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Tolerance Induction in HLA Disparate Living Donor Kidney Transplantation by Donor Stem Cell Infusion: durable chimerism predicts outcome¹

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Abstract

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Background—We recently reported that durable chimerism can be safely established in mismatched kidney recipients through nonmyeloablative conditioning followed by infusion of a facilitating cell (FC)-based hematopoietic stem cell transplant termed FCRx. Here we provide intermediate-term follow-up on this phase 2 trial.

Methods—Fifteen HLA mismatched living donor renal transplant recipients underwent low intensity conditioning (fludarabine, cyclophosphamide, 200cGyTBI), received a living donor kidney transplant on day 0, then infusion of cryopreserved FCRx on day +1. Maintenance immunosuppression(IS),consisting of tacrolimus and mycophenolate, was weaned over one year.

Results—All but one patient demonstrated peripheral blood macrochimerism post-transplantation. Engraftment failure occurred in a highly sensitized (PRA of52%) recipient. Chimerism was lost in 3patients at 2, 3, and 6 months post transplantation. Two of these subjects had received either a reduced cell dose or incomplete conditioning; the other 2 had PRA >20%. All demonstrated donor-specific hyporesponsiveness and were weaned from full dose immunosuppression. Complete immunosuppression withdrawal at one year post-transplant was successful in all patients with durable chimerism. There has been no GVHD or engraftment syndrome. Renal transplant loss occurred in 1 patient who developed sepsis following an atypical viral infection. Two subjects with only transient chimerism demonstrated subclinical rejection on protocol biopsy despite donor-specific hyporesponsiveness.

Conclusions—Low intensity conditioning plus FCRx safely achieved durable chimerism in mismatched allograft recipients. Sensitization represents an obstacle to successful induction of chimerism. Sustained T cell chimerism is a more robust biomarker of tolerance than donor-specific hyporeactivity.

Keywords

hematopoietic chimerism; facilitating cell; kidney transplant; nonmyeloablative conditioning; T cell chimerism; tolerance testing

Introduction

The toxicity of immunosuppressive agents that remain the current mainstay of solid organ transplantation has prompted investigators to pursue the induction of donor-specific tolerance. It has been known since the early reports of Owen in 1945(1) and Billingham et al in 1953(2) that chimerism induces tolerance to organ and tissue transplants. Owen observed that genetically disparate “freemartin” cattle twins sharing a common placenta were red blood cell chimeras, suggesting that each was reciprocally tolerant to the other sibling as evidenced by persistent chimerism after birth. Billingham, Brent and Medawar extended these findings to demonstrate that infusion of hematopoietic-derived cells into newborn mice resulted in chimerism and was associated with acceptance of donor skin grafts. In the ensuing years, significant effort was focused on overcoming obstacles preventing this approach from successful translation to the clinic: graft-versus-host disease (GVHD), the requirement for HLA-matched bone marrow donors, and the toxicity of myeloablative conditioning.

We have developed a nonmyeloablative reduced-intensity conditioning approach to establish high levels of donor chimerism without GVHD or engraftment syndrome in recipients of combined kidney/hematopoietic stem cell transplants (HSCT) (3). We recently reported the outcomes on the first eight subjects enrolled who were at least one year post-transplantation. Five of six recipients transplanted with the optimum facilitating cell/HSCT product (FCRx) and who received the full reduced intensity conditioning regimen demonstrated durable whole blood and T cell chimerism and were successfully weaned completely off

immunosuppression. The other chimeric subject developed viral sepsis at three months following transplantation, lost his kidney to vascular thrombosis and was subsequently successfully re-transplanted. No patient developed GVHD or engraftment syndrome. We now present an interim follow-up for 15 subjects currently enrolled and transplanted. We have prospectively evaluated functional assays of donor specific hyporesponsiveness (DSH), comparing durably chimeric recipients to those with transient macrochimerism and the one subject who failed FCRx engraftment in order to identify potential disparities in biomarkers to predict the ability to reduce or successfully discontinue immunosuppression. We report here that mixed lymphocyte reaction proliferative (MLR) and cytotoxicity (CML) assays were not reliable predictive biomarkers, as two subjects who exhibited transient macrochimerism had evidence of subclinical rejection on protocol biopsy at the time they exhibited DSH *in vitro*. The most predictive endpoint for tolerance was durable T cell and whole blood macrochimerism.

