

Donor Chimerism, Kir Ligand Mismatch and Cytokine Levels (Tnf α , Il17, Gmcsf, Il1 β) and their Association with Graft Rejection in Liver Transplant Recipients

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Abstract

Background: The aim of the study is to design a predictive model of graft rejection in liver transplant recipients based on donor chimerism (DC) quantification, functional study of Natural Killer cells and cytokine levels.

Methods: Seventy-four liver transplant recipients and their respective donors were studied, providing a total of 468 post-transplantation samples in the chimerism study. A total of 23 Liver Transplant patients and their respective donors took part in HLA typing and KIR genotyping, phenotyping, and cytotoxicity studies. 62 liver transplant recipients were analyzed in this cytokine study (62 pre-transplant and 109 post-transplant serum samples). Chimerism study was carried out by quantitative real time PCRs and 7 indels and 14 SNPs were detected with TM probes. A study of NK-cell subsets was performed on fresh samples by multiparametric flow cytometry (Becton Dickinson). KIR genotyping and KIR ligand were analyzed by PCR with a KIR typing Kit (Milteny Biotec). Cytotoxicity assays were monitored in a conventional 2-hour europium-TDA release assay (Perkin-Elmer). Biorad "17-plex Kit on a luminex" platform was used for cytokines measurement.

Results and conclusions: High chimerism levels during the first months after transplantation were related to a lower probability of rejection. A CD56 bright NK cell subset appeared to dominate the early post-transplant period following liver transplantation. A tendency to high frequency of graft rejection was observed in cases with KIR-ligand mismatches. Donors lacking C1 ligands exhibited increased risk of acute rejection. Cytokine levels predicted events in liver transplant recipients: low TNF α levels in pre-transplant recipient samples were associated with liver disease relapse and low IL17 and TNF α levels and high GMCSF and IL1 β levels were associated with low rejection free survival. Finally IL6 levels showed an adverse impact on recipient overall survival.

Keywords: Chimerism; Tolerance; NK cells; Cytokines; Liver transplantation

Abbreviations: LT: Liver transplantation; DC: Donor Chimerism; DDLT: Deceased donor liver transplant; HSCT: Hematopoietic stem cell transplantation; TAC: Tacrolimus; CyA: Ciclosporin

Background

Long-term acceptance of transplanted organs without requirement for indefinite immunosuppression remains the ultimate goal of transplant clinicians and scientists [1]. Currently, more than 90% of all liver transplant recipients are treated with the calcineurin inhibitors cyclosporine or tacrolimus [2]. Patients are faced with severe complications of immunosuppressant therapy such as infections, metabolic disturbances, malignancies [3], and drug-specific toxicity such as nephrotoxicity when treated with these inhibitors [4,5].

The incidence of chronic rejection is much lower in liver transplantation cases, compared to other organ grafts [6], and approximately 20% of stable recipients can be weaned off immunosuppressive medication without developing graft rejection.

New recommendations on this subject, included in a meta-analysis study, have shown that randomized trials are needed to evaluate calcineurin inhibitor reduction versus continuation of calcineurin inhibitor treatment; but these randomized trials should only be performed in carefully selected patients [2].

The proposal that microchimerism induces tolerance was first put forward by Starzl et al. [7]. Starzl and Zinkernagel hypothesized that organ engraftment is a form of chimerism-dependent partial tolerance [7-13] and this tolerance can be inferred from the amount of immunosuppression necessary to maintain the stable function and structure of the graft.

Previously we have reported that "Stable, high levels of donor chimerism (DC), in the absence of other major clinical events, may be a marker of transplantation tolerance" [14] in a cohort of forty-two liver transplant recipients without lowering the amount of immunosuppression. Nonspecific genomic (NK, CD T cell genes),

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immunophenotypic (CD4+CD25highFOXP3+, T cells), and HLA (soluble HLA-G) signatures also appear to be ready for testing as tolerance predictors in prospective studies [15-18].

It has been demonstrated that donor NK cells may promote engraftment, prevent graft- versus host disease, control infections, and reduce the risk of leukemia relapse in hematopoietic stem cell transplantation (HSCT). It has also been established that NK cell alloreactivity is affected by many determinants, including donor versus recipient KIR ligand compatibility, KIR-HLA receptor ligand mismatch, and the milieu of cytokine such as IL-12 and IL-18 [19-21]. KIR genotype and KIR-HLA-C ligand compatibility play roles in recurrence and progression of hepatitis C [22] and graft rejection [23] in liver transplant recipients.

In the field of hematology, the killer immunoglobulin-like receptor /HLA ligand matching plays a part in T-cell complete DC in hematopoietic stem cell transplantation. And Natural Killer cells can exert a graft vs. tumor effect in haploidentical stem cell transplantation for pediatric solid tumors. We hypothesized that NK function and KIR/HLA matching could modify DC levels in liver recipients. Taking into account the immunological aspects, we also studied cytokine levels as potential biomarkers to predict acute cellular rejection. We also examined these cytokines in relation to DC and the NK function.

The aim of the study is to develop a predictive model of graft rejection in liver transplant recipients based on DC quantification, NK cell study and cytokine levels (IL1β, IL5, IL7, IL12, IL13, IL17, GCSE, MCP1, MIP1B, IL2, IL4, IL6, IL8, IL10, GMCSF, IFNγ, TNFα) in a cohort of liver recipients and their donors in a prospective study.

Materials and Methods

Chimerism study

Characteristics of recipients and donors: A total of 80 Deceased Donor Liver Transplant (DDLT) recipients and their respective donors took part initially in this study, but eventually it was conducted with 74 LT recipients. Six LT cases were not studied, non-informative polymorphism between recipient and donor was found in two of them, two patients had died before the first post-transplantation samples were collected, and in two cases, donor samples were not available. All patients underwent transplantation at the General Surgery of the Alimentary Tract and Abdominal Organ Transplantation Department of the 12 de Octubre Hospitals (Madrid, Spain) between October 2008 and January 2011. The study was approved by the Ethical Institutional Review Board of 12 de Octubre Hospital and all patients gave their consent to participate in the study in compliance with the Declaration of Helsinki. The characteristics of patients and immunosuppressive regimen are summarized in Table 1.

Sample collection and DNA preparation: A total of 792 samples were studied. 156 were pre-transplantation from both donors and recipients; 468 were post-transplantation peripheral blood samples from recipients (from 74 recipients) and 168 were post-transplantation serum samples from recipients (from 25 recipients).

