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Early and Limited Use of Tacrolimus to Avoid Rejection in an Alemtuzumab and Sirolimus Regimen for Kidney Transplantation: Clinical Results and Immune Monitoring

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Alemtuzumab induction with 60 days of tacrolimus treatment and continuous sirolimus treatment prevented acute rejection in nine of 10 consecutive renal allograft recipients. All patients are alive with a functioning kidney graft at 27–39 months of follow-up. Extensive immune monitoring was performed in all patients. Alloantibody detection, cytokine kinetics assay (CKA), and trans vivo delayed-type hypersensitivity (DTH) assay were performed every 6 months showing correlation with clinical evolution. Despite alloantibody presence in five patients, eight patients remain without the need for specific treatment and only sirolimus monotherapy in decreasing dosage. Four patients take only 1 mg sirolimus daily with levels of 3–4 ng/mL. One patient showed clinical signs of rejection at month 9 post-transplant, with slow increase in serum creatinine and histological signs of mixed cellular (endarteritis) and humoral rejection (C4d positivity in peritubular capillaries and donor-specific antibody (DSA)). In summary, the addition of tacrolimus therapy for 2 months to a steroid-free, alemtuzumab induction and sirolimus maintenance protocol limited the previously shown acute rejection development. Nevertheless, alloantibody was present in serum and/or C4d present on 1-year biopsy in half the patients. The combination of CKA and DSA monitoring or the performance of transvivo DTH correlated with immune status of the patients.

Key words: Alemtuzumab, immune monitoring, immunosuppression, kidney, transplantation

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Introduction

Evidence derived from a nonhuman primate model suggests that profound and durable T-cell depletion achieved using an anti-CD3 immunotoxin substantially reduce the risk of acute rejection and the need for maintenance immunosuppression, and in many cases lead to donor-specific tolerance (1). In humans, lymphocyte depletion at the time of renal transplantation with induction with alemtuzumab (Campath-1H), a humanized anti-CD52 monoclonal antibody that depletes T and B lymphocytes, natural killer cells and some monocytes and macrophages, has allowed allografts to be maintained with reduced immunosuppression (2). As an alternative to cyclosporine, sirolimus has the potential advantages of reducing nephrotoxicity, preventing graft fibrosis (3), and perhaps enhancing the potential for tolerance induction (4). A pilot study by our group demonstrated that a majority of renal allograft recipients treated with alemtuzumab induction therapy maintained good graft function while on low-dose sirolimus monotherapy, but 28% had a rejection episode and 63% of the episodes correlated with a strong humoral component (5,6).

Convinced that alemtuzumab induction therapy and low-exposure sirolimus monotherapy may be a useful combination to further establishment of unresponsiveness in a fraction of patients treated, we sought to avoid early rejection with the use of a limited course of tacrolimus. Herein we describe results in 10 consecutive patients, with protocolized mechanistic and histological studies.

Methods

Immunosuppressive protocol

Alemtuzumab (Campath-1H), a humanized anti-CD52 monoclonal antibody (Ilex, Inc., San Antonio, TX), was administered intraoperatively on the day of transplant (day 0, 30 mg) and additional doses of 30 mg were given

on days 1 and 2. Prior to infusion, patients were administered 500 mg of methylprednisolone. Rapamycin (sirolimus, Rapamune®, Wyeth, Philadelphia, PA) was administered at a dose of 2 mg orally starting on the day after the transplant, adjusted to achieve blood levels in the 6–10 ng/mL range. Tacrolimus (Prograf®, Astellas, Deerfield, IL) was administered at a dose of 2 mg orally twice a day starting on the day after the transplant, adjusted to achieve blood levels in the 6–10 ng/mL range, and abruptly withdrawn after day 60 posttransplantation. The criterion for withdrawal of tacrolimus at 60 days was absence of clinical evidence of rejection. Criteria for tapering of sirolimus at 1 year included absence of clinical or biopsy evidence of rejection, glomerular filtration rate (GFR calculated by MDRD) >50 mL/min, and informed consent.

Recipient and donor selection

Patients were enrolled under an IRB-approved protocol at the University of Wisconsin, Madison, under ITN surveillance in collaboration with the NIAID and included review by a data safety and monitoring board. Primary one-haplotype living donor or zero-mismatched deceased donor adult renal transplant recipients (ages 18–60 years) were selected based on: current PRA <10%, historical peak PRA <25%, and body mass index <32. Patients with an HLA-identical living donor were excluded, as were patients who were cytomegalovirus (CMV) seronegative but had a CMV seropositive donor. Donor kidneys from nonheart-beating donors were excluded, as were kidneys from donors older than 55 years or kidneys preserved for >36 h. A negative NIH and AHG crossmatch test was required prior to transplantation.

Postoperative monitoring/infection prophylaxis/pathology

Renal allograft biopsies were performed at the time of the transplant and in patients with graft dysfunction (elevation of baseline SCr > 20%), and per protocol at 12 months. Biopsies were scored according to Banff criteria (7–9). Immunostaining for the C4d complement component was performed in all biopsies. All biopsies were interpreted at the University of Wisconsin (J.T.) and Massachusetts General Hospital (R.C.).

Patients underwent testing for donor-specific antibody (DSA) using Luminex® xMAP® multiplex technology. Single antigen class I and class II specificities were detected with specific beads (LABScreen® Single Antigen class I and class II, One Lambda Inc., Canoga Park, CA).

