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The Knife's Edge of Tolerance: Inducing Stable Multilineage Mixed Chimerism But With A Significant Risk of CMV Reactivation and Disease in Rhesus Macaques

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Abstract

While stable mixed-hematopoietic chimerism induces robust immune tolerance to solid organ allografts in mice, the translation of this strategy to large animal models and to patients has been challenging. We have previously shown that in MHC-matched NHP, a busulfan plus combined belatacept and anti-CD154 -based regimen could induce long-lived myeloid chimerism, but without T cell chimerism. In that setting, donor chimerism was eventually rejected and tolerance to skin allografts was not achieved. Here we describe an adaptation of this strategy, with the addition of low-dose total body irradiation (TBI) to our conditioning regimen. This strategy has successfully induced multilineage hematopoietic chimerism in MHC-matched transplants that was stable for as long as 24 months post-transplant, the entire length of analysis. High-level T cell

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

chimerism was achieved and associated with significant donor-specific prolongation of skin graft acceptance. However, we also observed significant infectious toxicities, prominently including CMV reactivation and end-organ disease in the setting of functional defects in anti-CMV T cell immunity. These results underscore the significant benefits that multilineage chimerism-induction approaches may represent to transplant patients as well as the inherent risks, and emphasize the precision with which a clinically successful regimen will need to be formulated and then validated in NHP models.

Introduction

One of the prevailing hypotheses in the field of solid organ transplantation is that if stable mixed chimerism could be reproducibly induced, then immune tolerance to both skin and solid organ allografts from the same donor would follow. In murine models, this strategy has met with widespread success.(1–15) The critical importance of attaining and maintaining *multilineage* chimerism for tolerance-induction has also been demonstrated in mice, where it has been shown that in the absence of T cell chimerism, tolerance-induction could not be achieved. (16, 17) The significance of T cell chimerism to multilineage donor allogeneic hematopoietic cell chimerism stability has also been observed in clinical transplantation, where patients that lack substantial (>50–75%) donor T cell engraftment even in the setting of significant myeloid chimerism, are at high risk for loss of the donor hematopoietic graft. (18–21) While the mechanisms controlling T cell chimerism-mediated tolerance are not completely understood, they likely include the migration of both donor T cells and T cell precursors to the thymus to promote central tolerance.(16, 17, 22)

While chimerism-based strategies for tolerance-induction have achieved widespread success in mice, the translation of the intentional induction of stable multilineage mixed-chimerism to NHP models and to the clinic has been more challenging. This is especially the case after lower intensity non-myeloablative pre-transplant conditioning, with most work focused on inducing transient chimerism (17, 23–27). Alternatively, others have chosen to significantly escalate the intensity of pre-transplant conditioning to establish the donor graft (28–31) or to induce full-donor rather than mixed chimerism. (32–34) Our group has built on our experience with reduced-intensity pre-transplant conditioning and T cell costimulation-blockade-mediated chimerism- and tolerance-induction in mice (1–3, 5, 6) as the platform for translation to NHP. Using an MHC-defined NHP model, we first sought to determine if we could directly translate the success in mice, using a busulfan-based conditioning regimen and either anti-CD154 or anti-CD40 antibodies plus CD80/86-directed costimulation blockade-based immunomodulation to NHP. Our previous work demonstrated the successful induction of long-lived (> 1 year) myeloid-predominant chimerism with this regimen.(1, 35–38) However, unlike in mice, reduced-intensity busulfan-only conditioning in NHP did not lead to significant T cell chimerism (35, 36) and without significant T cell chimerism, the donor hematopoietic graft was eventually rejected by host T cells (35, 36, 39) (at a pace directly related to the degree of MHC disparity).(36) This transient chimerism did not induce tolerance to renal allografts.(38) Here we report an adapted transplant strategy that can lead to long-lived multilineage mixed chimerism, and in the setting of significant T cell chimerism, is associated with tolerance to donor hematopoiesis and prolonged skin graft

acceptance. Importantly, however, these transplants also provide an important cautionary tale: this tolerance-induction regimen was associated with a significant risk of infectious disease complications (prominently including CMV reactivation and disease), along with evidence for functional deficits in anti-viral protective immunity. Further refinements of this transplant strategy designed to 'thread the needle' of allograft tolerance while maintaining intact protective immunity, are needed for successful translation to the clinic.

