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Myeloma Responses and Tolerance Following Combined Kidney and Nonmyeloablative Marrow Transplantation: *In Vivo* and *In Vitro* Analyses

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Six patients with renal failure due to multiple myeloma (MM) received simultaneous kidney and bone marrow transplantation (BMT) from HLA-identical sibling donors following nonmyeloablative conditioning, including cyclophosphamide (CP), peritransplant antithymocyte globulin and thymic irradiation. Cyclosporine (CyA) was given for approximately 2 months posttransplant, followed by donor leukocyte infusions. All six patients accepted their kidney grafts long-term. Three patients lost detectable chimerism but accepted their kidney grafts off immunosuppression for 1.3 to >7 years. One such patient had strong antidonor cytotoxic T lymphocyte (CTL) responses in association with marrow rejection. Two patients achieved full donor chimerism, but resumed immunosuppression to treat graft-versus-host disease. Only one patient experienced rejection following CyA withdrawal. He responded to immunosuppression, which was later successfully withdrawn. The rejection episode was associated with antidonor Th reactivity. Patients showed CTL unresponsiveness to cultured donor renal tubular epithelial cells. Initially recovering T cells were memory cells and were enriched for $CD4^+CD25^+$ cells. Three patients are in sustained complete remissions of MM, despite loss of chimerism in two. Combined kidney/BMT with nonmyeloablative conditioning can achieve renal allograft tolerance and excellent myeloma responses, even in the presence of donor marrow rejection and antidonor alloresponses *in vitro*.

Key words: Bone marrow transplantation, kidney, limiting dilution assay, mixed chimerism, multiple myeloma, tolerance

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Introduction

Nonmyeloablative mixed allogeneic chimerism induction achieves transplantation tolerance in adult animals (1–3). Transient mixed chimerism induced using nonmyeloablative conditioning and a 4-week course of cyclosporine (CyA) is associated with tolerance to simultaneously-grafted donor kidneys in nonhuman primates (4,5).

Donor lymphocytes given several months following bone marrow transplantation (BMT) can convert mixed to full donor chimerism without inducing graft-versus-host disease (GVHD) (6,7). Such lymphohematopoietic GVH reactions mediate potent graft-versus-tumor (GVT) effects (8), which are maximal in mixed chimeras, due to the presence of professional host-type antigen-presenting cells (APC) (9). Based on a murine model involving conditioning with pretransplant anti-CD4 and -CD8 mAbs, cyclophosphamide (CP) and thymic irradiation (7), we have developed clinical protocols that have demonstrated safety and efficacy for the treatment of advanced hematologic malignancies (10–13).

While allogeneic hematopoietic cell transplantation (HCT) is the only known cure for multiple myeloma (MM) (14–16), this approach is not widely applied because the age and general status of the myeloma population make these patients particularly susceptible to the complications of standard allogeneic BMT (17). These patients might therefore benefit from less toxic allogeneic HCT followed by delayed donor leukocyte infusions (DLI) to achieve GVT.

A complication of MM, renal dysfunction induced by urinary light chain excretion, afflicts about 50% of patients.

Because of their poor prognosis, myeloma patients with renal failure are not generally considered to be candidates for renal allotransplantation. Combined renal transplantation with BMT from the same donor, using nonmyeloablative, relatively nontoxic conditioning, might cure the underlying malignancy while allowing acceptance of a donor renal allograft without chronic immunosuppression. Based on our previous experience in patients with malignancies and on the observed tolerance induction with combined kidney/BMT in nonhuman primates (4), we initiated a clinical trial of combined kidney/nonmyeloablative BMT followed by DLI. The initial two patients achieved donor allograft acceptance without chronic immunosuppressive therapy (18,19). We now present clinical results on all six patients who have received this treatment and report on their immune recovery and *in vitro* alloreactivity.

Patients and Methods

Patients

Six patients with end-stage renal failure due to advanced (≥stage II) kappa (n = 4) or lambda (n = 2) light chain MM received BMT and kidney transplants from HLA-identical related siblings between September 1998 and December 2003, following nonmyeloablative conditioning as described (18,19). A seventh patient was withdrawn prior to transplantation due to a severe adverse event related to the conditioning treatment. Patients 1 and 2 were transplanted according to an IRB-approved innovative treatment plan, and their initial clinical outcomes have been previously reported (18,19). The subsequent four patients received the same treatment under an Immune Tolerance Network (ITN)-sponsored IRB-approved protocol. Prior therapies and myeloma status at the time of transplant are shown in Table 1.

Flow cytometric (FCM) analyses of lymphocyte recovery and chimerism analyses were performed every 1–2 weeks for the first 100 days, then at approximately 6, 12 and 24 months post-transplant when possible. Functional assays on peripheral blood mononuclear cells (PBMC) were performed, whenever cell numbers were sufficient, on samples obtained on approximately days 35, 70, 100, 180 and 365 post-transplant.

Conditioning regimen

Conditioning included CP, 60 mg/kg/day, intravenously on days 5 and 4, thymic irradiation (700 cGy) on day 1 and equine antithymocyte globulin 15–20mg/kg/day on day 1, 1, 3 and 5 as described (18,19). CyA was administered as described (18,19) and tapered rapidly in patients without GVHD, with a goal of discontinuation by day 60. The marrow and kidney were obtained from an HLA-identical related donor and transplanted on day 0 as described (18,19).

Donor leukocyte infusion

Protocol DLI (containing 10^7 $CD3^+$ T cells/kg recipient body weight) was performed following CyA discontinuation if there was no evidence of GVHD. Additional, 'therapeutic' DLI was given if disease progressed after BMT, even if previous GVHD had disqualified patients from protocol DLI.

FCM analysis

Red blood cells were lysed from whole blood and white cells were stained in four-color combination with mAbs against CD3, CD4, CD8, CD19, CD45RA, CD45RO, CD25 and CD56 (Becton Dickinson, Mountain View, CA, USA), then analyzed on a Becton Dickinson (San Jose, CA) LSR II. Data analysis was performed using Cell Quest (Becton Dickinson) or WinList (Verity Software House, Inc., Topsham, ME, USA) software.

Microsatellite analysis for chimerism

$CD3^+$ cells were isolated from ficoll separated PBMC with MACS beads (Miltenyi Biotech, Sunnyvale, CA, USA). Chimerism was determined for $CD3^+$ and $CD3^-$ PBMC, or for $CD3^+$ cells and granulocytes obtained after ficoll separation. In later studies, $CD3^+$ and $CD33^+$ enriched cells obtained by negative selection (RosetteSep, StemCell Technologies, Vancouver, Canada) were used. DNA was extracted from separated cells and chimerism was determined by PCR using primers for a variable number of tandem repeats locus or with a multiplex short tandem repeats kit (Profiler Plus, Applied Biosystems, Foster City, CA, USA) as described (10,20) (see online Supplementary Information).

