Analysis of $CD8^+CD28^-$ T-suppressor cells in living donor liver transplant recipients

Yi-Xin Lin, Lan-Lan Wang, Lu-Nan Yan, Pei Cai, Bo Li, Tian-Fu Wen and Yong Zeng

Chengdu, China

BACKGROUND: **Human $CD8^+CD28^-$ T-suppressor (Ts) cells have been considered to indicate a reduced need for immunosuppression in pediatric liver-intestine transplant recipients and recipients of deceased heart-kidney transplants. However, in adult-to-adult living donor liver transplantation (A-A LDLT) little information is available and the clinical significance is still unknown.**

METHODS: **Flow cytometry was used to detect the population of $CD8^+CD28^-$ Ts cells present in peripheral blood in A-A LDLT recipients (n=31), patients with end-stage liver disease (n=24) and healthy controls (n=19). Meanwhile, we tested the graft function and trough levels of immunosuppression in recipients. The clinical and follow-up data of 31 transplant recipients were analyzed.**

RESULTS: **Compared with diseased controls (P=0.007) and healthy individuals (P=0.000), a notable expansion of $CD8^+CD28^-$ Ts cells was found in recipients of A-A LDLT. This was associated with graft function, levels of immunosuppression and rejection episodes.**

CONCLUSIONS: **To monitor the $CD8^+CD28^-$ Ts cells levels is important to evaluate the immune state of recipients. Meanwhile, it is also important to promote expansion of $CD8^+CD28^-$ Ts cells in recipients of A-A LDLT, not only to sustain good graft function and decrease the dosage of immunosuppressants, but also to reduce the occurrence of rejection.**

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KEY WORDS: **T-suppressor cells; CD8-positive; living donor; liver transplantation; clinical analysis**

Introduction

The emergence of living donor liver transplantation (LDLT) is as an important option for patients with end-stage liver diseases (ESLD) and it has partially relieved the shortage of cadaveric donor grafts. Human regulatory $CD8^+CD28^-$ T-suppressor (Ts) cells exhibit suppressive functions and inhibit T-helper cell activation and proliferation by allogeneic cells.[1] Emerging evidence suggests that the appearance of $CD8^+CD28^-$ Ts cells is associated with reduced need for maintenance of immunosuppression, not only in pediatric liver-intestine transplant recipients but also in recipients of deceased heart-kidney transplants.[2, 3] However, in adult-to-adult living donor liver transplantation (A-A LDLT) little information is available and the clinical significance is still unknown.

In this article, we present data about $CD8^+CD28^-$ Ts cell populations in recipients of A-A LDLT and analyze the clinical significance of these cells.

Methods

Patient characteristics

This consecutive series included 31 patients who underwent A-A LDLT from June 2005 to November 2007. They had operations utilizing right lobe liver grafts without the middle hepatic vein. The donors who had emotional or genetic relationships in 31 cases served as ABO blood group compatible with the recipients. The diseased controls included 24 patients with ESLD who were on the waiting list. Nineteen healthy volunteers who donated blood samples were included in the study to establish normal reference

Author Affiliations: Center of Liver Transplantation and Department of Surgery (Lin YX, Yan LN, Li B, Wen TF and Zeng Y), Division of Clinical Immunology and Department of Laboratory Medicine (Wang LL and Cai P), West China Hospital, Sichuan University, Chengdu 610041, China

Corresponding Author: Lu-Nan Yan, MD, Center of Liver Transplantation and Department of Surgery, West China Hospital, Sichuan University, Chengdu 610041, China (Tel: 86-28-85422475; Fax: 86-28-85422077; Email: yanlvnan_phd@126.com)

ranges for $CD8^+CD28^-$ Ts cells. The detailed data of donors, recipients and patients are shown in Table 1.