Genotyping and chimerism analysis: Seven insertion-deletion bi-allelic polymorphisms (GSTM, GSTT, SRY, Xq28, FVII, RhD, and rs4399) were detected with Hyb probes, and 14 SNPs (S1a, S1b, S4a, S4b, S5a, S5b, S7a, S7b, S8a, S8b, S9a, S9b, S11a, and S11b) were detected with TM probes. Quantitative real time PCRs and chimerism quantification were performed as described previously by Ayala et al. [14,24,25].

| | |
|--|--------------|
| Sex (n, %) | |
| Males | 63(78.8) |
| Females | 17(21.2) |
| Age (years) | |
| Mean ± SD | 54.9±9.71 |
| Median (range) | 54.0 (26-71) |
| Blood Type | |
| O | 34 (42.5) |
| A | 34 (42.5) |
| B | 9 (11.2) |
| AB | 3 (3.8) |
| Rh blood group | |
| Positive | 66 (82.5) |
| Negative | 14 (17.5) |
| Cause of transplantation (n, %) | |
| HBV | 9 (11.25) |
| HCV | 29 (36.25) |
| Alcoholic Cirrhosis | 21 (26.25) |
| Autoimmune Cirrhosis | 3 (3.75) |
| Drug Toxicity | 1 (1.3) |
| Cryptogenic | 3 (3.8) |
| HCV and Alcoholic Cirrhosis | 10 (12.5) |
| HBV and Alcoholic Cirrhosis | 2 (2.5) |
| Budd-Chiari Syndrome | 1 (1.25) |
| Other | 1 (1.25) |
| Type of Transplant (n, %) | |
| Split | 4 (5) |
| No Split | 76 (95) |
| Brain-dead donor | 70 (87.5) |
| Non Heart beating donor | 10 (12.5) |
| Global Survival (n, %) | |
| Alive | 64 (80) |
| Dead | 16 (20) |
| Immunosuppressive Regimens | |
| Mono therapy | 19 |
| Dual therapy | 41 |
| Triple therapy | 19 |
| Quadruple therapy | 1 |
| Type of CIN | |
| Tacrolimus | 75 |
| Ciclosporin A | 3 |
| No CIN | 2 |
| Nº Samples obtained during an immunosuppressive regimen containing: | |
| Tacrolimus | 445 |
| Ciclosporin A | 22 |
| Mycophenolate | 173 |
| Prednisone | 247 |
| Sirolimus | 58 |
| Azathioprine | 3 |
| Outcome (n, %) | |
| No events after LT | 17 (21.2) |
| Events after LT | 63 (78.8) |
| Events (n, %) | |
| Thrombosis | 6 (7.5) |
| Acute Rejection | 29 (36.2) |
| Chronic Rejection | 2 (2.5) |
| Toxicity | 21 (26.2) |
| Recurrent Disease | 27 (33.8) |
| Tumors | 5 (6.25) |
| Graft Dysfunction | 8 (10) |
| Infections | 40 (50) |

| | |
|---------------|-----------|
| HCV | 22 (27.5) |
| HBV | 2 (2.5) |
| CMV | 3 (3.8) |
| HCV+CMV | 1 (1.2) |
| HBV+CMV | 2 (2.5) |
| Others | 10 (12.5) |

Table 1: Liver Transplant Recipient Characteristics in a peripheral chimerism study.

DC was expressed as a percentage of donor DNA in the post-transplant recipient sample in this study.

HLA typing and KIR genotyping, phenotyping, and cytotoxicity

Characteristics of the recipients and donors: A total of 23 LT patients and their respective donors took part in this study. All patients underwent transplantation at the 12 de Octubre Hospital during the period of time already stated above. In these cases ethical issues were completed as well. The characteristics of patients and immunosuppressive regimen are summarized in Table 2.

Sample collection and DNA preparation: KIR phenotype was studied in 10 peripheral blood donor samples, and 11 pre-transplantation and 8 post-transplantation peripheral blood recipient samples. The cytotoxicity assay was made in 9 donor samples, and 11 pre-transplantation and 11 post-transplantation recipient samples. KIR genotyping and KIR ligand (HLA-I typing) were studied in 13 donor samples and 23 pre-transplantation and 13 post-transplantation recipient samples.

Antibodies and flow cytometry analysis: The following fluorochrome-labeled monoclonal antibodies (mAbs) against human antigens were obtained from Becton Dickinson (Franklin Lakes, NJ, USA): CD3 PE-Cy7, CD20-PE, CD45-FITC, CD158b1FITC, NKG2D-APC. HLA Lineage-FITC, HLADR-APC-Cy7, CD11c-PECy5, CD45RA-PE-Cy5, CD158 a,h-PE, and CCR7-PE. Fluorochrome-labeled mAbs against CD19-PE, CD56-APC were obtained from Beckman Coulter (Fullerton, CA, USA).

NK cell, NK cell subsets, T cell, T cell subsets, B cell, and NKT cell phenotyping was performed on fresh samples of whole blood by multiparametric flow cytometry (Becton Dickinson, FACSCanto II). NK cells were defined as CD3-negative CD56-positive cells. The NK dim cell subset was defined as a moderate expression of CD56 on NK cells, and the NK bright cell subset was defined as a high expression of CD56 on NK cells.

KIR genotyping and KIR ligand (HLA-I typing): Fifteen human KIR genes and two pseudo genes were analyzed by PCR with a KIR typing kit (Miltenyi Biotec, Bergisch Gladbach, Germany). KIR A and B haplotype were performed as described previously by A. Pérez-Martínez et al (BMT 2012) KIR ligand HLA-C allotypes (C1 and C2) and HLA-B allotypes (Bw4) were determined using high-resolution PCR-sequence-based typing.

Cytotoxicity assays: The natural cytotoxicity of NK cells was evaluated as described formerly by Perez-Martinez et al. (BMT 2012) The number of NK cells was calculated by multiplying the lymphocyte counts by the percentage of CD3-CD56+ NK cells.

The equations used to calculate spontaneous and specific cytotoxicity were also previously defined by Perez-Martinez et al. [26].

Luminex assay for cytokine measurement: Recipient pre (n=62) and post (n=109) transplantation serum samples were analyzed by

| | |
|--|--------------|
| Sex (n, %) | |
| Males | 12(46.15) |
| Females | 14(53.75) |
| Age (years) | |
| Mean ± SD | 54.25±9.98 |
| Median (range) | 56.0 (26-71) |
| Blood Type | |
| 0 | 11 (42.4) |
| A | 14 (53.8) |
| B | 1 (3.8) |
| Rh blood group | |
| Positive | 20 (76.9) |
| Negative | 6 (23.1) |
| Cause of transplantation (n, %) | |
| HBV | 6 (23.1) |
| HCV | 6 (23.1) |
| Alcoholic Cirrhosis | 10 (38.5) |
| Cryptogenic | 1 (3.8) |
| HCV and Alcoholic Cirrhosis | 2 (7.7) |
| HBV and Alcoholic Cirrhosis | 1 (3.8) |
| Type of Transplant (n, %) | |
| Split | 2 (7.7) |
| No Split | 24 (92.3) |
| Brain-dead donor | 24 (92.3) |
| Non Heart beating donor | 2 (7.7) |
| Global Survival (n, %) | |
| Alive | 21 (80.8) |
| Dead | 5 (19.2) |
| Outcome (n, %) | |
| No events after LT | 9 (34.6) |
| Events after LT | 17 (65.4) |
| Events (n, %) | |
| Thrombosis | 2 (7.7) |
| Acute Rejection | 10 (38.8) |
| Chronic Rejection | 2 (7.7) |
| Toxicity | 6 (23.1) |
| Recurrent Disease | 7 (26.9) |
| Graft Dysfunction | 2 (7.7) |
| Infections | 9 (34.6) |
| HCV | 4 (15.4) |
| CMV | 2 (7.7) |
| HCV+CMV | 1 (3.8) |
| HBV+CMV | 2 (7.7) |

Table 2: Liver Transplant Recipient Characteristics in a NK cell study.

