Reconstitution of T-Cell Subsets Following Thymoglobulin-Induced Depletion in High Immunologic Risk and Donation After Cardiac Death Renal Transplant Recipients

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ABSTRACT

Introduction. Depletion therapy in high immunologic risk (HR) patients by antithymocyte globulin (rATG) induces lymphopenia and subsequent compartmental repopulation of T-cell subsets. rATG is also given to patients receiving kidneys from donations after cardiac death (DCDs) to mitigate innate immune activation associated with the DCD process.

Methods. We compared the T-cell response with rATG in both HR and DCD kidney recipients. We examined the reconstitution of T-cell subsets after rATG treatment in HR and DCD recipients ($n = 19$ per group) by multicolor flow cytometry.

Results. Following treatment, there was a rapid drop in the frequency of T cells in both groups, which persisted over 28 days. HR patients had an early surge in the frequency of $CD4^+$ naïve, effector-memory, and regulatory T cells. Although we found a significant proliferation of the T cells in both groups, the DCD cohort had a blunted response as well as reduced $CD4^+$ T-cell immune-reactivity compare with the HR group.

Conclusions. Our data suggest that there is a lack of significant homeostatic proliferative response in DCD recipients following rATG, and $CD4^+$ T cells may be less reactive in the DCD group than previously thought, indicating that rATG treatment may not have to be considered a first-line induction therapy in DCD recipients.

THE RISE in the incidence of end-stage renal disease (ESRD) is a worldwide concern. Compared with dialysis, renal transplantation has the best optimal longevity and enhances the quality of life of ESRD patients [1]. Unfortunately, there is a widening disparity between the availability of transplantable organs and the number of patients on the waiting list [2]. To compensate, the transplant community has made strides to expand the availability of donor organs through the use of expanded criteria for donor and donation after cardiac death (DCD) kidneys, which considerably increased the number of deceased donors [3,4]. Maximizing the use of DCD kidneys, however, causes a higher risk of delayed graft function (DGF) and graft loss compared with standard criteria donor organs [4,5]. In addition, DCD organs experience prolonged warm ischemia that elicits a greater innate immune response to transplantation, which portrays a higher risk of acute cellular rejection [6]. Consequently, recipients of DCD kidneys receive depletion induction therapy to mitigate the potential immune response induced by ischemia-reperfusion injury.

Because of the increased use of marginal organs and the increasing incidence of early graft loss, transplant

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programs are seeing a greater proportion of retransplanted or highly sensitized patients with a higher immunologic risk (HR) at the time of their next transplant. The HR individuals have higher rates of acute antibody and T-cell mediated rejection, earlier graft loss, and chronic allograft nephropathy [7]. In this study, we compared the effect of induction depletion immunotherapy in HR and DCD recipients, which are 2 very different groups of recipients.

One of the mainstays of induction immunotherapy is using agents that target circulating T cells in the blood, such as antithymocyte globulin (Thymoglobulin or ATG) and basiliximab (Simulect) [8,9]. In most transplant centers, all HR (panel reactive antibody >40%) and DCD renal transplant recipients [10,11] routinely receive Thymoglobulin (rATG) as the preferred agent for T-cell depletion [8,12,13]. However, some of these patients still undergo severe acute rejection that can be resistant to rescue therapy [14]. Additionally, evidence from animal studies suggests that various T-cell subsets may be more sensitive to induction therapy than others and that their rapid repopulation may lead to imbalances in functions of the immune system [15,16]. However, the impact of rATG on the kinetics of T-cell repopulation following primary depletion, specifically in HR and DCD renal transplant recipients, is not thoroughly understood. This study aims to assess early T-cell reconstitution and functional features in HR and DCD kidney transplant recipients who received rATG induction therapy.

MATERIAL AND METHODS

A total of 38 kidney transplant recipients who received rATG induction therapy were enrolled in accordance with the guidelines of the University of Western Ontario's Research Ethics Board (MEC–2010–022) and in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. All patients gave their informed consent prior to their inclusion in the study.

The characteristics of the recipients (including the number [n = 19 per group], age, sex, body mass index, pretransplant creatinine, HLA mismatch, previous transplants, cytomegalovirus and Epstein-Barr virus mismatch, similar distribution of ESRD causes, and maintenance immunotherapy; excluding the source of donated kidneys and panel reactive antibody, values did not differ between HR and DCD recipients (Table 1). Both groups were administered the same triple regimen of immunosuppressants (mycophenolate mofetil, tacrolimus, and prednisone) at a very narrow therapeutic range as shown in Table 2. Both groups of recipients received 2.0 mg/kg rATG (Sanofi-Genzyme, Paris, France) intravenously on days 1, 2, and 3 after transplantation. Bloodwork was conducted to assess the lymphocyte populations perioperatively and postdischarge on days 2, 7, 14, and 28 posttransplantation (Fig 1A). During each visit, heparinized blood (60.0 mL) was collected to isolate the peripheral blood mononuclear cells (PBMCs) by gradient density centrifugation and stored at −80°C until analysis. The numbers of red blood cells, white blood cells, T cells, and platelets were calculated, and the immune response of leukocytes after rATG therapy was evaluated.

