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Early high levels of regulatory T cells and T helper 1 may predict the progression of recurrent hepatitis C after liver transplantation.

GHAZAL K ^{1,2}, MORALES O ³, BARJON C ^{1, 5}, DAHLQVIST G ^{1,4}, AOUDJEHANE L ^{1,6}, OUAGUIA L ³, DELHEM N ^{3*}, and CONTI F ^{1,7*}.

*FC and ND contributed equally to this work.

1- Sorbonne Universités, UPMC Univ Paris 06 & INSERM, UMR_S 938, INSERM, UMR_S 938, Saint-Antoine Research Center, Paris, FRANCE.

2- APHP, Bicêtre Hospital, Biochemistry Laboratory, 94275 Le Kremlin- Bicêtre Cedex, France.

3- CNRS UMR8161, Institut de Biologie de Lille, Université de Lille, Institut Pasteur de Lille, IFR 142, 59021 Lille cedex, France

4- Cliniques Universitaires Saint-Luc, 1200 Woluwe-Saint-Lambert, Belgium.

5- De Duve Institute, Université catholique de Louvain, B-1200 Brussels, Belgium

6- Institute of Cardiometabolism and Nutrition (ICAN), Paris, France.

7- APHP, Pitié-Salpêtrière hospital, Unité Médicale de Transplantation Hépatique, Paris, France.

Corresponding author: Dr Khaldoun GHAZAL: Sorbonne Universités, UPMC Univ Paris 06, UMR_S 938, CDR Saint-Antoine, F-75005, Paris, France.

Current adress : APHP, Hôpital Bicêtre, Laboratoire de Biochimie, Hôpital Bicêtre, 94275 Le Kremlin-Bicêtre Cedex, France.

Tel: + 33 613414454 ; Email: khaldoun.ghazal@aphp.fr

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Abbreviations:

ATG: antithymocyte; **CCR7:** C₂C₂ chemokine receptor type 7; **CD:** Cluster of Differentiation; **CD40L:** CD40 ligand; **CTLA-4:** Cytotoxic T-lymphocyte Antigen 4; **CXCR4:** C₂X₂C₂ Chemokine receptor type 4; **G3PDG:** Glyceraldehyde 3-phosphate dehydrogenase; **GATA3:** Trans-acting T-cell-specific Transcription factor 3; **GITR:** glucocorticoid-Induced Tumour Necrosis Factor receptor Family-Related Gene; **HCV:** Hepatitis C Virus; **ICAM-1:** Intercellular Adhesion Molecule 1; **IL:** Interleukin; **IL-10Ra/B:** Subunit a or B of the interleukin 10 receptor; **IFN:** Interferon; **LAG-3:** lymphocyte activation gene; **LT:** Liver Transplantation; **MMF:** mycophenolate mofetil; **nTreg:** natural Regulatory T-cell; **PBMC:** Peripheral Blood Mononuclear cell; **PD-1:** Programmed cell Death protein 1; **PDGF:** Platelet-Derived Growth Factor; **PDL-1/2:** PD-1 Ligand; **Tac:** tacrolimus; **TBX21/Tbet:** T-box transcription factor; **TGFβ:** Transforming growth factor beta; **Th1:** T Helper 1; **Th2:** T Helper 2; **Th17:** T Helper 17; **Tr1:** Type 1 Regulatory T-cell; **VEGF:** Vascular Endothelial Growth Factor; **28S:** ribosomal RNA of the 28S subunit.

Abstract:

Background: Immune response failure against hepatitis C virus (HCV) has been associated with an increased regulatory T-cell (Treg) activity. After liver transplantation (LT), 80% of patients experience an accelerated progression of hepatitis C recurrence. The aim of this work was to assess the involvement of Tregs, T helper (Th) 1, 2 and 17 cells in recurrent hepatitis C.

Methods: Peripheral blood cells obtained before and one month after LT from 22 recipients were analysed. Forty-four key molecules related to Treg, Th1, 2 and 17 responses, were evaluated using qRT-PCR. Liver recipients were classified in two groups according to graft fibrosis evaluated by the METAVIR score on the biopsy performed one year after LT (mild: $F \leq 1$, $n=13$; severe: $F > 1$, $n=9$). Patients developing a severe recurrence were compared with patients with a mild recurrence.

Results: mRNA levels of Treg markers obtained one month after LT were significantly increased in patients with a severe disease course when compared to patients with a mild recurrence. Markers of the Th1 response were elevated in the same group. No differences in the markers determined before LT were observed.

