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Altered balance between effector T cells and FOXP3$^+$HELIOS$^+$ regulatory T cells after thymoglobulin induction in kidney transplant recipients

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Conflicts of Interest
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Summary

This study examined the effect of thymoglobulin induction therapy on leukocyte population dynamics in kidney transplant patients. Patients receiving standard immunosuppression were compared with those who received additional thymoglobulin at the time of kidney transplantation. Thymoglobulin induction led to an immediate and significant decrease of all T cells and NK cells, but not B cells or monocytes. CD8$^+$ T cells recovered to near pretransplant level by 4 weeks post-transplant. CD4$^+$ T cells remained at less than 30% of pretransplant level for the entire study period of 78 weeks. Both CD4$^+$ and CD8$^+$ T cells showed reduced cytokine production after recovery. Deletion of CD4$^+$FOXP3$^+$HELIOS$^+$ regulatory T cells (Tregs) was less profound than that of CD4$^+$FOXP3$^-$ cells, thus the relative percentage of Tregs elevated significantly when compared with pretransplant levels in thymoglobulin-treated patients. In contrast, the percentages of Tregs and their expression of FOXP3 in the standard immunosuppression group decreased steadily and by 12 weeks after transplant the average percentage of Tregs was 56% of the pretransplant level. Thus, thymoglobulin-induced deletion of T cells led to significant and long-lasting alterations of the T-cell compartment characterized by a preservation of Tregs and long-lasting reduction in CD4$^+$, and potentially pathogenic, T cells.

Introduction

Thymoglobulin induction therapy reduces acute rejections in kidney transplantation and improves patient's quality-adjusted life years in long-term follow-ups, particularly in patients with higher immunological risk patients, such as retransplants and recipients with high panel reactive antibodies [1]. Short-term prophylaxic use of thymoglobulin at the time of transplant allows delayed introduction and reduction in maintenance dose of cyclosporine and the associated nephrotoxicity [2]. Thymoglobulin induction was also reported to allow early steroid withdraw in and steroid-free maintenance immunosuppression in kidney transplant patients [3,4]. Among high immunological risk patients, thymoglobulin is superior to daclizumab in preventing acute rejections [5]. In addition to being an efficacious induction agent, thymoglobulin can reverse acute renal graft rejection and is routinely used for controlling

acute rejection [6]. The efficacy of thymoglobulin in controlling graft rejection is thought to be as a result of its ability to delete various immune cells, especially T cells [6–12]. Emerging data suggest that thymoglobulin induction may preferentially kill conventional T (Tconv) cells resulting in an increased proportion of regulatory T cells (Tregs) [10,12]. The alteration of balance between Tconv and Treg cells may explain the long-term protection afforded by short-term thymoglobulin treatment. On the other hand, massive deletion of T cells and long-term alteration of the balance between effector T cells and Tregs may increase risks of infections and malignancy, such as post-transplant lymphoproliferative disease [13,14].

Repopulation of T cells after severe deletion is mediated by homeostatic proliferation of residue T cells and increase in thymic output [15]. As T cells expand in the lymphopenic environment, the cells may acquire an activated phenotype and manifest effector functions such as secreting IFN-γ [16–20]. Indeed, it has been reported that T cells recovered after thymoglobulin treatments have increased proportion of memory cells [10]. Yet, a recent study showed that patients' peripheral blood mononuclear cells (PBMCs) were broadly hyporesponsive to donor and third party antigen presenting cells [21].

In this study, we analyzed the population dynamics of various leukocyte subsets, including Tregs, memory and effector T cells in seven kidney transplant patients who received thymoglobulin induction because of delayed graft function (DGF). We found that thymoglobulin induction led to transient depletion of NK and $CD8^+$ T cells and prolonged reduction in total $CD4^+$ T cell counts. Effector $CD4^+$ T cells were significantly reduced when compared with the pretransplant levels. Lastly, Treg percentages and their expression of FOXP3 were sustained in the thymoglobulin-treated patients, but steadily decreased in patients on conventional maintenance therapy without induction. Our results demonstrate that thymoglobulin induction can effectively change the balance between effector T cells and Tregs.

Patients and methods

Kidney transplant patients

All patients in this study received primary renal transplant from either living or deceased donors at University of California, San Francisco Medical Center, or at California Pacific Medical Center. Patients who had DGF that necessitated thymoglobulin induction were enrolled in the thymoglobulin arm of the study. Patients without any induction therapy were enrolled in the control arm. Patients in both groups received standard maintenance immunosuppressive therapy consisted of mycophenolate mofetil, Tacrolimus, and prednisone. All procedures are approved by the Committee on Human Research at UCSF and are in accordance with the ethical guidelines by the Transplant Society.

Peripheral blood mononuclear cell collection and storage

Heparinized blood was collected just before transplant and at 1, 4, 13, 26, and 78 weeks after transplant. PBMC were isolated from recipient blood samples using ficoll density gradient centrifugation. The cells were frozen in human AB serum containing 10% DMSO and stored in aliquots in liquid nitrogen until use.

Flow cytometric analysis of PBMC

Flow cytometry panels were used to profile the patient's PBMC. The ''leukocyte panel'' contained fluorochrome-conjugated antibodies to CD3, CD4, CD8, CD14, CD19, CD45, and CD56. The ''Treg panel'' contained fluorescent antibodies to CD4, CD25, CD127, and FOXP3 as previously described [22]. Some samples were analyzed using a modified Treg panel with an additional antibody to HELIOS, a marker co-expressed with FOXP3 in Tregs [23]. The ''CD4 effector/memory panel'' consisted of fluorescent antibodies to CD3, CD4, CD25, CD27, CD28, CD45RA, and FOXP3. The ''CD8 effector/memory panel'' consisted of fluorescent antibodies to CD8, CD27, CD28, CD45RA, and perforin. All antibodies were purchased from BD Biosciences (Mountainview, CA, USA), except pacific blue conjugated anti-CD45 (clone H30) and eFluor450 conjugated anti-FOXP3 (clone 236/E7) were purchased from eBiosciences (San Diego, CA, USA), and phycoerythrin conjugated anti-HELIOS and PerCP-Cy5/5 conjugated Perforin (clone dG9) were purchased from Biolegend (San Diego, CA, USA). Stained PBMC were analyzed on FACSCalibur (BD) or LSRII (BD). FlowJo (Tree Star, Inc., Ashland, OR, USA) and FACS Diva software (BD) were used to analyze the acquired data.