In recipients of A-A LDLT, the regimen for immunosuppression included 500 mg methylprednisolone at the time of liver reperfusion. Tacrolimus was administered at a dose of 0.06-0.08 mg/kg per day by mouth or through a nasogastric tube starting later than 24 hours after the operation, but always within 48 hours from liver reperfusion, and adjusted to achieve trough levels in the range of 5-10 ng/ml. At 30 days posttransplantation, the target trough level was lowered to 5-8 ng/ml. Cyclosporine A (CsA) was administered at a dose of 6.5-7.0 mg/kg per day in the same way, and adjusted to achieve trough levels in the range of 150-200 ng/ml. At 30 days after transplantation, the target trough level was lowered to 100-150 ng/ml. Mycophenolate mofetil (MMF) was administered at a dose of 10-20 mg/kg per day by mouth or through a nasogastric tube within 48 hours from liver reperfusion. Corticosteroids were administered in a rapid taper regimen for the first month of methylprednisolone at a dose of 50 mg i.v. every 6 hours on day 1; 40 mg i.v. every 6 hours on day 3; 30 mg i.v. every 6 hours on day 5; 20 mg i.v. every 6 hours on day 7; and 20 mg i.v. every 8 hours on day 8; and 20 mg of prednisone by mouth or through a nasogastric tube on days 9-15; then 10 mg/d for 1 week; and 5 mg/d for an additional week. We weaned from corticosteroids in 1-2 months. Follow-up ranged from 11 to 37 months after transplantation (mean 20.09 months, SD±7.64). All studies were approved by the Human Rights Committee of the West China Hospital of Sichuan University (Chengdu, China).

Data groups

We used flow cytometry to detect $CD8^+CD28^-$ Ts cells in peripheral blood mononuclear cells from transplant recipients, diseased controls and normal individuals. Meanwhile, we determined the trough levels of immunosuppression and monitored the graft function in transplant recipients. We defined stable graft function by the following criteria: total bilirubin (TB) $\leqslant$28.0 μmol/L, alanine aminotransferase (ALT) $\leqslant$60 IU/L, and aspartate aminotransferase (AST) $\leqslant$ 55 IU/L. Depending on these criteria, we divided the recipients into two groups: stable graft function (SG) and unstable graft function (USG). According to the occurrence of acute rejection which was histologically proven, we divided the recipients into two groups: rejection (RG) and no rejection (NRG). According to the primary disease of recipients, we divided them into two groups: benign liver disease (BLG) and malignant liver disease (MLG). We analyzed the correlation between the levels of immunosuppression and the frequency of $CD8^+CD28^-$ Ts cells in recipients of A-A LDLT.

Statistical analysis

The Kolmogorov-Smirnov test was used to check for normality. Continuous variables were presented as the mean±SD, and categorical variables as rates. Student's *t* test and one-way analysis of variance (ANOVA) were performed in the appropriate conditions and bivariate correlations test was used to compare the correlation between $CD8^+CD28^-$ Ts cell levels and the trough level of immunosuppression

Table 1. Characteristics of donors, recipients and ESLD patients

	Donors	Recipients	ESLD patients
n	31	31	24
Sex (male/female)	26/5	24/7	18/6
Median age (years)	32.5 (24-41)	43.5 (32-59)	46.5 (28-63)
Median body height (cm)	176 (168-180)	168 (155-177)	171 (163-180)
Median body weight (kg)	65 (56-84)	61 (43-78)	63.5 (55-77)
Median Child-Pugh score		9 (5-13)	8 (5-13)
Median MELD score		24 (6-45)	17 (8-41)
Cirrhotic hepatitis B		17	13
Fulminant hepatitis B		2	2
Hepatocellular carcinoma		12*	9
Tacrolimus		28	
CsA		3	
MMF		31	
Corticosteroid		31	
Time from LTx (month) to $CD8^+CD28^-$ Ts cell assay		13.05±9.50	

*: Match to Milan criteria; LTx: liver transplant.

