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Decreased Treg/Th17 ratio in Bulgarian patients after liver transplantation: a single-center experience

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Abstract

Objective: **The unique mechanisms of immune regulation in the liver allow it to provide local and systemic immune tolerance to own and foreign antigens as a homeostatic condition via different T cell subsets and after liver transplantation (LT) while preserving an effective immune response against pathogens. Therefore, we aimed to analyze the Th17 and Treg subsets in the post-transplant period in patients with LT on immunosuppressive therapy and Treg/Th17 cell ratio and soluble CD30 (sCD30) as possible additional markers for characterizing the immune status of transplanted patients.**

Patients and Methods: **The study group consisted of 17 patients after LT on immunosuppressive therapy and 10 healthy controls. We assessed Th17 (CD3+CD4+CD183–CD194+CD196+CCR10–), Treg cells (CD3+CD4+CD25+CD127$^{-/low}$ and CD4+CD25+ Foxp3+), Treg/Th17 ratio in peripheral blood by flow cytometry, and soluble CD30 (sCD30) in serum by ELISA.**

Results: **The mean percentage of T regs (mean ± SD% of CD4+cells) were 5.26±2.21 and 5.17±2.12, assessed by peripheral and cytoplasmic staining, respectively (p<0.0001, r=0.9588). LT patients showed significantly higher percentages of Th17 in peripheral blood than healthy controls (7.23% vs. 3.55%, resp.) and reduction in Treg - 5.26% vs. 7.82%, resp. (p<0.05). Treg/Th17 ratio in healthy controls averaged 2.31, while in the LT group, it was significantly lower - 0.73 (p<0.05). We also observed significantly higher sCD30 levels in patients 45.47±23.62 ng/ml compared to the healthy controls 15.63 ±5.69 ng/ml (p<0.05). No significant associations were found between the CD4+ subsets with serum CD30 levels were demonstrated.**

Conclusions: **This pilot study showed both reduced immune tolerance and increased activation of the immune system in LT patients compared to healthy controls. However, additional studies are needed to confirm and expand these results because the immune balance in our transplanted patients is a complex interaction between a tolerogenic liver, an immune response against liver graft and immunosuppressive therapy.**

Introduction

The unique mechanisms of immune regulation in the liver allow it to provide local and systemic immune tolerance to own and foreign antigens as a homeostatic condition via different T cell subsets and after liver transplantation (LT) while preserving an effective immune response against pathogens[1]. This is done by synergic actions of immune regulatory cells and conventional and unconventional antigen-presenting cells, incl. dendritic, Kupffer, stellate, various lymphocytes, etc. Populations of lymphocytes in the normal liver include both classical MHC-restricted CD4+ and CD8+ T cells and B cells. The liver is particularly rich in CD8+

T cells, activated T cells and memory T cells[2]. Additionally, the accumulation of these T lymphocyte subsets in the liver has been associated with T cell apoptosis and depletion, which is why some authors describe the liver as a "graveyard" for T cells[2].

The liver has unique mechanisms of immune tolerance extend to liver transplantation[3]. Therefore, the determination of T-helper subsets, such as Th17 (pro-inflammatory) and T regulatory (Treg) cells (tolerogenic), might be one of the keys to characterize the immunological balance in the liver especially after LT[3].

Currently, there are two areas of practical interest in transplantology: finding adequate biomarkers for monitoring and predicting immune responses, including rejection, and the search for new therapeutic approaches and protocols[4]. However, since CD4+ T (Th) helper cells play an essential role in graft rejection by secreting various cytokines and providing help for other effector cells that cause inflammation and destruction of graft tissue and its rejection, that is why it is worth it to be investigated. Furthermore, pro-inflammatory Th17 and regulatory Treg FoxP3+ cells have opposite effects on the development of immunological tolerance and graft acceptance[5]. These phenomena were documented in experimental models as well as in clinical observations. For these reasons, the ratio between them could be used to monitor immunological homeostasis after transplantation and predict rejection. Moreover, data on Th17 lymphocytes and their critical role in LT occurred in recent years[6].

In contrast, Treg cells have an anti-inflammatory function and are essential for recipient tolerance to graft after LT. The relationship between the two lymphocyte subsets is thought to play a critical role in long-term success after LT[7,8]. In this regard, it is also interesting how immunosuppressive therapy affects these lymphocyte subsets.

Based on this background, we aimed to analyze the Th17 and Treg subpopulations in the post-transplant period in patients with LT and immunosuppressive therapy (IST) and their ratio as a possible additional marker for characterizing the immune status of patients.

Patients and Methods

Participants of the study

We included 17 patients after liver transplantation (LT group) on IST and 10 healthy controls in this pilot study, conducted between March 2020 to September 2021.