Activation of PBMC and Tconv cells with CD40L-stimulated allogeneic B cells

PBMCs were labeled with anti-CD4-PerCP, anti-CD25 allophycoerythrin and anti-CD127 phycoerythrin (all from BD) and $CD4^+CD25^+CD127^{low}$ Tconv cells were sorted on a BD FACS Aria II to greater than 99% purity. To generate CD40L-stimulated allogeneic B cells, a previously published protocol was followed [24]. In brief, B cells were isolated using a no-touch B-cell isolation kit (Invitrogen, Grand Island, NY) and stimulated with irradiated 3T3 cells stably expressing human CD40L with additional IL-4. The B cells were initially allowed to expand for 7 days and then were restimulated with 3T3-CD40L every 3–4 days

and used between 10 and 30 days after the culture initiation. To stimulate PBMCs or Tconv cells, the stimulated B cells were irradiated (1000 rads) and mixed with PBMCs or Tconv cells at a ratio of 2 B cells per PBMC or Tconv cell. Expression of HELIOS and FOXP3 in stimulated T cells was analyzed on day 4 postallogeneic B cell stimulation using flow cytometry as described above.

Intracellular IFN-γ analysis

Stored PBMC were thawed and plated in complete medium (RPMI1640 with 10% human AB serum, 1% penicillin and streptomycin) containing 2.5 μg/ml phorbol myristate ester (PMA), 250 μg/ml ionomycin, and 0.5 mg/ml brefeldin A (leukocyte activation cocktail with GolgiPlug, BD Biosciences). The cells were stimulated for 4 h and then stained with fluorochrome-conjugated antibodies to CD3, CD4, and CD8 (all from BD Biosciences) before washing and fixation using fixation/permeabilization buffer (BD Biosciences), and labeling with fluorochrome-conjugated antibody to IFN-γ (BD Biosciences).

Data analysis

Total number of each leukocyte subset was calculated by multiplying the lymphocyte counts provided by the clinical lab with the percentage of a particular leukocyte population derived from flow cytometric analysis. Statistical analyses of the data were performed with the aid of Prism Graphpad software (La Jolla, CA, USA).

Results

Patient information: demographics, medication, and clinical outcome

A total of seven patients in the thymoglobulin arm and four patients in the control arm completed the study and their demographic and clinical data are summarized in Table 1. All patients received three-drug immunosuppressive regimen consisted of Tacrolimus (Prograf, Astellas Pharma US, Northbrook, IL), mycophenolate morfetil (Cellcept, Genentech, South San Francisco, CA), and Prednisone. Trough levels of Tacrolimus at 1, 3, and 6 months post-transplant are shown in Table 1. Mycophenolate morfetil was given at 1000 mg BID, and in some patients, Myfortic 720 mg BID was given as an alternative. Prednisone taper consisted of decreasing doses from 500 mg on Day 1 to 30 mg/day by Day 10 post-transplant, and further reduction to a final dose of 5 mg/day by Day 30. Six patients in the thymoglobulin arm received 6 mg/kg body weight thymoglobulin in the first week of postoperative period. One patient in the thymoglobulin arm received 3 mg/kg body weight thymoglobulin, and results from this patient were consistent with those from other patients; therefore are presented together with the group. All patients in the thymoglobulin arm developed DGF as indicated by low urine output during the 24-h postoperative period. Five patients required one dialysis during the first week, one required three dialyses, and one did not require dialysis. None in the control arm developed DGF. The difference in DGF risk between patient populations should have no impact on circulating lymphocyte phenotypes beyond the first week when graft function (serum creatinine) between the two patient populations were become indistinguishable (Table 1). None of the patients in either arm experienced rejection based on clinical assessment; all showed comparable low creatinine levels post-transplant (Table 1). Both patient populations were screened for CMV, EBV, and BKV. In the thymoglobulin arm, no CMV or EBV reactivation was observed; one patient developed transient BKV viremia 27 months after transplant and biopsy ruled out BK infection in the transplanted kidney. The BK viremia resolved 6 weeks later with reduction in tacrolimus and MMF without other interventions. In the control arm, no BKV or EBV reactivation was detected; one patient developed CMV viremia 4 months post-transplant, which cleared 1 month later.

Table 1. Study patient demographics.

	Thymoglobulin $n = 7$	Control $n = 4$
Age (years, average ± SD)	48 ± 13	56 ± 13
Male sex (%)	71	75
Deceased donor	7	2
Living-unrelated donor	0	1
Living-related donor	0	1
% PRA	15 ± 32.3	0
Months of pre-Tx dialysis	80.7 ± 19.0	25.3 ± 35.0
Creatinine		
Pre-Tx	9.3 ± 3	7.6 ± 3
3-month post-Tx	1.4 ± 0.3	1.4 ± 0.3
Tacrolimus trough		
1-month post-Tx	6.8 ± 2.3	12.7 ± 4.7
3-month post-Tx	7.8 ± 2.5	8.5 ± 3.3
6-month post-Tx	8.6 ± 2.6	8.4 ± 1.7
Graft loss	0	0

Alternation of leukocyte populations after thymoglobulin induction

To assess the effect of thymoglobulin induction on the numbers of various leukocytes, we performed flow cytometric analyses on the PBMC collected just before transplant and at 1, 4, 13, 26, 39, and 78 weeks after transplant (Fig. 1a). We calculated the total numbers of circulating CD4, CD8 T cells, NK cells, B cells, and monocytes per milliliter of blood (Fig. 1b–f). Consistent with previous

