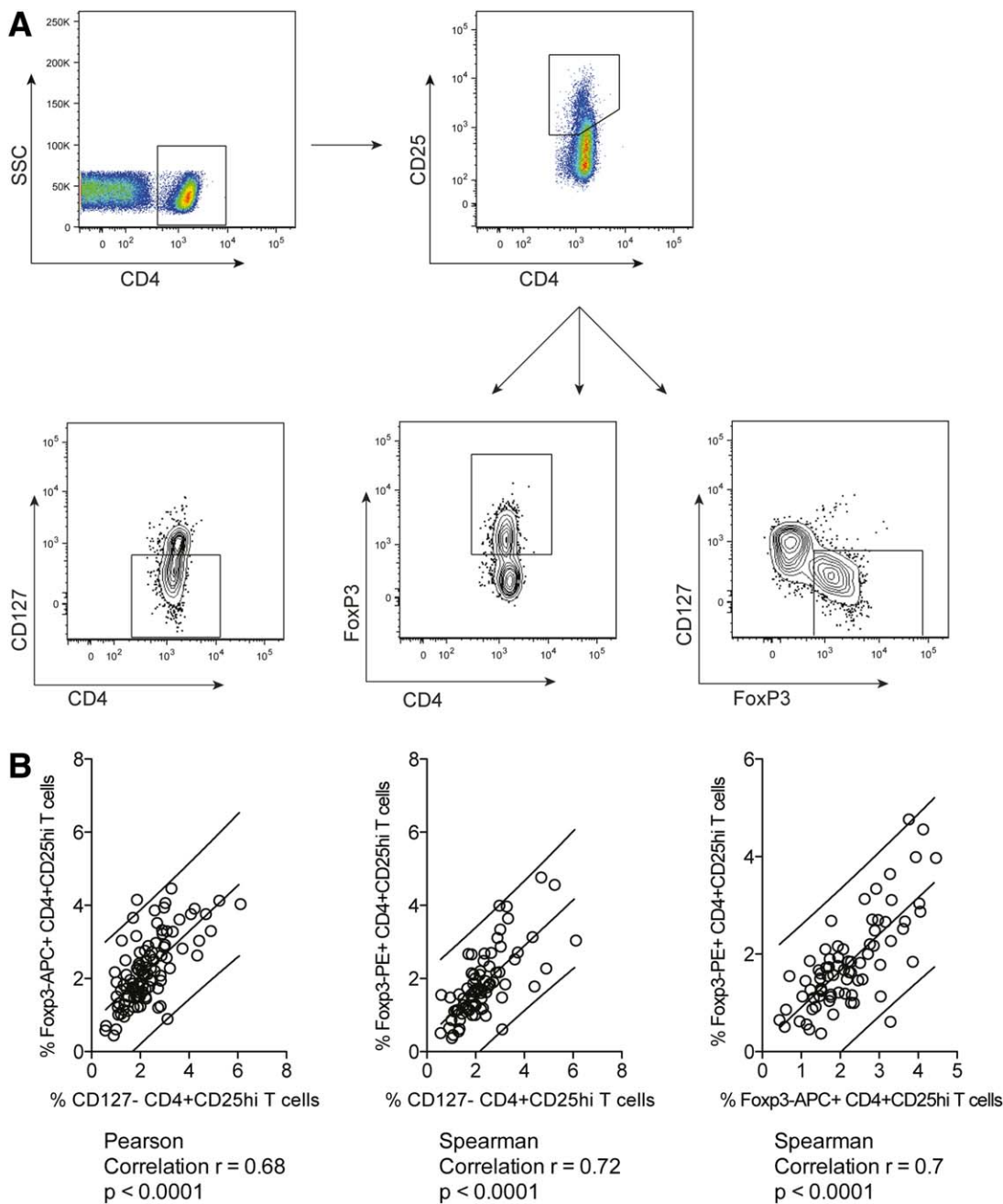
Figure 1.   Phenotypical characterization of Tregs. (A) Representative gating strategy for the characterization of Tregs. Lymphocytes were gated on the basis of their FSC-A/SSC-A location (not shown). Afterwards, CD4^{+} and CD25^{hi} were gated. For CD4^{+}CD25^{hi} cells, 3 different Treg gating strategies were applied (CD127^{−}, FoxP3^{+} or CD127^{−} and FoxP3^{+}). (B) Comparison of surface and intracellular definitions of Tregs. CD4^{+}CD25^{hi}CD127^{−} Tregs were correlated with CD4^{+}CD25^{hi}FoxP3^{+} Tregs, and CD4^{+}CD25^{hi}FoxP3^{+} was correlated for 2 different fluorochromes. When the data followed a normal distribution, a Pearson correlation was applied; if not, a Spearman correlation was used. 102, 80, and 80 pairs, respectively, were tested after they were stained for the combinations listed in Table 4.

interpreted in terms of interrelationships among samples. It helps to identify parameters that differentiate sample groups or to categorize new groups of samples that share 1 or more characteristics.

In order to compare parameters with different ranges of values, results were log_2-converted, and the variance was normalized to 1. Missing values were imputed with k-nearest neighbors. Thresholds of 0.05 for P and 0.2 for the false discovery rate (q) were applied throughout the study.

RESULTS

Tregs were extensively phenotypically characterized in order to better define their role and function in the context of LT, with a particular focus on patients infected with HCV. Three separate staining panels with 4 parameters defining Tregs (CD4, $CD25^{hi}$, $CD127^-$, and FoxP3) were applied for each sample in addition to HLA-DR, CCR7, CD45RA, CD62L, CD49d, CD39, ICOS, and LAP-TGFβ staining (Fig. 1A and Supporting Fig. 1). The different staining panels are described in Table 4. For this study, Tregs were defined as $CD4^+CD25^{hi}CD127^-$ or $CD4^+CD25^{hi}FoxP3^+$ as previously described by Liu et al.[7] The strong correlation between $CD127^-$ and $FoxP3^+$ was also confirmed for LT recipients, as shown in Fig. 1B. The combination of 3 different panels enabled a very robust characterization of the samples and allowed high quality control.

To further characterize Tregs and their subpopulations, groups were defined on the basis of the surface markers CD45RA and CCR7,[32] and naive Tregs were additionally described as $CD45RA^+CD62L^+$.[33] HLA-DR^+ Tregs have been previously described as a functionally distinct population with a high suppressive effect that might play a role in organ rejection.[34-36] Additionally, earlier publications have shown that $CD49d^-$ Tregs are free of contaminating activated T cells and result in purer populations of $FoxP3^+$ Tregs.[37]

The overall frequency of Tregs (regardless of the panel used for their definition) remained stable over time after transplantation, as shown in Supporting Fig. 3. This holds true also for their further characterization (depending on CD45RA and CCR7), with the exception of naive Tregs, which were slightly more frequent in the first 6 months after transplantation in comparison with later time points (Supporting Fig. 4). The percentage of HLA-DR^+ Tregs showed a slight increase over time, but $CD49d^-$ Tregs remained stable (Supporting Fig. 5).