Flow Cytometry

Multicolor flow cytometry (Becton Dickinson LSR II; Becton, Dickinson and Company, Franklin Lakes, NJ, United States) or FACSCalibur (BD-Biosciences, Mountainview, CA, United States) were used to identify various T-cell subsets of PBMCs. Cells were stained with LIVE/DEAD staining kit to exclude the dead cells for more robust analysis (LIVE/DEAD Fixable Aqua Dead Cell Stain Kits, ThermoFisher, Waltham, MA, United States). PBMCs were stained with specific fluorophore-tagged antibodies CD3 (Alexa 700), CD4 (HRZN V450), and CD8 (APC-H7). Then they were counterstained with markers associated with a naïve or memory phenotype [17], including CD45RO (PECy7), CD45RA (NHP-PE), and CD27 (FITC) as well as the activation marker CD25 (PerCP-Cy5.5). Memory $CD4^+$ and $CD8^+$ T cells were identified from naïve cells by expression of CD45RO (vs CD45RA in naïve cells). Regulatory T cells (Tregs) have been designated as $CD4^+CD25^+$ T cells. In addition, to distinguish from activated $CD4^+$ T cells, which also express $CD25^+$ from Tregs, we used the absence of the CD127 (PE-CY5) [18] and intracellular expression of Forkhead box protein P3 (FoxP3) (eFluor450) as previously described [19]. The percentages of $CD3^+CD4^+CD127^-CD25^+FoxP3^+$ and $CD3^+CD8^+CD127^-CD25^+FoxP3^+$ T-cells were quantified using flow cytometry as $CD4^+$ and $CD8^+$ Tregs, respectively. The $CD4^+$ effector and memory panel consisted of fluorescent antibodies to CD3, CD4, CD25, CD27, CD28, and CD45RA. Pacific-Blue–conjugated anti-CD45 (clone HI30) and anti-FoxP3 (clone 236A/E7) were purchased from eBiosciences (San Diego, CA, United States), and phycoerythrin-conjugated anti-Helios was purchased from Biolegend (San Diego, CA, United States). All other antibodies were purchased from BD-Biosciences. Isotype-matched control beads and monoclonal antibodies were used as compensation controls (Anti-Mouse Ig, κ/Negative Control Compensation Particles Set; BD-Bioscience). FlowJo (Tree Star, Inc, Ashland, OR, United States) and FACS Diva software (BD-Bioscience) were used to analyze the acquired data.

We assessed the proliferation of the various T-cell subtypes by staining for Ki67, a nuclear antigen that expresses during all active stages of the cell cycle (growth 1, synthesis, growth 2, and mitosis) except the resting phase (quiescent state) [20]. After surface staining, PBMCs were intracellularly stained with anti-Ki67 (Alexa Fluor 488) antibody. Percentages of proliferating lymphocyte subsets, as defined by expression of Ki67, were determined by flow cytometry.

Immune Cell Function

Immune cell function was measured using the Cylex ImmuKnow assay (Cylex Inc, Columbia, MD, United States). This assay measures the production of adenosine triphosphate (ATP) in response to stimulating patients' blood samples for 15 to 18 hours at 37°C with phytohemagglutinin, an antigen that induces the activation and proliferation of a subset of phytohemagglutinin-specific $CD4^+$ T cells. Monoclonal anti-CD4 antibodies (BD-Bioscience) attached to magnetic particles were used to facilitate the selection of $CD4^+$ lymphocytes [21]. The separated cells were lysed, and the level of ATP in the solution was measured by luminescence resulting from the oxidation of luciferin by the luciferase enzyme in the presence of ATP (emission maximum ∼560.0 nm).

Statistics

Statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA, United States). To determine statistical significance, we used 2-sided probability values according to the Mann-Whitney *U* and Fisher exact tests. P values $< .05$ were considered statistically significant.

RESULTS

rATG Treatment Efficiently Reduced T-Cells Numbers after Transplantation

Blood levels of mononuclear cells in HR and DCD recipients were examined by flow cytometry before and during the 28 days post-transplantation. The mononuclear cells were gated in the samples isolated from healthy volunteers by CD45 expression in combination with side and forward scatter. The frequencies of $CD3^+$ T cells in both groups were similar at the baseline (HR 65% ± 5.8% and DCD 59.8% ± 16%). However, by day 2 after induction therapy by rATG, the relative amount of $CD3^+$ T cells declined significantly in both groups (HR 13.8% ± 1.2% and DCD 21.8.8% ± 21% in comparison with baseline [day 0], $P < .05$). The number of T cells in both groups was suppressed until day 28. However, in the DCD group, T cells showed a trend toward proportional recovery of the lymphocyte population after day 7 (Fig 1B). Overall, the decline in the number of T cells demonstrated an expected lymphocyte depletion in both HR and DCD recipients over the 28 days after rATG treatment.

