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Tolerance and Withdrawal of Immunosuppressive Drugs in Patients Given Kidney and Hematopoietic Cell Transplants

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Sixteen patients conditioned with total lymphoid irradiation (TLI) and antithymocyte globulin (ATG) were given kidney transplants and an injection of CD34+ hematopoietic progenitor cells and T cells from HLA-matched donors in a tolerance induction protocol. Blood cell monitoring included changes in chimerism, balance of T-cell subsets and responses to donor alloantigens. Fifteen patients developed multilineage chimerism without graft-versus-host disease (GVHD), and eight with chimerism for at least 6 months were withdrawn from antirejection medications for 1–3 years (mean, 28 months) without subsequent rejection episodes. Four chimeric patients have just completed or are in the midst of drug withdrawal, and four patients were not withdrawn due to return of underlying disease or rejection episodes. Blood cells from all patients showed early high ratios of CD4+CD25+ regulatory T cells and NKT cells versus conventional naive CD4+ T cells, and those off drugs showed specific unresponsiveness to donor alloantigens. In conclusion, TLI and ATG promoted the development of persistent chimerism and tolerance in a cohort of patients given kidney transplants and hematopoietic donor cell infusions. All 16 patients had excellent graft function at the last observation point with or without maintenance drugs.

Key words: Bone marrow transplantation, kidney transplantation

Abbreviations: ATG, antithymocyte globulin; CMV, cytomegalovirus; DCs, dendritic cells; EBV, Epstein Barr virus; FSGS-focal segmental glomerulosclerosis; GVHD, graft-versus-host disease; MLR, mixed leukocyte reaction; NKT cells, natural killer T cells; STR, short tandem repeats; TLI, total lymphoid irradiation.

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Introduction

Kidney transplantation is the treatment of choice for patients with end-stage renal failure. Immune rejection of allotransplants remains a major problem, and transplant recipients require the lifelong use of immunosuppressive drugs (1,2). Sideeffects associated with maintenance immunosuppression include diabetes, heart disease, cancer and infection (2–4). Although advances in immunosuppressive drug therapy have markedly reduced the incidence of early graft loss, gradual long-term graft loss remains an unsolved problem (4,5). Immune tolerance to the organ graft holds the promise of eliminating the sideeffects of maintenance antirejection drugs, and of preventing long-term graft loss due to chronic rejection and/or renal toxicity of these drugs.

The induction of tolerance and chimerism can be achieved in a variety of preclinical studies (6–15). A successful preclinical approach that required the injection of donor hematopoietic cells to induce tolerance was recently used in clinical studies (16–19). However, complications of graft versus host disease (GVHD) in HLA-matched patients who developed persistent chimerism, and pulmonary capillary leak syndromes, prolonged severe neutropenia, humoral rejections and graft loss in HLA haplotype-matched patients have limited the application of this approach (17,18). In order to minimize complications in this study, 16 HLA-matched kidney transplant patients were given a donor cell infusion of highly enriched $CD34^+$ progenitor cells mixed with $CD3^+$ T cells, following a conditioning regimen of total lymphoid irradiation (TLI) and anti-T-cell antibodies.

This conditioning regimen has been shown to be safe and protect against GVHD in preclinical models, and in clinical trials of 111 patients with leukemia and lymphoma followed for up to 8 years after hematopoietic cell transplantation from HLA-matched related or unrelated donors (20,21). We used this regimen in the HLA-matched kidney transplant patients as a proof of concept study to determine the

safety and feasibility of this protocol before proceeding to HLA-mismatched patients. Immunosuppressive drugs were withdrawn from 11 patients and 8 have been observed for at least 1–3 years thereafter without evidence of rejection. No severe complications were observed in the study patients, and all had excellent graft function at the last observation point. The first recipient was the subject of a case report, and a brief summary of the outcomes of the first 12 patients was the subject of a letter (19,22). We now provide the details of the outcomes and monitoring of an expanded group of 16 patients.

Materials and Methods

Patients

Sixteen patients with end-stage renal failure who were candidates for kidney transplantation, and who had donors matched for HLA A, B, C, DR, DQ and DP antigens by high-resolution DNA typing with the exception of patient #6 with one mismatch at the DP locus were enrolled in the study between 2005 and 2011. Details of each patient and causes of renal failure are shown in Table 1.

Conditioning of recipients and collection of donor hematopoietic cells

TLI was administered to recipients as 10 doses of 80 or 120 cGy each to the supradiaphragmatic lymph nodes, thymus, subdiaphragmatic lymph nodes and spleen during the first 10 days posttransplant as described previously (19). Rabbit antithymocyte globulin (ATG; Thymoglobulin, Genzyme) was given intravenously (1.5 mg per kilogram for each of 5 daily doses) starting with an intraoperative infusion. The protocol was approved by the Institutional Review Board of Stanford University (Protocol 13746; IRB # 5136), and all recipients and donors provided written informed consent.

Donors received a 5-day course of granulocyte colony stimulating factor at a dose of 16 mcg per kilogram per day, and mononuclear cells were recovered by one apheresis for the first 4 recipients and by two aphereses for the last 12 recipients to achieve the target dose of 10×10^6 /kg $CD34^+$ hematopoietic progenitor cells. $CD34^+$ cells were enriched with the use of an Isolex column (Baxter, Deerfield, IL, USA) or a CliniMax column (Miltenyi Biotec, Auburn, CA, USA) and cryopreserved until infusion into recipients. Column flow through cells were added back to $CD34^+$ cells to achieve a defined dose of 1×10^6/kg $CD3^+$ T cells in the infusion based on preclinical studies (12) except in one patient with systemic lupus. In the latter patient, 10×10^6/kg $CD3^+$ T cells were given to facilitate chimerism in the presence of immune hyperreactivity.

Donor cell infusion and chimerism

The number of infused donor hematopoietic progenitor cells and T cells are given in Table 1 for each patient. Serial chimerism measurements were performed using DNA from blood mononuclear cells enriched for T cells, B cells, NK cells and granulocytes on immunomagnetic beads (Miltenyi Biotec) coated with monoclonal antibodies to CD3, CD19, CD56 and CD15, respectively (19–21). Purity of enriched subsets of cells was at least 95% as judged by flow cytometric analysis. The percentage of donor-type cells was determined by analysis of polymorphisms in the lengths of short tandem repeats (STR) (19–21). The threshold for detection of chimerism by STR analysis is $\geq$1% of donor-type cells.

Peritransplant steroids and treatment of rejection episodes or underlying disease

All patients received intravenous methylprednisolone as premedication for the five daily infusions of ATG. Prednisone was administered thereafter, and discontinued on day 10 after kidney transplantation in 15 patients who were not receiving prednisone pretransplant or adjusted to return to the pretransplant dose in one patient with lupus. Tapering of immunosuppressive drugs in patients with recrudescence of underlying disease such as focal segmental glomerulosclerosis (FSGS) or lupus was interrupted in order to treat the disease activity, and resumed if activity resolved. Patients who developed rejection episodes were treated with corticosteroids, and ATG in one case (#6). The latter patients were placed on maintenance immunosuppressive drugs after resolution of the rejection episode and no further attempts at discontinuation were made. Recipients were given prophylactic medications against fungal, bacterial and viral infections.

