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T helper1/T helper2 and Interferon-γ/Interleukin-10 Ratios in Liver Transplant Rejection

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A B S T R A C T

Introduction: Immune monitoring in transplant recipients, examining lymphocyte subsets and cytokine levels, is pivotal for advancing individualized medicine in transplantation. This study aims to assess T helper 1 and 2 cells in acute liver transplant rejection.

Material & Methods: Thirty liver transplantation candidates were enrolled pre- and six months post-transplantation under stable condition. Additionally, fifteen recipients with acute rejection, matched for age and transplantation duration, were included. Flow cytometry and ELISA assessed TCD4+CXCR3+IFN-γ (T helper 1) and TCD4+CCR4+IL-4+ (Th2) cell frequencies, as well as serum IFN-γ and IL-10 levels.

Results: Stable recipients showed significant decreases in Th1 and Th2 cell percentages six months post-transplant (both $p < 0.0001$), maintaining a comparable Th1/Th2 ratio. Serum IFN-γ levels also decreased. Conversely, the rejection group exhibited higher Th1 cell proportions and increased IFN-γ concentration compared to stable recipients ($P = 0.03$ and 0.001, respectively). IL-10 levels slightly decreased in both groups. Consequently, the IFN-γ/IL-10 ratio significantly increased during acute rejection ($p < 0.0001$). Th1 cell frequency and IFN-γ levels negatively correlated with allograft function.

Conclusion: Six months post-transplant, stable recipients experienced decreased Th1 and Th2 cell percentages, maintaining Th1/Th2 balance. The IFN-γ/IL-10 ratio significantly increased during acute rejection, indicating an immunological imbalance. Elevated Th1 frequency and IFN-γ levels were negatively correlated with allograft function, emphasizing their potential role in rejection dynamics.

Keywords: Liver Transplantation, Graft Rejection, T-Lymphocytes, Interleukin-10, Interferon-gamma

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Introduction

Liver transplantation is the standard treatment of acute and chronic hepatic failure as well as incurable hepatic cancers and infections (1). In spite of substantial advances in surgical techniques and post-operative care, the growing number of liver transplant recipients requiring lifelong administration of immunosuppressive (IS) drugs and the permanent risk of allograft dysfunction pose a serious challenge for post-transplant surveillance. The long-term side effects of IS drugs including metabolic dysregulation (e.g., hyperglycemia and dyslipidemia), gastrointestinal complications, osteoporosis, opportunistic infections, and increased risk of lymphoproliferative disorders and skin malignancies might reduce allograft and patient survival (2). The other obstacle in the way of successful transplantation is subclinical rejection, which eventually leads to clinical acute rejection or chronic allograft dysfunction. Indeed, pathological evaluation of tissue biopsy is the only way to detect the primary tissue damages caused by alloimmune responses, but it has not yet been accepted as a routine follow-up test in practice (3).

Immune monitoring of transplant recipients might be considered a solution for both of the abovementioned problems. Describing the immune profile of tolerant recipients, including the frequency of immune cells and mean levels of critical cytokines, may help establish standard ranges of immune components in transplant patients. Such standards might be beneficial in two ways: first, by avoiding excessive immunosuppression through dose adjustment according to the individual immune profile of recipients; second, by the early non-invasive diagnosis of allograft rejection when significant alterations are observed in the immune profile of patients (4).

T lymphocytes, as the most influential cells of the immune system, are able to develop alloimmune responses. Many T cell subsets have already been demonstrated to be involved in the acceptance or rejection of the allograft. For instance, T helper 1 (Th1) cells are supposed to provoke inflammatory responses in allograft tissue (5), while the Th2 subset seems to promote tolerance induction (6). Similarly, the Th17/T regulatory (Treg) cell ratio has been shown to have a predictive value for rejection or acceptance of allograft. Our previous study also showed lower Th17/Treg ratios in stable recipients compared to the patients with acute rejection (7).

IFN-γ is mainly produced by Th1, cytotoxic T lymphocytes, and natural killer cells, playing a critical role in developing cell-mediated immune responses (8). It has been shown that CD4+CD69+ and CD8+CD69+ lymphocytes of the liver transplant recipients express more IFN-γ upon stimulation compared to the normal controls (9). Moreover, an experimental model displayed increased levels of IFN-γ and IL-2 during the rejection episodes (10). In contrast, IL-10, secreted by Th2 and regulatory subsets, appears to have positive effects on allograft tissue since increased expression of IL-10 by genetically engineered cells resulted in tolerance induction towards liver transplant (11). On the other hand, IL-10 has been found to enhance antibody-mediated alloimmune responses (12).