Materials and Methods

Experimental animals

This study used Indian-origin rhesus macaques (18 animals) from the Yerkes National Primate Research Center, the Washington National Primate Research Center and the NIH-sponsored rhesus macaque colony operated by Alphagenesis Inc. The study was conducted in strict accordance with USDA regulations and the recommendations in the Guide for the Care and Use of Laboratory Animals. It was approved by both the Emory University, and the University of Washington Institutional Animal Care and Use Committees.

MHC Typing

Details of the MHC typing and disparity between donors and recipients are described in detail in Supplementary Methods.

Transplant preparation and immunosuppression strategy

The transplant strategy is shown in Figure 1, and is based on our previously published costimulation-blockade-based immunosuppression strategy (35, 36), with the addition of low-dose (200 or 300 cGray; cGy) total body irradiation (TBI, delivered with a linear accelerator (Varian) at a dose-rate of </=7cGy/min) to the busulfan-based pre-transplant conditioning regimen. Details of the strategy and dosing regimens are described in Supplementary Methods.

Hematopoietic stem cell transplant (HCT) protocol

HCT utilized leukopheresis-derived hematopoietic stem cells (HSC), harvested using a COBE Spectra or Spectra Optia apparatus from donors that underwent GCSF-based mobilization (50mcg/kg x 5 days SQ) as previously described (35, 36). The total nucleated cell dose/kg, total CD3+ T cell dose/kg and total CD34+ cell dose/kg for each transplant recipient is shown in Table 1.

Chimerism determination

Bone marrow, whole blood and flow-cytometry-sorted myeloid cells (CD3−/CD20−/CD14+), T cells (CD3+/CD20−/CD14−) and B cells (CD3−/CD20+/CD14−) were analyzed for donor chimerism based on divergent microsatellite markers at the UC Davis veterinary genetics laboratory as previously described.(35, 36)

CMV monitoring, Primary Prophylaxis and Treatment

Cytomegalovirus (CMV) monitoring, primary prophylaxis and treatment are described in Supplementary Methods.

ELISPot Analysis of CMV-specific T cell Immunity

ELISPot analysis was performed as previously described and detailed in Supplementary Methods.

Longitudinal flow cytometric analysis of T cell phenotype

Immune cell subpopulations were quantified flow cytometrically as previously described (details supplied in Supplementary Methods).

Skin allografting

Details of skin allografting are supplied in the Supplementary Methods.

Statistical analysis

Anova and Kaplan-Meier analysis was carried out using GraphPad Software Version 6. To determine Anova for multiple parameters, a post-hoc Tukey HST test was used to determine significant differences for pair-wise comparisons.

Results

Multilineage mixed chimerism in NHP

In this study we designed a stringent multi-year transplant protocol using MHC-matched transplant pairs, in which our previously published busulfan/costimulation blockade/sirolimus-based regimen(35, 36) was modified in order to enhance donor lymphoid chimerism (40) with low-dose (200–300 cGy) TBI.(41–43) As shown in Figure 1, completion of the entire experimental protocol required at least 25 months of follow-up: This included a pre-transplant conditioning phase (2 weeks) and on-therapy phase (10 months) during which recipients were first conditioned with non-myeloablative doses of busulfan + TBI, and then treated with sirolimus/5C8/belatacept, followed by sequential discontinuation in the following order: sirolimus→5C8→belatacept. After all immunosuppression was weaned, recipients were monitored for at least 12 months for chimerism stability, followed by skin allografting. As shown in Figure 2A,B, this conditioning regimen in conjunction with costimulation blockade/sirolimus-based post-transplant immunosuppression, was successful in creating multilineage chimerism in all transplant recipients. However, only 3 of the 9 transplanted animals completed the entire transplant protocol; the remaining animals reached protocol endpoint criteria due to clinical evidence of disease not responsive to treatment (on Days 32–202 post-transplant), each with infectious toxicities (discussed in detail below). For two of the three surviving animals, R.51 and R.52, multilineage mixed chimerism was sustained for the entire experiment. For the third transplant recipient, R.53, who received a sub-standard dose of donor cells after a problematic apheresis procedure [including a ~10-fold lower dose of total nucleated cells (TNC) CD34+ and CD3+ cells, Table 1], the initial chimerism set-point was substantially

