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ORIGINAL ARTICLE

An investigation to assess the potential of $CD25^{high}CD4^{+}$ T cells to regulate responses to donor alloantigens in clinically stable renal transplant recipients

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$CD4^{+}$ T cells, $CD25^{high}CD4^{+}$ T cells, kidney, rejection, regulatory T cells, tolerance, transplantation.

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Summary

Regulatory T cells are enriched within $CD25^{high}CD4^{+}$ leukocytes, however their role in renal transplant recipients with stable function vs. recipients with biopsy-proven chronic allograft dysfunction remains unclear. We therefore studied the number, phenotype, and function of $CD25^{high}CD4^{+}$ cells in the peripheral blood of 30 renal transplant recipients of living-related grafts, comprising 15 rejection-free recipients with stable graft function (Group A) and 15 with biopsy-proven chronic graft dysfunction (Group B). A higher absolute number of $CD25^{high}CD4^{+}$ cells were present in the peripheral blood of rejection-free recipients (Group A) vs. those recipients with chronic graft dysfunction (Group B) ($P = 0.019$); but there was no significant difference with healthy volunteers ($P = 0.084$). In carboxyfluorescein diacetate succinimidyl ester-mixed leukocyte culture assays, depletion of $CD25^{high}CD4^{+}$ revealed active regulation in 11 (74%) of 15 rejection-free recipient samples (Group A) in response to donor- but not third party-leukocytes, whereas no regulatory activity was observed in any samples from recipients with chronic graft dysfunction (Group B). In conclusion, these data provide evidence for the presence of an increased number of $CD25^{high}CD4^{+}$ T cells with donor-specific regulatory activity in the peripheral blood of renal transplant recipients with stable graft function compared with recipients with chronic graft dysfunction.

Introduction

Although transplantation has become the mainstay of therapy for end-stage organ failure and renal transplant recipients have benefited from dramatic improvements in short-term graft survival, long-term success continues to be limited by the ongoing need for nonspecific immunosuppressive therapy that is accompanied by unwanted effects, such as increased susceptibility to infection, malignancy, and failure to prevent chronic rejection [1]. Reducing the total load of immunosuppressive therapy, while maintaining stable graft function, is one of the main goals of clinical transplantation.

Immune unresponsiveness to a transplant can arise through different immunologic mechanisms including active regulation or suppression and/or deletion of donor-reactive leukocytes. Active regulation by T cells has been identified as a key mechanism for the induction and maintenance of unresponsiveness to donor alloantigens after organ transplantation [2–4]. Several investigators have confirmed the existence of a unique population of T cells that are enriched amongst T cells expressing both

CD4 and CD25 (the IL-2 receptor α chain), express the transcription factor Foxp3, are hypo-responsive upon stimulation with antigen *in vitro* and have potent immune regulatory effects both *in vitro* and *in vivo* [5,6]. Although first identified in mice, it is now clear that a population of T cells expressing high levels of CD25 with same functional properties also exists in humans [7,8]. Human $CD25^{high}CD4^{+}$ cells have regulatory activity that can inhibit both T-cell proliferation as well as the generation of effector cells with cytotoxic activity [9]. In experimental models, it is well documented that $CD25^{+}$ $CD4^{+}$ regulatory T cells can maintain transplantation tolerance [10,11]. In clinical transplantation, $CD25^{high}CD4^{+}$ T cells with regulatory activity responsive to donor alloantigen stimulation have been identified in immunosuppression (IS)-free liver transplant recipients [12,13]. In renal transplant recipients, $CD25^{high}CD4^{+}$ T cells have been identified in different clinical situations [14–17], but more data are required to understand their potential role in recipients with stable graft function.

Reliable and validated assays that can be used to monitor the immune status of transplant recipients, in particular their response to both donor alloantigens as well as other antigen challenges are an important, unmet need in clinical transplantation. A number of assays are currently being investigated including the multi-parameter immunophenotyping of peripheral blood lymphocytes in conjunction with an assessment of lymphocytes function. Proliferation assays have long been used to test the functionality of lymphocytes. The mixed lymphocyte reaction (MLC) using tritiated thymidine incorporation for quantifying cell division does provide an estimate of the number of cells capable of proliferating when co-cultured with either donor or third party alloantigen-presenting cells [18]. Flow cytometric analysis of cell division by assessing the loss of the fluorescence intensity of the intracellular carboxyfluorescein diacetate succinimidyl ester dye (CFSE) has recently been used as an alternative approach for measuring T-cell proliferation in MLC [19]. CFSE analysis provides detailed information and direct visualization of lymphocytes subpopulation as they proliferate in response to donor- and third party-alloantigens in the MLC [20]. Here, we have used the CFSE-MLC assay to assess the ability of $CD25^{high}CD4^{+}$ T cells to regulate responses to donor- and third party-alloantigens in renal transplant recipients with either stable function or chronic allograft dysfunction to assess the potential of $CD25^{high}CD4^{+}$ T cells in facilitating long-term graft survival.