Similar Frequencies of Tregs in LT Patients and Healthy Subjects

We first compared Tregs from LT patients and healthy controls. Peripheral blood mononuclear cells from LT patients and healthy donors showed no differences in their Treg frequencies. The 2 groups had similar mean frequencies for $CD127^-$ and $FoxP3^+$ Tregs (Fig. 2A,B). LT patients and healthy donors presented with similar distributions and frequencies of Treg subpopulations (Fig. 2C). No differences in naive $CD45RA^+CD62L^+$

Tregs were observed between LT patients and healthy controls (Fig. 2D). When the percentages of HLA-DR^+ Tregs in the LT patients and the healthy individuals were compared, again, no difference was observed (Fig. 2E). LT patients showed a lower frequency of $CD49d^-$ Tregs in comparison with healthy donors (Fig. 2F).

Overall, Tregs showed similar frequencies and phenotypes in patients who had undergone LT and healthy subjects, although there were some differences in the subpopulations. This suggests a lack of major disturbances of Treg populations in LT recipients.

Higher Effector Memory Tregs in HCV LT Patients Versus Non-HCV LT Patients

The next aim of this study was to compare HCV LT patients to LT recipients without recurrence of the initially underlying liver disease (non-HCV LT patients) in order to identify possible differences due to the active HCV infection. The frequencies of Tregs in HCV LT patients and non-HCV LT patients did not differ significantly. No differences in the overall percentages of $CD4^+CD25^{hi}CD127^-$ and $CD4^+CD25^{hi}FoxP3^+$ Tregs were found in HCV LT patients versus non-HCV LT patients (Fig. 3A,B). Treg subpopulations in HCV LT patients showed lower percentages for naivelike Tregs gated as $CD45RA^+CCR7^+$ but not as $CD45RA^+CD62L^+$ (Fig. 3C,D). HCV LT patient samples also had fewer Tregs with a memory phenotype (defined as $CD45RA^-CCR7^+$) in comparison with samples from non-HCV LT patients ($P = 0.09$). Tregs that showed an effector phenotype ($CD45RA^-CCR7^-$) were present at a higher percentage in the HCV LT patients. No differences could be found when the percentages of HLA-DR^+ Tregs or $CD49d^-$ Tregs were compared in HCV LT patients and non-HCV LT patients (Fig. 3E,F). In summary, no difference in the overall frequency of Tregs could be observed between LT patients with a persistent HCV infection and those without one. Nevertheless, distinct subpopulations of Tregs showed different distributions in the 2 groups: although naive and memory phenotypes were found at lower frequencies in the HCV LT patients, these showed a significantly increased frequency of Tregs with a $CD45RA^-CCR7^-$ effector phenotype.

Treg Phenotypes Differed Between Chronic HCV Patients and HCV-Infected Patients After LT

Because Tregs from HCV LT patients showed different phenotypes, we next aimed to investigate whether they would differ from those in HCV-infected individuals (HCV CHR patients). Treg frequencies based on the $CD4^+CD25^{hi}CD127^-$ definition were significantly lower in HCV LT patient samples versus samples from chronic HCV patients (Fig. 4A). This difference could no longer be observed when the frequencies of $CD4^+CD25^{hi}FoxP3^+$ Tregs were compared (Fig. 4B). When both definitions were combined and $CD127^-$ and $FoxP3^+$ Tregs were analyzed, chronic HCV
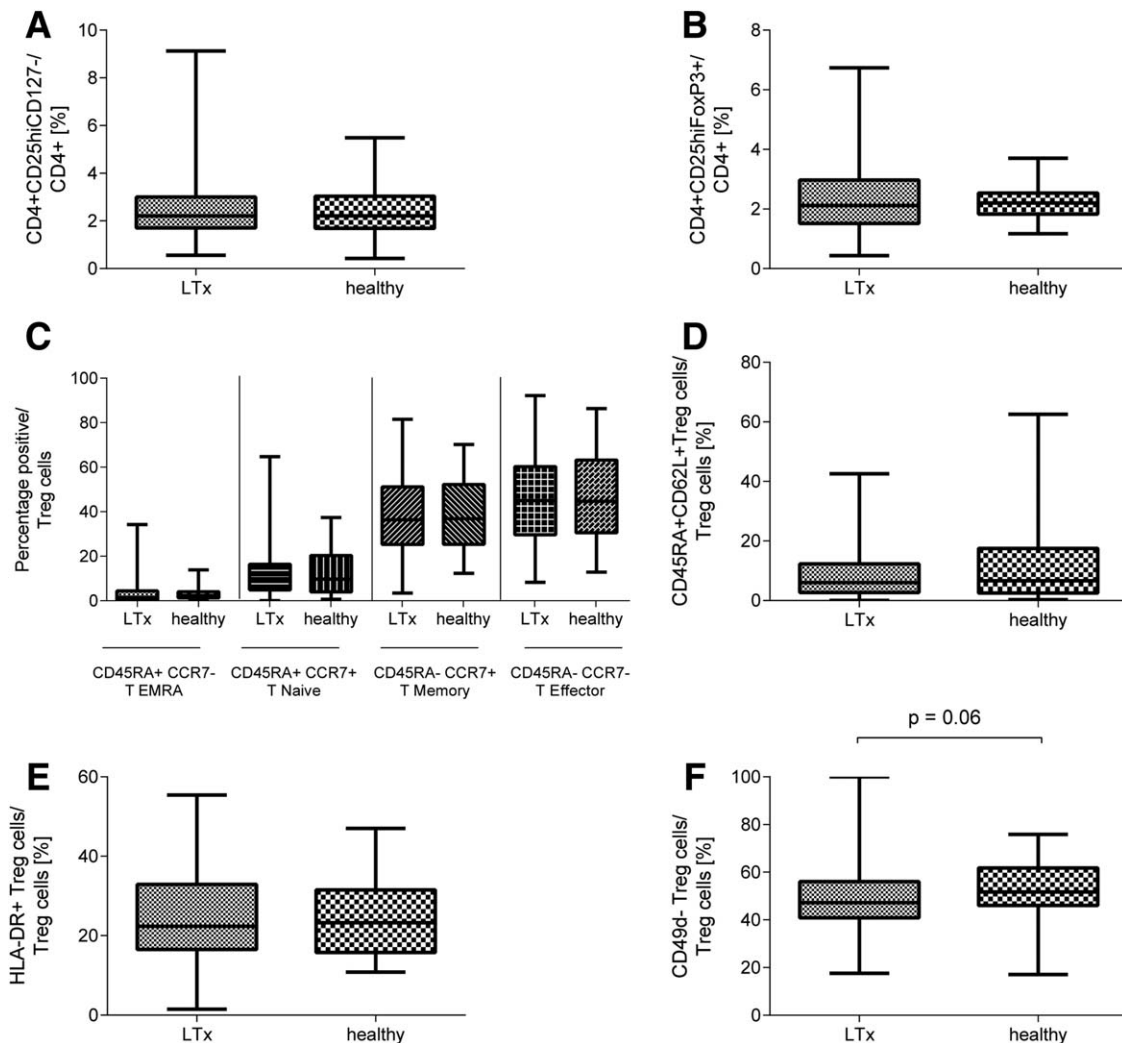
Figure 2. Comparison of Tregs in LT patients (83 patients and 121 samples) and healthy individuals (34 individuals). Percentages of Tregs stained with (A) surface markers as $CD4^+CD25^{hi}CD127^-$ and (B) intracellular markers as $CD4^+CD25^{hi}FoxP3^+$ are compared for samples from LT patients and healthy individuals. On Tregs (gated as $CD4^+CD25^{hi}CD127^-$), (C) different subpopulations according to a CCR7/CD45RA classification and (D) $CD45RA^+CD62L^+$ naive Tregs are compared between the 2 groups. On Tregs (gated as $CD4^+CD25^{hi}FoxP3^+$), (E) $HLA-DR^+$ and (F) $CD49d^-$ percentages in LT patients and healthy controls are shown. An unpaired t test was performed. Scatter plots show means and SDs.

