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Different phenotypes of $CD4^+CD25^+Foxp3^+$ regulatory T cells in recipients post liver transplantation

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ABSTRACT

$CD4^+$ regulatory T cells (Tregs) play an important role in inducing immune tolerance in organ transplantation, which can be divided into $CD45RA^+$Tregs (resting Tregs, rTregs) and $CD45RO^+$Tregs (activated Tregs, aTregs). Currently, the expressions and phenotypic changes of Tregs in recipients after liver transplantation (LT) is unknown. We therefore investigated the expression and transformation of rTregs and aTregs in 83 cases of recipients with normal status post-LT. The percentages of CD45RA, CD45RO, CD31 in $CD4^+$Tregs were detected by flow cytometry and the effective factors were analyzed. In LT recipients, the percentage of $CD45RO^+$Tregs in $CD4^+$Tregs was higher than that of $CD45RA^+$Tregs. There was significant difference in the ratio of positive Foxp3 between $CD45RA^+$Tregs and $CD45RO^+$Tregs. Percentage of $CD45RA^+$Tregs was higher in pediatric group than that in adult group, whereas percentage of $CD45RO^+$Tregs was lower in the pediatric group. However, it was different only in $CD45RO^+$Tregs in various survival periods post-LT. In conclusion, Tregs pool in human was heterogeneous post-LT and contained different subsets in phenotypes. Upon stimulation by donor graft, percentages of $CD4^+$Tregs and $CD45RO^+$Tregs were increased post-LT and most of rTregs was transformed into aTregs in peripheral blood, and rTregs and aTregs were both related to recipients' ages.

1. Introduction

In recent years, more and more researches have shown that regulatory T cells (Tregs) play an important role in transplantation immunity [1,2]. As one kind of cells with immune regulatory capability, Tregs were found to be constituted by different subsets, such as $CD4^+CD25^+$Treg, antigen induced T regulatory cell type 1 (Trl) or T helper cell 3 (Th3), antigen specific $CD4^+$Treg, $CD8^+$Treg, $CD8^+CD28^+$Treg, γδ Treg, NK Treg and $CD4^+$ $CD25^-$Treg [3–5].

In previous studies, Tregs represented a heterogeneous population of $CD4^+$ T cells. Generally, Tregs could be divided into natural Tregs (nTregs) and inducible Tregs (iTregs) or peripheral Tregs (pTregs). nTregs develop from the precursor single-positive CD4 cells in the thymus and develop into Tregs before entering the periphery, while iTregs are induced from $CD4^+CD25^-$ naive T cells by the stimulation of antigens in peripheral blood, which express Foxp3 upon activation by TGF-β [6,7]. There are some differences in the stability of Foxp3 expression and regulation between nTregs and iTregs. In cell culture, iTregs often lose their regulatory function after stimulation, and pTreg cells' capability of regulating immune tolerance are impaired after being induced activated in the periphery [8]. However, capability of Foxp3 expression of nTregs is relatively stable and will not be deprivated in the same conditions [9].

The phenotypes within $Foxp3^+$ cells could be the reflection of Tregs' experience in individuals. Based on the expression of CD45, nTregs can be further divided into naïve Tregs or resting Tregs (rTregs) expressing CD45RA and memory Tregs or activated Tregs (aTregs) expressing CD45RO [10]. Naïve Tregs are in resting state after being released into peripheral blood from thymus without presence of alloantigens [11], whereas, they will turn into the memory Treg cells when activated in peripheral lymphatic organs. Tregs in the circulation contains only a small fraction of $CD45RA^+$ Tregs and are mostly composed of

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$CD45RO^+$ Tregs, since most $CD45RA^+$ Tregs are converted into $CD45RO^+$ Tregs during proliferation [12]. Thus, $CD45RA^+$ Tregs and $CD45RO^+$ Tregs can be deemed as same cells in different growing stages [13]. It was found that rTregs were rich in umbilical cord blood and were the major subgroup of Tregs in uterus [14]. In younger adults (< 40 years), the proportion of rTregs in $CD4^+$ T cells was smaller than that in older adults (> 90 years), however the proportion of aTregs is opposite in the two groups [13]. The number of Tregs in the periphery is maintained and actually increases with age, though the thymic output was reduced [15,16]. The mechanism behind it may be the homeostasis sustained by rTregs and aTregs.

It is different in the aspects of differenciation, ability of immune suppression and proliferative stability between $CD45RA^+$Tregs and $CD45RO^+$Tregs [13,17,18]. However, change of Tregs' phenotypes in patients post-LT is not clear, and investigation on expression of CD45 subset Tregs in liver transplantation (LT) patients may contribute to further understanding of the regulation of transformation, function and mechanism of Tregs.

2. Patients and methods

2.1. Patients

Subjects: The study was initially conducted by including data from all recipients who received LT at our hospital from December 2010 to April 2012. Patients were in the period of post-LT longer than 6 months and with normal graft liver function, and those with post-LT period shorter than 6 months or found to have rejections or infections were excluded. In order to avoid the interference of different immunosuppressant, patients taking rapamycin were excluded and all patients included in our research were taking tacrolimus (FK506). Patients who diagnosed of hepatocellular carcinoma generally met Milan criteria pre-LT.

A total number of 15 males and 5 females were included in healthy control group with average ages of (48.6 ± 10.5) years old. There were no differences in gender ($P = 0.223$) and age ($P = 0.052$) between adult post-LT group and the healthy control group.

