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Mixed Chimerism, Lymphocyte Recovery and Evidence for Early Donor-Specific Unresponsiveness in Patients Receiving CKBMT to Induce Tolerance

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Abstract

Background—We have previously reported operational tolerance in patients receiving HLA-mismatched combined kidney and bone marrow transplantation (CKBMT). We now report on transient multilineage hematopoietic chimerism and lymphocyte recovery in five patients receiving a modified CKBMT protocol, and evidence for early donor-specific unresponsiveness in one of these patients.

Methods—Five patients with end-stage renal disease received CKBMT from HLA-mismatched, haploidentical living related donors following modified non-myeloablative conditioning. Polychromatic flow cytometry (FCM) was used to assess multilineage chimerism where evaluable and lymphocyte recovery post-transplant. Limiting dilution analysis was used to assess helper-T-lymphocyte reactivity to donor antigens.

Results—Transient multilineage mixed chimerism was observed in all patients but chimerism became undetectable by 2 weeks post-CKBMT. A marked decrease in T and B lymphocyte counts immediately following transplant was followed by gradual recovery. Initially recovering T cells were depleted of CD45RA⁺/CD45RO⁻ “naïve-like” cells, which have shown strong recovery in two patients and CD4/CD8 ratios increased immediately following transplant but then declined markedly. NK cells were enriched in the peripheral blood of all patients following transplant.

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Specific Contributions: Samuel A. LoCascio wrote the manuscript, coordinated sample acquisition, analyzed data and prepared specimens. Tatsuaki Morokata assisted with data analysis. Meredith Chittenden and Giovanna Andreola performed the limiting dilution assay and aided in flow cytometry panel design. Frederic I. Pfeffer and David M. Dombkowski provided expert advice on and performed flow cytometry. Kerry Crisalli supervised and coordinated research sample acquisition. Susan L. Saidman supervised and provided data from PCR chimerism analysis. Tatsuo Kawai, Thomas Spitzer, Nina Tolkoff-Rubin, A. Benedict Cosimi and David H. Sachs designed, supervised and carried out the clinical protocol and patient care. Megan Sykes participated in the design of the clinical protocol, participated in and oversaw design of all laboratory studies, data analysis and interpretation, and preparation of the manuscript.

For Subject 2, a pre-transplant limiting dilution assay revealed T helper cells recognizing both donor and third-party PBMCs. However, the anti-donor response was completely undetectable by Day 24, while third-party reactivity persisted.

Conclusion—These results characterize the transient multilineage mixed hematopoietic chimerism and recovery of lymphocyte subsets in patients receiving a modified CKBMT protocol. The observations are relevant to the mechanisms of donor-specific tolerance in this patient group.

Keywords

Bone marrow transplantation; tolerance; immunosuppression; kidney; mixed chimerism

Introduction

Two major barriers to solid organ transplantation are chronic graft rejection and complications that arise from long-term global immunosuppression, including increased incidence of opportunistic infections and malignancies. Induction of mixed chimerism by nonmyeloablative conditioning followed by bone marrow transplantation (BMT) has the potential to overcome these barriers by inducing a state of donor-specific unresponsiveness while leaving the recipient's immune system intact. The validity of this approach has been supported by studies in murine and large animal models (1). Recently, the approach has been used to induce long-term acceptance of HLA-mismatched renal allografts without maintenance immunosuppression for 4-7 years in four of five patients (2). These patients exhibited transient multilineage chimerism that lasted no more than 21 days, and gradual lymphocyte recovery following transplant (Andreola et al, manuscript submitted).

We report here on multilineage chimerism and recovering lymphocyte subsets in an additional 5 patients undergoing a modified CKBMT protocol by methods of polychromatic FCM. We also describe the rapid development of donor-specific tolerance among IL-2 producing T cells in one of these patients.

Results

Development of a flow cytometric method for reliable assessment of lineage-specific chimerism

An optimal recipient HLA antigen-specific mAb was identified for Patients 1, 2, 4 and 5, while no reliable donor or recipient HLA antigen-specific antibody was identified for Patient 3. Any cells negative for the HLA antigen-specific antibody were thus considered to be of donor origin.