Kinetics, Phenotype, and Proliferation of the T-Cells Reconstituted After rATG Induction Therapy

Next, we aimed to assess whether different T-cell subsets were affected by this treatment. We investigated the proportion and kinetics of peripheral blood T-cell subsets during the first 28 days post transplant in HR and DCD recipients (Fig 2). The $CD4^+$ T-cell proportion declined by day 2 in both groups (HR 35.9% ± 21.7% and DCD 53.9% ± 11.3% in comparison with baseline [day 0], $P < .05$), and from day 7 until day 28, proportions were similar between groups (HR 34.9% ± 21% and DCD 24.4% ± 4.6%) (Fig 2A). In contrast, the percentage of $CD8^+$ T cells increased gradually between days 2 to 28 in both groups (Fig 2B). To investigate specific changes in $CD4^+$ T-cell subsets, we assessed populations of effector-memory T cells and Tregs. Interestingly, the proportion of effector-memory T cells in HR recipients significantly increased by day 2 post transplantation (43.9% ± 17%) vs baseline (12% ± 10.3%) ($P < .05$), which persisted until day 28 with a reduction by the end of the period (24.7% ± 17.2%). In contrast, the effector-memory $CD4^+$ T cells did not proliferate in response to induction therapy in the DCD group and were proportionately lower vs the HR group at all time points (Fig 2C). Furthermore, the percentages of both $CD4^+$ and $CD8^+$ Tregs increased post transplant in HR recipients. However, only $CD8^+$ Tregs showed a sizeable proportional increase in $CD8^+$ Tregs (60% ± 3.1% vs 16.6% ± 7.3%) by day 2 vs baseline (1.6% for both) ($P < .05$) (Fig 2D, E). The levels of $CD4^+$ and $CD8^+$ Tregs returned to baseline in both groups by day 28 (Fig 2D, E). These data suggest that the $CD4^+$ T cells and not $CD8^+$ T cells are the major subset of depleted T cells in both treated groups. Additionally, the proportions of effector-memory $CD4^+$ T cells increased in HR recipients during the postdepletion period, suggesting that the $CD4^+$ T cells in HR recipients may have a higher turnover and proliferation rate after rATG treatment than in DCD recipients.

$CD4^+$ Effector-Memory and Treg Cells Proliferated in rATG-treated HR Recipients

Increased proportions of $CD4^+$ T cells, especially in HR recipients, after rATG treatment suggest that the $CD4^+$ T cells may proliferate despite depletion during induction. To evaluate this hypothesis, we directly assessed the proliferation of $CD4^+$ T cells using Ki67 [20]. The Ki67 assay showed that the proliferation rate of $CD4^+$ T cells on day 2 following rATG was more than 8-fold greater in comparison with HR recipients (43.8% ± 37.5% vs 4.8% ± 1.3%) ($P < .05$). In contrast, DCD recipients demonstrated a modest proliferation rate at the same time point over baseline levels (10.6% ± 0.2% vs 1.8% ± 0.14%) (Fig 3A). Similar proliferation patterns were seen for $CD4^+$ effector-memory T cells (Fig 3B) but not in $CD4^+$ Tregs (Fig 3C). This suggests the proliferation of $CD4^+$ T cells is due to the proliferation of effector-memory $CD4^+$ T cells. A slight but significant increase in the proliferation of $CD8^+$ Tregs was seen in HR recipients in comparison with the DCD group ($P < .05$) between days 2 and 28 (Fig 3D), consistent with the greater overall numbers (Fig 2E). In contrast, proliferation was minimal in subsets of $CD4^+$ and $CD8^+$ T cells in DCD recipients (Fig 3A–D). These results showed that the proliferation of $CD4^+$ T cells and especially $CD4^+$ effector-memory T cells upon depletion by rATG led to an increased number of $CD4^+$ T cells in HR recipients but not in DCD recipients.

$CD4^+$ T-Cells Following rATG Treatment Showed Less Alloreactivity in DCD Patients In Vitro

To evaluate the function of $CD4^+$ T cells in HR and DCD recipients on the T cells' recovery after rATG treatment, we used the Cylex ImmuKnow assay to measure the alloreactivity of $CD4^+$ T cells at baseline, 1 week, and 28 days after transplantation. $CD4^+$ T cell recall responses were determined by the levels of released ATP. As expected, both groups had similar baseline rates of ATP release prior to rATG treatment (Fig 3E). However, after rATG treatment, both groups had a significant decline in the immune reactivity on days 7 and 28 post transplantation compared with the baseline ($P < .05$) (Fig 3E). Interestingly, there was a significantly higher response in HR recipients compared with DCD recipients on days 7 and 28 ($P < .05$) (Fig 3E), suggesting that the $CD4^+$ T cells in DCD recipients are not only less proliferative but also showed less immune reactivity following rATG induction therapy.

Naïve $CD4^+$ T-Cells in the HR Group Were Proliferative and Gave Rise to Effector and Memory Cells Following rATG Treatment

It is reported that naïve T cells can proliferate and convert to effector and memory phenotypes after induction therapy [15]. We hypothesized that proliferation and conversion of naïve $CD4^+$ T cells upon depletion may explain the increased

Table 1. Demography and Measured Variables in ATG-Treated Recipients

Patient Characteristics	Group HR	DCD	*P* Value
Number	19	19	ns
Age	53 ± 10	52 ± 8	ns
Sex			
Male	9	8	ns
Female	10	11	
Body mass index, kg/m^2	29 ± 2	30 ± 3	ns
Pretransplant creatinine, μmol/L	614 ± 247	585 ± 141	ns
Pretransplant dialysis			
Hemodialysis	17	16	ns
Peritoneal dialysis	2	3	
% Panel reactive antibody			
<40%	0	17	< .01
>40%	19	2	< .01
HLA mismatch–total	3.93 ± 1.28	4.5 ± 0.88	ns
Previous transplant			
Yes	6	3	ns
CMV mismatch			
Yes	4	6	ns
EBV mismatch			
Yes	6	7	ns
Cause of end-stage renal disease			
Hypertension	8	7	ns
Diabetes	7	9	
Glomerular disease	3	1	
Obstructive uropathy	1	2	
Source of donor kidney			
Living	3	0	-
Deceased donor	16	19	ns
DCD	0	19	-
ECD	7	4	ns
SCD	9	15	.02
Delayed graft function			
Yes	3	12	.03
Maintenance immunotherapy			
MMF	19	19	-
Tacrolimus	19	19	-
Prednisone	19	19	-
Acute rejection			
Yes	1	2	ns
Serum creatinine, μmol/L			
Day 2	487 ± 177	548 ±102	ns
Day 7	371 ± 190	487 ± 184	ns
Day 14	166 ± 60	317 ± 136	.05
Day 28	65 ± 8	165 ± 26	.04