The LT group consisted of 17 patients who underwent LT at Lozenetz Hospital, Sofia, Bulgaria, from 2006 to 2020; one being retransplanted. The LT group consisted of 13 men and 4 women, mean age of 50 years (range 19 to 70 years). The average post-transplant follow-up period was 7 years (2 – 17 years). The underlying diseases for transplantation were decompensated liver cirrhosis of various etiologies (i.e., alcohol liver diseases - 4, viral - 4, autoimmune - 2 and 7 patients – end-stage liver diseases with unknown etiology).

All patients received complex therapy, including conventional immunosuppressive agents such as tacrolimus, everolimus, cyclosporine and mycophenolate mofetil (MMF) as follows. Fourteen patients were administered on tacrolimus (eight with tacrolimus + MMF, and 6 people – on tacrolimus alone), everolimus - 2 patients (one with everolimus + MMF, and one with everolimus + cyclosporine (sandimun)) and one with ciclosporin (neoral). In addition, routine clinical and laboratory tests have been performed during the follow-up period.

The control group enrolled 10 healthy controls - 7 women and 3 men, at a mean age of 52 years (range: 26 to 71 years).

The study was conducted following the Declaration of Helsinki of 1975 (revised in 2013). The Ethics Committee of the Sofia University "St. Kliment Ohridski" approved the study protocol. All participants were informed about the purpose of the study and written informed consent for inclusion was given before inclusion in the study.

Methods

Peripheral blood with Na heparin and serum samples were investigated.

Flow cytometry
T regulatory cells

To evaluate and compare the populations of so-called classical FoxP3+ Treg and CD4+ /CD127$^{-/low}$ Tregs, we determined them in parallel by two methods.

CD4+CD25+ FOXP3+ T regulatory cells

We used a commercial kit, "Regulatory T Cell Staining Kit (eBioscience, Thermo Fisher, Waltham, MA, USA)

and isolated BPMC samples were evaluated according to the manufacturer's instructions. The cells were labeled for surface and intracellular proteins by a combination of anti-human monoclonal antibodies (Mabs)[9,10].

CD3+CD4+CD25+CD127-/low T reg cells

Treg cells population was identified from whole peripheral blood samples according to published validated protocols[11-13]. We used the following mouse Mabs: CD3 FITC (clone SK7), CD127 PE (clone HIL-7R-M21), CD25 PE-Cy7 (clone M-A251), and CD4 APC-H7 (clone SK3) (Becton Dickenson, Brea, CA, USA). The determination of T regulatory cells was carried out by the direct surface immunofluorescence staining of whole blood samples using a Lyse/Wash Procedure[14].

Th17 cell subset determination

We used four surface chemokine receptors for the identification of CD4+ subsets. Based on this protocol, the phenotypic profile of Th17 was CD3+CD4+CD183-CD194+ CD196+CCR10–.

The Th17 determination was done according to EuroFlow standard operating procedures (SOPs) for sample preparation and staining of surface markers[15-17]. Were used 6-colors combination of monoclonal antibodies:CD3 FITC (clone SK7), CD194(CCR4) PE (clone 1G1), CD196 (CCR6) PerCP (clone 11A9), CD183(CXCR3) PE-Cy7 (clone 1C6), CCR10APC (clone 1B5) and CD4APC-H7 (clone SK3), Becton Dickenson. Briefly, the appropriate volume of fluorochrome-conjugated mouse Mabs directed against cell surface markers was added to 100 μL whole blood and incubated for 30 minutes at room temperature, followed by lysing with 2 mL lysing solution for 10 minutes at room temperature (RT), then washing and resuspending in 500 μL of PBS containing 0.2% Bovine Serum Albumin (PBS-BSA). The gating strategy and analysis of Th17 cells are presented in Figure 1.

All samples were acquired and analyzed in a BD Canto II flow cytometer and Diva 8.0.1 software.

Soluble CD30 (sCD30) in serum

Serum sCD30) was determinate by enzyme-linked immunosorbent assay (Human sCD30 Platinum ELISA, BMS240, Bender MedSystems GmbH, eBioscience). The procedures were performed according to the manufacturer's instructions.

Statistical analysis

The nonparametric Mann–Whitney U test and the Spearman rank correlation test were used to evaluate the differences in the distribution of T-helper subsets in the LT group *vs.* healthy controls and for similarity association between the two parallel samples of Tregs. The GraphPad Prism 6 software package (La Jolla, CA, USA) was used. A level of significance $p < 0.05$ was accepted.

Results

T regulatory cells measured by two flow cytometry approaches

In the literature, there are sometimes contradictive data about the identity and functions of CD3/FoxP3+ and CD4/CD127- Treg cells. For us, it was important to understand how this happens in liver transplantation settings. We evaluated Treg cells by simultaneously identifying whole blood samples with direct staining for cell surface markers (CD3+CD4+CD25+CD127-/low) and isolated PBMC with membrane plus intracellularly staining for CD4+CD25+Foxp3+. We achieved similar, nearly identical, individual values for Tregs by the two applied methods. The mean values of subsets (X ± SD % of CD4+cells) were 5.26±2.21 for CD3+CD4+CD25+CD127-/low *vs.* 5.17±2.12, CD4+CD25+Foxp3+. A significant correlation between both determinations was found ($r=0.9588$, $p<0.0001$) (Figure 2). The results show that both methods detect similar values for Treg cells and can be used expediently under our experimental conditions.

