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Long-Term Results in Recipients of Combined HLA-Mismatched Kidney and Bone Marrow Transplantation Without Maintenance Immunosuppression

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We report here the long-term results of HLA-mismatched kidney transplantation without maintenance immunosuppression (IS) in 10 subjects following combined kidney and bone marrow transplantation. All subjects were treated with nonmyeloablative conditioning and an 8- to 14-month course of calcineurin inhibitor with or without rituximab. All 10 subjects developed transient chimerism, and in seven of these, IS was successfully discontinued for 4 or more years. Currently, four subjects remain IS free for periods of 4.5–11.4 years, while three required reinstitution of IS after 5–8 years due to recurrence of original disease or chronic antibody-mediated rejection. Of the 10 renal allografts, three failed due to thrombotic microangiopathy or rejection. When compared with 21 immunologically similar living donor kidney recipients treated with conventional IS, the long-term IS-free survivors developed significantly fewer posttransplant complications. Although most recipients treated with none or two doses of rituximab developed donor-specific antibody (DSA), no DSA was detected in recipients treated with four doses of rituximab. Although further revisions of the current conditioning regimen are planned in order to improve consistency of the results, this study shows that long-term stable kidney allograft survival without maintenance IS can be achieved following transient mixed chimerism induction.

Keywords: Chimerism, immunobiology, kidney transplantation, living donors, tolerance

Abbreviations: AKI, acute kidney injury; BAFF, B cell activating factor; CKBMT, combined kidney and bone marrow transplantation; CML, cell-mediated lympholysis; Cr, creatinine; CyA, cyclosporine A; DBM, donor bone marrow; DBMT, donor bone marrow transplantation; DSA, donor-specific antibody; DSH, donor-specific hyporesponsiveness; DSN, donor-specific nonresponsiveness; GBM, glomerular basement membrane; GVHD, graft versus host disease; IS, immunosuppression; ITN, Immune Tolerance Network; MGH, Massachusetts General Hospital; MLR, mixed lymphocyte reaction; MMF, mycophenolate mofetil; mod NKD03, modified NKD03; MPGN, membranoproliferative glomerulonephritis; NHP, nonhuman primate; TBI, total body irradiation; TLI, total lymphoid irradiation

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Introduction

Short-term results following organ transplantation have been significantly improved by the use of increasingly efficacious immunosuppressive agents. However, their chronic administration results in significant morbidity, especially from an increased incidence of cardiovascular disease (1), infection (2), malignancies (3), *de novo* diabetes (4) and other metabolic derangements. Moreover, the potent immunomodulatory effects of current therapeutic protocols do not prevent the development of chronic rejection, despite their administration being pushed to toxic levels. Therefore, induction of tolerance, defined as the absence of destructive immune responses to a transplanted tissue without ongoing immunosuppressive therapy, remains the ultimate goal of organ transplantation.

Since the seminal work reported by Billingham, Brent and Medawar on neonatal tolerance in 1956 (5), numerous

tolerance induction strategies have been defined in rodents. However, only a very limited number of these strategies have been successfully translated to large animals and even fewer to primates. Among the few protocols that have been applied successfully in humans, induction of donor chimerism, either transient or durable, currently appears to be the most promising strategy to achieve renal allograft tolerance. Initial results of currently ongoing clinical trials for tolerance induction in three centers have so far been reported. Using total lymphoid irradiation (TLI) and donor bone marrow transplantation (DBMT), the Stanford group reported successful induction of stable chimerism and renal allograft survival following immunosuppression (IS) withdrawal in the majority of *HLA-identical* kidney transplant recipients (6–8). More recently, Leventhal et al (9) at Northwestern have reported the use of an intensive conditioning regimen and donor hematopoietic stem cells for induction of full donor chimerism and successful IS withdrawal in *HLA-mismatched* kidney transplant recipients. Although the follow-up of these patients is still relatively brief, persistent donor chimerism without graft versus host disease (GVHD) has been reported, allowing weaning from all maintenance IS by 1 year in more than half of the patients at this point.

At Massachusetts General Hospital (MGH), based on decades-long basic studies in animal models (10–14), we have applied combined kidney and donor bone marrow transplantation (CKBMT) for induction of transient donor chimerism and renal allograft tolerance in both HLA-matched (15–17) and HLA-mismatched (18) kidney transplant recipients. We now report more detailed postconditioning evaluation of anti-T cell responses, B cell depletion and B cell activating factor (BAFF) levels and their potential relationship with long-term humoral responses. Clinical results of the study subjects were also compared with immunologically similar living donor kidney recipients treated with conventional IS during the same time period. Our observations emphasize the importance of adequate B cell depletion during the initial 6 months to inhibit *de novo* donor-specific antibody (DSA).

Methods

Study subjects

A total of 10 subjects, age 22–46, 6 males and 4 females, were enrolled into these studies. Their original kidney diseases include Alport's syndrome (n = 4), polycystic kidney disease (n = 2), membranoproliferative glomerulonephritis (MPGN) type 1 (n = 2), reflux uropathy (n = 1) and focal glomerulosclerosis (n = 1) (Table 1). The first three subjects (1–3) received the NKD03 conditioning regimen; the next two subjects (4 and 5) received the modified NKD03 (mod NKD03) regimen. The last five subjects (6–10) received the ITN036 protocol detailed in Figure 1. To compare the long-term results of the subjects who entered the tolerance protocol with subjects who underwent kidney transplantation with conventional IS, 32 consecutive recipients of comparable age (20–45) who received ABO blood type compatible HLA haploidentical living donor kidney transplants between 2002 and 2007 at the MGH were evaluated. Eleven of these subjects were

Table 1: Subject profiles and results

	Age	Sex	Original disease	Time discontinued immunosuppression (months)	Graft survival	Current pathology	Current creatinine (mg/dL)	Urine protein (mg/L)	Immunosuppression
NKD03									
1	22	F	Alport's disease	9	>11.3 years	No rejection	1.1	12	None
2	22	M	MPGN type 1	14	>10.6 years	No rejection MPGN recurrence	2.1	210	Low-dose MMF (after 8th year for MPGN)
3	39	M	PCKD	–	10 days	Acute humoral rejection	–	–	NA (retransplanted)
Modified NKD03									
4	25	M	Alport's disease	9	>9.0 years	CR (after 5th year)	2.8–3.2	1190	MMF, belatacept (after 6th year)
5	46	M	PCKD	9	>8.0 years	Borderline CR (after 7th year)	2.3–2.8	490	Belatacept (after 7th year)
ITN036									
6	35	M	Alport's disease	8	>5.0 years	No rejection	1.5	10	None
7	37	F	Reflux uropathy	8	>4.8 years	No rejection	0.8	20	None
8	36	F	FGS	–	0.5 years	TMA	–	–	NA (on CAPD)
9	22	F	MPGN type 1	8	>4.4 years	No rejection	1.1	50	None
10	26	M	Alport's disease	8	3 years	Post-ACR	–	–	NA (retransplanted)

ACR, acute cellular rejection; CAPD, continuous ambulatory peritoneal dialysis; CR, chronic rejection; FGS, focal glomerulosclerolis; MMF, mycophenolate mofetil 250 mg twice a day; MPGN, membranoproliferative glomerulonephritis; NA, not applicable; PCKD, polycystic kidney disease; TMA, thrombotic microangiopathy.