samples also showed higher Treg numbers than samples from HCV LT patients ($P = 0.07$, data not shown). The differences between these 2 groups were most clear when we analyzed the different subpopulations of Tregs with CD45RA and CCR7. HCV LT patients not only had lower numbers of Tregs but also had a lower percentage of Tregs showing a naive phenotype ($CD45RA^+CCR7^+$) in comparison with chronic HCV patients (Fig. 4C). This reduced percentage in HCV LT patients was observed in $CD45RA^+CCR7^+$ and $CD45RA^+CD62L^+$ Tregs. Likewise, Tregs with a memory phenotype ($CD45RA^-CCR7^+$) were lower in HCV LT patients versus nontransplant patients with chronic HCV. Effector-like $CD45RA^-CCR7^-$ Tregs were significantly increased in HCV LT patients versus chronic HCV patients. No difference could be observed between the 2 groups when we analyzed $HLA-DR^+$ Tregs (Fig. 4E). In line with the findings for Treg frequencies (Fig. 4A), LT HCV patients had lower percentages of $CD49d^-$ Tregs in comparison with nontransplant chronic HCV patients (Fig. 4F).

Collectively, HCV LT patients showed a lower percentage of Tregs than nontransplant individuals persistently infected with HCV (both $CD4^+CD25^{hi}CD127^-$ and $CD4^+CD25^{hi}FoxP3^+CD49d^-$ Tregs). The Tregs from HCV LT patients showed a lower percentage of naive Tregs and memory-like Tregs and a higher percentage of Tregs with a $CD45RA^-CCR7^-$ effector phenotype.

Different Distributions of Functional Parameters on Tregs in Samples From Healthy Patients, HCV LT Patients, Non-HCV LT Patients, and HCV CHR Patients

Tregs assert their suppressive capacity through different mechanisms. In this study, CD39, ICOS, and
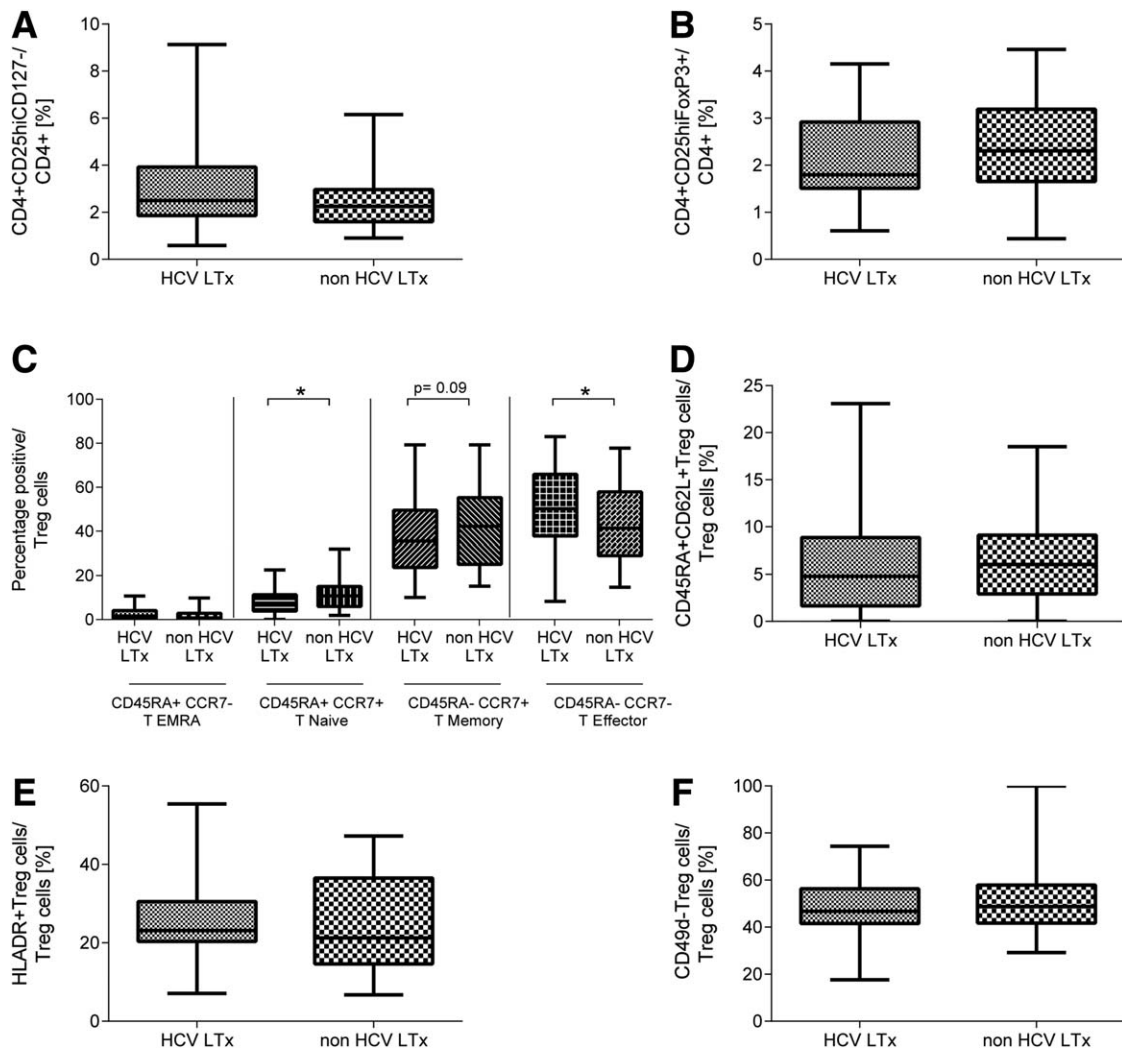
Figure 3. Comparison of Tregs in HCV LT patients (35 patients and 51 samples) and non-HCV LT patients (24 patients and 31 samples). Percentages of Tregs stained with (A) surface markers as $CD4^+CD25^{hi}CD127^-$ and (B) intracellular markers as $CD4^+CD25^{hi}FoxP3^+$ are compared for samples from HCV LT patients and non-HCV LT patients. On Tregs (gated as $CD4^+CD25^{hi}CD127^-$), (C) different subpopulations according to a CCR7/CD45RA classification and (D) $CD45RA^+CD62L^+$ naive Tregs are compared between the 2 groups. On Tregs (gated as $CD4^+CD25^{hi}FoxP3^+$), (E) HLA-DR$^+$ and (F) CD49d$^-$ percentages in HCV LT patients and non-HCV LT patients are shown. An unpaired t test was performed. Statistically significant differences are marked with asterisks ($P < 0.05$). Scatter plots show means and SDs.