For Patients 1 and 2, no leukocyte marker was used to distinguish between lymphocytes and erythrocytes in the lymphocyte forward/side-scatter gate area. Thus, lymphocyte chimerism was not evaluable, since MHC-I-negative erythrocytes present in the lymphocyte gate were indistinguishable from donor lymphocytes (Figure 1A). This was especially problematic due to a high percentage of erythrocytes resulting from lymphopenia during the immediate post-transplant period. The subsequent addition of an anti-CD45 antibody allowed us to analyze lymphocyte chimerism in Patients 4 and 5 (Figure 1B).

For Patient 4, a positive shift in MHC-I expression on leukocytes occurred during the post-transplant period (data not shown), making the donor-recipient HLA cell distinction somewhat unclear. Thus, a pan-MHC-I antibody was added to the chimerism analysis for Patient 5 to allow improved separation of cells staining with the antigen-specific mAb. For Patient 5, the use of anti-CD45 as well as pan-MHC-I mAbs allowed highly reliable

chimerism analysis in monocyte, granulocyte and lymphocyte lineages, as well as analysis of rare lymphocyte subsets such as natural killer (NK) and natural killer T (NKT)-like cells, defined as MHC-I⁺/CD45⁺/CD56⁺/CD3⁻ and MHC-I⁺/CD45⁺/CD56⁺/CD3⁺, respectively (Figure 1C).

Specific T-cell subsets were evaluable for chimerism in all patients by virtue of CD3, CD4 and CD8 markers distinguishing them from MHC-I-negative erythrocytes. Monocytes (CD14⁺/CD45⁺) were also evaluable for chimerism in all patients.

Transient Multilineage Mixed Chimerism

For all four patients for whom a suitable recipient-specific mAb was identified, transient multilineage chimerism was observed. Monocyte chimerism was detected at peak levels of 20.7 (Day 2), 65.1 (Day 7), 55.5 (Day 10) and 60.1% (Day 7) for Patients 1, 2, 4 and 5, respectively. Donor T-cell chimerism was also detected in all patients, for both CD3⁺/CD4⁺ and CD3⁺/CD8⁺ T cells. In all four patients, donor T-cell chimerism peaked on Day 7 post-transplant, and was undetectable by Day 14. Donor CD4⁺ T cells peaked at 11.8, 37.5, 6.1 and 4.5%, and CD8⁺ T cells peaked at 22.6, 9.5, 3.8 and 5.2% for Patients 1, 2, 4 and 5, respectively.

NK and NKT cell-like chimerism was also evaluable and detected in Patient 5, at levels of 48.0 and 21.4%, respectively, on Day 7 (Figure 1D).

Although chimerism was not evaluable by FCM in Patient 3, total WBC chimerism was confirmed by PCR assays of variable short tandem repeats as previously described (3-4), and followed a similar time-course to the other patients.

Lymphocyte Subset Recovery

Due to the conditioning treatment, a dramatic decrease in T-cell counts occurred in all patients immediately following transplant, reaching 0-12 cells/ μ L by Day 10. Four patients then showed a transient increase in T cells that peaked at Week 2 for Patients 1 and 5, and at Week 3 for Patients 3 and 4, at 151 ± 58 cells/ μ L (data not shown). All patients had a subsequent decline in and then gradual recovery of T cells, with most recent values of 267 (Day 499), 143 (Day 364), 776 (Day 388), 691 (Day 317) and 461 (Day 234) cells/ μ L for Patients 1-5, respectively (Figure 2).

At one week post-transplant, there was a transient increase in the CD4/CD8 ratio that peaked on Day 7 for all patients (Figure 3A). The ratios increased from pre-transplant (Day 0 for Patient 1) values of 1.11, 2.50, 0.71, 1.01 and 1.68, to Day 7 levels of 3.59, 11.07, 7.13, 1.81 and 2.75, respectively, for Patients 1-5, although this increase just missed statistical significance ($P=0.067$). The CD4/CD8 ratios then declined to below pre-transplant levels for all patients by Day 14 ($p<0.05$). In Patients 2 and 4, this inverted ratio has been sustained until the present (Days 364 and 317). Patient 1 recovered to Day 0 values by Day 27, and maintained a stable ratio at most points thereafter, while Patient 3 did not recover until Day 245. After the decline by Day 14, the CD4/CD8 ratio in Patient 5 was dramatically increased on Days 21, 31 and 57, largely because CD8 T cells were completely undetectable at the latter two time points, while small numbers of CD4 cells were spared following administration of ATG.