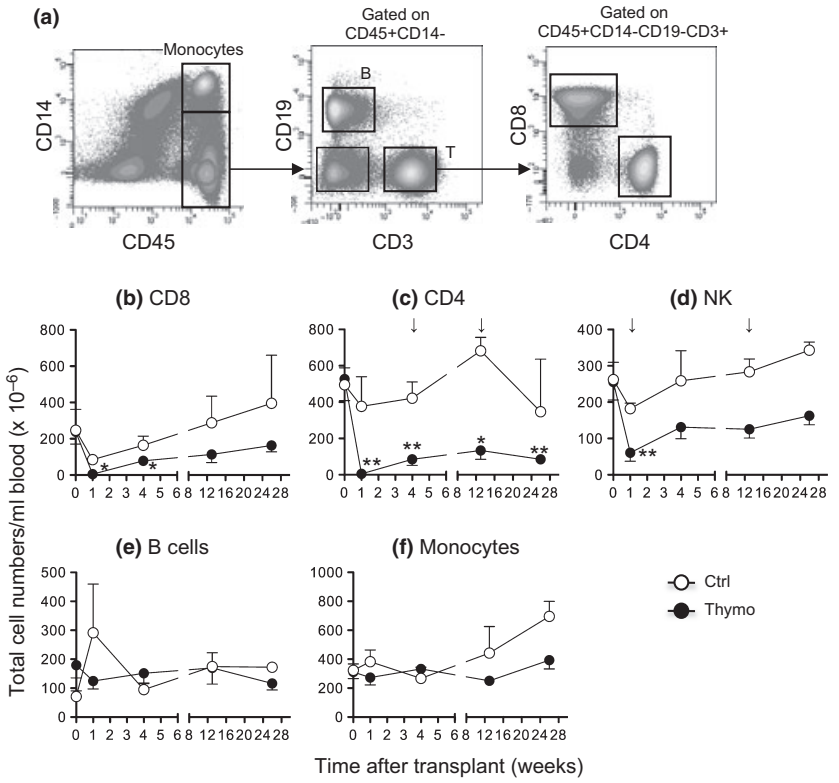

Figure 1 Effect of thymoglobulin induction on numbers of leukocyte subsets in peripheral blood. Percentages of leukocyte subsets in the PBMC were determined using multiparameter flow cytometry (a). Numbers of each leukocyte subset at various time points after transplant were normalized to the patient's own pretransplant numbers. The average cell numbers of CD8 (b), CD4 (c), NK (d), B cells (e), and monocytes (f) are then compared between patients in the control arm (open symbols, $n = 4$)) and those received thymoglobulin induction (filled symbols, $n = 7$). Error bars represent standard error of the mean. Asterisks indicate statistically significant differences between the data point and the pretransplant values of the same treatment group (*$P < 0.05$; **$P < 0.01$). Arrow indicates statistically significant differences between control and thymoglobulin groups at a particular time point ($P < 0.05$).

reports, thymoglobulin induction led to a rapid loss of $CD4^+$ and $CD8^+$ T cells in the peripheral blood. The numbers of $CD4^+$ and $CD8^+$ T cells were lowest at 1-week post-transplant, with a clear trend of recovery at 4-week post-transplant. The recovery of $CD8^+$ T cells was more complete, and, on average, the $CD8^+$ T cell counts were not statistically significantly different from the pretransplant levels by 3 months (13 weeks) after transplantation (Fig. 1b). In comparison, the recovery of $CD4^+$ T cells was incomplete and remained, on average, 50% below the pretransplant level at one and a half years (78 weeks) after transplant. We also observed a significant reduction in NK cells immediately after thymoglobulin induction, with slow and incomplete recovery (Fig. 1d). No change in B cells and monocytes was observed after thymoglobulin induction when compared with patients' own pretransplant baseline levels (Fig. 1e and f).

Sustained decrease in effector $CD4^+$ T cells after thymoglobulin induction

To determine if the T cells recovered after thymoglobulin induction express an activated phenotype in our patients, we analyzed cells in the lymphocyte gate surface expression of CD45RA, CD28, and CD27 on $CD4^+$ Tconv and $CD8^+$ T cells before and after transplant with or without thymoglobulin induction. Naïve $CD4^+$ T cells are defined as $CD45RA^+CD27^+$, memory $CD4^+$ T cells are defined as $CD45RA^-CD27^+$, and the remaining cells in the $CD4^+$ gate are grouped together as effector $CD4^+$ T [25] (Fig. 2a). Prior to transplant, effector T-cell populations were similar between the patients in thymoglobulin and control arms, but the control patients had significantly higher naïve cells and lower memory cells when compared with patients in the thymoglobulin arm (Fig. 2b). This difference is not as a result of thymoglobulin treatment because the pretransplant samples were collected before thymoglobulin administration.

After transplantation, we observed a trend of steady decline of the proportion of naïve $CD4^+$ T cells in control and thymoglobulin-treated patients, while the proportion of memory $CD4^+$ T cells did not change significantly (Fig. 2c and d). We also observed a trend of increase in effector $CD4^+$ T cells after transplant in control patients, but not in patients who received thymoglobulin induction (Fig. 2e). The $CD45RA^-CD27^-$ effector population contained two major cell subsets with cell surface phenotype of $CD28^-$ and $CD28^+$ (Fig. 2a, right panel). The $CD28^-$ subset was shown previously to have a IFN-γ expression Th1 bias, whereas the $CD28^+$ subset had a Th2 bias [25]. We observed a sharp drop of proportion of the $CD45RA^-CD27^-CD28^-$ subset in thymoglobulin-treated