Immunofluorescent staining and analysis of T-cell subsets

Blood mononuclear cells were stained with fluorochrome conjugated monoclonal antibodies against CD3, CD4, CD8, CD62L, CD45RA, CD45RO, CD25, CD19, CD127 (BD Pharmingen, San Diego, CA, USA), FoxP3 (eBiosciences, San Diego, CA, USA), and Vα24 and Vβ11 (Beckman Coulter, Brea, CA, USA). Multicolor flow cytometry was used to identify T-cell subsets with the use of standard techniques and equipment (LSR and FACS Vantage cytometers, BD Biosciences, Franklin Lakes, NJ, USA) (23).

In vitro *responses to alloantigens and recall antigens—patient selection and timing*

Transplant patients were monitored using mixed leukocyte reaction (MLR) assays once every 6 months posttransplant starting at month 12. When proliferative responses of cryopreserved posttransplant blood mononuclear cells to third-party irradiated stimulator mononuclear cells in the MLR were at least 10-fold above background, the responses to donor dendritic cells (DCs) were compared to those of cryopreserved pretransplant mononuclear responder cells (24). Donor DCs were used as stimulators instead of mononuclear cells, since the latter HLA-matched cells did not stimulate responses using pretransplant patient responder cells. Responses to recall antigens were performed at the same time. Posttransplant proliferative responses of patients #10 through 15 who stopped immunosuppressive drugs are pending recovery of posttransplant responses to third-party stimulator cells. Posttransplant proliferative responses of patients #6 and #9 who are maintained on immunosuppressive drugs are also pending recovery.

Statistical analysis

Comparisons of mean 3H-thymidine incorporation of replicate assays in the MLR using pretransplant versus posttransplant recipient samples stimulated with either irradiated donor DCs or irradiated third-party blood mononuclear cells were made using the paired Student's *t*-test. Similarly comparisons of pretransplant versus posttransplant mean 3H-thymidine incorporation of recipient blood mononuclear cells after stimulation with recall antigens were performed using the paired Student's *t*-test. Comparisons of absolute numbers, percentages and ratios of pre- and posttransplant lymphocytes, T cells and T-cell subsets for all patients used the paired Wilcoxon signed rank test. The Welch two-sample *t*-test was used to compare ratios of subsets between eight patients followed for more than 18 months who discontinued drugs and four patients followed more than 18 months who did not at each time point.

Table 1: Patient characteristics, conditioning and donor cell composition

Patients[1]	Age/Gender	ESRD cause	Total dose TLI (cGy)	CD34+ cell dose (×106/kg)	CD3+ cell dose (×106/kg)	Serum creatinine at last observation (mg/dL)	Duration off drugs[4]
1 (42 months)	48/M	Unknown	800	8.0	1	1.3	36 months
2 (72 months)	39/F	FSGS	800	8.4	1	0.9	–
3 (66 months)	24/M	Dysplasia	800	12.5	1	1.6	–
4 (50 months)	52/M	Unknown	1,200[2]	4.9	1	1.4	32 months
5 (48 months)	34/M	IgA	1,200	12.8	1	1.2	40 months
6 (47 months)	61/F	DM	1,200	12.2	1	1.2	–
7 (43 months)	23/F	SLE	1,200	16.5	10[3]	0.7	32 months
8 (40 months)	33/M	Reflux	1,200	16.6	1	0.9	34 months
9 (34 months)	29/F	Unknown	1,200	17.5	1	1.0	–
10 (33 months)	52/F	PKD	1,200	14.4	1	0.9	21 months
11 (25 months)	37/F	IgA	1,200	14.4	1	1.0	18 months
12 (22 months)	36/F	PKD	1,200	10.1	1	1.4	16 months
13 (14 months)	26/M	Unknown	1,200	6.6	1	1.0	1 month
14 (13 months)	22/F	Unknown	1,200	14.4	1	0.8	1 month
15 (12 months)	40/F	IgA	1,200	10.0	1	1.1	1 month
16 (4 months)	42/M	DM	1,200	6.0	1	1.5	TE

ESRD = end-stage renal disease; FSGS = focal segmental glomerulosclerosis; IgA = IgA nephropathy; DM = diabetes mellitus; SLE = systemic lupus erythematosus; PKD = polycystic kidney disease.

[1]Parentheses show duration of follow-up from kidney transplant.

[2]Dose increased to facilitate persistent chimerism.

[3]Dose used to facilitate persistent chimerism in SLE patient.

[4]Duration off antirejection drugs at last observation, dashes indicate patients on maintenance drugs; TE, too early to evaluate.

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Table 2: Patient safety outcome: leukopenia, GVHD, rejection episodes and infections

Patient #	Infection[1]	WBC nadir[4] ($\times 10^3$/mm^3)	Acute or chronic GVHD	Acute cellular[1] rejection episodes
1	None	0.6 (day 21)	0	0
2	None	1.7 (day 90)	0	0
3	None	1.6 (day 3)	0	1 (month 6)
4	None	1.1 (day 60)	0	0
5	None	1.2 (day 3)	0	0
6	V. zoster (month 4)	1.1 (day 4)	0	1 (month 2)
7	V. zoster (month 5) EBV[2](month19)	1.1 (day 5)	0	0
8	CMV[3] (month 2) V. zoster (month 18)	1.4 (day 5)	0	0
9	None	3.4 (day 70)	0	1 (month 12)
10	None	1.9 (day 24)	0	0
11	Pyelonephritis (month 2)	2.6 (day 24)	0	0
12	None	1.0 (day 4)	0	0
13	None	1.4 (day 45)	0	0
14	None	1.2 (day 5)	0	0
15	None	2.4 (day 28)	0	0
16	None	2.1 (day 5)	0	0

[1]Parentheses show time posttransplant of diagnosis of clinical rejection episode.
[2]Detected by PCR, associated with lupus flare.
[3]Primary infection with fever and malaise, confirmed by PCR.
[4]Nadir absolute neutrophil count-range (0.48–2.9 $\times$ 10^3 /mm^3); median (1.0 $\times$ 10^3 /mm^3).

