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Prof. Ferdinand Muehlbacher
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RE: TRI-OA-12-0140

Dear Professor Ferdinand Muehlbacher:

Thank you and the reviewers for the very fair and constructive review of our manuscript. We have considered all the reviewers' comments carefully and revised our manuscript accordingly. We believe that the revision has greatly improved the clarity of the manuscript and we hope that you and reviewers agree and consider it acceptable for publication in Transplant International.

Sincerely,

Qizhi Tang, Ph.D.

Altered balance between effector T cells and ~~Sustained decrease in effector cytokine production and increase in~~ F~~ox~~OXpP3^{+}HELIOS^{+} regulatory T cells after Thymoglobulin induction in kidney transplant recipients

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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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Running title: Thymoglobulin induction in kidney transplantation

Abstract:

This study examined the effect of Thymoglobulin induction therapy on leukocyte population dynamics in kidney transplant patients. Patients receiving standard immunosuppression were compared to 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 pre-transplant level by 4 weeks post transplant. $CD4^+$ T cells remained at less than 30% of pre-transplant 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~~FOXP3$^+$HELIOS$^+$ regulatory T cells (Tregs) was less profound than that of $CD4^+$ ~~Foxp3~~FOXP3$^-$ cells, thus the relative percentage of Tregs elevated significantly when compared to pre-transplant levels in Thymoglobulin-treated patients. In contrast, the percentages of Tregs and their expression of ~~Foxp3~~ FOXP3 in the standard immunosuppression group decreased steadily and by 12 weeks after transplant the average percentage of Tregs was 56% of the pre-transplant 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 of $CD4^+$, and potentially pathogenic, T cells.

Keywords:
Effector T cells,
~~Foxp3~~FOXP3,
HELIOS,
Regulatory T cells,
Renal transplant,
Thymoglobulin

Abbreviations:

Tregs	Regulatory T cells
Tconv	conventional T cells
PBMC	Peripheral blood mononuclear cells
PMA	phorbol myristate ester
DGF	delayed graft function

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 re-transplants and recipients with high panel reactive antibodies [1, 2]. Short-term prophylaxic use of Thymoglobulin at the time of transplant allows delayed introduction and reduction of maintenance dose of cyclosporine and the associated nephrotoxicity [3, 2]. Thymoglobulin induction was also reported to allow early steroid withdraw in and steroid-free maintenance immunosuppression in kidney transplant patients [3, 4, 5]. Among high immunological risk patients, Thymoglobulin is superior to daclizumab in preventing acute rejections [6, 5]. In addition to being an efficacious induction agent, Thymoglobulin can reverse acute renal graft rejection and is routinely used for controlling acute rejection [7, 6]. The efficacy of Thymoglobulin in controlling graft rejection is thought to be due to its ability to delete various immune cells, especially T cells [7, 6, 13, 12]. Emerging data suggest that Thymoglobulin induction may preferentially kill conventional T (Tconv) cells resulting in increased proportion of regulatory T cells (Tregs) [11, 10, 13, 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 [14, 15]. As T cells expand in the lymphopenic environment, the cells may acquire an activated phenotype and manifest effector functions such as secreting IFNγ [15, 16, 19, 20]. Indeed, it has been reported that T cells recovered after

Thymoglobulin treatments have increased proportion of memory cells [~~11~~10]. Yet, a recent study showed that patients' peripheral blood mononuclear cells were broadly hyporesponsive to donor and third party antigen presenting cells [~~20~~21].

In this study, we analyzed the population dynamics of various leukocyte subsets, especially 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 to the pre-transplant levels. Lastly, Treg percentages and their expression of ~~Foxp3~~ 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 ~~eliminate~~ change the balance between effector T cells ~~while preserving~~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 ~~delayed graft function~~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 ~~carried with patients~~ 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 (PBMC) collection and storage:

Heparinized blood was collected ~~from patients~~ 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 ~~Foxp~~FOXP3 as previously described [~~21~~22]. Some samples were analyzed using a modified Treg panel with an additional antibody to HELIOS, a marker co-expressed with ~~Foxp~~FOXP3 in Tregs [~~22~~23]. The "CD4 effector/memory panel" consisted of fluorescent antibodies to CD3, CD4, CD25, CD27, CD28, CD45RA, and ~~Foxp~~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), except pacific blue conjugated anti-CD45 (clone H30) and eFluor450 conjugated anti-~~Foxp~~FOXP3 (clone 236/E7) were purchased from eBiosciences (San Diego, CA) and phycoerythrin conjugated anti-HELIOS and PerCP-Cy5/5 conjugated Perforin (clone dG9) were purchased from Biolegend (San Diego, CA). Stained PBMC were analyzed on FACSCalibur (BD) ~~for the Treg panel and~~or LSRII (BD) ~~for the other panels~~. FlowJo ~~software~~ (Tree Star, Inc., Ashland, OR) 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 [~~23~~24]. In brief, B cells were isolated using a no-touch B cell isolation kit (Invitrogen) and stimulated with irradiated 3T3 cells stably expressing human CD40L with additional IL-4. The B cells were initially allowed to expanded for 7 days and then were restimulated with 3T3-CD40L every 3 to 4 days and used between 10 to 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 2 B cells per PBMC or Tconv cell ratio. Expression of HELIOS and ~~Foxp~~FOXP3 in stimulated T cells was analyzed on day 4 post allogeneic B cell stimulation using flow cytometry as described above.