LAP-TGFβ were analyzed and quantified with flow cytometry. It has been shown that ICOS, LAP-TGFβ, and CD39[38-40] play a vital role in mediating the suppressive function of Tregs. These markers on their own did not possess a high resolution power for differentiating between the patient groups studied here. Figure 5 shows the frequency of Tregs that were positive for CD39 (Fig. 5A), ICOS (Fig. 5B), and LAP-TGFβ (Fig. 5C) in the 4 analyzed groups (healthy individuals, HCV LT patients, non-HCV LT patients, and nontransplant chronic HCV patients). No difference or pattern could be observed.

A multiparameter analysis was performed; single positive subsets were grouped with a Boolean analysis. The groups were ranked according to the number of functional parameters (CD39, ICOS, and LAP-TGFβ) present on their Tregs (Fig. 5D). This classifica-

tion revealed that most of the Tregs of healthy donors expressed no functional markers (64%) or only 1 functional marker (27%). Similarly, 78% of all Tregs from non-HCV LT patients were positive for none of the tested functional markers. In contrast, HCV LT patients had a higher percentage of Tregs positive for 1 or more of the functional parameters. HCV LT patients and chronic HCV patients showed very similar expression patterns of functional markers on Tregs. In both groups, most of the Tregs were positive for 1 or 2 functional markers. Thus, this multiparameter analysis as a surrogate of functionality (ICOS, LAP-TGFβ, and CD39) revealed similarities between the HCV LT patients and the nontransplant chronic HCV group with respect to the distribution of functional markers on Tregs. To the same extent, Tregs from healthy individuals and non-HCV LT patients
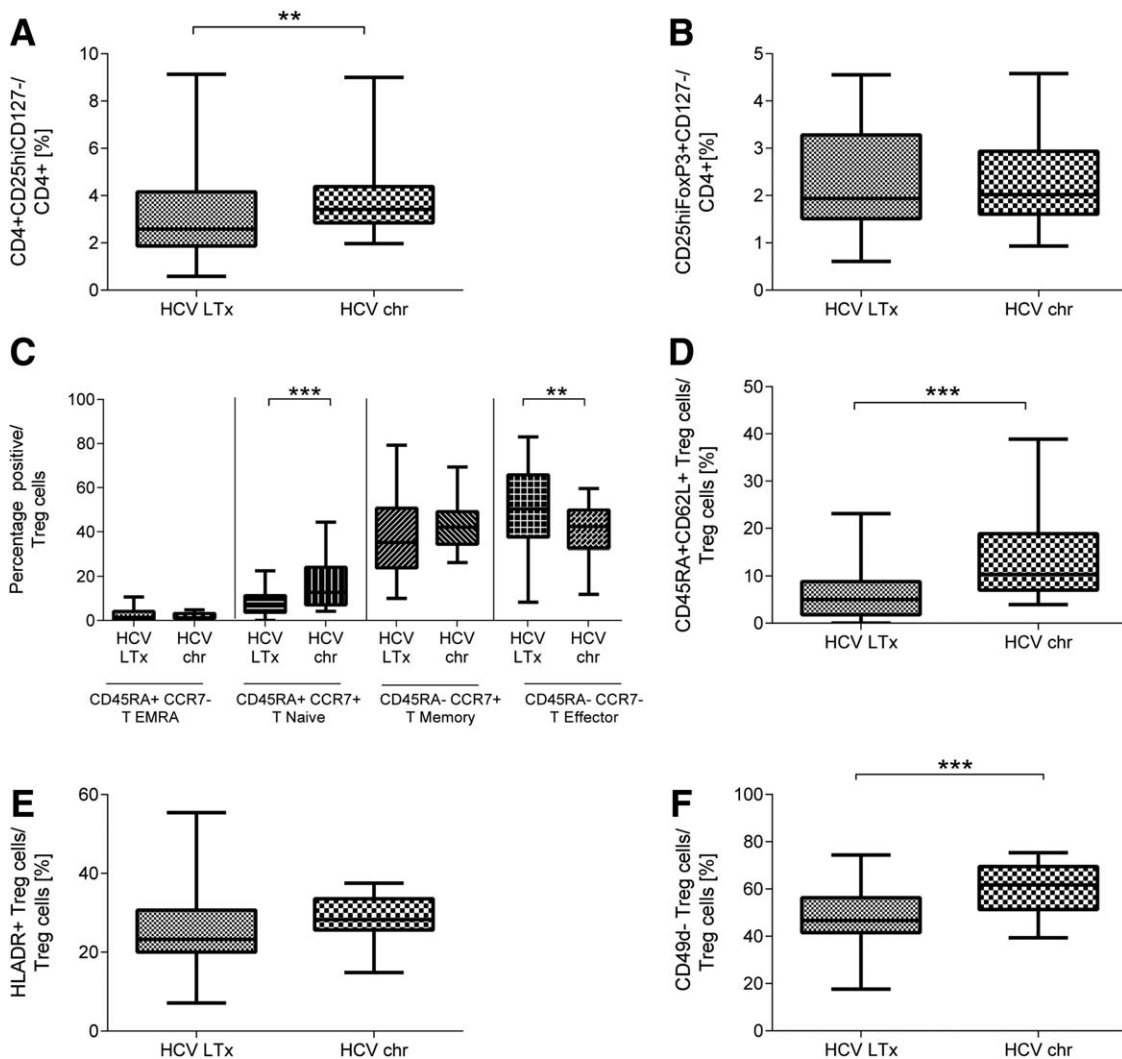
Figure 4. Comparison of Tregs in HCV LT patients (35 patients and 51 samples) and HCV CHR patients (24 patients). Percentages of Tregs stained with (A) surface markers as $CD4^+CD25^{hi}CD127^-$ and (B) intracellular markers as $CD4^+CD25^{hi}FoxP3^+$ are compared for samples from HCV LT patients and HCV CHR patients. On Tregs (gated as $CD4^+CD25^{hi}CD127^-$), (C) different subpopulations according to a CCR7/CD45RA classification and (D) $CD45RA^+CD62L^+$ naive Tregs are compared between the 2 groups. On Tregs (gated as $CD4^+CD25^{hi}FoxP3^+$), (E) HLA-DR$^+$ and (F) CD49d$^-$ percentages in HCV LT patients and HCV CHR patients are shown. The groups were compared with (A,D) the Mann-Whitney test and (B,C,E,F) an unpaired t test. Statistically significant differences are marked as follows: **$P < 0.01$ and ***$P < 0.001$.