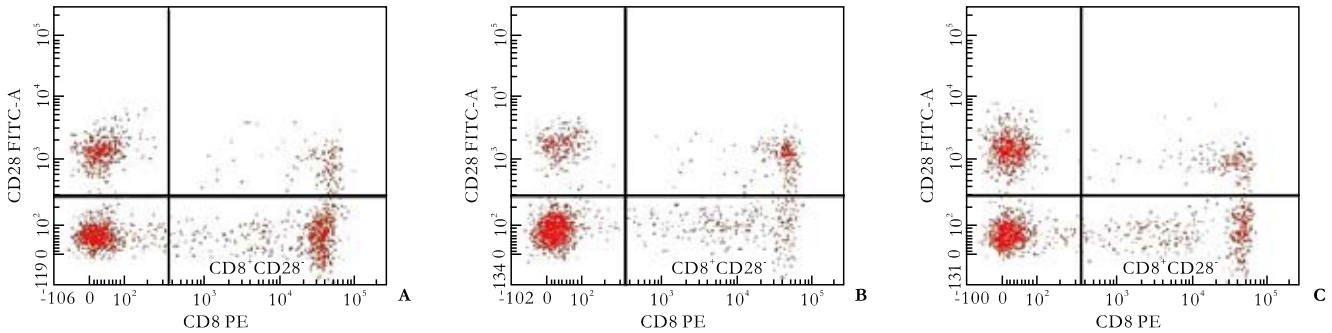

Fig. 1. Frequency of CD8$^+$ CD28$^-$ Ts cells in a recipient of A-A LDLT (**A**), in a patient with cirrhotic hepatitis B (**B**), and in a healthy individual (**C**). The recipient was a patient with cirrhotic hepatitis B, who received a liver transplant 17 months before CD8$^+$ CD28$^-$ Ts cell assay. PE: phycoerythrin. FITC: fluorescein isthiocyanate.

(SPSS 13.0; SPSS Inc., Chicago, USA). A *P* value of less than 0.05 was considered statistically significant.

Results

Three deaths occurred during follow-up, one due to cerebral hemorrhage 9 months after transplantation and two due to recurrence of hepatocellular carcinoma 15 and 21 months posttransplantation. Statistical analysis of recipient and control groups indicated that there was a significant expansion of the CD8$^+$ CD28$^-$ Ts cell population in recipients of A-A LDLT compared to diseased controls and healthy individuals (Table 2, Fig. 1).

A significant expansion of CD8$^+$ CD28$^-$ Ts cells was present in SG compared with USG and diseased or healthy groups (Table 3). There was a similar finding in NRG compared with RG and diseased or healthy controls (Table 4). The frequency of CD8$^+$ CD28$^-$ Ts cells was significantly negatively correlated with the trough levels of tacrolimus (Table 5, Fig. 2). However, there were no significant differences between BG and MG, according to the level of CD8$^+$ CD28$^-$ Ts cells (Table 6).

Table 4. Frequency of CD8$^+$CD28$^-$ Ts cells in NRG, RG, diseased controls and healthy controls

	n	CD8$^+$CD28$^-$ Ts (%)	*P* value*	*P* value$^\#$
NRG	25	27.87±8.06		
RG	6	19.04±7.18	0.028	
Diseased controls	24	18.05±8.79	0.000	>0.5
Healthy controls	19	10.17±2.77	0.000	0.000

*: RG versus diseased controls and healthy controls; #: NRG versus RG, diseased controls and healthy controls.

Table 2. Frequency of CD8$^+$CD28$^-$ Ts cells in recipients of A-A LDLT, diseased controls and healthy controls

	n	CD8$^+$CD28$^-$ Ts (%)	*P* value
LDLT recipients	31	26.13±9.36	
Diseased controls	24	18.05±8.79	0.007$^\#$
Healthy controls	19	10.17±2.77	0.000*

*: Recipients versus diseased controls; #: recipients versus healthy controls.

Table 5. Correlation between the trough level of immunosuppression and frequency of CD8$^+$CD28$^-$ Ts cells

	n	CD8$^+$CD28$^-$ Ts (%)	Trough levels (ng/ml)	Correlation coefficients	*P* value
Tacrolimus	28	26.20±10.15	6.52±2.70	-0.761	0.009
CsA	3	25.49±12.44	93±48.98	-0.246	0.072

Table 3. Frequency of CD8$^+$CD28$^-$ Ts cells in SG , USG, diseased controls and healthy controls

	n	TB (μmol/L)	ALT (IU/L)	AST (IU/L)	CD8$^+$CD28$^-$ Ts (%)	*P* value*	*P* value$^\#$
SG	21	22.19±5.90	51.05±21.80	47.71±24.06	28.89±9.13		
USG	10	34.27±10.40	97.53±41.37	112.81±57.62	21.08±10.01	0.032	
Diseased controls	24	30.27±8.66	80.11±39.30	89.49±45.05	18.05±8.79	0.000	0.356
Healthy controls	19				10.17±2.77	0.000	0.000

*: USG versus diseased controls and healthy controls; #: SG versus USG, diseased controls and healthy controls.