ELISA according to the manufacturer's instructions: Biorad™ 17-plex kit on a Luminex™ platform. The selected mediators included Th1 cytokines (IL-2; IL-12p70); Th2 cytokines (IL-4; IL-5; IL-10; IL-13); Th17 cytokines (IL-6; IL-17); growth factors (GM-CSF; GCSF); chemokines (IL-8; MCP-1; MIP-1b), and also IL-7. All samples and standards were run in duplicate. Data analyses of all assays were performed with the Bio-Plex manager software (BIORAD, CA, USA). For all cytokines tested, any sample falling below the lowest concentration on the standard curve was assigned to a level of 0 ng/mL.

Statistical analyses

Statistical analyses were performed with SPSS statistical software, version 15.0 (SPSS, Chicago, IL). To analyze associations between discrete and categorical numerical variables, the χ^2 test and Fisher test were used. A comparison of the mean values of different groups was carried out with the Student unpaired *t* test, the Wilcoxon test,

and the analysis of variance test. A forward stepwise regression logistic analysis was performed to find variables associated with graft rejection. Survival curves were calculated according to the Kaplan-Meier method, and differences between curves were evaluated with the log-rank test. A *P* value less than 0.05 was considered to be significant. We defined “graft rejection” as liver damage diagnosed by biopsy or that had good evolution with an increased immunosuppressant therapy. We only considered acute rejection episodes in this work.

The diagnosis of liver toxicity was made by excluding other potential causes, including infections, congestive heart failure, liver disease relapse or graft rejection, and was considered highly probable if the patients showed elevated liver enzymes (>10 fold increase in γ-glutamyl transferase ± aspartate aminotransferase ± alanine aminotransferase) without associated infections or without response to antirejection therapy.

Results

High levels of donor hematopoietic chimerism were associated with non-rejection liver recipients

An informative polymorphism to differentiate recipient and donor was found in 74 of 76 cases (97.4%). DC was detected in 241 of the 468 samples (51.5%) with a median of 0.02% (range 0.0045-175%). DC was expressed as a percentage of donor DNA in relation

to total DNA in the post-transplant recipient samples. No difference in mean DC in peripheral blood was detected between the rejection and non-rejection group, when we compared mean DC of all patient samples (in log) (Figure 1A). Nevertheless, high levels of DC in serum samples were associated with non-rejection liver recipients (Figure 1B). Donor/recipient chimerism was studied along 30 months in peripheral blood and serum samples (Figures 1C and 1D). The analysis of post-transplant evolution by time intervals showed high DC during the first three months in the group without graft rejection in peripheral blood and serum samples ($p=0.005$ and $p=0.016$, respectively) (Figure 2). Higher DC levels were detected in serum samples than in peripheral blood samples ($7.23\% \pm 24.86$ vs a $1.77\% \pm 13.59$; $p=0.006$) (Figures 2A and 2B).

Chimerism levels were correlated with platelet count ($r=0.234$, $p<0.001$); or inversely with leucocyte count ($r=-0.139$; $p=0.005$) and lymphocyte count ($r=-0.188$; $p<0.001$). We observed higher DC in female recipients and male donors than other combinations ($p=0.003$), and higher DC in RH+ recipients and RH- donors than other combinations ($p<0.001$). There were no differences in distribution depending on gender or blood group between recipients and donors. In the same way, significant differences were not detected in relation to split graft or non-heart-beating donor. Similar to bone marrow transplantation, DC level was correlated inversely with Mycophenolate level ($r=-0.173$; $p=0.022$), but no correlations were found for TAC or CYA levels.

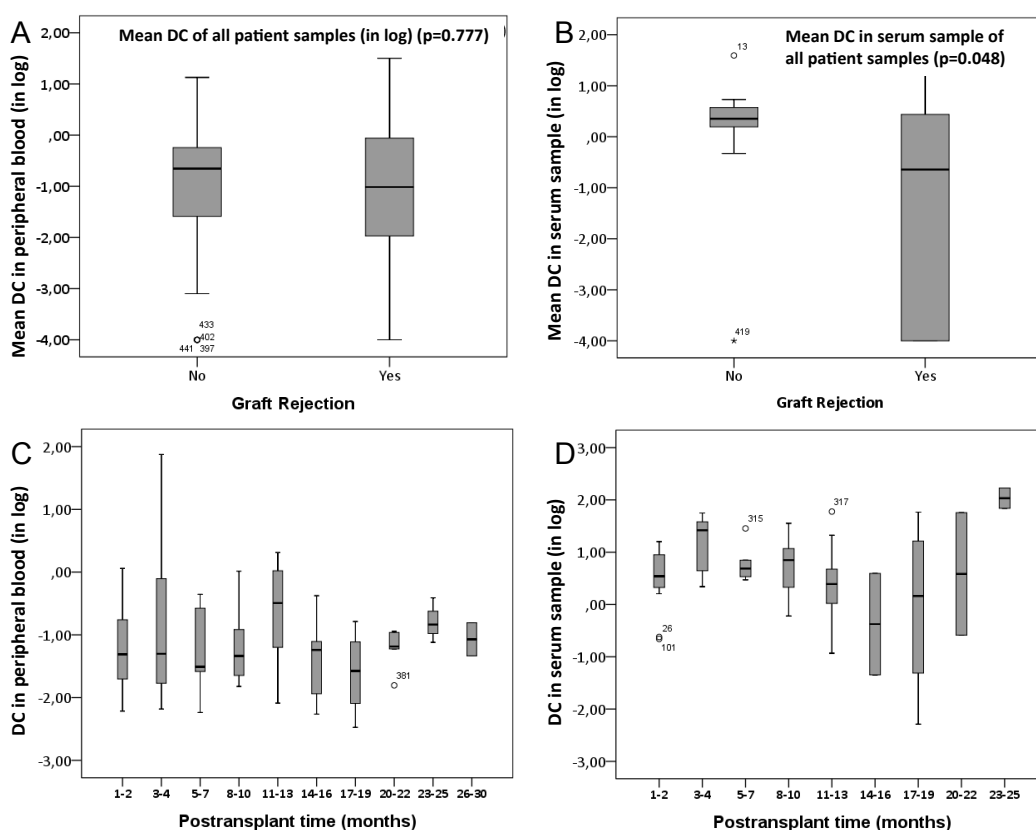


Figure 1: Donor chimerism in peripheral blood and serum samples in liver transplant recipients. Graph A and B show the donor chimerism (DC) mean in peripheral blood or serum samples, respectively, versus liver rejection ($n=47$) or non-rejection cases ($n=27$). C and D graphs show the stability of donor chimerism in peripheral blood or serum samples versus time after liver transplantation. The numbers indicating the outliers in the figures are the sample identifications.