Patient demographics and measured variables in those patients who received ATG induction immunosuppression at the time of renal transplantation because of their HR category or because they received kidneys from donors after cardiac death (DCD).

Data are expressed as mean ± SD.

Abbreviations: CMV, cytomegalovirus; DCD, donation after cardiac death; EBV, Epstein-Barr virus; ECD, expanded criteria donor; HR, high immunologic risk; MMF, mycophenolate mofetil; ns, no significance; SCD, standard criteria donor.

proliferation and number of CD4$^+$ T-cell subsets (Figs 2 and 3). To examine this hypothesis, we investigated the proliferative response of naïve CD4$^+$ T cells upon rATG treatment. In both groups, the percentage of total naïve CD4$^+$ and CD8$^+$

Table 2. Concomitant Immunosuppression Therapy

Immunosuppressant	Dose	Administration	Blood Level
MMF	750 mg	Twice a day	Not measured
Tacrolimus	0.12–0.15 mg/kg	Twice a day	5–7 ng/mL
Prednisone	5 mg	Daily	Not measured

Abbreviations: The immunosuppression therapy for both HR and DCD groups was the same triple regimen of MMF, tacrolimus, and prednisone and was kept at a very narrow therapeutic range as shown in this table.

Abbreviations: DCD, donation after cardiac death; HR, high immunologic risk; MMF, mycophenolate mofetil.

T cells during the 28-day period was not significantly different from the baseline (Fig 4A). However, compared with total CD4$^+$ T cells, CD4$^+$ naïve T cells in HR recipients on days 2 and 7 had a higher proportion of effector-like cells in comparison with baseline and also to the DCD group at all timepoints ($P < .05$) (Fig 4B). Interestingly, we found that the amount of naïve CD4$^+$ T cells with a memory and memory-effector phenotype increased significantly in HR recipients

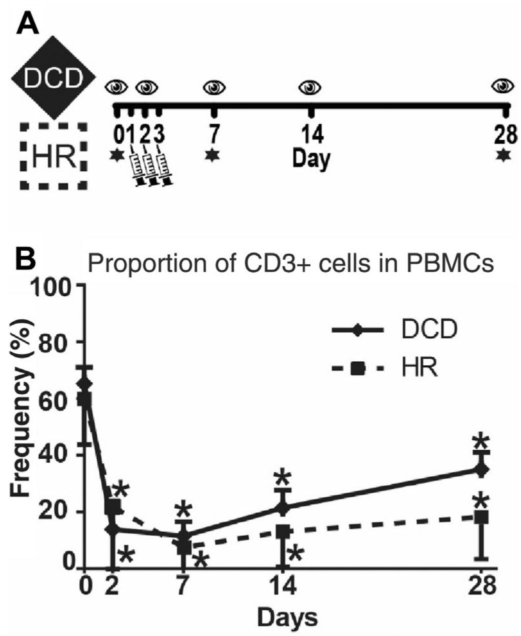

Fig 1. ATG treatment depleted CD4$^+$ T cells in both recipients of DCD and HR kidneys. Blood samples were collected from the transplant recipients from HR and DCD groups at the time of transplantation (day 0), then at day 2, 7, 14, and 28 after transplantation (indicated by eye symbols) for flow cytometry analysis; stars show days of ImmuKnow assay **(A)**. Percentage of CD3$^+$ cells in PBMCs before and during ATG treatment were measured at various time points **(B)**. The dashed line represents high immunologic risk recipients (HR, n = 19), whereas black represents recipients of kidneys obtained from donations after cardiac death (DCD, n = 19). * shows the significant difference of that group in the time point to the baseline (day 0) ($P < .05$), and † indicates the significant difference between 2 groups at that time point ($P < .05$). ATG, antithymocyte globulin; DCD, donations after cardiac death; HR, high immunologic risk; PBMC, peripheral blood mononuclear cells.