Results

Transplantation protocol

Sixteen patients were conditioned with 10 doses of TLI and 5 doses of rabbit ATG during the first 10 days after kidney transplantation in order to facilitate engraftment of the donor cells and organ. Hospitalization for transplantation surgery was between 4 and 7 days (median 5 days). Donor CD34$^+$-selected cells and a defined dose of 1×10^6/kg T cells were injected intravenously on day 11 in the outpatient infusion center (Table 1). The rationale for the inclusion of donor T cells was based on our preclinical studies that demonstrated facilitation of chimerism after nonmyeloablative conditioning when T cells were included in bone marrow transplants as compared to when they were depleted (25). All patients were given a posttransplant immunosuppressive regimen that was adapted from previous single- and multicenter trials of hematopoietic cell transplantation for hematologic malignancies (20,21,26), and included 1 month of mycophenolate mofetil (2 g per day after cell infusion) and at least 6 months of cyclosporine starting at day 0 (blood levels at 2 h of 800 to 1200 mg/mL or trough levels of 250–300 mg/mL). As in the previous trials of hematologic malignancies (21,26), cyclosporine was tapered starting at 3 months and discontinued at about 6 months if patients had stable chimerism (<50% decline

Table 3: Patient safety outcomes: hospitalizations

		Hospital readmissions in first year			
Patient #	Transplant hospitalization (days)	Number	Time	Days	Diagnosis
1	6	1	Week 4	2	Neutropenic fever
2	7	0	–	–	–
3	4	0	–	–	–
4	7	1	Week 5	4	Ureteral stricture
5	5	0	–	–	–
6	4	1	Week 5	8	Rejection episode
7	5	0	–	–	–
8	5	0	–	–	–
9	6	0	–	–	–
10	4	0	–	–	–
11	4	1	Week 7	4	Pyelonephritis
12	5	0	–	–	–
13	4	0	–	–	–
14	4	0	–	–	–
15	4	0	–	–	–
16	5	0	–	–	–

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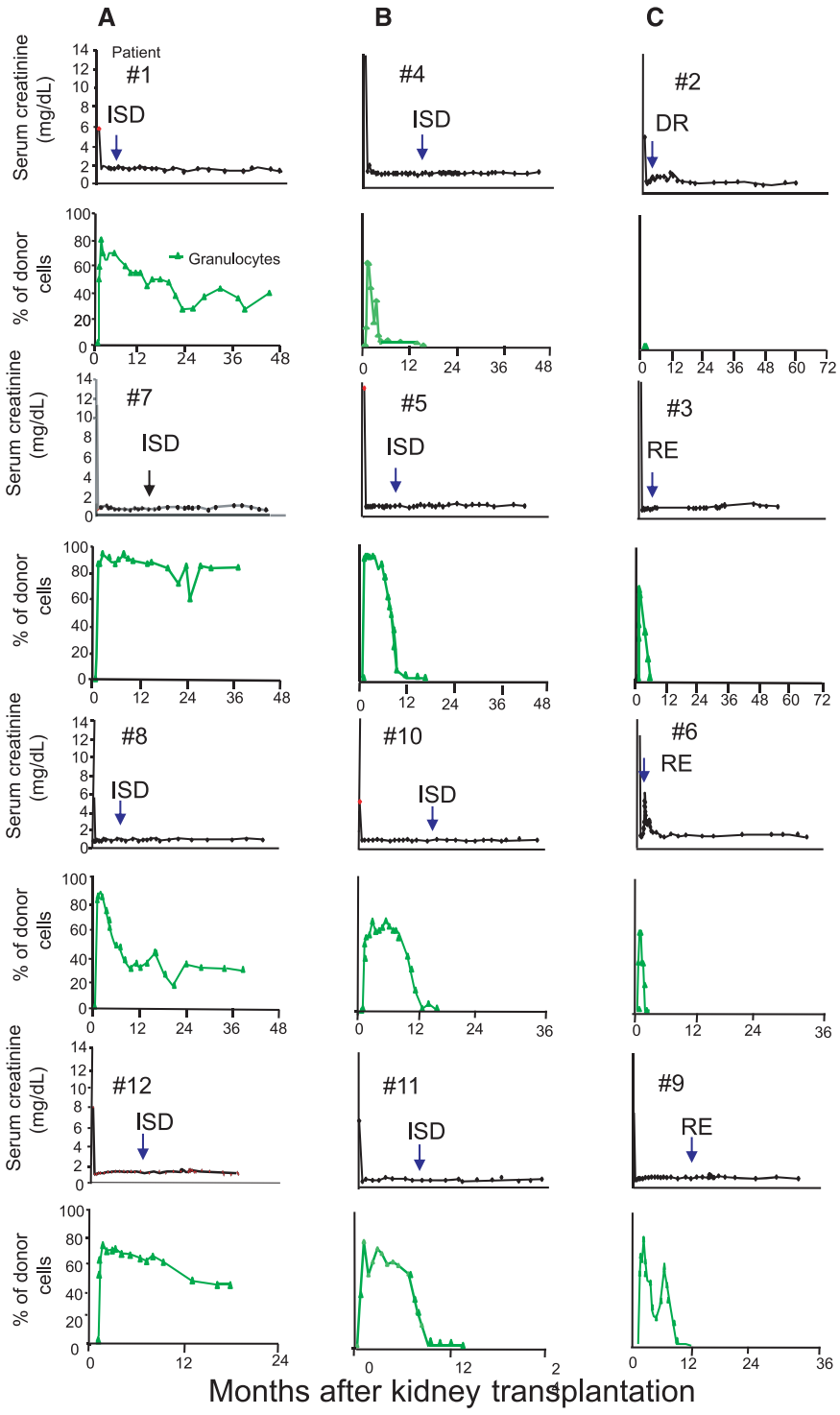

Figure 1: Serum creatinine concentrations, immunosuppressive drug withdrawal and chimerism among granulocytes in the blood of 12 patients followed for at least 18 months. (A) Shows serum creatinine concentrations, and percentage of donor-type cells measured by STR analysis at serial time points in patients #1, 7, 8 and 12 with persistent chimerism throughout the observation period who were withdrawn from immunosuppressive drugs. Arrows show time points at which immunosuppressive drugs were discontinued (ISD). (B) Shows the data for patients #4, 5, 10 and 11 with loss of chimerism (<1% donor-type cells) during the second year posttransplant who were withdrawn from immunosuppressive drugs before or after the loss of chimerism. (C) Shows the data for patient #2 with FSGS disease recurrence (DR) who never developed chimerism, and for patients #3, 6 and 9 who lost chimerism during the first or second year, and had an associated rejection episode (RE). Maintenance therapy for patient #3 is cyclosporine alone, for #9 is tacrolimus alone, for patient #2 is mycophenolate mofetil and cyclosporine and for patient #6 is tacrolimus and mycophenolate mofetil. Testing for chimerism was stopped in B and C after failure to detect chimerism in two consecutive blood samples.

in peak percentage of donor-type cells among white blood cells), and no evidence of GVHD. In the current trial, cyclosporine withdrawal was completed if there was no evidence of clinical rejection or of rejection in the surveillance biopsy at the time of withdrawal. Cyclosporine withdrawal was delayed in patients with declining chimerism (>50% decline from peak) during the first 6 months to ensure that a decline was not a harbinger of graft rejection.