Flow cytometry

Blood was collected pretransplant, and every 6 months and sent ambient to the Immune Tolerance Network central flow cytometry facility for immediate surface staining. Samples were stained using a wash-lyse whole blood staining method previously described (10,11). The following panels were used for whole blood evaluations (in FITC/PE/PerCP5.5 or PECy5.5/PECy7/APC configuration): (1) CD11c/CD80/CD3,56,19,14/HLA-DR/CD123; (2) CD11c/CD86/CD3,56,19,14/HLA-DR/CD123; (3) CD45RA/CD45RO/CD8/CD4/CD62L; (4) CD8/CD25/CD4/CD3/CD62L; (5) CD57/CD56/CD8/CD3/CD14; (6) CD8/CD69/CD4/CD3/HLA-DR; (7) CD52 (Campath)/CD56/CD8/CD3/CD20; (8) CD52 (Rat)/CD56/CD8/CD3/CD20.

Frozen PBMCs from pretransplant and 12 months posttransplant (visits 0 and 28) were used for staining with following panels: (1) FoxP3/CD127/CD3/CD39/CD25,CD4; (2) Vdelta1/Vdelta2/Gamma delta/CD3, (3) CD1c/IgD/CD27/CD19/IgM. Cytofluorometric analysis was performed using either a FACSCanto, LSR II, or FACSCalibur (BD BioSciences, San Jose, CA) flow cytometer and FlowJo software (Treestar, Inc., Ashland, OR).

Cytokine kinetics assay

PBMCs were purified by Ficoll gradient separation and frozen viably. Upon thawing, cells were washed once in complete media, and used as responder cells in an MLR in which both proliferation and kinetics of cytokine expression were measured in response to irradiated donor, third party, or autologous stimulator PBMCs. The number of class I and class II MHC mismatches between host/donor and host/third party were mimicked using available third parties. A total of 2×10^5 responder and 2×10^5 stimulator cells/well were added to a 96-well round-bottom plate, in a total of 200 uL/well complete RPMI with 10% FCS. Cultures were set up in quintuplet wells such that the supernatants could be collected every 24 h during the 5-day MLR. Cytokine expression levels in the MLR supernatants were measured using a multi-cytokine fluorescent bead detection system (Bioplex Th1/Th2, Bio-Rad, Inc.). Fifty microliters of day 1–5 supernatants were used to analyze nine cytokines: IL-2, 4, 5, 10, 13, GM-CSF, TNF-α, IFN-γ and IL-β. Fluorescence was measured using Luminex XMAP technology (Qiagen, Inc., Valencia, CA).

Transvivo DTH assays

To evaluate indirect T-cell allorecognition, transvivo delayed-type hypersensitivity (DTH) assay was used as described previously (12–14). Briefly, PBMCs (cryopreserved) were injected, along with donor antigen (sonicated donor cells) into the footpads of CB-17 SCID mice purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). To test the linked suppression, a recall antigen Epstein-Barr virus (EBV Viral Antigens, Inc., Memphis, TN), was co-injected with donor antigen. The recall antigen alone was used as a positive control. Antigen-driven swelling was measured after 24 h using a dial thickness gauge. Postinjection measurements were compared with preinjection measurements to obtain specific swelling. DTH reactivity was expressed as the change in footpad thickness, using units of 10–4 inches, subtracting for background thickness changes due to the injection of PBMC with phosphate buffered saline (PBS) alone. Percentage of inhibition was determined using the following formula:

$$1 - [(\text{Recall} + \text{donorAg})/(\text{Recall})] \times 100,$$

where the values in parentheses are the net swelling (PBS/PBMC background subtracted) values for that response.

Statistics

Values are given as means ± standard deviation. For flow cytometry, data was analyzed by comparing baseline to 1-year values using paired *t*-tests. For each transvivo DTH test performed, the swelling response (or net swelling response) values were averaged from a minimum of two determinations. Mixed model analysis of variance (ANOVA) models were used to test for differences within total swelling response and net swelling. The p-values less than 0.05 were considered significant. Statistical analysis was performed with SPSS Software version 14.0 (Chicago, IL).

Results

Clinical evolution

Ten renal transplants were performed from nine living-related donors, and one deceased donor. General demographics are depicted in Table 1 for each patient. Five patients were transplanted before starting dialysis. Preimplantation donor biopsies were completely normal in five cases and presented minor alterations in the other five cases (minimal fibrosis [n = 4], minimal hyalinosis [n = 1]). All living donors were 1-haplotype mismatched and the deceased donor had 0-mismatches with the recipient.

Table 1: Patient characteristics

Patient	1	2	3	4	5	6	7	8	9	10
Age in years at transplantation	57	46	55	37	52	42	56	54	31	30
Gender	Female	Male	Male	Male	Male	Female	Female	Male	Male	Male
Race	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Native American	Caucasian	Caucasian	Caucasian
Underlying renal disease	PCK	IgAN	PCK	IgAN	DM	PCK	PCK	Obstructive	Unknown	Unknown
Time on dialysis	0	0	0	4 months	4 months	0	50 months	0	4 months	4 months
ABO group	O	B	A	A	A	O	B	AB	A	O
BMI at Tx (kg/m^2)	31.6	37.3	25.8	27.9	20	30.6	36.1	25.9	30.5	24.6
Donor type	Living related	Living related	Deceased	Living related	Living related	Living related	Living related	Living related	Living related	Living related
Donor age (yr), sex, race	43, Male, Caucasian	43, Female, Caucasian	28, Male, Caucasian	32, Male, Caucasian	54, Female, Caucasian	48, Female, Caucasian	56, Female, Native American	30, Female, Caucasian	40, Female, Caucasian	52, Male, Caucasian
Donor biopsy	Minimal hyalinosis	Minimal fibrosis	Normal	Normal	Minimal fibrosis	Minimal fibrosis	Normal	Normal	Normal	Minimal fibrosis
Pre-Tx PRA (%)	3	0	0	0	0	3	0	0	0	0
HLA MM AII/DR	3/1	2/1	0/0[1]	2/0	3/1	3/1	3/1	3/1	2/0[2]	2/0
Cold ischemia time	0	0	15 hours[1]	0	0	0	0	0	0	0
Follow-up (months)	39	39	37	30	30	30	28	28	27	27

[1] CAD RTx, 0 HLA mismatch.
[2] 0 DR mismatch.