All subjects signed the Informed Consent Form. All treatments and operations were authorized by Ethics Committee of Tianjin First Center Hospital. The study was conducted in accordance with the principles delineated in the Declaration of Helsinki. The donors were voluntary to donate their livers, both transplantations with donation after cardiac death donor livers and living donor liver transplantations were included. None of the organs were from executed prisoners in our study.

2.2. Operations and immunosuppressant post-LT

Donation after cardiac death liver grafts in adult recipients were generally implanted with classic orthotopic technique, while pediatric recipients received living donor liver transplantation with left lateral segment, and piggy-back technique was used.

A combined regimen of immunosuppressants was tacrolimus + mycophenolate mofetil + methylprednisolone in the first 3–6 months post-LT and tacrolimus + mycophenolate mofetil in the following 6–12 months. In long-term maintenance, mono-therapy of tacrolimus was used. Trough concentration of tacrolimus was maintained at 6–8 ng/ml in the first year post-LT, 4–6 ng/ml in the second year and 2–4 in the following years, respectively. Patients received IL-2Rα antibodies 20 mg (Simulect, Novartis, Switzerland) 2 h before LT and on the 4th day post-LT (10 mg in pediatric patients).

Patients diagnosed with hepatitis B pre-LT were given nucleoside analogues combined hepatitis B immunoglobulin therapy to prevent hepatitis B recurrence post-LT.

2.3. Grouping criteria

Patients were divided into pediatric group and adult group according to patients' ages, and early survival group and long-time survival group according to survival time post-LT (Fig. 1).

2.4. Blood samples collection

Peripheral blood samples of recipients were collected into EDTA-K2 anticoagulant tubes before taking immunosuppressant, and samples were stored and transported at 4 °C. All samples were analyzed by flow cytometry on the day of blood collection.

2.5. Flow cytometric analysis

Blood samples were treated with specific antibodies, and followed by incubating with red blood cell lysate for 15 min. Monoclonal antibodies (mAbs) including CD4-FITC, CD25-APC, IgG1 κ Isotype CD25-APC, CD45RO-APC-H7, CD45RA-PE-Cy™7, CD31-PerCP-eFluor 710 (BD Biosciences, San Diego, USA) and Foxp3-PE, as well as Foxp3 Staining Buffer Set (eBioscience, San Diego, USA) were used.

Cell analysis by flow cytometry was performed with LSR II cytometer (BD Biosciences, San Diego, USA). Data were analyzed with FlowJo software (Treestar Inc., Ashland, USA).

2.6. Statistics

Data with normal distribution were expressed as mean ± standard deviation, and were analyzed by independent sample *t*-test, compared *t*-test and One-Way ANOVA. If data do not conform to normal distribution, median and quartile *M* (*Q25*, *Q75*) were applied, and non-parametric test was applied to compare the differences between groups. Linear correlations were computed in Pearson test or spearman test. All analyses were performed with SPSS version 19.0 (IBM Inc., Armonk, NY, USA). Data were considered to be significant when the *P* value was < 0.05.

3. Results

3.1. Characteristics of recipients pre- and post- LT

According to diagnosis, diseases status for most recipients was benign. 83 recipients were included in the research, with 73 adult recipients and 10 pediatric recipients. Patients' characteristics were shown in Table 1. In 27 cases of patients diagnosed with hepatocellular carcinoma, 20 cases were combined with hepatitis B pre-LT. Concentrations of immunosuppressant were at a low level in both adult and pediatric recipients, with no significant difference ($P = 0.083$), which was however enough to maintain patients' normal state without rejection. Post-LT follow-up period was longer in adult recipients than that in pediatric recipients ($P = 0.011$).

3.2. Expression of different phenotypes of $CD4^+CD25^+Foxp3^+$ Treg in adult group post-LT

Percentage of $CD4^+CD25^+Foxp3^+$ Tregs in $CD4^+$ T cells was (7.13 ± 3.14)% in adult LT, and (6.66 ± 2.41)% in healthy control group, respectively. There was no difference between the two groups ($P = 0.537$).

In LT group, percentage of $CD45RO^+$ Tregs in $CD4^+CD25^+Foxp3^+$ Tregs was (74.46 ± 12.21)% and that of $CD45RA^+$ Tregs was (16.42 ± 7.95)%, and level of $CD45RO^+$ Tregs was higher than that of $CD45RA^+$ Tregs ($P = 0.000$) (Fig. 2). Similarly, percentage of $CD45RO^+$ Tregs in healthy control group was higher than that of $CD45RA^+$ Tregs (63.59% ± 9.01% *vs* 23.89% ± 6.26%, $P = 0.000$).