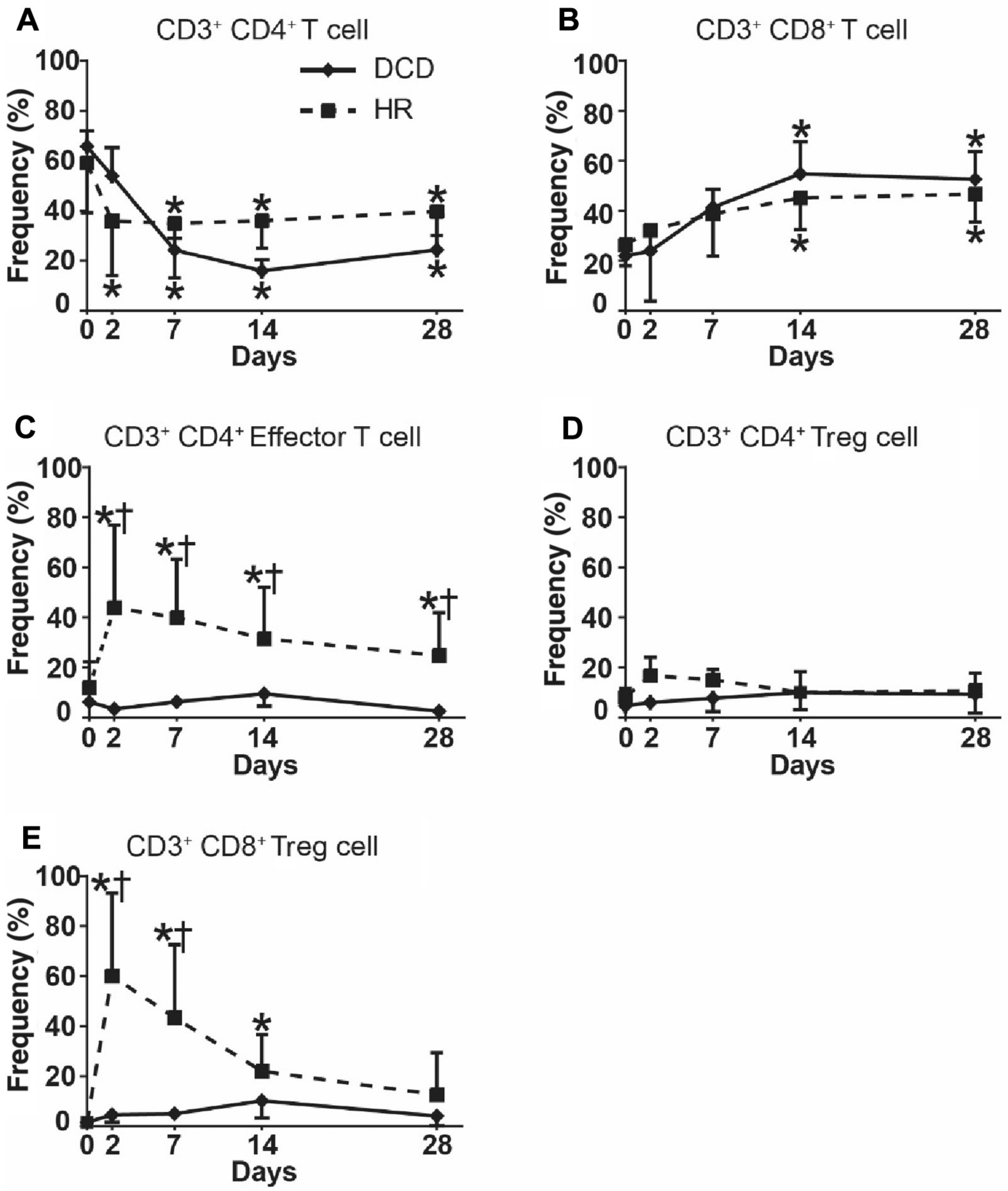

Fig 2. $CD4^+$ T cells are affected by ATG induction therapy. The effect of Thymoglobulin induction therapy on the frequency of circulating $CD4^+$ **(A)**, $CD8^+$ **(B)**, $CD4^+$ effector-memory **(C)**, $CD4^+$ regulatory **(D)**, and $CD8^+$ regulatory **(E)** T cells in HR (dashed line) and DCD (black) renal transplant recipients prior to kidney transplantation (day 0) and at various time points after, as determined by flow cytometry. Values are shown as mean ± SD. * shows the significant difference of that group in the time point to the baseline (day 0) ($P < .05$), and † indicates the significant difference between 2 groups at that time point ($P < .05$). ATG, antithymocyte globulin; DCD, donations after cardiac death; HR, high immunologic risk; SD, standard deviation; Treg, regulatory T cell.

by day 2 post transplantation and then gradually returned to baseline by day 28 (Fig 4C, D). In contrast, the amount of naïve effector, memory, and effector-memory $CD4^+$ T cells in the DCD group did not show any difference to the baseline during this 28-day period (Fig 4B–D). The increase in the number of $CD4^+$ naïve cells in HR recipients since day 2 after transplantation was associated with an increased incorporation of Ki67 at all time points from day 2 to day 28 (Fig 4E, F). These data suggest that naïve $CD4^+$ T cells in HR recipients have a higher turnover after depletion by rATG and may convert nonspecifically to effector and memory phenotypes as seen before in our animal model [15].