Table 2: Tacrolimus and sirolimus treatment

Patient	1	2	3	4	5	6	7	8	9	10
Tacrolimus (daily mg/ng/mL)[1]										
Day 2	4/7	4/2	4/10	4/2	4/4	4/4	4/–	4/3	4/3	4/<1
Day 14	2/3	6/8	4/6	2/3	3/3	2/3	4/8	3/5	6/5	3/6
Month 1	4/9	6/9	3/7	4/6	3/5	4/8	4/12	4/8	7/6	3/8
Month 2	4/8	6/10	3/8	4/11	3/5	4/9	4/4	4/5	5/3	3/7
Sirolimus (daily mg/ng/mL)										
Day 2	2/3	2/2	2/<1	2/1	2/2	2/1	2/3	2/2	2/2	2/-
Day 14	5/3	3/5	6/18	2/7	2/5	5/13	3/10	4/7	1/2	2/8
Month 1	5/11	3/11	5/22	2/6	3/8	3/8	3/11	4/17	2/2	2/7
Month 2	4/12	3/6	3/15	2/5	5/8	3/9	5/11	4/16	7/6	2/9
Month 3	6/16	5/11	2/11	4/13	5/13	3/8	5/16	3/10	9/6	3/8
Month 6	4/8	4/11	2/10	5/10	4/8	3/9	4/12	4/9	9/10	2/5
Month 12	4/9	4/7	2/11	3/10	4/7	3/9	4/9	3/10[2]	7/8	2/9
Month 18	4/11	4/8	1/4	2/7	4/9	2/7	5/11	0/0	5/9	2/9
Month 24	3/10	4/7	1/4	1/4	4/10	1/3	0/0[3]	0/0	5/9	2/9
Month 36	1/3	3/8	1/4							

[1]All patients stopped tacrolimus after two months post-transplantation.
[2]Myfortic 720 bid and tacrolimus added. Two months later, sirolimus withdrawn (see text).
[3]Myfortic 720 bid and tacrolimus added, sirolimus withdrawn (see text).

All patients are alive and well and the grafts are functioning after 27 to 39 months. Immunosuppressive drug doses and levels are shown in Table 2. All patients received tacrolimus and sirolimus for 60 days, and tacrolimus was abruptly stopped at day 61. Four patients were considered to meet criteria for sirolimus tapering. Patients 1, 3, 4 and 6 were weaned to sirolimus 1 mg daily as their sole immunosuppressive drug with levels of 3–4 ng/mL. They have maintained stable renal function for at least 13 months on this regimen. Another four patients are currently on sirolimus monotherapy at doses of 3–6 mg daily (blood levels 6–10 ng/mL). Patient 7 was converted from sirolimus to Myfortic plus steroids due to proteinuria at 2-year follow-up. Patient 8 experienced rejection at 9–12 months and required addition of steroids and enteric-coated mycophenolic sodium, with sirolimus withdrawal (see below). All patients showed weight gain of variable degree (median 10% at 1-year-posttransplantation). All but one patient were receiving antihypertensive drugs (7 of them one drug, 2 of them two drugs) before transplantation, and blood pressure levels and antihypertensive needs did not change during the whole evolution. Only one patient was taking a statin (atorvastatin) before transplantation, and at the end of follow-up, seven patients were receiving statins to control dyslipidemia, and three were taking gemfibrozil.

No malignancies have been encountered and there have been no systemic viral or fungal infections. Two out of the 10 patients received treatment for infection, one left foot gangrenous infection in a diabetic patient (patient 5) and one Klebsiella bacteremia of unknown origin that required admission and parenteral antibiotics (patient 6) (Table 3). Another four patients developed adverse events requiring hospitalization: patient 4, who soon after transplantation developed progressive hip pain and ultimately was diagnosed as having advanced avascular necrosis of the hips related to pre-transplant steroid use and underwent consecutive bilateral hip replacement; patient 7, who needed surgical repair of an incisional hernia; and patient 8, after an episode of right leg deep venous thrombosis with a mild-associated pulmonary defect possibly caused by a small embolism. The patient with proteinuria mentioned above was also considered an SAE.

Immune cell monitoring

T cells, B cells, and monocytes were substantially depleted immediately following alemtuzumab induction as shown in previous studies (5, 15) and the kinetics of repopulation are summarized in the supporting figures. Although total white blood cell counts did not substantially change compared with baseline, lymphocyte counts dropped profoundly, remaining only 58% of baseline at 1 year. CD3 cells returned to 54% of baseline levels at month 18 after alemtuzumab treatment. While CD4 cells remained significantly depleted beyond 18 months after treatment, CD8 cells reached 75% of baseline levels by month 12. Moreover, CD4 or CD8 cells expressing CD45RA (naïve phenotype) showed faster reconstitution kinetics than cells expressing CD45RO (memory phenotype). The absolute numbers for Treg population defined as CD3CD4CD25hi were also significantly reduced. NKT cells defined as CD3CD8CD56 underwent profound depletion with only modest recoveries reaching about 20% 2 years after treatment. NK cells (CD8CD56) reached about 80% depletion 1 month after treatment but reconstituted completely by month 6 after treatment. Moderate depletion of monocytes was observed and baseline levels were reached by month 3. There was profound B cell