lower compared to the other recipients (27% BM chimerism at Day +30 compared to 88% +/− 8.8% in the remaining 8 animals, Figure 2A). As shown in Figure 2B, R.53 lost chimerism at Day 383 post-transplant.

High incidence of CMV reactivation and other infectious disease complications amidst defects in anti-viral T cell function

While the creation of multilineage mixed chimerism that was capable of lasting more than 2–3 years (Figure 2B) is a striking result, one of the most important observations made in this study was the high incidence of infections that occurred. As shown in Table 1 and Figure 3, transplant recipients all experienced serious infectious disease complications. These prominently included CMV reactivation and disease (despite the fact that all recipients received primary prophylaxis and secondary CMV treatment using a regimen that has successfully prevented high-level CMV reactivation and disease in our other NHP studies) (36) but also included bacterial complications, including Giardia enterocolitis, Bacillus bacteremia, and multi-drug resistant E. coli bacteremia. To quantify the impact of the transplant regimen on T-cell mediated anti-CMV immunity, we used ELISPot assays directed at CMV antigens in eight animals for which we had available samples. These included two transplant recipients who survived long-term (R.51 and R.53) and two recipients who died early in the setting of CMV reactivation (R.55) and with CMV disease (R.58) (Figure 4). For each transplant, we assayed T cell responses pre-transplant for both the recipient and the donor, and at the time of necropsy in the transplant recipient. These results showed significant functional deficits of anti-CMV immunity in R.55 and R.58 at the time of necropsy. In contrast to the results with R.55 and R.58, at the time of necropsy, two of the long-term survivors (R.51 and R.53) had greater CMV-specific responses when compared to their responses at pre-transplant. It is important to note that the necropsy samples for the sick animals (R.55 and R.58) were obtained when they were still on immunosuppression, while the necropsy samples from R.51 and R.53 were obtained after immunosuppression withdrawal but with ongoing mixed chimerism.

Impact of sirolimus and costimulation blockade on hematologic reconstitution after transplantation

To investigate the hematologic associations with transplant course, a detailed longitudinal investigation of immune reconstitution was performed. Figure 5 shows the White Blood Cell Count (WBC), Absolute Neutrophil Count (ANC) and Absolute Lymphocyte Count (ALC) for all recipients. These data demonstrate that generalized lymphopenia developed and persisted in the setting of triple immunosuppression with sirolimus/5C8/belatacept. While the high risk of infectious complications led to early experimental termination in 6 recipients, as noted above, three animals (R.51, R.52 and R.53) were evaluable for the entire 25-month experiment. In each of these recipients, lymphopenia persisted after sirolimus withdrawal (during continued costimulation blockade with 5C8 and belatacept), and continued until discontinuation of 5C8, at which time there was a significant increase in lymphocyte counts, despite ongoing treatment with belatacept. There were no further increases in total lymphocyte counts when belatacept was discontinued.

