

## ORIGINAL

# Clinical role of Foxp3<sup>+</sup>regulatory T cell in Living donor related liver transplantation for prediction of life-threatening complications

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**Abstract : Purposes :** It is no doubt that regulatory T cells (Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>T cells : Treg) play important roles in transplant immunity. We investigated the significance of Treg expression in acute stage of living donor-related liver transplantation (LDLT) for the possibility of the sensitive marker for immunological state and homeostatic stress after liver transplantation. **Methods :** Peripheral blood was drawn from 5 recipients of LDLT preoperatively and on post operative 1, 4, 7, and 14 days. The peripheral blood mononuclear cells (PBMCs) were stained with CD4, CD25, Foxp3, and were analyzed with FACScan. This data was compared with clinical output of LDLT. **Result :** The populations of Treg were significantly decreased in all patients on day 1 after LDLT and significantly increased in patients who had early postoperative complications compared with patients who had no complications. **Conclusions :** The population of Treg in peripheral blood may reflect the surgical stress such as life-threatening complications after LDLT. *J. Med. Invest.* 62 : 37-40, February, 2015

**Keywords :** Liver transplantation, regulatory T cells, Foxp3, Transplant immunology

## INTRODUCTION

Living donor related liver transplantation (LDLT) is one of the ultimate options for irreversible liver failure and developed some countries such as Japan. The main reason LDLT is an extreme limitation is because of donor sources and severe shortage of cadaveric donors in such countries. In these circumstances, the graft loss is a major issue because additional liver transplantation is substantially impossible in these countries. Thus, precise biomarker for estimating immunological state of the graft or clinical status of the recipients is important.

Regulatory T cell (Treg) was reported an autoimmune disease (1, 2) and was also considered as a key immunological effector in transplant immunity (3, 4). Treg is one of the T cell subsets which are an immunosuppressive function against auto/alloreactive T cells. Recently, many reported that Forkhead box P3 (Foxp3) is a master key gene for regulatory activity of Treg cells, and Treg subset is defined as CD4<sup>+</sup>CD25<sup>+</sup> cells that express high levels of the transcription factor Foxp3 (5, 6). This regulatory activity of Treg plays important roles in various fields such as allergic and autoimmune diseases, antigen-specific immune response, tumor immunity, and modulation of inflammatory response (4, 7-9). This Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>T cell was recently reported it was also important in the transplant immunity. The population of Treg was increased in the liver graft and recipient spleens in early post operative period after LDLT (10). It is also reported that the populations of Treg in the peripheral blood decreased at three months after liver transplantation and rose up to about 3% from 1% (Tregs of CD4<sup>+</sup>T cells) in one year after transplantation (11). These Treg populations did not vanish by the administration of anti-CD25

antibody in the short term after transplantation and there was little influence on differentiation and production of Treg (12).

Thus, we investigated the change of the Treg expression (the populations of Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>T cell) after LDLT whether Treg could be a precise and useful biomarker for estimating graft status and recipient's immunological status.

## PATIENTS AND METHODS

### Patients

Five Patients were enrolled who underwent LDLT at our institution from 2008 to 2010. An immunological suppression was taken according to our protocol using Basiliximab (20 mg/day at 4 post-operative day ; POD), mycophenolate mofetil (2,000 mg/day from operation day), tacrolimus (4 mg/day from 7POD), and methylprednisolone (1,000 mg/day from operation day and tapering until postoperative day 7). They had no deviation of protocol. All experiments were conducted according to the Helsinki statement, and this study was approved by the institutional ethics committee (approved #283).