Intracellular IFNγ 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 hrs 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 ~~. The fixed and permeabilized cells were~~ labeled~~ed~~ling with fluorochrome-conjugated antibody to IFNγ (BD Biosciences) ~~analyzed on a BD LSRII flow cytometer~~.

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. ~~Because of variation in baseline cell numbers, we normalized all post-transplant cell counts to patients' own pre-transplant values to determine change of cell counts over time. Similarly, percentages of T cell subsets (naïve, memory, effector, Treg) were also normalized to patients' pre-transplant percentages before assessing their changes over time.~~ Statistical analyses of the data were performed with the aid of Prism Graphpad software (La Jolla, CA).

Results:

Patient information: demographics, medication, and clinical outcome

A total of 7 patients in the Thymoglobulin arm and 4 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), mycophenolate morfetil (Cellcept), 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 post-operative 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-hr 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 of tacrolimus and MMF without other interventions. In the control arm, no BKV or EBV reactivation was detected; one patient developed CMV viremia four months post-transplant, which was cleared one month later.

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 (Figure 1A). We calculated the total numbers of circulating CD4, CD8 T cells, NK cells, B cells, and monocytes per milliliter of blood (Figure 1B-F). Consistent with previous reports, Thymoglobulin induction led to a rapid loss of $CD4^+$ and $CD8^+$ T cells in the peripheral blood ~~(Figure 1B and C)~~. The numbers of $CD4^+$ and $CD8^+$ T cells were lowest at 1-week post transplant, with a clear trend of recovery at 4 weeks 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 pre-transplant levels by 3 months (13 weeks) after transplantation (Figure 1B). In comparison, the recovery of $CD4^+$ T cells was incomplete; and remained, on average, 50% below the pre-transplant level at one and a half year (78 weeks) after transplant ~~(Figure 1C)~~. We also observed a significant reduction in NK cells immediately after

Thymoglobulin induction, with slow and incomplete recovery (Figure 1D). No change in B cells and monocytes was observed after Thymoglobulin induction when compared to patients' own pre-transplant baseline levels (Figure 1E and F).

Sustained decrease of 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 [~~24~~25](Figure 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 to patients in the Thymoglobulin arm (Figure 2B). This difference is not due to Thymoglobulin treatment because the pre-transplant 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 (Figure 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 (Figure 2E). The CD45RA$^-$CD27$^-$ effector population contained two major cell subsets with cell surface phenotype of CD28$^-$ and CD28$^+$ (Figure 2A, right panel). The CD28$^-$ subset was shown previously to have a IFNγ expression Th1 bias, whereas the CD28$^+$ subset had a Th2 bias [~~24~~25]. We observed a sharp drop of proportion of the CD45RA$^-$CD27$^-$

CD28$^-$ subset in Thymoglobulin-treated patients in the first month post-transplant period (Figure 2F). The percentage of these cells recovered partially at 3 months after transplant and remained at a level lower than pre-transplant 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 pre-transplant levels in control patients. The CD45RA$^-$CD27$^-$CD28$^+$ population did not change significantly after transplant and Thymoglobulin induction (Figure 2G). In contrast, their percentages significantly and progressively increased in control patients who did not receive Thymoglobulin induction (Figure 2G). To directly determine the effect of Thymoglobulin induction on effector cytokine production, we activated the PBMCs using PMA and ionomycin for 4 hours and analyzed IFNγ expression by CD4$^+$ T cells. Consistent with our observation of decrease in Th1-biased CD45RA$^-$CD27$^-$CD28$^-$ effector cells, IFNγ production by CD4$^+$ T cells in all patients who received Thymoglobulin remained at or below pre-transplant levels. Our results demonstrate that the CD4$^+$ T cell recover incompletely following Thymoglobulin treatment. The limited recovery of CD4$^+$ T cells may explain the lack of increase of effector or memory CD4$^+$ T cells secondary to homeostatic proliferation. When compared to 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](Figure 3A).

Among these subpopulations, naïve and memory cells express the lowest level of perforin, which is followed by RA^- effectors, and RA^+ effectors expressed significantly higher amount of perforin than any other subpopulations (Figure 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 ~~significantly~~ markedly higher level in patients in control arm than in patients in the Thymoglobulin arm (Figure 3C). During the first 12 weeks after transplantation, no dramatic change in the percentages of $CD8^+$ T cell subsets was observed (Figure 3D to 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 (Figure 3F and G). This was associated with a drop of the naïve subset (Figure 3D). The rise of effector $CD8^+$ T cells in the Thymoglobulin patient was not statistically significant due to large variations that ranged between 50% and 455% of pre-transplant 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 (Figure 3H). Overall, we found rapid recovery $CD8^+$ T cells with delayed rise of effector $CD8^+$ cells after Thymoglobulin treatment.

Thymoglobulin induction increases relative Treg frequency and maintains their ~~Foxp~~FOXP3 expression

To assess the effect of Thymoglobulin induction on Tregs, with stained PBMC with antibodies to CD4, CD25, CD127, and ~~Foxp~~FOXP3 and determined the percentages of Tregs using flow cytometry. The percentages of $CD4^+$~~Foxp~~FOXP3^+ Tregs prior to transplant were similar between the two patient groups (Figure 4A). After Thymoglobulin induction, the percentages of Tregs increased above the pre-transplant level for at least 26 weeks (Figure 4A and B). In

contrast, a trend of progressive decline in Treg percentages was observed in control patients (Figure 4A and B).