Figure 6 shows a detailed analysis of the impact of immunosuppression withdrawal on B and T cells in R.51. As shown in this Figure, as with the total lymphocyte counts (Figure 5), the post-transplant expansion of the T cell compartment was significantly suppressed during triple-agent immunomodulation. Control of T cell expansion persisted despite the discontinuation of sirolimus, (in the setting of combined costimulation blockade with 5C8 and belatacept). In contrast, the withdrawal of 5C8 (with continued treatment with belatacept) resulted in both CD4+ and CD8+ T cell counts significantly rebounding, with no further increase after the discontinuation of belatacept (Figure 6A). The withdrawal of sirolimus and 5C8 also resulted in a significant shift in the T cell subset balance, with a shift in the proportion of both CD4 and CD8 T cell subsets away from the CD28+/CD95− naïve phenotype (Figure 6B). In CD4+T cells this was accompanied by both a proportional and absolute expansion of CD28+/CD95+ central memory T cells (Tcm). In CD8+ T cells, the loss of the Tnaive phenotype was accompanied by a significant shift towards both CD28+/CD95+ Tcm and CD28−/CD95+ effector memory (Tem) phenotype, with Tcm > Tem expansion. As shown in Figure 6 A,B, discontinuation of belatacept, and thus, the total withdrawal of all maintenance immunosuppression, resulted in no further shift in subpopulation balance or further expansion of conventional T cell numbers. Analysis of Treg homeostasis demonstrated a different pattern, with a proportional drop in these cells after withdrawal of sirolimus and 5C8, which did not recover until withdrawal of belatacept (Figure 6C). Moreover, the absolute number of Tregs did not significantly expand until after triple immunosuppression withdrawal.

Development of graft-versus-host disease (GVHD) in R.52

While none of the other animals in this study developed clinically apparent graft-versus-host disease, on Day 255 post-transplant, animal R.52 developed a skin rash covering 60% of his body surface area (BSA), that, when biopsied, showed histologic evidence of GVHD. This animal had previously experienced an episode of vomiting for which GVHD was considered in the differential diagnosis but developed neither diarrhea nor hyperbilirubinemia post-transplant. The diagnosis of GVHD prompted us to treat this recipient with oral prednisone from Day 271 to Day 550 post-transplant (with weaning and full discontinuation of prednisone prior to skin graft placement). Of note, T cell chimerism was stable prior to, during, and after the development and treatment of R.52 for GVHD (see Figure 2B).

Prolongation of Donor Skin Graft Acceptance in Transplant Recipients with Significant T Cell Chimerism

The survival of R.51, R.52 and R.53 to the 2+ year experimental endpoint allowed us to test the impact of T cell chimerism on skin graft survival. We had tested this on a previously reported animal (‘RQq9, conditioned with Busulfan alone, without concomitant TBI), who had developed very low T cell chimerism, and who had demonstrated donor skin graft rejection that occurred within 30 days.(36) As shown in Figure 7, in the current study, we observed a direct relationship between the percent T cell chimerism and the length of skin allograft survival. Thus, as shown in Figure 7A, R.51, who demonstrated stable, high-level T cell chimerism (~70% at the time of skin-graft placement), demonstrated long-term acceptance of both autologous and donor skin (>100 days) despite rapid rejection of unrelated, third-party skin (rejection by 9 days post-skin graft placement, Figure 7A). R.51

is thus the first NHP in our experience to meet the formal definition of long-term stable multilineage mixed-chimerism and donor-specific tolerance, having maintained mixed donor-recipient hematopoiesis for over 700 days, and donor-specific acceptance of skin allografts for over 100 days. R.52, who demonstrated significant levels of T cell chimerism, but which were lower than in R.51, also demonstrated significant prolongation of donor skin graft acceptance. In this recipient, third-party skin was rejected promptly (9 days) while donor skin was accepted for significantly longer (~69 days, Figure 7B). Importantly, however, R.52 eventually rejected the donor skin while autologous skin was accepted long term. It should be noted, as discussed above, that R.52 was also the animal who developed skin GVHD. Recipient R.53, who lost donor chimerism prior to skin graft placement, showed rejection of the donor skin graft at a similar tempo to previous recipients who had never developed T cell chimerism, showing signs of rejection by Day 40 post-skin graft placement (Figure 7C). This observation is important, as it provides evidence to support the hypothesis that significant T cell chimerism, rather than the conditioning regimen (which was identical in R.51, R.52 and R.53), was associated with skin graft tolerance. Of note, a fourth recipient, RDe9, who had been previously reported as the one recipient with stable chimerism in an earlier experimental cohort not-receiving TBI,(36) (for whom the donor was unfortunately not available for skin grafting), has now been followed for more than 10 years post-transplant, and has been a stable multi-lineage mixed chimera for the entire follow-up period, in the setting of substantial T cell chimerism (currently at 30%, with follow up for >10 years, Figure 2D). Taken together, these results are consistent with the acquisition of T cell chimerism as highly associated with long-term allograft acceptance, including both acceptance of bone marrow and skin graft tolerance. Indeed, although the numbers of recipients analyzed was small, three important observations arise from the skin allograft experiments, shown in Figure 7. First, we found that it was possible to create a scenario (R. 51) in which tolerance to both bone marrow and skin was created. Second, in the 4 animals on whom skin grafts were placed (three in the current study, one in a previous study(36), there was a direct correlation between the percent T cell chimerism and donor allograft survival (Figure 7D). Finally, in each of these animals, immune competence to allografts were maintained, given the rapid rejection of third party skin in 3/3 animals tested.