Results

Patient demographics

A total of 17 subjects have been enrolled and 15 have been transplanted in this phase II FDA regulated study (IDE 13947). They ranged in age from 18 to 60 years. All subjects were HLA-disparate from their living kidney/HSCT donors, ranging from five of six matched related to 0 of 6 matched unrelated. The patient demographics are shown in Table 1. Subject #13 developed *de novo* donor-specific antibody just prior to conditioning and was not transplanted as part of our study. Subject #16 developed positive serologies for coccidioides (residing in a high risk endemic area) after enrollment but also prior to conditioning and is undergoing antifungal therapy.

Chimerism testing and outcomes

Subjects have segregated into different categories with respect to observed patterns of donor stem cell chimerism. Nine patients developed high level (>90%) donor whole blood and T cell chimerism beginning at one month post-transplant. This chimerism has been durable in all but one patient (NW2) who developed an atypical viral infection at three months post-transplant accompanied by bone marrow failure. Despite successful recovery after infusion of stored autologous stem cells, he developed intercurrent sepsis which contributed to kidney allograft loss and loss of chimerism; this patient has been successfully re-transplanted with a living donor kidney and currently has normal renal function. Six of eight patients with high level donor chimerism have been fully withdrawn from immunosuppression without loss of engraftment. The remaining two have undergone successful weaning to tacrolimus monotherapy with planned full withdrawal at one year.

Transient donor chimerism lasting several months was observed in three subjects. Two (NW1 and NW4) received an FCRx with a reduced cell dose. In NW1, technical issues with FCRx processing of the donor leukopheresis product required a subsequent iliac crest bone marrow harvest to derive the FCRx. In addition, no post-transplant dose of cyclophosphamide conditioning was given. In Subject NW4, a deliberately reduced FCRx dose was used in response to the development of a generalized body rash concerning for GVHD in the previously transplanted subject (NW3). As skin biopsy results were pending at the time of product infusion for NW4, we elected to reduce the FCRx cell dose infused per clinical trial design; the biopsy was consistent with bacrim photosensitivity, not GVHD. The third transiently chimeric subject (NW11) was notable for being highly sensitized, with an historic maximum HLA Class I PRA of 64%, and an actual PRA of 33% at the time of transplant (Table 1A).

Two subjects (NW12 and NW17) have exhibited a picture of more sustained, mixed chimerism. In NW12, initial whole blood chimerism at one month of 77% was detected, but no measurable T cell chimerism was present. A gradual loss of whole blood chimerism occurred over the next nine months. Maintenance immunosuppression was weaned to tacrolimus monotherapy at six months as this subject exhibited DSH, had not developed DSA, showed measurable donor chimerism, and normal protocol kidney biopsy. Of interest, low level (8%) whole blood and T cell chimerism re-emerged at one year post-transplant. Further weaning of immunosuppression is pending future demonstration of stable mixed chimerism, as the loss of measurable chimerism in other subjects (as discussed below) has been predictive of the development of subclinical allograft rejection. In NW17, mixed whole blood (87%) and T cell (48%) donor chimerism was initially established, and has been stable for the first three months of post-transplant follow-up.

Complete failure of donor stem cell engraftment has occurred in only one subject, (NW9). This was the recipient of a completely HLA mismatched, living unrelated stem cell/kidney allograft. This subject was highly sensitized at the time of transplant (Class I PRA of 52%). This patient developed tacrolimus-induced hemolytic uremic syndrome early post-transplant requiring conversion to sirolimus. At six months he had a normal protocol biopsy and no evidence of DSA, which prompted weaning of immunosuppression to sirolimus monotherapy. At one year post-transplant he presented with new onset proteinuria and his kidney allograft biopsy showed evidence of recurrent IgA nephropathy. *In vitro* DSH was no longer present. He has been treated with a course of oral corticosteroids and maintained on sirolimus for maintenance immunosuppression with evidence of stable renal allograft function.