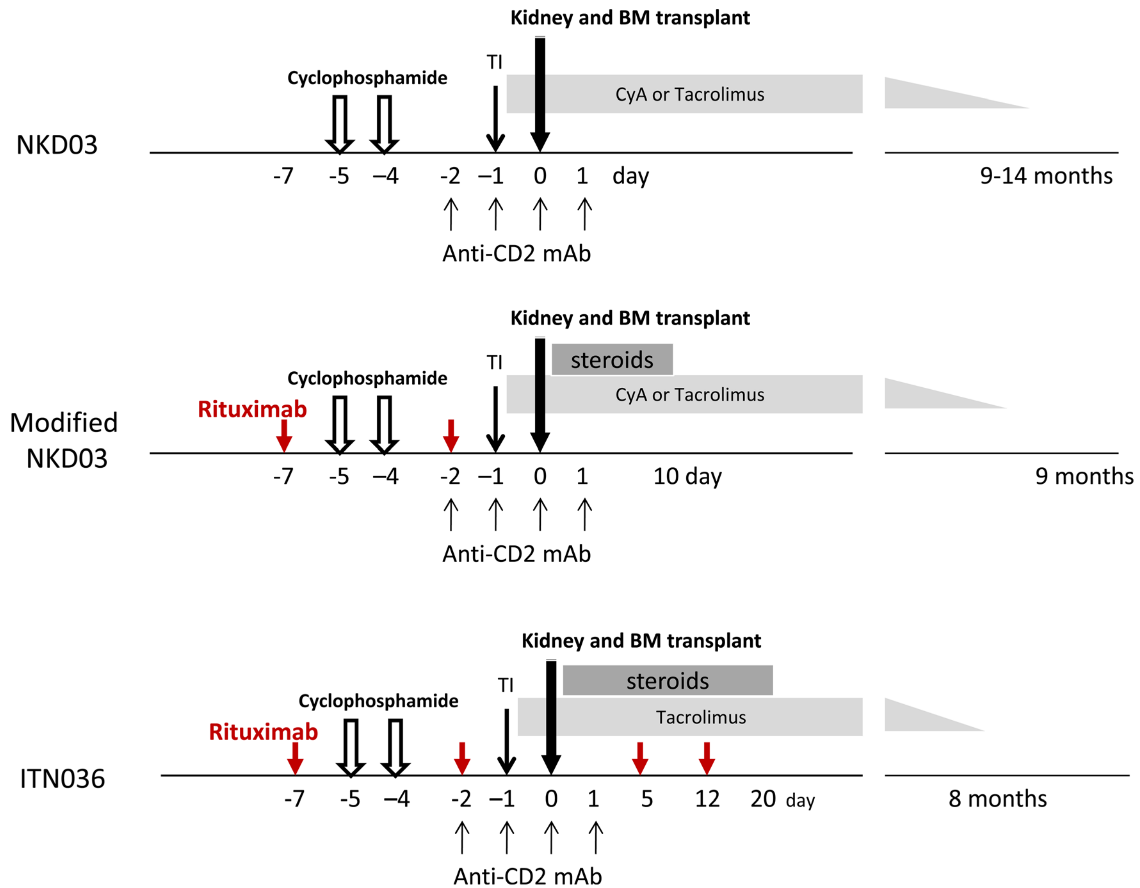

Figure 1: Nonmyeloablative conditioning regimens. The initial conditioning regimen (Figure 1, NKD03) consisted of cyclophosphamide (60 mg/kg) administered i.v. on Days −5 and −4 with respect to transplantation; humanized anti-CD2 mAb (MEDI 507) (0.6 mg/kg/dose) on Days −2, −1, 0 and +1; cyclosporine A (CyA) (5 mg/kg) i.v. on Day −1 and thymic irradiation (700 cGy) on Day −1. Hemodialysis was performed 14 h after each dose of cyclophosphamide. On Day 0, kidney transplantation was followed by i.v. infusion of unprocessed donor bone marrow (DBM; 2–3 $\times$ 10^8 mononuclear cells/kg). Oral CyA (Neoral) was administered postoperatively at 8–12 mg/kg/day with target trough blood levels of 250–350 ng/mL, then tapered and discontinued over several months. The protocol was modified after treatment of the third subject (see the Results section), with the addition of rituximab, 375 mg/m^2/dose on Days −7 and −2 (red arrows); and prednisone, 2 mg/kg/dose starting on the day of transplantation and tapering to withdrawal over the next 10 posttransplant days (mod NKD03). Since subjects treated with this mod NKD03 still developed donor-specific antibodies (DSAs) after discontinuation of immunosuppression, the regimen was further modified (Figure 1) to add two more doses of rituximab (375 mg/m^2/dose) on Days 5 and 12 (red arrows), plus a more prolonged course of prednisone until Day 20, and tacrolimus in place of CyA (ITN036). Tacrolimus was slowly tapered over several months and completely discontinued at 8 months after confirming no rejection by a 6-month protocol biopsy.

excluded from the study; seven had pretransplant insulin-dependent diabetes and four were followed by other institutions. The incidence of posttransplant complications and the number of medications required in the remaining 21 subjects, who were closely followed up at the MGH under identical institutional oversight/guidance, were compared to those in the study subjects.

Statistical analysis of two groups was performed using Fisher's test.

Conditioning regimen

The initial conditioning regimen (Figure 1, NKD03) consisted of cyclophosphamide (60 mg/kg) administered intravenously (i.v.) on Days −5 and −4 with respect to transplantation; humanized anti-CD2 mAb (MEDI 507; MedImmune, Inc., Gaithersburg, MD) at a test dose of 0.1 mg/kg on Day −2 followed by 0.6 mg/kg/dose on Days −1, 0 and +1; cyclosporine A (CyA; Novartis Pharmaceuticals, Inc., East Hanover, NJ) 5 mg/kg i.v. on Day −1; and thymic irradiation (700 cGy) on Day −1. Hemodialysis was performed before and 14 h after each dose of cyclophosphamide. On Day 0, kidney transplantation was followed by i.v. infusion of unprocessed donor bone marrow (DBM; 2–3 $\times$ 10^8 cells/kg). Oral CyA (Neoral; Novartis Pharmaceuticals, Inc.) was administered postoperatively at 8–12 mg/kg/day with target trough blood levels of 250–350 ng/mL, then tapered and discontinued over several months.

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more doses of rituximab (375 mg/m^2/dose) on Days 5 and 12, a more prolonged course of prednisone until Day 20, and tacrolimus in place of CyA (ITN036). Tacrolimus was subsequently tapered over several months and completely discontinued at 8 months after confirming no rejection by a 6-month protocol biopsy. All treatment regimens were approved by the MGH Institutional Review Board and developed in collaboration with the Immune Tolerance Network (ITN).

Biopsies

Protocol kidney allograft biopsies were taken at Day 0 and at months 6, 12, 24 and 36 (NKD03) and at months 6, 12 and 24 (ITN036). Some subjects had later protocol biopsies after 5–8 years of follow-up. Indication biopsies were taken for any episode of unexplained renal allograft dysfunction. All biopsies were processed for routine light microscopy, immunofluorescence (including C4d stains) and electron microscopy by techniques previously reported (18,19).

In vitro *immunologic assays*

Standard mixed lymphocyte reaction (MLR) and cell-mediated lympholysis (CML) assays were performed using the methods detailed previously (16). Limiting dilution assay to quantify cytotoxic T-lymphocyte precursor frequencies and IL-2-producing T helper frequencies were performed as described (20). Lymphocyte subsets were monitored by flow cytometry as previously described (21).