Since ~~Foxp~~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 [~~22~~23], but later reported to be also expressed in some adaptive Tregs [~~26~~27] and even recently activated Tconv cells [~~27~~28]. We found that the combination use of ~~Foxp~~FOXP3 and HELIOS identified a distinct population of cells among $CD4^+$ cells that co-expressed the two transcription factors (Figure 5A, top left panel). $CD4^+CD25^-CD127^{hi}$ Tconv did not contain this ~~Foxp~~FOXP3$^+$HELIOS$^+$ population (Figure 5A, top right panel), consistent with the notion that the ~~Foxp~~FOXP3$^+$HELIOS$^+$ cells identified Tregs. When we activated PBMCs with CD40L-activated allogeneic B cells, the ~~Foxp~~FOXP3$^+$HELIOS$^+$ population remained distinct in spite clear increases of ~~Foxp~~FOXP3$^+$HELIOS^{low} and ~~Foxp~~FOXP3^{low}HELIOS$^+$ cells (Figure 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 inducted robust proliferation of Tconv cells (data not shown), induced upregulation of ~~Foxp~~FOXP3 and HELIOS on separate cells but did not induce the emergence of ~~Foxp~~FOXP3$^+$HELIOS$^+$ cells (Figure 5A lower right panel). These results demonstrate that co-expression of ~~Foxp~~FOXP3 and HELIOS together is more reliable at distinguishing Tregs from recently activated Tconv cells than using ~~Foxp~~FOXP3 alone. We, therefore, re-analyzed samples from four patients from each group using these markers. Consistent with results in Figure 4B, we found persistence of $CD4^+$~~Foxp~~FOXP3$^+$HELIOS$^+$ Treg cells in Thymoglobulin-treated patients and decreased of these cells in control patients (Figure 5B).

In addition to decreases in the percentages of Tregs, we observed a slow and steady decline of ~~Foxp3~~ FOXP3 expression levels in the Tregs of control patients, whereas FOXP~~oxp~~3 expression in Tregs was preserved in patients who received Thymoglobulin induction (Figure 5B and 6A). Expression of CD25, a component of high affinity IL-2 receptor complex critical for Treg survival [~~28~~29, ~~29~~30], was significantly reduced in control patients as early as one week after transplant (Figure 6B). In contrast, Tregs in Thymoglobulin-treated patients showed a trend of slight increase in CD25 expression. The drop in the percentage of $CD25^+$ T cells preceded that of FOXP~~oxp~~3, 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 ~~with standard immunosuppression~~. 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 ~~Foxp~~FOXP3 expression ~~in Tregs~~. Taken together, our results ~~show~~ suggest that Thymoglobulin induction alters ~~the $CD4^+$ T cell compartment by suppressing~~balance between effector cells and ~~preserving~~ 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 [~~8~~7, ~~10~~9, ~~11~~10, ~~13~~12, ~~14~~13, ~~20~~21]. Results from our study is consistent with these previous reports in demonstrating that Thymoglobulin induction leads to long-terms changes in the T cell compartment~~, particularly in the incomplete recovery of CD4$^+$ T cells and the persistence of Tregs~~. Our study also makes several new observations ~~and advances~~. First, ~~we found that~~ NK cells sharply decreased after Thymoglobulin induction and did not fully recover in the 18-month study period. Second, we ~~monitored frequencies of effector CD4$^+$ and CD8$^+$ T cells in Thymoglobulin-treated patients using cell surface phenotypes and intracellular cytokine expression analysis and~~ found no evidence of effector T cell increase during T cell reconstitution after Thymoglobulin induction. Third, we have improved the method for flow cytometric analysis of human Tregs by using HELIOS as an additional marker. This ~~improved~~ protocol allowed more definitive identification of Tregs than using ~~Foxp~~FOXP3 alone or in combination with CD25~~, because both of these markers can be co-expressed on recently activated T cells~~. Our results showed that some Tconv cells upregulated ~~Foxp~~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 ~~Foxp~~FOXP3^{low} cells, thus co-expression of ~~Foxp~~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 of sequential loss of CD25 and ~~Foxp~~FOXP3 in Tregs of the control patients.

One concern with severe lymphodepletion is the nonspecific generation of memory and effector T cells due to homeostatic proliferation ~~of T cells~~ during reconstitution of the lymphoid compartment [~~16~~17, ~~17~~18]. ~~-~~ Patients received ~~Our analyses showed contrasting effects of~~

~~Thymoglobulin induction on CD4+ versus CD8+ T cells.~~ Thymoglobulin had ~~induction~~ significantly reduced ~~the~~ percentage of effector $CD4^+$ T cells, ~~,~~ when compared to ~~that observed on~~ control patients. This effect is a combined result of an increase in ~~effector CD4+~~ 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, thus less 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 [~~11~~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. Since most of the CD8 recovery occurred in the first three months after Thymoglobulin induction, and the rise of effector cells was seen after 6 months, ~~we do not think~~ 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~~, but likely a result of the immunological experience after the transplant~~.

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 [30-32]. 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 ~~induction~~ arm ~~also received~~similar dose of the standard three-drug maintenance immunosuppression ~~similar to that received by the control patients~~, yet the progressive decline of Tregs was only observed ~~on~~ 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 [~~33~~34-~~35~~36].