Patients and methods

Transplant recipients and donors

A cohort of 30 patients who underwent their first renal transplantation from living, related donors and who were followed at the Mansoura Urology and Nephrology Centre were the subject of this study (Table 1). Blood samples were collected from each transplant recipients during the period from November 2002 to January 2003 and from healthy volunteers with the informed consent. Peripheral blood mononuclear cells (PBMC) were isolated, adjusted to a concentration of 1×10^7 cells/ml/cryovial in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% human AB serum and 10% dimethyl sulphoxide and then stored in liquid nitrogen tanks (at −180 °C) upto the time of analysis. All patients were metabolically stable at the time of making the assay. Scheduled weaning of oral steroids was performed according to the span of time after transplantation and graft function, reaching a dose of 5–10 mg steroids, 500 mg azathiorpine and cyclosporine A trough level within the range of 60–120 μg/ml.

The renal transplant recipients were divided into two groups according to their graft function and history of

Table 1. Demography and patients characteristics.

	Group A	Group B	*P*-value
Number of patients	15	15	
Recipient age (year)	27.46 ± 8.9	28.8 ± 9	0.703
Recipient sex (male:female)	9:6	10:5	0.719
Post-transplantation time to assay (years)	7.6 ± 3	6 ± 3.7	0.167
Recipient creatinine chemical clearance (ml/min)	68 ± 28.8	41.7 ± 13.99	0.005
Original kidney disease end stage			
End-stage renal disease (histopathologic diagnosis)	11	13	
Chronic pyelonephritis	2	1	
Mesangio-capillary	1	1	
Hypoplasia	1	0	
Immunosuppression regimen			
Steroids, cyclosporine A (CsA), azathiorpine (Aza)	12	13	
Steroids, CsA	2	0	
Steroids, Aza	1	2	
Donors age (year)	36.13 ± 8.5	30.73 ± 9.38	0.132
Donor sex (male:female)	5:10	5:10	
Tissue mismatching			
Number of HLA (A and B) mismatches			
Zero mismatch	1	2	0.432
One mismatch	3	4	
Two mismatch	7	8	
Three mismatch	3	1	
Four mismatch	1	0	
Number of HLA DR mismatches			
One mismatch	15	15	

Values are expressed as means ± SD.

rejection episodes; patients with stable graft function for more than 2 years with no history of rejection were designated as group (A), and included nine males and six females, age (27.46 ± 8.9: 12–48 years), and they had received their graft 5–17 years previously at the time peripheral blood sample was taken. Serum creatinine (Cr) levels were 1.2 ± 0.25: 0.8–1.6 mg/dl and Cr chemical clearance was 68 ± 28.8: 40–130 ml/min. Recipients with a history of one or more of rejection episode and/or gradual increase of Cr of 0.2 mg/dl above the baseline that was taken as an indication of graft dysfunction were assigned to group (B) that included 10 males and five females, age (28.8 ± 9: 10–42 years), Cr levels were (1.9 ± 0.6: 1.2–3.2 mg/dl), Cr chemical clearance (41.7 ± 13.99: 20–70 ml/min); they received their graft 3–17 years previously at the time the peripheral blood sample was taken. Graft biopsies were performed to determine the probable diagnosis of chronic allograft dysfunction histologically and were assessed according to Banff97 working classification (22).

Cell culture, reagents, and flow cytometry

Phenotyping

Peripheral blood mononuclear cells were thawed and labeled with monoclonal, mouse anti-human antibodies including phycoerythrin (PE)-conjugated CD25 (CALTAG Laboratories, Burlingame, CA, USA), FITC-conjugated CD4 (Sigma-Aldrich, St Louis, MO, USA), APC-conjugated CD45RO (CALTAG Laboratories), PE-IgG1 and FITC-IgG1 (CALTAG Laboratories) as negative controls. Samples were seeded with a known number of fluorescent beads (Calibrate, BD Biosciences, Oxford, UK) for calculation of absolute cell number at the time of analysis. Flow cytometry was performed on a FACSCalibur dual laser cytometer incorporating CELL QUEST software (Becton Dickinson, Oxford, UK).