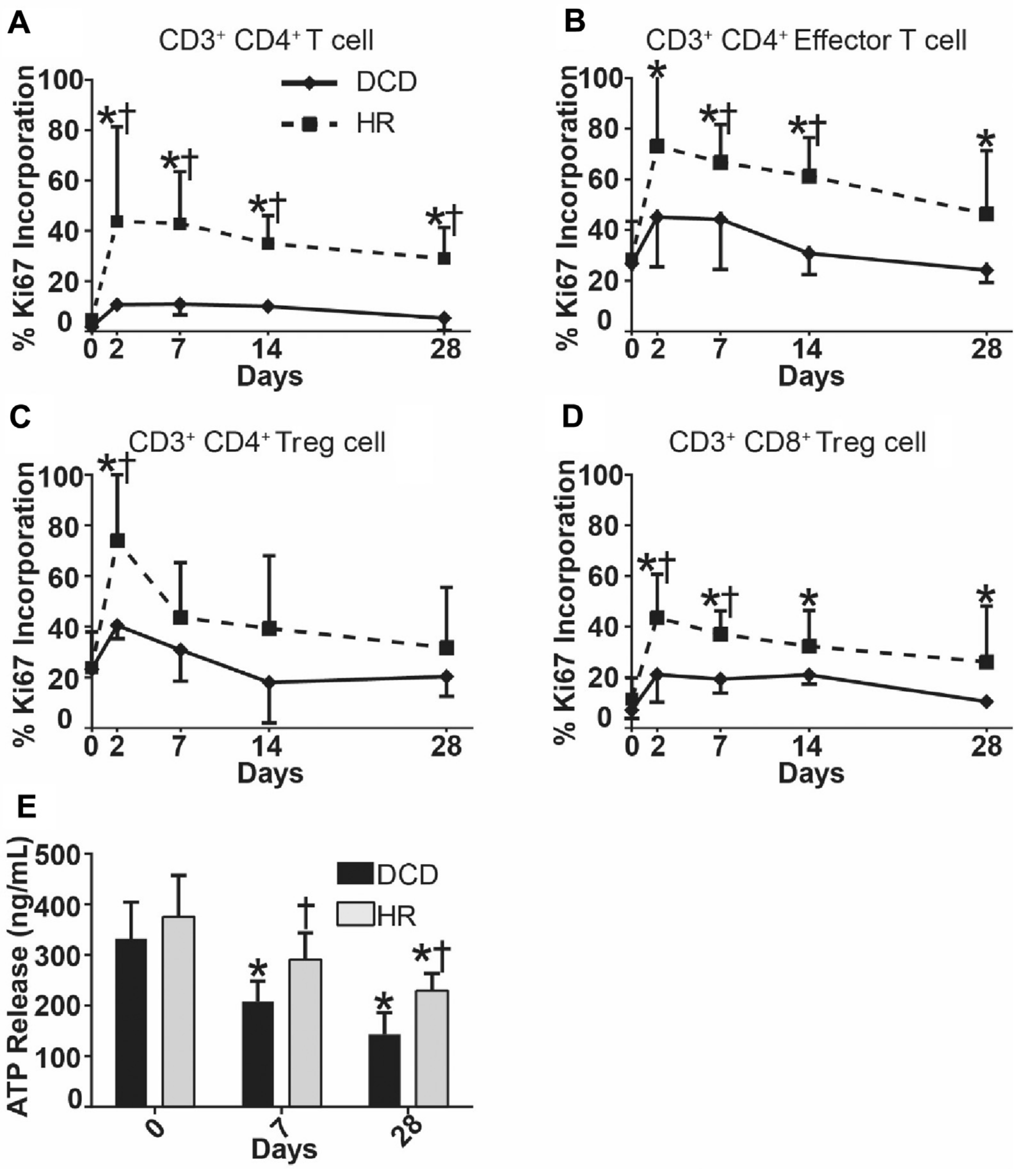

Fig 3. $CD4^+$ T cells are more proliferative and alloreactive in HR recipients. The effect of ATG induction therapy on the rate of Ki67 incorporation **(A–D)** as an index of cell proliferation in various subtypes of $CD4^+$ (naïve, memory, and Treg) T cells as well as of the $CD8^+$ regulatory T cells prior to HR and DCD kidney transplantation (day 0) and at various time points after, as determined by flow cytometry. The effect of ATG induction therapy on ATP release **(E)**. The dashed line represents high immunologic risk recipients (HR, n = 19), whereas the black represents recipients of kidneys obtained from donations after cardiac death (DCD, n = 19). Values are displayed as mean ± SD. * shows the significant difference of that group in the time point to the baseline (day 0) ($P < .05$), and † indicates the significant difference between 2 groups at that time point ($P < .05$). ATG, antithymocyte globulin; ATP, adenosine triphosphate; DCD, donations after cardiac death; HR, high immunologic risk; SD, standard deviation; Treg, regulatory T cell.

DISCUSSION

The current study evaluates T-cell homeostatic proliferation following depletion therapy with rATG in HR and DCD renal transplant recipients. We compared the reconstitution, degree of differential proliferation, and recall responses of the newly reconstituting T cells with alloantigens after rATG induction. We examined the early post-transplant period and different subsets of both $CD4^+$ and $CD8^+$ T cells to evaluate rapid changes in T-cell kinetics and to better understand the role of rATG in the prevention of DGF. We found that rATG treatment affected naïve, memory, and regulatory T-cell numbers and induced a preferential upregulation and phenotypic conversion from a naïve to a memory phenotype. To the best of our knowledge, this is the first study to describe