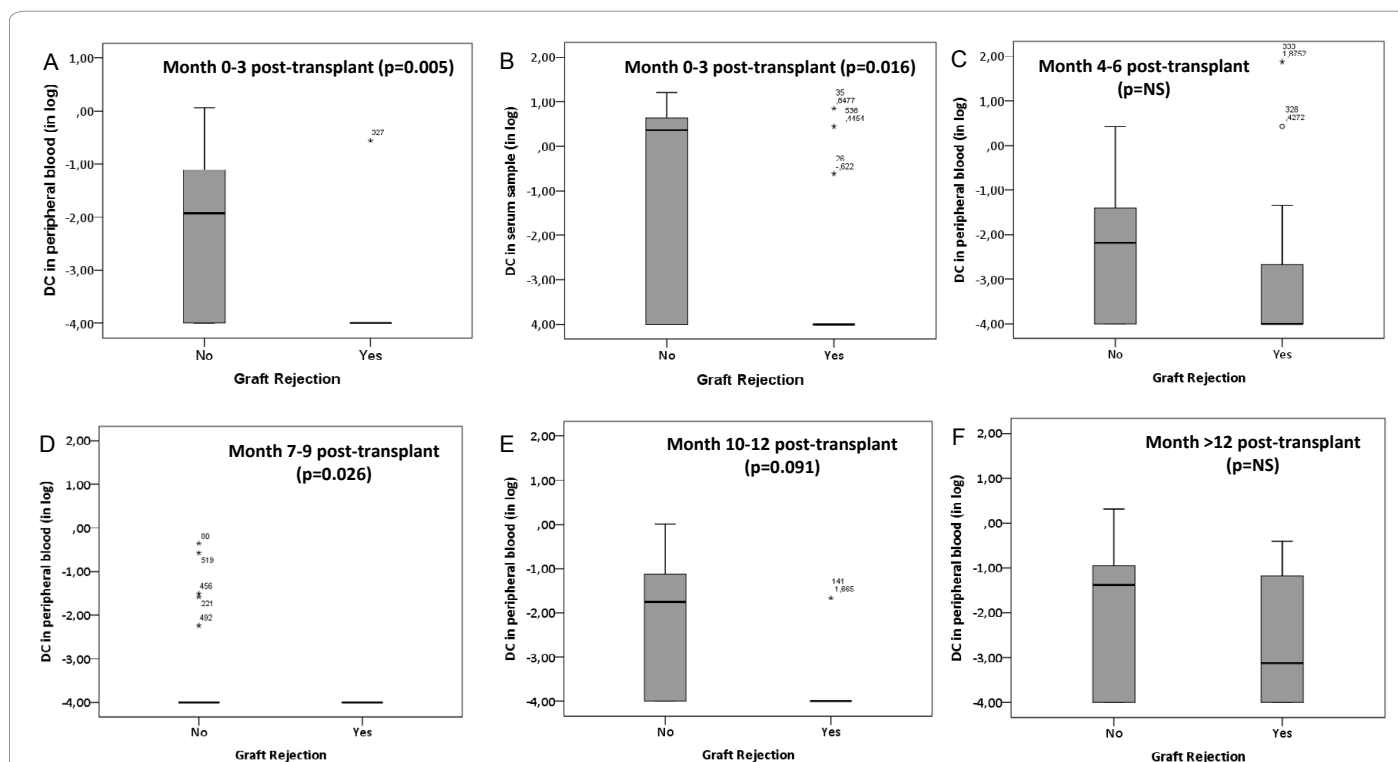


Figure 2: Donor chimerism in serum and peripheral blood in time intervals during liver post-transplant.

Donor/recipient chimerism follow-up versus graft rejection: Graphic A: n=32 non-rejection; 10 rejection. Graphic B: n=14 non-rejection; 14 rejection. Graphic C: n=25 non-rejection; 11 rejection. Graphic D: n=21 non-rejection; 6 rejection. Graphic E: n=20 non-rejection; 5 rejection. Graphic F: n=35 non-rejection; 16 rejection. The number indicating the outliers in the figures are the sample identifications.

High levels of donor hematopoietic chimerism in serum samples were associated with liver disease relapse

No differences in levels of chimerism in peripheral blood were observed related to liver disease relapse. However higher levels of chimerism in serum samples were detected in the group which had

liver disease relapse (p=0.018 in the second sample and p=0.008 in the third sample post-transplant) than the non-relapse group.

A CD56 Bright NK cell subset dominates the early post-transplant period following liver transplantation

Among liver recipients, median pre-transplant and one month

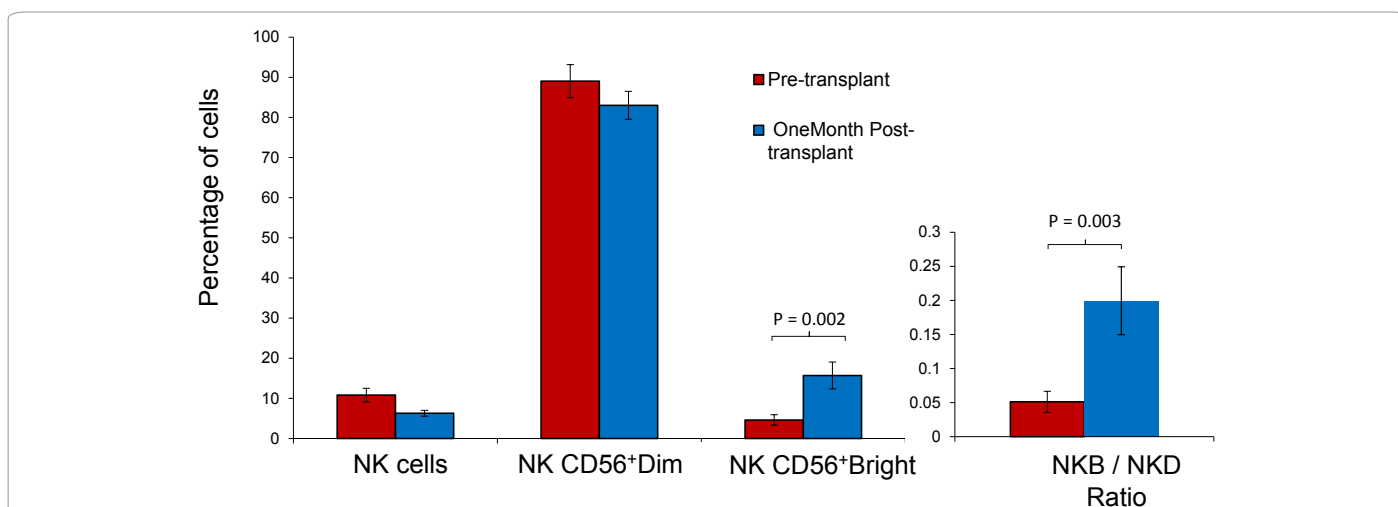


Figure 3: NK populations in liver recipients.