Functional assays

At the time of making the assay, PBMCs (responder cells) were thawed and divided into two; the first sample was left un-manipulated, while the second was depleted of $CD25^{high}$ cells by positive selection using magnetic beads (Miltenyi-Biotec Inc., Bergisch Gladbach, Germany). The level of depletion for each sample was determined by FACS. Depletion of $CD25^{+}$ $CD4^{+}$ using magnetic beads resulted in the depletion of the majority of $CD25^{high}CD4^{+}$ cells (mean depletion, 96.5% ± 1.7) and 35–45% depletion of $CD25^{low}CD4^{+}$ (mean depletion, 40.9% ± 7) as shown in Fig. 1a.

^{3}H-thymidine (^{3}H-Thy) incorporation

Peripheral blood mononuclear cells acting as responders were resuspended at 1×10^{5} cells/well and were

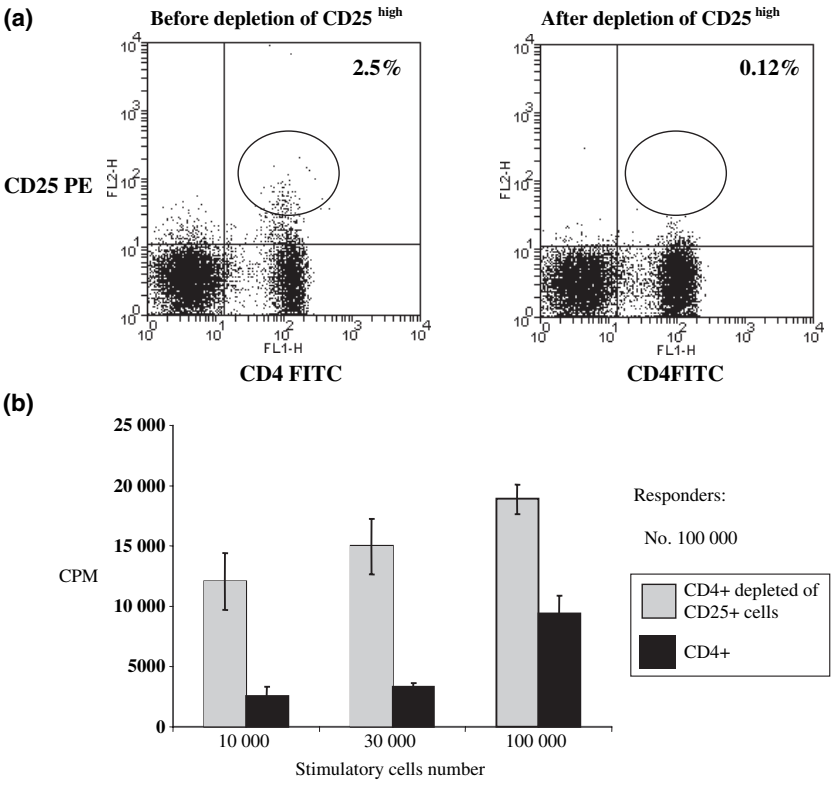

Figure 1 (a) Flow cytometry analysis of the percentage of $CD25^{high}CD4^{+}$ T cells before and after depletion of CD25 cells. Peripheral blood mononuclear cells (PBMC) were either left un-manipulated or depleted of $CD25^{high}$ cells by positive selection using magnetic beads. The level of depletion for each sample was determined by fluorescence activated cell sorter (FACS). The data shown are representative of over 30 assays performed in the course of this study. (b) Impact of depletion of $CD25^{+}CD4^{+}$ T cell on T-cell proliferation after alloantigen stimulation using a ^{3}H-thymidine to measure proliferation. PBMCs before or after depletion of $CD25^{high}$ cells were resuspended at 1×10^{5} cells/well and were co-cultured with increasing concentrations of stimulator cells in 96-well plates for 5 days. Stimulator cells (donor and third party PBMC; donor cells shown) were irradiated before use in the assay. ^{3}H-thymidine was added for the last 16–18 h of culture and incorporation determined.

co-cultured with increasing concentrations of stimulator cells in 96 well plates for 5 days. Stimulator cells (donor and third party PBMC) were irradiated (3132 RAD over 12 min) before use in the assay. ^{3}H-thymidine was added for the last 16–18 h of culture, before cells were harvested onto filter mats (Skatron, Sterling, VA, USA) with a Skatron 12-well semiautomatic cell harvester. The c.p.m. of the filter membrane was measured in scintillation liquid (Cytoscint; ICN Biomedical, Costa Mesa, CA, USA) on an LS 6000IC (Beckman Coulter, Palo Alto, CA, USA). The optimal proliferation response was observed at a 1:1 (responder:stimulator) cell ratio Fig. 1b.