Table 3: Clinical, analytical, histological, and immunological patient monitoring

Patient	1	2	3	4	5	6	7	8	9	10
Adverse events requiring hospitalization	No	No	No	Hip replacement × 2	No	Gram-negative bacteremia	Large incisional hernia	No	No	No
Kidney Bx before Month 12	No	No	No	No	No	Yes, day +35 (Normal)	No	Yes, day +270 (No ACR, C4d+)	No	No
1-yr protocol Bx										
ACR	Negative	Negative	Negative	Negative	Negative	Negative	Borderline	IIA	Borderline Focally+	Borderline Negative
C4d	Negative	Diffusely +	Negative	Negative	Negative	Negative	Focally +	Diffusely +	No	No
Neutrophils in PTC	No	No	No	No	No	No	No	Yes (Score I)	II	I
IF/TA	I	I	I	I	I	I	I	I-cg3		
Estimated GFR (mL/min) Mo 3										
Mo 6	61	46	61	66	52	38	23	40	37	40
Mo 12	55	46	48	61	48	48	29	33	35	44
Mo 18	61	49	61	66	45	58	27	23	44	47
Mo 24	54	49	61	66	–	–	–	–	–	–
	54	43	61	–	–	–	–	–	–	–
Proteinuria (mg/l)										
Mo 3	Neg	Neg	Neg	Neg	1280	Neg	Neg	388	647	Neg
Mo 6	Neg	Neg	Neg	Neg	864	Neg	Neg	Neg	647	Neg
Mo 12	Neg	Neg	Neg	Neg	628	Neg	Neg	19701	576	270
Mo 18	Neg	Neg	Neg	Neg	–	–	–	–	–	–
Mo 24	Neg	Neg	Neg	–	–	–	–	–	–	–
Donor-specific antibodies at 12 mo										
	Negative	Anti-B62 Anti-DR13	ND (0MM)	Negative	Anti-B37 weak	Negative	Anti-A3	Anti-A3 Anti-DQ7	Anti DQ7	Anti-A24 weak
Transvivo DTH Regulator	Yes (M6,12)	No (M6) Sensitized M12	Yes (M6,12,18)	No (M6) Yes (M12)	No (M6,12)	No (M6) Yes (M12)	No (M12)	No (M12)	Yes (M6) No (M12)	Yes (M6) No (M12)
Cytokine kinetics assay at month 12	Unresponsive	Hyperresponsive	Unresponsive	Hyporesponsive	Hyporesponsive	Hyporesponsive	Hyperresponsive	Hyporesponsive (unresponsive M 6)	Hyporesponsive	Hyporesponsive

[1] Increased to 6170 mg/L Mo 13 and decreased to 450 after sirolimus withdrawal and increase in lisinopril dose (see text).