In the present study, we aimed to examine Th1 and Th2 lymphocyte frequency and the serum levels of IFN-γ and IL-10 before and after transplantation in stable liver transplant recipients to describe optimal percentages and amounts of these subsets and their cytokine levels in stable graft function. Besides, the Th1/Th2 and IFN-γ/IL-10 ratios were evaluated to make a comparison between stable and rejection status.

Materials and methods

Participant Recruitment and Exclusion Criteria

Liver transplantation candidates from a single center (Imam Khomeini Hospital, Liver

Transplantation Department) were recruited between March 2019 and July 2021. Peripheral blood sampling took place before transplant surgery and six months post-transplant if the recipient was in stable condition according to clinical and para-clinical criteria. The donated livers were harvested from brain-dead unrelated individuals. The study excluded patients with active infection and those with a history of acute rejection episodes. Almost all recipients were under tacrolimus monotherapy [(Prograf) 0.06 ± 0.02 mg/Kg/day] as maintenance immunosuppressive therapy.

In the second phase, to make a comparison between rejecting patients and stable recipients, 15 independent patients with the clinical diagnosis of acute liver allograft rejection were recruited (prior to high-dose corticosteroid therapy). The rejection group was selected from age/sex-matched patients who had been transplanted for less than one year. All participants signed informed consent. General and laboratory data of the study population are presented in Table 1 (stable recipients before and after transplantation) and Table 2 (comparing acute rejection and stable recipients).

Table 1. Demographic, Clinical, and Paraclinical Data of Stable Recipients Before and After Transplantation

	Pre transplantation	Post transplantation	P. value
Recipients Age (Yr.)	47.1 ± 11		
Donors Age (Yr.)	41 ± 13.3		
Recipients Gender (male/female)	18/12		
Donors Gender (male/female)	19/11		
Underlying Liver Disease	NASH Cirrhosis 6 HCV Cirrhosis 5 HBV Cirrhosis 4 BCS Cirrhosis 4 AIH Cirrhosis 3 PSC Cirrhosis 3 ASH Cirrhosis 2 PBC Cirrhosis 2 Acute hepatic failure 1		
WBC (/mm^3)	6213 ± 2906	5360 ± 2061	0.19
AST (IU/L)	66.23 ± 40	18.5 ± 7	<0.0001
ALT (IU/L)	46.5 ± 29	21.5 ± 15.2	<0.0001
Bili Total (mg/dL)	4 ± 3.4	0.76 ± 0.51	<0.0001

NASH: non-alcoholic steatohepatitis; ASH: alcoholic steatohepatitis; HCV: hepatitis C; HBV: hepatitis B; BCS: budd-chiari syndrome; PBC: primary biliary cholangitis ; PSC: primary sclerosing cholangitis; AIH: autoimmune hepatitis; WBC: white blood cells; AST: aspartate aminotransferase; ALT: alanine aminotransferase; Bili: bilirubin

Table 2. Demographic, Clinical, and Paraclinical Data of Stable Recipients (Post-Transplant) and Acute Rejection Group

	Stable recipients	Acute rejection	P. value

Recipients Age (Yr.)	47.1 ± 11	43.4 ± 10.2	0.65
Donors Age (Yr.)	41 ± 13.3	44.5 ± 8	0.58
Recipients Gender (male/female)	18/12	9/6	1
Donors Gender (male/female)	19/11	10/5	0.7
Underlying Liver Disease	NASH Cirrhosis 6 HCV Cirrhosis 5 HBV Cirrhosis 4 BCS Cirrhosis 4 AIH Cirrhosis 3 PSC Cirrhosis 3 ASH Cirrhosis 2 PBC Cirrhosis 2 Acute hepatic failure 1	NASH Cirrhosis 6 HCV Cirrhosis 2 HBV Cirrhosis 1 BCS Cirrhosis 2 AIH Cirrhosis 2 PSC Cirrhosis 0 ASH Cirrhosis 1 PBC Cirrhosis 1 Acute hepatic failure 0	
TX duration (months)	6	9.4 ± 2.3	0.78
WBC (/mm^3)	5360 ± 2061	5658 ± 2317	0.88
AST (IU/L)	18.5 ± 7	55.5 ± 14.6	<0.0001
ALT (IU/L)	21.5 ± 15.2	44.7 ± 10.7	<0.0001
Bil Total (mg/dL)	0.76 ± 0.51	4.39 ± 2.1	<0.0001
IS drugs	Tac 27 CsA 1 Tac+MMF 2	Tac 14 Tac+MMF 1	

