Editor's Summary

Teaching Tolerance

According to Greek mythology, the Chimera was a fire-breathing creature made of parts from different animals: the body of a lioness, a snake's head at the end of the tail, and the head of the goat. Sightings of this fearsome beast portended any of a number of terrible disasters. In the context of organ transplantation, a "chimera" can indicate both desirable and disastrous outcomes. For example, hematopoietic chimerism, in which the immune cells in the graft recipient come from both the host and the donor, may promote graft tolerance, but may also cause graft-versus-host disease (GVHD), in which the donor immune cells attack the healthy tissue of the host. Leventhal et al. now report mixed chimerism and tolerance without the negative side effects of GVHD or engraftment syndrome in a phase 2 clinical trial of combined kidney and hematopoietic transplantation.

Leventhal et al. used a combination of mobilized cells enriched for hematopoietic stem cells and graft-facilitating cells—which are composed largely of plasmacytoid precursor dendritic cells—with nonmyeloablative conditioning in conjunction with kidney transplant from major histocompatibility complex−mismatched, nonrelated donors and recipients. Five of eight kidney transplant recipients exhibited durable chimerism and were weaned off immunosuppressive therapies by 1 year after transplantation, with no signs of GVHD or engraftment syndrome. If confirmed in larger patient cohorts, this approach to transplantation could free some patients from the difficulties associated with lifelong immunosuppression and add transplantation as a viable option for patients for whom nonmatched donors exist. As with the Chimera of legend, mixed chimerism may be a harbinger of things to come — albeit hopefully a brighter future for transplant patients.
Chimerism and Tolerance Without GVHD or Engraftment Syndrome in HLA-Mismatched Combined Kidney and Hematopoietic Stem Cell Transplantation


The toxicity of chronic immunosuppressive agents required for organ transplant maintenance has prompted investigators to pursue approaches to induce immune tolerance. We developed an approach using a bioengineered mobilized cellular product enriched for hematopoietic stem cells (HSCs) and tolerogenic graft facilitating cells (FCs) combined with nonmyeloablative conditioning; this approach resulted in engraftment, durable chimerism, and tolerance induction in recipients with highly mismatched related and unrelated donors. Eight recipients of human leukocyte antigen (HLA)–mismatched kidney and FC/HSC transplants underwent conditioning with fludarabine, 200-centigray total body irradiation, and cyclophosphamide followed by posttransplant immunosuppression with tacrolimus and mycophenolate mofetil. Subjects ranged in age from 29 to 56 years. HLA match ranged from five of six loci with related donors to one of six loci with unrelated donors. The absolute neutrophil counts reached a nadir about 1 week after transplant, with recovery by 2 weeks. Multilineage chimerism at 1 month ranged from 6 to 100%. The conditioning was well tolerated, with outpatient management after postoperative day 2. Two subjects exhibited transient chimerism and were maintained on low-dose tacrolimus monotherapy. One subject developed viral sepsis 2 months after transplant and experienced renal artery thrombosis. Five subjects experienced durable chimerism, demonstrated immunocompetence and donor-specific tolerance by in vitro proliferative assays, and were successfully weaned off all immunosuppression 1 year after transplant. None of the recipients produced anti-donor antibody or exhibited engraftment syndrome or graft-versus-host disease. These results suggest that manipulation of a mobilized stem cell graft and nonmyeloablative conditioning represents a safe, practical, and reproducible means of inducing durable chimerism and donor-specific tolerance in solid organ transplant recipients.

INTRODUCTION

The disappointing long-term results for solid organ transplantation have motivated investigators to pursue the clinical induction of immunologic tolerance. The toxicities associated with nonspecific immunosuppressive agents remain major causes of morbidity in organ transplant recipients, including hypertension, opportunistic infections, diabetes, and renal compromise (1). Moreover, these agents do not prevent chronic rejection, the primary cause of late graft loss. Chimerism has been shown to prevent chronic rejection and induce drug-free graft survival in several experimental animal models (2, 3). Two recent studies demonstrated the feasibility of this approach in humans (4–6). In one study, although recipients did not achieve durable chimerism, most recipients were successfully tapered off immunosuppression (5, 6). The other study limited to human leukocyte antigen (HLA)–identical related living donor/recipient transplants (4, 7).