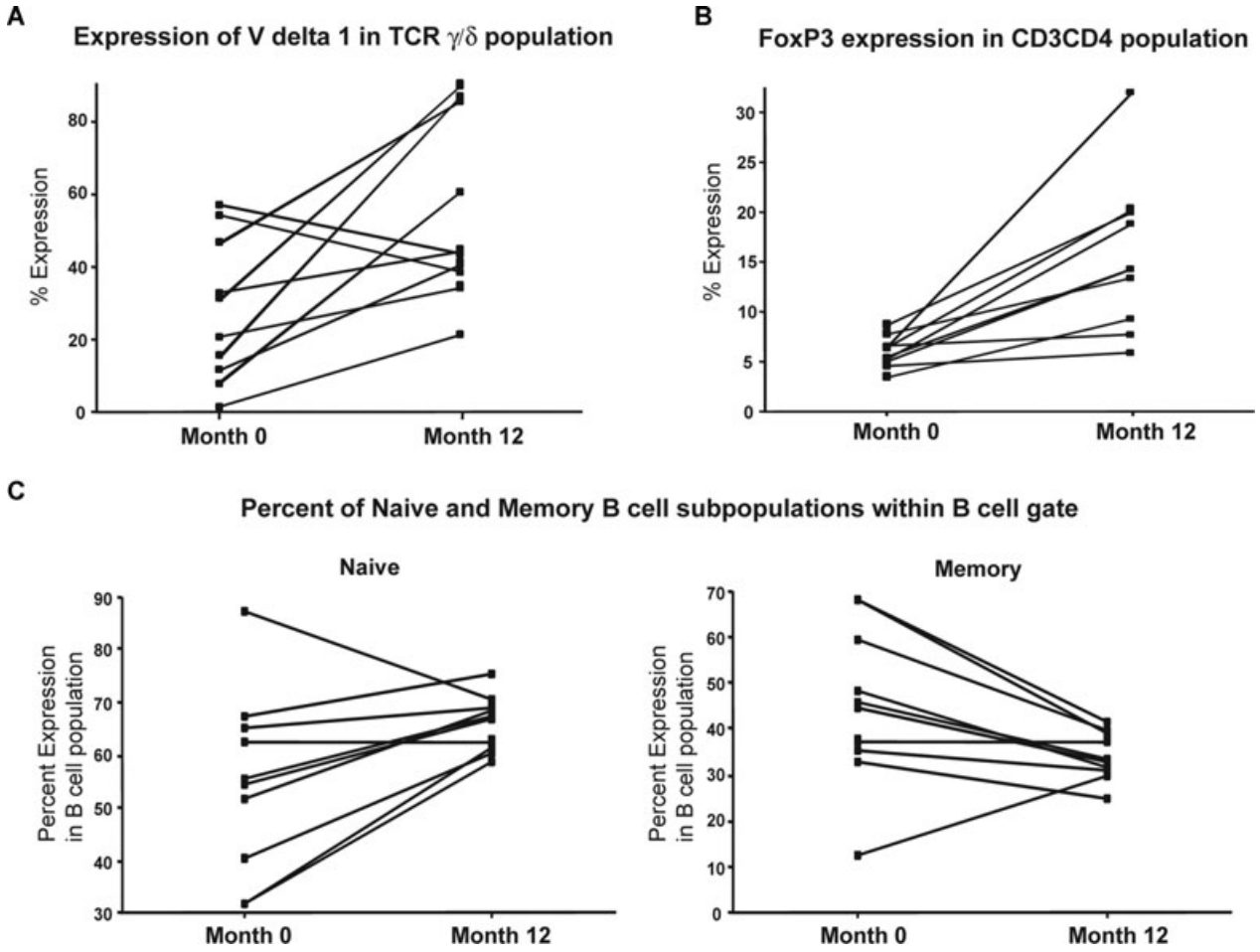

Figure 1: Expression of various cell populations before and after treatment with Campath–1H. (A) Frequencies of V delta 1 positive cells with γ/δ population. (B) Intranuclear expression of FoxP3. (C) Distribution of CD27 negative (naïve) and CD27 positive (memory) cells within B-cell population.

depletion after alemtuzumab treatment, but the absolute B cell numbers returned to baseline levels by month 6 and continued to increase thereafter (see supporting data for summary of depletion and repopulation kinetics for immune cells).

There were no significant differences in the percentage of overall gamma delta CD3 cells except for one patient. However, the contribution of V delta 1 and V delta 2 expressing cells in TCR γ/δ repertoire was significantly different (Figure 1A). We observed higher expression of V delta 1 positive cells within the CD3γ/δ compartment with concomitant reduction of V delta 2 expressing cells.

Expression of CD25 in the CD3CD4 population increased after Campath 1-H induction when measured 1-year-post Campath 1-H treatment. The percent of CD25 expressing cells in CD4 population increased from 2.79 ± 1.43 pre-transplant to 6.72 ± 4.10 at 1 year ($p = 0.0203$).

The percent of FoxP3 expressing cells in the CD3CD4 population increased from 5.95 ± 1.53 pre-transplant to 15 ± 7.61 at 12 months ($p = 0.0022$, Figure 1B). There was no difference in the level of FoxP3 expression in CD25hi cells between day 0 and 12 months (85.63% ± 4.03 vs. 87.10% ± 6.35).

The B cell compartment with B cells expressing mostly the naïve phenotype 12 months after transplant, as defined by CD19+ CD27- increasing from an mean average of 54.74% ± 17.17 to 65.93% ± 5.18 ($p = 0.03$, Figure 1C).

Kidney biopsies, acute rejection and allo-antibodies

No patient developed a typical clinically evident acute rejection. The only diagnosed T-cell acute rejection developed in patient 8, with progressive subacute kidney function deterioration (SCr 2.8 mg/dL) and a biopsy showing absence of histological signs of cellular rejection but with a C4d

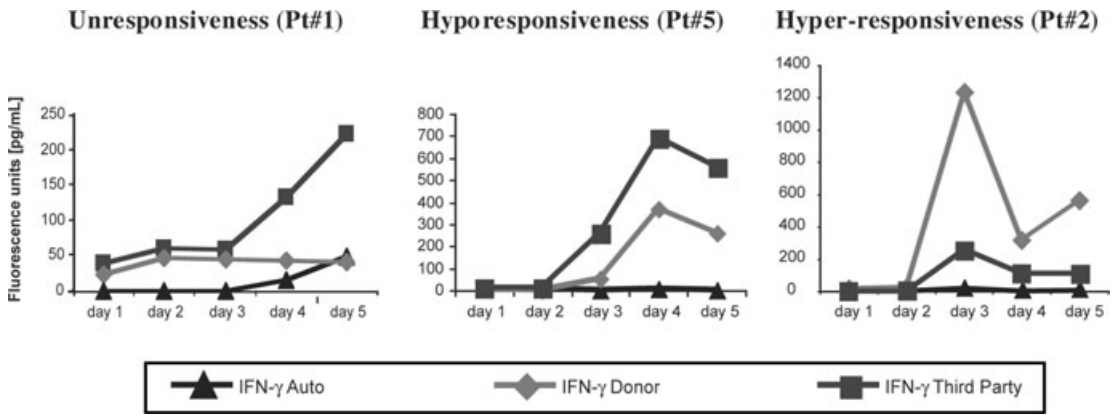

Figure 2: Cytokine kinetics assay patterns 1 year after transplantation.

staining diffusely positive in peritubular capillaries. Low antidonor HLA titers (class I anti-A3 and class II anti-DQ7) were detected in the serum at the time of this biopsy. The patient received treatment with IVIG at that time. Three months later, SCr was 2.9 mg/dL and a new biopsy showed: diffuse staining of C4d in PTCs, grade III chronic transplant glomerulopathy and grade IIA T-cell rejection, with intimal arteritis in a medium-sized artery. Given that chronic lesions were present and no acute kidney deterioration was observed, the patient was treated with a steroids, intravenous immunoglobulin (6 weekly doses of 100 mg/kg) and addition of mycophenolic sodium 720 mg twice a day to the sirolimus. At the time of the 1-year biopsy, proteinuria was 1.97 g/L, and it increased to 6.1 g/L 1 month later but resolved after stopping sirolimus.