CFSE labeling

Peripheral blood mononuclear cells were resuspended, 1×10^8 responder cells/ml, in RPMI 1640 medium, 1 μM 5-(and 6)-CFSE (Molecular Probes, Inc., Eugene, OR, USA) was added and cells were gently mixed and incubated for 10 min in a 37 °C water path, protected from light. Labeling of cells was stopped by adding cold RPMI 1640 medium; cells then washed and resuspended in culture medium.

CFSE-MLC assays

Carboxyfluorescein diacetate succinimidyl ester-labeled responder cells were resuspended at 1×10^5 cells/well and were co-cultured with stimulator cells at 1:1 ratio. After 5 days, the cells were harvested, labeled with PE mouse anti-human CD4 (BD Biosciences) and CD8 Biotin (Sigma-Aldrich, St Louis, MO, USA) and streptavidin APC (BD Biosciences), dead cells were labeled with 7AAD (BD Biosciences) and were gated out. Samples were seeded with a known number of fluorescent beads (Calibrate, BD Biosciences) for calculation of absolute cell number at the time of analysis. Flow cytometry was performed on a FACSCalibur dual laser cytometer incorporating CELL QUEST software (Becton Dickinson).

Quantifying proliferation of $CD4^+$ and $CD8^+$ T cells

Carboxyfluorescein diacetate succinimidyl ester curve fitting was done using WEASEL software v 2.0 (Walter & Eliza Hall Institute of Medical Research, Melbourne, Australia). Divisions of reactive cells, which were identified and determined by their CFSE intensities, were labeled from 0 to n as dividing time. A single cell dividing n times will generate $2n$ daughter cells. With use of this mathematical relationship, the number of division precursors was extrapolated from the number of daughter cells of each division and from proliferation events in $CD4^+$ and $CD8^+$ cell subsets. Stimulation index was calculated by dividing stimulation index of allogeneic combinations by those of self-control.

Statistical analysis

Data were recorded and analyzed using SPSS for windows (SPSS Inc., Chicago, IL, USA). Quantitative data were described in terms of arithmetic mean ± SD. Qualitative data were measured by chi-squared test. Significance differences between data sets were tested using the Mann–Whitney 2-tailed U approximation. P-value < 0.05 was considered significant.

Results

Characteristics of the patients enrolled in the study

The characteristics of the patients enrolled in the study are summarized in Table 1. The data show that the two groups of renal allograft recipients analyzed ($n = 15$ per group) were similar with regard to their demographic and tissue-matching characteristics.

Phenotypic analysis

There were no significant differences between the absolute number of the white blood cells, lymphocytes, and $CD4^+$ cells in the renal transplant groups (A and B) and healthy volunteers were enrolled in the study as shown in Table 2.

Differential analysis of $CD25^+CD4^+$ T population revealed that the intensity of staining for CD25 ranged from low to high levels. The absolute number of $CD25^{high}CD4^+$ T cells in renal transplant patients allocated to groups A (44 ± 26 cells/μl) and B (23 ± 19 cells/μl) was significantly different ($P < 0.01$), while there were no significant difference between group A, recipients with stable graft function and no evidence of rejection, and the healthy volunteers (33 .8 ± 17 cells/μl) ($P = 0.084$) as shown in Table 2.

Table 2. The absolute number of peripheral blood mononuclear cells subsets in the peripheral blood.

	Group A	Group B	Healthy donors
White blood cells (×10^9/l)	9.25 ± 1.87	9.37 ± 2	9.28 ± 1.67
Lymphocytes (/μl)	2601 ± 744	2782 ± 993	2744 ± 987
$CD4^+$ (/μl)	867 ± 248	927 ± 397	914 ± 329
$CD4^+CD25^-$ (/μl)	639 ± 186	722 ± 335	697 ± 247
$CD4^+CD25^{low}$ (/μl)	183 ± 97	179 ± 105	188 ± 99
$CD4^+CD25^{high}$ (/μl)	44 ± 26*	23 ± 19	33 .8 ± 17*

*Denotes $P < 0.01$ compared to Group B.

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Table 3. Results of CFSE-mixed leukocyte culture (MLC) – renal Allograft recipients with stable graft function and no history of rejection (Group A).