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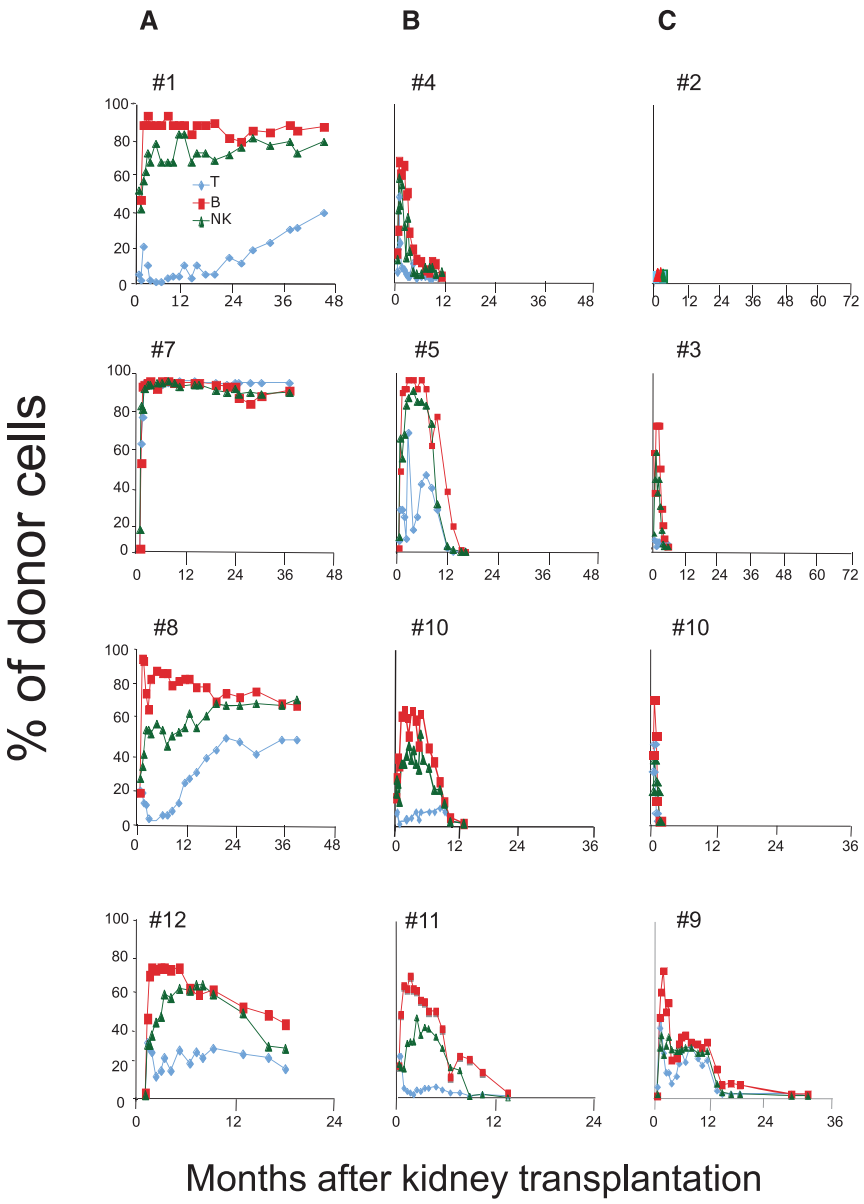

Figure 2: Chimerism among lymphocytes in the blood of 12 patients followed for 18 months. The percentage of donor-type cells among purified T cells, B cells and NK cells is shown before and at serial time points after kidney transplantation.

Assessment of safety

None of the 16 patients developed severe leukopenia (<500 cells/mm^3) (Table 2) or acute or chronic GVHD, pulmonary capillary leak syndromes or early humoral rejections. Four had return hospitalizations for either neutropenic fever, ureteral stricture, acute cellular rejection or pyelonephritis during the first year (Table 3). During the same interval, infection was diagnosed in one patient with cytomegalovirus (fever and malaise), three with varicella zoster, one with pyelonephritis and one with Epstein Barr virus (EBV) viremia associated with a flare of lupus (Table 2). Treatment of viral infections was given without hospitalization. Patient #10 was diagnosed with early-stage breast cancer, and after excision of a single tumor nodule there is no evidence of relapse. Patient #7 with lupus had immunosuppressive drug withdrawal delayed to 12 months due to a lupus flare, and reinstituted as needed to treat disease activity. Patient #1, who had a history of myocardial infarction and stent placement, died suddenly 42 months after transplantation during a mountain bicycle tour. No autopsy was performed. All other patients are alive and well at last follow-up.

Graft function, chimerism and immunosuppressive drug withdrawal after transplantation

Figure 1 shows the percentage of donor-type cells among blood granulocytes and serum creatinine concentrations at serial time points after transplantation in 12 patients who were followed for more than 18 months. Four patients with

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Table 4: Creatinine clearances, urinary protein excretion and surveillance biopsy grades

Patient #	Creatinine clearance (mL/min/1.73m^2)[1]	Urinary protein (mg/24 h)[1]	Surveillance biopsy at drug withdrawal (AR/IFTA)[2]	Follow-up surveillance biopsy (AR/IFTA)[2]
1	–	–	0/I	0/I
2	55	186	NA	NA
3	63	<150	NA	NA
4	57	<150	0/I	0/I
5	97	<150	0/0	0/0
6	58	<150	NA	NA
7	107	<150	0/0	0/0
8	99	<150	0/I	0/I
9	95	<150	NA	NA
10	86	231	0/0	0/0
11	85	<150	0/0	0/I
12	71	224	0/I	0/I
13	93	158	0/I	–
14	105	<150	0/0	–
15	96	<150	0/I	–
16	83	411	NA	NA

[1]Creatinine clearance and urinary protein within 12 months of last observation point or biopsy. Patient # 2 had recurrence of FSGS. Surveillance biopsies were not performed on patients # 2, 3, 6 and 9 on maintenance drugs.

[2]Banff '07 classification of renal allograft pathology. AR (acute rejection) Grade-0 indicates no evidence of rejection. IF/TA (interstitial fibrosis and tubular atrophy) Grade-0 indicates no evidence of fibrosis or atrophy. I—indicates lowest score with less than 25% of cortical area affected on a I–III scale. Biopsies were obtained at time of immunosuppressive drug discontinuation in patients # 1, 4, 5, 7, 8 and 10–15, and repeated 6 months later in # 1, 12 months later in # 4, 7, 8, 10, 11 and 12 and 18 months later in # 5, and all were unchanged. There was no evidence of tubulitis, intimal arteritis, mononuclear cell interstitial inflammation, glomerulitis or chronic allograft glomerulopathy in any of these biopsies, and all were scored as Grade 0 for these features.

NA, not applicable in patients without antirejection medication withdrawal.

stable mixed chimerism (column A) and 4 patients with declining chimerism (column B) had antirejection drugs discontinued. Granulocyte chimerism levels were representative of levels in white blood cells. The duration of granulocyte chimerism was shorter than that for lymphocytes including NK cells, T cells and B cells as shown in Figure 2. Chimerism of DCs was not analyzed. The lowest levels of chimerism were found among T cells. The mean time point of drug discontinuation was 9 months. These patients had no evidence of rejection before immunosuppressive drug discontinuation (ISD), and at 16, 18, 21, 32, 32, 34, 36 and 40 months (median 32 months) after discontinuation with serum creatinine levels in the range of 0.8–1.6 mg/dL at the last observation point (Figures 1A and B and Table 1). Table 4 summarizes data showing lack of rejection as judged by creatinine clearances, proteinuria and histopathologic scoring of tubular atrophy, fibrosis and injury on surveillance biopsies.