Despite stable kidney function, the 12-month protocol biopsies showed diffusely positive C4d in PTCs in patient 2 and focally positive in patients 7 and 9 (Table 3). Patient 7 showed borderline acute cellular rejection on 1-year protocol biopsy. Finally, most patients showed mild interstitial fibrosis and tubular atrophy. No other patients showed clinical acute rejection during follow-up.

Patients 1, 3, 4, 5, 6 (group A) showed normal, C4d negative protocol biopsies, and did not develop relevant DSA production whereas patients 2, 7, 8, 9, 10 (group B) showed either subclinical signs of acute rejection in protocol biopsies, and/or C4d positivity in PTC and/or DSA. Total lymphocyte count at month 3 was significantly higher in patients not meeting weaning criteria 487.38 $\pm$ 193.07 cells/μL versus 213.19 $\pm$ 19.19 in patients that did ($p = 0.0159$). As expected, SCr was significantly lower and eGFR significantly higher in patients meeting weaning criteria at 1 year at all previous time points from month 2 to month 12.

T-cell alloresponse measurement with cytokine kinetics assay

The 5-day CKA was assessed by collecting supernatants at 24-hour intervals and analyzing them for the expression of 9 different TH1 and TH2 cytokines (see materials and methods). The kinetic patterning was categorized into three distinct groups as previously described (15): (1) patients who were hypo-responsive to donor, (2) those who were hyperresponsive to donor, and (3) those who were unresponsive to donor but responsive to third-party antigen. As depicted in Table 3, patients 4, 5, 6, 8, 9, 10 were hyporesponsive to donor, patients 2 and 7 were hyper-responsive, and patients 1 and 3 were completely unresponsive to the donor but responded to a third-party antigen (Figure 2). Of note, patient 8 was unresponsive by month 6 but hyporesponsive by month 12, suggesting an increase in alloreactivity.

Transvivo DTH

Transvivo DTH study results in these 10 patients are summarized in Table 4. Three main patterns of DTH response to recall and donor antigens may be defined (Figure 3). Patient 1 showed the regulator pattern, characterized by a weak response to donor antigen ($\leq 20 \times 10^{-4}$ in.) coupled with a 75% inhibition of the recall antigen response in the presence of donor antigen. This pattern of DTH response was previously found to be associated with organ allograft tolerance in mice, monkeys, and humans (12, 14, 16). In contrast, patient 8 exhibited a nonregulator pattern, which has the feature of a low response to donor but low or absent bystander suppression (in this case, 0%). This pattern has been frequently observed in transplant patients taking maintenance immunosuppressive drugs. Its basis is unknown, but may reflect a failure of allospecific memory T cell development due to the drug therapy. Lastly, patient 2 exhibited the donor-sensitized pattern, featuring a response to donor antigen similar to the recall antigen response. Bystander suppression was not tested due to lack of sufficient cell numbers at this timepoint, but was 0% at all subsequent timepoints (18, 30, and 36 months, data not shown). In summary, at 12 months patients 1, 3, 4, and 6 (50–75% inhibition) were regulators, patients 5, 7, 8, and 9 (0–25% inhibition) were nonregulators, and patient 2 was sensitized. Patient 10 was a non-regulator, but since

Table 4: Transvivo DTH studies at 6 and 12 months after transplantation

Patient	Months after transplant	Donor antigen	Recall antigen	Donor + recall antigens	Donor + recall antigen (%linked suppression)	Type of overall DTH pattern (R, NR, or S)
1	6	0	35	15	57	Regulator
	12	10	40	10	75	Regulator
2	6	20	50	40	20	Nonregulator
	12	30/40	40	N/A	N/A	Sensitized
3	6	0	50	20	60	Regulator
	12	0	40	10	75	Regulator
4	6	10	30	20	33	Nonregulator
	12	0	40	20	50	Regulator
5	6	10	40	30	25	Nonregulator
	12	N/A	40	40	0	Nonregulator
6	6	0	30	20	33	Nonregulator
	12	10	30	15	50	Regulator
7	6	0	30	30	0	Nonregulator
	12	10	30	30	0	Nonregulator
8	6	N/A	N/A	N/A	–	Not tested
	12	10	30	30	0	Nonregulator
9	6	0	30	10	67	Regulator
	12	20	40	30	25	Nonregulator
10	6	5	30	15	50	Regulator
	12	N/A	40	40	0	Nonregulator

Net mice footpad swelling is expressed in 10^{-4} inches (after negative control substraction).

response to donor antigen alone was not tested due to lack of sufficient cell numbers, a donor-sensitized phenotype in this patient could not be ruled out. As shown in Table 3, the four regulators did not develop rejection. In contrast, the five nonregulators and the sensitized patient developed DSA or showed C4d positivity on kidney biopsy.

Discussion

Our previous trial of alemtuzumab induction and sirolimus monotherapy was characterized by a relatively high incidence of early cellular and humoral acute rejection (5,6). Nonetheless, all but one of the early acute rejection episodes were reversed and, remarkably at 3-year follow-up, there was 93% graft survival (6). Studies of antibody production in that cohort showed that 10 of 24 patients developed alloantibody at some point post-transplant, although antibody later disappeared from the circulation in many of these (17). The high incidence of humoral injury led to a protocol modification, adding 2 months of tacrolimus treatment during the early post-transplant phase, and selecting well-matched donor–recipient pairs. This study is the result of applying that modification to 10 new patients, extensively monitored under sirolimus monotherapy. Nevertheless, we acknowledge that the small size of the current clinical series and the lack of a control group represent significant limitations to the study. The most notable findings in the current study are: (1) conventional acute rejection is rare with a limited course of tacrolimus, but some patients develop subacute humoral alloactivity and graft function deterioration, and (2) retrospective CKA *in vitro* in combination with DSA measurement by Luminex, and transvivo DTH reactions in mice were able to distinguish those patients with suboptimal evolution and alloreactivity. The safety profile of the immunosuppressive combination was remarkably good, particularly with respect to the absence of opportunistic infections and other adverse events despite profound lymphocyte depletion.