In conclusion, our study demonstrates that Thymoglobulin induction together with standard immunosuppression induces prolonged reduction of 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 [~~36~~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 [~~9~~8], would induce similar immunological changes.

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Figure legends:

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 pre-transplant numbers. The average ~~normalized~~ 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 pre-transplant 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 ($p<0.05$).

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 $CD4^+$~~Foxp~~FOXP3$^-$ conventional T cells (Tconv) 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 pre-transplant levels. The normalized levels of naïve (C), memory (D), and effector (E) CD4 T cells after transplant in control and Thymoglobulin-treated patients compared. Average percentages of $CD28^-$ effectors (F) and $CD28^+$ effectors (G) after transplant in control (n=4) and Thymoglobulin patients (n=7) are shown. IFNγ expression by $CD4^+$ T cells after 4 hr 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 pre-transplant values of the same treatment group (*, $p<0.05$; ** $p<0.01$).

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Figure 4. Tregs persist in patients received Thymoglobulin induction. A. Average percentages of CD4$^+$~~Foxp3~~FOXP3$^+$ Tregs in CD4$^+$ ~~gate before transplant (A) and~~ at various time points after transplant ~~(B)~~ were compared between patients in the control arm and Thymoglobulin arm. B. Percentages of Tregs after transplantation were normalized to patients' own pre-transplant levels. Error bars represent standard error of the mean. Asterisks indicate statistically significant differences between the data point and the pre-transplant 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 ($p<0.05$).

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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~~
pre-Tx	9.3±3	7.6±3
~~Creatinine~~ 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

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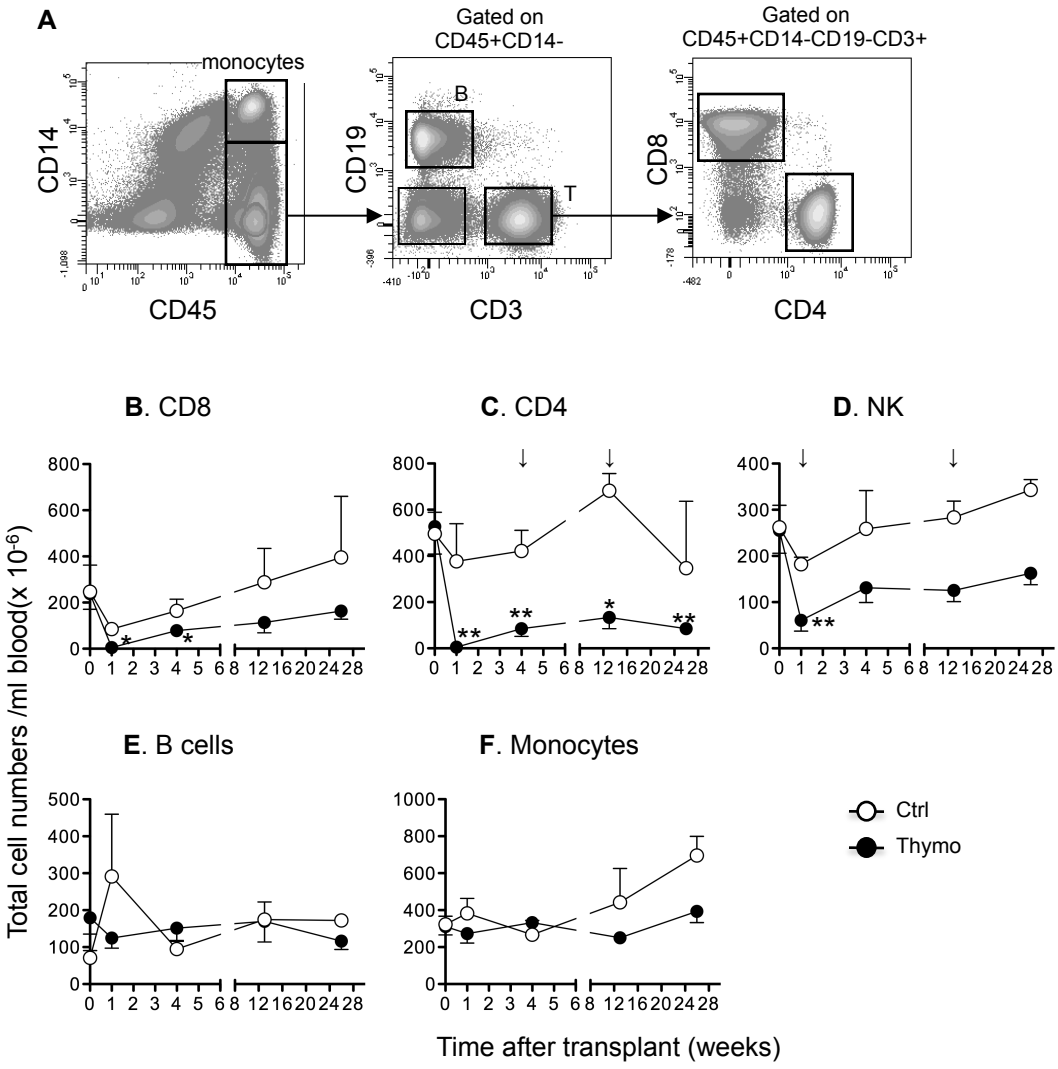