Conclusion: These findings suggest that Treg, induced by a multifactorial process, which could include a strong Th1 response itself, may play a role in suppressing the early antiviral response, leading to a severe recurrence of hepatitis C.

Introduction

Hepatitis C virus (HCV) infects over 170 million people worldwide and the WHO estimates in the Global Hepatitis Report 2015 that roughly 71 million people are living with chronic infection worldwide(1, 2), leading to cirrhosis and hepatocellular carcinoma; furthermore, cirrhosis due to HCV is one of the principal indications for liver transplantation (LT). When chronic HCV infection is established, it induces a wide range of chronic liver injuries but does not appear to be cytopathic. Liver lesions seem to result from locally driven immune responses that are mainly non-specific. Local inflammation triggers fibrogenesis, in which hepatic stellate cells play a major role (3, 4). The mechanisms by which HCV evades the immune surveillance system remain unclear, and persistent infection appears to be due to weak CD4⁺and CD8⁺ T-cell responses during acute infection, which fail to control viral replication (5, 6). The impairment of T-cell responses is multifactorial (7, 8), including skewed T-cell differentiation (T helper1 (Th1) deficiency, Th2 dominance) (9), the elevated secretion of inhibitory cytokines such as interleukin-10 (IL-10) and TGF- β which create an immunosuppressive environment (10, 11), an up regulation of inhibitory pathways (Programmed death-1 (PD-1), Cytotoxic T-lymphocyte Antigen 4 (CTLA-4, CD152)) (12, 13), impairment of the allostimulatory capacity of both myeloid and plasmacytoid dendritic cells (14), and the induction of regulatory T-cells (Tregs) (15). Attention has recently focused on Tregs and their contribution to HCV disease. Natural Tregs account for 5-10% of peripheral CD4⁺ T-cells; they constitutively express CD25 (16), and can suppress host immune responses in the setting of autoimmune diseases, transplantation, and anti-tumour immunity (17). An accumulation of Tregs at the infection site is a common characteristic of most chronic viral infections; they may significantly suppress antiviral CD4⁺ and CD8⁺ T-cell responses (18, 19). Tregs also

constitutively express surface markers such as the glucocorticoid-induced Tumour Necrosis Factor receptor Family-Related Gene, (GITR, CD133) (20), CTLA4 (21) and the transcription factor Foxp3 (22), which is characteristic of this sub-population. Other subsets of regulatory T-cells are also present, such as IL-10-secreting Tr-1 cells that express CD18, CD49b and the lymphocyte activation gene (LAG-3) (23-25).

In the setting of chronic hepatitis C virus (HCV) infection, the activation of virus-specific T-cells may be suppressed by Tregs (26), which might contribute to an inadequate immune response to HCV. An increased frequency of Tregs was found in the blood of patients with persistent HCV infection, compared with those who had cleared HCV (15). The *in vitro* depletion of CD25⁺ T-cells results in increased HCV-specific T-cell responsiveness, and CD4⁺CD25⁺ cells may contribute to HCV persistence by suppressing HCV-specific T-cell responses (27). Some studies have also shown a correlation between a reduced HCV-specific T-cell response and the secretion of TGF- β by liver-infiltrating Tregs (28), and ability for Tregs to inhibit HCV-specific T-cell activity, independently of IL-10 and TGF- β (29). In chronically infected patients, IL-10 secreting Tr1 cells circulate concomitantly with interferon (IFN)- γ -secreting Th1 cells, and these two subtypes of CD4 T-cells recognize the same epitopes on HCV core protein (30), suggesting that Tr1 cells may also be implicated in HCV pathogenesis.

Following LT for HCV cirrhosis, graft infection is universal and graft damage is often accelerated, leading to cirrhosis in 30% of patients within five years, and reduced patient survival compared to other indications (31, 32). The mechanisms of accelerated HCV-induced liver damage after LT are poorly understood. The virus and the host immune system, as well as the donor's age and graft ischemia, may contribute to the prognosis in terms of viral recurrence. From an immunological point of view, a weak or

impaired CD4⁺ immune response, as well as a reduction in plasmacytoid and myeloid dendritic cells, may facilitate the recurrence of hepatitis C (33, 34).