Th17 and Treg cells in healthy persons and patients with LT

The levels of Th17 and Tregs in peripheral blood were measured in 10 healthy controls and 17 LT patients. The mean values (X ±SD % of CD4+ cells) and the ratio between these two populations - Treg/Th17- were evaluated and presented in Table 1. In addition, the individual values and the nonparametric statistical comparison of the LT group and healthy controls are shown in Figure 3.

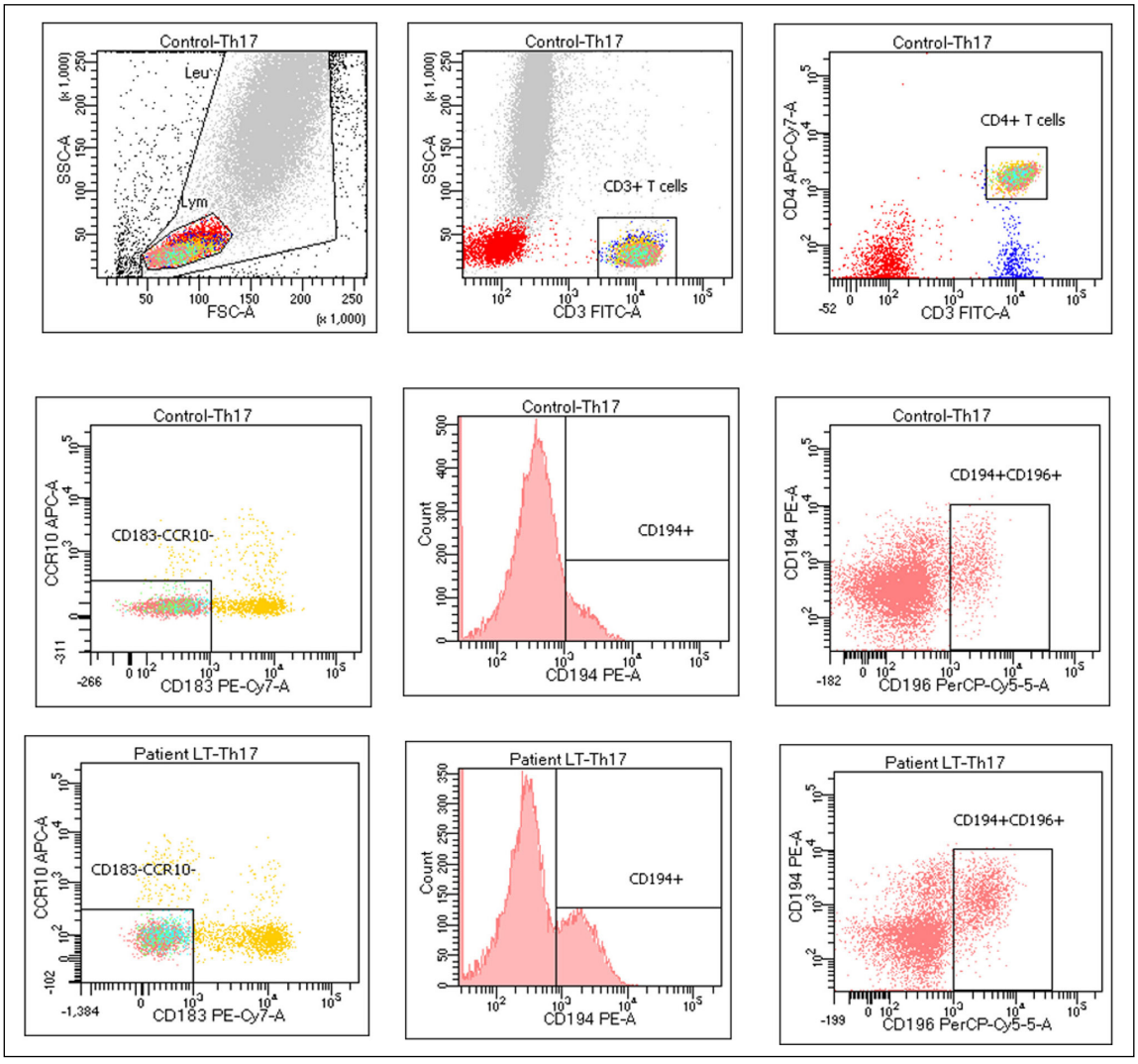

Figure 1. Analysis of Th17 cells with phenotype CD3+CD4+ CD183–CD194+ CD196+ CCR10–. The lymphocytic population was visualized on a dot plot FSC vs. SSC. The T-cells (CD3+) were outlined on a dot plot CD3 vs. SSC, and from them were defined T-hepper cells – CD4+. The analysis of Th17 cells as a percentage of CD4+ cells was carried out as follows: 1) CD183- CCR10-gated cells; 2) CD194+ cells on a histogram; 3) dot plot CD194 vs. CD196 Th17 cells are CD4+CD183-CD194+CD196+CCR10-.