				CFSE-MLC stimulation index							
				$CD4^+$T cells stimulated with				$CD8^+$T cells stimulated with			
	HLR-DR			Donor cells		Third party cells		Donor cells		Third party cells	
Recipient ID	Patient (P)	Donor (D)	Third party (T)	P+D	P-25 + D	P+D	P-25 + D	P+D	P-25 + D	P+D	P-25 + D
1	4,11	2,11	3,4	3.3	36.3	8.4	31.9	2.7	28.1	4.5	8.1
2	3,4	2,3	3,11	6.1	216.6	6.6	71.9	5.2	255.3	5.5	91.9
3	7,11	2,11	3,13	2.0	3.6	1.1	1.1	1.4	1.5	1.2	1.2
4	3,11	3,8	13,14	7.5	59.3	8.3	26.6	9.8	108.8	1	3.6
5	11,13	13,1	2,3	1.2	6.72	1.1	2.1	1.3	18.5	1	1.6
6	13,-	13,4	3,7	6.1	256.8	6.2	22.9	4.7	72.4	5.6	33.6
7	2,3	2,1	3,13	7.5	16.5	1.0	1.5	2.6	3.6	2	2.8
8	1,13	3,13	3,7	1.6	8.2	1.5	5.9	8.6	117.0	8.2	18.9
9	7,11	4,11	2,11	1.1	1.5	2.5	3.0	2.1	8.4	1.5	1.5
10	2,3	3,4	4,1	2.0	1.4	5.1	3.6	7.4	9.6	5.5	6.6
11	3,11	3,4	7,11	2.0	2	9.2	8.3	9.2	11.0	9	11.7
12	2,3	3,11	13,1	3.1	3.1	2.8	10.1	2.3	471.5	2.1	55.9
13	3,9	3,8	3,13	7.8	9.4	6.1	7.3	7.1	5.7	5.7	4.6
14	1,11	4,11	3,4	9.8	7.8	4.1	3.7	7.9	37..9	9	7.2
15	3,4	3,1	3,7	5.7	8.0	6.4	9.6	3.7	4.8	3.6	5.4

Monitoring the function of $CD25^{high}$ $CD4^+$ T cells

Proliferation of 100 000 CFSE-labeled PBMC from each transplant recipient was assessed upon challenge with either donor or third party antigen-presenting cells in MLC, before and after depletion of $CD25^{high}$ T cells. Half of the cells were used in a ^{3}H-thymidine assay and the other half in CFSE-MLC assay.

In the CFSE-MLC assay, PBMC from recipients with stable graft function and no history of rejection (Group A) were hypo-responsive to stimulation by both donor- and third party-leukocytes (Table 3). Depletion of $CD25^{high}$ cells resulted in an increase in stimulation index in response to donor but not third leukocytes, in 11 (74%) of 15 recipients. The CFSE-MLC enabled evaluation of the impact of depletion of $CD25^{high}$ cells on the proliferative response of $CD4^+$ and $CD8^+$ T cells individually (Fig. 2). In six (40%) recipients, depletion of $CD25^{high}$ cells resulted in a marked increase in the proliferative response of both $CD4^+$ and $CD8^+$ T cells, while in five (33%) other recipients an increase in the proliferative response was only observed in either $CD4^+$ or $CD8^+$ T cells but not both populations after depletion of $CD25^{high}$ cells. In two recipients, depletion of $CD25^{high}$ cells resulted in the increased proliferation of $CD4^+$ T cells and was accompanied by a dramatic increase of the production of intra-cellular INF-γ. In three (20%) recipients, the increase in proliferation was observed in $CD8^+$ T cells and was accompanied by an increase in expression of intra-cellular granzyme B (Table 3). Data from one transplant patient in group A are shown in Fig. 2a as an example.

Depletion of $CD25^{high}CD4^+$ from PBMC of the recipients with renal allograft dysfunction (Group B) resulted in no selective increase in the proliferative response to donor stimulation in any of the 15 assays performed (Table 4). In four of the 15 recipients, there was an increase in proliferation to both donor and third party stimulations after depletion of $CD25^+$ cells. Data from one transplant recipient in group B are shown in Fig. 2b as an example.

In the ^{3}H-thymidine assay, PBMC from recipients with stable graft function with no history of rejection (Group A) were also hypo-responsive to stimulation to both donor and third party antigen-presenting cells. After depletion of $CD25^{high}$ T cells, 11 (74%) of 15 recipients showed increased proliferation to donor cells but not to third party stimulation. In contrast, no change in the initial response was detected after depletion of $CD25^{high}CD4^+$ in PBMC from recipients with chronic allograft dysfunction (Group B) (data not shown). The data obtained using the ^{3}H-thymidine assay were consistent with that from the CFSE-MLC.