Detection of DSAs

Serially collected pretransplant and posttransplant sera samples were tested for the presence of HLA antibodies using ELISA kits (LAT Class I and II; One Lambda, Canoga Park, CA).

Serum BAFF measurement

Concentration of BAFF in the serum was measured using a Quantikine ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Serum samples were diluted 1:8 in phosphate buffered saline.

Results

Induction of transient chimerism

All subjects developed transient multilineage mixed chimerism, which became undetectable by 2–3 weeks post-CKBMT (18,22).

Acute kidney injury 10–14 days after DBMT

We previously described the cytokine syndrome-like manifestations observed after CKBMT as ''engraftment syndrome'' (18,19). The symptoms have been temporally associated with the loss of peripheral chimerism as well as of the return of host-derived hematopoietic elements. The most troublesome manifestation of this syndrome, and the only manifestation not alleviated by addition of steroids to the posttransplant treatment regimen, was acute kidney injury (AKI), which was observed after Day 10 in all patients, except for Subject 1. The biopsies taken during AKI showed severe capillary endothelial injury with CD8$^+$ and CD68$^+$ cells in peritubular and glomerular capillaries, as reported previously (19). The peak serum creatinine (Cr) level ranged from 3.5 to 15.4 mg/dL during Days 10–20. Among the nine subjects who developed AKI, three recovered without additional treatment. Renal allograft function was normalized in two subjects in conjunction with Thymoglobulin and plasma exchange, in one with Thymoglobulin administration alone and in one with additional steroid therapy. Two kidney allografts failed to recover, one in association with humoral rejection possibly due to preformed anti-donor antibody (Subject 3) and one that progressed to thrombotic microangiopathy associated with high tacrolimus levels (Subject 8).

Long-term clinical course of NKD03 subjects (1–5)

Initial results, after follow-up periods of 2–5 years, in the first five patients, were previously reported (18). Except for Subject 3, who suffered early graft failure due to acute antibody-mediated rejection, followed by successful retransplantation with conventional IS, all original renal allografts are functional with the longest survival now exceeding 11 years (Subject 1). Four protocol biopsies performed in Subject 1 over the first 7.5 years after CKBMT showed no evidence of rejection, including the absence of C4d deposition and normal electron microscopy (Figure 3A–C). Subject 2 was successfully tapered off of IS by 14 months, after treatment for suspected humoral rejection (transient C4d deposition without detectable serum DSA) at around Day 45. He remained stable until Year 7 following IS withdrawal (Figure 2). At that point, microalbuminemia first became detectable. Allograft biopsy revealed no rejection but recurrence of his original disease, MPGN Type I (Figure 3D–F). Despite inconsistent compliance with the low-dose mycophenolate mofetil (MMF; 250 mg twice a day) monotherapy recommended for recurrent MPGN after the 7th year, he is currently stable (serum Cr 2.1 mg/dL) (Figure 2) with minimal urinary protein (200–300 mg/L) more than 10 years after CKBMT. Subject 3 had shown high panel reactive antibody (50%) but did not show DSA by ELISA and crossmatch was negative before transplantation. However, he lost his renal allograft with severe acute antibody-mediated rejection on Day 10 (Figure 2). More recent analysis with single antigen beads suggested preformed DSA against HLA Class I (mean fluorescence intensity >10,000, data not included), leading us to conclude that his humoral rejection may have been due to presensitization. This subject subsequently underwent successful retransplantation with conventional IS. Subject 4 received the mod NKD03 regimen (Figure 1). Although he developed DSA to donor Class II (DR17) shortly after IS withdrawal, protocol biopsies as early as 11 months and up to Day 731 did not have features of active rejection despite remaining positive for C4d (18). However, his 5-year protocol biopsy revealed glomerular basement membrane duplication by light and electron microscopy with capillaritis, continued C4d deposition, and minimal interstitial fibrosis (Figure 3G–I), meeting the criteria for chronic active antibody-mediated rejection (23). This process was subclinical with his renal function remaining stable (serum Cr 1.6 mg/dL) and no proteinuria (Figure 2). Nevertheless,

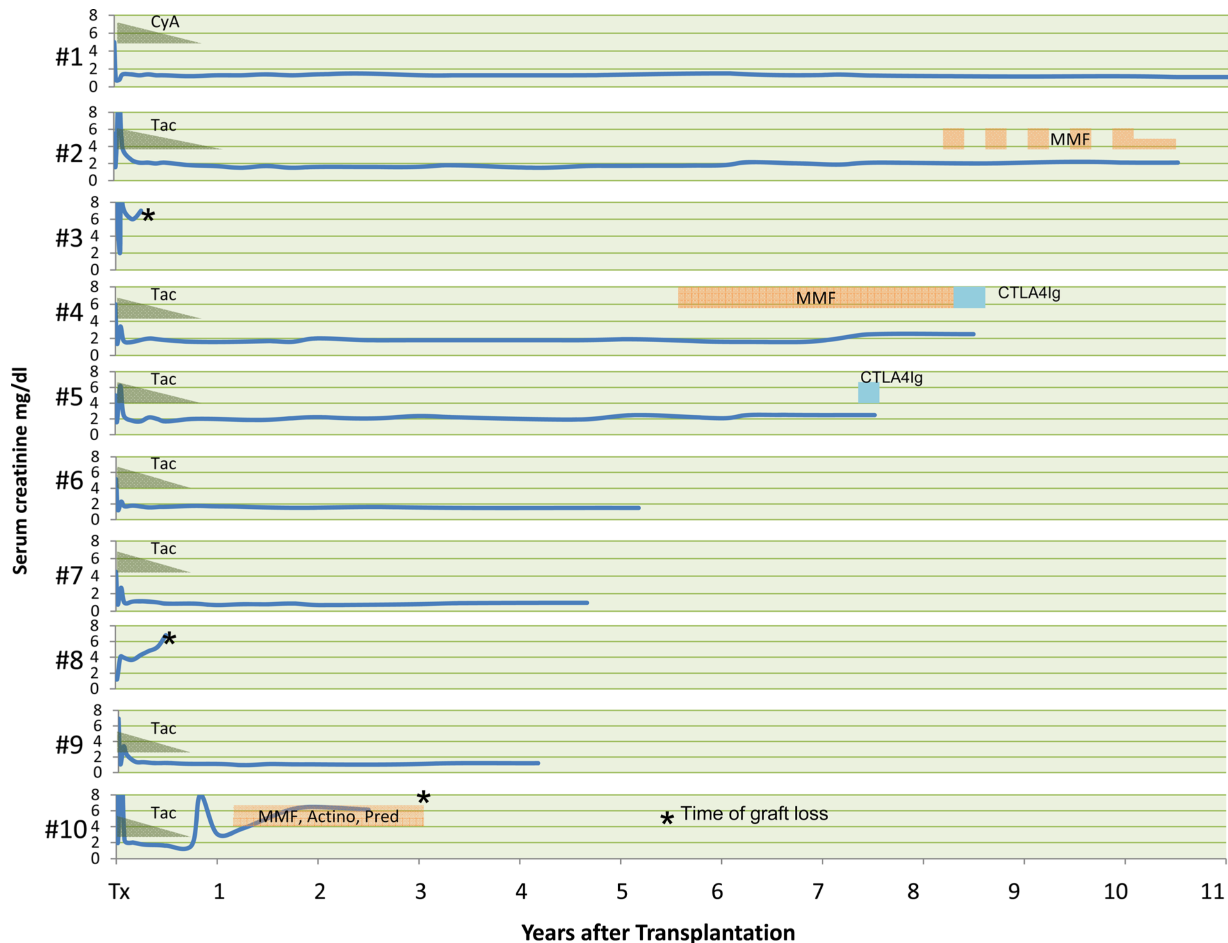

Figure 2: Clinical course after combined kidney and bone marrow transplantation. Blue lines indicate serum creatinine levels (mg/dL). Green bars indicate induction immunosuppression (CyA: cyclosporine, Tac: tacrolimus). Orange/blue bars indicate reinstitution of maintenance immunosuppression (MMF: mycophenolate mofetil, actino: actinomycin, Pred: prednisone), CTLA4Ig (belatacept).