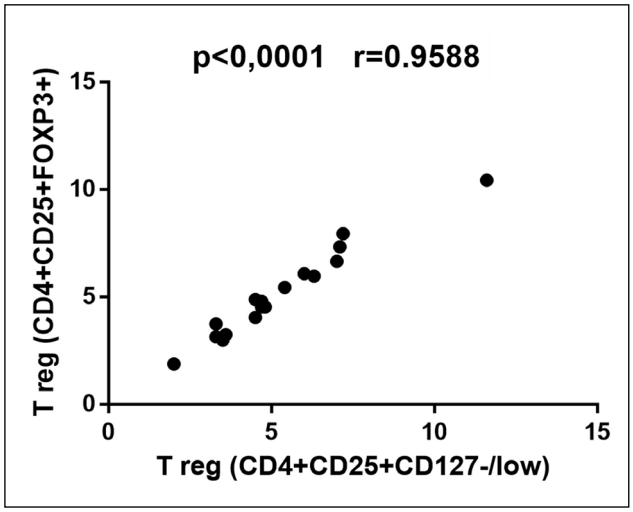

Figure 2. Correlation between T reg values identified simultaneously by two methodical approaches in the liver transplantation (LT) group (% of CD4+ cells).

Table 1. T-helper subpopulations and sCD30 of liver transplantation (LT) patients and healthy controls - mean values with SD in peripheral blood.

Parameters/Groups	Treg cells (CD4+CD25+CD127low/) % of CD4+	Th17 cells % of CD4+	Treg/Th17 Ratio	sCD30, ng/ml
Healthy controls (n=10)	7.82±1.60	3.55±1.09	2.31	15.63 ±5.69
LT patients (n=17)	5.26± 2.21	7.23±2.74	0.73	45.47±23.62

It was found that the changes of all parameters of T cell subsets between healthy controls and the LT group were significantly different. Patients with LT showed higher mean percentages of Th17 cells than the healthy controls: 7.23±2.74% *vs.* 3.55±1.09%, respectively, and a reduction in Treg percentages - 5.26±2.21% *vs.* 7.82±1.60%, respectively ($p<0.05$).

We decided to check the relationship of appearance of the Th17 and Treg cells in healthy controls and in the LT group. For this purpose, we made a Spearmen correlation testing in the two groups) (Figure 4). We found similar models of appearance of the Th17 and Treg in our healthy controls and LT group. Both presented significant positive correlations between Treg and Th17 cells as follows – in the healthy controls group r=0.7190, $p=0.0191$ and in the LT group r=0.5562, $p=0.0222$.

Treg/Th17 ratio estimation in peripheral blood

We decided to evaluate the ratio of Treg/Th17 subsets in peripheral blood as an additional complex marker for the balance of these two cellular populations. The data are presented in Table 1. It shows quite a variety of the indexes between healthy controls, and the mean ratio value was 2.31±0.53 (range 1.72 – 3.23). However, suppose we accept this as a "homeostatic" model of the Treg/Th17 ratio in healthy controls. In that case, it appears quite different in the LT group on IST. In our LT group, the Treg/Th17 ratio was clustered around the mean 0.74±0.18 (range 0.49 – 0.99), and it was significantly lower than the control group ($p<0.05$) (Figure 3).