Phenotypic characterization of CD56+bright and CD56+dim NK cell subsets using flow cytometry in 11 pre-transplant and 11 post-transplant recipient samples. CD3-CD56+ NK cells were separated into two subsets, CD56+bright and CD56+dim based on the cell surface density of CD56. Y-line represents the mean of percentage of lymphocytes in the NK subsets (first column), or percentage of CD3-CD56+ NK cells in the NK CD56 dim and NK bright subsets (second and third columns), in peripheral blood of liver recipients. NK bright/dim ratio is shown in pre-transplant and at 1 month post-transplant recipientsamples (graph at righth).

post-transplant CD56 bright NK cells were 4.61% vs 15.70% (p=0.0029) (Figure 3). No differences were observed at other times or NK subsets. Only data from one pair of donor/recipient samples corresponding to a liver rejection case was available. CD56 dim NK percentage for this recipient was similar to non-rejection median (98.60% vs 87.89% vs) and CD56 bright NK was 1.51% (median 11.49%).

Disparities in both NK cell subsets were found when comparing liver recipient and donor percentages. Median CD56dim NK cells in recipients was 89.034% vs 56.86% in donors (p=0.009). Median CD56bright NK cells in recipients was 4.61% vs 2.94% in donors (p=0.015); Median NK bright/NK dim ratio was 0.0512 vs 0.17 in recipients and donors respectively (Figure 4).

Impact of KIR-ligand mismatch on allograft outcome in liver transplantation

In the study of alloreactivity, a higher NK activity was shown in

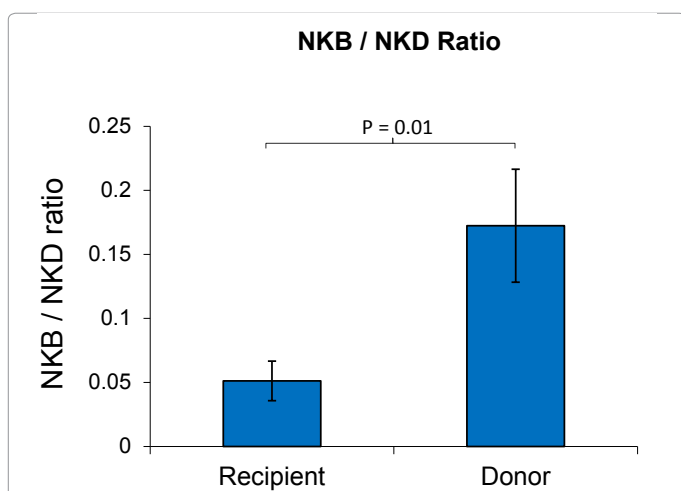


Figure 4: NK populations in donors vs liver recipients. Phenotypic characterization of CD56+^{bright} and CD56+^{dim} NK cell subsets using flow cytometry in 11 recipients and 9 donor samples. NK bright/dim ratio is shown in donor samples and at 1 month post-transplant in recipient samples.

pre-transplant recipients than in post-transplant recipients or in donor samples (Figure 5). KIR-ligand mismatch/match was defined according to van Bergen et al. [27]. KIR ligand mismatch was detected in 12/22 cases (54.5%) and a tendency to association was observed between acute rejection and KIR ligand mismatch: patients with alloreactivity, 5 cases in the group with no rejection and 7 in the rejection group; patients without alloreactivity, 8 cases in the group with no rejection and 2 in the rejection group (p=0.069). We investigated the impact of the number of active or inhibitory KIR genes in recipients. The presence of KIR haplotype B (containing multiple activating KIR genes) has been reported to affect outcomes in hematopoietic stem cell transplantation [28,29]. But we did not observe influence of recipient and donor KIR genotypes on liver rejection.

Donors lacking C1 ligands (C2/C2) exhibited increased risk of acute rejection: We observed no impact of HLA-C2 (lacking C1 ligands) of liver recipients on graft rejection rates (p=0.831), but the existence of lacking C1 ligands (C2/C2) in the donors was associated with graft rejection (17 cases with donor HLA-C2, 7 of them in the non-rejection group and 10 of them in the rejection group). 6 cases without donor HLA-C2 were in the non-rejection group (p=0.012). We observed no association between activating recipient KIR genes and graft rejection. We found 9 cases with activating KIR genes in recipients (7 cases non-rejection and 2 cases with graft rejection); and 14 cases without activating KIR genes (6 cases non-rejection and 8 cases with rejection, p=0.099).

Lower cytokine levels in pre-transplant recipient samples were associated with liver rejection (IL10), liver toxicity (MIP1b) and liver disease relapse (TNFα): Lower cytokine levels in pre-transplant recipient samples were associated with a tendency to graft rejection (mean IL10 was 1.14 vs 7.91 pg/ml, p=0.076). No relation was observed with chronic graft rejection possibly due to the low number of cases available (2 cases) (Figure 6A). Lower MIP1b levels in pre-transplant recipient samples were associated with graft toxicity (mean MIP1b was 66.43 vs 131.81 pg/ml, p=0.017) (Figure 6B). Lower TNF alpha levels in pre-transplant recipient samples were associated with liver disease relapse (mean TNFα was 5.015 vs 62.102 pg/ml, p=0.020) (Figure 6C). Lower GMCSF levels in post-transplant recipient samples were associated with graft rejection (mean GMCSF as 28.89 vs 81.7 pg/ml; p=0.006) (Figure 6D).