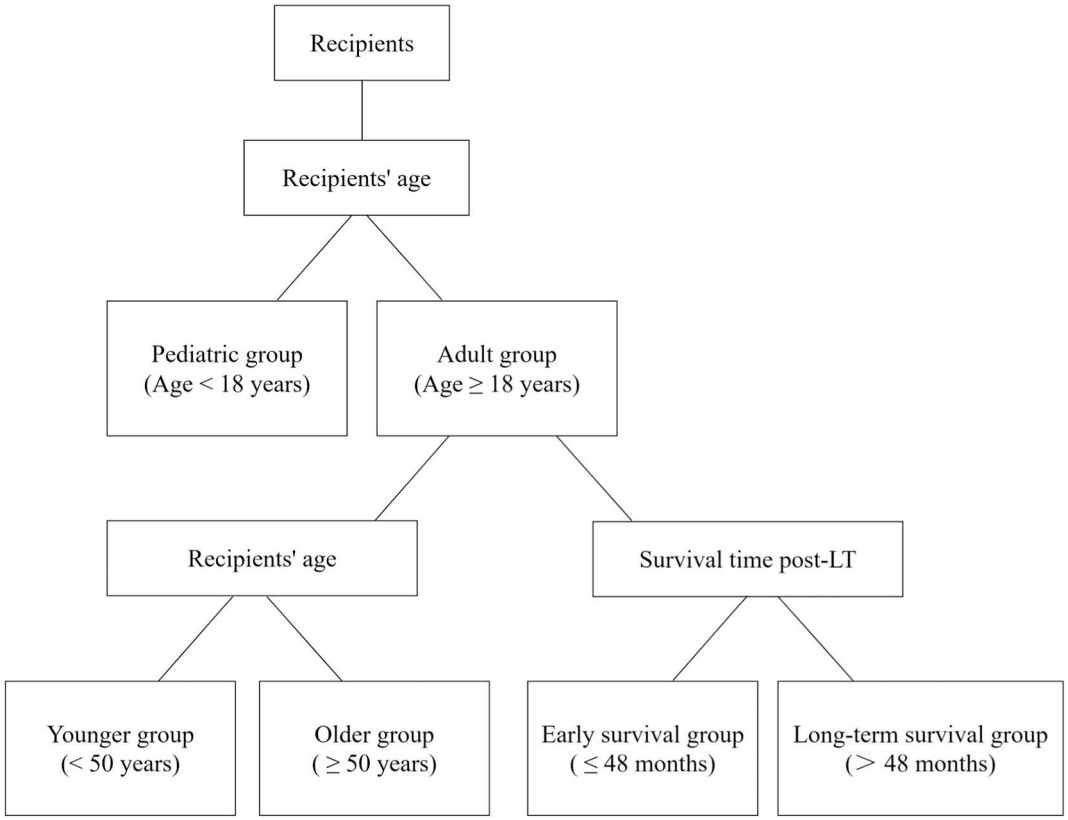

Fig. 1. Schematic illustration of grouping criteria in the research. Recipients were divided into pediatric group and adult group by age of 18 years old. Most of the pediatric recipients were < 9 years old, with only one 11 years old. Adult recipients were divided further into the younger group and older group by age of 50 years old. In addition, adult recipients were divided into early survival group and long-term survival group by survival time post-LT of 48 months.

Table 1
Conditions of patients in the research.

Subjects	Adults	Pediatrics	Controls
Ages (year)	53.5 ± 9.9	3.3 (1.6–7.2)[a]	48.6 ± 10.5
Gender (female/male)	10/63	3/7	5/15
BMI (kg/m^2)	23.99 ± 3.08	17.11 ± 1.70	22.18 ± 2.25
Diagnosis pre-LT (case)			–
Cirrhosis related hepatitis B	26	–	–
Hepatocellular carcinoma	27	–	–
Re-LT	8	–	–
Alcoholic cirrhosis	2	–	–
Cirrhosis related autoimmune hepatitis	5	–	–
Cirrhosis related hepatitis C	1	–	–
Polycystic disease of liver	2	–	–
Hepatolith	1	–	–
Wilson's disease	1	–	–
Biliary atresia	–	10	–
Mode of operation			–
Classic orthotopic LT	73	–	–
Piggy-back	–	10	–
Duration post-LT (month)	30.8 (16.7–70.3)[a]	14.6 (7.1–29.5)[a]	–
Immunosuppressant			–
Dosage (mg/d)	2.5 (1.0–4.0)[a]	1.25 (0.75–2.06)[a]	–
Concentration (ng/ml)	3.70 (2.55–5.05)[a]	4.95 (3.45–7.53)[a]	–

Note:
[a] Median.

3.3. Positive expression of Foxp3 in $CD4^+CD25^+CD45RA^+$ T cells and $CD4^+CD25^+CD45RO^+$ T cells in adult post-LT group

In adult LT group, the expression ratio of Foxp3 in $CD4^+CD25^+CD45RA^+$ T cells was (76.82 ± 20.26)%, while the one in $CD4^+CD25^+CD45RO^+$ T cells was (70.54 ± 19.58)%. There was statistically significant difference between the two subpopulations ($P = 0.002$). $CD4^+CD25^+CD45RA^+$ T cells contained a higher proportion of $Foxp3^+$ Tregs (Fig. 3).

Similarly, in healthy control group, ratio of $Foxp3^+$ in $CD4^+CD25^+CD45RA^+$ T cells and $CD4^+CD25^+CD45RO^+$ T cells was (87.05 ± 11.42)% and (71.64 ± 22.58)% respectively. There was significant difference between the two subpopulations ($P = 0.003$).