showed functional similarities. As a result, these data might suggest that HCV infection, but not transplantation, is associated with multifunctional Tregs.

PCA of Treg Markers Distinguished Different Patient Cohorts

In order to further validate the results and explore relationships among samples and patient groups, PCA was performed with the various Treg subpopulations and surface markers studied here. Each point on a PCA plot represents a blood sample, and its position with respect to the axis is determined by the combined effects of all parameters measured for that sample. The distance between sample points represents the Euclidean distance. The main 3 component

vectors are displayed as axes, and the percentage signifies the proportion of the variability in the data for which each component accounts.

Notably, PCA did not identify any statistically significant parameters with an acceptable q value that could differentiate LT patients from healthy individuals (Fig. 6A).

In contrast, HCV LT patients and non-HCV LT patients showed partial clustering on a PCA plot (Fig. 6B). Samples of the 2 patient groups could be distinguished by variations in the frequencies of Tregs (CD127$^-$), Tregs with a CD45RA$^-$CCR7$^-$ effector phenotype, and CD45RA$^+$CCR7$^+$ Tregs. As shown in Fig. 6C, samples from HCV LT patients could be differentiated from those of nontransplant patients with chronic HCV. Naive Tregs (CD62L$^+$CD45RA$^+$), CD49d$^-$ Tregs, Tregs with a CD45RA$^-$CCR7$^-$ effector
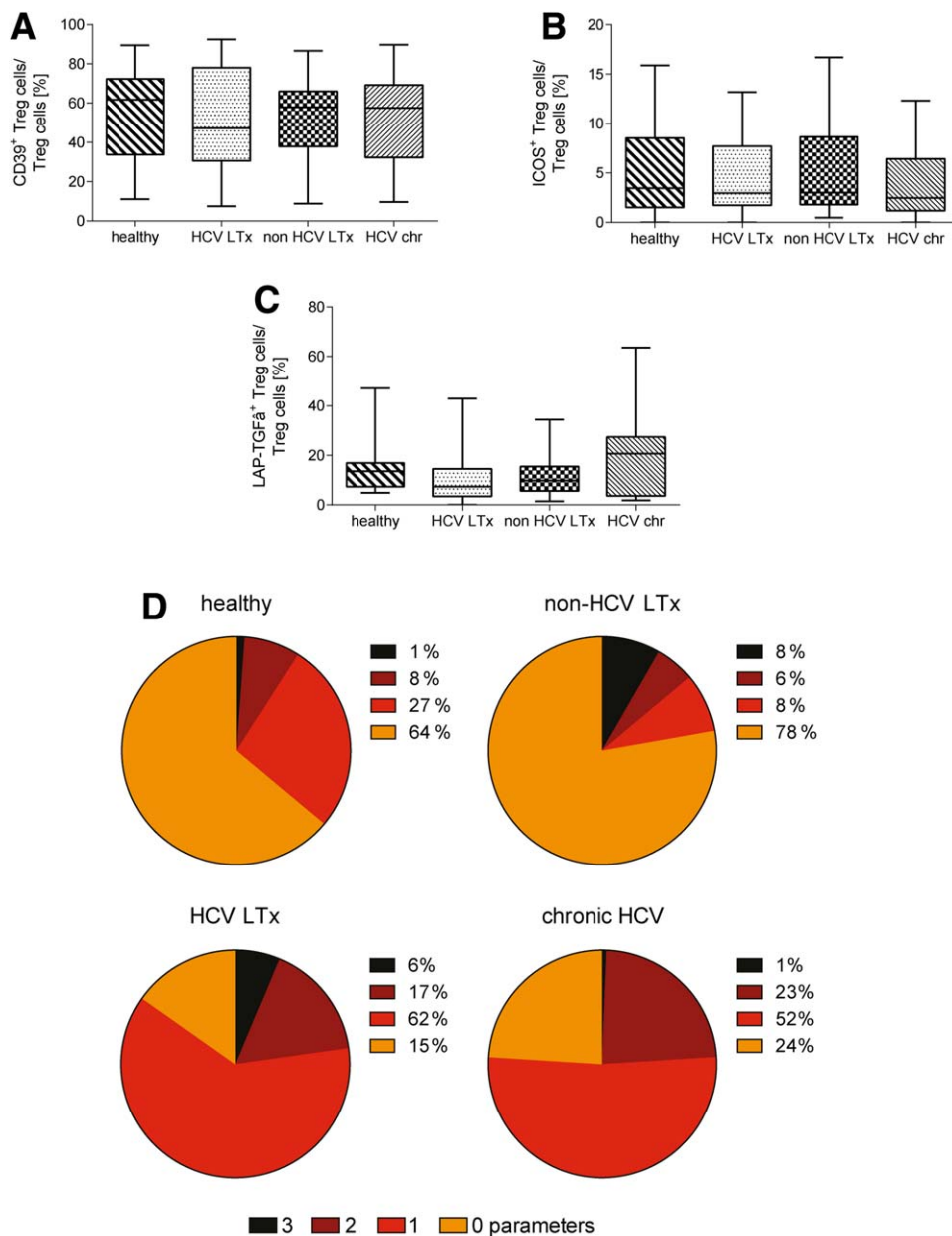
Figure 5.   Comparison of functional associated markers on Tregs from healthy patients, HCV LT patients, non-HCV LT patients, and HCV CHR patients. Tregs were defined as $CD4^+CD25^{hi}FoxP3^+$ cells. Percentages of (A) positive $CD39^+$ Tregs, (B) positive $ICOS^+$ Tregs, and (C) positive $LAP-TGF\beta^+$ Tregs were analyzed. The scatter plots show means and SDs. (D) Multiparameter analysis of Tregs. $ICOS^+$, $LAP-TGF\beta^+$, and $CD39^+$ Tregs were grouped, and percentages of Tregs expressing no markers (0 parameters), 1 marker, 2 markers, or all 3 markers are shown. The groups included healthy individuals (n = 34), HCV LT patients (n = 35), non-HCV LT patients (n = 24), and HCV CHR patients (n = 24).

phenotype, $CD45RA^+CCR7^+$ Tregs, $LAP-TGF\beta^+$ Tregs, and memory Tregs varied significantly between the 2 groups.