because of the protocol biopsy, MMF was initiated 5 years after transplantation. Subsequently, proteinuria became detectable 7 years posttransplant and his kidney function began to slowly deteriorate. A brief course of intravenous immunoglobulin and rituximab was administered and belatacept has recently been added (Figure 2) to try to prevent further progression. Subject 5 remained stable for 6 years (Figure 2) with no evidence of rejection or C4d deposition, despite intermittent detection of weak DSA after 3 years (Figure 4). Subsequently, after suffering multiple severe episodes of gout, his renal function deteriorated and C4d deposition transiently became detectable in the biopsy at 6 years.

A subsequent protocol biopsy at 6.8 years showed transplant glomerulopathy (cg3) and glomerulitis (g3) (Figure 3J and K) with no detectable C4d deposition (Figure 3L), meeting the 2013 Banff criteria for early stage of C4d-negative antibody-mediated chronic rejection (23). Renal function continues to fluctuate with serum Cr between 2.3 and 2.8 mg/dL (Figure 2) and belatacept was recently initiated after the 7th year.

Clinical course of ITN036 (6–10)

Subject 6 developed the typically observed AKI after Day 10 and recovered without additional treatment. His IS was slowly tapered and completely discontinued at 8 months after CKBMT. Although DSA has never been detectable by ELISA, his protocol biopsy at 2 years showed C4d deposition in peritubular capillaries (C4d3) with little or no glomerulitis (g0) or peritubular capillaritis (ptc0) and no GBM duplication by electron microscopy (Figure 3M–O). These findings fit the Banff category of C4d deposition without active rejection (23). He is currently doing well with a serum Cr level of 1.5 mg/dL over 5 years after transplantation, without ongoing IS (Figure 2).

Immunosuppressive therapy for Subject 7 was discontinued at 8 months. She is currently almost 5 years after

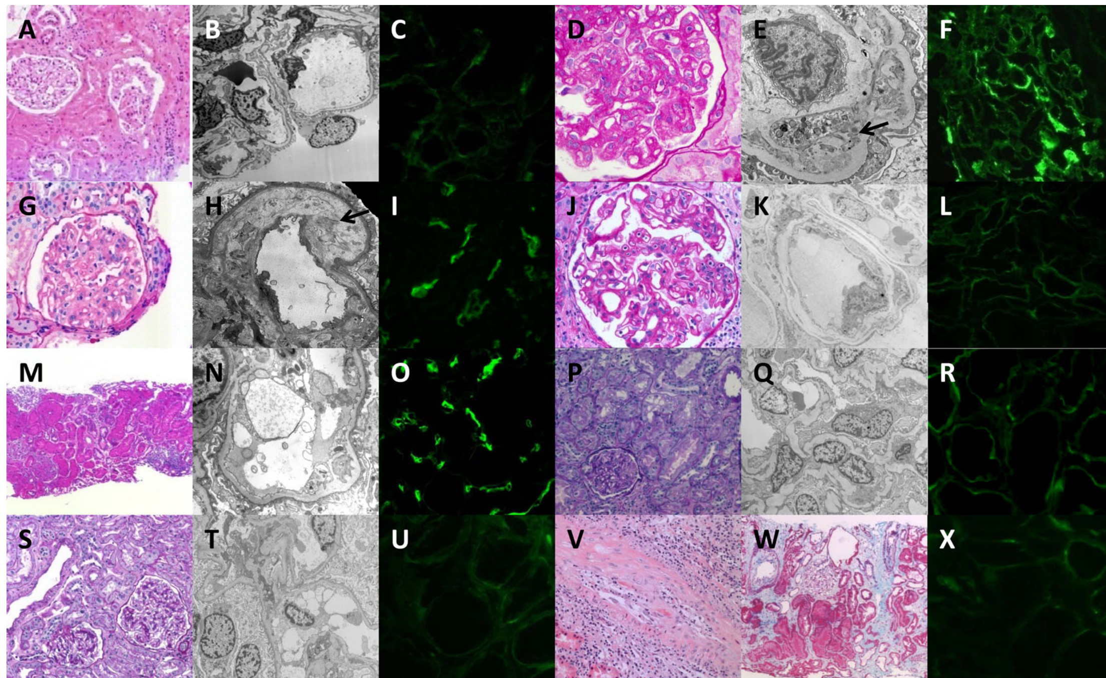

Figure 3: Late renal allograft biopsies (all protocol biopsies unless noted). (A, B, C [Subject 1 at 7.5 years]) Protocol biopsy shows no evidence of rejection by light microscopy (LM) (A). The glomeruli are normal by electron microscopy (EM) (B) and no C4d staining is detectable by immunofluorescence (IF) (C). (D, E, F [Subject 2 at 8 years]) An indication biopsy for proteinuria shows prominent lobular mesangial expansion with widespread glomerular basement membrane (GBM) duplication (D). Granular dense deposits in the duplicated GBM are seen by EM (E, arrow). Granular and segmental staining for C3 along the GBM and in the mesangium is evident by IF, but no immunoglobulin was detected (F), indicative of recurrent C3 glomerulopathy (originally classified as membranoproliferative glomerulonephritis [MPGN], Type I). (G, H, I [Subject 4 at 5 years]) Widespread GBM duplication and glomerulitis are seen by LM (G). EM shows prominent duplication of the GBM without deposits and reactive endothelial cells (H, arrow). C4d deposition is detected in peritubular capillaries (I). These findings are indicative of chronic, active antibody-mediated rejection, which at this time was subclinical. (J, K, L [Subject 5 at 6 years]) The last protocol biopsy shows glomerulitis and widespread duplication of the GBM by LM (J) and EM (K) and no C4d deposition by IF (L). DSA for donor Class II was detected intermittently and a previous biopsy showed C4d deposition. These findings meet the criteria of subclinical, C4d negative, early-stage chronic active antibody-mediated rejection. (M, N, O [Subject 6 at 2 years]) LM shows normal glomeruli with rare foci of interstitial mononuclear inflammation affecting <5% of the cortex (M). EM shows minimal focal GBM duplication and normal endothelium (N). C4d was present in the majority of the peritubular capillaries (O). (P, Q, R [Subject 7 at 2 years]) LM is within normal limits (P). EM reveals a normal GBM and endothelium; foot process effacement is present in a minority of the capillaries (20%) (Q). There is no C4d deposition (R). (S, T, U [Subject 9 at 2 years]) The kidney biopsy is within normal limits and shows no evidence of rejection by LM (S) with normal glomeruli by EM (T). There is no evidence of recurrent MPGN. No C4d is detected (U). (V, W, X [Subject 10]) Indication biopsy at 9.5 months shows acute cellular rejection with endarteritis (V). A protocol biopsy 6 months later shows complete resolution of the inflammatory process and residual interstitial and intimal fibrosis (W). No C4d deposition is detectable at 9.5 months (X) or at other times. LM stains: H&E, A, M, V; PAS, D, G, J, P, S. Immunofluorescence stains: C4d, C, F, I, L, O, R, U, X.