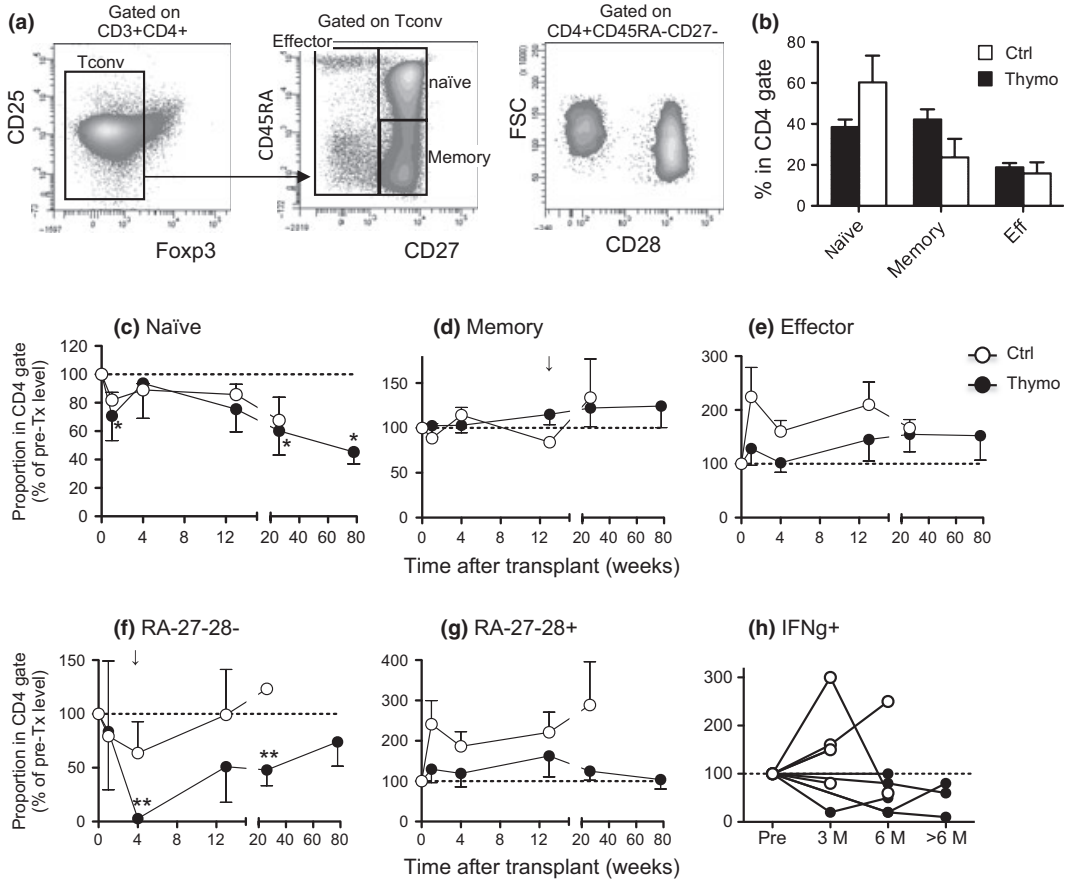

Figure 2 Effect of thymoglobulin induction on proportions of naïve, memory, and effector $CD4^+$ Tconv cells. The percentages of naïve, memory, and effector T-cell subsets in $CD4^+FOXP3^-$ Tconv cells were determined by sequential gating of $CD3^+CD4^+$ T cells (a). Compositions of $CD4^+$ T cells before transplant are shown (b). Percentages of each CD4 subset at various time points after transplant were normalized to the patient's own pretransplant levels. The normalized levels of naïve (c), memory (d), and effector (e) CD4 T cells after transplant in control and thymoglobulin-treated patients were compared. Average percentages of $CD28^-$ effectors (f) and $CD28^+$ effectors (g) after transplant in control (n = 4) and thymoglobulin patients (n = 7) are shown. IFN-γ expression by $CD4^+$ T cells after 4-h PMA and ionomycin treatment is shown in h (n = 4 in each group). Error bars represent standard error of the mean. Asterisks indicate statistically significant differences between the data point and the pretransplant values of the same treatment group (*P < 0.05; **P < 0.01). Arrow indicates statistically significant differences between control and thymoglobulin groups at a particular time point (P < 0.05).

patients in the first month post-transplant period (Fig. 2f). The percentage of these cells recovered partially at 3 months after transplant and remained at a level lower than pretransplant for the entire duration of the study (one and a half years). In contrast, the percentages of $CD45RA^-CD27^-CD28^-$ cells were at or even exceeding the pretransplant levels in control patients. The $CD45RA^-CD27^-CD28^+$ population did not change significantly after transplant and thymoglobulin induction (Fig. 2g). In contrast, their percentages significantly and progressively increased in control patients who did not receive thymoglobulin induction (Fig. 2g). To directly determine the effect of thymoglobulin induction on effector cytokine production, we activated the PBMCs using PMA and ionomycin for 4 h and analyzed IFN-γ expression by $CD4^+$ T cells. Consistent with our observation of decrease in Th1-biased $CD45RA^-CD27^-CD28^-$ effector cells, IFN-γ production by $CD4^+$ T cells in all patients who received thymoglobulin remained at or below pretransplant levels. Our results demonstrate that the $CD4^+$ T cells recover incompletely following thymoglobulin treatment. The limited recovery of $CD4^+$ T cells may explain the lack of increase in effector or memory $CD4^+$ T cells secondary to homeostatic proliferation. When compared with patients who did not receive thymoglobulin induction, patients in the thymoglobulin arm showed a trend of reduced effector cell frequency in the peripheral blood.

Increase of CD8$^+$ effector T cells in thymoglobulin-treated patients is not associated with homeostatic proliferation

To assess the proportions of naïve, memory, and effector CD8$^+$ T cells, we stained patients' PBMC with antibodies to CD8, CD27, CD28, CD45RA, and perforin. Naïve CD8$^+$ T cells are defined as CD45RA$^+$CD27$^+$Perforin$^-$, memory cells are defined as CD45RA$^-$CD27$^+$Perforin$^-$, and effector cells are CD27$^-$ that be further divided into RA$^+$ and RA$^-$ subsets [25,26] (Fig. 3a). Among these subpopulations, naïve and memory cells express the lowest