Clinical course: cytopenias and infections

All subjects were discharged on postoperative day 2 to a clinical research unit and thereafter managed as outpatients. Patients experienced an expected nadir period associated with conditioning, with an absolute neutrophil count (ANC) <500 cells/uL lasting for a mean of 10 days (range 2–14 days) and thrombocytopenia (platelet count of <50,000/uL) lasting for a mean of 12 days (range 0–21 days). Cytopenias were managed with neutropenic precautions, administration of G-CSF, and platelet transfusions for a platelet count of less than 20,000. No CMV infections have occurred. Three subjects developed single dermatome herpes zoster while still on conventional immunosuppression. No herpesvirus reactivations have occurred in patients taken off of immunosuppression. Additional significant infections have included histoplasmosis in one subject (NW14) at 5 months post-transplant; this patient who resides in a high risk endemic area has responded to appropriate antifungal therapy. An additional subject (NW12) developed persistent BK virus viremia at more than one year post-transplant in the absence of allograft dysfunction; viral titers have gradually improved with reduction of tacrolimus-based IS. Currently, 10 of the 14 subjects transplanted and remaining on study (and 10 of the 12 subjects who received the optimal product and both doses of cyclophosphamide) exhibit durable chimerism at the time of this publication. None of the chimeric subjects have developed anti-donor antibody or disease recurrence post-transplant (Table 1B). No GVHD or engraftment syndrome has been observed. Of the transiently chimeric subjects one has developed recurrent membranous (NW1) and one recurrent IgA nephropathy (NW9).

***In vitro* assays of DSH alone do not correlate with donor specific tolerance**

Our initial endpoints for tapering of immunosuppression included DSH *in vitro* and/or durable chimerism. However, as the study has progressed, in light of findings described below, we have elected to rely only on T cell and whole blood chimerism for the complete

withdrawal of immunosuppression. The most evaluable time points for comparison were at 6 months (all subjects have had mycophenolate mofetil [MMF] discontinued) and 12 months.

In vitro proliferative and cytotoxic assays (MLR and CML) were performed at selected time points on all subjects. Recipients who developed durable high level donor chimerism were reproducibly tolerant to their donor by MLR and CML. In addition, these “fully” chimeric subjects failed to respond to archived pre-transplant recipient cells in an MLR, consistent with the absence of clinical acute or chronic GVHD. In contrast, peripheral blood mononuclear cells (PBMC) obtained from the original stem cell/kidney donor showed robust proliferation in response to recipient stimulators (data not shown). Protocol biopsies at 6, 12, and 24 months post-transplant showed no evidence of rejection in all durably chimeric subjects (data not shown).

Recipients who exhibited transient donor chimerism in peripheral blood also demonstrated DSH, even after loss of chimerism. However, *in vitro* hyporesponsiveness did not predict histologic findings on protocol biopsy. The most representative of this is Subject NW4. At 6 months, after gradual loss of detectable peripheral blood chimerism (Fig. 1), MLR and CML assays and a protocol biopsy were performed (Fig. 2 and 3A). Evidence of DSH, combined with normal allograft histology, led to the discontinuation of MMF. Repeat testing for DSH at 9 months showed persistent hyporeactivity (Fig. 2B) and led to tapering of tacrolimus monotherapy. Despite evidence for DSH in MLR (Fig. 2A) and CML assays, which persisted up to 18 months (Fig. 2C and 2D), protocol biopsy at 12 months demonstrated a mononuclear cell infiltrate and inflammation consistent with subclinical Banff 1A rejection (Fig. 3B). This was treated with intravenous corticosteroids, increase in tacrolimus dosing to therapeutic range and the infiltrates completely resolved. At 24 months post-transplant anti-donor reactivity developed in MLR (Fig. 2E). A second subject (Subject NW11) who had transient chimerism exhibited similar subclinical Banff 1A infiltrates on his 6 month protocol biopsy despite stable renal function and coincident DSH *in vitro* that responded to anti-rejection therapy (data not shown). Notably, there has been no evidence for rejection or fibrosis in any of the subjects with durable chimerism. A representative series of protocol biopsies at implantation (Fig. 3C), 12 months (Fig. 3D), and 24 months after transplantation and one year off all immunosuppression (Fig. 3E) is shown for subject 3.