CKBMT with normal kidney function (serum Cr 0.8 mg/dL) (Figure 2) and a normal protocol biopsy at 2 years (Figure 3P–R). Subject 8 failed to fully recover after developing typical early AKI. Biopsy performed on Day 22 revealed arterial intimal matrix expansion with infiltrating mononuclear cells, red cell fragments and arterial fibrinoid necrosis. No C4d deposition or circulating DSA was detectable. Since the differential diagnosis included acute cellular rejection Type III, the tacrolimus dosage was initially increased. When subsequent interpretation concluded this was more likely thrombotic microangiopathy, possibly due to tacrolimus toxicity, tacrolimus was discontinued and three doses of anti-thymocyte globulin were administered together with MMF. Despite these treatments, her kidney function gradually failed and she was returned to continuous ambulatory peritoneal dialysis at 7 months after transplantation. Subject 9 developed transient AKI after Day 10 and recovered without additional treatment. Her IS was discontinued at 8 months after transplantation. She is currently well at over 4.5 years after CKBMT with normal kidney function (serum Cr 1.0 mg/dL) (Figure 2). The protocol biopsy at 2 years showed no evidence of rejection (Figure 3S–U). In Subject 10, kidney function gradually returned to normal by 2 months after AKI and a 6-month protocol biopsy did not show rejection. His IS was discontinued at 8 months. One month later, he developed acute pyelonephritis with moderately elevated serum Cr (2.2 from 1.6 mg/dL). This was treated with antibiotics

		pre	1 y	2 yrs	3 yrs	4 yrs	5 yrs	6 yrs	7 yrs	8 yrs	9 yrs	10 yrs
NKD03	#1	-	-	-	-			-				-
	#2	-	-	-	-					DQ9	-	-
	#3	-*	DR4/B44									
Mod NKD03	#4	-	DR17	DR17	DR17			DR17	DR17			
	#5	-	-	-	DR53	DR53	DR53	-				
ITN036	#6	-	-	-	-	-						
	#7	-	-	-	-	-						
	#8	-	-									
	#9	-	-	-	-							
	#10	-	-	-	-							

Figure 4: Detection of donor-specific antibody (DSA). DSAs as detected by ELISA. NKD03: Subject 1: DSA never detected. Subject 2: DSA transiently detected once at 8 years. Subject 3: *DSA was negative by ELISA but retrospective analysis with single antigen beads showed positive DSA against donor HLA Class I (mean fluorescence intensity > 10 000). The subject developed acute humoral rejection on Day 10 with ELISA detected DSA (B44 and DR4). Pretransplant DSA had been negative by ELISA, but more recent Luminex suggested that positive anti-Class I DSA (B44) before transplantation may have been present before transplantation. Mod NKD03: Subject 4: anti-donor DR17 antibody has been persistently positive since shortly after stopping immunosuppression. Subject 5: weak anti-donor DR53 antibody has been intermittently detectable. ITN036: in contrast to NKD03 subjects, no DSA has been detectable in any ITN036 subject including Subject 10 who developed severe acute cellular rejection.

without biopsy since the kidney function promptly recovered to baseline. However, 3 weeks after the resolution of his infection, he developed severe acute T cell–mediated rejection (Banff 2B) (Figure 3V). Several DSA assays remained negative as was C4d stain. He was treated with steroid pulses and anti-thymocyte globulin, following which his renal function improved but never fully recovered (Figure 2). A renal biopsy 6 months later showed interstitial and intimal fibrosis without active inflammation (Figure 3W). His kidney function remained compromised thereafter. He underwent preemptive living donor kidney retransplant with conventional IS at 3.3 years after CKBMT.

Anti-donor T cell responses

All NKD03 subjects developed donor-specific nonresponsiveness (DSN) by MLR, CML, CTLp and HTLp assays at 3–9 months, as previously reported (18,24). In the four ITN036 subjects who discontinued their IS, DSN or donor-specific hyporesponsiveness (DSH) also developed in MLR by 3–18 months. Anti-donor T cell responses became detectable again at 18–36 months in Subjects 7 and 9 (Table 2).

High incidence of de novo *DSA production in the mod NKD03*

De novo DSA measured by ELISA has never been detected in Subjects 1, 6, 7, 8, 9 and 10 after IS was discontinued. DSA was transiently detected only once at 8 years in Subject 2 with no evidence of rejection. Anti-HLA Class II DSA has been persistently positive in Subject 4, and was associated with persistent C4d deposition and glomerulopathy. In Subject 5, weak anti-Class II DSA has been detected intermittently after 3 years with transient C4d deposition (Figure 3). In ITN036 subjects, no DSA has been detectable even in Subject 10 who developed severe acute cellular rejection.

B cells ($CD3^-CD19^+$) were almost completely eliminated from the circulation for 6 months in ITN036 subjects

Recovery of $CD3^-CD19^+$ cells in the two subjects (1 and 2) after treatment with the original NKD03 regimen was observed by Days 50 and 100, respectively. With two doses of pretransplant rituximab (mod NKD03), depletion of $CD3^-CD19^+$ cells was extended to Day 150 (Subjects 4 and 5) but complete loss of $CD3^-CD19^+$ cells from the peripheral blood was not observed. In contrast, peripheral blood $CD3^-CD19^+$ cells were less than 1–2/mm^3 for 6 months in all five ITN036 subjects treated with four doses of peritransplant rituximab (Figure 5A) (22).

High serum BAFF levels in the recipients treated with rituximab

Serum BAFF levels were <2 ng/mL at all time points assessed in Subject 1. Subject 2 had high BAFF levels initially until Day 100 and then waned thereafter to <2 ng/mL after Day 100. In contrast, high BAFF levels were detected for over 300 days in the two subjects treated with the mod NKD03 regimen as well as in the ITN036 subjects (Figure 5B).

Table 2: Summary of MLR, CML and LDA–CTLp in ITN036[1]

	MLR	CML	LDA–CTLp
Pt6			
preTx	R	GN[2]	R
3 months	–	–	R
6 months	R	GN	DSH
9 months	DSH[3]	DSN[4]	DSN
12 months	R	DSN	R
18 months	DSH	DSN	DSH
Pt7			
preTx	DSN	R	DSH
3 months	–	–	GH[5]
6 months	GN	GN	GH
12 months	GN	GN	GH
18 months	DSN	DSN	GH
36 months	DSN	R	R
Pt9			
preTx	DSH	R	R
2 months	R	GN	GH
3 months	R	DSN	R
6 months	R	R	R
9 months	R	R	R
12 months	DSH	R	R
18 months	DSH	R	DSH
Pt10			
preTx	DSH	R	R
4 months	GN	GN	GH
6 months	DSH	DSN	DSN
9 months	DSN	DSN	DSH
12 months	DSN	GN	GH

CML, cell-mediated lympholysis; LDA–CTLp, limiting dilution assay cytotoxic T-lymphocyte precursor; MLR, mixed lymphocyte reaction; R, responsive to donor and third party; –, not done; GN, global nonresponsiveness; DSH, donor-specific hyporesponsiveness; DSN, donor-specific nonresponsiveness.