Discussion

Reducing the total load of immunosuppressive therapy, while maintaining stable graft function, is one of the

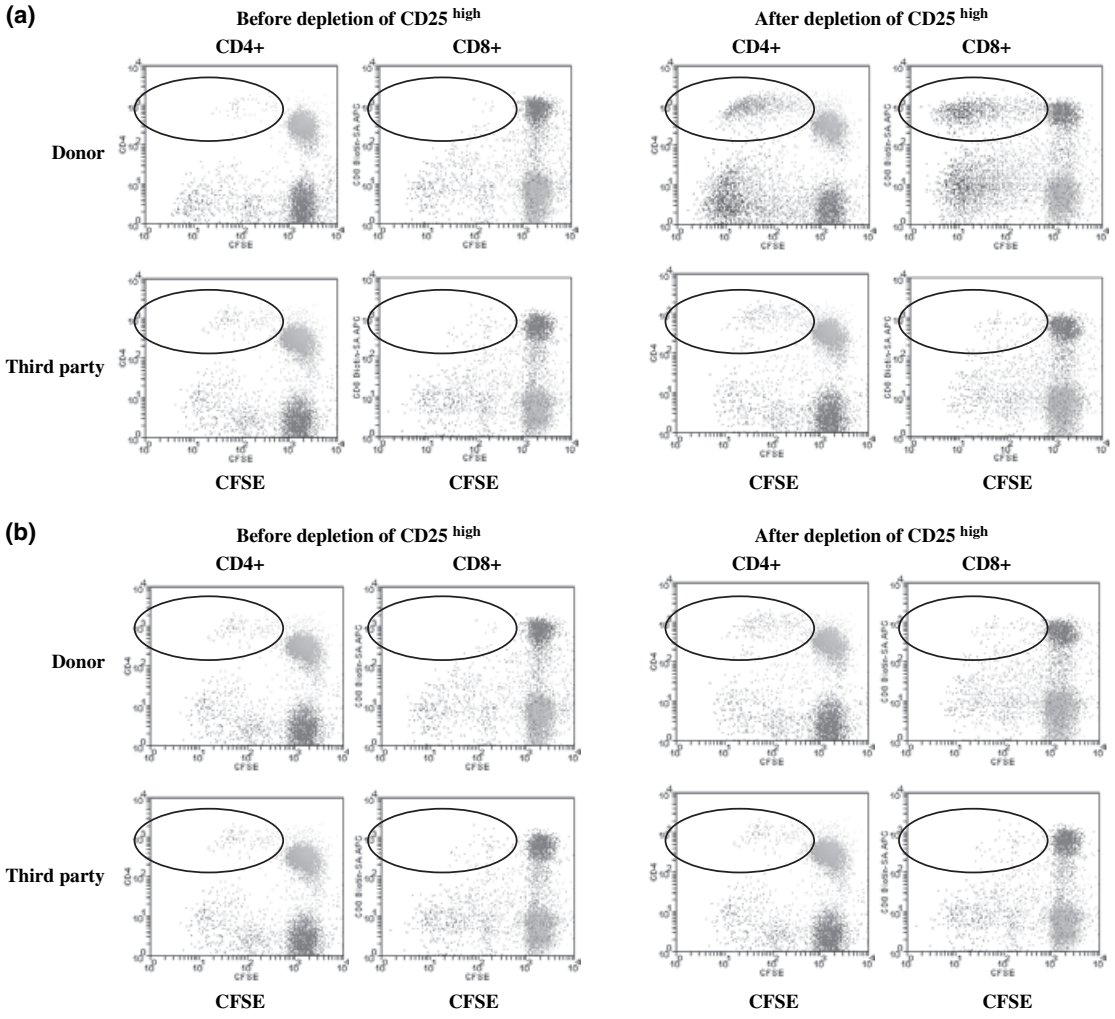

Figure 2 Carboxyfluorescein diacetate succinimidyl ester (CFSE)-mixed leukocyte culture assays result from a renal transplant patient with no history of rejection (group A) (a) and a renal transplant recipient with chronic allograft dysfunction. (b) CFSE-labeled responder cells before or after depletion of $CD25^{high}$ cells were resuspended at 1×10^5 cells/well and were co-cultured with stimulator cells at 1:1 ratio. After 5 days, the cells were harvested, labeled with phycoerythrin mouse anti-human CD4 (BD Biosciences, Oxford, UK) and CD8 Biotin and streptavidin APC; dead cells were labeled with 7AAD and were gated out. Samples were seeded with a known number of fluorescent beads for calculation of absolute cell number at the time of analysis. Flow cytometry was performed on a FACSCalibur dual laser cytometer incorporating CELL QUEST software.

main goals of clinical transplantation. Pilot clinical studies and trials to achieve a state of IS-free graft survival (operational tolerance) in clinically stable transplant recipients have become feasible for selected recipients in some situations. For example, weaning of IS in liver transplant recipients can be achieved in a subset of recipients without risk of rejection [12,13,21,22]. However, even with very careful monitoring of recipients who are enrolled in such studies, a considerable number experience rejection while undergoing weaning from IS [22]. Such observations highlight the fact that clinically stable transplant recipients are heterogeneous with respect to their immune status even when treated with the same IS protocol. Moreover, they emphasize the importance of developing and validating biomarkers and functional assays to identify the immunologic differences between clinically stable transplant recipients to ensure that weaning from IS can be carried out safely without risk to the transplant patient.