In this report of a Food and Drug Administration (FDA)–approved phase 2 study, we tested the hypothesis that a tolerance-promoting facilitating cell (FC)–based (8, 9) hematopoietic stem cell (HSC) graft (together designated as FCRx) would promote the establishment of durable chimerism and tolerance in HLA-mismatched living donor renal allograft recipients. These bone marrow–derived FCs, which are CD8+ but do not express the T cell receptor (TCR), potently enhance engraftment of allogeneic HSCs (8) and limiting numbers of syngeneic (10) HSCs in conditioned recipients. FCs are composed predominantly of a plasmacytoid precursor dendritic cell subpopulation (p-preDC FCs) (9). Although removal of p-preDC FCs abrogates facilitation, p-preDC FCs do not replace FCs in function. FCs induce the generation of antigen-specific regulatory T (Treg) cells in vitro (11) and in vivo (12) and potentially prevent graft-versus-host disease (GVHD) in the mouse (13). Hence, FCs may represent a clinically relevant cell-based therapy for tolerance induction.

We report here the translation of this work to the clinic. This therapy induced durable high levels of chimerism and stable renal function, and avoided induction of GVHD in HLA-mismatched kidney/FCRx recipients, who are now off all immunosuppression for periods ranging from 6 to 20 months. A safe approach to induce graft/host tolerance in mismatched donor and recipient combinations could be transformational not only in solid organ and cell transplant recipients but also for applications of HSC transplantation (HSCT) in general, including hemoglobinopathies, inherited metabolic disorders, and autoimmune diseases.

RESULTS

Subject clinical course

The demographics of the study subjects and composition of the FCRx infused are shown in Table 1. The conditioning consisted of two doses of cyclophosphamide (days +3 and −3), 200-cGy total body irradiation...
(TBI), and three doses of preoperative fludarabine (days −4, −3, and −2), followed by renal transplantation (day 0) and FC Rx infusion (day +1) as detailed in Fig. 1A. Immunosuppression after transplant consisted of mycophenolate mofetil (MMF) and tacrolimus. Subjects were discharged on day 2 after renal transplant and subsequently managed as outpatients. The characteristic nadir of absolute neutrophil counts (ANCs) occurred about 1 week after kidney/stem cell transplant, with median recovery of ANC to more than 500 per cubic milliliter at 9 days (range, 2 to 14). This was managed with neutropenic precautions. Multilineage chimerism was achieved in all subjects at 1 month after transplant (Table 1). Chimerism persisted in five of the eight subjects (Tables 2 and 3). None of the subjects demonstrated engraftment syndrome (Table 1). Chimerism was 30% at 1 month, but gradually decreased and was lost 6 months, and tapering of tacrolimus was initiated in light of donor-specific hyporesponsiveness in cell-mediated cytolytic assays in vitro. The subject presented with new-onset proteinuria at his 1-year standard of care visit. A kidney transplant biopsy revealed recurrent membranous nephropathy. Tapering of tacrolimus was halted and the subject was treated with rituximab (375 mg/m² weekly for 1 month). The proteinuria subsequently resolved. A protocol biopsy at 24 months after transplant was free of acute or chronic rejection and confirmed disease quiescence. At the time of publication, he remains on tacrolimus monotherapy with normal renal function (Fig. 1B).

Subject 2 is a 56-year-old male with end-stage renal disease (ESRD) caused by hypertension who underwent a three of six HLA-matched living donor renal/stem cell transplant in April 2009. He tolerated the conditioning and transplant well and was discharged on postoperative day 2. He experienced immediate renal allograft function and the expected nadir for white blood cells and platelets of less than 2 weeks. He achieved 95% chimerism at month 1 and 100% at month 2. He had no evidence of GVHD or engraftment syndrome. Three months after transplant, the subject developed a febrile illness of uncertain etiology that progressed to intercurrent sepsis and hypotension, requiring inotropic support and ventilation. Bone marrow chimerism testing showed 100% donor chimerism at onset of the illness. After several days, the patient was weaned from inotropic support and extubated. Serum creatinine was noted to be increasing, and a renal transplant biopsy showed extensive hemorrhagic necrosis. A nuclear medicine scan of the transplant showed absence of blood flow through the renal artery. He underwent renal transplant nephrectomy. He subsequently underwent a living-related renal transplant and continues to do well, with stable renal function on standard immunosuppression. A careful and thorough review by the investigators and data safety monitoring board (DSMB) for the study concluded that occult viral sepsis was the most likely explanation.