Pathology studies

Under the ITN protocol, protocol renal biopsies were taken at the time of transplantation and approximately 1 year later. Diagnostic biopsies were taken at times of graft dysfunction. Specimens were processed and analyzed as described in online Supplementary Information.

Lymphocyte NK cell depletion

PBMC aliquots were depleted of natural killer (NK) cells with CD56 microbeads using SuperMACS (Miltenyi Biotec), following the manufacturer's protocol. Less than 1% $CD56^+$ cells were detectable by FCM following depletion. Depleted PBMC were resuspended and frozen as described in online Supplementary Information.

Epstein-Bar virus (EBV) transformed B-cell lymphoblastoid cell

PBMC from donors and pretransplant recipients were incubated with EBV for 2 h at 37°C. EBV transformed B cells Epstein-Bar virus transformed B-cell lymphoblastoid cell lines (EBV-LCL) were maintained in RPMI-1640 (Cellgro, Herndon, VA) plus 6% fetal calf serum (FCS).

Culture of renal tubular epithelial cells from pretransplant donor kidney biopsies

Renal tubular epithelial cells (RTECs) obtained from donor kidney biopsies prior to revascularization were cultured as previously reported (21,22), and the cultured RTECs were used as targets in some CTL assays (details in online Supplementary Information). These biopsy samples were available only on Patients 3–6. Epithelial origin of cultured RTEC was demonstrated by flow cytometry using antihuman cytokeratin antibody (CAM 5.2, BD) (Figure 1A). They did not express CD31 (data not shown), an endothelial marker. RTECs demonstrated low class-I HLA expression (Figure 1B) and susceptibility to NK cell-mediated cytolysis (Figure 1C), measured as described (23). Culture in rhIFN-γ enhanced class-I HLA expression (Figure 1B) and reduced RTEC NK cell-mediated cytotoxicity susceptibility to the level of normal PHA blasts (Figure 1C). Class-I HLA-enhanced RTECs and NK cell-depleted responders (>99% depletion confirmed by FCM) were therefore used for all RTEC assays presented.

Mixed lymphocyte culture and cell mediated lysis assays

Frozen PBMC were thawed, resuspended, cocultured with stimulator cells, and analyzed as described (24). Cell mediated lysis (CML) results are presented as maximal PSL at 50:1 or 25:1 responder: target ratios. Third party controls were fresh or frozen PBMC from healthy volunteers. Positive control allogeneic responders were included in all assays, and, unless specifically stated otherwise, these yielded strong responses in all assays for which we report patient unresponsiveness.

RTECs were used as target cells in cytotoxicity assays following incubation with 1000 U/mL rhIFN-γ for 2 h. After a 24-h incubation in 5% CO_2 at 37°C, cells were recovered with 2 mM EDTA and labeled with ^{51}Cr, and 4×10^3 cells were added to each well and incubated for 4 h.

Table 1: Clinical status, treatments and transplant outcomes

Patient No.–age (years)	Prior therapies[1]	Pre-transplant myeloma	Chimerism	GVHD	Time since transplant (years)	Creatinine (mg/dL)[2]	Kidney rejection	Myeloma post-transplant	Antimyeloma treatment post-transplant	DLI days	Immuno-suppression
1–55	Mel Dex	BM 60% PC[3] Multiple LL[4]	Mixed < 105 days	None	7.3	0.9	None	CR[5]	None	67, 112	Off by day 73
2–51	VAD × 6	BM 48% PC	Mixed < 123 days	None	5.3	2.0	None	PR[6], then PD[7]	None × 35 mos chemotherapy[8]	78, 971	Off by day 76
3–34	Dox + Dex Mel	BM < 5% PC Multiple LL	Full day 62	Chronic	4.3	1.5	None	CR	None	None	MMF, Prednisone for GVHD[9]
4–35	VAD × 4	BM 50% PC	Mixed < 71 days	None	3.5	1.5	None	PD, Minimal residual disease after 2nd HSCT	None × 1.3 years chemotherapy HSCT at 2.1 years	129, 318	Off by day 60 Prednisone after 2nd HSCT for GVHD
5–35	Thal + Dex[10] CP × 4	BM 65% PC	Full day 90	Acute/chronic	2.8	5.6	None	PD	Chemotherapy[11] since 11 weeks	45	Prednisone for GVHD
6–56	Thal + Dex	BM 10% PC	Mixed < 98 days	None	2	1.7	Day 104	CR	None	None	Off by 1.8 years

[1]Mel = melphalan; Dex = dexamethasone; VAD = vincristine, actinomycin and dexamethasone; Thal = Thalidomide; Dox = doxorubicin; CP = cyclophosphamide.
[2]Most recent serum creatinine.
[3]PC = plasma cells.
[4]LL = lytic lesions.
[5]CR = complete remission.
[6]PR = partial remission.
[7]PD = progressive disease.
[8]Thalidomide/dexamethasone followed by VAD.
[9]Cyclosporine was discontinued on day 380.
[10]Thalidomide/dexamethasone followed by bortezomib/dexamethasone.
[11]Bortezomib, melphalan/dexamethasone followed by bortezomib/thalidomide.