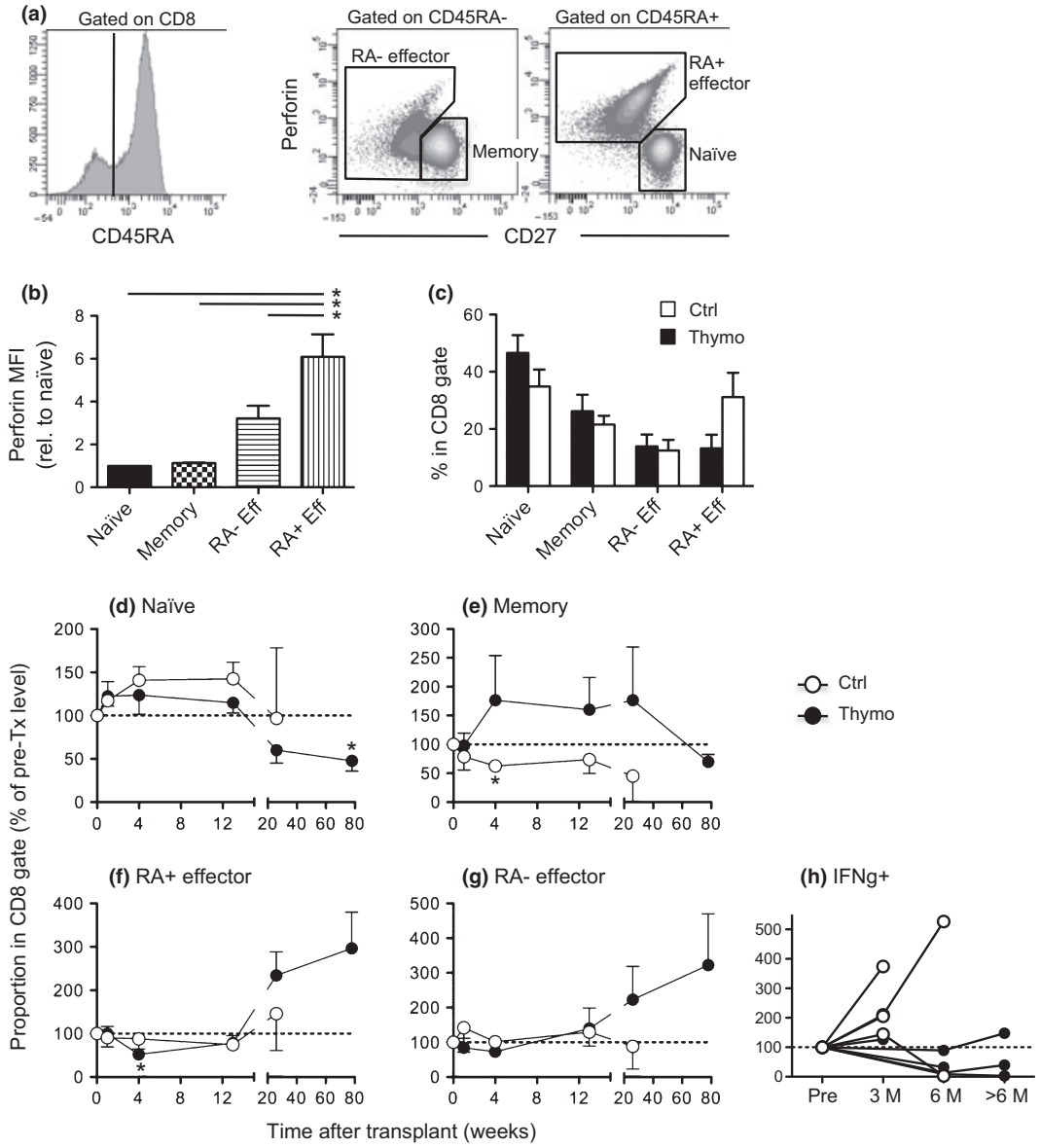

Figure 3 Effect of thymoglobulin induction on proportions of naïve, memory, and effector CD8$^+$ T cells. The percentages of naïve, memory, and CD45RA$^-$ and CD45RA$^+$ effector subsets were determined by after gating on CD8$^+$ T cells (a). The average mean fluorescence intensity (MFI) of perforin in each cell type in all study patients (n = 11) before transplant is compared (b). Asterisk indicates statistically significant difference in Perforin MFI between two subsets indicated by the line (*P < 0.05). Compositions of CD8$^+$ T cells before transplant are shown (c). Percentages of each CD8 subset at various time points after transplant were normalized to the patient's own pretransplant levels. The normalized levels of naïve (d), memory (e), CD45RA$^+$ effector (f), and CD45RA$^-$ (g) CD8$^+$ T cells after transplant in control (n = 4) and thymoglobulin-treated patients (n = 7) were compared. Error bars represent standard error of the mean. Asterisks indicate statistically significant differences between the data point and the pretransplant values of the same treatment group (*P < 0.05). IFN-γ expression by CD8$^+$ T cells after 4-h *ex vivo* PMA and ionomycin treatment is shown in h (n = 4 in each group).

level of perforin, which is followed by RA^- effectors, and RA^+ effectors expressed significantly higher amount of perforin than any other subpopulations (Fig. 3b). The percentages of the naïve, memory, and RA^- effector subsets before transplant were comparable in patients in the two study groups, whereas the RA^+ effectors were present at markedly higher level in patients in control arm than in patients in the thymoglobulin arm (Fig. 3c). During the first 12 weeks after transplantation, no dramatic change in the percentages of $CD8^+$ T-cell subsets was observed (Fig. 3d–g). We observed a dramatic rise of percentages of effector $CD8^+$ cells, both $CD45RA^+$ and $CD45RA^-$ more than 26 weeks after transplant in patients received thymoglobulin induction (Fig. 3f and g). This was associated with a drop of the naïve subset (Fig. 3d). The rise of effector $CD8^+$ T cells in the thymoglobulin patient was not statistically significant because of large variations that ranged between 50% and 455% of pretransplant levels. Interestingly, despite the marked increase in perforin$^+$ effector cells, percentage of IFNγ expressing $CD8^+$ T cells decreased or remained unchanged in thymoglobulin-treated patients (Fig. 3h). Overall, we found rapid recovery $CD8^+$ T cells with delayed rise of effector $CD8^+$ cells after thymoglobulin treatment.

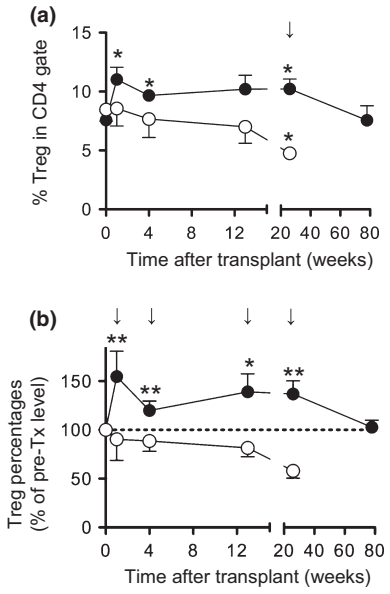

Figure 4 Tregs persist in patients who received thymoglobulin induction. (a) Average percentages of $CD4^+FOXP3^+$ Tregs in $CD4^+$ at various time points after transplant were compared between patients in the control arm and thymoglobulin arm. (b) Percentages of Tregs after transplantation were normalized to patients' own pretransplant levels. Error bars represent standard error of the mean. Asterisks indicate statistically significant differences between the data point and the pretransplant values of the same treatment group (*$P < 0.05$; **$P < 0.01$). Arrow indicates statistically significant differences between control and thymoglobulin groups at a particular time point ($P < 0.05$).

Thymoglobulin induction increases relative Treg frequency and maintains their FOXP3 expression

To assess the effect of thymoglobulin induction on Tregs, we stained PBMC with antibodies to CD4, CD25, CD127, and FOXP3 and determined the percentages of Tregs using flow cytometry. The percentages of $CD4^+FOXP3^+$ Tregs prior to transplant were similar between the two patient groups (Fig. 4a). After thymoglobulin induction, the percentages of Tregs increased above the pretransplant level for at least 26 weeks (Fig. 4a and b). In contrast, a trend of progressive decline in Treg percentages was observed in control patients (Fig. 4a and b).