Discussion

The definition of tolerance has had numerous interpretations and defined criteria. Organ transplant recipients who have been successfully weaned from immunosuppression and have maintained stable graft function for 1 year are referred to as functionally or operationally tolerant (4, 5). Although up to 20% of liver transplant recipients may be successfully withdrawn from immunosuppression (5–7), operational tolerance to renal allografts appears to be much less frequent (8, 9). In all of these studies a predictive biomarker for success vs. failure in weaning immunosuppression has not been reliably identified and validated so as to be used as a tool to discontinue immunosuppression. As a result, the subjects who fail immunosuppression weaning experience rejection episodes resulting in re-initiation of conventional immunosuppression after anti-rejection therapy. Moreover, it is currently not known whether they will experience compromised long-term graft survival.

Historically, MLR and CML assays have been considered gold standard tests for donor-specific tolerance. However, our own recent experience and that reported by the Massachusetts General Hospital (MGH) group (10–13) would suggest that in the absence of macrochimerism these tests are not uniformly reliable. In our own study, subjects exhibiting transient chimerism post-transplant were unresponsive to their donors by MLR and CML assays, sometimes for up to 18 months. However, protocol biopsies revealed cellular

infiltrates and inflammation indicating subclinical rejection requiring treatment and an increase in maintenance immunosuppression. This contrasts sharply with the histologic appearance of one year and two year protocol biopsies from the durably chimeric subjects, which are completely free of cellular infiltrates or manifestations of chronic rejection. Notably, durably chimeric subjects have had normal-appearing biopsies. This contrasts sharply with nonchimeric subjects who electively discontinued immunosuppression where the majority experienced severe rejection (14). Similarly, the MGH kidney/HSCT tolerance study, in which there was only transient microchimerism for < 21 days, reported DSH in their nonchimeric subjects, some of which went on to experience rejection episodes (11). Notably, 3 of 10 subjects enrolled in the MGH study experienced graft loss. One of these three subjects developed a viral infection 7 weeks after discontinuation of immunosuppression and experienced severe acute rejection and subsequent graft loss (15). Taken together, these findings suggest that when macrochimerism is present, subjects are tolerant to their donors; but in the absence of chimerism, a tolerant profile in MLR and CML is not always predictive of success in weaning immunosuppression without rejection episodes. The MGH group attributed the persistence of donor-specific tolerance following loss of chimerism to evolution from central tolerance to regulation in part mediated by antigen from the kidney allograft itself (12, 16). In light of the fact that infusion of donor bone marrow into unconditioned organ transplant recipients reduced the occurrence of chronic rejection (17–19), bone marrow itself may have immunoregulatory properties. Alternatively, it is possible that the presence of transient chimerism induces clonal deletion and that replacement of those clones is delayed after loss of the chimerism. The delayed immune recovery reported by Hakim et al, in subjects undergoing chemotherapy for malignancy, would support this hypothesis (20). As a result of these findings, we have elected to maintain our nonchimeric recipients on low dose tacrolimus or sirolimus monotherapy indefinitely rather than discontinue immunosuppression and risk rejection.