For this study, we chose to focus on one potential mechanism of immunologic unresponsiveness after renal transplantation, immunoregulation by $CD25^{high}CD4^{+-}$ $Foxp3^+$ T cells. The study reported here investigated the hypothesis that stable graft function in renal allograft recipients with no history of rejection would be associated with an increasing number of $CD25^{high}CD4^+$ T cells in

Table 4. Results of carboxyfluorescein diacetate succinimidyl ester (CFSE)-mixed leukocyte culture (MLC) – renal allograft recipients with chronic allograft dysgraft function (Group B).

				CFSE-MLC stimulation index								
				CD4 $^+$ T cells stimulated with				CD8 $^+$ T cells stimulated with				
Recipients'	HLR-DR			Donor		Third party		Donor		Third party		
ID	Patient	Donor	Third party	*P+D	P-25 + D	P+D	P-25 + D	P+D	P-25 + D	P+D	P-25 + D	Graft biopsy
1	9.11	4.11	3.9	2.4	2.2	1.1	1.1	4.5	5.4	5.4	8.1	Chronic rejection
2	3.1	3.5	4.1	1.4	1.5	1.5	1.5	1.6	2.4	1.5	3	Chronic rejection
3	2.4	4.11	4.11	4.9	6.9	4.5	5.0	3	2.7	3.2	2.2	Chronic rejection
4	1.4	4.3	4.8	2	1.0	2.0	1.0	2.0	1.0	2	1	Chronic allograft nephropathy
5	1.13	3.13	13.7	3.1	31	2.9	29.0	2.5	22.5	2.5	22.5	Border line rejection
6	1.7	1.4	4.7	3.6	3.2	3.2	3.8	6.2	6.2	6	6	Chronic tubular fibrosis
7	3.4	3.7	3.7	4.3	2.6	8.9	6.2	4.4	2.2	7.3	1.5	Chronic cyclosporine A (CsA) toxicity
8	3.4	3.13	3.13	1.8	1.4	2.6	1.3	1.3	2.2	1.8	15.5	Mild focal interstitial fibrosis
9	1.4	4.3	4.8	1.1	2.9	8.8	28.1	5.9	33.0	4.5	43.2	Chronic CsA toxicity
10	2.4	2.3	2.13	3.7	77.7	3.9	120.9	3.4	234.6	3.4	319.6	Chronic CsA toxicity
11	2.7	3.7	13.2	2.0	1.0	2.0	2.0	2.0	2.0	2.0	2.0	Chronic allograft nephropathy
12	7.1	3.7	1.13	5.2	0.5	3.6	0.4	2.9	0.3	2.8	0.3	Chronic tubulo in fibrosis
13	6.1	2.6	6.4	4.3	10.3	3.2	8	3.2	9.3	2.7	7.3	Border line rejection
14	16.1	4.16	4.16	2.2	2.4	7.3	6.6	6.4	6.4	8.7	8.7	Chronic rejection
15	4.8	3.4	3.4	2.3	0.2	2.1	0.2	2	0.2	2	0.2	Chronic rejection

*P+D, patient cells co-cultured with irradiated donor cells; P-25 + D, patient cells after depletion of CD25+ cells and co-cultured with irradiated donor cells; P+T, patient cells co-cultured with irradiated third party cells; P-25+T, patient cells after depletion of CD25+ cells co-cultured with irradiated third party cell.

the peripheral blood and that depletion of such cells would result in an increased proliferative response to donor alloantigens.