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<th>Donor relationship</th>
<th>Cause of renal failure</th>
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*Chimerism testing was performed by molecular analysis of STRs. †All cell dosing is calculated as follows: number × 10⁶/kg recipient body weight.
Subject 3 is a 43-year-old male who developed ESRD due to polycystic kidney disease. A detailed clinical course is presented because he represents our first complete success in this trial. He received a one of six HLA-matched living unrelated kidney/FCRx transplant in May 2009. The characteristic nadir, which is similar for all recipients, is shown in Fig. 2A. He had 95% donor chimerism at 1 month after transplant. Chimerism has fluctuated between 63 and 100% with no evidence of GVHD or engraftment syndrome (Fig. 2B and Table 2). At 6 months, peripheral blood multilineage chimerism testing revealed the presence of 100% donor B cell, T cell, and myeloid cell production (Fig. 2C). T cell chimerism has consistently been 100% (Table 3). Flow cytometric crossmatch for anti-donor antibodies was negative at 1 month, 6 months, 1 year, and 2 years after transplant. At month 3, the recipient began to exhibit donor-specific hyporesponsiveness but regained immunocompetence to respond to HLA-disparate third-party alloantigen in vitro mixed lymphocyte reaction (MLR) proliferative assays, which has persisted (Fig. 2D). Renal function has remained stable and within normal limits (Fig. 1B). Protocol biopsies at 6 and 12 months after transplant were histologically normal. MMF was discontinued at 6 months after transplant. Tacrolimus was reduced to subtherapeutic dosing at 9 months (trough, 0 to 3 ng/ml) and discontinued at 1 year. A subsequent protocol graft biopsy at 24 months after transplant (12 months of all immunosuppression) was histologically normal (Fig. 3, A to C). Donor chimerism has remained 100% more than 1 year after immunosuppression was withdrawn. Adverse events in this subject include an exanthema at 1 month after transplant. GVHD was included in the differential diagnosis, but a biopsy revealed a sulfa-based drug rash. The rash resolved spontaneously and has not recurred. In addition, the subject developed a single dermatome varicella zoster reactivation at 9 months after transplant, which resolved and has not recurred. At the time of publication, he was 32 months after transplant with stable renal function.

Subject 4 is a 29-year-old male with ESRD due to Alports syndrome who received an LRD kidney/FCRx transplant (three of six HLA match) in June 2009. He received a deliberately reduced aβ T cell dose (Table 1) because of unresolved concerns regarding the etiology of the rash in subject 3, described above. His course after transplant was complicated by a wound infection successfully treated with intravenous antibiotics. Donor chimerism was 6% at 1 month but was gradually lost by 3 months (Table 2). Donor-specific hyporesponsiveness persisted as assessed by MLR, and a protocol biopsy at 6 months was histologically normal. MMF was discontinued at 6 months, and tapering of tacrolimus was initiated. However, a protocol biopsy at 12 months showed subclinical Banff 1A rejection despite a normal serum creatinine. Staining for C4d and donor-specific antibody was negative. He was treated with a short course of intravenous corticosteroids and has been maintained on therapeutic tacrolimus monotherapy (trough, 5 to 8 ng/ml). Renal function has remained stable (Fig. 1B), no donor-specific antibody has been detected, and a follow-up allograft biopsy at 17 and 24 months after transplant was histologically normal.

The first four subjects represent a learning curve in this pilot study. Because of the loss of chimerism in subjects 1 and 4, who both received lower numbers of aβ T cells, FCs, and CD34+ cells, all subsequent subjects in our study have received processed stem cell infusions modeled after the product was administered in subject 3 (Table 1), as well as both doses of cyclophosphamide. This has resulted in durable high levels of whole-blood and CD3 cell chimerism in subjects 5 to 8 as shown in Tables 2 and 3. No subject has exhibited engraftment syndrome, none has developed anti-donor antibody, and none has exhibited acute or chronic GVHD. All have stable renal function (Fig. 1B).