[1]The details of the experiments upon which these assay results are based will be published elsewhere (Sykes et al, manuscript in preparation).

[2]Global nonresponsiveness (GN) defined in MLR as a stimulation index (SI) anti-donor <2.5 and SI anti-third party <2.5 and in CML as both maxPSL anti-donor and maximum percent-specific lysis (maxPSL) anti-third party $<5\%$.

[3]Donor-specific hyporesponsiveness (DSH) defined in MLR as an SI anti-donor >2 and SI anti-donor less than or equal to one-third SI anti-third party, in CML as NmaxPSL patient anti-donor 5–10% and less than one-fifth of maxPSL patient anti-third party and in CTLp as maximum percentage of positive wells (maxPPW) anti-donor between 5–20% and less than one-third of anti-third party.

[4]Donor-specific nonresponsiveness (DSN) defined MLR as an SI anti-donor ≤ 2 and SI anti-third party >3, in CML as maxPSL patient anti-donor $\leq 5\%$ and maxPSL anti-third party $>5\%$ and in CTLp as maxPPW anti-donor $<5\%$ and maxPPW anti-third $>30\%$.

[5]Global hypo-responsiveness (GH) was in CTLp defined as a maxPPW anti-donor $<20\%$ and maxPPW anti-third party $<20\%$.

Comparison of clinical course with that observed in immunologically similar kidney allograft recipients treated with conventional IS

Between 2002 and 2007, 32 consecutive recipients of comparable age (20–45) received ABO blood type compatible HLA haploidentical living donor kidney transplants at our own institution. Eleven of these patients were excluded from the study; seven had pretransplant insulin-dependent diabetes and four were followed by other institutions. The clinical course of the remaining 21 patients treated with conventional IS was compared with the 10 subjects who received the tolerance protocol. Of the 21, four suffered graft loss at 29 days, 3.9, 6.3 and 7.1 years due to rejection (Table 3). Seventeen recipients remain on conventional IS with serum Cr levels ranging from 0.9 to 2.3 mg/dL. Not unexpectedly, many of the conventionally treated recipients suffered significant posttransplant morbidity including hypertension requiring medical managements in 17/20 (85%, $p = 0.04$) and hyperlipidemia in 13/20 (65%, $p = 0.005$). New-onset insulin-dependent diabetes were observed in seven patients (35%). Two patients developed malignant skin lesions and approximately 25% of these patients required posttransplant hospital admission for treatment of various infectious complications including urinary tract infection, pneumonia and actinomycosis. Their maintenance medical regimen, in addition to the immunosuppressive medications, included more than five medications to treat these morbidities. In contrast, only three of the recipients (43%) from whom IS was withdrawn are taking anti-hypertensive medications. None have developed hyperlipidemia, *de novo* diabetes, malignancy or infection requiring hospitalization.

Discussion

We report here that induction of long-term (>11 years) stable renal allograft function without maintenance IS can be achieved after induction of transient lymphohematopoietic chimerism in recipients of HLA-mismatched CKBMT. Our clinical protocol is based upon decades-long studies in rodent, swine and nonhuman primate (NHP) models. In these studies, we found that durable mixed chimerism, which is readily inducible in rodents, is difficult to achieve in MHC-mismatched NHP conditioned with our nonmyeloablative regimen. Using a TLI-based regimen, the Stanford group has similarly reported that induction of durable mixed chimerism is difficult to achieve in HLA-mismatched recipients, although durable chimerism was observed in about two-thirds of HLA-matched kidney/bone marrow recipients (8). The Northwestern group recently reported induction of durable full donor chimerism and renal allograft tolerance in HLA-mismatched recipients treated with a more intensive conditioning regimen including fludarabine, total body irradiation (TBI) and cyclophosphamide (9). They, in fact, concluded that durable chimerism is a necessary requisite for successful tolerance induction (25). However, in our opinion, replacing the entire recipient hematopoietic population with donor cells is difficult to justify for patients without malignancies. In addition to concerns regarding GVHD, full donor chimerism may lead to immuno-incompetence (26) and various bacterial, viral and fungal infections were indeed reported