The data obtained from a single-time point analysis of the potential role of $CD25^{high}CD4^+$ T cells in 30 renal allograft recipients demonstrate that a higher absolute number of $CD25^{high}CD4^+$ cells were detectable in rejection-free renal transplant patients (Group A) when compared with renal transplant patients with chronic allograft dysfunction (Group B) ($P < 0.01$) (Table 2). In early studies, evaluating the total number of $CD25^+$ cells present in peripheral blood samples at serial time-points after renal transplantation as a potential marker of immune status has failed to predict graft function or outcome [23,24]. However, these studies did not discriminate between cells expressing different levels of CD25. On account of the observation that human T cells with regulatory activity are contained within the $CD25^{high}$ subpopulation of $CD4^+$ T cells [7,8], other studies have re-evaluated this parameter in transplant recipients. An IS-free liver allograft recipients were found to have an increased number of $CD25^{high}CD4^+$ T cells in the peripheral blood compared to controls remaining on IS [12,13]. In renal transplant recipients, no significant difference in the percentage of $CD25^{high}CD4^+$ T cells was found in 12 living-related transplants with stable graft function compared to healthy controls [15]; a finding consistent with the data reported here (Table 2) and that was obtained from the analysis of peripheral blood from drug-free renal transplant recipients [16]. In addition, Louis *et al.* [16] also found that the number of $CD25^{high}CD4^+$ T cells was lower in recipients with chronic rejection, as was observed in Group B here. Taken together, data from these studies support the hypothesis that renal allograft recipients who are clinically tolerant or have stable graft function during treatment with maintenance IS have 'normal' levels of $CD25^{high}CD4^+$T cells, i.e. equivalent to the levels found in healthy individuals, whereas that chronic rejection is associated with a decrease in the number of $CD25^{high}CD4^+$T cells in the peripheral blood.

Analyzing the functional activity of $CD25^{high}CD4^+$ T cells is an important additional parameter required to assess their potential role in transplant recipients. Here, we used two MLC assays to compare the proliferative response of PBMC to stimulation with either donor or third party antigen-presenting cells in the presence or absence of $CD25^+$ leukocytes. The depletion technique preferentially depleted $CD25^{high}$ leukocytes (Fig. 1a). During the course of the *in vitro* suppression assay, alloantigen reactive T cells have the potential to be activated irrespective of their functional capacity, i.e. both effector cells capable of proliferating in response to the

stimulation and regulatory cells capable of suppressing proliferation, and therefore it was not necessary to pre-activate the Treg to enable them to elaborate regulatory activity. Using the CFSE-MLC assay, 11 (74%) of the 15 renal transplant recipients with stable graft function and no history of rejection analyzed (Group A) showed increased proliferation to donor but not third party stimulators in the absence of $CD25^+$ cells. In six of these 11 recipients, both $CD4^+$ T cells and $CD8^+$ cells showed an increased proliferative response (representative data are shown in Fig. 2a); while in the remaining recipients, the difference was only observed in either the $CD4^+$ or the $CD8^+$ population that correlated with an increase of the production of intra-cellular INF-γ ($n = 2$) or upregulation of granzyme B respectively ($n = 3$). The ability of $CD25^{high}$ regulatory T cells to suppress the function of $CD8^+$ cells has been reported previously in experimental and human studies [25]. In contrast, in recipients with chronic allograft dysfunction (Group B), depletion of $CD25^+$ cells failed to reveal regulation that was specific for donor alloantigens; however, there was increase in the response of both $CD4^+$ cells and $CD8^+$ cells in four (26%) of the 15 recipients in response to both donor and third party stimulation after depletion of $CD25^+$ cells (Table 4; representative data are shown in Fig. 2b). These functional data are consistent with those reported by other studies [14,26], where the functional activity of PBMC from stable renal transplant patients was analyzed. In each study, only a subset of the recipients demonstrated a regulated proliferative response to donor alloantigens upon removal of $CD25^+$ cells. Several possible explanations for these findings include the possibility that T cells with regulatory activity were not contained within the $CD25^{high}CD4^+$ T cells in the peripheral blood of the patients where regulation was not detected as the cells were sequestered from the blood to either the draining lymph nodes or the graft or that these patients did not exhibit a regulated response to donor alloantigens, but rather the donor reactive leukocytes were depleted.

In conclusion, we have investigated the potential role that $CD25^{high}CD4^+$ T cells with regulatory activity may play in maintaining, long-term rejection-free graft survival in renal transplant recipients. Our data are consistent with $CD25^{high}CD4^+$ cells playing a role in controlling immune responsiveness to donor alloantigens in a subset of clinically stable renal transplant patients treated with maintenance IS. The data we obtained are consistent with those of other small studies highlighting the need for larger studies designed to identify the patients who develop T cells with regulatory activity after transplantation and investigated if such recipients may benefit from minimization of IS in the long term without compromising graft function.

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Authorship

AA performed the research and analyzed the data. NJ designed and developed one of the assays used for the study. NR performed the research and analyzed the data. MAB identified and enrolled the transplant recipients. AM identified and enrolled the transplant recipients. MES identified the transplant recipients enrolled in the study; analyzed the clinical data. MAG supervised the clinical components of the study. KW designed and supervised the research study and wrote the paper.

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