NASH: non-alcoholic steatohepatitis; ASH: alcoholic steatohepatitis; HCV: hepatitis C; HBV: hepatitis B; BCS: budd-chiari syndrome; PBC: primary biliary cholangitis ; PSC: primary sclerosing cholangitis; AIH: autoimmune hepatitis; WBC: white blood cells; AST: aspartate aminotransferase; ALT: alanine aminotransferase; Bil: bilirubin; Tac: Tacrolimus; CsA: Cyclosporin A; MMF: Mycophenolate mofetil

All the techniques carried out in the present study involving human participants were in accordance with the standards of the institutional research committee and with the Helsinki Declaration and its later amendments or comparable ethical standards. It has received ethical approval from Tehran University of Medical Sciences (Code: IR.TUMS.CHMC.REC.1396.2394). Informed consent was obtained from all participants after describing the process and goals of the study.

Cell separation and Flowcytometry

Ficoll-Hypaque (inno-train, Germany) gradient was utilized for the isolation of peripheral blood mononuclear cells (PBMC). The PBMCs were then adjusted to a concentration of 1x10^6 cells/ml and stained with PE/Cy5 anti-human CD4 (300510, Biolegend), PE anti-human CD183 (CXCR3) (353706, Biolegend), and intracellular FITC anti-human IFN-γ-conjugated antibodies (506504, Biolegend) to identify the Th1 subset. Th2 cells were stained using PE anti-human CD194 (CCR4) (359411, Biolegend), intracellular FITC anti-human IL-4 (500807 Biolegend) antibodies, and PE/Cy5 anti-human CD4 (300510, Biolegend). FACSCalibur (BD FacsCalibur, Becton Dickinson, USA) was used for Flow Cytometry analysis. The data were analyzed with FlowJo v10.7 software (TreeStar Inc., Ashland, OR, USA). To evaluate the

frequency of CD4+CXCR3+IFNG+ (Th1) cells in PBMC, CD4+ gating was performed after lymphocyte selection. CXCR3+IFNG+ cells were gated in the CD4+ population. CD4+CCR4+IL-4+ (Th2) cells were also gated as the CCR4+IL-4+ population within CD4+ lymphocytes. Unstained cells from each sample were used for the negative control.

ELISA

Human IL-10 (ZB-0102C-H9648, ZellBio) and Human IFN-γ (ZB-0105C-H9648, ZellBio) Kits were employed to assess the cytokine levels. ELISA tests were conducted in duplicate. The absorbance levels were determined using the Hiperion MPR4++ Microplate Reader (Medizintechnik GmbH & Co.KG) in accordance with the standard calibration curves.

Statistical Analyses

Data are presented as mean ± standard deviation (SD) or mean ± standard error of mean (SEM), along with the 95% confidence interval. To compare pre- and post-transplantation values, the Paired T-test was employed. Bivariate correlation and linear regression tests were performed to calculate the correlation between variables, presented by the coefficient of determination (r2) and Pearson correlation (r). Non-parametric variables were compared using the Mann-Whitney U test. Ratios were calculated using descriptive statistics and analyzed by the paired T-test. Graphs were presented using box plots (for non-parametric variables) or error bars (for parametric variables). P-values less than 0.05 were considered statistically significant (SPSS 28.0, SPSS Inc., USA).

Results

Th1 and Th2 subset percentages reduced significantly in stable recipients

The frequency of T helper 1 cells, determined as the CD4+CXCR3+IFN-γ+ population, decreased at six months post-transplant (12.87±3.65 vs. 8.31±2.6 mean±SD) (P-value<0.0001). The mean percentage of T helper 2 lymphocytes (CD4+CCR4+IL-4+) was 5.19±1.63 six months post-transplant versus 8.21±2.14 (mean±SD, P-value<0.0001) before transplantation (Figure-1). The mean Th1/Th2 cells ratio pre-transplantation was 1.52 with a coefficient of dispersion (COD) = 0.406, and 1.60 with COD of 0.417 post-transplant, showing no significant alteration in stable recipients (P-value=NS) (Figure 1).