Production of donor T cells has been used as a biomarker of graft durability in HSCT (21). In the present study this metric has successfully translated to predict stable graft function and absence of rejection to date. Obviously, more patients and longer follow-up will be required to confirm this observation. The dichotomy between chimerism and tolerance was first reported when knockout mice were used as donors for bone marrow. It was shown that T cells and antigen-presenting cells were necessary for tolerance induction (22–24). In spite of chimerism, donor-specific skin transplants were rejected. Our own group previously reported in a mouse model using nonmyeloablative conditioning that production of donor T cells is associated with donor-specific tolerance to skin allografts (25). Notably, although myeloid chimerism was present at high levels, donor skin grafts were rejected. Similar findings were recently reported in a nonhuman primate model (Invited commentary, manuscript in press, *Nature Reviews Nephrology* 2012)(26). Our clinical findings and these historic supporting studies have led us to use T cell chimerism as our primary endpoint for decisions regarding immunosuppression weaning and withdrawal.

In summary, we report here the interim follow-up for 15 subjects transplanted with bioengineered FC-based HSCT product. We have established durable macrochimerism in the majority of subjects treated with the target cell dose and conditioning, with PRA < 20%, without GVHD or engraftment syndrome. The conditioning is well tolerated and can be managed as an outpatient. Studies are currently underway to adapt this approach to deceased donors in which a sequential or delayed tolerance approach would be necessary, and for subjects who have already had a living donor renal transplant and have a donor willing and able to donate HSCT (IND 14900: www.clinicaltrials.gov).

Materials and Methods

All protocols were approved by the Northwestern University and University of Louisville Institutional Review Boards and the FDA (IDE 13947). Informed consent was obtained for all donors and recipients.

HSC/Kidney conditioning regimen

Conditioning consisted of 3 doses of fludarabine (30 mg/kg/dose) days -4, -3, -2; two doses of cyclophosphamide (50 mg/kg/dose) days -3 and +3; and 200 cGy TBI on day -1 relative to the renal transplant (day 0). Hemodialysis was performed 6–8 h after the administration of fludarabine and cyclophosphamide to avoid toxicities of these agents. Tacrolimus (target trough concentrations 8–12 ng/ml) and MMF (1 gm orally twice daily if recipient weight < 80 kg, 1.25 gm twice daily if weight > 80 kg) were started on day -3 and continued as maintenance immunosuppression. Kidney transplantation was performed without antibody induction therapy or oral corticosteroids. A bioengineered FDA regulated HSC product enriched for facilitating cells (FCRx) was infused intravenously on the day following kidney transplant.

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Abbreviations

CML	cell-mediated lymphocytolysis
FC	facilitating cell
GVHD	graft-versus-host disease
HSCT	hematopoietic stem cell transplant
MLR	mixed lymphocyte reaction
mPBSC	mobilized peripheral blood stem cell
STR	short tandem repeats
TBI	total body irradiation
PRA	panel reactive antibody
DSH	donor-specific hyporesponsiveness
G-CSF	granulocyte colony-stimulating factor

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Figure 1.

A. Stability of chimerism over time in subjects who demonstrated high level and/or mixed donor chimerism. **B** Serum creatinine vs. time for all clinical trial subjects.

A

B

Figure 2.

A MLR results for subject NW4 at 5 months post-transplant, showing DSH. **B** MLR results for subject NW4 at 9 months post-transplant showing persistent DSH. **C,D** NW4 exhibits cytolytic activity to third party but not donor target cells in CML at month 8 and month 18 following transplantation. **E** First presence of anti-donor proliferative response in MLR.

Figure 3.

Representative hematoxylin and eosin stained sections (10X) of kidney transplant biopsy at 6 months **A** and one year **B** post-transplant from subject NW4. Image A shows no acute or chronic rejection, minimal tubular atrophy, and minimal interstitial fibrosis. Image B shows Banff 1A rejection, with interstitial inflammation and lymphocytic infiltration associated with tubulitis. In contrast, all protocol biopsies on subjects with high levels of donor chimerism have been normal at all times tested. **C** thru **E**: Periodic acid-Schiff staining (20X magnification) of renal allograft from representative subject (#3) with stable donor chimerism and donor specific tolerance. **C** Implantation biopsy at time of live donor

nephrectomy; **D** One year post-stem cell/kidney transplant; **E** Two years post-FcRx/kidney transplant, one year off all immunosuppression.