B cells were also effectively depleted in all patients, reaching <1 cell/ μ L by Day 7, and remaining at nearly undetectable levels for >180 days. Strong B cell recovery has been observed in Patients 1 and 2, with most recent values of 273 (Day 499) and 163 (Day 364) cells/ μ L, respectively. Patient 3 has shown some recovery, with 38 cells/ μ L on Day 388. The

remaining two patients have yet to recover B cells at Days 317 and 234, respectively (Figure 2).

A trend to an increase (though not statistically significant) in blood NK cell counts was observed in all patients following conditioning, and this became the dominant lymphocyte population in all patients, peaking at 238 ± 136 cells/ μL ($67.0 \pm 17.9\%$ of lymphocytes) within the first 121 days, in comparison to 79 ± 37 cells/ μL ($7.2 \pm 3.9\%$ of lymphocytes) pre-transplant (Figure 2). The increase in percentage of NK cells from pre-transplant to peak values was statistically significant ($p < 0.01$). NK cells remained dominant for >280 days in Patients 1 and 2, 156 days in Patient 5, 39 days in Patient 3, and predominated at only one time point in Patient 4 (Figure 2).

“Memory” vs. “naïve-like” recovering T cells

CD4^+ T cells included a reduced percentage of “naïve-like” ($\text{CD45RA}^+/\text{CD45RO}^-$) cells following transplant in all patients except Patient 1, for whom pre-transplant data was not evaluable. This phenotype was paralleled by an increased proportion of “memory-like” $\text{CD3}^+/\text{CD4}^+$ cells ($\text{CD45RA}^-/\text{CD45RO}^+$), which has remained the dominant phenotype until the present in Patients 1-3, while the percentage of naïve CD4^+ T cells has increased to levels of 52.1% at Day 317 and 42.8% at Day 234 in Patients 4 and 5, respectively (Figure 3B). Consistently, in Patients 2, 4 and 5, the percentage of “naïve” $\text{CD3}^+\text{CD8}^+$ cells ($\text{CD45RA}^+/\text{CD45RO}^-/\text{CD62L}^+$) was decreased following transplant, though low numbers precluded reliable evaluation in Patient 5 between Days 14 and 108. Such changes were not evaluable in Patients 1 and 3, since pre-transplant data for this phenotype was not obtained.

Early Donor-Specific Unresponsiveness

Limiting dilution analysis was used to assess helper-T-lymphocyte (HTL) reactivity to donor and third-party PBMCs for samples obtained from Patient 2 pre-transplant and at Day 24. Because the frequencies of positive wells did not follow a Poisson distribution upon serial dilution, it was not possible to accurately determine the frequencies of reactive T cells. Nevertheless, marked changes in responses were evident upon analysis of frequencies of responding wells. HTLs were detected that were reactive to donor or third-party PBMCs pre-transplant. However, by Day 24, the anti-donor response was completely undetectable (no positive wells), while third-party reactivity persisted. A constant, low level of autoreactivity was observed both pre-transplant and at Day 24 (Figure 4).

Discussion

We describe here the development of a reliable polychromatic flow cytometric method for assessment of lineage-specific chimerism under lymphopenic conditions. By including anti-CD45 mAb in our gating scheme, we excluded erythrocytes that would have otherwise appeared as negative for the HLA antigen-specific mAb. Furthermore, the addition of an anti-pan-MHC-I mAb allowed us to compensate for overall shifts in MHC-I expression on leukocytes. Whether these shifts represented true up-regulation of MHC-I by leukocytes or simply increased fluorescence intensity due to a higher antibody-to-leukocyte ratio post-transplant is not clear. In either case, the modified method for detection of lineage-specific chimerism should permit improved characterization of chimerism in future patients undergoing CKBMT and HLA-mismatched hematopoietic cell transplantation (HCT) alone.