### Cell Preparation

Peripheral blood was drawn from all the patients on preoperative and on postoperative days 1, 4, 7, and 14. These samples were analyzed with FACScan for T cells stained by the manufacturer's guidelines as described before (14). Briefly, A 3 mL peripheral blood sample was taken from each patient. The peripheral blood mononuclear cells (PBMCs) were isolated using 4 mL of Lymphoprep (AXIS-SHIELD, Oslo, Norway). They were centrifuged at 1,800 rpm for 20 minutes at room temperature and then resuspended with 4 mL of FACS buffer. After the addition of anti-CD4 (Allophycocyanin (APC)-conjugated anti-human CD4 antibody ; e-Bioscience, San Diego, CA) and anti-CD25 (FITC conjugated anti-human CD25 antibody ; e-Bioscience) antibodies to the cell pellets, the mixture was centrifuged at 2,000 rpm for 5 minutes at 4°C,

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placed on ice for 15 minutes in the dark, and further incubated at  $-20^{\circ}\text{C}$  overnight. After 2-time washes, 4% paraformaldehyde in PBS (PH 7.4) 2 ml and 0.1% saponin (Wako Ltd, Tokyo, Japan) PBS were administrated and the mixture was incubated in the dark at room temperature for 15 minutes. Next, an anti-Foxp3 antibody (Phycoerythrin (PE)-conjugated monoclonal antihuman Foxp3 antibody; e-Bioscience) was added to the cell pellet in the dark and incubated at room temperature for 30 minutes. The cells were then washed twice with FACS buffer, centrifuged at 1,800 rpm for 5 minutes at  $4^{\circ}\text{C}$ , and analyzed using a FACSCalibur (BD Biosciences, San Jose, CA) (Fig. 1). The whole procedure was achieved within 24 hours after drawn blood samples.

### Statics

All statistical analysis was performed using statistical software (JMP 8.0.1., SAS Campus Drive, Cary, 27513 NC, USA). All results were presented as mean  $\pm$  SEM and analyzed with the Mann-Whitney U test and Wilcoxon signed-rank test. P value of 0.05 or less was considered significant.

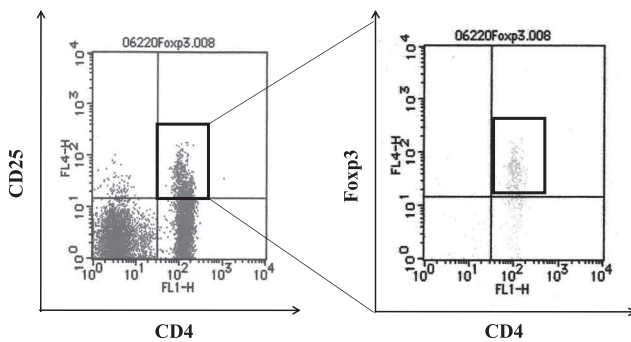


Fig. 1 The peripheral blood mononuclear cells (PBMCs) were subjected to FACScan analysis after labeling with anti-CD4, anti-CD25, and anti-Foxp3 antibodies. We ascribed Treg to  $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$  cells in  $\text{CD4}^+\text{CD25}^+$  cells.

## RESULT

### Patients' background

Five patients (two men and three women, aged 38-66 years) underwent LDLT. All patients had liver cirrhosis (LC) and two of those patients had hepatocellular carcinoma (HCC). Two recipients were infected with Hepatitis B virus (HBV) and two recipients were infected with Hepatitis C virus (HCV). Splenectomy was performed

on all patients. The liver graft used left and caudate lobe ( $n=4$ ), and left lobe ( $n=1$ ). There was no acute rejection. However, there were complications of patients 1 and 3. Patient 1 had an infection of abdominal cavity at 5POD. Patient 3 had respiratory failure at 3POD and needed reintubation (Table 1).

### Paradoxical movements of white blood cell counts and lymphocytes

In all patients, the postoperative white blood cell count increased gradually, reached the peak on 7POD, and it decreased afterwards. However, the number of the total lymphocytes increased after LDLT in a time dependent manner (Figure 2).

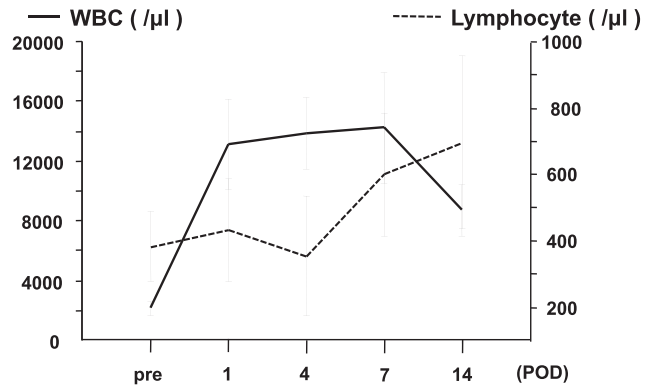


Fig. 2 In all patients, the postoperative white blood cell count increased gradually, reached the peak on 7POD, and it decreased afterwards. The number of the total lymphocytes increased after LDLT in a time dependent manner.