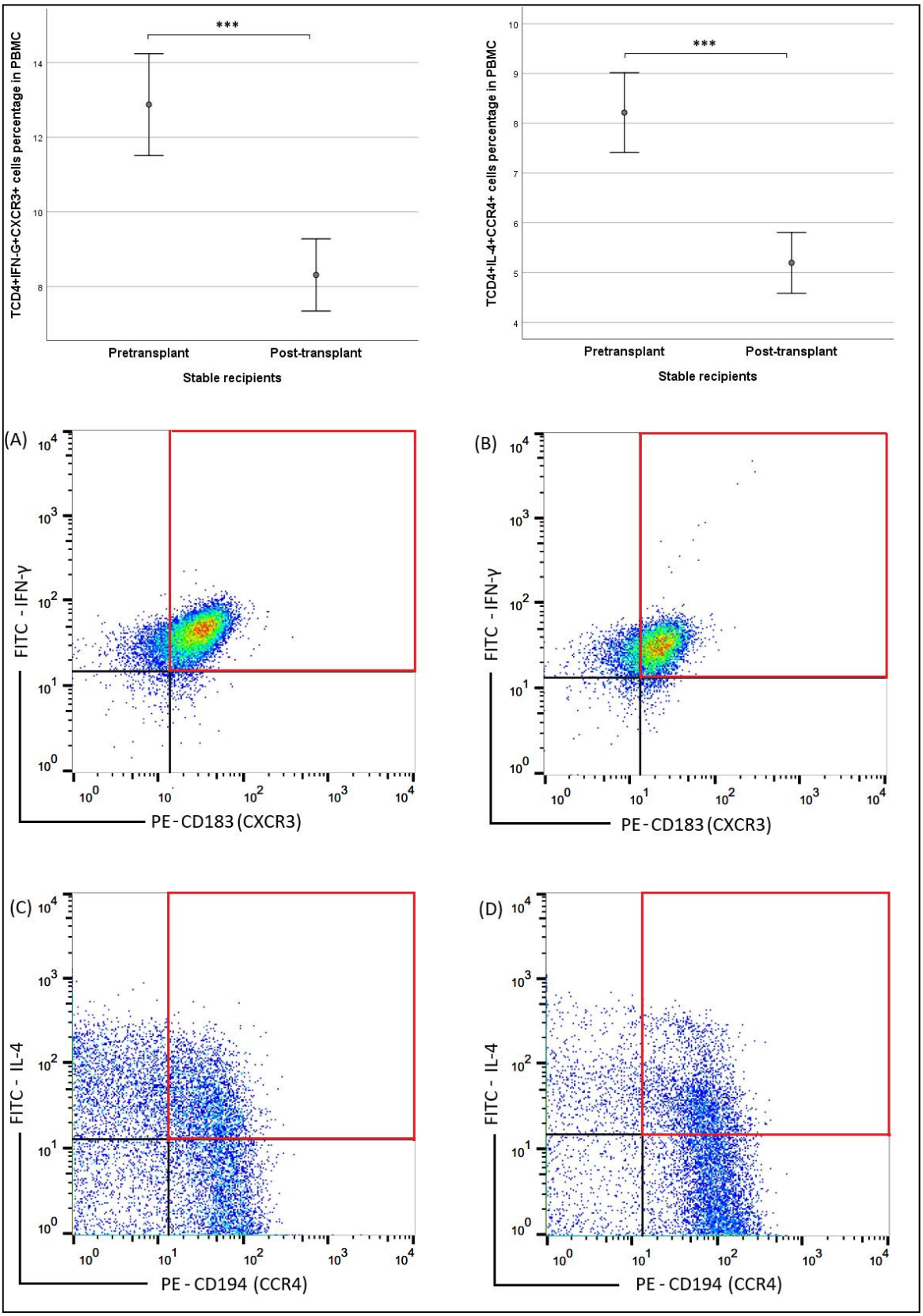

Figure 1. **Changes in Th1 and Th2 Cell Percentages Post-Transplant**. The percentage of CD4+CXCR3+IFN-γ+ (Th1) and CD4+CCR4+IL-4+ (Th2) cells significantly decreased 6 months post-transplant in stable recipients (***: P-value<0.0001). Representative images of Th1 cells (highlighted in red) in a stable recipient before **(A)** and after **(B)** transplantation. Th2 cells (highlighted in red) in a stable recipient before **(C)** and after **(D)** transplantation

IFN-γ levels reduced slightly in stable recipients but the IFN-γ/IL-10 ratio did not change

The serum level of IFN-γ decreased slightly six months after transplantation, but it was not statistically significant (6.67±2.7 vs. 5.82±2.1 mean ± SD) (P-value = NS). IL-10 concentrations showed no considerable change post-transplant in the stable group (3.49±2.08 vs. 3.21±1.7 mean ± SD) (P-value = NS) (Figure 3). The IFN-γ/IL-10 ratio was also comparable between pre and post-transplant evaluations (1.88 vs. 1.71) (p-value = NS) (Figure 2).