Multilineage chimerism was observed in the first two weeks in all evaluable patients and included monocyte, granulocyte, lymphocyte, T cell, NK cell and NKT or NKT cell-like chimerism. Because donor T cell chimerism was detected as early as 1 week post-transplant in all 4 patients in whom it was evaluable by FCM, it seems likely that these were donor T

cells that survived despite MEDI-507 treatment, since maturation and selection of T cells through a recently irradiated adult thymus could not take place in such a short time. The very transient nature of chimerism in all lineages raises the possibility that chimerism in all lineages reflects engraftment of transplanted progenitors but not of hematopoietic stem cells (HSCs). In such a scenario, the loss of chimerism would not be due to rejection of the donor marrow, but instead could reflect failure to create sufficient hematopoietic “space” for engraftment of the relatively small number of donor HSCs administered. Indeed, cyclophosphamide, the only marrow-toxic agent included in our regimen, is only mildly toxic to HSCs.

Although marrow rejection would be inconsistent with the long-term renal allograft survival and rapid development of tolerance in the HTL assay, the rapid loss of donor chimerism may be an immunological event, possibly mediated by recipient CD8 T cells. Several of the patients developed an engraftment syndrome early post-transplant that was associated with renal dysfunction and accumulation of expanding recipient CD8 T cells in the glomeruli, but did not produce a rejection pattern (Farris et al, manuscript in preparation). The early development of tolerance among peripheral blood HTLs and the long-term donor-specific unresponsiveness in *in vitro* assays (2) (Andreola et al, manuscript submitted) is also inconsistent with an early rejection mechanism in these patients. Further studies are needed to elucidate the mechanisms of early chimerism loss and the associated engraftment syndrome.

The ability of donor NK cells to rapidly repopulate the periphery from progenitors (5-7) may help to explain their high peak chimerism levels in Patient 5. Early detection of high levels of monocyte chimerism (as early as Day 2 in Patient 1) was probably supported by rapidly dividing monocyte progenitors (8) from the donor marrow. While we were able to detect CD3⁺/CD56⁺ cell chimerism in Patient 5, it is unclear whether these are NKT cells or conventional T cells that express CD56 (9-10), which comprise a significant portion of CD56⁺ cells in humans (9). Further studies should allow us to determine the level of reconstitution of “classical” invariant NKTs (iNKTs), which typically comprise the majority of NKT cells (11) and can be identified by their expression of the invariant TCR- α chain, V α 24J α 18. The differing level of donor chimerism in the CD3⁺/CD56⁺ subset compared to conventional T cell and NK cell chimerism levels in Patient 5 is consistent with the possibility that these represent a distinct cell lineage, such as NKT cells.

Clearly, the infusion of donor bone marrow at the time of kidney transplant has a tolerogenic impact on the host immune system, as is evident from our studies in monkeys (12), and from the fact that ten of eleven patients receiving our previous (2,13) and current CKBMT protocols underwent successful withdrawal from immunosuppression while maintaining stable renal allograft function. However, the mechanisms of tolerance in the current human protocol are not fully understood. In mouse models of durable mixed chimerism, the life-long contribution of donor hematopoiesis to APCs that mediate central deletion of donor reactive T cells by presenting donor antigen in the thymus is the major mechanism of long-term tolerance (1). The transient nature of peripheral chimerism in these patients and in monkeys undergoing similar conditioning regimens (12-14), on the other hand, suggests that alternate mechanisms of tolerance may be of equal or greater importance in this setting. Peripheral T regulatory cells (Tregs) have been implicated in allograft tolerance, and we have previously described their enrichment in recipients of allo-BMT with non-myeloablative conditioning that involved MEDI-507 (15) and in the first series of CKBMT patients (Andreola et al, manuscript submitted). Furthermore, a high level of FoxP3 expression in the kidneys of patients from our previous CKBMT protocol suggests the presence of Tregs in the graft itself (2), while flow cytometric analysis of the current

patients also suggests a marked enrichment for peripheral Tregs due to both recent thymic emigration and peripheral expansion (Morokata et al, manuscript in preparation).