Discussion

In this study, we demonstrate that a transplant strategy employing a non-myeloablative busulfan/TBI conditioning regimen and costimulation blockade +sirolimus-based immunomodulation can reliably produce multilineage mixed chimerism, and in the setting of substantial and stable T cell chimerism, donor-specific acceptance of a skin allograft is achievable. These results are notable, given the demonstration that stable multilineage mixed chimerism can be intentionally created in primates. This represents, to our knowledge, the longest duration of stable multilineage mixed chimerism-induction in NHP. As such, these data provide proof-of-concept that, even in outbred primates, stable T cell mixed-chimerism can be achieved by design. As we will discuss in detail below, the results of this study also underscore the significant risks of infectious disease complications that can accompany this transplant regimen, indicating that further modifications are necessary before translation to the clinic.

This study is distinguished from others in the field by two facts: (1) That it sought to intentionally create *mixed donor/recipient T cell chimerism* and (2) That it sought to create multilineage mixed chimerism that was *stable post-transplant.* Thus, the group at Massachusetts General Hospital (MGH) has developed a transplant regimen in NHP that leads to transient donor chimerism, with rejection of the donor BMT within one month of transplantation. (23–27) In NHP, this regimen is effective in achieving long-term renal allograft tolerance in ~half of the renal transplant recipients. Encouragingly, clinical trials of BMT plus renal transplant based on these NHP studies resulted in multiple patients being weaned off of immunosuppression. (28, 29, 44–46) However, a significant engraftment syndrome(44) developed in several patients, suggesting that loss of the donor hematopoietic graft may have untoward effects in humans that were not observed in NHP. An independent clinical transplant series from the Stanford group has led to sustained donor chimerism in the setting of intensive total lymphoid irradiation (TLI, 1200cGy) plus ATG during combined BMT plus renal transplant. This regimen has been successful in achieving stable mixed chimerism and tolerance to the renal allograft in a number of MHC-matched donor/recipient pairs. (28–31). Whether this regimen will be translatable to the more commonly encountered MHC-mismatched setting is not yet known. Finally, the Leventhal and Ildstadt group has reported full donor engraftment (ie not *mixed* chimerism, but rather full replacement with donor hematopoieisis) and long-term allograft acceptance in patients given combined hematopoietic and renal transplants in the presence of facilitating cells. While this trial is not a ‘mixed-chimerism’ trial per se, if long-term GVHD-free immunosuppression-free survival becomes a reality for these patients, it is of high significance to the field.