As FOXP3 expression can be induced in Tconv cells after activation, we reanalyzed some of the samples for the expression of the transcription factor, HELIOS. HELIOS is initially reported as a marker of natural Tregs [23], but later reported to be also expressed in some adaptive Tregs [27] and even recently activated Tconv cells [28]. We found that the combination use of FOXP3 and HELIOS identified a distinct population of cells among $CD4^+$ cells that co-expressed the two transcription factors (Fig. 5a, top left panel). $CD4^+CD25^-CD127^{hi}$ Tconv did not contain this $FOXP3^+HELIOS^+$ population (Fig. 5a, top right panel), consistent with the notion that the $FOXP3^+HELIOS^+$ cells identified Tregs. When we activated PBMCs with CD40L-activated allogeneic B cells, the $FOXP3^+HELIOS^+$ population remained distinct in spite of clear increases in $FOXP3^+HELIOS^{low}$ and $FOXP3^{low}HELIOS^+$ cells (Fig. 5a lower left panel). Furthermore, we isolated $CD4^+CD25^-CD127^{hi}$ Tconv cells using fluorescence activated cell sorting and activated them using a similar protocol. The CD40L-activated allogeneic B cells induced robust proliferation of Tconv cells (data not shown), and upregulation of FOXP3 and HELIOS on separate cells, but did not induce the emergence of $FOXP3^+HELIOS^+$ cells (Fig. 5a lower right panel). These results demonstrate that co-expression of FOXP3 and HELIOS together is more reliable at distinguishing Tregs from recently activated Tconv cells than using FOXP3 alone. We, therefore, reanalyzed samples from four patients from each group using these markers. Consistent with results in Fig. 4b, we found persistence of $CD4^+FOXP3^+HELIOS^+$ Treg cells in thymoglobulin-treated patients and decreased of these cells in control patients (Fig. 5b).

In addition to decreases in the percentages of Tregs, we observed a slow and steady decline of FOXP3 expression levels in the Tregs of control patients, whereas FOXP3 expression in Tregs was preserved in patients who

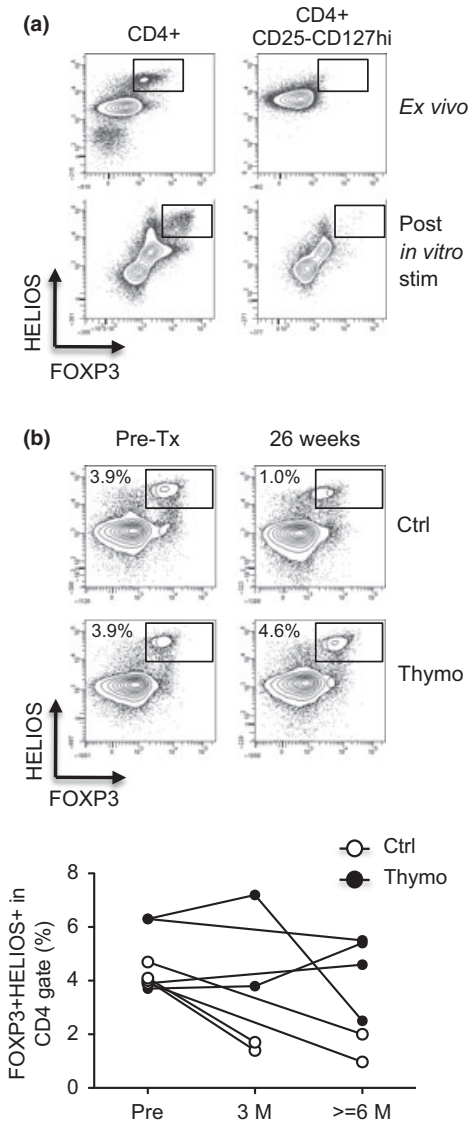

Figure 5 FOXP3 and HELIOS together distinguish Tregs from recently activated Tconv cells. Examples of flow cytometric plots of FOXP3 and HELIOS expression in $CD4^+$ T cells and $CD4^+CD25^-CD127^{hi}$ Tconv cells in freshly isolated PBMCs (a, top panels) and the same $CD4^+$ T cells and Tconv cells 4 days after activation with CD40L-stimulated allogeneic B cells (a, bottom panels). Results are representative of two independent experiments. Examples of flow cytometric plots of FOXP3 and HELIOS expression in $CD4^+$ T cells from study patients before and after transplant (b, top) and a summary graph of all analyzed samples are shown (b, bottom, $n = 4$ in each group).

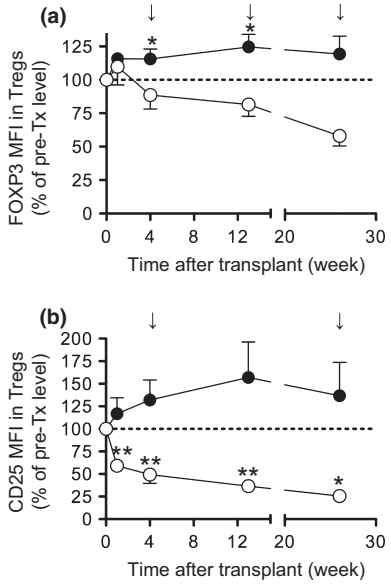

Figure 6 Thymoglobulin induction preserved FOXP3 and CD25 expression on Tregs. Normalized mean fluorescence intensities of FOXP3 are compared between control and thymoglobulin-treated patients (a). Mean fluorescence intensities of CD25 on Tregs are normalized against patient's own pretransplant levels and normalized values between control and thymoglobulin-treated patients were compared (b). Error bars represent standard error of the mean. Asterisks indicate statistically significant differences between the data point and the pretransplant values of the same treatment group ($*P < 0.05$; $**P < 0.01$). Arrow indicates statistically significant differences between control and thymoglobulin groups at a particular time point ($P < 0.05$).

received thymoglobulin induction (Fig. 5b and 6a). Expression of CD25, a component of high affinity IL-2 receptor complex critical for Treg survival [29,30], was significantly reduced in control patients as early as 1 week after transplant (Fig. 6b). In contrast, Tregs in thymoglobulin-treated patients showed a trend of slight increase in CD25 expression. The drop in the CD25 expression preceded that of FOXP3, which was followed by an overall decline of Treg percentages. These sequential changes are consistent with the interpretation that loss of CD25 expression is the primary trigger of Treg decline in control patients. Together, our results demonstrate that thymoglobulin induction selectively preserved the Tregs in transplant patients.