3.4. CD31 expression in different phenotypes of $CD4^+$ Tregs

3.4.1. Positive expression of CD31 in $CD45RA^+$ Tregs and $CD45RO^+$ Tregs

Expression of CD31 can be used as a marker to distinguish whether CD45 subpopulations are originated from thymus. There was no difference in CD31 expression between $CD45RA^+$ Tregs and $CD45RO^+$ Tregs in adult LT group, pediatric LT group or healthy control group (Table 2). Meanwhile, level of CD31 was not different between adult group and control group, not only in $CD45RA^+$ Tregs ($P = 0.551$), but also in $CD45RO^+$ Tregs ($P = 0.944$). Similarly, CD31 expression was not different in $CD45RA^+$ Tregs ($P = 0.563$) and $CD45RO^+$ Tregs ($P = 0.218$) in adult group and pediatric group.

3.4.2. Expression of Foxp3 in $CD4^+$ $CD25^+$ $CD31^+$ T cells

Foxp3 was expressed in most $CD4^+CD25^+CD31^+$ T cells, and the positive expression of Foxp3 was (65.16 ± 20.96)% in pediatric group, (68.36 ± 17.94)% in adult group and (69.82 ± 21.93)% in control

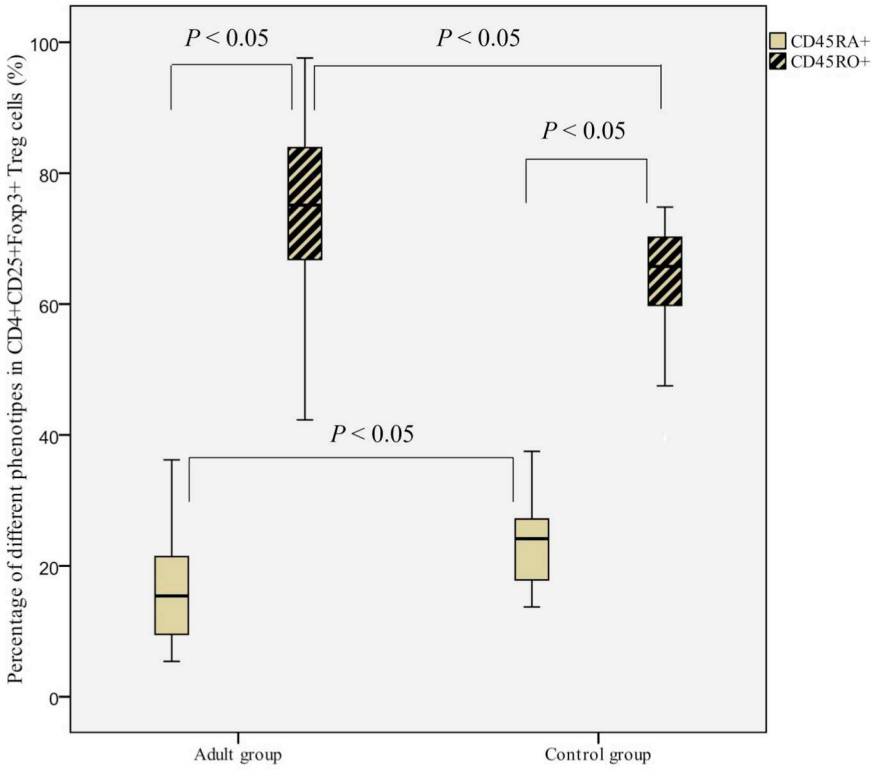

Fig. 2. Expression of CD45RO$^+$ and CD45RA$^+$ in CD4$^+$CD25$^+$Foxp3$^+$ Tregs. No significant difference was found in percentage of CD4$^+$CD25$^+$Foxp3$^+$ Tregs, but there was different in phenotypes between adult LT group and the healthy control group. Percentage of CD45RO$^+$ in CD4$^+$CD25$^+$Foxp3$^+$ Tregs was higher in LT group than that in the control group ($P = 0.000$), whereas percentage of CD45RA$^+$ was lower in LT group than that in control group ($P = 0.000$).

group, respectively. There was no difference among these groups ($P = 0.819$).

3.5. Effect of age on phenotypes of CD4$^+$ CD25$^+$ Foxp3$^+$ Tregs

3.5.1. Expression of CD45RA and CD45RO in CD4$^+$ Tregs in pediatric recipients

Our results showed that there was no difference in proportion of CD4$^+$CD25$^+$Foxp3$^+$ Tregs between pediatric group and adult group ($P = 0.257$), with the median proportion of 6.34% (2.19%–8.28%) and 6.74% (5.05%–8.05%), respectively. Similarly to that of adult LT recipients, percentage of CD45RA$^+$ Tregs was lower than that of CD45RO$^+$ Tregs in pediatric recipients (Fig. 4). Furthermore, both percentages of CD45RA$^+$ and CD45RO$^+$ were different between pediatric and adult group.

3.5.2. Expression of CD45RA and CD45RO in CD4$^+$ Tregs in different age groups in adult recipients

In adult recipients, it was not different in the proportions of CD45RA$^+$ Tregs and CD45RO$^+$ Tregs between younger group and the older one ($P > 0.05$), Foxp3 expression in the two phenotypic groups was the same (Table 3).