Moreover, in the post-transplant setting, numerous experimental studies have demonstrated that Tregs induce allograft tolerance (35). Tregs are influenced by immunosuppressive therapy; in particular, calcineurin inhibitors reduce Treg function in vitro (36, 37) while rapamycin appears to have a beneficial effect on the Treg count (38, 39). We recently demonstrated that classical CD4CD25⁺ Treg levels were significantly higher in patients with recurrent hepatitis C, and that Tr1 cell numbers and serum IL10 levels were higher at 1 and 5 years after LT in a context of severe recurrent hepatitis C, and associated with a more severe progression of recurrence and a weaker response to antiviral therapy (40, 41).

In the present study, we further analysed the correlations between mRNA Tregs and Tr1, and also other T-cell sub-populations, early after LT, as well as their possible predictive value relative to the progression of HCV recurrence. For the first time, we were able to show that Treg mRNA levels during the 30 days after LT were significantly higher in patients who would go on to develop a severe recurrence when compared to those developing a mild recurrence. These results suggest that Tregs are enhanced soon after LT in patients who will develop severe HCV recurrence and they may be predictive of the progression of HCV recurrence.

Patients and Methods

Patients

Our study population consisted of 22 patients who had been included in the BEFIRTH (Benefit of Immunoprophylaxis on Fibrosis to Reduce Viral Load After Liver Transplantation) study, an open-label, randomized, multicentre clinical study comparing three immunosuppressive regimens in HCV-positive recipients receiving their first liver transplant: (A) tacrolimus (Tac) and corticosteroids, (B) antithymocyte induction therapy (ATG) and full-dose Tac without corticosteroids, and (C) ATG with mycophenolate mofetil (MMF) and a reduced dose of Tac without corticosteroids. The

principal objective of the BEFIRTH study was to evaluate HCV recurrence through the determination of fibrosis in the different groups at least one year after LT. The Ethics Committee (CCPPRB) in Toulouse (France) approved both the BEFIRTH study and the present ancillary study to evaluate Treg sub-populations. All patients gave their written informed consent before undergoing any protocol-related procedures. PBMC samples were only available from 22 of the 80 patients enrolled in the BEFIRTH study, and they underwent RT-PCR analysis in the ancillary study. A liver biopsy was performed in all the recipients included, one year after LT, and the patients were classified in two groups as a function of their METAVIR fibrosis score: a mild recurrence when the score was $F \leq 1$, and a severe recurrence group when the score was >1 . All patients had detectable serum HCV RNA levels.

Primer pairs

All primers were designed for real-time PCR use and purchased from MWG-Biotech. The following genes were studied (Table 1): those representative of naturally occurring Treg cells such as CD4, CD25, Foxp3, CD18, the integrin beta chain beta 2, and CD49b; the integrin alpha subunit specific to induced Tr1; other key molecules related to Th1 antiviral responses (IFN γ , IL-2, and IL-23), and the activation status of the immune system (CD27, CD28, CD40, CD40L). The housekeeping genes ribosomal RNA 28S, β -actin and G3PDH were used as controls.

RT-PCR analysis

RT-PCR analysis of selected genes were performed on PBMC samples that were obtained before LT and on day 30 after LT by density gradient centrifugation over Ficoll-Paque PLUS (Amersham, Piscataway, NJ), as previously described (36), and stored in liquid nitrogen for subsequent RNA extraction. Total RNA was extracted from frozen PBMCs using the RNeasy midi kit (QIAGEN, Courtaboeuf, France), according to the manufacturer's instructions. Briefly, samples were first lysed then homogenized. Ethanol was added to the lysate to ensure ideal binding conditions. The lysate was then loaded onto the RNeasy silica membrane for RNA binding, and all contaminants were efficiently removed by washing. Pure, concentrated RNA was eluted in water. Overall RNA quality and quantity was assessed using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher scientific, MA, USA) and the RNA was then stored at -80°C . The same RNA concentration for all samples was used for reverse transcription experiments.

RNA were supplemented with the following mixture [oligo dT (Roche, Meylan, France) + RNAsin (PROMEGA, Charbonnières; France) + H₂O] and then incubated at 70°C for 10 minutes. After a further 5 minutes at room temperature, the following mixture [buffer 5X (Life Technologies) + DTT (Life Technologies) + dNTPs 10 mM + RNAsin (PROMEGA) + Superscript (Life Technologies)] was added. This reaction was followed by an initial incubation step at 45°C for 60 minutes and a second incubation at 95°C for 5 minutes. Finally, ultra pure DNase/RNase-free distilled water (Life Technologies) was added to obtain a concentration of 10 ng total RNA/1µl.