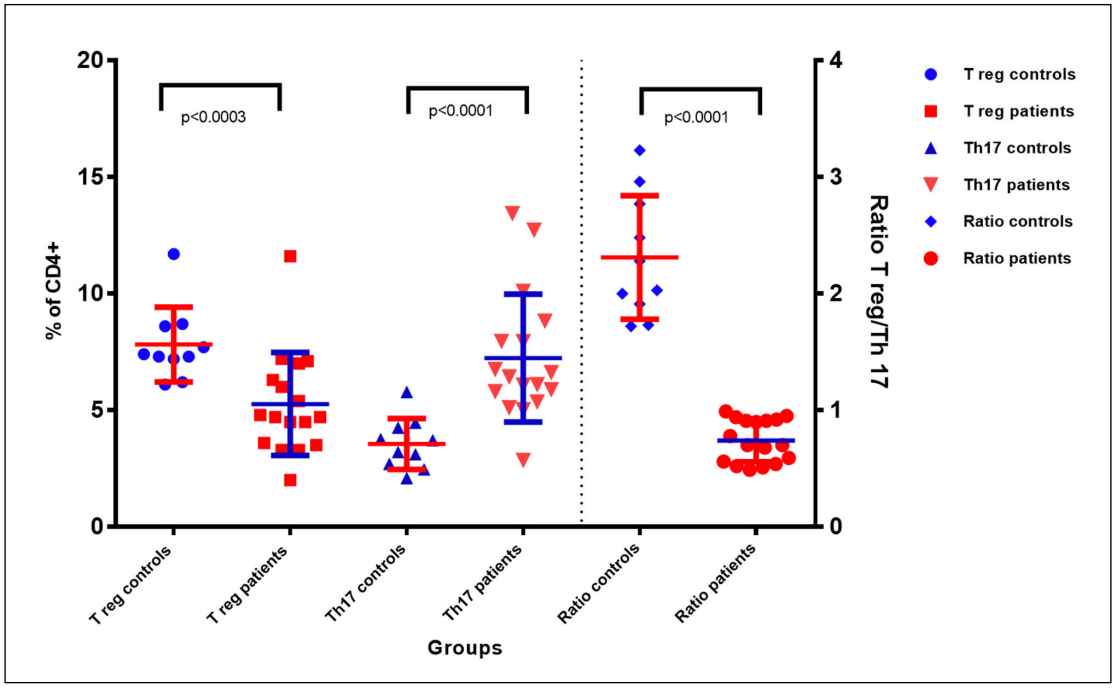

Figure 3. Comparison of individual and mean values of T cells subsets - T reg and Th17 and the Treg/Th17 ratio in healthy controls and patients after liver transplantation.

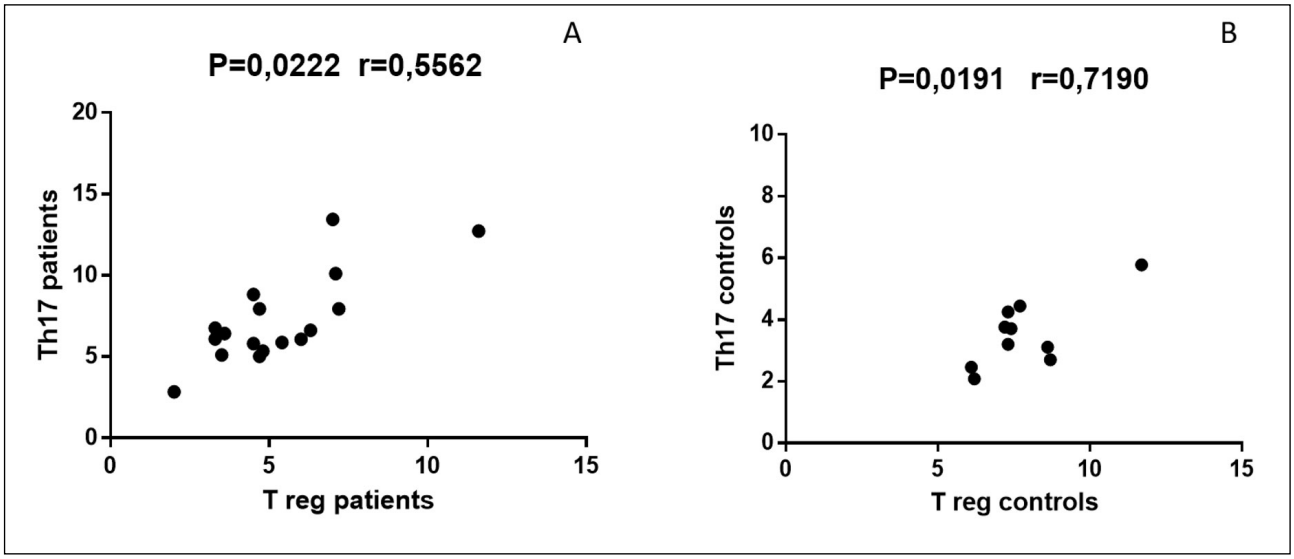

Figure 4. Correlation between T reg and Th17 values (% of CD4+ cells) in liver transplantation (LT) group (A) and healthy controls (B).

Serum levels of sCD30

The serum levels of soluble CD30 were measured in both groups by ELISA. We found a significantly higher sCD30 level in LT group (45.47 ± 23.62 ng/ml) compared to the healthy controls (15.63 ± 5.69 ng/ml) ($p<0.05$) (Table 1 and Figure 5).

No significant correlations were found between the CD4+ subsets, Treg/Th17 index and serum sCD30 levels. Furthermore, we did not find any significant correlations between other clinical and laboratory data evaluated at the enrollment of the LT patients into the study.

Associations of the Th17 cells, T regs and sCD30 with the treatment

The Treg/Th17 index expression model in patients with LT suggests that one possible reason might be the IST, which strongly suppresses T lymphocytes. Therefore, we tried to analyze differences in T helper subsets and sCD30 in the context of the regimes of IST applied to the LT patients. In Table 2, we put the different regimens of IST currently administered against the evaluated increased or decreased samples of the LT patients compared to healthy controls mean values plus or minus 1SD (Table 2). It can be seen that tacrolimus alone or in combination with MMF are the most pronounced inhibitor of T cell subsets. Only one patient on everolimus+MMF therapy showed an increase in Treg cells compared to healthy controls. Reduced levels of Th17 (2.85%) compared to the healthy controls (4.64%) were analyzed in an LT patient on tacrolimus. At the same time, lower sCD30 concentrations were found in both patients on tacrolimus and everolimus+ cyclosporine (sandimun). The results are shown in Table 2. However, the number of patients included in the study up to now is too small for any convincing conclusions.