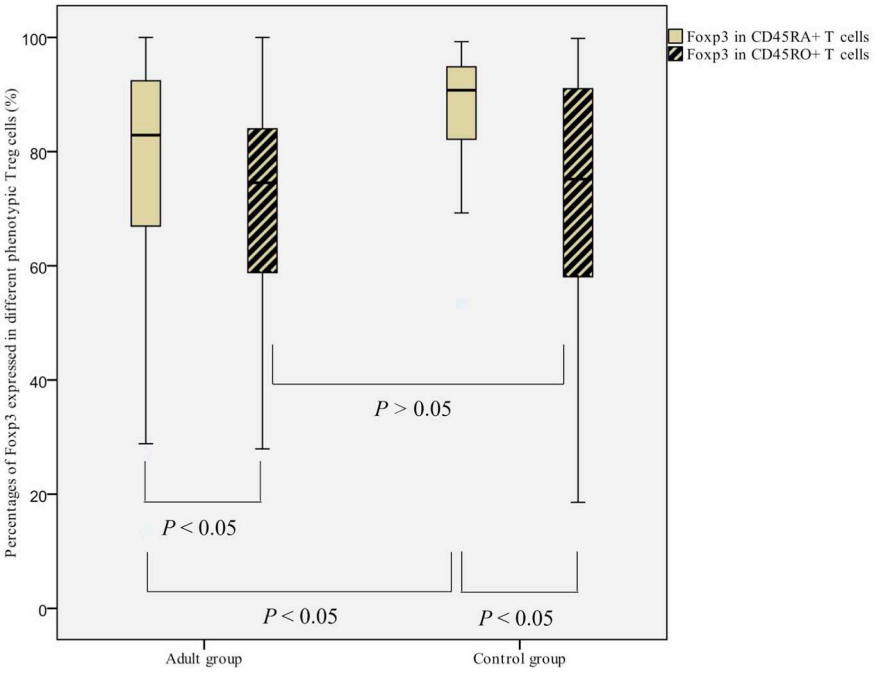

Fig. 3. Percentages of Foxp3 expression in CD4$^+$ CD25$^+$CD45RO$^+$ T cells and CD4$^+$CD25$^+$CD45RA$^+$ T cells. In both LT recipients and healthy controls, the ability of Foxp3 expression was stronger in CD45RA$^+$ T cells than that in CD45RO$^+$ T cells in adults. The positive expression ratio of Foxp3 in CD45RA$^+$ Tregs was lower in LT than that in healthy control group ($P = 0.043$), while there was no difference in percentage of CD45RO$^+$ Tregs between the two groups ($P = 0.829$).

Table 2
Proportions of different phenotypic $CD4^+CD25^+Foxp3^+$ Tregs originated from thymus.

Groups	Subpopulations		*P*-value
	$CD31^+CD45RA^+$ (%)	$CD31^+CD45RO^+$ (%)	
Adult LT group (n = 73)	72.25 ± 20.30	72.48 ± 16.21	0.897
Pediatric LT group (n = 10)	68.23 ± 22.01	65.43 ± 21.27	0.552
Control group (n = 20)	69.17 ± 20.69	72.79 ± 21.10	0.080

Table 3
Subsets expressed in $CD4^+CD25^+Foxp3^+$ Tregs between younger and older groups in adult recipients.

Phenotypes	Age groups		*P*-value
	< 50 years (n = 23)	≥50 years (n = 50)	
$CD4^+$ $CD25^+$ $Foxp3^+$ Treg (%)	5.94 (4.56–7.64)[a]	6.94 (5.20–8.16)[a]	0.296
$CD45RA^+$ Treg (%)	17.96 ± 8.08	15.71 ± 7.87	0.263
$CD45RO^+$ Treg (%)	70.05 ± 17.07	73.60 ± 15.86	0.169
$Foxp3^+$ in $CD45RA^+$ Treg (%)	83.33 ± 15.69	73.83 ± 21.53	0.062
$Foxp3^+$ in $CD45RO^+$ Treg (%)	69.12 ± 18.50	71.19 ± 20.20	0.678

Note:
[a] Median.

3.5.3. Correlations between ages and the expression of CD45RA and CD45RO in $CD4^+CD25^+Foxp3^+$ Tregs in LT recipients (including pediatric and adult patients)

There was no correlation between $CD4^+CD25^+Foxp3^+$ Tregs and ages (Fig. 5A). However, proportions of $CD45RA^+$ and $CD45RO^+$ in $CD4^+$ Tregs were correlated with recipients' ages, with negative correlation in $CD45RA^+$ Tregs and positive correlation in $CD45RO^+$ Tregs (Fig. 5B and C). There was no correlation between $CD4^+CD25^+Foxp3^+CD45RA^+CD31^+$ Tregs and ages (Fig. 5D), whereas $CD4^+CD25^+Foxp3^+CD45RO^+CD31^+$ Tregs were correlated with ages (Fig. 5E).

3.6. Effect of survival time post-LT on phenotypes of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in adult recipients

There were 47 cases in early survival group with mean survival time of 20.4 (10.5–30.6) months and 26 cases in long-term survival group with survival time of 80.4 (69.6–110.0) months. There was no statistical difference in age between the early survival group and long-term survival group ((52.5 ± 11.5) years and (57.2 ± 6.9) years ($P = 0.056$), respectively).

Our results showed that percentage of $CD4^+CD25^+Foxp3^+$ T cells in $CD4^+$ T cells didn't change following the time of post-LT (Table 4). As to percentage of $CD45RA^+$ Tregs, there was no difference between early survival group and long-term survival group (Fig. 6). However, expression of $CD45RO^+$ Tregs was lower in the early survival group than that in long-term survival group. Furthermore, $CD4^+CD25^+Foxp3^+CD31^+$ Tregs was significantly different between the two groups.