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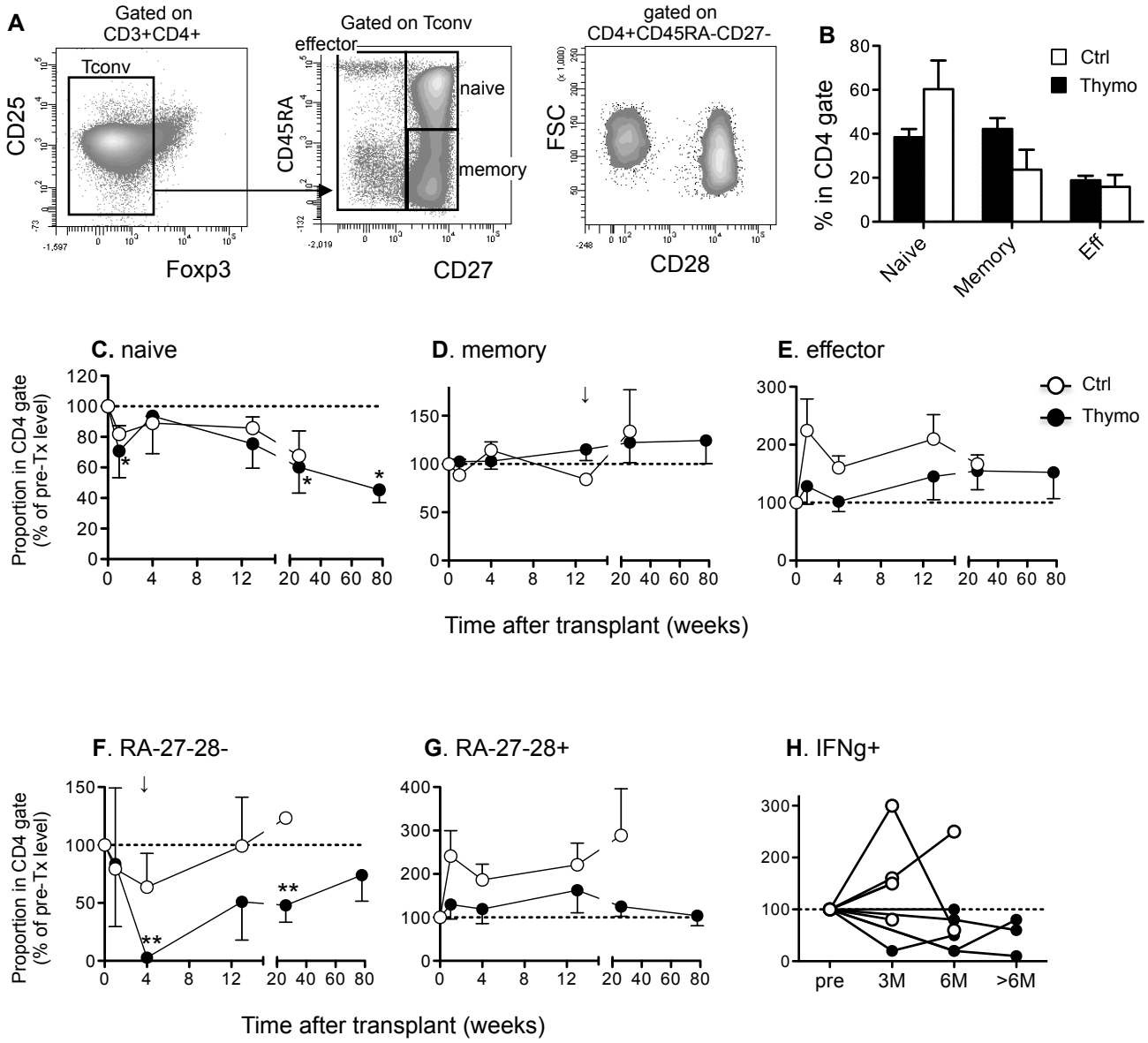