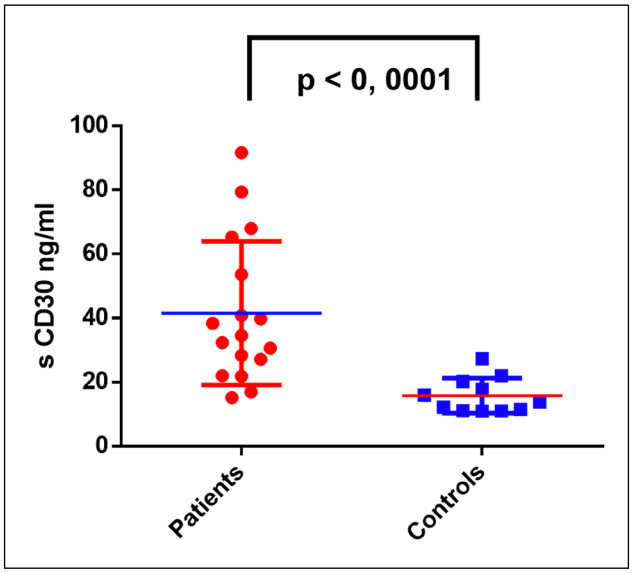

Figure 5. Comparison of individual and mean values of sCD30 in the liver transplantation (LT) group and healthy controls.

Table 2. CD4+ subsets change in relevance to liver transplantation (LT) patients' therapy. The healthy controls group values for Treg, Th17 cells and sCD30 were calculated as mean ± SD. Red arrows denote the LT patients that differ from the group. Upward arrows represent an increase, downward arrows represent a decrease.

Indicators/Therapy	LT patients (n=according to therapy)	Treg cells compared to healthy controls	Th17 cells compared to healthy controls	sCD30 compared to healthy controls
Tacrolimus	6	6 ↓	1 ↓ 5 ↑	1 ↓ 5 ↑
Tacrolimus +MMF	8	8 ↓	8 ↑	8 ↑
Everolimus+MMF	1	1 ↑	**1** ↑	**1** ↑
Everolimus+Cyclosporine (Sandimmun)	**1**	**1** ↓	**1** ↑	**1** ↓
Cyclosporine (Neoral)	**1**	**1** ↓	**1** ↑	**1** ↑

Discussion

Transplant research focuses on interactions between the immune system and organ grafting. Liver transplantation is a life-saving procedure in patients with liver failure[18]. LT differs from transplants of other solid organs due to the tolerogenic functions of the liver itself and, on the other hand, the hepatic immune system, both assured homeostatic driven better acceptance of LT, as mentioned above. However, acute graft rejection and severe infections remain the major complications[19].

Moreover, Th17 cells are thought to be involved in graft rejection after LT, but there is limited evidence to date. However, a study presented by Fábrega et al[20] describes a possible correlation between acute graft rejection and induction of Th17 lymphocytes after LT. In addition, researchers have also found an increase in serum levels of the cytokines IL-17 and IL-23 after transplantation and an even more pronounced IL-17/IL-23 response in acute graft rejection.

For this reason, research for and monitoring specific parameters of the immunological balance of inflammation/rejection and tolerance in patients after LT, most of which are on IST, is increasingly being demanded. It is also interesting to assess the balance between Th17 and regulatory T lymphocyte subpopulations in peripheral blood and whether it reflects actual processes in tissues and liver graft. Furthermore, it is of particular interest to state whether their ratio correlates with other clinical and laboratory data characterizing the transplanted patients' current clinical and immune status.

Several protocols have been developed using flow cytometry as a method to analyze these lymphocyte subpopulations in peripheral blood (before, during and after transplantation)[15]. Finally, it is crucial to examine the impact of IST on T cell subsets distribution.

There is evidence of a possible correlation between Th17 activation and acute graft rejection after LT[20]. This fact has been demonstrated in animal models and humans after LT. Recipients who experienced acute rejection had significantly increased Th17 cells in the peripheral blood than those without graft rejection.

In contrast, Treg cells and their functional properties are reduced during acute rejection in patients after LT[6,21-24]. Therefore, studying the balance of Treg/Th17 cells may be a valuable approach to predicting the outcome for the hepatic allograft. However, the exact effects of Th17/Treg cells on acute liver rejection are not yet fully understood, and other factors have been suggested.