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TABLE 1

A. PATIENT CHARACTERISTICS									
Patient	Age	Sex	KD Tx Date	Source	HLA match	Donor Relationship	Cause of Renal Failure		
NW1	50	M	2/09	IC *	5/6	Related	Membranous		
NW2	56	M	4/09	MPB **	3/6	Related	Hypertension		
NW3	43	M	5/09	MPB	1/6	Unrelated	Polycystic kidney disease		
NW4	29	M	6/09	MPB	3/6	Related	Alports Syndrome		
NW5	40	M	2/10	MPB	1/6	Unrelated	Chronic glomerulonephritis		
NW6	39	F	3/10	MPB	2/6	Unrelated	Reflux; 2 nd Tx		
NW7	35	M	4/10	MPB	3/6	Related	Hypertension		
NW8	46	F	7/10	MPB	1/6	Unrelated	Polycystic kidney disease		
NW9	28	M	9/10	MPB	0/6	Unrelated	IgA Nephropathy		
NW10	43	M	10/10	MPB	4/6	Related	IgA Nephropathy		
NW11	54	M	10/10	MPB	3/6	Related	Type II Diabetes Mellitus		
NW12	23	M	2/11	MPB	1/6	Related	IgA Nephropathy		
NW13	24	F	N/A	MPB	2/6	Related	Focal segmental glomerulosclerosis		
NW14	60	M	10/11	MPB	2/6	Unrelated	Hypertension		
NW15	46	M	12/11	MPB	2/6	Unrelated	Polycystic kidney disease		
NW16	23	M	N/A	N/A	5/6	Related	Urinary Reflux Nephropathy		
NW17	18	F	3/12	MPB	4/6	Related	Membranoproliferative GN		

B. PATIENT CHARACTERISTICS									
Patient	Highest Historic PRA [†]	PRA [†]	αβ T cells#	CD34 [#]	FC [#] TOTAL	% Chimerism at 1 month ^{††}	Anti-donor Alloantibody after Transplant	Time off all Immunosuppression	Durability of Chimerism
NW1		0	0.963	.896	.467	30	None	On Prograf	Transient
NW2		6	3.80	2.53	6.65	95	Off study	Off study	Off study
NW3		19	3.80	3.60	3.96	94	None	22 months	Durable
NW4		0	1.94	1.00	1.55	6	None	On Prograf	Transient
NW5		0	3.80	3.94	3.68	100	None	15 months	Durable

B. PATIENT CHARACTERISTICS									
Patient	Highest Historic PRA [†]	PRA [†]	αβ T cells [#]	CD34 [#]	FC [#] TOTAL	% Chimerism at 1 month ^{††}	Anti-donor Alloantibody after Transplant	Time off all Immunosuppression	Durability of Chimerism
NW6		14	3.80	8.59	12.30	100	None	14 months	Durable
NW7		0	3.80	16.90	12.20	100	None	13 months	Durable
NW8		16	3.80	12.60	8.06	100	None	10 months	Durable
NW9		52	3.80	5.07	6.32	0	None	On Sirolimus	None
NW10		0	3.80	4.28	6.04	90	None	6 months	Durable
NW11	64	33	3.80	6.78	7.88	20	None	On Prograf	Transient
NW12		0	3.80	5.41	5.36	77	None	Pending	Transient
NW13		18	NA	NA	NA	NA	Off study	Off study	Off study
NW14		0	3.80	3.06	14.8	98	None	Pending	Durable
NW15		0	3.80	2.29	4.15	98	None	Pending	Durable
NW16		0	NA	NA	NA	NA	NA	NA	NA
NW17		0	3.80	6.07	4.84	87	None	Pending	Durable

* Iliac crest;

** Mobilized peripheral blood;

[†] PRA=panel reactive antibody;

^{††} Chimerism testing was performed by molecular analysis of short tandem repeats (STR) Sensitivity 2% – 5%.

[#] All cell dosing is $\# \times 10^6$ /kg recipient body weight.