Analysis of lymphocyte subsets in all five patients revealed marked depletion of T cells post-transplant, followed by a transient early increase, decline, and then gradual persistent recovery. The use of ATG to treat engraftment syndrome complicated the T cell depletion observed in some patients and delayed T cell recovery. The initial recovery of T cells was likely due to lymphopenia-driven proliferation of recipient and donor T cells, as thymopoiesis was likely impaired following irradiation. This is further supported by the fact that both CD4 and CD8 T cells were predominated by a “memory-like” phenotype following transplant, consistent with data in a humanized mouse model definitively showing that naïve human T cells convert to the effector/memory phenotype when placed in a lymphopenic environment (16). Alternatively, memory-like T cells may have been preferentially spared by conditioning. The sustained memory phenotype in Patients 1-3, especially within the CD4 subset, suggests that these patients have not regained robust thymus-dependent generation of CD45RA⁺ T cells, whereas evidence for such recovery was obtained in the latter two patients. This is consistent with the age-dependent nature of thymic recovery in adults (17), considering that the first three patients were each over 35 years old at the time of transplant, in comparison to Patients 4 and 5, who were 22 and 26 years old, respectively. Despite CD4/CD8 ratio inversion, which can be associated with the occurrence of opportunistic infection due to low CD4 T cell counts (18-19), no serious infections have occurred in any of the patients to date.

Since NK cells were the dominant lymphocyte lineage for a prolonged period of time in four of five patients following transplant, it is important to understand the role of this subset in recipients of our protocol. Typically, NK cells are among the first lymphocytes to repopulate the periphery following HCT, when they are predominantly CD56^{high}CD16^{low}, with low cytotoxicity and a high capacity for cytokine production. In contrast, the peripheral blood NK cells in normal individuals are predominantly CD56^{dim}CD16^{high} and have high cytolytic activity (20-22). In our previous analysis of patients receiving HCT with MEDI-507, we found that early recovering NK cells lacked cytolytic function *in vitro* (5). However, they also expressed uncharacteristically low levels of CD2, a marker which has been associated with the more cytotoxic subset of NK cells (22). Anti-CD2 mAb (MEDI-507) treatment may have been responsible for this phenotype. While we have yet to extensively examine NK cells in the current study, we will better characterize these cells in future patients by refining our FCM methods to include CD2, CD3, CD16, CD45 and CD56 markers to distinguish among these subsets.

The strong recovery of NK cells in the current patients probably reflects an increased availability of IL-15 due to T-cell depletion, which has been reported to enhance their development and contribute to NK cell homeostasis (23-24). The presence and expansion of NK cells in this context might be beneficial for a number of reasons, one being that in a lymphopenic environment, they can inhibit homeostatic CD8 T cell proliferation through IL-15 competition, thereby potentially decreasing the risk of early T cell-mediated allograft rejection (25). A number of other NK-associated immunoregulatory properties have been described, including killing (26) and regulation (27) of activated T cells, as well as secretion of regulatory cytokines like TGF-beta, which has been induced *in vitro* by administration of anti-CD2 (28), as well as by exposure to apoptotic cells, which in turn suppressed CD4 T cell proliferation and activation (29). The presence of NK cells during lymphopenia may have also served as a protective barrier against opportunistic infections.

The *in vitro* assay we performed with early post-transplant PBMCs from Patient 2 provides evidence for the very early development of donor unresponsiveness among HTLs. While the

anti-third party response was also reduced at this time, the donor-specific response was completely undetectable, despite the recent loss of donor chimerism and the presence of donor antigen in the kidney graft. Whether this unresponsiveness was due to early deletion of donor-reactive CD4 T cells, specific anergy or the presence of peripheral regulatory mechanisms is not clear. Further functional and phenotypic analyses of the lymphocytes recovering early following CKBMT should help to clarify the mechanisms involved in the achievement of tolerance.