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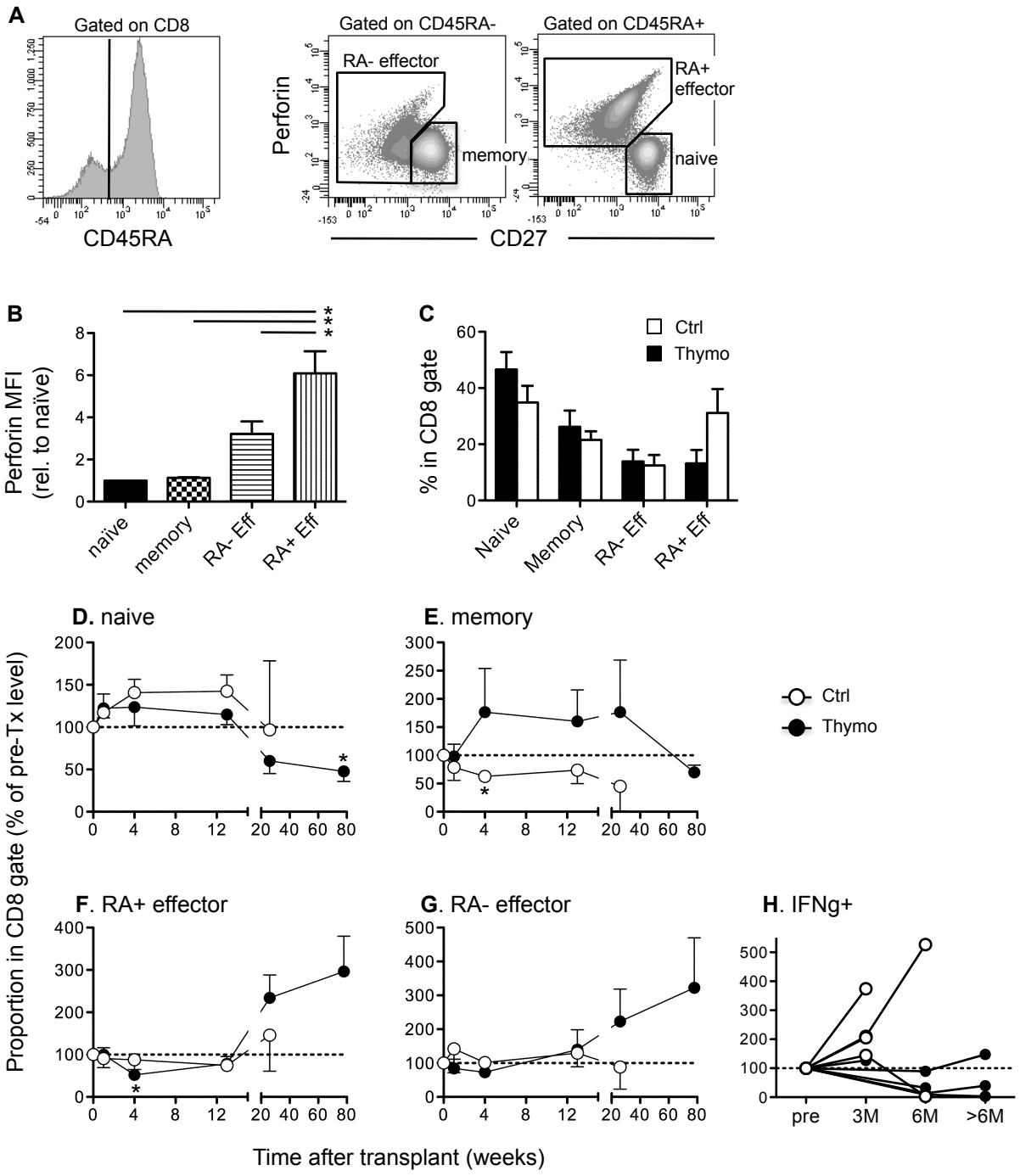

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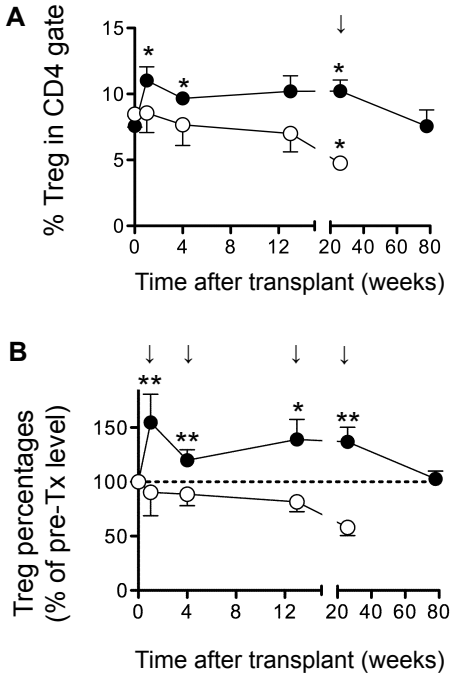

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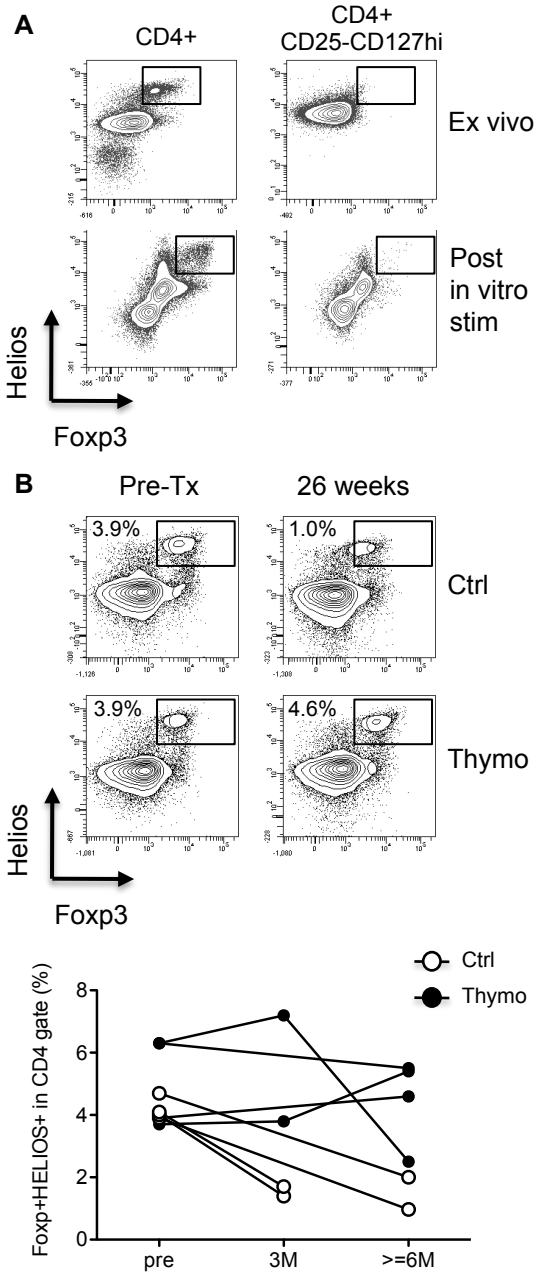