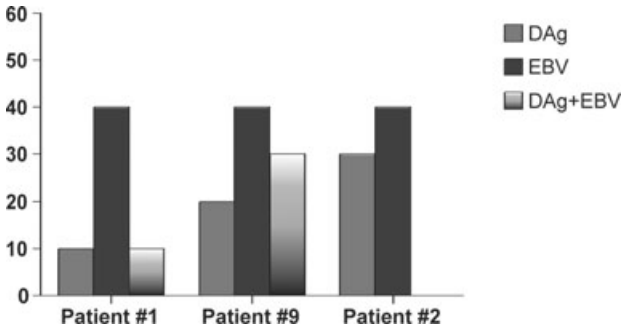

Figure 3: Transvivo DTH patterns 1 year after kidney transplantation measured by net mouse footpad swelling. Patient 1 is a Regulator, showing 75% of linked suppression after injection of combined donor antigen (dAg) and recall antigen (EBV). Patient 9 is a Nonregulator, with only 25% of linked suppression. Finally, Patient 2 is a sensitized patient, with intense response to donor antigen.

Alemtuzumab-induced depletion has been shown to result in a homeostatic expansion of memory T cells (18). This highly reactive T-cell subset, with the help of B-memory cells, may be responsible for an increased incidence of

antibody-mediated rejection, and tacrolimus was able to control that activity during the 2 months of treatment, with complete absence of rejection. Furthermore, 40% of the patients showed excellent graft function and complete absence of alloreactivity on low-dose sirolimus monotherapy. An additional 40% showed quite stable function, but subtle signs (asymptomatic C4d positivity in PTC, DSA class I and II production and/or borderline acute T-cell lesions) of instability and cellular and humoral alloreactivity. Reasons for this may be that immune cells that downregulate the alloantibody response are impaired by the treatment protocol, or that the immunosuppressive regimen permits upregulation of the alloantibody response in some patients, particularly those with equal response to donor antigen and third party by CKA or non-regulators by transvivo DTH. The significance of C4d staining in the PTC in patients without clinical or analytical graft function deterioration (patients 2 and 9) is not clear, although the detection of variable titers of DSA, the hyperresponsiveness by CKA and the sensitization or no regulation by DTH suggests relevant alloreactivity and bad prognosis, rather than 'accommodation' (19).

It was reported by Li et al. (20) and Martinez-Llordella et al. (21) that one of the markers of operational tolerance after living donor liver transplantation was an expansion of the V delta 1 positive cells. This is the first report showing alteration of CD3γ/δ repertoire after alemtuzumab induction in kidney transplant patients. One of the tolerogenic effects attributed to alemtuzumab treatment might be expansion of V delta 1 population. However, it is not known how V delta 1 expressing cells can contribute to induction of tolerance in kidney transplant patients.

The increase in CD4CD25+ cells was not related to reduced vulnerability of CD25 positive population to CD52-mediated lysis, but may reflect ongoing homeostatic expansion driven by IL-7. Increased frequency of cells expressing CD25 after alemtuzumab induction confirms previous observations in multiple sclerosis patients and kidney transplant patients (22,23). Bloom et al. (22) reported previously that FoxP3 Tregs significantly increase in patients treated with alemtuzumab.

Overall, alemtuzumab treatment promotes an increase of markers previously reported to be associated with tolerance induction in liver transplant patients where increased frequencies of V delta 1 and CD4/CD25/FoxP3 cells were observed. In addition to monitoring reconstitution in T-cell compartment, we looked at the reconstitution patterns in various B cell populations. It was previously shown that reconstitution of the memory compartment after treatment with B cell depleting antibody (Rituximab) might be also greatly delayed (24). In the study of Anolik et al. (24), patients with good clinical outcome had a B-cell repertoire dominated by the naïve B-cell population. Interestingly, SLE patients with shorter term or no clinical response to Rituximab treatment had faster memory B cell recovery. Reichardt et al. (25) showed that naïve B cells can play a role in generation of regulatory T-cells. We observed higher numbers of FoxP3 expressing cells along with higher percentages of B cells with naïve phenotype. In summary, we show that cell populations expressing FoxP3, V delta 1 g/d T cells, and naïve B cells, all implicated in tolerance maintenance, are increased in repopulating cell compartments after alemtuzumab treatment suggesting that the tested regimen causes changes at multiple levels that favor unresponsiveness.

The clinical attempts to develop protocols based on alemtuzumab induction and further maintenance minimization strategies are summarized in Table 5 (2,26–35). The absence of a control group in our pilot trial precludes any comparison between alemtuzumab-induced patients with further minimization and patients with more conventional immunosuppressive approaches, and represents a significant limitation of the current study. However, our objective was to test the hypothesis that short-term tacrolimus may avoid humoral rejection in this regimen, and our plan after these two uncontrolled initial experiences is to design a controlled trial with immune monitoring and selective, directed drug weaning and withdrawal only in those patients who do not appear to be at risk for alloantibody or T-cell alloresponses.