Real-time PCRs were performed using a Stratagene MxPro 3005P qPCR System. The mix was optimized for real-time PCR analysis using MESA GREEN qPCR MasterMix Plus for SYBR® Assay (Eurogentec) that contained: the hotstart enzyme "MeteorTaq", dNTPs with dUTP, MgCl₂, in a total volume of 20 µl. Each sample was run in a 96-well plate containing 47 primers by performing initial denaturation at 95°C for 5 minutes, after which the PCR reactions were cycled 45 times as follows: 15 seconds at 95°C and 1 minute at 60°C. Fluorescence intensity was measured at the end of each elongation phase. A melting curve analysis was performed immediately after amplification, in accordance with the manufacturer's instructions.

Light Cyclor-based PCR assay

Genes whose level of expression reached significance to discriminate between the two groups of patients (mild vs. severe viral recurrence) were further analysed using the Light Cyclor-based PCR assay. The quantification of transcripts from samples was confirmed by real-time quantitative RT-PCR using the Light Cyclor system (Roche Diagnostics, Meylan, France). The PCR mixture contained the following: Fast start Taq polymerase, 1X of LightCyclor-DNA master SYBRGreen I (Roche Diagnostics), 1,875 mM MgCl₂, 0.5 µmol/L of each primer and 2 µl of the cDNA preparation (patient cDNA samples), in a total volume of 20 µl. The samples were run in parallel by performing an initial denaturation at 95°C for 8 minutes, and then the PCR reactions were cycled 40 to 45 times as follows: 15 seconds at 95°C, 7 seconds at the appropriate annealing temperature, and 18 to 64 seconds at 72°C, depending on the length of annealing for the target sequence (40s at 58° C). Fluorescence intensity was measured at the end of each elongation phase. A melting curve analysis was performed immediately after amplification, following the manufacturer's instructions.

Data interpretation and statistics

The PCR results were analysed using the “Relative Gene Expression Method” as described by Livak et al (42). Briefly, individual CT values were normalised using the average CT values for housekeeping genes ($\Delta CT = CT - CTHKG$). Average ΔCT values for each group were then compared with the ΔCT values of the control group (mild recurrence group) ($\Delta\Delta CD = \Delta CT - \Delta CT_{MRgroup}$). An evaluation of $2^{-\Delta\Delta CT}$ then indicated the fold change in gene expression relative to the control group.

The relevance of the PCR results was validated using a statistical rank sum Mann-Whitney test on the ΔCT values, with GraphPad Prism version 5.00 for MAC OS X, GraphPad Software, San Diego California USA, www.graphpad.com". p values of <0.05 were considered to be significant.

Results

Characteristics of the study population

The study cohort comprised 22 patients who were included in the BEFIRTH study, and clinical data collected during that study were available for all of them. Table 2 shows the demographic parameters of this population of 15 men and 7 women, with a median age of 51 years. At the liver biopsy performed one year after LT, 13 patients had experienced a mild recurrence (fibrosis score $F \leq 1$) and nine had experienced a severe recurrence (fibrosis score >1). Table 3 shows the characteristics of the patients with mild and severe recurrences; viral load, immunosuppressive regimen and donor age were similar in the two groups.

Expression of the markers studied prior to LT

At baseline, immediately before LT, no significant difference was observed in the PBMC expression of Treg mRNA markers or any other studied population markers, between patients who would go on to develop a severe HCV recurrence one year after LT and those with a mild recurrence at 1 year (data not shown).

mRNA Expression of Treg markers one month after LT

At one month after LT, the PBMCs' mRNA expression of most of the studied Treg-associated markers (CD4, CD25, TGF β , CTLA-4, and the two subunits of IL-10 receptor, IL-10R α and β) was significantly higher in patients who would develop severe HCV

recurrence 1 year after LT than in those experiencing a mild recurrence (Figure 1a). The mRNA relative expression of CD25 and TGF β was 2.6 fold and 5.5 fold higher in the severe recurrence group than in the mild recurrence group ($p=0.045$ and $p=0.03$, respectively), and the relative expression of CTLA-4 was 4.2 fold higher ($p=0.016$). A slight increase in Foxp3 mRNA was observed in the severe recurrence group, but the difference was not significant ($p=0.18$) (Figure 1a). No significant difference was noticed for IL10, while the relative expression of both subunits of its receptor (IL10R α : 3.6-fold higher, and IL10R β : 3.3-fold higher) was significantly higher in patients with a severe recurrence ($p=0.02$ and $p=0.007$, respectively).