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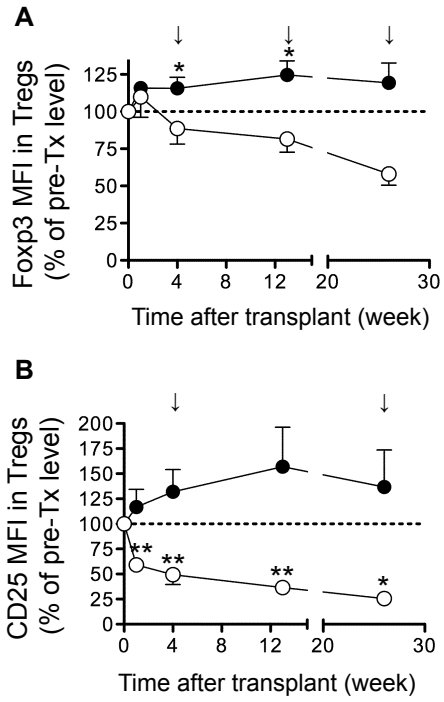

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Altered balance between effector T cells and FOXP3$^+$HELIOS$^+$ regulatory T cells after Thymoglobulin induction in kidney transplant recipients

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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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All authors declare no conflict of interest.

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Running title: Thymoglobulin induction in kidney transplantation

Abstract:

This study examined the effect of Thymoglobulin induction therapy on leukocyte population dynamics in kidney transplant patients. Patients receiving standard immunosuppression were compared to 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 pre-transplant level by 4 weeks post transplant. $CD4^+$ T cells remained at less than 30% of pre-transplant 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 to pre-transplant 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 pre-transplant 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 of $CD4^+$, and potentially pathogenic, T cells.

Keywords:
Effector T cells,
FOXP3,
HELIOS,
Regulatory T cells,
Renal transplant,
Thymoglobulin

Abbreviations:

Tregs	Regulatory T cells
Tconv	conventional T cells
PBMC	Peripheral blood mononuclear cells
PMA	phorbol myristate ester
DGF	delayed graft function

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 re-transplants and recipients with high panel reactive antibodies [1]. Short-term prophylaxic use of Thymoglobulin at the time of transplant allows delayed introduction and reduction of 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 due to 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 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 IFNγ [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 were broadly hyporesponsive to donor and third party antigen presenting cells [21].

In this study, we analyzed the population dynamics of various leukocyte subsets, especially 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 to the pre-transplant 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 (PBMC) 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), except pacific blue conjugated anti-CD45 (clone H30) and eFluor450 conjugated anti-FOXP3 (clone 236/E7) were purchased from eBiosciences (San Diego, CA) and phycoerythrin conjugated anti-HELIOS and PerCP-Cy5/5 conjugated Perforin (clone dG9) were purchased from Biolegend (San Diego, CA). Stained PBMC were analyzed on FACSCalibur (BD) or LSRII (BD). FlowJo (Tree Star, Inc., Ashland, OR) 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) and stimulated with irradiated 3T3 cells stably expressing human CD40L with additional IL-4. The B cells were initially allowed to expanded for 7 days and then were restimulated with 3T3-CD40L every 3 to 4 days and used between 10 to 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 2 B cells per PBMC or Tconv cell ratio. Expression of HELIOS and FOXP3 in stimulated T cells was analyzed on day 4 post allogeneic B cell stimulation using flow cytometry as described above.

Intracellular IFNγ 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 hrs 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 labelling with fluorochrome-conjugated antibody to IFNγ (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).

Results:

Patient information: demographics, medication, and clinical outcome

A total of 7 patients in the Thymoglobulin arm and 4 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), mycophenolate morfetil (Cellcept), 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 post-operative 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-hr 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 of tacrolimus and MMF without other interventions. In the control arm, no BKV

or EBV reactivation was detected; one patient developed CMV viremia four months post-transplant, which was cleared one month later.

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 (Figure 1A). We calculated the total numbers of circulating CD4, CD8 T cells, NK cells, B cells, and monocytes per milliliter of blood (Figure 1B-F). Consistent with previous 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 weeks 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 pre-transplant levels by 3 months (13 weeks) after transplantation (Figure 1B). In comparison, the recovery of $CD4^+$ T cells was incomplete; and remained, on average, 50% below the pre-transplant level at one and a half year (78 weeks) after transplant . We also observed a significant reduction in NK cells immediately after Thymoglobulin induction, with slow and incomplete recovery (Figure 1D). No change in B cells and monocytes was observed after Thymoglobulin induction when compared to patients' own pre-transplant baseline levels (Figure 1E and F).