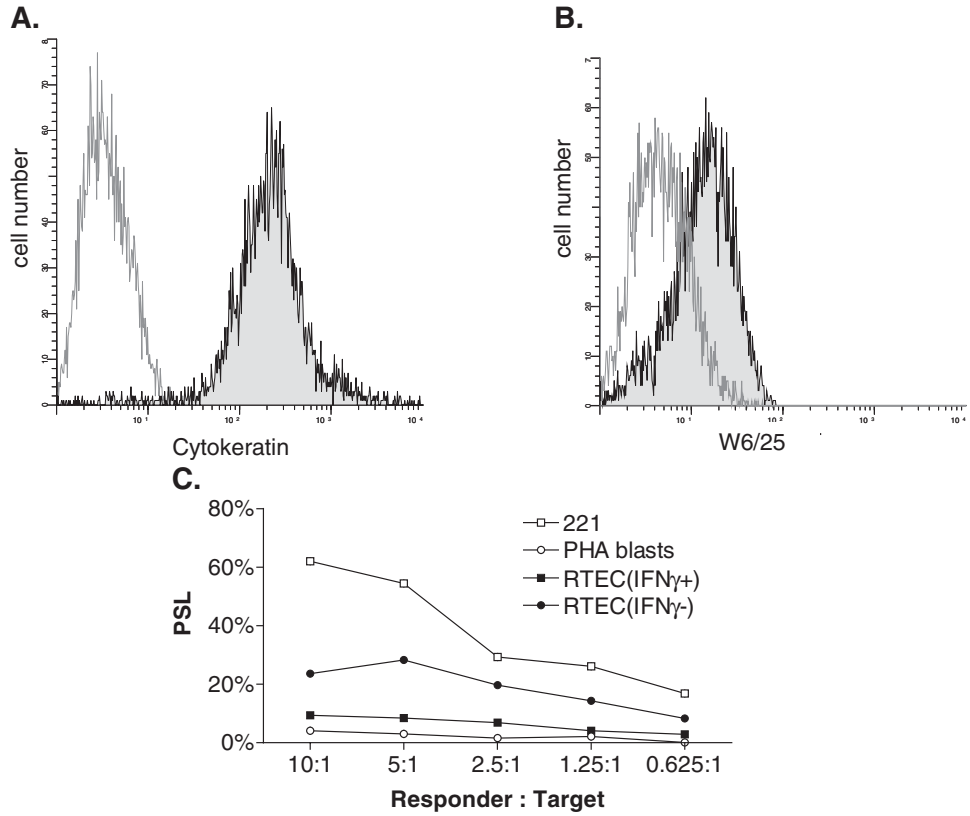

Figure 1: Analyses of cultured renal tubular epithelial cell lines as targets of NK cell cytotoxicity. (A) Expression of cytokeratin on cultured cells from renal biopsy (shaded histogram) and control staining with HOPC-1 Ab (open histogram); (B) cultured renal tubular epithelial cells (RTECs) express low levels of class-I HLA (open histogram). Class-I HLA expression on RTECs is increased following culture with recombinant human IFN-γ (shaded histogram); (C) direct NK cytotoxic assays using RTECs. Enhancement of class-I HLA expression using rhIFN-γ reduced the susceptibility of RTECs to NK cell-mediated cytotoxicity. Nonenhanced RTECs were targets of NK cell-mediated cytotoxicity, as was an HLA class I-deficient cell line (221).

Limiting dilution assay for cytotoxic T-lymphocyte precursor frequencies and IL-2-producing (helper) T-cell frequencies

Limiting dilution assays (LDA) provide a means of quantifying responder cells while diluting out the effects of suppressive populations (34–37). These were performed as described (24), using graded numbers (ranging from 4×10^4 to 312 per well) of responder cells in 24 replicate wells containing 4×10^4 PBMC or EBV-LCL (for CTLp assays only) stimulator cells irradiated with 30 or 100 Gy, respectively (see supplementary online information for details). Positive control allogeneic responders were included in all assays, and, unless specifically stated otherwise, these yielded strong responses.

Results

Chimerism

All subjects developed transient pancytopenia and recovered normal or near-normal counts by day 20 (not shown). All subjects achieved initial mixed chimerism; four subjects later lost chimerism and two converted to full donor chimerism, one spontaneously and one following DLI (Table 1). Loss of chimerism occurred while the four patients were still on CyA, 71–123 days post-transplant. Peak donor granulocyte chimerism levels ranged from about 80% (Patient 1, Figure 2A) to <20% (Patients 2, 4 and 6; Patient 6 shown in Figure 2B). Peak donor T-cell chimerism varied from about 80% (Patient 1, Figure 2A) to <20% (e.g. Patients 4 and 6; Patient 6 shown in Figure 2B).

Patient 3 converted spontaneously to full donor chimerism (Figure 2C). Patient 5 showed initial multilineage mixed chimerism (not shown). Because of rapid disease progression, CyA was discontinued by day 35, and a DLI was administered on day 45, converting her to full donor chimerism by day 90 (not shown).

Renal allograft acceptance

Details are provided in online Supplementary Information. Three of four patients with transient chimerism had no rejection episodes, despite being off all immunosuppression or any chemotherapeutic treatments for >1, >5 and >7 years (Table 1). Only one patient (Patient 6) experienced a rejection episode after discontinuation of

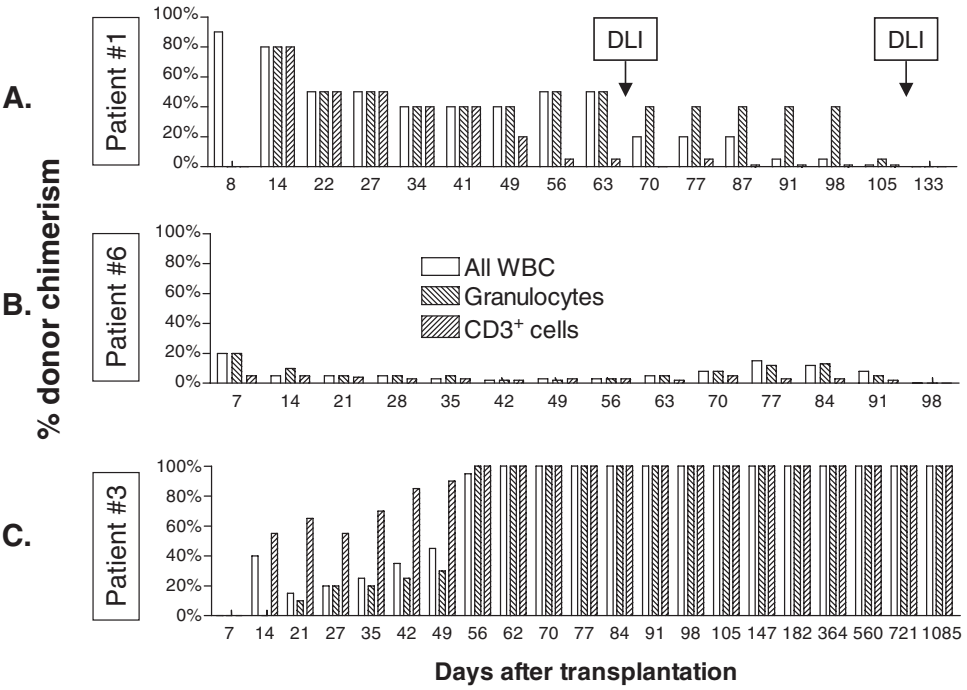

Figure 2: (A) Time course of chimerism in representative patients. Granulocytes and $CD3^+$ cells were not evaluated separately on Day 8. CD3 cell chimerism was not evaluable on day 70. DLIs were performed on days 67 and 112; (B) Patient 6; (C) Patient 3. Chimerism was not evaluable on day 7. Granulocyte chimerism was not evaluable on day 14.

CyA. He responded to anti-rejection treatment and after 1 year of stable renal function, immunosuppression was successfully withdrawn (Table 1). Among the two patients with full donor chimerism, who were treated at various times for GVHD, bronchiolitis obliterans and organizing pneumonia or myeloma recurrence, no rejection episodes occurred (Table 1). In summary, only one of six patients experienced a rejection episode, and he was later successfully removed from immunosuppression.