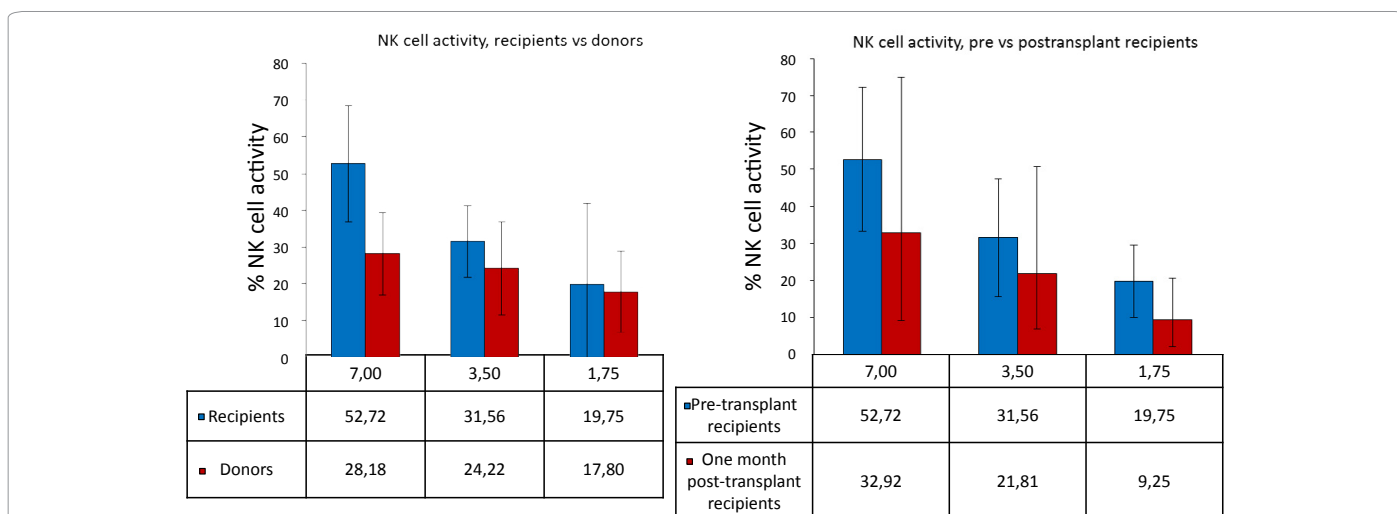
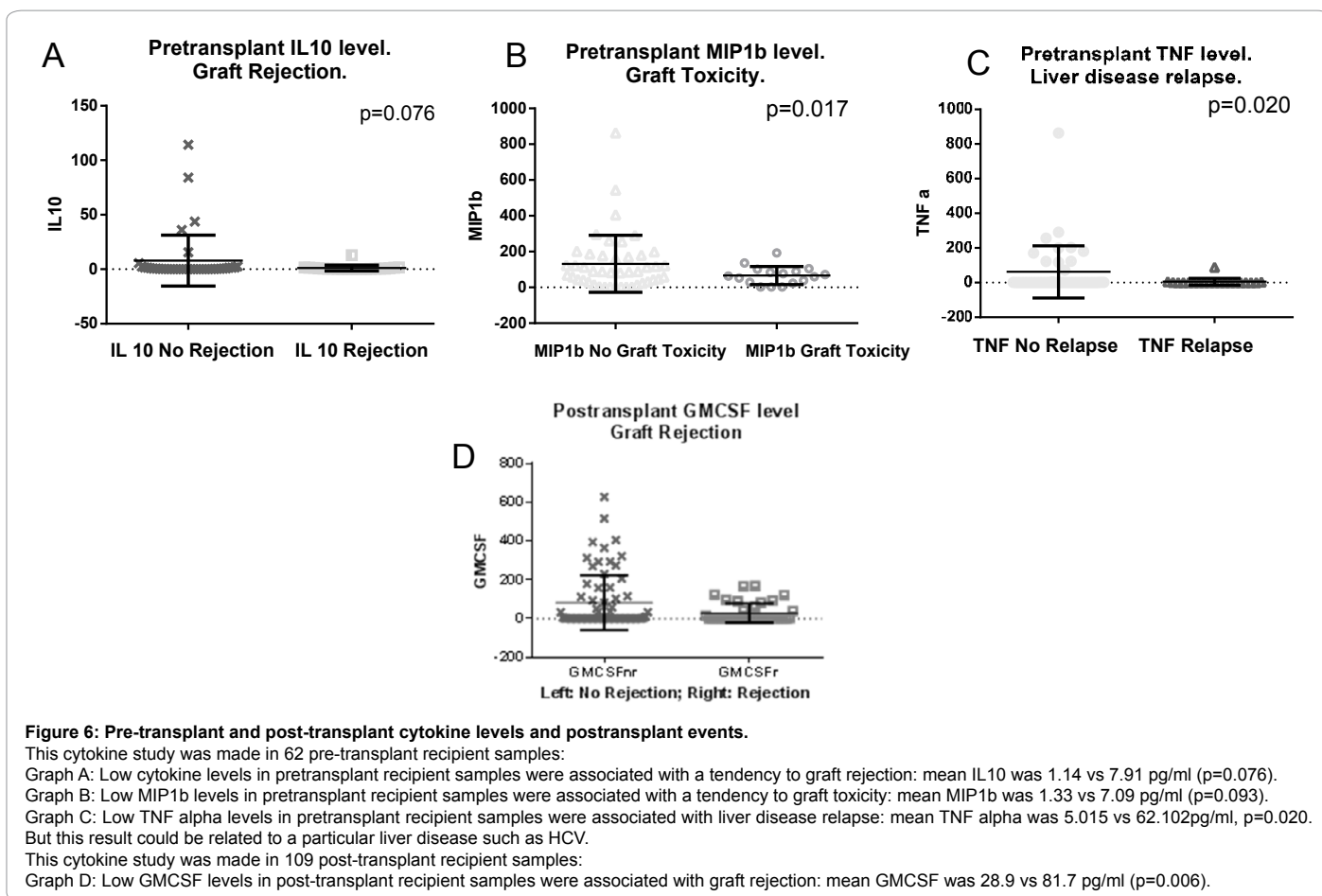


Figure 5: Alloreactivity studies in donor and liver recipients. NK cytotoxicity at different effector-to target ratios (E/T ratios). Effector cells (NK cells) were incubated with BATDA labeled K562 target cells at different effector-to-target (E : T) ratios from 7 : 1, 3.5 : 1, and 1.75 : 1. Flow cytometry-based cytotoxicity of K562 target cells by isolated NK cells (n = 31) from donors and liver recipients were studied. The cytotoxicity assay was made in 11 pre-transplant recipient, 9 donor and 11 post-transplant recipient samples.



Cytokine levels in post-transplant recipient samples were correlated with DC levels in peripheral blood of recipients: IL13 ($r=0.140$, $p=0.013$), MCP1 ($r=0.393$, $p<0.001$), IL2 ($r=0.206$, $p=0.034$), IL8 ($r=0.225$, $p=0.021$) and GMCSF ($r=0.229$, $p=0.018$). Other analyzed cytokines showed no correlation. The immunosuppressive regimen was associated with cytokine levels. IL4 serum levels decreased with the number of administered immunosuppressive drugs (2 or 3 drugs vs 1 drug) (Figure 7).

Pre and post-transplant IL6 levels showed an impact on recipient overall survival. Lower TNF and IL17 levels and higher GMCSF and IL1 β levels were associated with low rejection free survival: Only pre and post-transplant IL6 levels showed an impact on recipient overall survival (OS). Lower OS with high IL6 levels was observed in both determinations ($p=0.022$ and $p=0.022$) (Figures 8A and 8B).