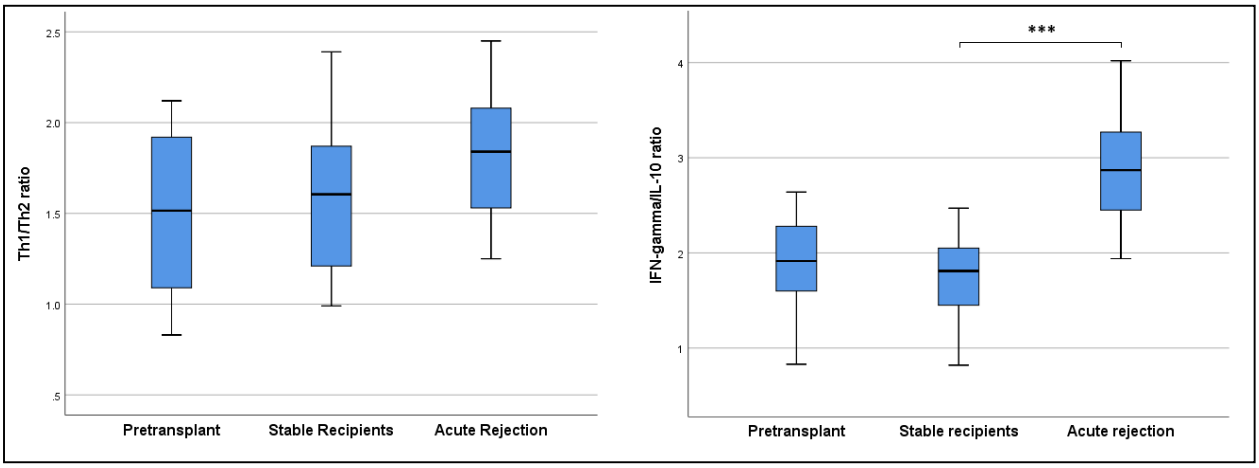

Figure 2. Th1/Th2 Cells and IFN-γ/IL-10 Ratios in Two Groups. Th1/Th2 cell ratios and IFN-γ/IL-10 ratios in two groups of stable recipients (before and six months after transplantation) and patients with acute rejection (***: P-value<0.0001)

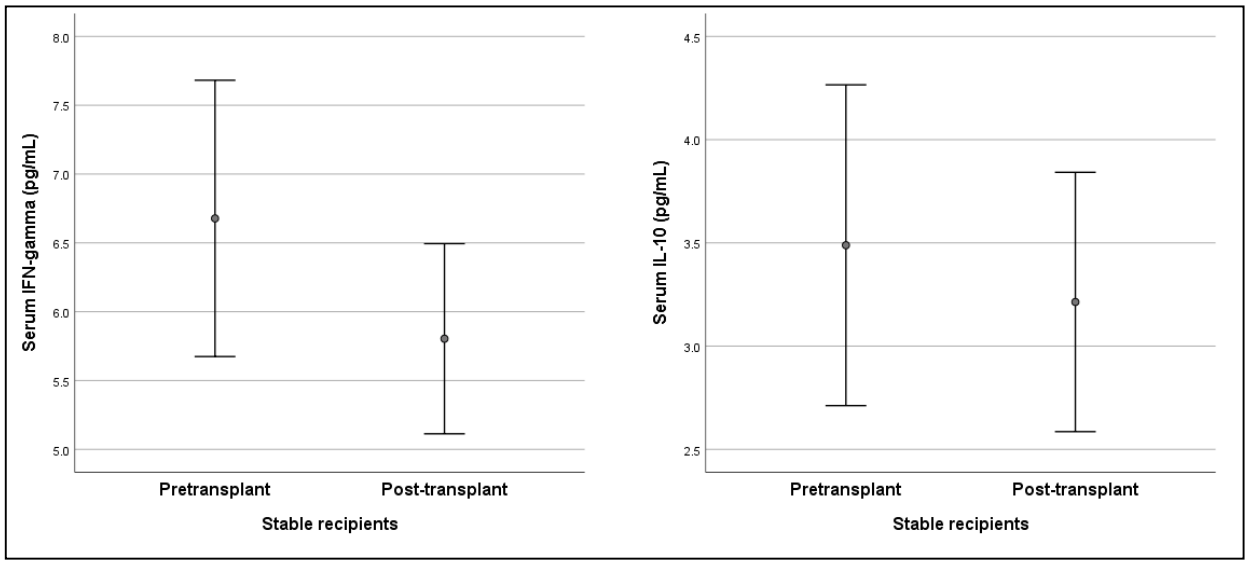

Figure 3. Changes in IFN-γ and IL-10 Cytokine Levels. IFN-γ and IL-10 cytokines showed a slight reduction in stable recipients six months post-transplant (P-value=NS).