In our pilot study, we identified Tregs by two established methods. Foxp3 is known as a proven marker of Tregs. In 2006 a new marker for identifying Tregs was introduced[25,26]. The low or lack of expression of CD127 in CD25+ T cells identify human Tregs with a potent suppressive function. The two methods for T reg determinations - from membrane only and surface with cytoplasmic staining were very closed and showed a significant positive correlation. Our results correspond to many other studies, which show that CD127 expression is an excellent marker of Treg cells, especially when combined with CD25. The combination of these markers identifies 6–8% of CD4+ Treg in healthy controls[27-29]. Like our results, a high correlation between two strategies to analyze Treg has been observed in many studies[27,30,31]. However, the determination of Tregs by CD127 staining is faster and easier to implement, and it is applicable in

laboratory practice. Therefore, with the same results of both approaches, surface staining is preferred.

As already mentioned, Treg lymphocytes are an essential factor, but probably not the only one important in preventing rejection and avoiding graft-*vs*.-host disease (GvHD). The Treg / Th17 ratio also plays a significant role in organ rejection[32]. Moreover, Th17 lymphocytes were increased during acute and chronic organ transplant rejection, with pro-inflammatory cytokines leading to Treg differentiation in Th17 cells by the expression of the RORγt transcription factor. Early reduction of Treg lymphocytes after transplantation has been shown to be a risk factor for acute rejection[22]. However, Th17 cells have their crucial role in host defense against specific pathogens. Simultaneously, they are potent inducers of autoimmunity and tissue inflammation. In addition, the differentiation factors responsible for their generation have revealed an interesting reciprocal relationship with Treg cells, which prevent tissue inflammation and mediate self-tolerance.

Determination of Th17 by membrane molecules is a new, innovative method proposed in 2020[15]. The EuroFlow Consortium, with partners from the Horizon 2020 / IMI project PERISCOPE and via highly reproducible multicenter results, offer chemokine receptor-based Th-cell phenotypes. According to EuroFlow immune monitoring T CD4 tube, Th17 phenotype CXCR3- CCR6+CCR4+ CCR10- is positively associated with *in vitro* cytokine secretion IL17A and with Gene Expression Patterns (GEP) - chemokine receptor CCR6 and the transcription factor RORC[33,34]. However, the addition of other markers - surface CD161 and intracellular transcription factor RORgt, do not improve the identification of the Th17 cells[15].

Determined in this way, Th17 values in our healthy controls group (mean 3.55%) are similar to the data published by other authors[35-37]. Significantly increased levels of this cells' population and decreased values of Treg cells, which we detected in LT patients, are also found in acute rejection of solid organs transplantation[7,38]. All these methodological details are essential because immune monitoring by flow cytometry is a fast and highly informative way of studying the effects of novel therapeutics to reduce transplant rejection or treat autoimmune diseases. To monitor tolerance, it is essential to develop biomarkers to non-invasively detect early signs of rejection as well[38]. Many authors, including us, believe that flow cytometric markers could serve this role.

Indeed, it was shown that at the onset of acute rejection, Tregs frequency and Tregs/Th17 ratio were sharply decreased, whereas Th17 cell frequency was dramatically increased. Interestingly, as the rejection subsided, Tregs and Th17 cells were both restored to levels close to those before rejection[7]. Thus, the predominance of the inflammatory responses of Th17 cells and the reduced Treg cells are among the causes for disease progression of many inflammatory and autoimmune diseases[37,39,40].

At the same time, existing findings showed enhanced responses of Th17 cells and decreased Treg cells in COVID-19 patients compared with healthy controls. These results demonstrated a strong relationship with hyper inflammation, lung damage, and disease pathogenesis. Also, the higher ratio of Th17/Treg cells indicates the critical role

of inflammation in the mortality of COVID-19 patients[39,40]. In many autoimmune and inflammatory diseases, the disturbance balance of Th17/Treg cells is due to the predominance of the inflammatory responses driven by Th1 and Th17 cells and the dysfunctional Tregs[40,41].

Studies carried out in healthy subjects guide us on the values and relationship between Treg and Th17 cell subpopulations in peripheral blood. Some generally accepted markers characterize the balance between inflammation and tolerance in various acute conditions - allergies, infections, autoimmune diseases, transplants of solid organs, etc. We found that in our healthy controls, the ratio averaged 2.31 (range 1.72 - 3.23). However, this balance in patients with LT was disturbed - on average 0.73. The imbalance can be due to various causes: peculiarities of the liver's immune response, immunosuppressive therapy, concomitant infections, or other diseases. Our LT patients were on the hospital's accepted IST regimens, so we sought a probable link to the treatment.

We have also evaluated the correlations between the parameters of the individual helper populations, clinical and laboratory data. Negative correlations were observed between Tregs/Th17 ratio and the levels of ALT (r = 20.668, p= 0.005), AST (r = 20.541, p= 0.031), ALP (r = 20.518, p= 0.039), and GGT (r = 20.764, p= 0.001) (Figure 2). These results indicated that the Tregs/Th17 ratio may be an alternative indicator for diagnosing liver damage in liver transplant recipients[7].