mRNA Expression of Type-1 regulatory cells markers one month after LT

For Tr1 markers at one month post-transplant, we analysed the mRNA of markers of Tr1 cells (ie: CD18, CD49b & LAG3). Relative expression of CD18 was 9.8-fold higher in the severe recurrence group than in the mild recurrence group ($p=0.04$); the same trend was observed for CD49b and LAG-3 but it was not statistically significant ($p=0.64$ and $p=0.54$, respectively) (Figure 1b). These results were confirmed by performing RT-PCR using the Light Cycler 480®.

mRNA expression of Th1 markers one month after LT

An increased mRNA relative expression of Th1 markers was observed one month after LT in PBMC from patients that would develop a severe HCV recurrence one year later. The relative expression of IFN γ was 20 times higher in the severe recurrence group than in the mild recurrence group ($p=0.019$), and an increased expression of IL-2 and IL-23 mRNA was also observed in the severe versus mild recurrence groups ($p=0.011$ and $p=0.04$, respectively) (Fig2.a). A significant increase was seen regarding the mRNA levels of activation markers of Th1 cells, such as CD27 (the relative expression of which was 2.6-fold higher in severe recurrence patients; $p=0.007$), CD28, (12.1-fold higher, $p=0.016$), CD40L (4.4-fold higher, $p=0.016$) and the T-box transcription factor (TBX21/T-bet) (Figure 2a).

mRNA expression of Th2 and Th17 markers 1 month after LT

The mRNA of Th2 response markers (IL-4) did not differ significantly between the severe and mild recurrence groups, one month after LT (data not shown), by contrast

the mRNA levels of the transcription factor GATA-3 were higher in the severe recurrence group (the relative expression of which was 2.7-fold higher in severe recurrence patients; $p=0.011$). PCR analysis of the cytokines associated with Th17 development and function revealed a slight decrease in IL-6 mRNA one month after LT in the severe recurrence group, and a slight increase in IL-17 mRNA, although this was not statistically significant ($p=0.59$ and $p=0.34$, respectively), while IL-23 mRNA, which is important for the expansion of already differentiated Th17 cells, was significantly higher in the severe recurrence group ($p=0.04$) (Figure 2b).

Discussion

It had previously been reported that Tregs are induced by HCV (43), and we had observed that Treg markers were significantly enhanced in a context of recurrent hepatitis C after LT, and that Tr1 cell levels were specifically elevated in severe recurrent hepatitis C when compared to a mild recurrence (41). The present study analysed the early correlation (one month after LT) between different T-cell populations (Treg, Tr1, Th1, Th2, Th17) and the future development of severe recurrent hepatitis C. The cohort study was extracted from an open-label, randomized, multicentre clinical study comparing different immunosuppressive regimens in HCV-positive liver transplant recipients. PBMC samples were available from 22 patients included in our centre, and all data collected prospectively before, during, and after LT were available for these patients.

The present study shows for the first time that PBMC Treg levels, assessed from the expression of their surface markers mRNA (CD4, CD25, CTLA-4), and mRNA of molecules associated with Treg activity such as TGF β , IL-10R α and β , determined one month after LT, were predictive of a severe recurrence one year post-LT. Patients with higher levels of Tregs one month after LT had a greater chance of developing a severe recurrence of hepatitis C than those with lower Treg levels. Our findings could represent an important tool for the management of HCV recurrence, which is associated with an accelerated progression of fibrosis when compared to immunocompetent patients, and with the impairment of patient and allograft survival (32, 44).

It still needs to be clarified whether high Treg mRNA levels are constitutive or due to immunosuppression. Because no difference in Treg levels was found between the two groups of patients immediately prior to LT, the immunosuppressive regimen or the

patient's individual response to immunosuppressive therapy was probably responsible for the results. The number of Tregs before LT may depend on the patient's characteristics or the severity of the disease, but it is not predictive of a progression of recurrent hepatitis C.