We investigated the relevance of cytokine levels on rejection free survival (RFS) and observed an impact on rejection related to IL17, TNF α , IL1 β and GMCSF levels. Lower IL17 ($p=0.008$) and TNF levels ($p=0.009$) and higher IL1 β ($p=0.010$) and GMCSF levels ($p=0.008$) were associated with low RFS (Figures 8C-8F).

In the multivariate analysis, older recipients at transplantation (OR 1.025; 95% 1.002-1.047; $P=0.032$), younger donors at transplantation (OR 0.971; 95% CI 0.960-0.983; $P<0.001$), negative donor RH (OR 3.8; 95% CI 1.644-6.951; $p=0.001$); positive recipient RH (OR 0.459; 95% CI 0.242-0.870; $p=0.017$), male recipient (OR 0.266; 95% CI 0.152-0.468; $p<0.001$), gender donor/recipient mismatch (OR 2.974; 95% CI 1.9-4.655; $p<0.001$) and heart-beating donor (OR 0.347; 95% CI 0.169-

0.713; $p=0.004$) were independently associated with the probability of graft rejection.

Finally, we performed a multivariate analysis with both clinical

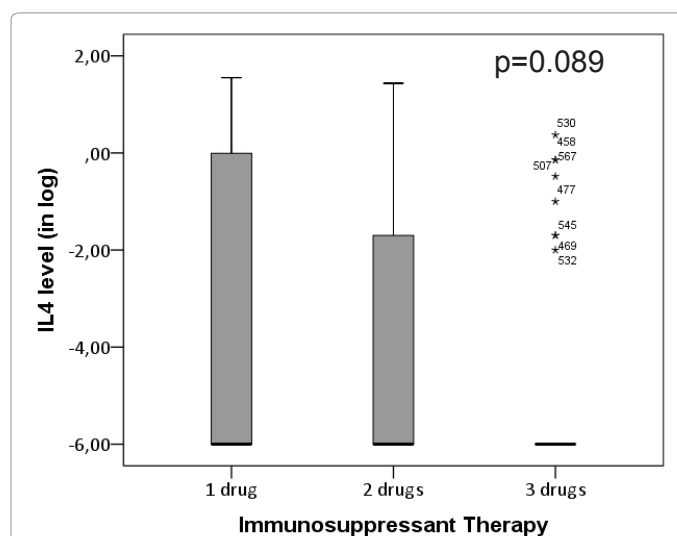
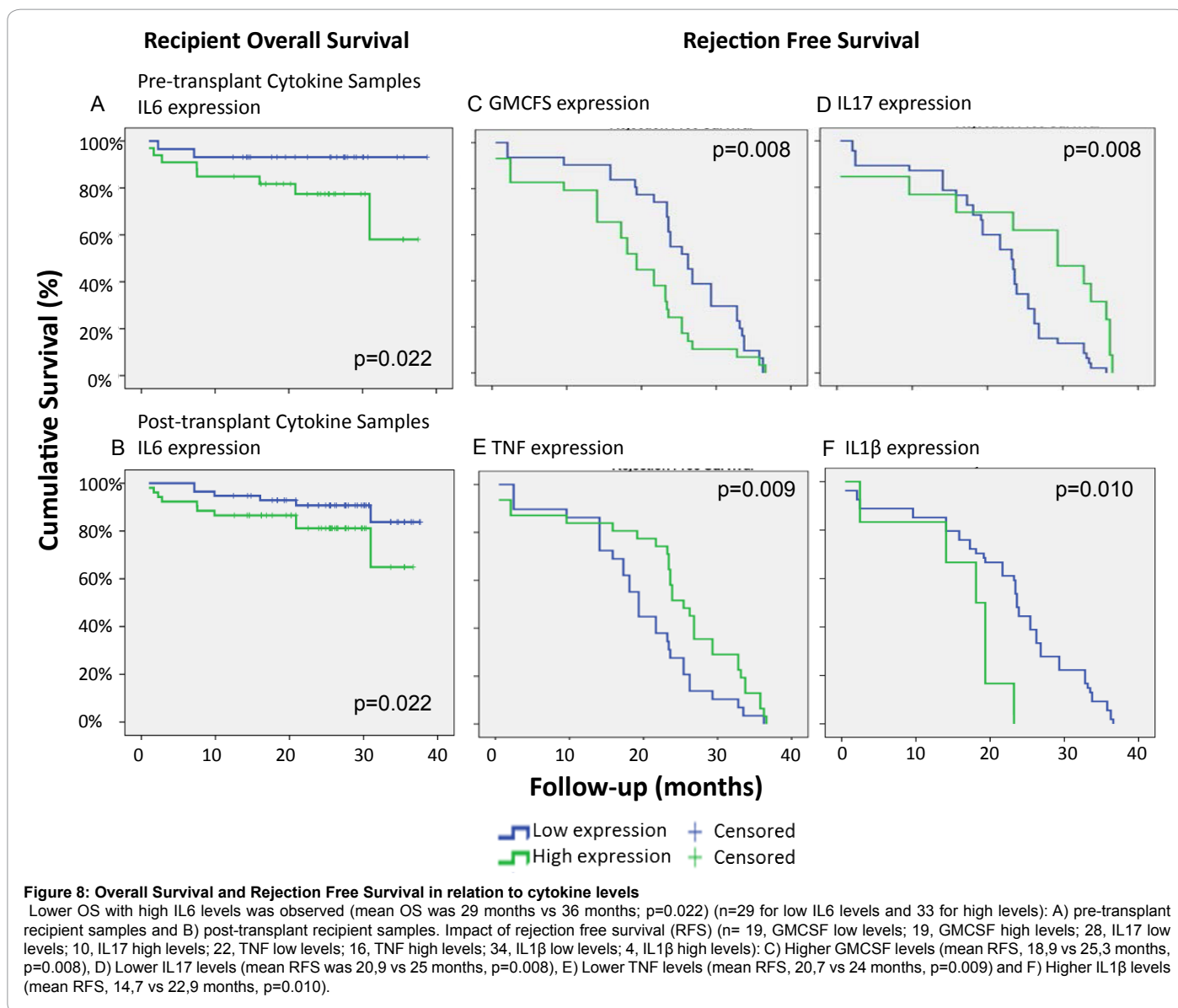


Figure 7: Post-transplant cytokine levels and graft rejection.
 The influence of immunosuppressive regimen (2 or 3 drugs vs 1 drug) on cytokine levels showed a tendency to lower the IL4 level. ($p=0.089$). This cytokine study was made in 109 post-transplant recipient samples.



and biological tolerance markers such as DC, HLA C2/C2, KIR ligand mismatch, haplotype A,B, pre-transplant IL1β, IL17, TNF, GMCSF, and IL6; but only gender donor/recipient mismatch (OR 2.303; 95% CI 1.006-5.274; $p=0.047$) was associated with graft rejection.