Methods

Conditioning and transplantation

In a study sponsored by the Immune Tolerance Network (ITN), five patients with end-stage renal disease received CKBMT from HLA-haploidentical living related donors on Day 0. BMT included an average of $2.5 \pm (\text{SD}) 0.3 \times 10^8$ nucleated cells, $22.0 \pm 7.5 \times 10^6$ CD3⁺ cells and $2.0 \pm 0.4 \times 10^6$ CD34⁺ cells per kilogram. Non-myeloablative conditioning included anti-CD20 mAb (Rituximab) (Biogen Idec Inc. and Genentech USA, Inc., South San Francisco, CA, USA) administered on Days -7, -2, 5 and 12, anti-CD2 mAb (MEDI-507) (MedImmune Inc., Gaithersburg, MD, USA) given on Days -2 (test dose), -1, 0 and 1, and 7 Gy thymic irradiation on Day -1. Cyclophosphamide (MeadJohnson, Princeton, NJ, USA), was administered on Days -5 and -4. Prednisone was started at approximately 2 mg/kg on Day 4 and then tapered off by Day 31 for all patients except Patient 3, who received an additional course from Days 31 to 215. Additional immunosuppression that was given in response to early clinical events will be described elsewhere and is indicated in Figure 2 only in order to facilitate interpretation of lymphocyte recovery data. Immunosuppression was successfully discontinued in patients 1, 2, 4 and 5 at days 238, 245, 256 and 244. The clinical details of and rationale for the treatments listed here will be described elsewhere (Kawai et al, manuscript in preparation).

Flow cytometry

Polychromatic FCM was performed with the use of an LSR II flow-cytometer (BD Biosciences, Mountain View, CA, USA), as described elsewhere (13), to analyze peripheral white blood cells (WBC) stained with fluorochrome-labeled anti-HLA class I antigen-specific mAbs (One Lambda, Inc., Canoga Park, CA, USA), and anti-CD3, CD4, CD8, CD14, CD19, CD25, CD45, CD45RA, CD45RO, CD56 (BD Biosciences) and CD62L (Invitrogen, Carlsbad, CA, USA) mAbs. Data analysis was performed with FlowJo (Tree Star Inc., Ashland, OR, USA). Gates were established using pre-transplant or late post-transplant samples, in which the large number of lymphocytes made separations between cell populations clear. Gates were kept constant throughout analysis for each patient where possible. Lymphocyte subset percentages were determined by dividing the percentage of cells gated for a given phenotype by the percentage of CD45⁺ cells within the lymphocyte gate, and then multiplying by 100. This formula was necessary to compensate for non-lymphocytes (CD45⁻ cells) present in the lymphocyte forward/side-scatter gate. Absolute numbers of lymphocyte subsets were calculated by multiplying the percentage of each subset out of all lymphocytes by the absolute lymphocyte count determined from the clinical differentials at each time point ($\% \text{ subset out of lymphocytes} \times ((\text{WBC}/\mu\text{L}) / (\% \text{ lymphocytes}))$). For chimerism analysis in specific cell lineages, the total gated cells in that lineage (defined by the indicated mAb combinations) were used as the denominator population.

Limiting dilution assay for helper-T-lymphocyte reactivity

Graded numbers of responder PBMCs were tested in 24 replicate wells each as described previously (30). Positive wells were defined as those producing cpm > 3 standard deviations above the mean of the 24 control wells containing stimulators alone.

Statistical Analysis

Statistical analysis was performed using Microsoft Excel (Microsoft Corporation, Redmond, WA). A two-tailed paired T-test was applied, except for analysis of % natural killer cells, where a two-tailed, two-sample equal variance T-test was applied, since pre-transplant data were not available for Patient 1.

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Abbreviations

ATG	anti-thymocyte globulin
BMT	bone marrow transplantation
CKBMT	combined kidney and bone marrow transplantation
FCM	flow cytometry
HSC	hematopoietic stem cell
HTL	helper T lymphocyte
PBMC	peripheral blood mononuclear cell