Regarding sCD30, a marker for Hodgkin's lymphoma, Primary Immune Thrombocytopenia[42,43], as well as for organ transplantation[32,44,45], we did not find any correlations between sCD30 and flow cytometric or other laboratory markers. Previously, we have

demonstrated that sCD30 and Tregs could be valuable parameters in the immunologic monitoring of liver and kidney transplanted patients. Furthermore, a decrease in sCD30 levels was observed in all patients, regardless of the transplanted solid organ[32].

All published data show that liver transplantation is, in many cases, the only way to cure the end-stage liver disease[1]. LT improves the patient's clinical course, but there is always a risk of chronic graft rejection and the side effects of immunosuppressive therawwpy[24]. Therefore, optimal immunosuppressive treatment to prevent rejection and toxicity while avoiding opportunistic infections should be closely monitored and individualized.

Recipients of LT often require long-term IST, which, although low dose, is associated with a risk of infection and severe side effects[22]. In addition, it is known that some patients after LT develop spontaneous immune tolerance. Although this is a prerequisite for reducing IST, practice shows that this is often impossible. For these reasons, a study of Th17 and Treg cell changes in patients after LT and continued IST is relevant. In addition, it may help characterize post-transplant tolerance. Early detection of patients with impaired tolerance and incipient rejection response is a prerequisite for increased survival of transplant patients.

Our pilot study shows that LT patients on IST in the late post-transplant period demonstrated an imbalance in T cell subpopulations, characterizing the immune response. Despite the small number of participants, data show that everolimus alone can stimulate Tregs[46,47].

Thus, even without evaluating the immunosuppressive drugs plasma levels, which is the subject of clinical laboratory, we can assess the degree of immunosuppression by determining the inflammation/tolerance balance[39,48].

In line with this, the two types of lymphocyte subpopulations can undoubtedly be used to assess conditions after LT. For example, we know that Th17 cells are needed for adequate pathogenic clearance in the liver. In contrast, Tregs coordinate the immune response in autoreactivity, autoimmune diseases and after LT[24].

Our pilot study is not without limitations. The number of patients analyzed with various immunosuppressive therapeutic schemes is too small. Therefore, it does not allow us to make reliable conclusions. Future studies are needed in a more significant number of patients.

We have also to admit that most of the data on the role of Th17 and Treg cells were obtained from experimental mouse models. However, the importance of these subpopulations in humans has been lesser emphasized in clinical trials. Many studies highlight the need to examine these cells in the liver tissue, as circulating cells may not reflect intrahepatic conditions. In addition to possible altered function and amounts in liver disease, Treg/Th17 ratio can be monitored for therapeutic purposes. Therefore, future studies should focus on the role of Th17 and Treg lymphocytes and their balance in the liver tissue to clarify the relationship between these subpopulations in the liver and peripheral blood. In addition, various signals that could affect the differentiation of naive T lymphocytes to the two opposing subpopulations, especially in the liver, need to be analyzed to better understand the liver-specific graft-specific pathogenic mechanism and select liver-specific therapy.

Conclusions

This pilot study showed both reduced immune tolerance and increased activation of the immune system in patients after LT compared to healthy controls. However, additional studies are needed to confirm and expand these results because the immune balance in our transplanted patients is a complex interaction between a tolerogenic liver, an immune response against liver graft and immunosuppressive therapy.

Furthermore, acute and chronic liver inflammation are likely to be associated with an imbalance in the T-helper immune response, leading to liver fibrosis and liver failure. Studies over the past 10 years have confirmed that the differentiation of Th17 cells favors pro-inflammatory responses in almost all tissues, reciprocal to the action of Treg cells in the direction of immune tolerance and homeostasis. However, the balance between the two subpopulations of lymphocytes is delicate and critical, especially in the already damaged liver, making restoring immune homeostasis challenging. Furthermore, the pathogenic contribution of Th17 and Treg cells in autoimmunity, acute infections and chronic liver damage is diverse and varies according to disease etiologies. Understanding the mechanisms underlying Th17 and Treg cell differentiation and functions makes it possible to develop new therapeutic strategies for liver disease and LT. This could include the active manipulation of the balance between pathogenic and regulatory processes in the liver, which in turn can help restore homeostasis, especially in liver inflammation or after LT.

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Author contributions:
Conception and design of the study: AM, TV, IA; Acquisition of data: MP, AK, MS-P, NY, II, AS; Analysis and interpretation of data: AM, TV, IA; Drafting the article or making critical revisions related to relevant intellectual content of the manuscript: AM, TV; Supervision: IA; Validation and final approval of the version of the article to be published: IA, TV.

Ethical approval:
The study was conducted following the Declaration of Helsinki of 1975 (revised in 2013). The Ethics Committee of the Sofia University "St. Kliment Ohridski" approved the study protocol.

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Conflict of Interest:
The authors declare that they have no conflict of interest to disclose.

Informed consent:
Written informed consent for inclusion was given by patients before inclusion in the study.

Data availability statement:
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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