In the current study, by closely monitoring the impact of sequential withdrawal of sirolimus, followed by anti-CD154 followed by belatacept, we gained important insights into the impact of triple, dual and single immunomodulation on T cell reconstitution and subpopulation balance. Our results demonstrate a significant suppression of absolute T cell counts and preservation of naïve T cell predominance during sirolimus and dual costimulation blockade. The suppression of T cell expansion and changes in T cell subpopulation balance was maintained despite discontinuation of sirolimus. When anti-CD154 treatment was also discontinued (during monotherapy treatment with belatacept), we observed both T cell expansion and a shift in the subpopulation balance, from a Tnaive predominance to a Tcm predominance for both CD4 and CD8 T cells (with CD8 T cells also expanding the Tem compartment). Importantly, while no further quantitative or qualitative changes occurred in the conventional T cell compartment after withdrawal of belatacept, discontinuation of this therapy was associated with expansion of Tregs. The shift in CD8+ T cells to a Tcm (rather than Tem)-predominance is a new finding that we have not observed with our previous mixed-chimerism experiments.(35–38) While this, along with the eventual expansion of Tregs is notable, it is not clear if these shifts represent causation or correlation with tolerance-induction. As such, this represents an important area for future study. Importantly, these data also indicate that reciprocal tolerance to both donor and recipient hematopoiesis could persist despite both the T cell expansion and the shift in the naïve/memory T cell balance that occurred after immunosuppression withdrawal, indicating robust control of both host-versus-graft and graft-versus-host vectors of T cell activation towards hematopoiesis with this transplant strategy.

The stability of bidirectional tolerance to hematopoiesis (permitting the coexistence of both donor and recipient T cells) also suggests that central tolerance mechanisms contributed to long-term acceptance of the hematopoietic graft in this model. The observation of donor chimerism in 3 of 4 isolated thymi is supportive of this hypothesis (Figure 2C). The significant duration of triple→double→single agent immunomodulation (with some form of immunosuppression present for 300 days post- transplant) may have facilitated the development of central tolerance, by providing sufficient immunosuppression-mediated immune restraint to permit trafficking of donor cells to the thymus.

One of the other central experiments performed in this transplant series was the placement of autologous, donor, and non-donor 'third-party' skin allografts a full year after withdrawal of sirolimus and costimulation blockade. This experiment demonstrated long-term acceptance (>100 days) in the recipient with the highest level of T cell chimerism, prolonged but limited skin graft acceptance in a recipient with lower, but still significant T cell tolerance, and prompt rejection of donor skin in two recipients with no T cell chimerism. These data suggest that as in mice, in primates, tolerance to tissues may require significant, stable T cell chimerism. One important caveat to the current studies is the well-recognized "high bar" for tolerance induction that skin grafts represent. It is possible that less immunogenic grafts, such as renal or liver allografts, could have a lower threshold for T cell chimerism required for tolerance-induction. Indeed, we also observed GVHD of the skin in R.52, which, while reversible with steroids, suggests that the difficulty in attaining tolerance to skin antigens occurred in both the host-versus-graft and graft-versus-host direction in this animal. Renal allograft studies (both proof-of-concept delayed renal transplantation as well as renal allografts placed concurrently with chimerism-induction) thus represent an important area for future research, once an optimized regimen, able to support stable mixed chimerism while minimizing infectious complications, is developed.

Despite the success in creating donor chimerism that was capable of lasting for several years, the extremely high rate of loss of our transplant recipients due to infectious disease complications represents a sobering reality. In the current study, 8/9 recipients experienced CMV reactivation, despite all recipients receiving primary prophylaxis with cidofovir. In 2 of these recipients, CMV end-organ disease was associated with recipient death, underscoring the seriousness of CMV disease in this transplant regimen, and the difficulty of reversing CMV disease in NHP, a phenomenon that has been documented previously by our group and others.(35, 36, 47–52) Whether the clinical challenges encountered with this virus is an inherent attribute of the NHP-specific CMV virus, or due to decreased efficacy of standard antivirals against rhesus CMV is not known. Delivering effective treatment for CMV is also considerably more challenging in NHP than patients, which could also have negatively affected our ability to control reactivated virus. Importantly, the most significant infectious disease complications occurred during the period of active immunosuppression, rather than during the period of sustained mixed chimerism, with R.52 and R.53 being much more stable from an infectious disease standpoint after immunosuppression withdrawal than they were on triple, double or single-agent therapy. This observation suggests that a rigorous step-wise elimination experiment is warranted, to determine which elements of the conditioning regimen and immunomodulatory strategy are required for stable multilineage mixed chimerism, and which produce the most severe functional defects in protective