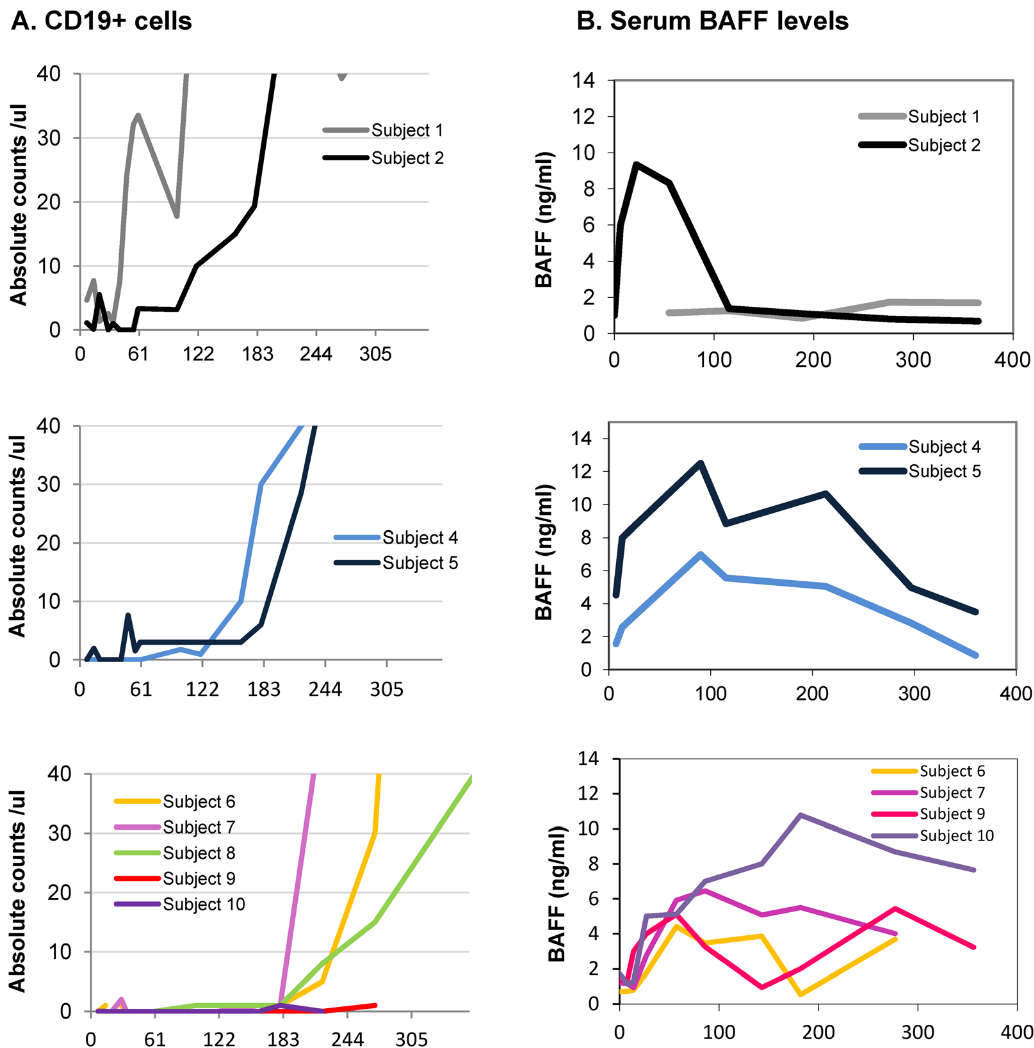

Figure 5: (A) $CD3^{-}CD19^{+}$ cell depletion after combined kidney and bone marrow transplantation. In two NKD03 subjects, recovery of $CD3^{-}CD19^{+}$ cells (B cells) was found by Day 50 (Subject 1, gray) and Day 120 (Subject 2, black). With addition of two doses of pretransplant rituximab (modified NKD03), B cells were partially depleted and recovered by Days 150 (Subject 4, dark blue) and 180 (Subject 5, light blue). With four doses of peritransplant rituximab (ITN036), B cells were nearly completely depleted (less than 1–2/mm^2) for 180 days. (B) Serum B cell activating factor (BAFF). Serial serum BAFF levels were measured by ELISA. Serum BAFF levels were <2 ng/mL at all time points assessed in Subject 1. Subject 2 had high BAFF levels initially until Day 100 and waned thereafter to <2 ng/mL after Day 100 and he successfully discontinued his immunosuppression. High BAFF levels were detected for over 300 days in the two subjects treated with the mod NKD03 regimen and in the ITN036 subjects.

in the Northwestern patients (9). Moreover, the long-term consequences of this treatment on autoimmunity or cancer surveillance have not been known.

Although induction of durable mixed chimerism might avoid these concerns, this state has been difficult to achieve in MHC-mismatched NHP and humans, in which chimerism tends to be either transient (with recipient cells replacing donor cells) or full (with donor cells replacing recipient cells), depending on the intensity of the conditioning regimen. Consistent with the fact that chimerism in our recipients was transient, there has been no GVHD observed in our preclinical (11,12) or clinical studies (18), which is an attractive attribute of this approach for tolerance induction. Apparently, the mechanism of tolerance induction after transient chimerism in NHPs and humans differs from that observed in murine mixed chimerism models, where central deletional pathways have been demonstrated, in conjunction with chimerism that persists indefinitely (10,13,27,28). Important to the mechanisms operative in this strategy, our NHP studies have consistently demonstrated the absolute necessity for DBM administration, resulting in at least transient measurable donor chimerism, especially in lymphoid lineages, for successful induction of

Table 3: Posttransplant complications in conventional immunosuppression versus tolerance

A. Conventional immunosuppression

No.	Age at Tx	Sex	Survival	Creat	HTN	Hyper-lipid	*De novo* DM	Malignancy	Infection[1]	No. of drugs IS + others[2]
1	33	F	>11.6 years	1.1	–	+	–	–	+	3 + 5
2	42	F	>11 years	1	+	+	+	–	+	3 + 7
3	36	F	>10.8 years	1.4	+	+	+	–	–	2 + 12
4	38	F	>10.8 years	2.3	+	+	–	–	+	2 + 8
5	40	F	>10.7 years	1.2	+	+	–	–	+	3 + 5
6	42	F	>10.6 years	1	+	–	–	SCC BCC	–	2 + 1
7	26	M	>10.3 years	2	+	+	+	–	–	2 + 6
8	23	M	>9.7 years	1.3	+	–	–	–	–	3 + 2
9	27	M	>9.5 years	1.5	+	+	+	Melanoma, SCC	–	2 + 3
10	40	M	>9.2 years	1.9	+	+	+	–	–	3 + 9
11	20	F	>8.5 years	1.1	–	–	–	–	–	3 + 4
12	42	M	>8.4 years	2.1	+	–	–	–	–	2 + 2
13	32	M	>8.3 years	1.3	+	+	+	–	–	3 + 8
14	26	M	>8.2 years	0.95	+	+	+	–	+	2 + 9
15	42	F	>8.1 years	1.4	–	–	–	–	–	3 + 3
16	43	M	>7.9 years	1.2	+	+	–	–	–	3 + 5
17	31	M	>7.7 years	1.6	+	+	–	–	–	3 + 5
Graft loss										
18	37	F	29 days	reTx	NA	NA	NA	NA	NA	NA
19	44	M	3.9 years	On HD	+	–	–	–	–	2 + 5[3]
20	45	M	6.3 years	On HD	+	+	–	–	–	2 + 12[3]
21	22	M	7.1 years	On HD	+	–	–	–	–	3 + 3[3]
Incidence of complication (p-value[4])					85% ($p = 0.04$)	65% ($p = 0.005$)	35% (ns[5])	10% (ns)	25% (ns)	

B. Tolerance protocol

No.[6]	Age at Tx	Sex	Survival	Creat	HTN	Hyper-lipid	*De novo* DM	Malignancy	Infection	No. of drugs IS + others
1	22	F	>11 years	1.1	–	–	–	–	–	0 + 1
2	22	F	>10.5 years	2.2	+	–	–	–	–	1 + 1[7]
4	28	F	>8.8 years	2.0	+	–	–	–	–	2 + 2[8]
5	46	F	>7.8 years	2.5	+	–	–	–	–	1 + 1[9]
6	35	F	>4.8 years	1.5	–	–	–	–	–	0 + 1
7	37	F	>4.6 years	0.8	–	–	–	–	–	0
9	22	M	>4.2 years	1.1	–	–	–	–	–	0
Graft loss										
3	39	M	10 days				NA			
8	36	F	6 months				NA			
10	26	M	3 years				NA (on IS after 10 months)			
Incidence of complication					43%	0%	0%	0%	0%	

BCC, basal cell cancer; Creat, serum creatinine (mg/dL); *De novo* DM, *de novo* diabetes that requires insulin therapy; HTN, hypertension; Hyper-lipid, hyperlipidemia; NA, not applicable (observation too short); SCC, squamous cell carcinoma; IS, immunosuppression; HD, hemodialysis.

[1]Required admission to Massachusetts General Hospital.
[2]Number of medications taking daily: IS + other drugs.
[3]Immediately before graft failure.
[4]Comparing with the incidence in tolerance induction.
[5]Statistically not significant, comparing with tolerance induction ($p > 0.05$).
[6]Patient no. in Table 1.
[7]Noncompliant.
[8]After 6th year.
[9]After 7th year.

tolerance (11,12,29). The NHP studies have also shown that co-stimulatory blockade, with agents directed against CD40/CD154 (12) or B7/CD28 (manuscript in preparation), are important in achieving consistent tolerance. In our clinical trials, we have used an anti-CD2 mAb (MEDI507) which provides both T cell depletion and co-stimulatory blockade via the CD2/LFA-3 interaction (18). Our preclinical studies have also emphasized that the kidney allograft itself plays a critical role in the induction and maintenance of tolerance. For example, the same conditioning regimen that achieves renal allograft tolerance after induction of transient chimerism, has consistently failed to induce tolerance of isolated heart allografts in monkeys (30). However, heart allograft tolerance is regularly achieved by co-transplanting the kidney from the same donor (31). Moreover, patients who developed transient chimerism after receiving the same conditioning regimen as NKD03 and BMT for hematologic malignancies, but without a kidney transplant, failed to develop DSH (20). This was in marked contrast to those receiving kidney transplantation with the same BMT regimen (24). We hypothesize that generation of donor-specific regulatory cells in the thymus during the initial chimeric state is a process that is maintained by continuing interaction with as yet undefined cells or antigens in the kidney allograft and is responsible for maintenance of the tolerance (24). One potentially important renal cell is the renal tubular epithelial cell that has been reported to participate in the induction of allospecific tolerance in rodents and by *in vitro* assays in humans (32–34). Studies are in progress in our laboratories to assess the possible role of this cell in the NHP tolerance model upon which these clinical studies are based.