Another observation of this study is that the glomerular filtration rate (GFR) of these predominantly haploidentical living donor kidney transplants varied from 23 to 66 at 3 months, and six of ten patients had GFR <50 mL/min at 1 year. In order to compare this outcome to patients receiving standard immunosuppression at our center, we reviewed GFR at 1 year in all other (n = 138) haploidentical living donor renal transplant recipients transplanted during the same 3-year period, and found that their mean GFR was 49.7 mL/min according to MDRD calculation. Thus, while the results of this study are slightly below the average GFR for comparable transplants, given the small sample size of the study cohort, the difference is not remarkable. This median GFR is below that reported by Velosa et al. in another cohort of haploidentical donor renal transplants where the mean was 70 mL/min/1.73 m^2 by direct measurement (36). Of note was that the GFR of 9/10 patients remained stable throughout this study, with significant deterioration in the one patient with chronic antibody injury. With respect to the incidence and severity of interstitial fibrosis and arteriopathy on biopsy, the degree of injury was not remarkable in the study patients except for the one patient with chronic antibody injury.

The 6–8 year follow-up of the original cohort of 29 renal transplant patients induced with alemtuzumab at the University of Wisconsin shows that 26/28 (93%) remain alive and 24/28 (86%) have functioning grafts suggesting successful rescue from rejection and alloantibody. A shortcoming of the presently reported protocol was that 50% of patients developed alloantibody to either HLA class I or

Table 5: Clinical trials with alemtuzumab (C1H) induction and immunosuppression minimization 1998–2007

Series	Design	Maintenance immunosuppression	n	Alemtuzumab dose	AR	Follow-up	Evolution	Conclusion
Calne et al. (1998, 1999 and 2005)[2,26,27]	Retrospective with historical controls	Low dose CsA (vs. conventional triple therapy)	33 vs. 68	20 mg × 2	31.5% (14% after the 1st year)	5 years	Similar to conventional protocols	C1H allows maintenance low-dose CsA monotherapy
Kirk et al. (2003)[35]	Prospective observational	None by protocol	7	0.3 mg/kg × 3	100%, all reversible	446–950 days	100% needed maintenance IS, mainly SRL monotherapy (one recurrent FSGS needed long-term steroids)	C1H alone is not enough for KT
Knechtle et al. (2003 and 2006)[5,6]	Prospective observational	Sirolimus monotherapy	29	20 mg × 2 (Pts 25–29: Thymoglobulin+ 14-d steroids)	28% 1 year (17% humoral, most of them in <45 yr-olds)	3 years	57% sirolimus monotherapy at 3 yr	C1H allows sirolimus monotherapy but high rates of rejection
Ciancio et al. (2004)[28]	Prospective observational	Tacrolimus-MMF low dose	44	0.3 mg/kg × 2	9%	1–19 months	100% patient and graft survival, 38/44 steroid free	C1H with further bitherapy with low-dose Tac-MMF is safe
Ciancio et al. (2005)[29]	Prospective, RCT	Tacrolimus-MMF in all groups, but half doses and only 7-day steroids in C1H arm	30 vs. 30 vs. 30	0.3 mg/kg × 2 vs. daclizumab vs. thymo	16.6% in all groups	Median 15 months	No differences among the groups, more Tregs in C1H group	C1H with further bitherapy with low-dose Tac-MMF is safe
Vathsala et al. (2005)[30]	Prospective, RCT	low-dose CsA (n = 20) vs full dose CsA-AZA-steroids (n = 10)	20 vs. 10	20 mg × 2	25 vs. 20% (biopsy proven)	6 months (planned 36)	More infections in C1H group	Similar efficacy and higher morbidity with C1H
Shapiro et al. (2005)[31]	Retrospective with historical controls	Tacrolimus monotherapy (90 C1H, 101 thymo) or Tac+ prednisone and often a third agent usually MMF or Sirolimus (n = 152)	90 vs. 101 vs. 152	30 mg × 1	With C1H or controls, lower incidence and later onset than with Thymo	12–18 months	High rate of weaning to tacrolimus low-dose monotherapy with low rejection rates	C1H is more effective than thymoglobulin for immuno-suppression weaning

Continued.

Table 5. Continued.

Series	Design	Maintenance immunosuppression	n	Alemtuzumab dose	AR	Follow-up	Evolution	Conclusion
Flechner et al. (2005)[32]	Prospective observational	Sirolimus (8–12 ng/mL)-MMF 500 mf bid	22	30 mg × 2	36.3% (2 humoral)	1 year	Acute respiratory distress syndrome (n = 2), 27% MMF stop due to leukopenia	C1H allows sirolimus monotherapy but high rates of rejection and morbidity
Kirk et al. (2005)[33]	Prospective observational	Deoxyspergualin	5	0.3 mg/kg × 4	100%	2 years	All needed SRL monotherapy (two converted to tacrolimus)	C1H plus DSG is not enough for KT
Tan et al. (2006)[34]	Retrospective with historical controls	Tacrolimus monotherapy	205 vs. 42 (liv don)	30 mg × 1	32 episodes in 22 patients at 12 mo (10.7%) vs. 21.3% in controls	Median 493 282 d	Similar to conventional protocols	C1H allows maintenance low-dose tacrolimus monotherapy
Present study	Prospective observational	Tacrolimus and sirolimus, after 60 d, sirolimus alone	10	20 mg × 2	10%	15–28 mo	90% on sirolimus monotherapy since day 61	C1H allows maintenance low-dose sirolimus monotherapy

II including two with diffuse C4d+ biopsies and two with focally C4d+ biopsies. The next step might be to prolong tacrolimus coverage and withdraw it only in those patients at low risk of developing alloactivation and humoral rejection as detected by immune monitoring. DSA, CKA, transvivo DTH and B cell phenotype may be useful indicators of the immune responsiveness.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1: The kinetics of repopulation of various immune cell compartments as determined by flow cytometry are shown. V0 and V28 correspond to pretransplant and 12-month timepoints, respectively.

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