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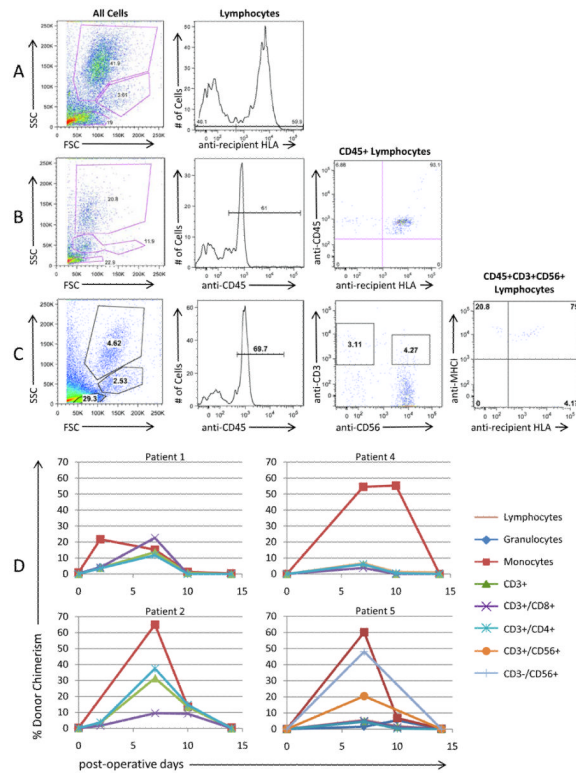


Figure 1.

Development of a flow cytometric method for reliable assessment of lineage-specific chimerism. (A) For Patients 1 and 2, gating of lymphocytes by forward/side-scatter followed by analysis of recipient HLA expression yielded unreliable levels of chimerism due to inclusion of HLA-negative erythrocytes in the gate. Monocyte and T-cell chimerism was interpretable (not shown). (B) The addition of anti-CD45 mAb for Patient 4 resulted in exclusion of erythrocytes and more reliable monocyte and lymphocyte chimerism. (C) The addition of anti-pan-MHC-I mAb for Patient 5 allowed for highly reliable chimerism analysis in rare subsets post-transplant, such as CD3⁺CD56⁺ NKT-like cells. (D) Levels of lineage-specific donor chimerism are shown for Patients 1, 2, 4 and 5 where evaluable by FCM.

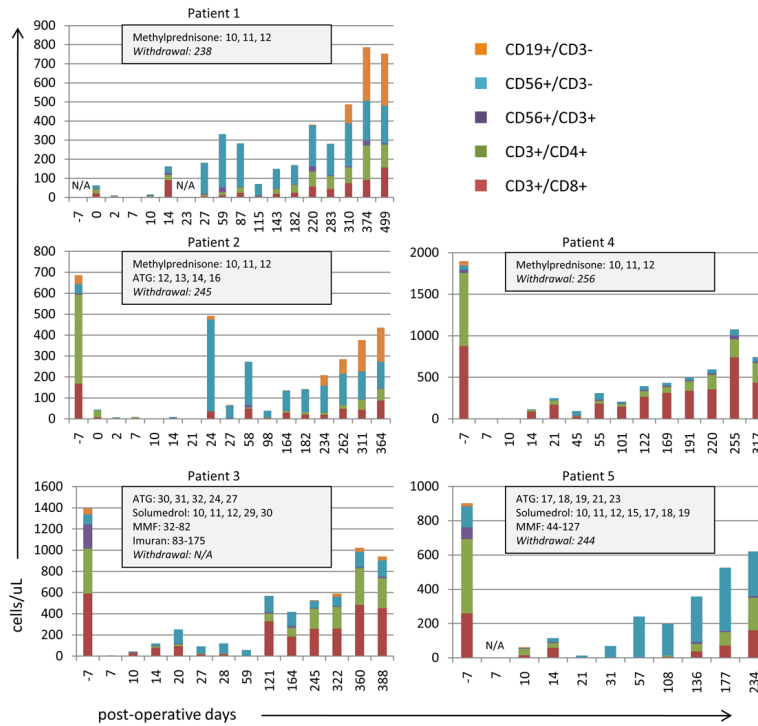


Figure 2. Absolute numbers of lymphocyte subsets (cells/μL) are shown for Patients 1-5. Time points at which the data were not evaluable are indicated by “N/A.” Administration of additional immunosuppression, as well as the time of complete withdrawal from immunosuppression is indicated in days post-transplant for each patient.