Myeloma outcomes

Three of six patients achieved sustained CRs of myeloma (at >2, >4 and >7 years), one achieved a partial remission, and two patients had progressive disease (see online Supplementary Information for details). Among the four patients with transient chimerism, two have achieved sustained CRs (Patients 1 and 6), despite having advanced disease, for >7 years and 2 years post-transplant, respectively (Table 1). Patient 2 had an initial partial response and in Patient 4 disease progression mandated chemotherapy at 1.3 years (Table 1). A myeloablative peripheral blood HCT from the same donor was performed at 2.1 years, and Patient 4 currently has normal renal function and minimal residual myeloma. The patient who spontaneously developed full donor chimerism has sustained a CR for >4 years (Table 1). Patient 5 had a very high tumor burden pretransplant and developed recurrent light chain nephropathy. A combination of chemotherapeutic and biologic regimens led to partial myeloma remission (Table 1).

Lymphocyte recovery

T cells: Post-transplant T-cell recovery varied markedly, as did CD4:CD8 ratios. In Patient 1, declining T-cell chimerism reflected a rapid increase in recipient CD8 T-cell recovery (Figure 3A). CD4 counts recovered slowly, and the CD4:CD8 ratios were inverted >2 years (Figure 3A). Other patients who lost chimerism (Patients 2, 4 and 6), showed earlier CD4 recovery and slower CD8 recovery, with early normalization of CD4:CD8 ratios (e.g. Patient 6 in Figure 3B). Among the two patients with durable chimerism, CD4 and CD8 T-cell counts recovered gradually over the first 6 months (e.g. Figure 3C), and CD4:CD8 ratios were variable.

As shown in Figure 4A, initially-recovering $CD3^+CD4^+$ cells in these patients were largely 'memory-type' $CD45RO^+CD45RA^-$ cells. $CD45RO^-CD45RA^+$ naïve-type CD4 cells recovered to age-appropriate proportions by approximately 6 months to 1 year post-transplant. Similarly, most initially-recovering $CD3^+CD8^+$ cells were $CD45RO^+RA^-$, and $CD45RA^+CD62L^+$ 'naïve-type' CD8 cell recovery varied over time and between patients.

Notably, a very high proportion of $CD3^+CD4^+$ cells expressed CD25 in the initial months in all patients compared to normal donors (Figure 4B). Although it declined over time, percentages of $CD25^+$ $CD3^+CD4^+$ cells remained elevated in some patients for >2 years. Among $CD3^+CD8^+$ cells, CD25 expression was usually low or undetectable (Figure 4B), but a few exceptions were seen (individual data not shown). In Patient 6, approximately 40% of $CD3^+CD8^+$ cells expressed CD25 throughout the first

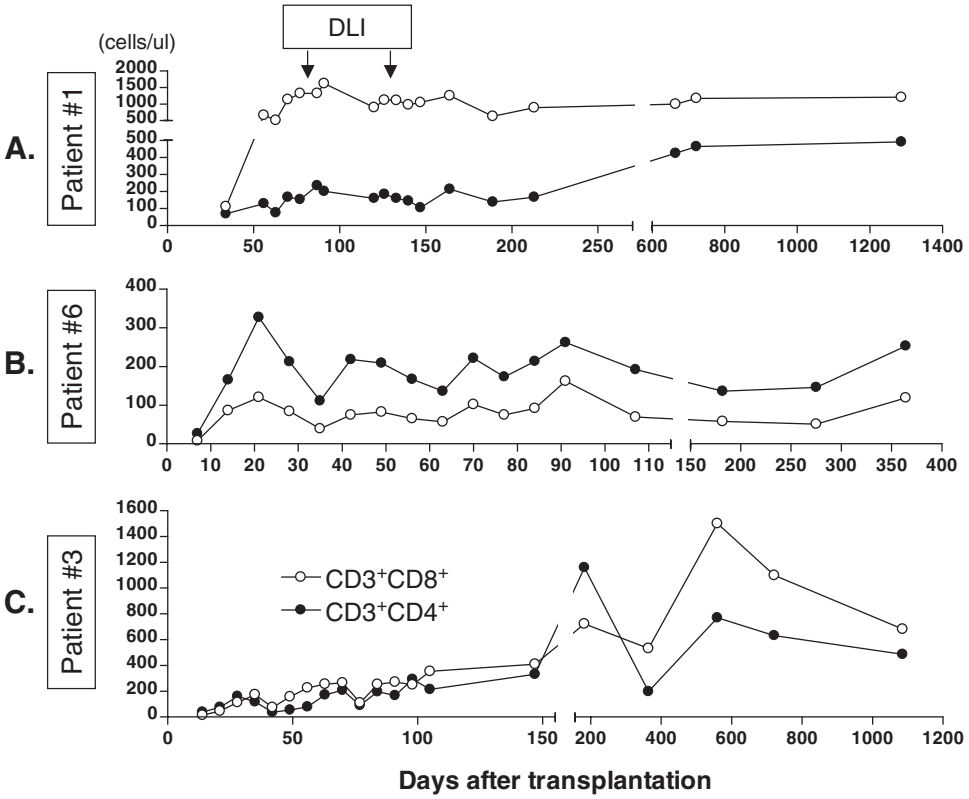

Figure 3: Time course of T cell recovery. (A) Patient 1. CyA was discontinued on day 73; (B) Patient 6; (C) Patient 3.

year post-transplant, with almost 100% CD25 positivity at 2 weeks.

B cells and NK cells: In all patients, NK ($CD3^-CD56^+$) cell counts recovered to normal or supra-normal levels within the first month post-transplant, then declined as T cells recovered (Figure 4C). B cell ($CD3^-CD19^+$) counts normalized at highly variable rates, achieving normal levels by 12 weeks to 2 years post-transplant (Figure 4C).

Correlations between outcomes and in vitro *alloresponses*

Patients with transient chimerism: Coincident with the loss of chimerism and expansion of recipient CD8 cells, Patient 1 developed a strong antidonor CML response by Day 67. The response persisted to Day 665 (Figure 5A) but disappeared by 2.5 years (not shown). Late LDA revealed a very high antidonor CTLp frequency (Figure 5B), which deviated from single-hit kinetics. The observed sawtooth pattern may be attributable to the presence of suppressive cells regulating CTLp (Figure 5B). These data fit with mathematical suppressor models developed by Bonnefoix et al. (25), which indicated that each suppressor cell had the ability to inhibit the cytotoxic function of 9 CTLp (see online Supplementary Information). Purification of CD25-negative cells did not increase antidonor cytotoxic T-lymphocyte precursor frequencies (CTLpf) or bulk CML responses at >2 years post-transplant (not shown). Significant antidonor (or antihost) mixed lymphocyte culture (MLR) responses were not detected at any time (not shown). These data implicate a CD8 cell-mediated marrow rejection that did not affect the donor kidney.

In contrast, Patients 2 and 4, who had slower host CD8 recovery, showed no early antidonor CTL responses in bulk culture or LDA (data not shown). By 22 and 32 months, a weak antidonor CTL response was measurable in both bulk culture and LDA in Patient 2. Purification of CD25-negative T cells did not enhance the response (not shown). Significant antihost CTL responses were never detected.