**Immunologic monitoring**

We conducted serial immunophenotypic analyses of lymphocyte and monocyte subpopulations in all subjects. The figures are provided in the Supplementary Materials (fig. S1, A to H). We observed a reduction in CD3+ T cells/aβ TCR+ lymphocytes early after transplant (fig. S1A). Immunologic reconstitution during the first year after transplant was notable for a blunted return of CD4+ T cells (fig. S1B) compared to...
CD8+ T cells (fig. S1C), with an inversion of the CD4/CD8 ratio (fig. S1D). In addition, immunologic reconstitution was characterized by robust recovery and/or increase in the numbers of CD19+ B cells (fig. S1E), CD14+ monocytes (fig. S1F), and CD56+ natural killer (NK) cells (fig. S1G). A marked reduction in CD19+ B cells was seen at 12 and 18 months after transplant in subject 1, reflecting the administration of the anti-B cell monoclonal antibody rituximab as therapy for his recurrent membranous nephropathy. Furthermore, although we noted an initial decrease in the absolute numbers of CD4+/CD25+FoxP3+/CD127− phenotypic Treg cells, an increase in the Treg-to-Teff (effector T) cell ratio was frequently observed in durably chimeric recipients compared to the two recipients who exhibited only transient macrochimerism (subjects 1 and 4) (fig. S1H). In the context of full donor chimerism without GVHD, this observation suggests that dynamic immune regulation by Treg cells likely contributes to the prevention of GVHD in our subjects.

In vitro proliferation assays performed in our subjects identified the development of donor-specific hyporesponsiveness and the ability to respond to third-party alloantigen. Of interest, donor-specific hyporesponsiveness was evident during the first year after transplant in subjects 1 and 4 even after donor chimerism was lost, and in subject 4 at the time that subclinical rejection occurred. The presence of full donor chimerism without GVHD was associated with tolerance to donor alloantigen as reflected by the absence of in vitro proliferative responses of the chimeric recipient peripheral blood lymphocytes (PBLs) against archived pretransplant recipient stimulators in subjects 3, 5, 6, 7, and 8 by MLR. Figure 4 for subject 5 is representative of this effect. In marked contrast, PBLs obtained from the living donor demonstrated robust proliferation against recipient stimulators. These data support the notion that the donor chimeric lymphocytes are rendered tolerant to recipient alloantigens after transplant.

Composition of FC total (CD8+ TCR−) subpopulations

We previously reported that the mouse FC subpopulations include NK FCs, p-preDC FCs, and CD19+ FCs (8). Multiparameter flow cytometry was performed to analyze the phenotype of FC subpopulations in human mobilized HSCT product. Two major FC subpopulations are CD56dim− and CD56bright. Using multicolor flow cytometric analysis, we found that most CD56dim− FCs are also positive for CD3e and HLA-DR and negative for the dendritic cell markers CD11c and CD123 (Fig. 5A). CD56dim− FCs comprise about 49% of FC total (n = 4 experiments). Most of the CD56bright FC subpopulation is CD19+, CD11c+, CD11b+, and CD3e− and comprises about 46% of FC total (Fig. 5B).

**DISCUSSION**

Transplantation of organs and cellular grafts has become widely accepted therapies. However, the toxicity of the immunosuppressive

### Table 2. Percentage of whole-blood chimerism at selected months after transplant. NT, not tested.

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### Table 3. Percentage of T cell chimerism at selected time points after transplant. NT, not tested.

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agents required for graft maintenance is substantial, resulting in end-organ damage and failure, opportunistic infections, hypertension, diabetes, and an increased rate of malignancy (15, 16). Chronic rejection remains the primary cause of late graft loss (17). Therefore, strategies to induce donor-specific tolerance are being actively pursued. One approach to achieve tolerance is through cell-based therapies resulting in chimerism. First described in mice (2), mixed chimerism has been shown to induce donor-specific tolerance in a number of species, including humans, and is the only approach that appears to be generalizable to all species in which it has been tested (18–20).