Interestingly, the elevated Treg markers mRNA levels in the severe recurrence group were associated with increased mRNA levels of Th1 response markers. This indicates the presence of an active immune response (effector and regulatory) during this early stage after LT. The concomitant increase in both Tregs and Th1 markers could be interpreted as being contradictory, but It has been shown that in response to INF-gamma, Tregs upregulated the Th1 specifying transcription factor T-bet and promoted the accumulation of Th1(45). Our hypothesis is that during early HCV recurrence, Th1 cells are activated to inhibit the evolution of HCV infection towards chronicity. In the group that would evolve to experience severe recurrence, Th1 were significantly increased and could induce a higher Treg response, probably due to the presence of higher IL-2 levels, produced during the expansion of T effector cells, but also necessary for the expansion of Tregs (46, 47), and under the effect of INF-gamma (45) Then, in severe recurrent forms, Th1 and Tregs would be simultaneously and significantly enhanced soon after LT and thus be predictive of severe progression.

Foxp3 mRNA was present in severe and mild recurrence groups, but results interpretation is difficult since FOXP3 expression is present in Tregs but also transiently in activated nonsuppressive T cells (48). Moreover, a murine study showed that Foxp3 RT-PCR analysis alone does not fully reflect Treg level (49). Furthermore, in the setting of LT associated with HCV infection, Foxp3 in mild recurrence group could be expressed by activated effector T cells involved in the control of HCV reinfection (other Treg markers were significantly lower in this group), while in severe recurrence group by both Tregs and other activated immune cells. Therefore, the insignificant difference of Foxp3 expression in the two groups does not exclude an increased number of Tregs in the severe recurrence group since other Treg functional markers such as CTLA-4 and TGF-beta were significantly over expressed.

Only a few non-specific Tr1 markers mRNA were significantly enhanced (CD4, CD28 and TGF β), while more specific Tr1 markers mRNA such as CD49b and LAG-3 were slightly

but not significantly elevated one month after LT. This might be explained by the need for IL10 and chronic contact with antigens to enable the development of Tr1 cells (24), and it may also be too early to detect a significant increase in these cells.

We hypothesize that Tregs contribute to failure of the antiviral immune response during HCV recurrence after LT. Several studies focusing on HCV infection in immunocompetent patients have previously shown a correlation between peripheral Tregs and the chronicity of HCV infection (15, 18, 19, 50). The role of natural or induced Tregs in HCV recurrence after LT has only been suspected hitherto, and we previously showed that high levels of natural and induced Tregs one year after LT was associated with more severe fibrosis and was predictive of subsequent fibrosis progression (41), but no data were available at an earlier stage.

mRNA levels of Th17 response markers were similar in the two groups and IL-6 levels were even lower in the group that would go on to present a severe recurrence; IL-6 lower mRNA levels may thus favour the further development of Tregs (51).

Thus Tregs, induced by a multifactorial process, which may include a strong Th1 response itself, probably play a role in the failure of a successful Th1 anti-HCV response, as well as in the severity of recurrence after LT. An early evaluation of Treg levels may constitute a tool, which could help clinicians to select liver transplant recipients for earlier and more intensive antiviral therapy. Indeed, high levels of Tregs may thus be predictive of severe recurrence, highlighting patients who warrant more intensive management. The small study size is a major limitation and further research is now needed in a larger patient population and using other techniques such as flow cytometer phenotyping in a prospective manner, in order to confirm these results.

Table 1: Primers used in real-time RT-PCR.