No significant antidonor or antihost MLR or HTL responses were detected over 2.8 years of follow-up in Patient 2 (data not shown). Patient 4 showed weak antidonor MLR responses on Days 99 and 211 (Stimulation Index [SI] 5.7 and 9.3, respectively). Thus, Patients 2 and 4 did not show host CD8 cell recovery or strong antidonor immune responses in association with loss of chimerism.

In Patient 4, donor RTEC targets were not killed by the patient's post-transplant NK cell-depleted PBMC stimulated with donor PBMC. Third-party NK cell-depleted PBMC strongly killed these cells (Figure 6). Surprisingly, NK cell-depleted donor PBMC also showed measurable killing of RTEC (Figure 6).

In Patient 6, no significant bulk MLR or CML responses to the recipient were detected at any time or to the

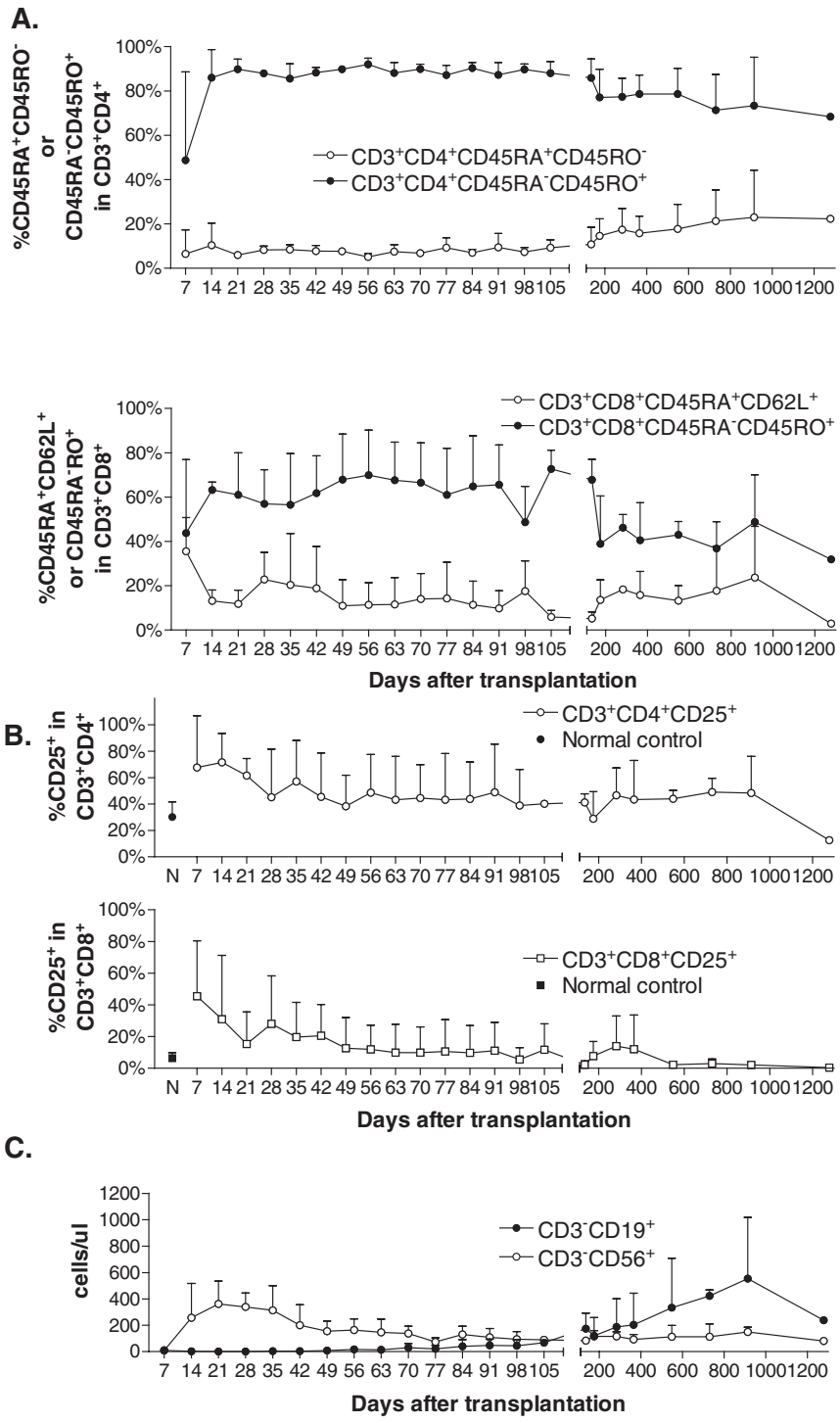

Figure 4: T-cell subset, B-cell and NK-cell recovery. (A) Time course showing mean % (+SD) of $CD45RA^+CD45RO^-$ and $CD45RA^-$ $CD45RO^+$ among $CD3^+CD4^+$ cells (top), and $CD45RA^+CD62L^+$, $CD45RA^-$ $CD45RO^+$ among $CD3^+$ $CD8^+$ cells (bottom); (B) time course showing mean (+SD) % of $CD25^+$ cells among $CD3^+CD4^+$ cells (top) or $CD3^+CD8^+$ cells (bottom). N; normal control; (C) time course showing mean B cell ($CD3^-CD19^+$) and NK cell ($CD3^-CD56^+$) recovery as cell concentrations per microliter blood. Data are expressed as mean + SD for all six patients.

donor in the first 70 days (not shown), but 3rd party controls also failed to respond to the donor at these times. However, on Day 107, during the rejection episode, a weak antidonor MLR response (SI $\approx$ 7), and very strong third-party responses (SI $>$ 600, data not shown) were detected. The antidonor (helper) T-cell frequencies (HTLf) increased markedly by Day 100, then became undetectable by 1-year post-transplant, when the patient had stable renal function (Figure 7A). Remarkably, a strong antihost HTL response appeared shortly before the