A number of reports from clinical protocols using combined HSC and renal transplantation have recently demonstrated the feasibility of using cell-based therapies to induce tolerance. One significant variable in these approaches is whether the researchers only achieved microchimerism, defined as very low levels of donor chimerism versus macrochimerism, where donor cells are typically present at least 1% of peripheral blood cells. In a series of 10 subjects with end-stage renal failure who underwent conditioning with cyclophosphamide, rituximab, 700 cGy of thymic irradiation, and anti-CD2 monoclonal antibody followed by transplantation of unmodified donor marrow, very low levels of donor chimerism were present for up to 21 days in the peripheral blood (5, 6, 20). Thereafter, chimerism was undetectable. A capillary leak syndrome associated with production of anti-donor antibody and elevated creatinine levels, recently termed “engraftment syndrome” (14, 21), developed in 9 of the 10 subjects (5, 20). Two subjects lost their kidneys to acute rejection (14). Seven of the 10 total subjects were successfully tapered off immunosuppression. However, one of these seven subjects developed acute rejection 7 weeks after immunosuppression was discontinued, and 6 months after reinitiation of immunosuppression, renal function declined to a level where dialysis was required (6). Another of these recipients has developed C4d positivity on biopsy and anti-class II HLA donor antibody, indicating antibody and complement deposition, which has been associated with an increased risk of chronic allograft nephropathy and graft loss (20). All seven recipients reportedly show donor-specific hyporesponsiveness in vitro. GVHD did not occur, perhaps because sustained engraftment was not achieved (22). The impact of antibody-mediated rejection on long-term renal allograft function remains a concern because it has historically been associated with impaired graft survival (23).

Fig. 2. Summary of nadir, donor chimerism, and clinical outcomes in subject 3. (A) Kinetics of nadir (ANC ≤500). The horizontal axis represents days after kidney transplant, and vertical axis represents ANC. (B) Whole-blood mononuclear cell chimerism using the STR molecular assay (LabCorp). The sensitivity and specificity for these assays were 1 to 2%. (C) Whole-blood mononuclear cells were isolated and sorted for B cells (CD19+), T cells (CD3+), or myeloid-derived cells (CD66B+). (D) Fresh recipient PBLs were exposed to PHA, Candida, recipient stimulators, donor stimulators, and third-party allostimulators in one-way MLR proliferative assays. Third-party alloresponder controls were performed. A stimulation index of ≥3 is positive. The stimulation index is calculated as the ratio of antigen-specific proliferation to unstimulated recipient lymphocytes. Error bars are means ± SD for triplicate assays.

Fig. 3. Representative sections of kidney transplant biopsy at 1 year after all immunosuppression was withdrawn. (A to C) Staining with (A) hematoxylin and eosin (20x), (B) Masson trichrome (10x), and (C) periodic acid–Schiff (20x) shows no acute or chronic rejection, minimal tubular atrophy, and minimal interstitial fibrosis.
approach did not achieve durable chimerism when it was expanded to HLA-mismatched recipients (7). The requirement for HLA matching would preclude the application of this approach to most solid organ recipients who are not HLA-matched to their donor, especially deceased donor transplants. Collectively, these studies demonstrate the feasibility and efficacy of using stem cell–based therapies to induce tolerance in the clinic.

The phase 2 study reported here is a translational research protocol based on the tolerogenic features of CD8+/TCR– graft FCs that were first discovered in the mouse (8). FCs are a heterogeneous population composed predominantly of p-preDC FCs (9). Removal of p-preDC FCs completely abrogates FC function in vivo and in vitro (9, 12). However, p-preDC FCs do not completely replace CD8+/TCR– FCs in function. CD8+/TCR– FCs enhance clonogenicity of HSCs in vitro (9, 25) and robustly prevent GVHD in vivo (13). They induce FoxP3+/CD4+/CD25+ T reg cells in vitro (26) and antigen-specific T reg cells in vivo (12). Hence, FCs may address the primary challenge in translating cell–based therapies to the clinic: to maintain the tolerogenic features after transplantation while avoiding GVHD (27). Here, we tested whether a stem cell–based cellular product engineered to be enriched for FCs plus HSCs while depleting cells that predispose to GVHD would allow the induction of reciprocal graft/host tolerance in renal allograft recipients. We report here a nonmyeloablative conditioning HSCT approach to establish chimerism and tolerance, while avoiding GVHD and engraftment syndrome, in renal transplant recipients. To our knowledge, this is the first report of durable macrochimerism without GVHD in HLA-mismatched related and unrelated stem cell/renal transplant recipients. We have observed that both HSCT composition and conditioning significantly influence outcome.