Sustained decrease of 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](Figure 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 to patients in the Thymoglobulin arm (Figure 2B). This difference is not due to Thymoglobulin treatment because the pre-transplant 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 (Figure 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 (Figure 2E). The $CD45RA^-CD27^-$ effector population contained two major cell subsets with cell surface phenotype of $CD28^-$ and $CD28^+$ (Figure 2A, right panel). The $CD28^-$ subset was shown previously to have a IFNγ 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 patients in the first month post-transplant period (Figure 2F). The percentage of these cells recovered partially at 3 months after transplant and remained at a level lower than pre-transplant 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 pre-transplant levels in control patients. The $CD45RA^-CD27^-CD28^+$ population did not change significantly after transplant and Thymoglobulin induction (Figure 2G). In contrast, their percentages significantly and progressively increased in control patients who did not receive Thymoglobulin induction (Figure 2G). To directly determine the effect of Thymoglobulin

induction on effector cytokine production, we activated the PBMCs using PMA and ionomycin for 4 hours and analyzed IFNγ expression by $CD4^+$ T cells. Consistent with our observation of decrease in Th1-biased $CD45RA^-CD27^-CD28^-$ effector cells, IFNγ production by $CD4^+$ T cells in all patients who received Thymoglobulin remained at or below pre-transplant levels. Our results demonstrate that the $CD4^+$ T cell recover incompletely following Thymoglobulin treatment. The limited recovery of $CD4^+$ T cells may explain the lack of increase of effector or memory $CD4^+$ T cells secondary to homeostatic proliferation. When compared to 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](Figure 3A). Among these subpopulations, naïve and memory cells express the lowest level of perforin, which is followed by RA^- effectors, and RA^+ effectors expressed significantly higher amount of perforin than any other subpopulations (Figure 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 (Figure 3C). During the first 12 weeks after transplantation, no dramatic change in the percentages of $CD8^+$ T cell subsets was observed (Figure 3D to 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 (Figure 3F and G). This was associated with a drop of the naïve subset (Figure 3D). The rise of effector CD8$^+$ T cells in the Thymoglobulin patient was not statistically significant due to large variations that ranged between 50% and 455% of pre-transplant 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 (Figure 3H). Overall, we found rapid recovery CD8$^+$ T cells with delayed rise of effector CD8$^+$ cells after Thymoglobulin treatment.

Thymoglobulin induction increases relative Treg frequency and maintains their FOXP3 expression

To assess the effect of Thymoglobulin induction on Tregs, with 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 (Figure 4A). After Thymoglobulin induction, the percentages of Tregs increased above the pre-transplant level for at least 26 weeks (Figure 4A and B). In contrast, a trend of progressive decline in Treg percentages was observed in control patients (Figure 4A and B). Since 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 (Figure 5A, top left panel). CD4$^+$CD25$^-$CD127^{hi} Tconv did not contain this FOXP3$^+$HELIOS$^+$ population (Figure 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 clear increases of FOXP3^{+}HELIOS^{low} and FOXP3^{low}HELIOS^{+} cells (Figure 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 inducted robust proliferation of Tconv cells (data not shown), induced upregulation of FOXP3 and HELIOS on separate cells but did not induce the emergence of FOXP3^{+}HELIOS^{+} cells (Figure 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, re-analyzed samples from four patients from each group using these markers. Consistent with results in Figure 4B, we found persistence of CD4^{+}FOXP3^{+}HELIOS^{+} Treg cells in Thymoglobulin-treated patients and decreased of these cells in control patients (Figure 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 received Thymoglobulin induction (Figure 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 one week after transplant (Figure 6B). In contrast, Tregs in Thymoglobulin-treated patients showed a trend of slight increase in CD25 expression. The drop in the percentage of CD25^{+} T cells 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 is consistent with these previous reports in demonstrating that Thymoglobulin induction leads to long-terms 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 cell increase during T cell reconstitution after Thymoglobulin induction. Third, we have improved the method for flow cytometric analysis of human Tregs by 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 of sequential loss of CD25 and FOXP3 in Tregs of the control patients.

One concern with severe lymphodepletion is the nonspecific generation of memory and effector T cells due to homeostatic proliferation during reconstitution of the lymphoid compartment [17, 18]. Patients received Thymoglobulin had significantly reduced percentage of effector CD4$^+$ T cells when compared to control patients. This effect is a combined result of an increase in 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, thus less 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. Since most of the CD8 recovery occurred in the first three 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 [30-32]. 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 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 of 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.

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Figure legends:

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 pre-transplant 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 pre-transplant 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 ($p<0.05$).

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 $CD4^+FOXP3^-$ conventional T cells (Tconv) 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 pre-transplant levels. The normalized levels of naïve (C), memory (D), and effector (E) CD4 T cells after transplant in control and Thymoglobulin-treated patients compared. Average percentages of $CD28^-$ effectors (F) and $CD28^+$ effectors (G) after transplant in control (n=4) and Thymoglobulin patients (n=7) are shown. IFNγ expression by $CD4^+$ T cells after 4 hr 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 pre-transplant 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 ($p<0.05$).

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 pre-transplant 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 pre-transplant values of the same treatment group (*, $p<0.05$). IFNγ expression by CD8$^+$ T cells after 4 hr ex vivo PMA and ionomycin treatment is shown in H (n=4 in each group).

Figure 4. Tregs persist in patients 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 pre-transplant levels. Error bars represent standard error of the mean. Asterisks indicate statistically significant differences between the data point and the pre-transplant 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 ($p<0.05$).

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 in are compared between control and Thymoglobulin-treated patients (A). Mean fluorescence intensities of CD25 on Tregs are normalized against patient's own pre-transplant 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 pre-transplant 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 ($p<0.05$).

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