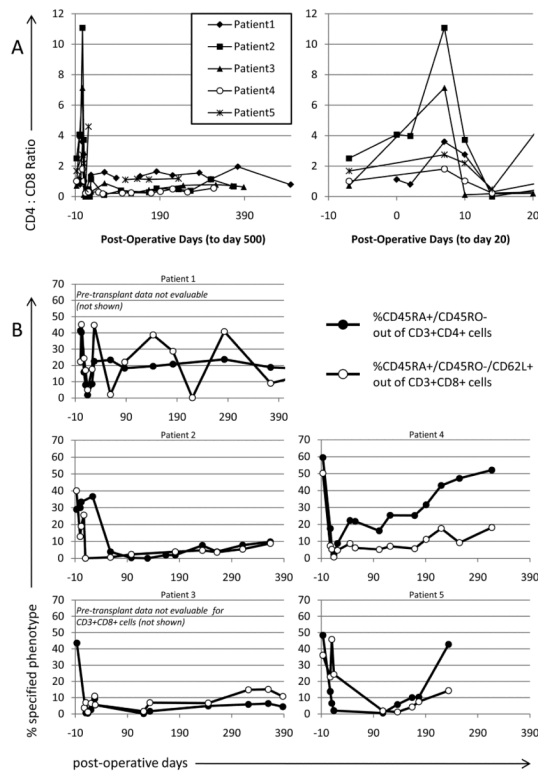
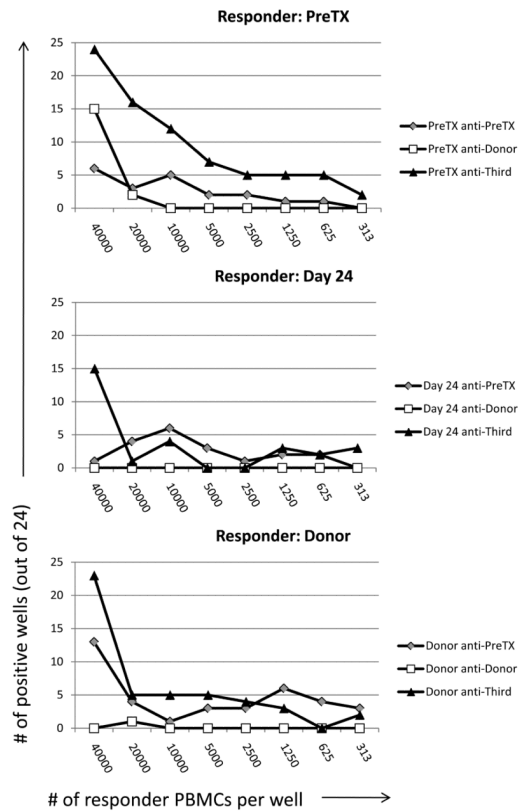


Figure 3.

Characterization of recovering T cells. (A) CD4/CD8 ratios for each of the five patients are shown for the duration of follow-up thus far (left panel). The x-axis is expanded to show the transient increase in the CD4/CD8 ratio at Day 7, followed by inversion to a CD8 dominant phenotype (right panel). Ratios for Patient 5 between Days 14 and 108 are not shown, since there were no detectable CD8 T cells at these time points. Other time points at which data were not evaluable are left blank. (B) Percentages of naïve-like CD4 T cells (CD45RA⁺/CD45RO⁻) and CD8 T cells (CD45RA⁺/CD45RO⁻/CD62L⁺) out of all CD4 and CD8 T cells are shown, respectively, for each of the five patients. Pre-transplant data for Patient 1 was not evaluable for CD4 or CD8 T cells, and CD62L analysis was not possible for Patient 3. These time points are left blank.

**Figure 4.**

The number of wells (out of 24 wells) in which reactive IL-2 producing T cells were detected are displayed for each responder/target combination, at each dilution (number of responder PBMCs per well). Responders included PBMCs obtained from Patient 2 pre-transplant (PreTX), Patient 2 at Day 24 post-transplant (Day 24) and donor. Irradiated targets included PBMCs obtained from Patient 2 pre-transplant (PreTX), donor and a third party. Donor-reactive IL-2 producing T cells were completely undetectable at Day 24, while third-party reactivity persisted.