A recent large series of haploidentical (three of six HLA match) transplants in subjects with hematologic malignancies and comorbidities that precluded ablative HSCT who were similarly conditioned with fludarabine, 200-cGy TBI, and two or four doses of cyclophosphamide before and after transplant demonstrated that the pre- and posttransplant cyclophosphamide was required for durable engraftment (22). This approach was first developed in a mouse model of nonmyeloablative conditioning and then translated to the clinic (22, 28). Building on this experience, we found that both doses of cyclophosphamide in the renal tolerance protocol were required for durable chimerism in this patient population. The cyclophosphamide is hypothesized to eliminate newly activated host-versus-graft and graft-versus-host reactive cells while sparing the HSCs and host and donor memory immune responses, resulting in reciprocal self-tolerance/allotolerance and superior immunologic recovery (22).

The patterns of immune reconstitution observed in our combined FCRx/kidney recipients resemble those previously described in adult recipients of HSCT (29). We observed a rapid recovery and increase in CD56– NK cells after FCRx in our subjects, consistent with previous published studies (30). Initial recovery of the T cell compartment has been shown to reflect peripheral expansion of memory T cells, driven by cytokines and the presence of alloreactive antigens, before the production of naïve T cells in the thymus begins. This is especially true for CD4+ T cells that reconstitute later than CD8+ T cells and rely more on thymic production of naïve T cells after HSCT, leading to an inversion of the CD4/CD8 ratio as we observed in our FCRx/kidney patients. Although initial expansion of T cells in our fully donor chimeric recipients may reflect proliferation of T cells contained in the FCRx product, versus FC-replete or unmodified grafts was deemed inadvisable by the investigators and the DSMB. Hence, a direct role for FCs in outcomes cannot be definitely concluded.

A clinical trial of mismatched recipients using unmodified bone marrow cells did not show the GVHD-sparing effect experienced in our subjects. In similarly conditioned haploidentical recipients of unmodified mobilized peripheral blood mononuclear cells who underwent HSCT for hematologic malignancy, the incidence of grade II to IV and II to III acute GVHD by day 200 was 34 and 6%, respectively (22). Chronic GVHD occurred in 25%, with most presenting by 100 days after transplantation and all by 300 days (22). The major difference in our study is the removal of effector GVHD-causing cells, the presence of FCs, and the renal transplant itself. Because GVHD normally occurs within 300 days after HSCT and while the recipients are still on tacrolimus/MMF GVHD prophylaxis, it is highly unlikely that our subjects will develop de novo GVHD in the future. Luznik et al. concluded that the posttransplant cyclophosphamide deleted alloreactive host-versus-graft and graft-versus-host reactive cells while sparing the HSCs and host and donor memory immune responses, resulting in reciprocal self-tolerance/allotolerance and superior immunologic recovery (22).

Fig. 4. Nonresponsiveness of chimeric recipients to archived pretransplant recipient stimulators. Subject 5 is shown as a representative MLR. The y axis denotes the mean stimulation index; the x axis denotes the antigenic challenge used. White bar demonstrates response of the transplant recipient; gray and black bars show response of third-party MHC-disparate individuals allo #1 and #2. Allo #1 and #2 were also used as stimulators in these experiments.


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recovery in our subjects who lost chimerism likely derives from cells that escaped the effects of the low-intensity conditioning regimen. Moreover, the fact that the PBLs from the chimeric recipients who were 100% donor do not respond to archived pretransplant recipient alloantigen would suggest that these donor-derived T cells are newly produced and/or have been tolerized. TCR rearrangement excision DNA circles (TRECs), as well as CD31 expression, have been used as markers for naïve T cell reconstitution occurring in the thymus. TRECs levels have been reported to remain low for months, even years, after allogeneic HSCT in adults. We plan to investigate the kinetics of thymic repopulation and the contribution of new thymic emigrants to repopulation of different T cell subsets in future studies.