4. Discussion

It is generally recognized that Tregs play a key role in transplant immunity, and it has been confirmed in clinical and animal models that Tregs could induce immune tolerance. Moreover, there are important biological differences between naïve and memory $CD4^+$ $CD25^+$ Tregs [19].

rTregs and aTregs showed different characteristics and functions [20]. As to the suppressive function, Hoffmann et al. [21] have reported that $CD45RA^+$ Tregs instead of $CD45RO^+$ Tregs were the dominant subset in Tregs that have higher Foxp3 expression when proliferated *in vitro*, though the proportion of $CD45RA^+$ Tregs was significantly lower than that of $CD45RO^+$ Tregs. In addition, the proportion of naive resting Tregs was positively correlated with Treg suppression [22]. However, Beriou and his colleagues found that aTregs had stronger inhibitory function *in vitro* [23]. In another research including transplant patients who received calcineurin inhibitor, $CD45RA^-$ Tregs retained a greater suppressive capacity when expanded with tacrolimus,

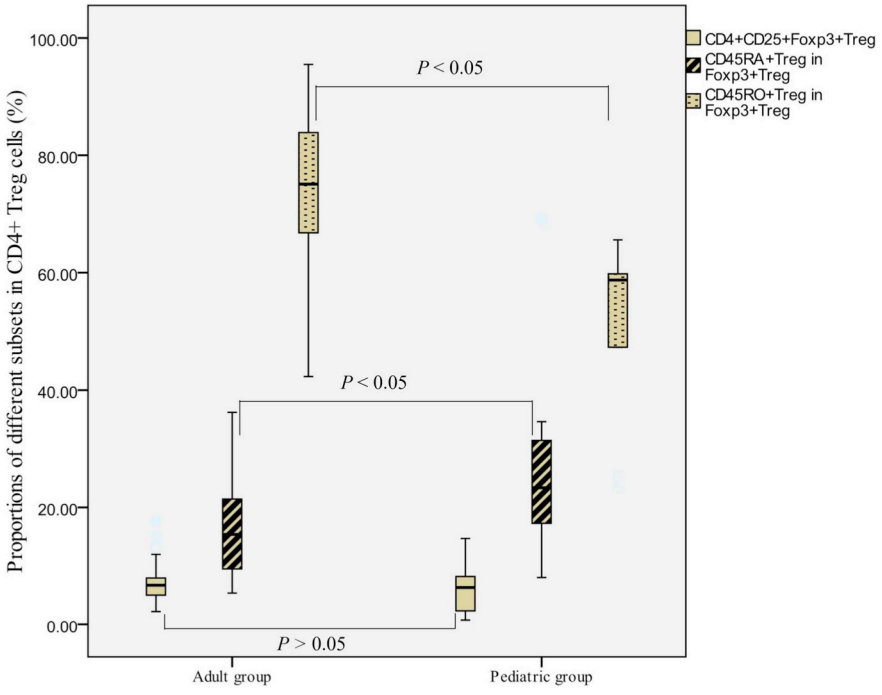

Fig. 4. The proportions of Tregs and subsets between pediatric group and adult group. Percentage of $CD45RA^+$ was higher in pediatric group than that in adult group ($P = 0.013$), with the median percentage of 23.35% (16.97%–32.20%) and 15.40% (9.51%–21.76%), respectively. On the contrary, percentage of $CD45RO^+$ was lower in pediatric group (74.46% ± 12.21% *vs* 51.64% ± 15.18%, $P = 0.000$).

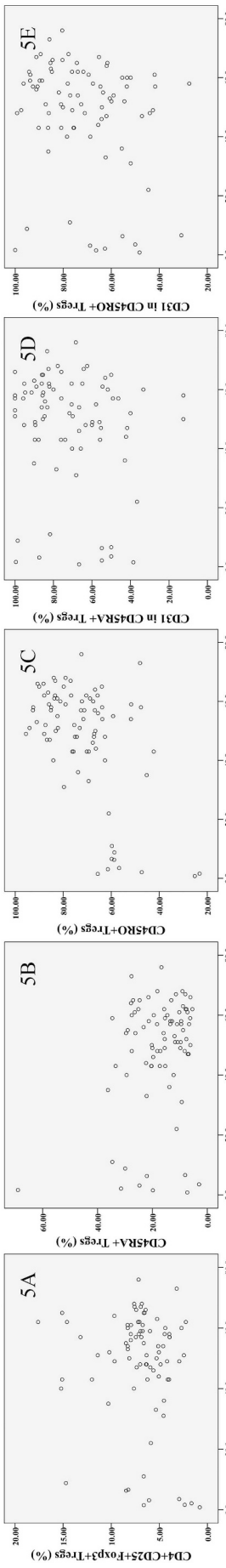

Fig. 5. Correlation between different subsets in $CD4^+$ Tregs and ages in LT recipients. Panel A: correlation between $CD4^+CD25^+Foxp3^+$ Tregs and ages, $P = 0.185$. Panel B: correlation between $CD4^+CD25^+Foxp3^+CD45RA^+$ Tregs and ages, $P = 0.015$. Panel C: correlation between $CD4^+CD25^+Foxp3^+CD45RO^+$ Tregs and ages, $P = 0.000$. Panel D: correlation between $CD4^+CD25^+Foxp3^+CD45RA^+CD31^+$ Tregs and ages, $P = 0.177$. Panel E: correlation between $CD4^+CD25^+Foxp3^+CD45RO^+CD31^+$ Tregs and ages, $P = 0.043$.

while they were likely to lose the T cell-specific demethylation region demethylated status which was related to the stability of Treg cells and the regulation of Foxp3 expression compared to $CD45RA^+$ Tregs with a stable T cell-specific demethylation region demethylated $Foxp3^+$ phenotype after expansion [24,25].