Patients in Figure 1, column C, were not withdrawn from immunosuppressive drugs because of underlying disease recurrence (DR) with failure to develop chimerism, or rejection episodes (RE) during the tapering of cyclosporine. Patient #2 had recurrence of FSGS with nephrotic range proteinuria during the first week after transplantation confirmed by the kidney biopsy. Patients #3 and 6 had rejection episodes (Banff IB and Banff IIA) associated with the rapid loss of chimerism in all lineages tested during immunosuppressive drug reduction in the first 6 months. Patient #9 had a rejection episode (Banff IA) during withdrawal of cyclosporine at about 12 months. All rejection episodes were cellular with negative complement fragment, C4d, staining and were reversed with standard medications. None of these patients with treated rejection episodes had evidence of graft dysfunction or chronic rejection thereafter (Table 4) with follow-up between 34 and 66 months after transplantation.

Four patients who were followed for less than 18 months were also monitored for changes in blood lymphocyte and granulocyte chimerism, and serum creatinine concentrations (Figure 3). Three of the four had declining chimerism, and it is too early to determine the stability of chimerism in patient #16. Patients #13–15 have just completed immunosuppressive drug withdrawal, and #16 is in the midst of withdrawal. Thus, a total of 11 patients were withdrawn, and none have had rejection episodes.

Changes in T-cell subsets after transplantation

Marked depletion of recipient naive T cells, and an altered balance of naive $CD4^+$ and $CD8^+$ T cells versus regulatory natural killer T (NKT) cells and $CD4^+CD25^+$ Treg cells (induced by TLI and ATG), favoring the regulatory cells were required for graft acceptance in the preclinical tolerance model (27). Figure 4(A) shows that there was a sharp reduction, at day 28 posttransplant, in the absolute lymphocyte count, the percentage of T cells among lymphocytes and the percentage of naive ($CD62L^+$

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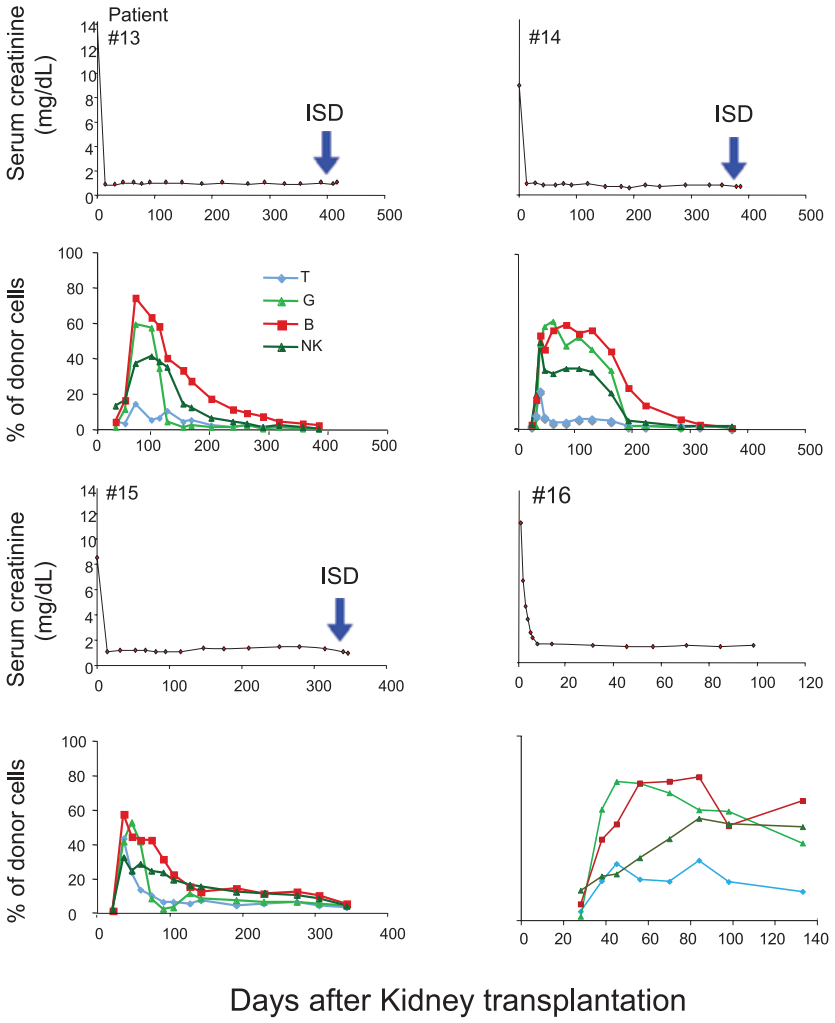

Figure 3: Serum creatinine concentrations, immunosuppressive drug withdrawal and chimerism among lymphocytes and granulocytes (G) in four patients followed for less than 18 months.

CD45RA$^+$) cells among T cells in all 12 patients followed for more than 18 months. The combination of reductions resulted in about a 200-fold and 30-fold reduction in the mean absolute number of naive CD4$^+$ and CD8$^+$ T cells, respectively (Figure 4A). In contrast to the reduced percentage of naive T cells, the percentage of CD4$^+$CD25$^+$ Treg cells among CD4$^+$ T cells increased at the same time point, and the mean ratio of Treg to naive CD4$^+$ T cells increased about 30-fold (Figure 4B). The Treg cells expressed the FoxP3$^+$CD127$^-$ phenotype (Figure 5B). Similarly, the percentage of $V_\alpha 24V_\beta 11$ NKT cells among CD3$^+$ T cells increased at day 28, and the ratio of NKT cells to naive CD3$^+$ T cells increased about 30-fold (Figures 4B and C). The changed ratios were due to the greater reduction of the absolute number of naive as compared to the reduction in the absolute number of regulatory T cells, and gradually shifted toward the pretransplant values during the first 6 months (Figures 4A and B). There were no expansions of the number of regulatory T cells above pretransplant levels. The gradual increase in the absolute number of CD4$^+$ and CD8$^+$ T cells continued during the second through fourth years in 10 patients with the longest follow-up (Figure 5A). There were no significant differences in the ratios of Treg and NKT cells to naive T cells on days 0, 28 and 190 between the patients successfully withdrawn from immunosuppressive drugs, and those who were not ($p > 0.05$–0.1). It is of interest that the recovery of T cells was slowest among the three patients with rejection episodes (# 3, 6, 9) who are on maintenance immunosuppressive drugs (Figure 5A).