Gene	Sens	Anti-sens
<i>IL-1</i>	TAGTAGCAACCAACGGGAAGGT	TGCTCAGGAAGCTAAAAGGTGC
<i>IL-2</i>	ACCAGGATGCTCACATTAAAGTTTAC	TCCAGAGGTTTGAGTTCTTCTCTAGA
<i>IL-4</i>	CACAAGCAGCTGATCCGATTTC	TTCCAAGAAGTTTCCAACGTACTC
<i>IL-6</i>	ATGTAGCCGCCACACACA	CCAGTGCCTCTTTGCTGCTT
<i>IL-8</i>	GAGTGGACCACACTGCGCCA	TCCACAACCTCTGCACCCAGT
<i>IL-10</i>	GAGAACCAAGACCCAGACATCAA	CCACGGCCTTGCTCTTGTT
<i>IL12</i>	CCTTCACCACTCCAAAACCT	TGGTAAACAGGCCTCCACTGT
<i>IL-15</i>	TTTCCATCCAGTGCTACTTGTGTT	CATTACCCAGTTGGCTTCTGT
<i>IL-17</i>	TCCTGGGAAGACCTCATTGG	AGAATTTGGGCATCTCTGGATT
<i>IL21</i>	GATCGCCACATGATTAGAATGC	AGGAAAAAGCTGACCACTCACAGT
<i>IL-23</i>	GTGGGACACATGGATCTAAGAGAA	AAATCAGACCTGGTGGATCCT
<i>IL-27</i>	GGAGGGAGTTCACAGTCAGC	GGCAGGAGGTACAGGTTTAC
<i>INFγ</i>	ATGTAGCGGATAATGGAATC	GACATTCAAGTCAGTTACC
<i>TGFβ1</i>	CGAGCCTGAGGCCGACTAC	CGGAGCTCTGATGTGTTGAAGA
<i>CD127</i>	TGGATCGCAGCATTCACTGA	CTTTACCTCCACGAGGGCC
<i>IL-10Rα</i>	CCGAGAGTATGAGATTGCCATTC	CAGATGGTTTACCTGGACACA
<i>IL-10Rβ</i>	TGGGAGTCACCTGCTTTTGC	TCCGTCAAGGTAGTATTCATGCA
<i>CXCR4</i>	TCATGGGTACCAGAAGAACTGA	GAAGTTCCCAAAGTACCAGTTTGC
<i>CXCR5</i>	TACCCGCTAACGCTGGAAT	AGGTGTCGTTATAGTTGTCCAATCTG
<i>CCR7</i>	GAGACAACACCACAGTGGACTACAC	GTCAACACGACCAGCCCAT
<i>VEGF</i>	TGCCGCCACCACACCATCAC	GCCCTCCGAGCCCAAAGTGC
<i>PDGF</i>	GCCGCCAGCGCCATTTTTCATT	CTTTCAGCGAGGCTGGAGGGT
<i>Gata3</i>	GATGGCACGGGACACTACCT	GGTCTGACAGTTTCGCACAGGA
<i>LAG3</i>	TGGCTTCAACGTCTCCATCA	CCCACCTGGAACCTGCT
<i>CD8</i>	CCCTGAGCAACTCCATCATGTAC	GGCGTCGTGGTGGGC
<i>CD4</i>	GGGAAATCAGGGCTCCTTCTTA	TGGTCCCAAAGGCTTCTTCTT
<i>CD11a (hINT-αL)</i>	TACCTCTTCCGCCAGAATCTG	GCAAGCTCATCGAACCATCA
<i>CD18 (hINT-β2)</i>	ATGCTTGATGACCTCAGGAATGT	ACGGTCTTGTCCACGAAGGA
<i>CD25</i>	GGGACTGCTCAGTTCATCA	TTCAACATGGTTCCTTCTTGTAG
<i>CD27</i>	CCTTCTCTCTGACCACCACA	CGACAGGCACACTCAGCATT
<i>CD28</i>	GCTGCTCTTGGCTCTCAACTATT	CCGCATTGTCGTACGCTACA
<i>CD40</i>	TCCAGAACCACCACTGCAT	CACCGCAAGGAAGGCATT
<i>CD40L</i>	GAAAGAAAACAGCTTTGAAATGCA	TTTTTCAGCCCACTGTAACACAGA
<i>CD49b (hINT-α2)</i>	CAACGGGTGTGTGTCTGACA	TCATCACACACAACCACAACATCT
<i>CD71</i>	CGTCGGGATATCGGGTGGCGG	CCCGCAGGATGAAGGGAGGACA
<i>PD1</i>	GCTACAACCTGGGCTGGCG	ATGTGTTGGAGAAGCTGCAGGT
<i>PDL1</i>	TTTATATTCATGACCTACTGGCATTG	AATTGTCATATTGCTACCATACTTACCA
<i>PDL2</i>	AGCCTGGAATTGCAGCTTCA	AAGTTGCATTCCAGGGTCACAT
<i>ICAM1</i>	CCCTGATGGGCAGTCAACA	GCAGCGTAGGGTAAGGTTCTTG
<i>CTLA4</i>	TTCTTCTCTTCATCCCTGTCTTCTG	GAGATGCATACTCACACAAAGCT
<i>GITR</i>	TGTGTCCAGCCTGAATTCCA	CCGAGGCACAGTCGATACACT
<i>OX40</i>	ACGACGTGGTCAGCTCCAA	AGCGGCAGACTGTGTCTCTG
<i>Foxp3</i>	GAAACAGCACATTCCCAGAGTTC	ATGGCCAGCGGATGAG
<i>tBet</i>	GATGCGCCAGGAAGTTTCAT	CTCTCCGTCGTTCACTCAAC
<i>Bactine</i>	CACGGCATCGTCACCAACT	AGCCACACGCAGCTCATTG
<i>GAPDH</i>	CCATCAATGACCCCTTCATTG	CTTGACGGTGCCATGGAATT
<i>28S</i>	TTGAAAATCCGGGGGAGAG	ACATTGTTCCAACATGCCAG