Furthermore, difference in proliferative activity was also found between rTregs and aTregs. rTregs was considered to have stronger expanding capability *in vitro*, while aTregs were easier to lose suppressive capability after expansion [21]. In patients diagnosed of Crohn's disease, it was found that $CD45RA^+$ Tregs were more likely to retain phenotypic stability and were less likely to acquire an effector phenotype than $CD45RA^-$ Tregs [26]. On the contrary, Lei et al. found that $CD45RA^-$ $CD62L^+$ central memory Tregs had stronger suppressive activity on conventional T cells at early stage of immune activation and the suppressive ability was equal to naive Tregs after expansion with mTOR inhibition *in vitro* [27]. Additionally, differences in function and proportion between rTregs and aTregs are determined by their fate. rTregs are a reservation pool to supplement aTregs when activated and aTregs are the main part in Tregs, both of them together maintain the dynamic balance of Tregs. Booth et al. found rTregs began to express CD45RO instead of CD45RA when being activated [17], and it was shown that $CD45RO^+Foxp3^{hi}$ Tregs were differentiated from the naïve $CD45RA^+Foxp3^{low}$ Tregs [28]. Thus, phenotypes of Tregs can be considered as biomarkers of different stages in differentiation.

The pool of Tregs is stably maintained by a dynamic conversion from rTregs to aTregs, although it is a heterogeneous population contained different proportional and functional subsets. In our research, we found that both $CD45RA^+$ Tregs and $CD45RO^+$ Tregs were correlated with recipients' ages, the former and latter was negatively and positively correlated with age, respectively. It is a natural conversion process in Tregs when $CD45RA^+$ Tregs are exposed to alloantigens and convert into $CD45RO^+$ Tregs after being secreted into periphery from thymus post-LT. Thus, the proportion of $CD45RO^+$ Tregs is consistently higher than that of $CD45RA^+$ Tregs in LT recipients and healthy people. However, the proportion of $CD45RO^+$ Tregs in LT recipients was higher than healthy control group, while the proportion of $CD45RA^+$ Tregs was lower than that of the control group in our study. We suspected that the difference was related to the sustained stimulation of a new graft to the immune system after LT, especially the stimulation to $CD45RA^+$ Tregs. In Whitehouse's research, rTregs and aTregs were decreased by treatment of calcineurin inhibitor through increasing cellular turnover and apoptosis, although aTreg subsets exhibited higher proliferation capability in LT recipients than in healthy controls [29]. In a study of renal transplantation, $CD4^+CD45RA^-Foxp3^{hi}$ memory Tregs showed greater suppressive properties exclusively in tolerant patients, which may contribute to the maintenance of graft tolerance [30].

A number of $CD45RA^+$ Tregs were converted into $CD45RO^+$ Tregs post-LT under constant stimulation, which was the main power to maintain immune balance, and that could be the reason why proportion of $CD45RO^+$ Tregs was higher in long-term survival group. Both in LT patients and in the controls, expression of Foxp3 were lower in $CD45RO^+$ Tregs than that in $CD45RA^+$ Tregs, which indicated that $CD45RA^+$ Tregs played an important role in the maintenance of inhibition and $CD45RO^+$ Tregs apoptosized or lose expression of Foxp3 after contacting antigens or proliferation. However, expression of Foxp3 in $CD45RA^+$ Tregs was lower in patients than that in the controls, which was due to the long-term effects of immunosuppressants, especially tacrolimus.

CD31 expression in Treg cells was a biomarker for thymus origination, which could discriminate whether Tregs come from thymus or peripheral lymphocyte organs. $CD31^+$ Tregs was expressed in aTregs at a low level, which could exert strong suppression *in vivo* [11]. Such differentiation of $CD45RA^+CD31^+$ Tregs ensures the enhanced suppressive activity of the rTregs pool [31]. Similar to rTregs from thymus, percentage of $CD31^+CD45RA^+CD4^+$ T cells were negatively correlated

Table 4
Subsets expressed in $CD4^+CD25^+Foxp3^+$ Tregs between early and long-term survival groups in adults post-LT.

Phenotypes	Survival groups (months post-LT)		*P*-value
	≤48 group (n = 47)	> 48 group (n = 26)	
$CD4^+$ $CD25^+$ $Foxp3^+$Treg (%)	6.54 (4.56–8.47)[a]	7.01 (6.07–7.67)[a]	0.717
$CD4^+$ $CD25^+$ $Foxp3^+CD45RA^+$Treg (%)	17.56 ± 8.58	14.35 ± 6.29	0.099
$CD4^+$ $CD25^+$ $Foxp3^+CD45RO^+$Treg (%)	72.36 ± 12.21	78.25 ± 11.47	0.048*
$Foxp3^+$in $CD4^+CD25^+CD45RA^+$Treg (%)	78.31 ± 20.12	74.14 ± 20.62	0.404
$Foxp3^+$in $CD4^+CD25^+CD45RO^+$Treg (%)	70.94 ± 19.33	69.80 ± 20.38	0.814
$Foxp3^+CD31^+$ in $CD4^+CD25^+$Treg (%)	42.60 ± 12.40	35.50 ± 15.78	0.037*

Note:
[a] Median.
* $P < 0.05$.