Table 6. Frequency of $CD8^+CD28^-$ Ts cells in BLG, MLG, diseased controls and healthy controls

	n	$CD8^+CD28^-$ Ts (%)	*P* value*	*P* value#
BLG	19	26.63±11.05		
MLG	12	24.87±9.07	>0.5	
Diseased controls	24	18.05±8.79	0.014	0.043
Healthy controls	19	10.17±2.77	0.000	0.000

*: MLG versus diseased controls and healthy controls; #: BLG versus MLG, diseased controls and healthy controls.

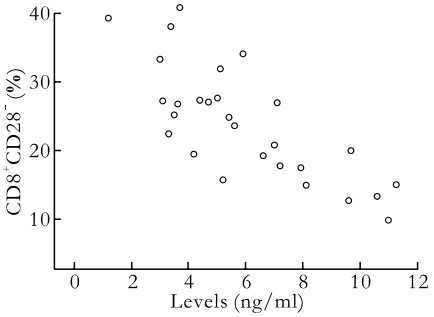

Fig. 2. Scatterplot of the frequency of $CD8^+CD28^-$ Ts cells in recipients who took tacrolimus. The Y axis shows the frequency of $CD8^+CD28^-$ Ts cells and the X axis shows the trough levels of tacrolimus.

Discussion

Liver transplantation is an established therapy for patients with ESLD. However, its application is hindered by the scarce supply of cadaveric donors. The increasing shortage of organs and growing waiting list place inherent pressure on transplant programs to expand the donor pool. A-A LDLT offers a realistic hope of new life for thousands of patients worldwide, especially in Asian countries.[4] To guarantee donor safety, we have utilized right lobe liver grafts not containing the middle hepatic vein.

Basically, regulatory T cells (Tregs) contain two main cell populations: $CD4^+CD25^{high}$ T cells and $CD8^+CD28^-$ Ts cells. The mechanisms by which $CD4^+CD25^{high}$ T cells mediate their function seem to require cell-cell contact through the binding of cell surface molecules such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4).[5] $CD4^+CD25^{high}$ T cell-mediated transplant tolerance is dependent on both interleukin-10 and CTLA-4 because blockade of either pathway completely abrogates transplant survival.[6] Furthermore, $CD4^+CD25^{high}$ Tregs may also down-modulate antigen presenting cell (APC) functions, thereby making APCs unable to activate effector T cells.[7] $CD8^+CD28^-$ Ts cells suppress the activation and proliferation of $CD4^+$ T-helper cells by preventing the up-regulation of B7 molecules and inhibiting the CD40 signaling pathway of APCs.[8, 9] Studies in heart transplant patients indicate that $CD8^+CD28^-$ Ts cells up-regulate immunoglobulin-like transcripts 3 and 4 on dendritic cells.[10]

However, Game et al[11] detected no regulation of direct immune reactivity by peripheral $CD4^+CD25^+$ Tregs in stable kidney transplant patients some years after transplantation. Braudeau et al[12] observed no differences in the suppressive capacity of peripheral $CD4^+CD25^+$ Tregs from these patients compared with those from clinically stable kidney transplant patients. To our knowledge, there is little information about $CD8^+CD28^-$ Ts cells in A-A LDLT recipients and their clinical significance is still unknown.

Our study demonstrated that, there was a significant expansion of $CD8^+CD28^-$ Ts cells in recipients of A-A LDLT compared with diseased and healthy controls. The clinical and follow-up data revealed significant associations among the levels of $CD8^+CD28^-$ Ts cells, graft function, acute rejection episodes and the trough levels of tacrolimus. However, no significant differences were found in the frequency of $CD8^+CD28^-$ Ts cells between BLG and MLG (Table 6). Posttransplant time seems to have no association with the circulating population of $CD8^+CD28^-$ Ts cells (data not shown).

Graft function

Significant differences in $CD8^+CD28^-$ Ts cell levels were found in recipients with stable graft function compared with those whose graft function was abnormal. Accordingly, good graft function was associated with significantly higher levels of $CD8^+CD28^-$ Ts cells. Despite the quantitative changes, a significant expansion of the frequency of $CD8^+CD28^-$ Ts cells was present in SG compared with USG and diseased or normal controls (Table 3).