IL: Interleukin; INF γ : Interferon gamma; TGF β 1: Transforming Growth Factor beta-1; CD: Cluster of Differentiation; IL-10Ra/B: Subunit a or B of the interleukin 10 receptor; CXCR4: C_X_C Chemokine receptor type 4; CCR7: C_C chemokine receptor type 7; VEGF: Vascular Endothelial Growth Factor; PDGF: Platelet-Derived Growth Factor; GATA3: Trans-acting T-cell-specific Transcription factor 3; LAG3 Lymphocyte Activation Gene 3; CD40L: CD40 ligand; PD-1: Programmed cell Death protein 1; PDL-1/2: PD-1 Ligand; ICAM-1: Intercellular Adhesion Molecule 1; CTLA-4: Cytotoxic T-Lymphocyte Antigen 4; GITR: Glucocorticoid-Induced TNFR-Related protein; G3PDG: Glyceraldehyde 3-phosphate dehydrogenase; 28S: ribosomal RNA of the 28S subunit.

Table2. Demographic data on the cohort

<i>Characteristics</i>	<i>Distribution</i>
Cohort study	22 patients
Inclusion date	From June 2006 to January 2009
Age at selection*	51 (46-66)
Gender	
Male	15
Female	7
HCV genotype**	Gen 1: n=18; Gen 4 : n=4
Immunosuppression	Tac + corticosteroids: n=7 (31,8%) ATG+Tac: n=4 (18,2%) ATG + MMF + Tac (tapered dose): n=11 (50%)
Metavir F score 1 year after LT	F1 ≤n=13, F>1 n=9
* Median value (min, max)	
** n= number of patients	

Table 3. Characteristics of patients with a mild or severe HCV recurrence one year a

<i>Characteristics</i>	Mild recurrence	Severe	p
	(F≤1 one year post-LT)	recurrence	
	N=13	(F>1 one year post-LT)	
		N=9	
% of patients	59%	41%	
Age (years)	50±13	53±7	0.57
Weight (kg)	74.7±12.7	70.3±14.3	0.32
HCV genotype	Gen 1: n= 11	Gen 1: n= 7	
	Gen 4: n= 2	Gen 4: n= 2	
Basal Creatinine (μmol/L)	71,8±24.9	93.3±39.9	NS
Creatinine 1 year after LT	121±18.6	125±35.2	NS
Immunosuppressive regimen			
Tac +corticosteroids: n=7	4	3	
ATG+Tac: n=4	3	1	
ATG + MMF + Tac (tapered dose):			
n=11	6	5	
Viral load before LT (log)	4.2±0.5	4.8 ±0.8	NS
Viral load 1 year after LT (log)	6.2±0.7	6.9±1.2	NS
Donor age >40 yrs.	58%	62%	NS

Figure legends

Figure 1: Relative expression results of qRT-PCR one month after LT obtained on PBMC from study patients expressed using the $2^{-\Delta\Delta CT}$ methods, with the mild recurrence group as a reference. The severe recurrence group was defined by a METAVIR score F>1 at the one year follow-up biopsy. a) Relative expression of Treg-associated markers. b) Relative expression of induced regulatory T-cell (Tr1)-associated markers. (Calculation of Standard Deviation and statistics are based on ΔCT values) (* $p<0.05$, ** $p<0.01$).

Figure 2: Relative expression of a) the Th1 response, and b) Th17-associated molecules in PBMC obtained one month after LT using RT-PCR in the mild and severe HCV recurrence groups. Results are expressed using the $2^{-\Delta\Delta CT}$ method with the mild recurrence group as a reference. (Calculation of Standard Deviation and statistics are based on ΔCT values) (* $p<0.05$, ** $p<0.01$)

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Figure 1:

a) Treg associated genes

b) Tr1 associated genes

Figure 2:

a)

Th1 response associated genes

b)

Th17 response associated genes