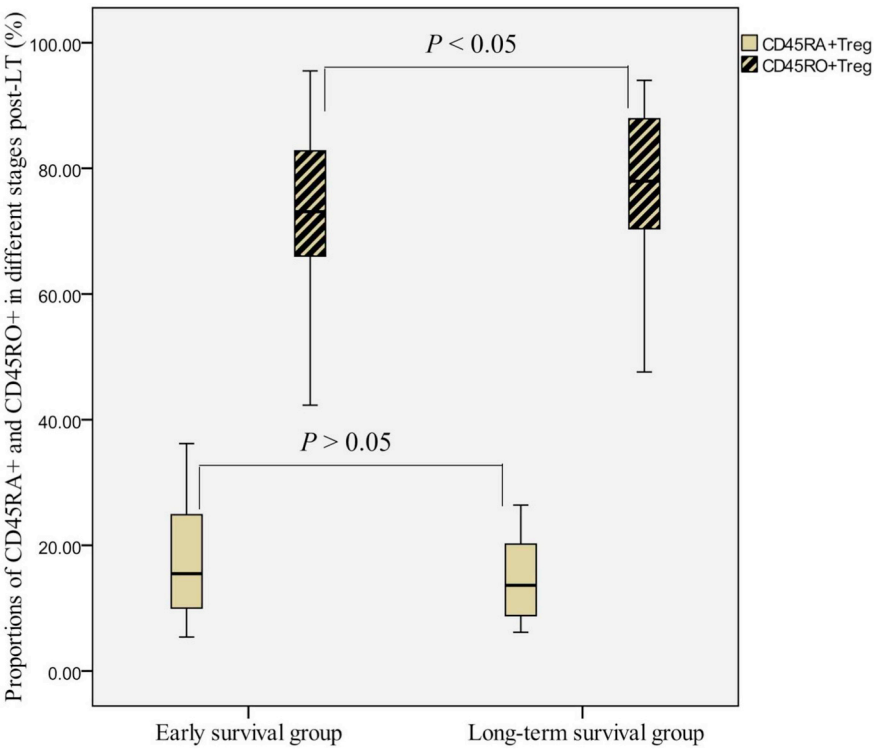

Fig. 6. $CD45RA^+$ and $CD45RO^+$ expression in $CD4^+CD25^+Foxp3^+$ Tregs between early and long-term survival groups in adults post-LT. Excluding the influencing factor of ages in adult LT, percentage of $CD45RO^+$ Tregs was higher than that of $CD45RA^+$ Tregs, both in early survival and long-term survival groups. $CD45RO^+$ Tregs in long-term survival group was higher than that in early survival group, and no difference in $CD45RA^+$ Tregs between the two groups was detected.

with age [32]. However, our finding was different from other researches. There was no difference in the proportion of positive CD31 expression between $CD45RO^+$ Tregs and $CD45RA^+$ Tregs in recipients in our study, which may due to the fast conversion of phenotype from $CD31^+CD45RA^+$ Tregs to $CD31^+CD45RO^+$ Tregs stimulated by allograft. In addition, e proportion of positive CD31 expression in Tregs in different age groups of recipients and healthy control people was not different. Majority of $CD4^+CD25^+CD31^+$ T cells express Foxp3 both in recipients and the healthy controls. However, expression of Foxp3 in $CD31^+$ Tregs was lower in long-term survival group than that in early survival group.

There are inevitably some limitations in our study. First, to what extent $CD45RA^+$ Tregs contribute to the maintenance of the total amount of Tregs post-LT is not clear. Second, whether aTregs are newly converted from rTregs or derived from responder cells could not yet be distinguished. Third, the sample size was limited, which could have been expanded if more pediatrics and recipients who have withdrawn immunosuppressants were included.

In conclusion, our study demonstrated that Treg pool in post-LT patients was heterogeneous and contained different subsets which have various phenotypes with distinct characters and functions. Although being suppressed by immunosuppressants, the subset of Treg originated from thymus was increased. Furthermore, majority of $CD45RA^+$ Tregs transformed into $CD45RO^+$ Tregs in peripheral blood post LT and both of them were related to recipients' age, and percentage of $CD45RO^+$ Tregs increased with post-LT period. Change or conversion of Treg subsets was in a relatively stable homeostasis, which was affected by the stimulation of alloantigen from donor grafts. These findings have important implications for exploring the adoptive transfer of Tregs in transplant immunity in future clinical trials.

Authorship

Wei Gao proposed the study. Kai Wang conducted the research, analyzed the data and wrote the first draft. Zhuo-Lun Song collected the data and revised the draft. Bin Wu and Wei Liu performed culture of cells. Chun-Lei Zhou performed the detection of Treg and Th17 cells by FACS. All authors contributed to the design and interpretation of the study and to further drafts.

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Conflict of interest statement

The authors declare no conflicts of interest. We would like to thank all authors who contributed to this research and all authors are in agreement with the content of the manuscript. We certify that we have participated sufficiently in the work to take public responsibility for the experimental design and method, and the analysis and interpretation of the data.

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