### The movements of Treg populations after transplantation

$\text{CD4}^+\text{CD25}^+$ T cell population decreased from 1POD compared to preoperative population ( $5.6 \pm 1.5\%$  at preoperative day,  $3.0 \pm 1.2$  at 1POD, N.S.). The ratio of Treg showed similar change and significantly decreased from 1POD compared to preoperative ratio ( $3.2 \pm 0.81$  at preoperative day,  $0.80 \pm 0.35$  at 1POD,  $p < 0.05$ ) (Figure 3).

$\text{Foxp3}^+\text{CD4}^+\text{CD25}^+$ T cell ratio was dramatically increased with the case with serious postoperative lethal complications such as respiratory failure, infection in the abdominal cavity ( $4.9 \pm 0.25$  at 7POD) (Figure 4), compared to the case with no complications ( $0.55 \pm 0.087$  at 7POD) (Figure 5).

Table 1 Patient's Background

Patient	Age (years)	Sex	Disease	Hepatitis virus	Operation	Acute rejection	Complications
1	50	M	LC, HCC	HBV	LDLT (Left and caudate lobe) Splenectomy	(-)	Infection of abdominal cavity at 5POD
2	57	F	LC	(-)	LDLT (Left lobe) Splenectomy	(-)	(-)
3	66	M	LC, HCC	HCV	LDLT (ABO incompatibility, Left and caudate lobe) Splenectomy	(-)	Respiratory failure at 2POD
4	61	F	LC	HCV	LDLT (Left and caudate lobe) Splenectomy	(-)	(-)
5	38	F	LC	HBV	LDLT (Left and caudate lobe) Splenectomy	(-)	(-)

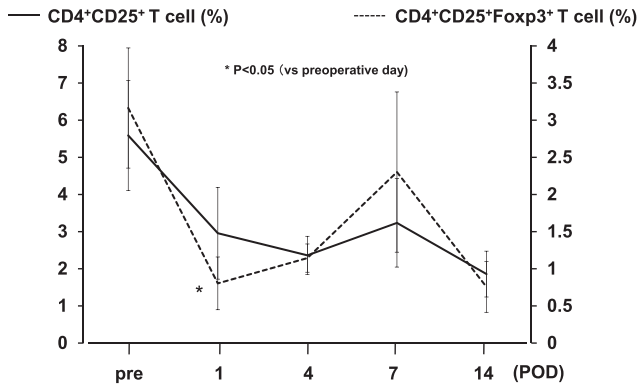


Fig. 3 CD4<sup>+</sup>CD25<sup>+</sup>T cell population decreased from 1POD ( $3.0 \pm 1.4\%$ ) compared to preoperative population ( $5.6 \pm 1.5\%$ ). The ratio of Treg showed a similar change ( $3.2 \pm 0.81$  at preoperative day,  $0.80 \pm 0.35$  at 1POD)

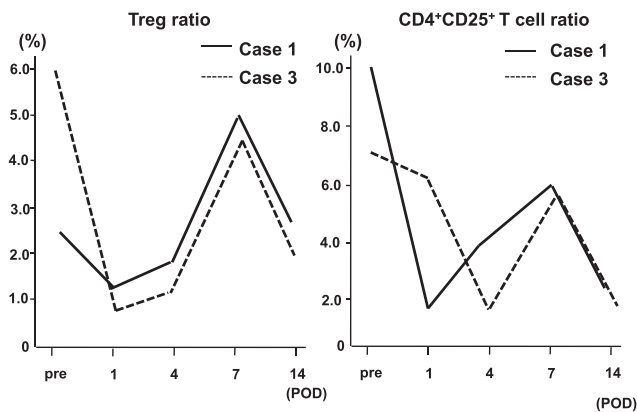


Fig. 4 Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>T cell ratio was dramatically increased at case 1 and 3 with serious postoperative lethal complications such as respiratory failure, infection in the abdominal cavity ( $4.935 \pm 0.245$  at 7POD), compared to the case with no complications ( $0.55 \pm 0.087$  at 7POD) ( $p < 0.05$ ). CD4<sup>+</sup>CD25<sup>+</sup>T cell population showed a similar change.