Discussion

Starzl [7] was the first to make a strong argument about allogeneic microchimerism, in which the donor passenger leukocytes that migrate widely into the recipient's lymphoid tissues are essential for the maintenance of clonal exhaustion-deletion. This is induced by the initial flood of passenger leukocytes during the first few weeks after transplantation, and the survival of passenger leukocytes is associated with long-term acceptance of the graft.

We reported in a previous work [14] that stable levels of DC, in the absence of other major clinical events, may be a marker of transplantation tolerance, and may help to tailor immunosuppressive treatment in liver transplantation. We have confirmed, in this prospective work, the

relevance of DC observed in the first ten post-transplantation months. However, other variables might influence the levels of DC, especially when they are determined in serum samples. High chimerism levels in serum samples in cases with liver disease relapse can be related to DNA released from the graft where the graft has an endothelial injury. In this sense, our DC results in peripheral blood would reflect more appropriately the positive effect on tolerance.

Natural killer (NK) cell function can be modulated by killer cell immunoglobulin-like receptors (KIR), which interact with human leukocyte antigen (HLA) class I molecules on target cells. KIR-ligand mismatching has been shown by van Bergen et al. [27] to be a significant risk factor for long-term graft loss in HLA-A, -B and -DR compatible kidney transplants. Nevertheless, Tran et al. [30] studied this finding, and performed genotyping on HLA-A, -B and -DR of 608 deceased-donor kidney graft recipients, and found no impact of KIR-ligand mismatch on allograft outcome in HLA-compatible kidney transplantation. Recently, Minguela et al. [31] studied 402 liver recipients and found that KIR gene-gene mismatch is associated with

acute rejection and KIR2DS4+/-C ligands also influence short-term graft survival. Our results support the findings that KIR mismatch has influence on acute rejection although the difference is not significant, probably because of the very small cohort in our study. Other authors [22] described that KIR genotype and KIR-HLA-C ligand compatibility play roles in the recurrence and progression of hepatitis C in liver transplant recipients but our results have not shown this.

The expansion of the cytokine-producing CD56 (bright) NK cell subset is a main feature of lymphocyte reconstitution after allogeneic hematopoietic stem cell transplantation (HSCT) [32]. We observed an increase of CD56(bright) NK cells during first post-transplant month compared to the pre-transplant period. Despite our cohort to study CD56 NK subtypes only included a graft rejection recipient, it should be noted that a clearly lower NKbright/NKdim ratio was obtained in this case.

We detected lower cytokine levels in pre-transplant recipient samples associated with liver toxicity (MIP1b) and liver disease relapse (TNFalpha). These results have not been previously published. Papers with correlations between graft outcome and pre-transplant cytokine determinations are few in liver transplantation, but Friedman's work [33] showed in multivariate analyses that patients experiencing early allograft dysfunction had lower pre-operative IL-6 and higher IL-2R levels.

Nader Najafian et al. published [34] a paper aimed at determining the utility of a noninvasive cytokine assay in screening of acute rejection and their analyses revealed a strong association between post-transplant IL-6 levels and acute rejection after multivariate adjustment for clinical characteristics ($P < 0.001$). Although six cytokines (IL-1B, IL6, TNF, IL4, GM-CSF and MCP-1) distinguished acute rejectors in the training cohort, logistic regression modeling identified one IL-6 as the best predictor. But we found no relation between post-transplant IL-6 levels and acute rejection cases although we observed lower OS with high IL6 levels. We detected lower post-transplant GMCSF levels to be associated with graft rejection. Nevertheless, lower IL17 ($p = 0.008$) and TNF levels ($p = 0.009$) and higher IL1β ($p = 0.010$) and GMCSF levels ($p = 0.008$) were associated with low RFS in the survival analysis. A possible origin for the high GMCSF secretion pointed at the increased CD56 Bright NK population [35]. Elevated GMCSF might in turn induce macrophage differentiation and as result an increased IL1β production. This hypothesis would imply an important role for CD56 Bright NK cells on graft rejection.

IL17 is a cytokine which acts as a potent mediator in delayed-type reactions, with a pro-inflammatory function and acts synergistically with TNF and interleukin-1. IL17, secreted by Th 17 cells, is a highly inflammatory cytokine with a robust effect on stromal cells in many tissues [36-38] and its expression is increased in bronchoalveolar lavages in patients with lung transplantation rejection. Hsieh et al. [39] reported that IL-17 could serve as a predictive parameter for borderline subclinical renal allograft rejection in the future. TNF alpha is an adipokine involved in systemic inflammation and is a member of a group of cytokines which stimulate the acute phase reaction. The ratio of IL10/TNFa expression in regulatory B cells may be a better indicator of regulatory function than IL10 expression alone for the prediction of immunologic human allograft injury [40]. Our results agree with previously published papers and also detected lower TNFα and IL17 levels associated with low RFS.

On the other hand, cytokine levels could be influenced by the particular immunosuppressant therapy. Patients on calcineurin

inhibitors had impaired inflammatory cytokine production in response to TLR2, TLR4, and TLR7/8 stimulation compared with healthy controls [41]. In our work, the influence of immunosuppressive regimen (2 or 3 drugs vs 1 drug) on cytokine levels showed a tendency to decrease the level of IL4.

Gender donor/recipient mismatch was independently associated with the probability of graft rejection even when clinical and biological tolerance markers were considered. A higher risk of graft loss in male recipients after liver transplantation from female donors has been reported before by other authors [42]. Also male-to-female liver transplantations seem to present in some cases complications during surgery intervention with a negative impact in the patient outcome [43]. As Rustgy et al. remarked immunologic factors are influenced by gender; anyhow, additional studies are required to confirm this finding and to clearly uncover its causes. Although only gender donor/recipient mismatch was associated with graft rejection in multivariate analysis, DC in peripheral blood/serum samples, KIR ligand mismatch and cytokine levels (TNFα, IL17, GMCSF, IL1β and IL6) should be incorporated into the evaluation of liver recipients in the screening of operational tolerance programs.

In conclusion, we have confirmed that high chimerism levels in peripheral blood during the first months after transplantation are related with a lower probability of rejection. A CD56 bright NK cell subset dominates the early post-transplant period following liver transplantation. A tendency to high frequency of graft rejection was observed in cases with KIR-ligand mismatches. Donors lacking C1 ligands exhibited increased risk of acute rejection. Cytokine levels predict events in liver transplant recipients: low TNFα levels in pre-transplant recipient samples were associated with liver disease relapse and low IL17 and TNFα levels and high GMCSF and IL1β levels are associated with low rejection free survival. Finally IL6 levels show an adverse impact on recipient overall survival.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

RA, EM and JML designed research, analyzed data, and wrote the paper; IR and SB analyzed the data; and SG, AP, JLV, MS, JAM, CL, and CJ performed the research. All authors read and approved the final manuscript.

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