Specific unresponsiveness to donor alloantigens in patients off drugs

In order to determine whether there was a specific loss of immune responses to donor alloantigens in patients off drugs, the *in vitro* immune responses to third-party and donor alloantigens and to microbial recall antigens were determined during the second year in four patients who discontinued immunosuppressive drugs. Details concerning selection of patients for assays and assay timing are described in the Materials and Methods section. Figure 6(A) shows representative examples of two of the four patients (#4 and 8). Posttransplant responses to donor

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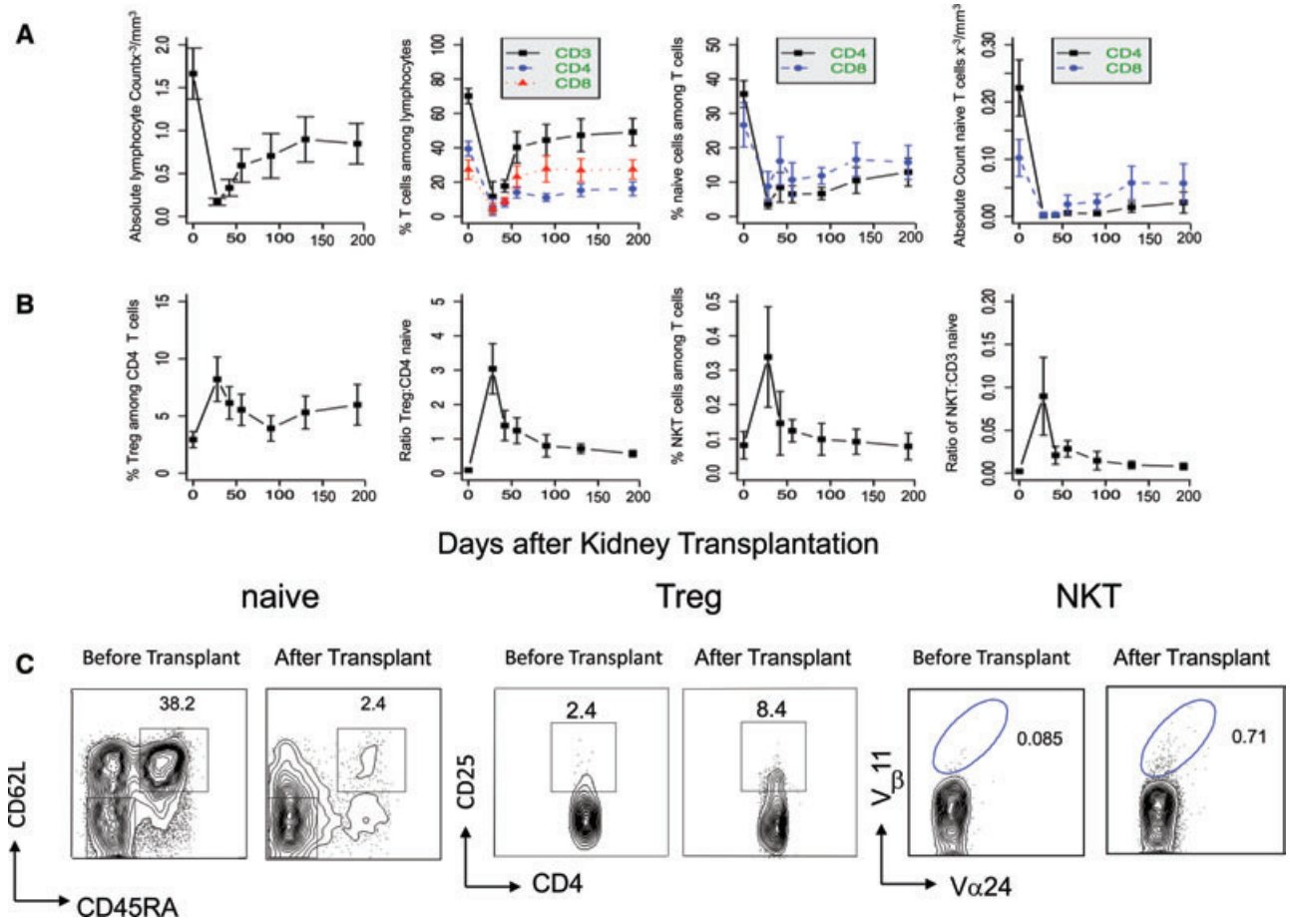

Figure 4: Early changes in T-cell subsets in the blood of 12 transplant patients observed for at least 18 months. The posttransplant conditioning from days 1 through 10 induced severe lymphopenia, and there were insufficient cells to perform subset monitoring in all patients until day 28. (A) Shows the changes in mean absolute numbers of lymphocytes, mean percentages of $CD3^+$, $CD4^+$ and $CD8^+$ T cells among lymphocytes, mean percentages of naive ($CD62L^+CD45RA^+$) cells among $CD4^+$ and $CD8^+$ T cells, and absolute numbers of naive $CD4^+$ and $CD8^+$ T cells at serial time points for patients before and at 28, 56, 90, 130 and 190 days after transplantation. Brackets show 90% confidence limits. (B) Shows the mean percentages of $CD4^+CD25^+$ Tregs among $CD4^+$ T cells, the mean ratios of Treg to naive $CD4^+$ T cells, mean percentages of NKT cells among $CD3^+$ T cells and mean ratios of NKT cells to naive $CD3^+$ T cells. All reductions in the day 0 versus day 28 and 190 means were significant for ALC ($p = 0.0004$ and 0.006), percentage T cells ($p = 0.003$ and 0.012), percentage naive among $CD4^+$ cells ($p = 0.004$ and 0.002), percentage naive cells among $CD8^+$ ($p = 0.001$ and 0.02), absolute number of naive $CD4^+$ T cells ($p = 0.0004$ and 0.0004) and absolute numbers of naive $CD8^+$ T cells ($p = 0.0004$) with the exception of the latter at day 190 ($p = 0.12$). All increases in the day 0 versus day 28 and 190 means were significant for percentage Tregs ($p = 0.002$ and 0.03), ratio of Tregs:naive ($p = 0.0004$ and 0.0004), ratio of NKT:naive ($p = 0.0009$ and 0.01) and percentage NKT cells ($p = 0.01$) with the exception of the latter at day 190 ($p = 0.72$). (C) Shows representative examples of two color flow cytometry analyses at days 0 and 28 for naive (CD62L vs. CD45RA) T cells among gated $CD4^+$ T cells, Treg (CD25 vs. CD4) cells among gated $CD4^+$ T cells and NKT cells ($V_\beta 11$ vs. $V_\alpha 24$) among gated $CD3^+$ T cells. Boxes or ellipses enclose naive, Treg and NKT cells, and percentages of enclosed cells are shown.

alloantigens were significantly reduced ($p = 0.0007$–0.01) as compared to the pretransplant values, and posttransplant responses to third-party alloantigens and microbial antigens (tetanus, cytomegalovirus and influenza) were not significantly reduced ($p > 0.05$). Two of four patients (#2 and 3) who were not withdrawn from drugs were also tested during the second year while on maintenance immunosuppressive drugs (Figure 6B). In contrast to patients off drugs, their pre- and posttransplant responses to donor alloantigens were not significantly different ($p = 0.12$–0.75).