Treg Phenotypes Differentiated Graft Rejection From Graft HCV in LT Patients

We finally asked whether Treg phenotypes could differentiate patients suffering from graft rejection from transplant recipients with histologically confirmed recurrent HCV. The 2 patient subgroups did not differ in key characteristics, including age, sex, time after transplantation, HCV genotype, and immunosuppression (data not shown). As shown in a PCA plot (Fig. 7A), Treg phenotypes of HCV LT patients with acute rejection clustered together and could be clearly separated from Tregs derived from individuals with graft HCV. HCV-infected patients with rejection were not differentiated in the PCA from HCV-negative patients with rejection (data not shown). The parameters that differed between the 2 groups were $CD62L^+CD45RA^+$ Tregs, $HLA-DR^+$ Tregs, and

Variable	p-value	q-value
Tregs with CD45RA-CCR7-effector phen.	0.019	0.122
CD45RA+CCR7+ Treg cells	0.024	0.122
Treg cells (CD4+CD25hiCD127-)	0.033	0.122

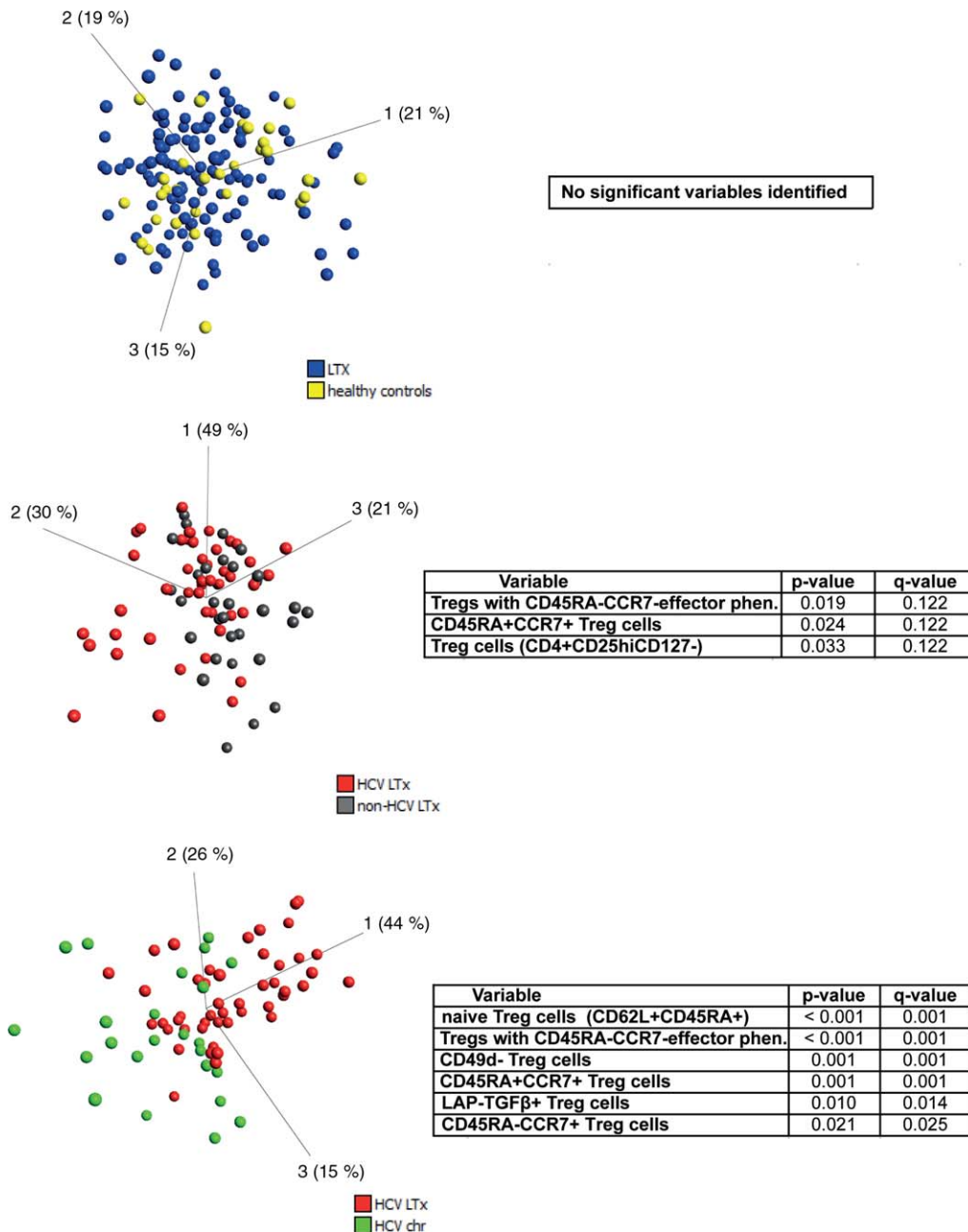| Variable	p-value	q-value
naive Treg cells (CD62L+CD45RA+)	< 0.001	0.001
Tregs with CD45RA-CCR7-effector phen.	< 0.001	0.001
CD49d- Treg cells	0.001	0.001
CD45RA+CCR7+ Treg cells	0.001	0.001
LAP-TGFβ+ Treg cells	0.010	0.014
CD45RA-CCR7+ Treg cells	0.021	0.025

Figure 6.   PCA plots generated with Treg subpopulations and surface markers in order to find characteristics varying between the different patient groups. (A) No major differences in the quality of the Tregs were observed between LT patients and healthy controls. (B) Samples from HCV LT patients showed partial clustering on the PCA plot and could be slightly distinguished from those from non-HCV LT patients on the basis of the variations in the frequencies of Tregs (CD127^{low}), Tregs with a CD45RA$^-$CCR7$^-$ effector phenotype, and CD45RA$^+$CCR7$^+$ Tregs, which were statistically significant. (C) Points representing HCV LT patients clustered together, and they could be separated from HCV CHR patients. Naive Tregs (CD62L$^+$CD45RA$^+$), CD49d$^-$ Tregs, Tregs with a CD45RA$^-$CCR7$^-$ effector phenotype, CD45RA$^+$CCR7$^+$ Tregs, LAP-TGFβ$^+$ Tregs, and memory Tregs varied significantly between the 2 groups.