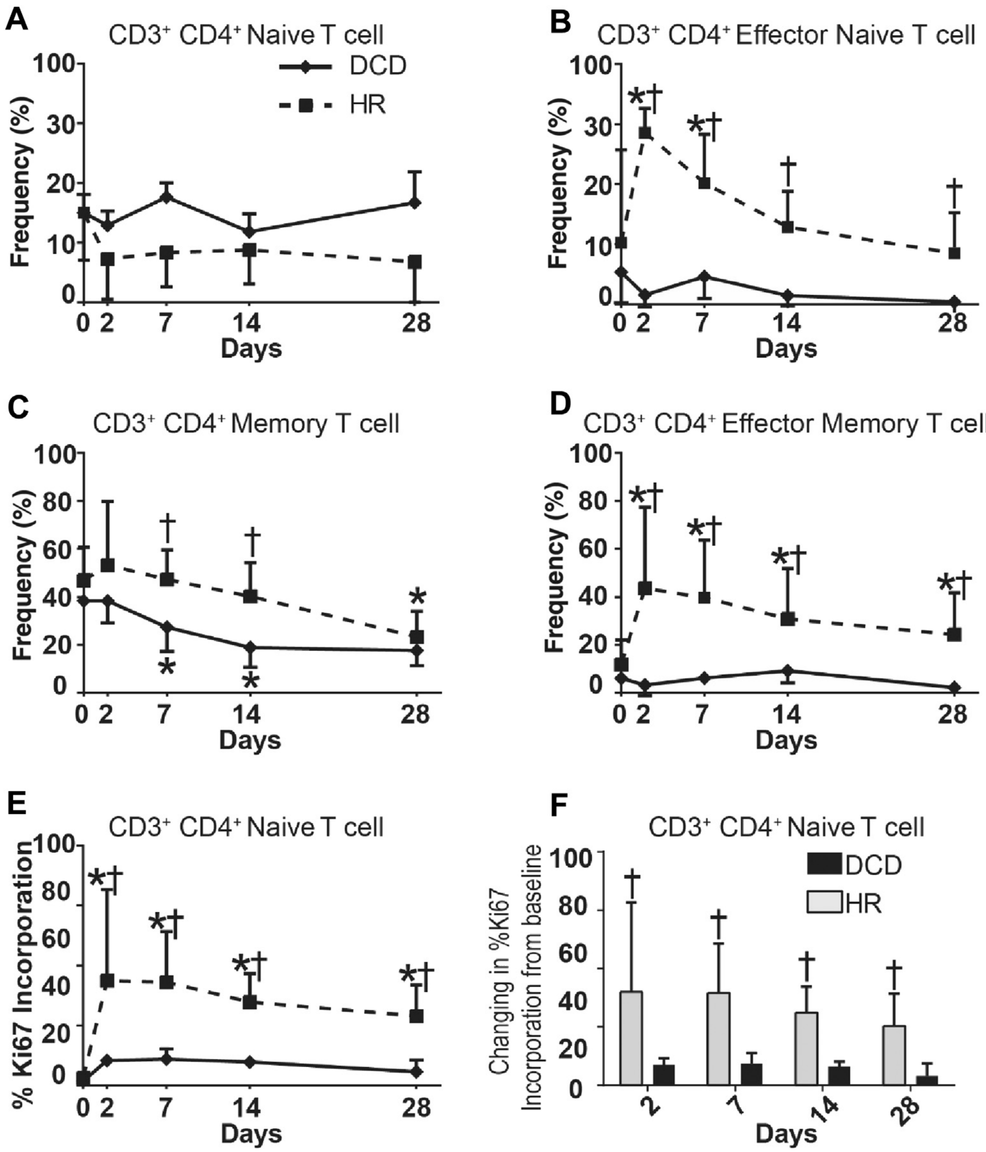

Fig 4. Naïve CD4$^+$ T cells proliferate after ATG treatment and convert to effector and memory CD4$^+$ T cells. The effect of ATG induction therapy on the frequency of circulating CD4$^+$ naïve **(A)**, CD4$^+$ effector naïve **(B)**, CD4$^+$ memory **(C)**, and CD4$^+$ effector-memory **(D)** T cells taken prior to kidney transplantation (day 0) and at various time points after, as determined by flow cytometry. The effect of ATG induction therapy on the rate of Ki67 incorporation in naïve CD4$^+$ T cells is shown by the proportion of Ki67$^+$ cells at each time point **(E)** and their changes than the baseline at each time point **(F)**. The dashed line represents high immunologic risk recipients (HR, n = 19), whereas the black represents recipients of DCD kidneys (DCD, n = 19). Values are expressed as mean ± SD. * represents the significant difference of that group in the time point to the baseline (Day 0) ($P < .05$), and † shows the significant difference between 2 groups at that time point ($P < .05$). ATG, antithymocyte globulin; DCD, donations after cardiac death; HR, high immunologic risk; SD, standard deviation.

the kinetics of T-cell reconstitution and function early after transplantation in a cohort of HR and DCD recipients.

Lymphocyte homeostasis and reconstitution following depletion have been the focus of several clinical and animal studies [8,15,22–26]. Previously, Sener et al [15] showed the susceptibility of memory CD4$^+$ T cells to lymphodepletion and that the postdepletion T-cell compartment is repopulated to a significant extent by homeostatically expanded naïve T cells in a mouse model. Several studies have evaluated the effect rATG treatment in the later phases after transplant, ranging from 5 months [22] to 5 years, but only in HR recipients, 24 showing that rATG treatment reduced the rate of biopsy-proven acute rejection episodes. Interestingly, Kho et al [26] reported that rATG treatment during the first 3 days post transplantation decreased the proportion of B and T cells in patients; however, they did not investigate T-cell subsets and their immune reactivity.