Th1 percentages and IFN-γ levels were higher in patients with acute rejection than in the stable recipients

The percentage of Th1 cells in patients with acute rejection was significantly greater compared to stable recipients (10.8±3.75 vs. 8.31±2.6 mean ± SD) (P-value = 0.03). Additionally, the serum level of IFN-γ in the rejection group was 8.5±2.2, which was remarkably higher than in stable patients (5.82±2.1) (P-value = 0.001) (Figure 4).

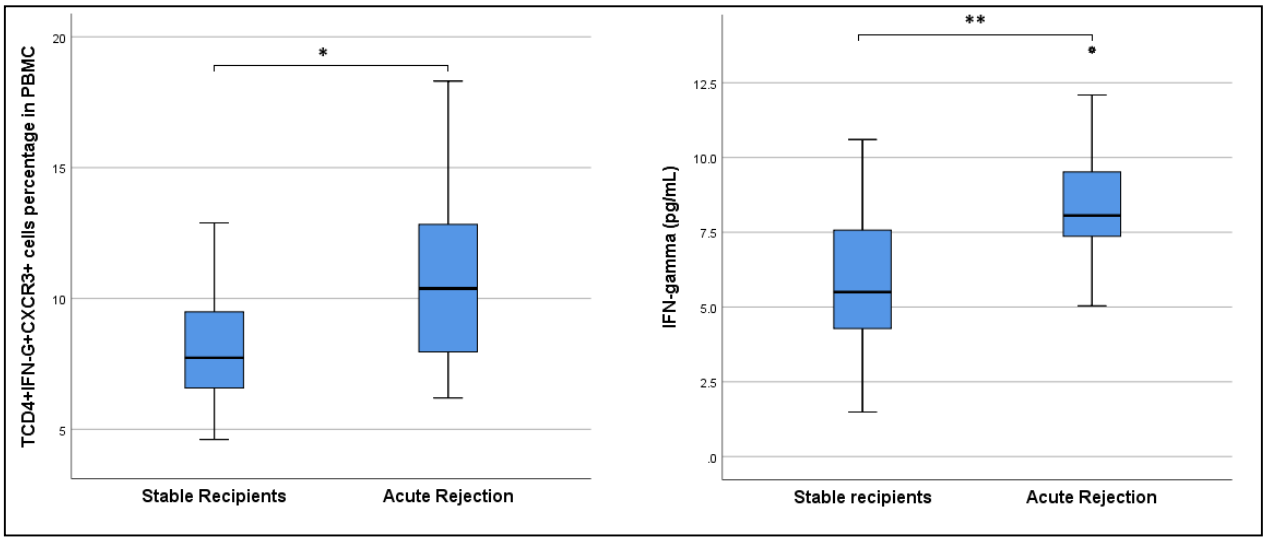

Figure 4. Elevated Th1 Cells Percentage and IFN-γ Levels in Acute Rejection. Th1 cells percentage and IFN-γ levels were higher in patients with acute rejection compared to stable recipients (*: P-value<0.05) (**: P-value<0.001)

Th2 percentages and IL-10 amounts did not show significant differences between stable and rejection groups

The frequency of Th2 cells in patients with acute rejection was marginally higher than in stable recipients, but the difference was not significant (5.19±1.63 vs. 6.06±1.72) (P-value = NS); however, IL-10 levels were slightly lower in the rejection group (3.21±1.7 vs. 2.35±1.11 mean ± SD) (P-value = NS) (Figure 5).