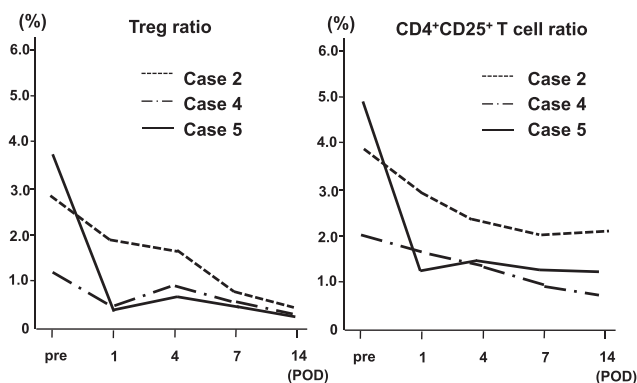


Fig. 5 There was not significant change in case 2, 4, and 5 with no complications ( $0.55 \pm 0.087$  at 7POD).

DISCUSSION

We have previously reported that Treg cells are strong regulators for T cells, which can induce a tolerant state in transplantation (13). On the other hand, peripheral Foxp3<sup>+</sup>CD4<sup>+</sup>T cell population is a precise biomarker for metastasis detection in pancreatic cancer patients, especially at advanced stages of the disease with

immunological state (14). Therefore, we considered these peripheral Treg population may be a new biomarker for estimating the recipients' immunological status in LDLT.

After LDLT, the Treg populations decreased at postoperative day 1 compared to those of preoperative day in all patients. At first, it is considered the influence of anti-CD25 antibody as immunosuppressive protocol. Secondly, this had occurred with an accumulation of Treg in inflammatory tissues as already reported (15). In other words, Treg cells were increased significantly in synovial fluid compared to paired peripheral blood from rheumatoid arthritis patients. Thus, the decreased Treg population in peripheral blood on postoperative day 1 may reflect variation in body distribution of Treg.

Moreover, after LDLT, the Treg population in PBMC showed an extremely interesting trend. Even though the anti-CD25 antibody is administered as an immunosuppression, the number of the lymphocytes in peripheral blood increased in time depending manner. More interestingly, the Treg populations suddenly increased in cases with lethal complications even though the CD4<sup>+</sup>CD25<sup>+</sup>T cell populations decreased in response to immunosuppression. Under the administration of anti-CD25 antibody as an immunosuppression, it was shown that the Treg populations increased in the life-threatening situations. We can not reach any concrete conclusions because n-number of this study is too small, however, conventional CD4<sup>+</sup>CD25<sup>+</sup>T cell population changes at the perioperative period seemed similar to Foxp3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>T cell population (data not shown). Thus it may reach an interesting immunological discovery if conventional CD4<sup>+</sup>CD25<sup>+</sup>T cell population is fully investigated in such situations because that may be the trigger of the increase Treg for the life-threatening events (16). Even though the accumulation of further investigation is required, it is considered the sudden increase of the Treg populations may predict the existence of life-threatening complications from an immunological point of view.

Furthermore, there were no acute rejections in our cases, but it is still unknown whether these Treg populations reflected immunological state of the transplanted grafts. Many reported there are various methods for estimating recipients' immunological state such as Immuknow (17). However, we still did not have a more precise tool for estimating the immunological status for transplanted grafts than a biopsy. Generally immunosuppression is required to be diminished when serious complications occur in transplant recipients, thus immunological estimation can be important in the meaning of avoiding graft loss. Therefore, our data is important that the population of Treg in peripheral blood may reflect the surgical stress such as life-threatening complications after LDLT.

CONFLICT OF INTEREST STATEMENT

Michihito Asanoma and other co-authors of this study, as far as they are aware, have no conflict of interest in performing and reporting the subsequent outcomes of this research.

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