We observed a relative increase in numbers of CD19+ B cells in nearly all of our FCRx/kidney subjects during the first year after transplant. Recent studies in operationally tolerant kidney transplant recipients who have been tapered off immunosuppression after conventional organ transplantation have identified a particular blood B cell phenotype, with an expansion of memory activated B cells and increased expression of inhibitory molecules, suggesting a role for B cells in maintaining graft tolerance (31). Whether so-called regulatory B cells contribute to immune modulation, helping to control graft-versus-host responses in our chimeric subjects, and/or operational tolerance and graft acceptance in subjects where chimerism is lost will require future studies.

Chimeric recipients exhibited responses to phytohemagglutinin (PHA) and third-party alloantigen in vitro. It is not surprising that standard immunocompetence testing using PHA and alloantigens revealed immunocompetence, because these responses are polyclonal and a crude measure of immune function. Previous experiments using chimeric animals suggested that the thymic epithelium was imprinting major histocompatibility complex (MHC) restriction. Viral challenge in ablatively conditioned fully chimeric mice was lethal, whereas mixed chimeras eliminated the virus and survived (32). That would lead one to question whether 100% donor chimeric humans would be immunologic cripples. In recent studies in mice, this has not proven to be the case. Elegant studies using transgenic mice prepared as tetraparental aggregation chimeras have demonstrated that TCR–APC (antigen-presenting cell) interactions are more important than MHC class I or class II, and that T cell repertoire selection is independent of thymic MHC (33). These studies demonstrated that the MHC of nonthymic epithelial cells efficiently selects a functional T cell repertoire. We would therefore hypothesize that non-myeloablative conditioned chimeric humans would be immunocompetent to respond to infectious challenge. It is likely that there are sufficient numbers of professional and nonprofessional APCs.

![Flow Cytometric Analysis](https://stm.sciencemag.org)
remaining to allow robust immunocompetence and tolerance in the nonmyeloablative conditioned subjects with high levels of donor chimerism (33, 34). Note that all severe adverse events experienced by the eight subjects occurred while they were on conventional maintenance immunosuppression.

Studies that empirically wean immunosuppression in nonchimeric individuals rely on the concept of “operational tolerance,” a term that has evolved to refer to subjects who have been off immunosuppression for at least 1 year. One major limitation in studies of operationally tolerant recipients is that there is a paucity of biomarkers or assays that predict which subjects will do well with minimization or cessation of immunosuppression and which are prone to chronic or even acute rejection (35). Clearly, standard in vitro proliferative responses are not reliable, as reflected by the one subject in our study who lost chimerism, remained tolerant to his donor in proliferative assays, yet experienced a subclinical rejection episode (Banff 1A) on protocol biopsy that responded to increased immunosuppression. It is of interest that this subject has since gone on to lose donor-specific hyporesponsiveness at 2 years after transplant (fig. S2). Similarly, one subject reported in the Massachusetts General Hospital tolerance study experienced acute rejection 7 weeks after cessation of immunosuppression despite in vitro evidence for tolerance (20). Levitsky reported that although 80% of liver transplant recipients can be successfully weaned from immunosuppression, only 20% are successfully maintained off immunosuppression long-term without experiencing rejection (35). Notably, there was no reliable biomarker/endpoint identified as a predictor of success versus failure in tapering and maintaining rejection-free graft survival off immunosuppression. Some subjects were off immunosuppression for more than 1 year before rejection occurred. The persistence of high levels of donor chimerism observed in the present study appears to represent a reliable, easily evaluable biomarker for donor-specific tolerance induction and the ability to safely wean subjects from immunosuppression. The chimerism testing was performed by an independent CLIA (Clinical Laboratory Improvement Amendments)– and FDA-approved laboratory (LabCorp) as per standard of care for HSCT (36). It has been shown in mice (37) and humans that T cell chimerism exceeding 50% for more than 6 months reliably predicts durable graft acceptance after HSCT (38). In our own renal/FCRx recipients, T cell chimerism was associated with persistent donor chimerism and the ability to successfully wean subjects from immunosuppression while maintaining stable renal function. This has now been adapted as our primary clinical endpoint.