Despite the encouraging results of successful long-term IS-free renal allograft survival in our initial clinical trials, AKI remains an important obstacle to be overcome in order for this approach to achieve more widespread application. AKI has been observed at approximately Day 10 in all subjects except for Subject 1. We have previously reported this complication as ''engraftment syndrome,'' a term coined for the reaction that sometimes follows autologous or allogeneic BMT as the donor cells begin to repopulate the recipient (35). However, in the present setting, ''engraftment syndrome'' may not adequately describe the phenomenon we have observed, since it was always associated both with recovery of host hematopoietic cells, potentially including homeostatic recovery of memory T cells, and with rapid loss of chimerism. We have noted that the AKI has never been observed in our MHC-mismatched NHP model, in which chimerism is also lost, but at a more gradual pace, usually over 1–2 months (11,12,36). Our interpretation of this observation is that the pretransplant cyclophosphamide-based regimen chosen for the clinical trials (18), in place of low-dose TBI (1.5 Gy $\times$2) used in the NHP preparative regimen (11,12,36,37), may promote more rapid and consequently deleterious homeostatic recovery of host memory T cells, which, when rapidly expanding, may have had destructive effector function at the level of donor renal endothelial cells. This hypothesis is consistent with the observation of CD8 T cell activation immediately following conditioning (19) and the appearance of CD8 T cells in peritubular capillary endothelial cells during the period of AKI. For this reason, in a current modification of our CKBMT protocol, we are now evaluating the effect of substituting low-dose TBI in place of pretransplant cyclophosphamide in HLA-mismatched kidney and bone marrow transplant recipients. In the first treated patient, chimerism was successfully induced but its disappearance and homeostatic recovery of T cells were delayed until 4 weeks after transplantation. Of potential importance, no significant AKI was observed in this recipient. The patient is currently doing well at 4 months after transplantation with his IS being tapered. This encouraging result must be reproduced in additional patients, but it supports our hypothesis that AKI and rapid loss of chimerism seen in the patients reported here may indeed result from inadequate lymphocyte suppression by cyclophosphamide. AKI has not been observed with either the Stanford or the Northwestern protocols. Although the Stanford protocol does not include cyclophospamide, the Northwestern protocol includes cyclophosphamide before and after transplantation. Absence of AKI despite cyclophosphamide in their protocol may be attributed to more severe T cell depletion/suppression concomitantly administered in their conditioning regimen.

We did not anticipate the frequent development of either transient or persistent *de novo* DSA, which was observed among the first five subjects, despite specific loss of anti-donor T cell responses. Since anti-HLA antibodies are thought to represent T cell–dependent responses, we investigated the relevance of anti-T cell responses to DSA production. Surprisingly, there was no relationship observed between DSA production and anti-donor T cell responses in these clinical studies. Mod NKD03 subjects developed DSA despite undetectable anti-donor T cell responses. On the other hand, Subjects 7 and 9 in ITN036 have shown no DSA development despite persistent anti-donor CTL responses.

As depletion of B cells enhances BAFF production, which has been found to significantly enhance the differentiation of marginal zone B cells into immunoglobulin secreting cells in a T cell–dependent manner (38), we monitored serial peripheral blood B cell ($CD19^+$) and serum BAFF levels. In the two mod NKD03 subjects, persistently high serum BAFF levels were detected while B cells were not completely depleted and recovered within 6 months. In these patients, residual or recovering B cells might have been activated under high BAFF environment. In contrast to these NKD03 subjects, peripheral blood B cells were essentially nondetectable for more than 6 months in ITN036 subjects and no DSA production was observed, despite similarly high BAFF levels to those observed in the mod NKD03 patients. Thus, high BAFF levels may not be a risk factor for DSA production if B cells are profoundly

depleted. Of note, transient IgG reactive to multiple HLA alleles were observed before or around 6 months posttransplant for Subjects 6, 7 and 9 (data not shown). These IgG displayed characteristics of polyreactive antibodies (39) and were not accompanied by any clinical manifestation. A detailed analysis of B cell reconstitution in ITN036 subjects, including early polyreactive IgG secretion, will be presented in a separate study. Our clinical observations therefore suggest that B cell depletion for 6 months by rituximab, as achieved in the ITN036 subjects, may avoid the late DSA obstacle to tolerance induction via the mixed chimerism approach. More detailed analysis of B cell responses in failed and successful cases may elucidate mechanisms of DSA development in some recipients, which will lead to the development of novel anti-B cell treatment for consistent inhibition of DSA.

Finally, to compare the results observed in the current study with long-term outcomes of patients who received conventional IS, we identified 21 recipients who underwent HLA haploidentical living donor kidney transplantation between 2002 and 2007 and were closely followed up at MGH. These patients were selected as being most similar to the study subjects in age, histocompatibility and original disease. Among these 21 patients, four recipients lost their kidney allograft at 29 days to 7 years posttransplant due to either acute or chronic rejection. As anticipated, recipients treated with conventional IS developed significantly higher incidence of various metabolic derangements including hypertension, hyperlipidemia and *de novo* diabetes. The majority of patients (85%) require anti-hypertensive medications and 65% of recipients were treated with statins for their dyslipidemia. Despite their relative youth at the time of transplantation, a high incidence (35%) of *de novo* diabetes and skin cancers (melanoma or squamous cell carcinoma) was observed. As in all chronically immune-suppressed patients, infectious complications were common, with 25% of patients repeatedly admitted for treatment. In contrast, in the recipients successfully treated for tolerance induction, only 45% of patients required single anti-hypertensive medication and no other metabolic derangements were observed. There have been no admissions for infectious disease treatment. Of note, human polyoma virus–induced systemic warts that had complicated postoperative course of Subject 1 after her first transplant with conventional IS, completely disappeared after her second transplant with tolerance induction. It is also encouraging that no malignancy has been observed. These results support the criticisms of our current standard of care in organ transplantation as being far from ideal.

In conclusion, our clinical studies have shown that IS-free stable renal allograft function can be achieved in HLA-mismatched donor–recipient pairs, with follow-up times now of >11 years, by induction of transient chimerism through DBMT. We hope that further modifications of the protocol as well as development of more reliable tolerance assays will avoid the remaining obstacles to more widespread application of this technology for clinical tolerance induction.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Authors' Contributions

Tatsuo Kawai, A. Benedict Cosimi, David H. Sachs, Megan Sykes, Thomas R. Spitzer and Robert B. Colvin performed study design, clinical management, data analyses and manuscript writing. Peter Sayre performed study design. Nina Tolkoff-Rubin, Waichi Wong, Winfred W. Williams Jr., Kerry Crisalli and Rex-Neal Smith participated in clinical management. Susan L. Saidman performed DSA assays. Fred Preffer performed lymphocyte subset assays, Baoshan Gao and Emmanuel Zorn performed serum BAFF measurement. Ben Sprangers, Heather Morris, Brittany Shonts and Samuel A. LoCascio, with supervision from Megan Sykes, performed *in vitro* assays and data analyses.

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