Discussion

In this study, we examined the effects thymoglobulin induction on leukocyte populations after kidney transplantation. We observed a significant deletion of T cells and NK cells and no effect on B cells and monocytes after thymoglobulin treatment. Among the T cells that recovered after depletion, effector $CD4^+$ T cells remained low through the entire study period of 18 months, whereas effector $CD8^+$ T cells increased 6 months after transplant and thymoglobulin induction. We observed a significant and prolonged increase in the percentages of Tregs, associated with a preservation of CD25 and FOXP3 expression. Taken together, our results suggest that

thymoglobulin induction alters balance between effector cells and Tregs. The patient numbers in both arms of this study are low, and future studies with more patients are needed to verify the conclusions.

Effects of thymoglobulin therapy on T-cell subset dynamics in kidney transplant recipients have been reported previously [7,9,10,12,13,21]. Results from our study are consistent with these previous reports in demonstrating that thymoglobulin induction leads to long-term changes in the T-cell compartment. Our study also makes several new observations. First, NK cells sharply decreased after thymoglobulin induction and did not fully recover in the 18-month study period. Second, we found no evidence of effector T cells increase during T-cell reconstitution after thymoglobulin induction. Third, we have improved the method for flow cytometric analysis of human Tregs using HELIOS as an additional marker. This protocol allowed more definitive identification of Tregs than using FOXP3 alone or in combination with CD25. Our results showed that some Tconv cells upregulated FOXP3 after activation, but they did not co-upregulate HELIOS. In addition, we found that de novo induction of HELIOS expression in Tconv cells were restricted to the $FOXP3^{low}$ cells, thus, co-expression of FOXP3 and HELIOS unequivocally identified Tregs even after T-cell activation. Lastly, we report that the relative preservation of Tregs in thymoglobulin-treated patients was associated with persistent high expression of CD25 on Tregs, which was in sharp contrast to the sequential loss of CD25 and FOXP3 in Tregs of the control patients.

One concern with severe lymphodepletion is the non-specific generation of memory and effector T cells as a result of homeostatic proliferation during reconstitution of the lymphoid compartment [17,18]. Patients who received thymoglobulin had significantly reduced percentage of effector $CD4^+$ T cells when compared with control patients. This effect is a combined result of an increase in control patients and a reduction in thymoglobulin-treated patients. The lack of increase in memory and effector $CD4^+$ T cells after thymoglobulin-induced depletion is likely due to the incomplete reconstitution, and therefore the reduced homeostatic proliferation, of the CD4 compartment. The partial CD4 reconstitution is likely a result of inhibition of $CD4^+$ T-cell activation by the maintenance immunosuppression. A previous report noted that different immunosuppressive regimens affected the rate and the magnitude of $CD4^+$ T-cell reconstitution [10]. In contrast to that for $CD4^+$ T cells, reconstitution of $CD8^+$ T cell was more rapid and complete, followed by a delayed rise of effector $CD8^+$ T cells. This delayed change in CD8 compartment was unexpected and our study did not include control patient samples at late time points; therefore, we could not determine whether the increase in percentage of effector $CD8^+$ T cells was specific to thymoglobulin-treated patients. As most of the CD8 recovery occurred in the first 3 months after thymoglobulin induction, and the rise of effector cells was seen after 6 months, it is likely that the appearance of effector CD8 T cells is a result of the immunological experience after the transplant, not a direct consequence of homeostatic proliferation.

Our analysis of Tregs confirmed previous reports that Tregs are more resistant to depletion by thymoglobulin leading to their increased percentages after thymoglobulin treatment. Previous studies reported the decline of Tregs in patients receiving calcineurin inhibitors [30–33]. The loss of Treg may be secondary to the inhibition of TCR signaling and/or the reduction in steady-state IL-2 production resulted from maintenance immunosuppression. It is noteworthy that patients in the thymoglobulin arm have similar dose of the standard three-drug maintenance immunosuppression, yet the progressive decline of Tregs was only observed in control patients. The reason for the sustained Treg homeostasis in thymoglobulin-treated patients is not clear. It is possible that the incomplete reconstitution of T cells and NK-cell compartments may indirectly contribute to the persistence of Tregs by reducing competition for common gamma chain cytokines such as IL-7 and IL-15 that can partially substitute IL-2 in maintaining Tregs [34–36].

In conclusion, our study demonstrates that thymoglobulin induction together with standard immunosuppression induces prolonged reduction in effector $CD4^+$ T cells and persistent elevation of Tregs. This alteration of immune profile to favor immune suppression may underlie the efficacy of thymoglobulin induction in controlling transplant rejection, but raises concerns of increased infection and neoplasm with this therapy [37]. More studies are needed to determine if lower dose thymoglobulin induction, reported to be equally effective in controlling rejection without increasing risks of infection [8], would induce similar immunological changes.

Authorship

FV: designed the clinical trial. SMK and VMP: contributed patient samples. QT: designed and supervised the experiments. JL, KM, KL, EC, and WL: collected patient samples and performed the experiments. JL, QT, SMK, JAB, and FV analyzed data. QT and FV: wrote the manuscript.