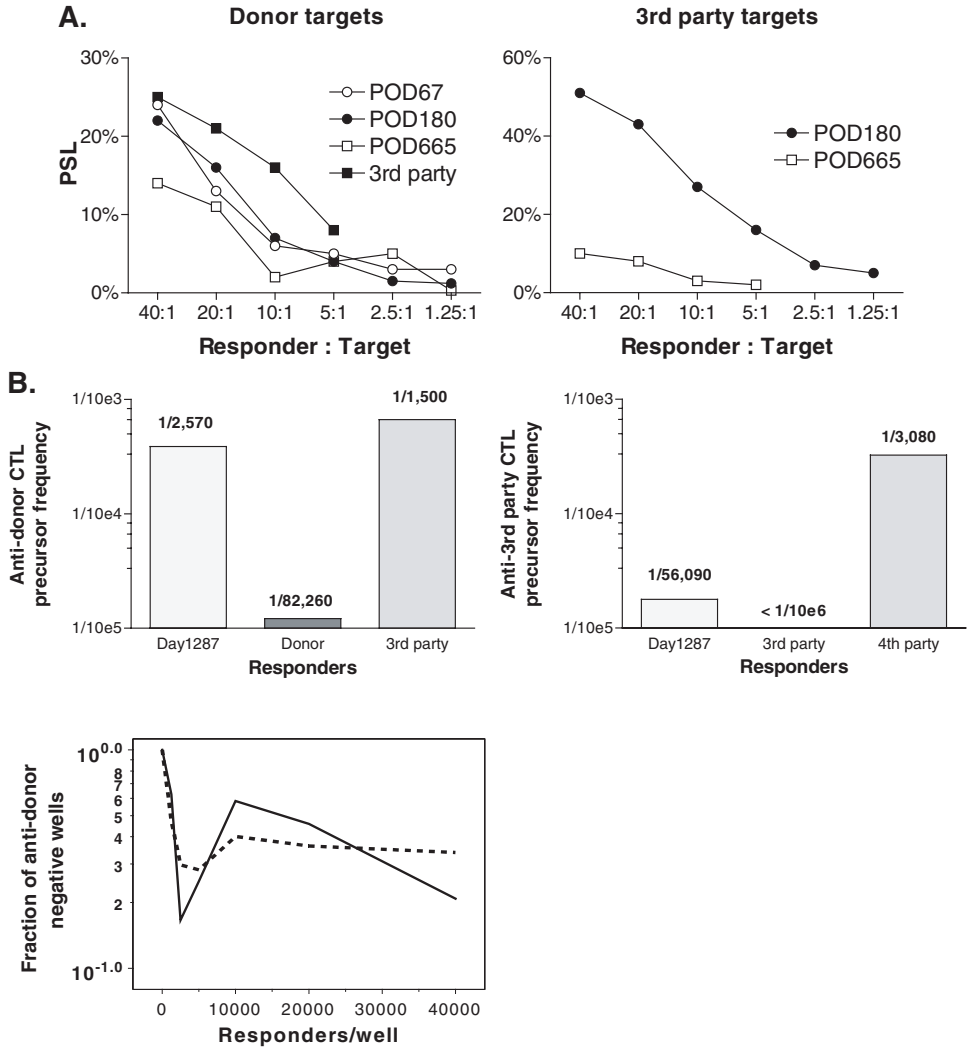

Figure 5: *In vitro* responses of Patient 1. (A) CML assays on Patient 1 revealed strong post-transplant antidonor responses (left) and anti-third-party responses (right); (B) Antidonor (top panel, left) and anti-third-party (top panel, right) cytotoxic T-lymphocyte precursor frequencies from limiting dilution assays performed on Patient 1 on Day 1287 post-transplant. The antidonor response produced a 'sawtooth' pattern (bottom). Data were modeled according to the following model:
$F_i = \exp(-f_{CTLp}x_i) + \sum_k \left[\sum_{n=k\Psi+1}^{(k+1)\Psi} \frac{(f_{CTLp}x_i)^n}{n!} \exp(-f_{CTLp}x_i) \left\{ 1 - \sum_{m=0}^{k} \frac{(f_{reg}x_i)^m}{m!} \exp(-f_{reg}x_i) \right\} \right]$, where i is the ith group of replicate wells, F is the fraction of negative wells, x is the number of cells per well, f_{CTLp} is the frequency of CTLp per well, f_{reg} is the frequency of regulatory (suppressor) cells, Ψ is a stoichiometric parameter that is defined as the maximum ratio at which CTLp and suppressor cells must be simultaneously present in the same well to ensure that this well will be negative for growth, $\Psi > 1$. The suppressor model was fitted to experimental data by using the quasi-Newton method to maximize the likelihood of the data. A standard chi-square test was used as goodness-of-fit test to evaluate the adequacy of the model to the experimental data. Solid line = experimental data; dotted line = theoretical (fitted) data.

rejection episode (Figure 7A), coincident with an increase in donor T-cell chimerism (approximately 5% on day 70) (Figure 2B). Thus, an increase in T-cell chimerism associated with a marked expansion of GVH-reactive HTL immediately preceded the loss of chimerism, expansion of antidonor HTL, and renal allograft rejection episode.

No antidonor or antihost CTLp were detected in LDA performed pre-BMT, on days 35, 70, 100 or 107. NK-depleted post-transplant PBMC showed markedly reduced antidonor RTEC precursor frequencies compared to NK cell-depleted donor and third-party PBMC (Figure 7B). Together, the early recipient CD4 recovery and coincidence of antidonor HTL response and rejection,

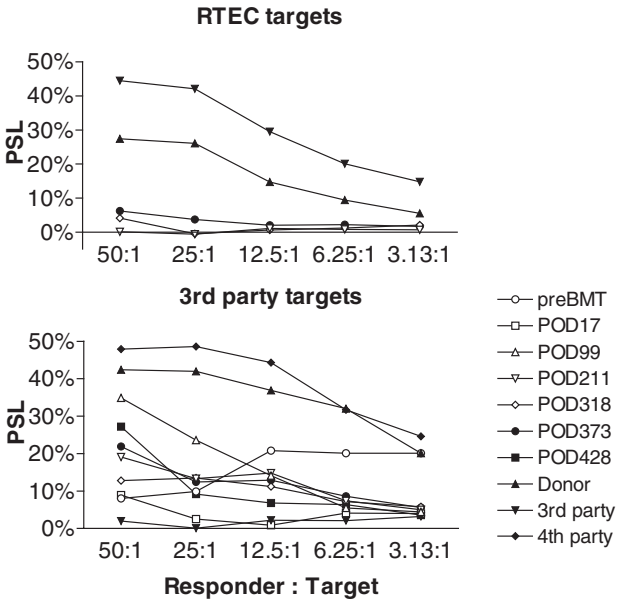

Figure 6: Antidonor RTEC (top) and anti-third-party (bottom) CML assays from Patient 4. All responders in anti-RTEC CML assays were CD56-depleted PBMC stimulated with donor PBMC, and class-I HLA enhanced donor RTECs were used as targets.

without anti-donor CTL, suggest that the rejection episode may have been mediated by Th rather than CTL effector mechanisms.

Thus, one patient rejected the marrow in association with strong antidonor CTL responses, without renal allograft rejection. Two patients showed loss of chimerism without strong antidonor responses without renal allograft rejection, and showed unresponsiveness to donor RTEC. The one rejection episode was associated with antidonor Th sensitization without a CTL response to donor lymphoblasts or RTEC.

Patients with durable chimerism

Patient 3 had early antidonor CTL reactivity when host T cells persisted, and antirecipient CTL in association with conversion to full donor chimerism and GVHD. Antidonor CTLp peaked on day 35 (Figure 8), when mixed T cell chimerism was present, and disappeared by day 70 (Figure 8), following conversion to full donor chimerism (Figure 2C). Antirecipient CTLp were detectable in the donor at high levels pretransplant, were measurable on Days 35 and 70, then fluctuated over time (Figure 8). Early responses in this patient were revealed only in LDA, when putative suppressive cell populations were diluted out. Bulk antidonor (including RTEC) or -recipient MLR or CML responses were not detected at any time (not shown).