CD39$^-$ICOS$^+$LAP-TGFβ$^-$ Tregs. Individual differences in these markers were confirmed with an unpaired t test or the Mann-Whitney test, as shown in Fig. 7B-D. The percentage of HLA-DR$^+$ Tregs was reduced in HCV LT patients suffering rejection versus those with graft HCV (Fig. 7B). This difference was independent of the time of the investigation after transplantation (before and after 6 months after transplantation; data not shown). On the contrary, the percentage of CD62L$^+$CD45RA$^+$ Tregs was increased in HCV LT patients with rejection versus patients with graft HCV (Fig. 7C). HCV LT patients with liver rejection had a higher number of naivelike Tregs. The difference in CD39$^-$ICOS$^+$LAP-TGFβ$^-$ Tregs between the 2 patient groups was not statistically significant (Fig. 7D).
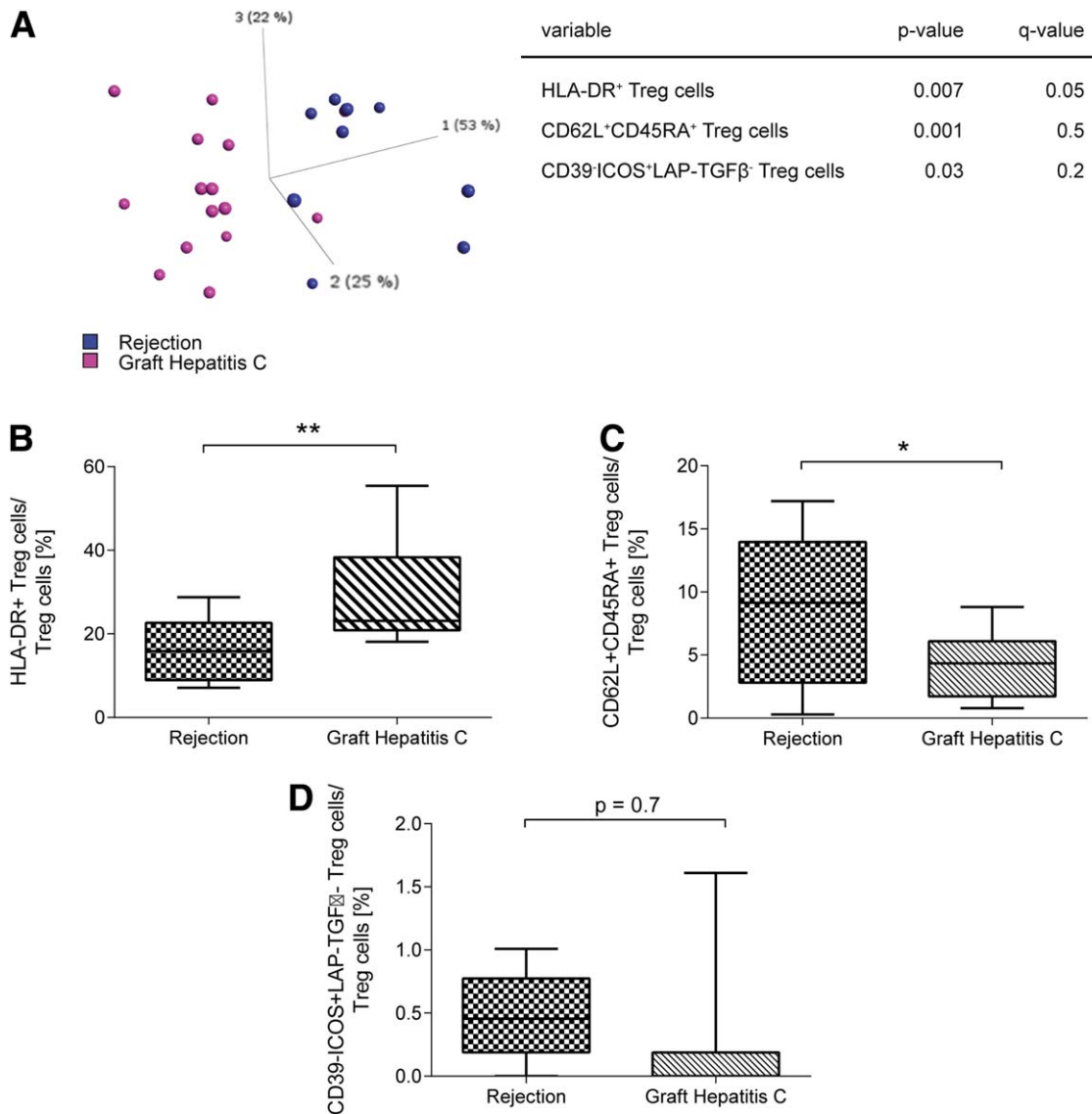
Figure 7.   Comparison of Tregs in HCV LT patients with rejection or graft HCV. (A) Samples from HCV LT patients with rejection clustered together and could be clearly differentiated from the samples of those who suffered graft HCV on the basis of differences in CD62L$^+$CD45RA$^+$ Tregs, HLA-DR$^+$ Tregs, and CD39$^-$ICOS$^+$LAP-TGFβ$^-$ Tregs. (B) The percentage of HLA-DR$^+$ Tregs was reduced in HCV LT individuals suffering rejection versus those with graft HCV. (C) CD62L$^+$CD45RA$^+$ Tregs showed an increase in HCV LT patients with rejection versus patients with graft HCV. (D) The difference in CD39$^-$ICOS$^+$LAP-TGFβ$^-$ Tregs between the 2 patient groups was not statistically significant. Statistically significant differences are marked by * $P < 0.05$, ** $P < 0.01$.

DISCUSSION

This study represents, to our knowledge, the largest and broadest phenotypic analysis of Tregs in LT recipients so far. Tregs were analyzed in 3 independent staining panels with different markers, which allowed stringent quality control. We, therefore, believe that the presented data set is very robust with limited confounding factors. The first—and not trivial—finding from this data set is that FoxP3 and CD127 expression shows a strong negative correlation also in cells derived from organ transplant recipients receiving various medications. When we consider potential

therapeutic strategies for isolating Tregs from these patients,[41] surface CD127 expression seems to be a reliable marker that can be included also in sorting strategies for purifying Tregs in patients treated with immunosuppressive drugs.

Several key findings have come from this study. We have shown that (1) the overall frequencies of Tregs and Treg subpopulations do not differ between LT recipients and healthy individuals, (2) Tregs with a CD45RA$^-$CCR7$^-$ effector phenotype are enriched in the peripheral blood of LT recipients with chronic HCV versus HCV-negative transplant patients, (3) HCV infection, rather than LT, alters the expression

levels of functional markers on Tregs, and (4) Treg phenotypes (particularly HLA-DR and markers indicating naivelike cells) may differentiate HCV-infected LT recipients with rejection from patients with recurrent graft HCV.