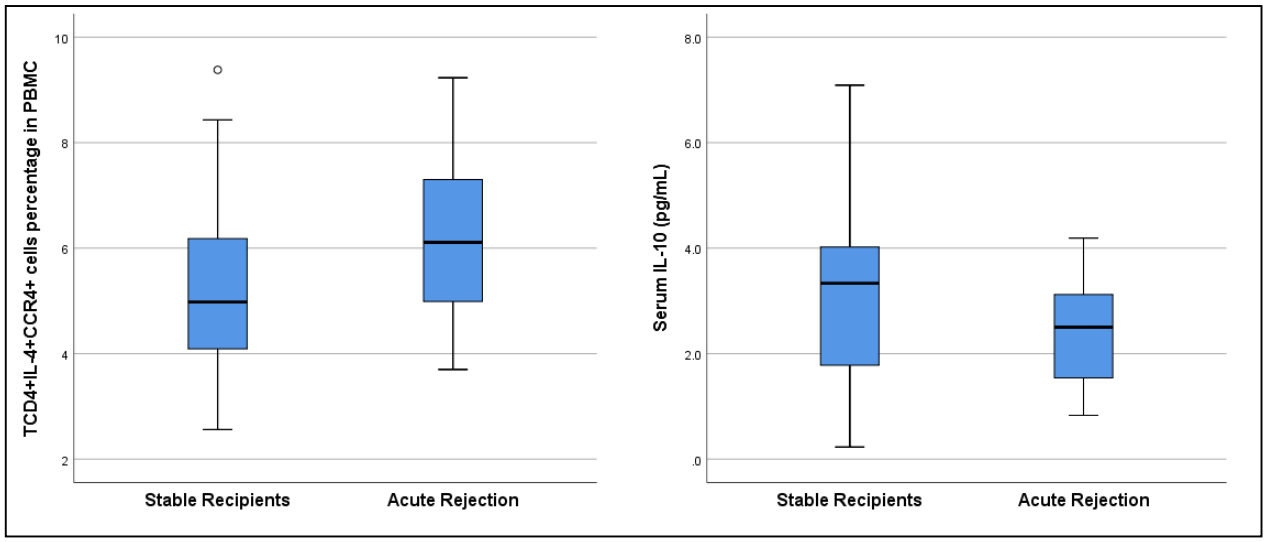

Figure 5. Comparable Th2 Cells Proportions and IL-10 Levels. Th2 cells proportions and IL-10 levels were comparable between acute rejection and stable groups (P-value=NS)

Th1/Th2 ratios were comparable between rejection and stable groups but IFN-γ/IL-10 ratios were significantly higher in patients with rejection

The mean ratio of Th1/Th2 in acute rejection patients was 1.82 with a coefficient of dispersion (COD) of 0.382, and comparable to the mean ratio of stable recipients (1.60 with COD of 0.417) (P-value = NS). Remarkably, IFN-γ/IL-10 ratios were

significantly higher in patients with acute rejection than in the stable group (2.9 vs. 1.71) (p-value<0.0001) (Figure 2).

Significant correlation of Th1 and IFN-γ with allograft function

Th1 cells' percentage in the whole group of recipients showed a significant association with Aspartate transaminase (AST) [Pearson correlation=0.365 and r2= 0.135 (P-value=0.002)], Alanine transaminase (ALT) [Pearson correlation=0.247 and r2= 0.062 (P-value=0.039)], and total bilirubin [Pearson correlation=0.284 and r2= 0.081 (P-value=0.017)] levels. There was also a positive correlation between IFN-γ and AST [Pearson correlation=0.477 and r2= 0.22 (P-value<0.001)], ALT [Pearson correlation=0.354 and r2= 0.122 (P-value=0.003)], and total bilirubin [Pearson correlation=0.261 and r2= 0.06 (P-value=0.029)]. However, neither Th2 cell frequency nor IL-10 levels were correlated with allograft function.

Discussion

T lymphocytes, playing a key role in the initiation and expansion of alloimmune responses, have a particular significance in describing the immune profile of transplant recipients. Th1 cells are responsible for direct and indirect allorecognition. After priming by autologous or allogeneic dendritic cells (DCs) in the lymph node, Th1 cells migrate to the allograft tissue and recruit the effector cells, including TCD8+ cytotoxic cells, B cells, natural killer cells, and macrophages, activating them with cytokine secretion and co-stimulatory molecules expression (e.g., CD40 and CD28). Therefore, it is conceivable that the increased frequency of Th1 cells can be detrimental to transplant outcome (13, 14). On the other hand, Th2 cells, which are mainly involved in antibody-mediated rejection (rarely observed in liver transplantation), produce anti-inflammatory cytokines such as IL-10 and IL-4 that contribute to tolerance induction (15).