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References

1. Hardinger KL, Schnitzler MA, Miller B, *et al.* Five-year follow up of thymoglobulin versus ATGAM induction in adult renal transplantation. *Transplantation* 2004; **78**: 136.
2. Malaise J, Kuypers DR, Claes K, *et al.* Immunosuppressive drugs after simultaneous pancreas-kidney transplantation. *Transplant Proc* 2005; **37**: 2840.
3. Kandaswamy R, Melancon JK, Dunn T, *et al.* A prospective randomized trial of steroid-free maintenance regimens in kidney transplant recipients – an interim analysis. *Am J Transplant* 2005; **5**: 1529.
4. Woodle ES, Peddi VR, Tomlanovich S, Mulgaonkar S, Kuo PC. A prospective, randomized, multicenter study evaluating early corticosteroid withdrawal with thymoglobulin in living-donor kidney transplantation. *Clin Transplant* 2009; **24**: 73.
5. Noel C, Abramowicz D, Durand D, *et al.* Daclizumab versus antithymocyte globulin in high-immunological-risk renal transplant recipients. *J Am Soc Nephrol* 2009; **20**: 1385.
6. Carter JT, Melcher ML, Carlson LL, Roland ME, Stock PG. Thymoglobulin-associated Cd4+ T-cell depletion and infection risk in HIV-infected renal transplant recipients. *Am J Transplant* 2006; **6**: 753.
7. Muller TF, Grebe SO, Neumann MC, *et al.* Persistent long-term changes in lymphocyte subsets induced by polyclonal antibodies. *Transplantation* 1997; **64**: 1432.
8. Wong W, Agrawal N, Pascual M, *et al.* Comparison of two dosages of thymoglobulin used as a short-course for induction in kidney transplantation. *Transpl Int* 2006; **19**: 629.
9. Ciancio G, Burke GW, Gaynor JJ, *et al.* A randomized trial of three renal transplant induction antibodies: early comparison of tacrolimus, mycophenolate mofetil, and steroid dosing, and newer immune-monitoring. *Transplantation* 2005; **80**: 457.
10. Morelon E, Lefrancois N, Besson C, *et al.* Preferential increase in memory and regulatory subsets during T-lymphocyte immune reconstitution after thymoglobulin induction therapy with maintenance sirolimus vs cyclosporine. *Transpl Immunol* 2010; **23**: 53.
11. Zand MS, Vo T, Huggins J, *et al.* Polyclonal rabbit antithymocyte globulin triggers B-cell and plasma cell apoptosis by multiple pathways. *Transplantation* 2005; **79**: 1507.
12. Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N. A novel mechanism of action for anti-thymocyte globulin: induction of CD4+CD25+FOXP3+ regulatory T cells. *J Am Soc Nephrol* 2006; **17**: 2844.
13. Opelz G, Naujokat C, Daniel V, *et al.* Disassociation between risk of graft loss and risk of non-Hodgkin lymphoma with induction agents in renal transplant recipients. *Transplantation* 2006; **81**: 1227.
14. Kirk AD, Cherikh WS, Ring M, *et al.* Dissociation of depletional induction and posttransplant lymphoproliferative disease in kidney recipients treated with alemtuzumab. *Am J Transplant* 2007; **7**: 2619.
15. Gurkan S, Luan Y, Dhillon N, *et al.* Immune reconstitution following rabbit antithymocyte globulin. *Am J Transplant* 2010; **10**: 2132.
16. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 2004; **22**: 745.
17. Surh CD, Sprent J. Homeostasis of naive and memory T cells. *Immunity* 2008; **29**: 848.
18. Wu Z, Bensinger SJ, Zhang J, *et al.* Homeostatic proliferation is a barrier to transplantation tolerance. *Nat Med* 2004; **10**: 87.
19. Moxham VF, Karegli J, Phillips RE, *et al.* Homeostatic proliferation of lymphocytes results in augmented memory-like function and accelerated allograft rejection. *J Immunol* 2008; **180**: 3910.
20. Sener A, Tang AL, Farber DL. Memory T-cell predominance following T-cell depletional therapy derives from homeostatic expansion of naive T cells. *Am J Transplant* 2009; **9**: 2615.
21. Sewgobind VD, Kho MM, van der Laan LJ, *et al.* The effect of rabbit anti-thymocyte globulin induction therapy on regulatory T cells in kidney transplant patients. *Nephrol Dial Transplant* 2009; **24**: 1635.
22. Bluestone JA, Liu W, Yabu JM, *et al.* The effect of costimulatory and interleukin 2 receptor blockade on regulatory T cells in renal transplantation. *Am J Transplant* 2008; **8**: 2086.
23. Thornton AM, Korty PE, Tran DQ, *et al.* Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced FOXP3+ T regulatory cells. *J Immunol* 2010; **184**: 3433.
24. Zand MS, Bose A, Vo T, *et al.* A renewable source of donor cells for repetitive monitoring of T- and B-cell alloreactivity. *Am J Transplant* 2005; **5**: 76.
25. Okada R, Kondo T, Matsuki F, Takata H, Takiguchi M. Phenotypic classification of human CD4+ T cell subsets and their differentiation. *Int Immunol* 2008; **20**: 1189.
26. Takata H, Takiguchi M. Three memory subsets of human CD8+ T cells differently expressing three cytolytic effector molecules. *J Immunol* 2006; **177**: 4330.
27. Haribhai D, Williams JB, Jia S, *et al.* A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity. *Immunity* 2011; **35**: 109.
28. Akimova T, Beier UH, Wang L, Levine MH, Hancock WW. Helios expression is a marker of T cell activation and proliferation. *PLoS One* 2011; **6**: e24226.
29. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in FOXP3-expressing regulatory T cells. *Nat Immunol* 2005; **6**: 1142.
30. Demirkiran A, Sewgobind VD, van der Weijde J, *et al.* Conversion from calcineurin inhibitor to mycophenolate mofetil-based immunosuppression changes the frequency and phenotype of CD4+FOXP3+ regulatory T cells. *Transplantation* 2009; **87**: 1062.

31. Presser D, Sester U, Mohrbach J, Janssen M, Kohler H, Sester M. Differential kinetics of effector and regulatory T cells in patients on calcineurin inhibitor-based drug regimens. *Kidney Int* 2009; **76**: 557.
32. Kim SH, Oh EJ, Ghee JY, *et al.* Clinical significance of monitoring circulating CD4+CD25+ regulatory T cells in kidney transplantation during the early posttransplant period. *J Korean Med Sci* 2009; **24**(Suppl.): S135.
33. San Segundo D, Fernandez-Fresnedo G, Gago M, *et al.* Number of peripheral blood regulatory T cells and lymphocyte activation at 3 months after conversion to mTOR inhibitor therapy. *Transplant Proc* 2010; **42**: 2871.
34. Vang KB, Yang J, Mahmud SA, Burchill MA, Vegoe AL, Farrar MA. IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern CD4+FOXP3+ regulatory T cell development. *J Immunol* 2008; **181**: 3285.
35. Bayer AL, Lee JY, de la Barrera A, Surh CD, Malek TR. A function for IL-7R for CD4+CD25+FOXP3+ T regulatory cells. *J Immunol* 2008; **181**: 225.
36. Yates J, Rovis F, Mitchell P, *et al.* The maintenance of human CD4+ CD25+ regulatory T cell function: IL-2, IL-4, IL-7 and IL-15 preserve optimal suppressive potency in vitro. *Int Immunol* 2007; **19**: 785.
37. Liu Y, Zhou P, Han M, Xue CB, Hu XP, Li C. Basiliximab or antithymocyte globulin for induction therapy in kidney transplantation: a meta-analysis. *Transplant Proc* 2010; **42**: 1667.