The ability to establish high levels of donor multilineage chimerism in haploidentical and highly mismatched unrelated donor recipients without GVHD or engraftment syndrome could have revolutionary therapeutic implications for treatment of disorders for which HSCT can provide a “functional cure,” including inherited metabolic disorders, hemoglobinopathies, and autoimmune diseases (39). It could also address the significant numbers of individuals who are candidates for a bone marrow transplant for hematologic malignancy but do not have a suitably matched donor.

**MATERIALS AND METHODS**

All protocols are approved by the Northwestern University and University of Louisville Institutional Review Boards and the FDA [Investigational Device Exemption (IDE) 13947]. Informed consent was obtained for all donors and recipients. Donor and recipient eligibility criteria are detailed in the Supplementary Materials.

**Cell dosing algorithm**

Most bone marrow cell processing devices leave residual T cells after depletion. A dose escalation for maximum allowable αβ T cell content was used in phase 1 of this protocol, with the intent to administer as many FCs and HSCs as possible in that context. Historically, beginning in 1996, the starting maximum allowable αβ T cell dose was 2 × 10° per kilogram of recipient body weight. If engraftment and GVHD did not occur, the T cell dose was increased by 4 × 10°/kg in the next recipient. Macrochimerism did not occur in any subjects transplanted until the current conditioning, and cell dosing was adopted for the subjects reported here. All non-engrafting subjects resumed endogenous hematopoiesis and were not tapered from immunosuppression.

**MPBSC collection and FCRx preparation**

At least 2 weeks before the renal transplant, donors were mobilized with granulocyte colony-stimulating factor (10 μg/kg, twice daily), and apheresis was performed on day +4. The product was diluted in nutrient-rich ex vivo cell medium (BioWhittaker) and immediately transported by courier, in a controlled-temperature container, to the Institute for Cellular Therapeutics at the University of Louisville and processed in a Foundation for the Accreditation of Cellular Therapy–accredited Good Manufacturing Practice facility by a proprietary approach under FDA IDE 13947 with the CliniMACS (Miltenyi Biotec) to remove mature GVHD-producing and APCs while retaining HSCs, FCs, and progenitor cells. Cells treated in this way are available from the authors, who will process them with cost recovery under FDA approval. The product was then shipped back to Northwestern University for intravenous infusion on the day after living donor kidney transplant. Seven of eight subjects received cryopreserved product. Recipients underwent similar mobilization, and autologous cryopreserved MPBSCs were stored in case hematologic rescue was required.

**Immunologic monitoring**

Recipient responses to PHA, Candida, tetanus toxoid, donor, and third-party alloantigens were tested monthly as previously described (40). Flow crossmatch assays were performed at 1 and 6 months. Protocol biopsies were performed at 6 months and 1 year after transplant per standard of care.

**Chimerism testing**

Chimerism was determined by genotyping of simple sequence-length polymorphisms encoding short tandem repeats (STRs) at an independent laboratory (LabCorp) (36). For lineage chimerism testing, CD19⁺ (B cells), CD3⁺ (T cells), and/or CD66B⁺ (myeloid) cells were sorted from whole blood and then analyzed by molecular STR typing. This assay is sensitive to about 1 to 2%. Internal controls to define the sensitivity are performed for each assay.

**Weaning of immunosuppression and clinical endpoints**

Tacrolimus and MMF were continued at therapeutic levels until 6 months after transplant (Fig. 1). If renal function and protocol biopsy were normal, and if chimerism or donor-specific hyporesponsiveness were present, the MMF was discontinued. The tacrolimus was tapered at 9 months to subtherapeutic trough levels (0 to 3 ng/ml).
Immunosuppression was discontinued at 1 year if the following criteria were met: durable whole-blood macrochimerism, T cell chimerism, stable renal function, no anti-donor antibody, and a normal protocol renal allograft biopsy.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/4/124/124ra28/DC1

Materials and Methods

Detailed subject clinical course descriptions

References and Notes


10. H. L. Grimes, C. L. Schanie, Y. Huang, D. Cramer, F. Rezzoug, I. Fugier-Vivier, S. T. Ildstad, Graft facilitating cells are derived from hematopoietic stem cells and functionally require CD3, but are distinct from T lymphocytes.


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