Discussion

The goal of this study was to determine the safety and feasibility of planned withdrawal of immunosuppressive drugs after combined kidney and hematopoietic cell transplantation in HLA-matched patients. The low-intensity conditioning regimen of TLI and ATG did not extend the conven-

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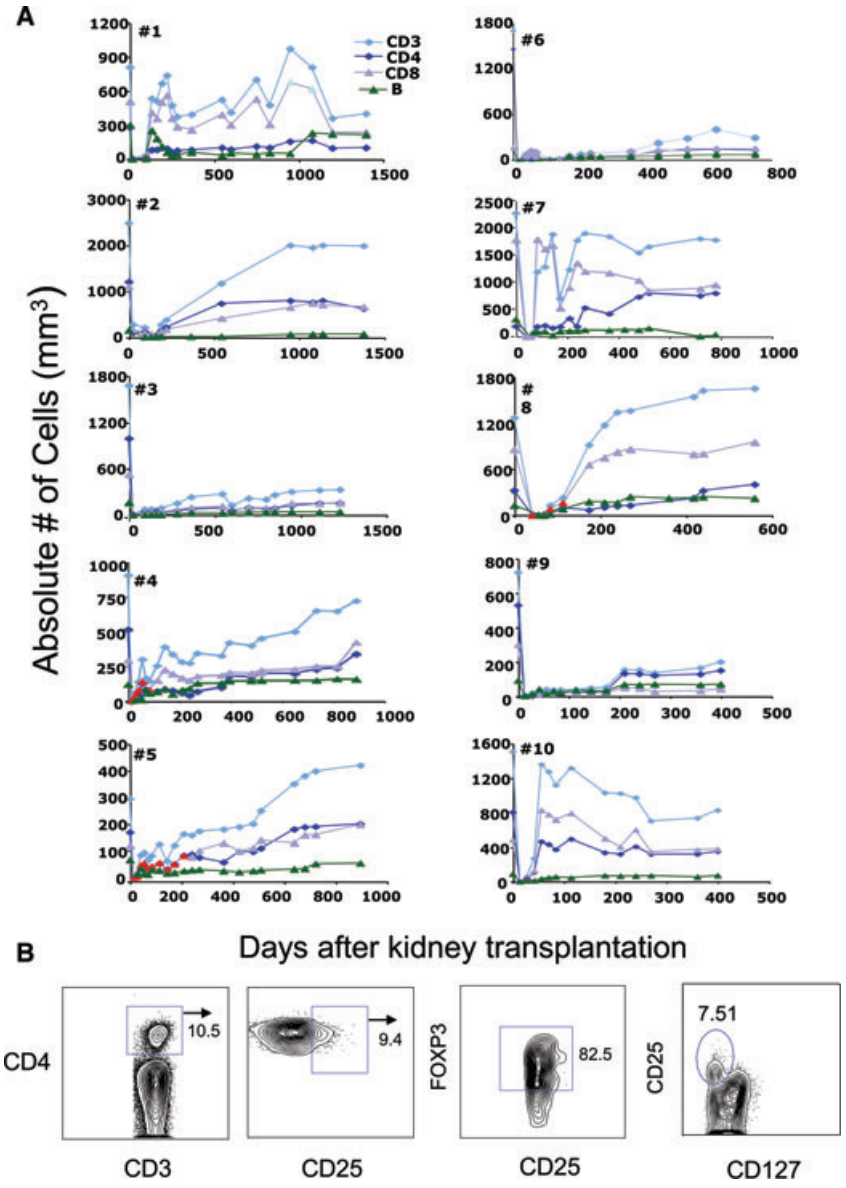

Figure 5: Changes in the absolute numbers of T-cell subsets and B cells for 1–4 years, and representative example of CD127 and intracellular FoxP3 staining of $CD4^+CD25^+$ Treg cells. (A) The absolute numbers of total $CD4^+$, $CD8^+$ and $CD3^+$ T cells and $CD19^+$ B cells in the blood before and at serial time points after transplantation with follow-up from 400 to 1400 days in the first 10 patients are shown. Y-axis labels are variable to reflect variability in starting absolute counts. (B) Shows representative staining of Treg cells. CD4 versus CD3 among gated $CD3^+$ T cells in a posttransplant blood sample with a high Treg/CD4 naive ratio is shown on the left panel, for CD4 versus CD25 among gated $CD4^+$ cells in the adjacent panel, for CD25 versus FoxP3 among gated $CD4^+CD25^+$ cells in the adjacent panel and for CD127 versus CD25 on gated $CD4^+$ cells in the far right panel. Percentage of cells enclosed in each box or ellipse is shown.

tional transplant hospitalization (median 5 days), and can be adapted for the use of deceased donor grafts because it is administered posttransplant. Although successful immunosuppressive drug withdrawal after kidney transplantation with hematopoietic cell transplantation has been reported previously, severe adverse events including GVHD in HLA-matched patients with persistent chimerism, graft loss, capillary leak syndromes and prolonged severe neutropenia in HLA haplotype-matched patients, have limited the use of these protocols with intensive pretransplant conditioning (17,18).

In this study these adverse events were not observed in the 11 patients who completed withdrawal or in one who is in the midst of withdrawal of antirejection drugs or in the four patients who failed to be withdrawn. Viral infections with EBV, varicella zoster and CMV were observed, and effectively treated without hospitalization. At the last observation point, all 16 patients had good graft function without evidence of rejection up to 72 months after transplantation, and up to 40 months off antirejection drugs. Persistent mixed chimerism has been shown to promote tolerance by inducing clonal deletion in preclinical models (11,12), and was observed for at least 6 months in all patients successfully withdrawn from immunosuppressive drugs. However, a requirement for chimerism for successful drug withdrawal was not proven, since we did not have a control patient group without hematopoietic cell infusion. In the preclinical model, hematopoietic cell infusion was required for tolerance, and grafts were uniformly rejected in recipients without infusion (11,12). Chimerism was not sufficient to prevent rejection during withdrawal in three