Previous studies reported reduced Treg frequencies in the blood of patients after transplantation versus healthy controls,[42,43] and this contrasts with the findings presented here, which show similar overall Treg frequencies in the 2 populations. We also need to acknowledge that calcineurin inhibitors have been shown to decrease the frequency of Tregs.[44] Individual treatments and even the differences between cyclosporine A and tacrolimus probably would need to be taken into account to pinpoint changes. Overall, the immunosuppressive regimen did not show a demonstrable effect in the bulk of the LT patients analyzed. Even though minor differences were also found in our study for some Treg subpopulations (eg, CD4$^+$CD25^{hi}FoxP3$^+$CD49d$^-$), the data do not indicate major general alterations of the Treg pool in LT recipients. Still, changes over time may occur.

Indeed, a study from Rotterdam reported initial declines of Treg populations after LT, particularly during rejection episodes, with subsequent re-increases until month 12 after transplantation.[45] This could suggest that a Treg homeostasis might establish itself only some time after transplantation. In the present cross-sectional data set, Treg frequencies did not differ in samples obtained during the first 6 months after transplantation and samples obtained later. It is striking that LT recipients and healthy individuals showed not only rather similar Treg frequencies but also no major alterations in Treg functional markers. This further supports the conclusion that despite ongoing immunosuppression, Tregs do not suffer general disturbances after LT, and the Treg population adjusts itself within the network of immune responses. This is also supported by the observation that neither the frequency of Tregs nor the phenotype of Tregs after LT was related to any pathological correlates of liver damage or viral loads.

Even if the Treg frequency and phenotype seemed to be rather robust in the LT recipients, distinct profiles were observed between HCV LT patients and non-HCV LT patients. Naive and memory phenotype Tregs were reduced, whereas Tregs with a CD45RA$^-$CCR7$^-$ effector phenotype were more frequent in HCV LT patients. Moreover, a broader repertoire of functional parameters was observed on Tregs from HCV-infected LT recipients and on cells derived from nontransplant control individuals with chronic HCV. Collectively, these findings may indicate that the ongoing viral infection triggers antiviral immune responses, which force the Treg population to engage in higher functionality and effector-like phenotypes. In contrast, transplantation and immunosuppression per se have less impact on Tregs.

Various previous studies demonstrated a clear impact of HCV infection on Treg function in otherwise immunocompetent patients, with generally increased Treg frequencies seen with chronic HCV.[28,29,46,47] The increase in Treg numbers with an HCV infection seems to be ameliorated in the context of LT because the HCV-infected transplant patients showed lower Treg frequencies than the nontransplant individuals with chronic HCV. This reduced frequency of Tregs in the posttransplant HCV group held true for the CD4$^+$CD25^{hi}CD127$^-$ population and especially for CD49d$^-$ Tregs. CD49d$^-$ Tregs may be free of contaminating activated T cells and result in purer populations of FoxP3$^+$ Tregs.[37] However, their suppressive capacity is still under debate, with some results showing a lower capacity[38] and others showing a highly suppressive capacity in vitro.[48]

Distinct profiles of CD45RA, CCR7, and CD62L have been linked to differences in Treg functionality. For example, Tregs expressing a naive phenotype are highly suppressive.[49,50] Subsequently, a protective effect of CD45RA$^+$CD62L$^+$ Tregs in avoiding rejection has been suggested in patients after stem cell transplantation.[33] In this context, it is interesting that Tregs from transplant patients with chronic HCV were more likely to have an effector CD45RA$^-$CCR7$^-$ phenotype. Future studies need to investigate whether these cells are also less functional than naivelike Tregs in the context of HCV and whether this shift in Treg populations may make HCV-infected patients more susceptible to rejection episodes.[51] Moreover, it would be interesting if functionally reduced Treg subpopulations are a characteristic feature of HCV infection after LT or if other persistent infections lead to similar disturbances of the Treg pool.

A major clinical challenge in the management of posttransplant HCV is the differentiation between rejection and HCV-induced graft hepatitis because the 2 conditions lead to basically opposite treatment decisions.[4] Following the aforementioned alterations of the Treg phenotype and function in HCV-infected LT patients, we wondered whether the 2 clinical conditions lead to particular changes in Treg profiles. To address this question, an unbiased approach was applied with a PCA that included all parameters determined for Tregs. Strikingly, patients with histologically proven rejection could clearly be separated from individuals with HCV-associated graft injury on the basis of Treg phenotypes in the PCA. HCV-infected patients with rejection were not differentiated in the PCA from HCV-negative patients with rejection (data not shown). Markers potentially associated with higher immunosuppressive function, such as a naive-like phenotype or the expression of CD39, ICOS, and LAP-TGFβ, were not lower but instead were higher in patients with rejection. This is well in line with a previous finding by some of us demonstrating higher frequencies of Foxp3$^+$ Tregs in grafts of patients with higher rejection activity indices.[27] These findings could indicate that changes in Treg frequencies and function are a response to an increased alloimmune response rather than the cause of rejection. However, individual parameters may be of limited clinical diagnostic value because a reliable separation of the 2 groups was possible only with combined PCA.

Even though several aims were achieved in this work, the study has obvious limitations that need to be acknowledged. Although the overall number of investigated samples was extensive, the numbers for the subgroups (especially transplant recipients with HCV) were still limited. In addition, the amount of cells obtained from patients receiving immunosuppressive medications was limited, and because of this, no functional assays could be performed. However, functional experiments are needed to also prove functional alterations of Tregs in HCV-infected LT recipients, even though the value of the functional surrogate markers investigated here is well established. These experiments would also include a broader analysis of the Treg cytokine profile. Furthermore, only peripheral blood was tested because the number of cells obtained from liver biopsies was too small to apply large flow cytometry–based phenotyping panels. To understand better the role of Tregs over time after transplantation, a longitudinal analysis should follow our cross-sectional approach, and a parallel investigation of HCV-specific effector immune responses would be of major interest. Finally, the effects of antiviral therapy on Tregs remain to be investigated. Novel interferon-free treatment approaches are reaching the market, and thus a large proportion of patients could be cured during the next years.[52-54] It will be very interesting to see whether the HCV imprint on the Treg phenotype and function can be reversed by these therapies.

In summary, Treg phenotypes but not Treg frequencies may differentiate HCV-infected patients after LT. An investigation of Tregs could help us to distinguish the clinically challenging differential diagnosis of recurrent HCV from graft rejection and to better understand the immunopathophysiology of HCV infection after LT.

ACKNOWLEDGMENT

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