Existing research also suggests that pan-lymphocyte depletion and rapid reconstitution may have significant clinical consequences on the effectiveness of post-transplant immunotherapy [15,27–29]. In a study evaluating the late responses of $CD4^+$ T-cell, $CD8^+$ T-cell and Tregs in response to rATG, the authors found that naïve and memory T cells diminished until 3 months, followed by a rise in effector and central memory phenotypes while the naïve population remained depressed [27]. When taken together with previous reports of the predominance of memory T cells following lymphodepletion [22–24,30], the apparently diminished susceptibility of Tregs to rATG treatment may be a protective phenomenon that occurs because of the conversion of the rapidly expanding naïve T-cell pool to a regulatory phenotype [28]. The functional capacity of these de novo Tregs is unknown and they may be contributing to immune dysregulation following transplantation. Our findings in HR and DCD graft recipients is congruent with these studies and adds to the understanding of T-cell reconstitution following induction therapy.

In adults, homeostatic proliferation is thought to be the major contributor to immune reconstitution after rATG induction therapy [31]. Instead of measuring homeostatic proliferation indirectly by studying the absolute number of memory T cells like earlier studies, we directly measured proliferation with Ki−67 to understand the role of proliferating naïve T cells adopting a memory phenotype. The gradual loss of T-cell effector functions, starting with a decrease in their proliferative capacity at day 7 post transplant, might be due to exhaustion of T cells. A similar declining pattern was observed by Bouvy et al [31]; however, because of starting their evaluation at 1 month post transplant, they may have missed the early dynamic changes and phenotypic transitions we observed in our study.

The increased release of cytokines during brain death and during the period before the procurement of DCD organs makes allografts susceptible to DGF and acute rejection. Induction therapy is imperative for minimizing the release of danger signals during this ischemia-reperfusion period, which can cause rapid activation of the innate immune system in the recipient upon transplantation [32]. The use of rATG is the standard approach for depletion of lymphocytes [33], and basiliximab is the most commonly used non−lymphocyte-depleting monoclonal (anti-interleukin [IL]−2 receptor) antibody [34–36]. Data from the United Network for Organ Sharing from 2003 to 2008 reveal that HR recipients who received rATG treatment had the lowest rate of acute rejection after 1 year compared with the group who received anti–IL−2 receptor therapy [37]. Our data showed that DCD recipients treated with rATG have significantly lower overall levels of homeostatic T-cell proliferation compared with their high-risk transplant cohorts. These data suggest that using IL−2 receptor antagonists could be the first-line therapy in low-risk DCD recipients, followed by rATG if the need arose. Given that DGF rates can be as high as 60% [38] in DCD grafts, many centers may be using ATG to minimize confusing DGF with acute rejection immediately after transplantation. Given that both groups had similar rates of acute rejection ($n = 1$) in the small sample sizes we were working with, these conclusions highlight important points about current immunosuppressive strategies that need to be evaluated further using larger cohorts.

Furthermore, ATP released by the reconstituted T cells suggests that they were functional after rATG treatment in both HR and DCD recipients, with greater potency in the HR group. Since ATP is mainly synthesized during oxidative phosphorylation in the mitochondria and is used as the core energy source within cells, any rise in intracellular ATP levels induced by mitogens or antigens in lymphocytes is related to cell activation and subsequent cell proliferation [39]. Likewise, Kowalski et al [39] described the ATP release by repopulated T cells after ATG induction in 76 renal allograft recipients. The authors demonstrated that although low ATP levels identify patients with an increased risk for infection, high ATP values do not correlate with rejection and do not justify increased immunosuppression [39]. Myslik et al [40] showed that an ImmuKnow level before kidney transplantation could scale the recipients into moderate- and high-risk for rejection. Recipients with low ATP pretransplant have less chance of rejection than those with high ATP [40].

In this study, we were unable to evaluate long-term T-cell profiling because of budget limitations. However, other studies show increased long-term graft survival in HR recipients, including more hypersensitized, younger patients, second transplants, and deceased donors treated with rATG [41]. We analyzed the stored frozen PBMCs obtained from patients over several days, and because the variation in the proportion of live cells among samples affected the absolute numbers, it made it impossible to make a reliable comparison among absolute numbers of T-cells subsets; therefore, we used the proportions of cell subsets in the figures instead of the absolute numbers. Another limitation of our findings is the day-to-day variability in the CD4+ T-cell reactivity, as measured by the ImmuKnow assay. Instead, the ELISpot assay could be used to detect cytokine production by the activated T cells. Importantly, whether slow thymic regeneration and output in the DCD group may be attributed to the weaker recovery of T cells remains unstudied. Nonetheless, a positive immunologic response upon rATG induction in HR recipients may indicate its beneficial usage in this subgroup for achieving a steroid-free immunosuppression regimen without decreasing graft and patient survival. However, the use of rATG as a first-line therapy in DCD recipients could be reconsidered.

CONCLUSION

Our study provides important novel insight into the early postdepletion T-cell milieu and may lead to new immunosuppressive strategies to promote short-term and long-term graft function in kidney transplant recipients. Interestingly, although HR recipients behaved as expected concerning T-cell depletion and reconstitution, DCD recipients displayed a much less active T-cell reconstitution and proliferative profile, which suggests that their T-cell compartment may not be as active as we once thought. The results of this study should be evaluated prospectively in larger trials to further evaluate the ideal induction therapy in recipients of DCD grafts. Additionally, the results of this study may help future transplant professionals in predicting the occurrence of new-onset immune disorders in the early period after transplantation.

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