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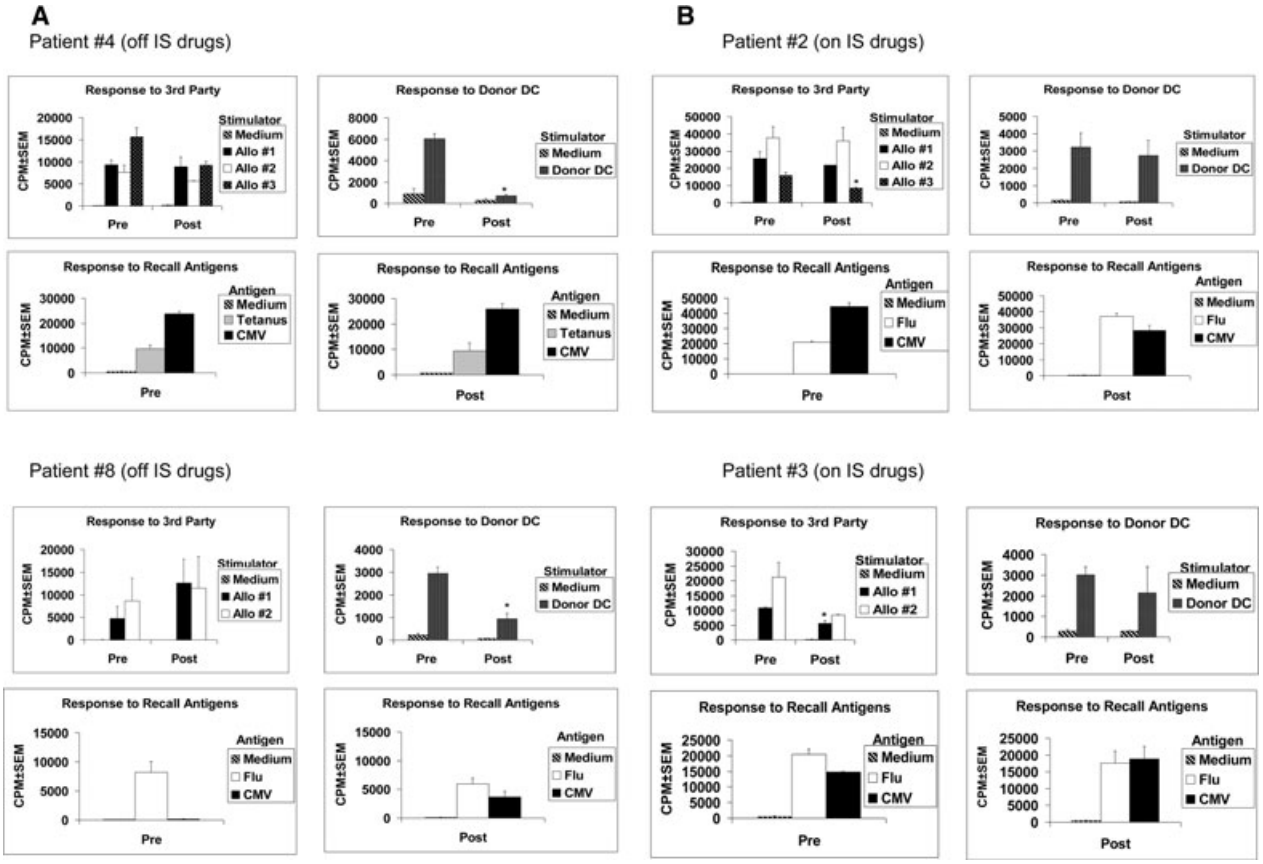

Figure 6: *In vitro* immune responses of patients to donor alloantigens, third-party alloantigens and to microbial recall antigens. The Panels in (A) show representative responses from two (#4 and 8) of four patients who were withdrawn from immunosuppressive drugs, and had *in vitro* assays performed during the second- or third-year posttransplant. Assays in patients #4 and 8 were performed 17 and 13 months, respectively, posttransplant. The patient responses to HLA-unmatched third-party stimulator cells from normal individuals (allo#1–allo#3) had recovered such that there were no statistically significant decreases ($p > 0.05$) in the 3H-thymidine incorporation (mean cpm+/− SE) from 3–6 wells when the posttransplant samples were compared to the pretransplant samples as judged by the paired Student's t-test. In contrast, the posttransplant responses to irradiated HLA-matched donor dendritic cells (DCs) were significantly decreased ($p = 0.0007$–0.01) as compared to pretransplant responses (right upper panels, asterisks show significant differences). The left and right lower panels show the mean counts per minute (cpm) for responses of patient mononuclear cells obtained pre- or posttransplant, respectively, to recall antigens including tetanus toxoid, influenza (Flu), and/or cytomegalovirus (CMV). There were no significant decreases ($p > 0.3$) in posttransplant as compared to pretransplant recall responses. Control cultures with responder cells without stimulator cells or recall antigens (Medium) are shown for each assay. Note that the range of cpm values for stimulation with HLA-matched DCs is lower than that for stimulation with HLA-unmatched third-party mononuclear cells. The pattern of responses from patients #4 and 8 were similar to those from patients #1 and 5 (not shown). Panels in (B) show responses from two (#2 and 3) patients who were not withdrawn from immunosuppressive drugs. The left upper panels for patient #2 show that during month 17 posttransplant, the patient's responses to third-party stimulator cells from normal individuals (allo#1–allo#2) were not statistically significantly decreased ($p = 0.5$–0.9), but the response to allo#3 was significantly decreased ($p = 0.02$) as compared to the pretransplant responses. The posttransplant response to irradiated donor DCs was not significantly decreased ($p = 0.5$) as compared to the pretransplant response (right upper panels). The left and right lower panels show the mean cpm for responses of patients' mononuclear cells obtained pre- or posttransplant, respectively, to recall antigens. There were no significant decreases in posttransplant as compared to pretransplant recall responses ($p > 0.3$). The left upper panels for patient #3 show that during month 20 posttransplant, the response to third-party allo#1 stimulator cells was significantly decreased ($p = 0.01$), but the response to allo#2 was not ($p = 0.08$). The posttransplant response to donor DCs was neither significantly decreased ($p = 0.8$) nor were responses to recall antigens ($p > 0.3$).

patients in this study. It remains unclear whether successful drug withdrawal in this study requires a minimum duration and/or level of chimerism. Since we measured chimerism only in the blood, it is possible that chimerism persisted in the thymus, lymph nodes and/or spleen after loss in the blood.

In the preclinical studies of combined organ and bone marrow graft acceptance using TLI and antithymocyte antibod-

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ies, there was a much greater reduction of immunocompetent host naive T cells as compared to host regulatory $CD4^+CD25^+$ T cells and NKT cells. The latter regulatory T-cell subsets were required to prevent donor cell and organ rejection in the early posttransplant period (12,27,28). A similar change was observed in this study of patients, however, a requirement for the host regulatory cells to prevent early rejection could not be proven, and the change was not sufficient to prevent rejection in some patients. In previous preclinical and clinical studies (8–10,18,19), and in this clinical study, the development of specific unresponsiveness to donor alloantigens in the mixed leukocyte reaction was observed in recipients with long-term graft acceptance without immunosuppression.

The TLI and ATG conditioning regimen was used previously to induce tolerance in recipients of HLA haplotype-mismatched kidney and hematopoietic cell transplants from related living donors as well as in recipients of HLA-mismatched transplants from unrelated living donors (16). Six recipients were enrolled in this protocol in 2000–2004, and criteria for drug withdrawal were development of chimerism regardless of duration, and specific unresponsiveness to donor cells in the MLR. Two recipients met criteria, and were completely withdrawn, but were returned to maintenance drugs 4–6 months later due to rejection episodes (Scandling, J., et al. unpublished data). Chimerism in the latter patients was lost by 3 months. However, the induction protocol used in the mismatched study differed from that in this study in three ways; in this study the total dose of TLI was increased from 800 to 1200 cGy, the target dose of donor $CD34^+$ progenitors was increased from 5 to 10×10^6 cells/kg and the target dose of donor $CD3^+$ T cells was increased from 1×10^4 to 1×10^6 cells/kg. All of these increases were designed to promote the persistence of chimerism in this study and have been adapted to a follow-up study of HLA haplotype-mismatched patients. It is of interest that there has been no kidney transplant loss due to rejection or graft dysfunction among the 6 HLA-mismatched and 16 HLA-matched patients enrolled in the TLI and ATG protocols with up to 10 years follow-up in the combined group of 22 patients. In conclusion the TLI and ATG protocol was well tolerated, and the majority of HLA-matched recipients could be withdrawn from immunosuppressive drugs. The protocol has the potential to improve the quality of life of transplant patients by eliminating the side effects of immunosuppressive drugs and to reduce annual health-care costs by eliminating the considerable costs of these drugs.

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Disclosure

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