Indeed, it has been shown that Th1 and Th17 subsets, and their cytokines IFN-γ, TNF-α, IL-2, and IL-17 are implicated in allograft rejection (16). Ganschow and Chen, in separate investigations, showed a considerable correlation between Th1 cytokine levels and the risk of rejection, whereas Th2 cytokine concentration was associated with improved graft survival (17, 18). Likewise, Wang et al. found that high IFN-γ expression was considerably associated with acute rejection; nonetheless, there was no significant difference in IL-4 and transforming growth factor β (TGF-β) levels between stable recipients and the rejection group in early post-transplant evaluations (19). A recent study showed that prolonged warm ischemia time is associated with elevated IFN-γ, IL-2, and IL-17 levels, and shifts Th1/Th2 balance towards Th1 dominance in liver allograft recipients. Therefore, inhibition of Th1 cells might promote graft survival (20). However, the altered counts of T cell populations post-transplant due to the administration of immunosuppressive drugs disturb the Th1/Th2 balance. For instance, the percentages of TCD4+ and TCD4+CD25+ cell populations have been shown to decrease dramatically in most recipients 12 months after transplantation (21). Our study also demonstrated reduced percentages of both Th1 and Th2 subsets at six months after transplantation in stable recipients, but the Th1/Th2 ratios were similar to that of the pre-transplant period. In addition, the Th1 cell proportion in acute rejection was significantly greater than in stable recipients. Th2 cells of rejecting patients were slightly more than in the stable group, but this was not statistically significant. Therefore, there was not any significant difference between the mean Th1/Th2 ratio of stable recipients and the acute rejection group. This ratio has not been investigated in human liver transplantation; nonetheless, an experimental model of corneal transplant reported increased ratios of Th1/Th2 cells in the lymph nodes and spleens of the rejecting mice (22). Taken together, this ratio does not appear to have

considerable value in the prediction of clinical rejection.

The role of cytokines in liver transplantation outcome has already been studied, and certain cytokines have been found to exert considerable influence on allograft (23). For instance, IFN-γ seems to be implicated in cell-mediated rejection, as IFN-γ-expressing cells were more frequently detected among the stimulated T cells of liver transplant recipients with acute rejection (9). Moreover, the protein expression of IFN-γ reached a peak during the acute rejection of rat liver allograft (10).

IL-10 is an immunomodulatory cytokine secreted by lymphocytes and macrophages, which might inhibit alloimmune responses; however, it has also been shown to contribute to the pathogenesis of antibody-mediated rejection (24). Elevated levels of IL-10 during liver transplant surgery and at the first hours of transplantation suggest a regulatory role for this cytokine in limiting the inflammatory responses elicited by ischemia-reperfusion injury (25). Our results showed slightly decreased levels of IFN-γ and comparable IL-10 amounts before and after transplantation in stable recipients. IL-10 is secreted by a wide range of immune cells, including lymphocytes, monocytes, dendritic cells, and granulocytes; therefore, the number of Th2 cells could not be directly correlated to the serum level of IL-10. However, IFN-γ levels of patients with acute rejection were remarkably higher than in stable patients, whereas IL-10 was slightly lower. As a result, the IFN-γ/IL-10 ratios were significantly greater in the rejection group.

The significance of the IFN-γ/IL-10 ratio has already been investigated in infectious diseases (26), autoimmunity (27), and certain malignancies (28). In transplantation, higher frequencies of donor-specific IFN-γ-producing cells were found among stimulated lymphocytes of patients with renal transplant rejection. On the other hand, the proportion of cells expressing IL-10 was greater in stable recipients. Therefore, IFN-γ/IL-10 ratios were significantly higher in the rejection than in good graft function (29). Likewise, another study demonstrated increased amounts of IFN-γ, reduced levels of IL-10, and higher IFN-γ/IL-10 ratios in chronic renal allograft rejection compared to stable patients (30). Furthermore, Chen et al. also showed higher mRNA expression of IFN-γ in rat hepatic allograft tissue but more IL-10 mRNAs in the isograft, both associated with the serum levels. Thus, the IFN-γ/IL-10 ratios were lower in the tolerant state (31). These and other findings suggest the IFN-γ/IL-10 ratio as a predictor of allograft rejection with considerable sensitivity and specificity.

Finally, we observed a positive correlation between Th1 percentages and IFN-γ levels, as well as liver enzyme levels, particularly AST, which further indicates the significance of Th1 in the prediction of transplant outcome and allograft function. Such an association was absent in the case of Th2 or IL-10.

Conclusion

In conclusion, describing the immune profile of stable liver transplant recipients, particularly T lymphocyte subsets' proportion and function, can help establish individual and evidence-based post-transplant care for patients. By defining the optimal range of immune cells and cytokines in blood circulation, the administration of immunosuppressive drugs can be adjusted without posing secondary immunodeficiency. Additionally, any deviation from these ranges might be considered as an early sign of unfavorable events such as subclinical rejection, opportunistic infections, infection recurrence, or reactivation of preliminary autoimmune disorders. The present study suggests the IFN-γ/IL-10 ratio as a predictor of transplantation outcome in liver allograft recipients.

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Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

SA and NS: Data analysis, writing manuscript; MNT: Patient selection and evaluation; BA: Flow cytometry; HM: ELISA tests; MS and BMM: Serum and cell extraction; FF and BM: Sampling; MHN: Manuscript revision, supervision.

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