Patient 5 showed high anti-donor HTL and CTLp frequencies on day 35 when mixed chimerism was present, with a suppressive 'sawtooth' pattern in CTLp LDA (not shown). These responses disappeared after conversion to full donor chimerism and the development of GVHD, which was associated with an increase in antihost HTL and later in CTLpf. Patient antidonor lymphoblast and RTEC CTL responses were undetectable, whereas NK cell-depleted donor PBMC killed autologous RTEC (not shown).

Recovery of anti-third-party responses is described in online Supplementary Information.

Discussion

Patients with renal failure due to advanced MM received a novel nonmyeloablative conditioning regimen and simultaneous BMT plus kidney transplantation from HLA-identical sibling donors. Excellent myeloma responses and renal allograft tolerance were achieved with acceptable toxicity. Four of the six patients had only transient chimerism, yet 3 of these showed renal allograft acceptance for a prolonged period off immunosuppression, which may be regarded as 'operational tolerance'. The results are consistent with studies in a nonhuman primate model, in which transient donor chimerism induced with a nonmyeloablative, ATG-based regimen is associated with tolerance when donor kidneys and marrow are grafted simultaneously (4).

Immune responses to minor histocompatibility antigens (mHA) are usually weak in naïve hosts. Strong responses suggesting sensitization in some of our patients correlated with clinical events, including rejection, GVHD and changes in chimerism. In Patient 1, CTL responses against donor mHA were associated with loss of chimerism without kidney graft rejection. Rapid recovery of recipient CD8 T cells was observed, consistent with results in patients who lost chimerism following BMT without kidney transplantation (24). While our data do not rule out persisting microchimerism in this patient, the strong bulk antidonor CTL response associated with loss of chimerism strongly suggests sensitization against donor mHA and rejection of the donor marrow. The surprising long-term acceptance of the donor kidney without immunosuppressive therapy led to the hypothesis that these responses targeted mHA expressed by hematopoietic cells that may not be expressed by the kidney. Several human mHA have been reported to be expressed primarily on hematopoietic cells and not on other cell lineages (26). We hypothesize that this patient was tolerant to mHA shared by the donor kidney and hematopoietic cells and that rejection was directed against mHA expressed only on hematooipetic cells and not the kidney. In subsequent patients, we evaluated cytotoxicity against donor RTECs in addition to lymphoblast targets. Lack of donor RTEC cytolysis in two patients who lost chimerism was consistent with a form of split tolerance.

Detection of autologous RTEC killing in normal donors was surprising. Although NK cells were depleted from the re-

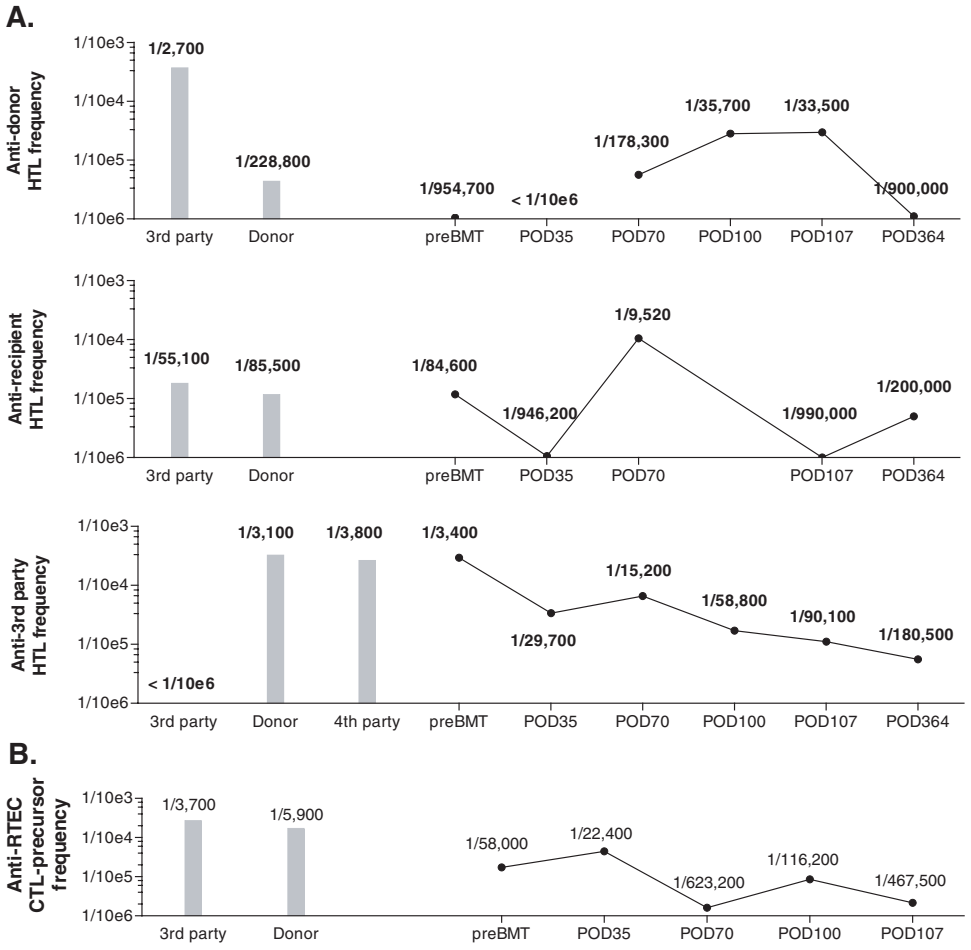

Figure 7: LDA studies on patient. (A) Antidonor (top), antirecipient (middle) and anti-third-party (bottom) helper T-lymphocyte frequencies from limiting dilution assays performed on Patient 6; (B) anti-RTEC cytotoxic precursor frequencies from limiting dilution assays performed on Patient 6. All responders were CD56-depleted PBMC, and class-I HLA enhanced donor RTECs were used as targets.

sponder cell populations and class-I HLA was upregulated on the RTECs by culture with IFN-γ, a small (<1%) contaminating population of NK cells might be responsible. Alternatively, renal tubular cell-specific antigens may not be expressed in the thymus, and self 'tolerance' under normal circumstances may reflect failure of T cells to traffic to the kidney parenchyma. Renal tubular cells have been reported to express class-I MHC *in vivo* (27) and class-I induction with IFN-γ promotes RTEC presentation of mHA to CTL (28). The absence of antidonor RTEC responses in all four patients tested raises the possibility that transplantation allows T-cell exposure to these renal antigens, promoting CTL tolerance.