This finding indicated that the $CD8^+CD28^-$ Ts cells play an important role in keeping graft function stable. Though the specific mechanism needs further study, it may benefit recipients of A-A LDLT to promote the expansion of their $CD8^+CD28^-$ Ts cells to sustain good graft function.[13-15]

Acute rejection

Acute rejection of liver transplants is a frequent cause of hepatic dysfunction in the early phase after surgery. Current data have shown that in acute rejection the liver is characterized by an environment displaying a significant number of activated T cells with a preferential T-helper-like cytokine pattern.[16-18] In our study, we found that these events may be

correlated with a lower level of $CD8^+CD28^-$ Ts cells compared to recipients without rejection. It is suggested that monitoring the $CD8^+CD28^-$ Ts cell levels is helpful to evaluate the immune status of recipients.

In our opinion, detection of $CD8^+CD28^-$ Ts cells in allograft rejection is a tool for the early diagnosis of possible liver rejection in recipients of A-A LDLT. It is also suggested that $CD8^+CD28^-$ Ts cells are novel targets for immunological intervention.

Levels of immunosuppression

All patients in our cohort were treated with calcineurin inhibitors (Tacrolimus or CsA), which modulate the number of circulating Tregs.[19] An excessive load of tacrolimus induced a significant decrease in $CD8^+CD28^-$ Ts cells (Fig. 2). There was an association between the blood level of tacrolimus and the frequency of $CD8^+CD28^-$ Ts cells (Table 5). However, we cannot make such a conclusion for recipients who took CsA.

Our data suggest that immunosuppressive therapies, in particular the use of tacrolimus, decrease the levels of $CD8^+CD28^-$ Ts cells, at least in peripheral blood. In A-A LDLT recipients, it may be helpful to lower immunosuppression in view of the high frequency of potentially tolerant cells in peripheral blood.[20, 21]

Evidence has shown that the appearance of $CD8^+CD28^-$ Ts cells is associated with a reduced need for maintenance of immunosuppression in recipients of cadaveric heart-kidney transplants and pediatric liver-intestine transplant recipients.[2, 3] In our center, the initial immunosuppression regimen in A-A LDLT was a standard triple-drug combination including tacrolimus or CsA, MMF and methylprednisolone. The initial dosages of tacrolimus, CsA and MMF were 0.06-0.08 mg/kg per day, 6.5-7.0 mg/kg per day and 10-20 mg/kg per day, respectively. The target trough levels of tacrolimus and CsA were in the range of 5-10 ng/ml and 150-200 ng/ml, respectively within 1 month posttransplantation, and then decreased to the ranges of 5-8 ng/ml and 100-150 ng/ml, respectively at 30 days after transplantation. Our initial dosages and target trough levels of immunosuppressants in A-A LDLT were lower than in other centers.[22-27] Accompanied with the low dosage and target trough level of tacrolimus or CsA, $CD8^+CD28^-$ Ts cells were highly expanded in peripheral blood from recipients of A-A LDLT. In our opinion, administration of low dosage of tacrolimus or CsA in A-A LDLT may be helpful to promote expansion of $CD8^+CD28^-$ Ts cells, which may allow maintainance of low dosages of immunosuppressants or even decreasing or withdrawing them. $CD8^+CD28^-$ Ts cells may be a useful index to adjust the administration of immunosuppression in recipients of A-A LDLT. However, the small numbers of subjects and cross-sectional nature of this study prevent us from making such a conclusion.

Our study has indicated that it is helpful to monitor $CD8^+CD28^-$ Ts cell levels in evaluating the immune status of recipients. Meanwhile, it is important to promote expansion of $CD8^+CD28^-$ Ts cells in A-A LDLT, not only to sustain good graft function and decrease the dosage of immunosuppression, but also to reduce the occurrence of rejection. This partly illustrates the current dilemma of immunosuppressive treatment — on the one hand preventing rejection, but on the other hand inhibiting Tregs and thereby possibly interfering with the development of transplant tolerance. In our opinion, further evaluation is required to choose either $CD8^+CD28^-$ Ts cells or $CD4^+CD25^{high}$ T cells as the suitable monitoring target for A-A LDLT recipients.

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Contributors: YLN proposed the study. LYX wrote the first draft. WLL analyzed the data. All authors contributed to the design and interpretation of the study and to further drafts. YLN is the guarantor.

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