Two patients ultimately developed full donor chimerism, but showed sensitized antidonor CTL and Th responses early posttransplant, when mixed T-cell chimerism was present. These early antidonor responses were not associated with renal allograft rejection and disappeared in association with chimerism conversion, after which antihost responses increased. These studies show that T cells with reactivity to both donor and host can coexist in mixed T-cell chimeras. This mutual alloreactivity *in vivo* may be responsible for the suppressive LDA pattern and the inability to detect alloresponses in bulk culture, as suggested by previous studies in mice (6). Such suppression might mitigate the ability of sensitized donor-reactive T cells to cause allograft rejection and may attenuate GVHD.

Two patients lost chimerism without measurable antidonor responses, perhaps reflecting inadequate donor stem cell engraftment due to insufficient myelosuppression and/or insufficient donor stem cells, rather than immunological rejection.

Patient 6 showed sensitization of antidonor Th in association with donor marrow rejection, and this was associated with the only renal allograft rejection episode. This patient did not demonstrate antidonor CTL against lymphoblasts or RTECs, despite autologous RTEC killing by the donor. Although confirmation is needed in larger numbers of patients, these results suggest that a previously unknown Th-mediated pathway for renal allograft rejection without CTL responsiveness may exist. We have previously described data suggesting Th-mediated loss of chimerism in patients receiving this BMT regimen without kidney transplantation (24).

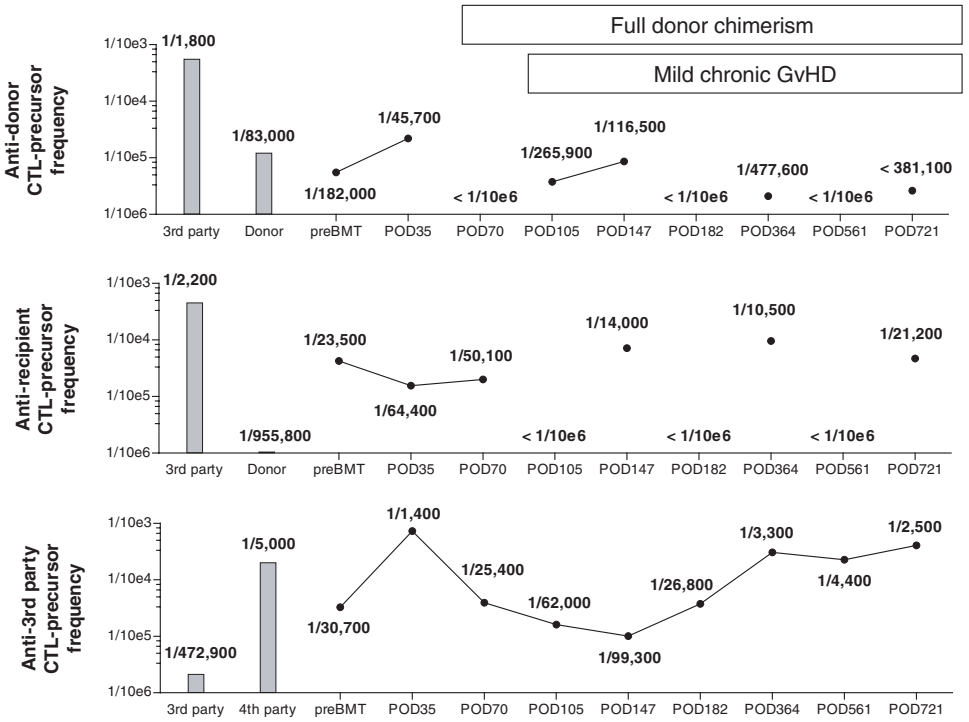

Figure 8: Antidonor (top), antirecipient (middle) and anti-third-party (bottom) cytotoxic T-lymphocyte precursor frequencies from limiting dilution assays performed on Patient 3.

The apparently Th-mediated rejection episode in Patient 6 followed the transient appearance of an antihost Th response at the time when donor T-cell chimerism peaked. This scenario remarkably resembles a pathway we have described in the murine model upon which this model was based. Administration of GVH-reactive CD4 cells without CD8 cells led to a paradoxical loss of donor chimerism apparently mediated by residual host-anti-donor cytokine-producing cells rather than CTL (29). Similar mechanisms may be implicated in this patient.

Three of six patients with advanced myeloma in our series achieved durable CRs and one patient achieved a prolonged PR. Surprisingly, three of these excellent responses occurred among four patients who lost donor chimerism. We and others have described this phenomenon in hematologic malignancy patients receiving nonmyeloablative HCT without renal transplantation (30,31). In mice, intentionally-induced rejection of donor marrow in mixed chimeras leads to significant responses against recipient tumors in association with generation of tumor-specific cytotoxicity (32,33).

The current study confirms the increased sensitivity of LDA compared to bulk CML and MLR assays in measuring post-BMT alloresponses (24). LDA frequently reveal responses only after dilution to low responder numbers per well, consistent with the existence of suppression at higher responder concentrations (34–37). Of the two patients who lost chimerism in association with strong antidonor responses, the patient with operational tolerance of her kidney graft demonstrated a suppressive pattern in LDA, whereas the patient experiencing a rejection episode did not show this pattern in his sensitized antidonor Th response (not shown). Depletion of $CD25^+$ cells did not enhance antidonor responses or abrogate the suppressive pattern in the tolerant patient, suggesting an alternative phenotype for the putative suppressive population. Consistently, cellular infiltrates and foxp3+ cells were not detected in renal biopsies of operationally tolerant patients. While an extraordinarily high proportion of CD4 cells recovering initially posttransplant expressed CD25 in all patients, this may denote early recovery of $CD25^+$ regulatory cells, activation of CD4 cells, or possibly lymphopenia-driven expansion.

T-cell recovery kinetics varied considerably, with prolonged predominance of 'memory-type' T cells, consistent with the relatively advanced ages of the patients in this study and possibly the prior chemotherapeutic treatments they had received. B-cell recovery was also highly variable, whereas NK cells recovered relatively early, consistent with other HCT studies (38–40). Anti-third-party alloreactivity generally recovered quite early when measured via LDA, and was frequently associated with a 'suppressive' pattern, possibly explaining the frequently weak simultaneous bulk culture alloresponses.

In conclusion, combined renal and BMT with this nonmyeloablative protocol can lead to excellent tumor responses in advanced MM while achieving operational tolerance to the donor renal allograft. Since patients with

renal failure due to myeloma are typically ineligible for either renal transplantation or allogeneic HCT, the only known cure for this disease, this approach offers significant potential benefit to the patient. Even in patients who lose chimerism and experience myeloma relapse, a second, potentially curative hematopoietic cell transplant from the same donor becomes possible if renal function has been restored (e.g. Patient 4). The achievement of renal allograft tolerance with minimal toxicity in these patients has important implications for tolerance induction in the absence of malignant disease. However, the risk of GVHD and the rejection episode experienced by one patient following CyA withdrawal mandates caution and the need for greater understanding of the mechanisms and markers associated with operational tolerance in recipients of this treatment.

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