immunity. If successful, this approach may yield a regimen that can succeed in producing tolerance without the defects in protective immunity observed in this transplant series. While it is not yet clear which of the elements of pre-transplant conditioning or post-transplant immunomodulation are the most important to eliminate/alter, our results suggest that a regimen that drives less severe lymphopenia while maintaining a positive Treg:Tcon balance would potentially tip the balance in favor of specific tolerance to the donor with less global defects in anti-viral protective immunity.

In summary, this transplant series has demonstrated a series of important milestones, as well as challenges to tolerance induction. We describe a transplant regimen that is consistently able to induce multilineage mixed chimerism as well as proof-of-concept that in the setting of significant T cell mixed chimerism, donor-specific tolerance, even to a highly immunogenic skin graft, is possible. We also demonstrate an important pitfall with this regimen, encapsulated by the significant clinical risks of infectious disease-related morbidity and mortality that these transplant recipients faced. These risks provide strong rationale for further iteration of this tolerance induction strategy to identify the minimally-effective immunomodulation strategy. Standing at the precipice of intentional multilineage mixed-chimerism and tolerance-induction, these studies underscore both the significant benefits that these approaches may represent to patients as well as the risks, and emphasize the precision with which clinically successful regimens will need to be formulated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALC	absolute lymphocyte count
ANC	absolute neutrophil count
CMV	cytomegalovirus
GVHD	graft-versus-host disease
HCT	hematopoietic stem cell transplant
HSC	hematopoietic stem cell
MGH	Massachusetts General Hospital
TBI	total body irradiation

TLI	total lymphoid irradiation
TNC	total nucleated cell
WBC	white blood cell count

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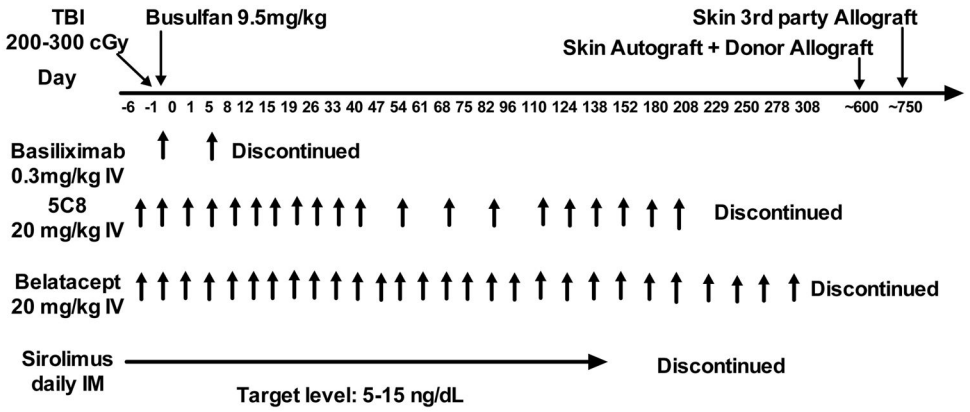

Figure 1. Transplant and Immunosuppression Strategy

Transplant strategy with details of conditioning regimen, doses of immunosuppression given and immunosuppression discontinuation end points. Busulfan was given on day −1 at a dose of 9.5mg/kg. The hematopoietic stem cell transplant took place on day 0. The immunosuppressive regimen was given as shown by the arrows in the figure, with each arrow representing an individual dose of drug, at the following concentrations: Belatacept (20mg/kg), Basiliximab (0.3mg/kg), Anti-CD154 (20mg/kg), sirolimus (once daily dosing was begun at 0.025mg/kg and adjusted to achieve a serum trough level of 10–15mg/mL). Autologous and donor skin grafts were placed as shown. Third party grafts were placed approximately 150 days after the placement of the donor and autologous skin grafts.

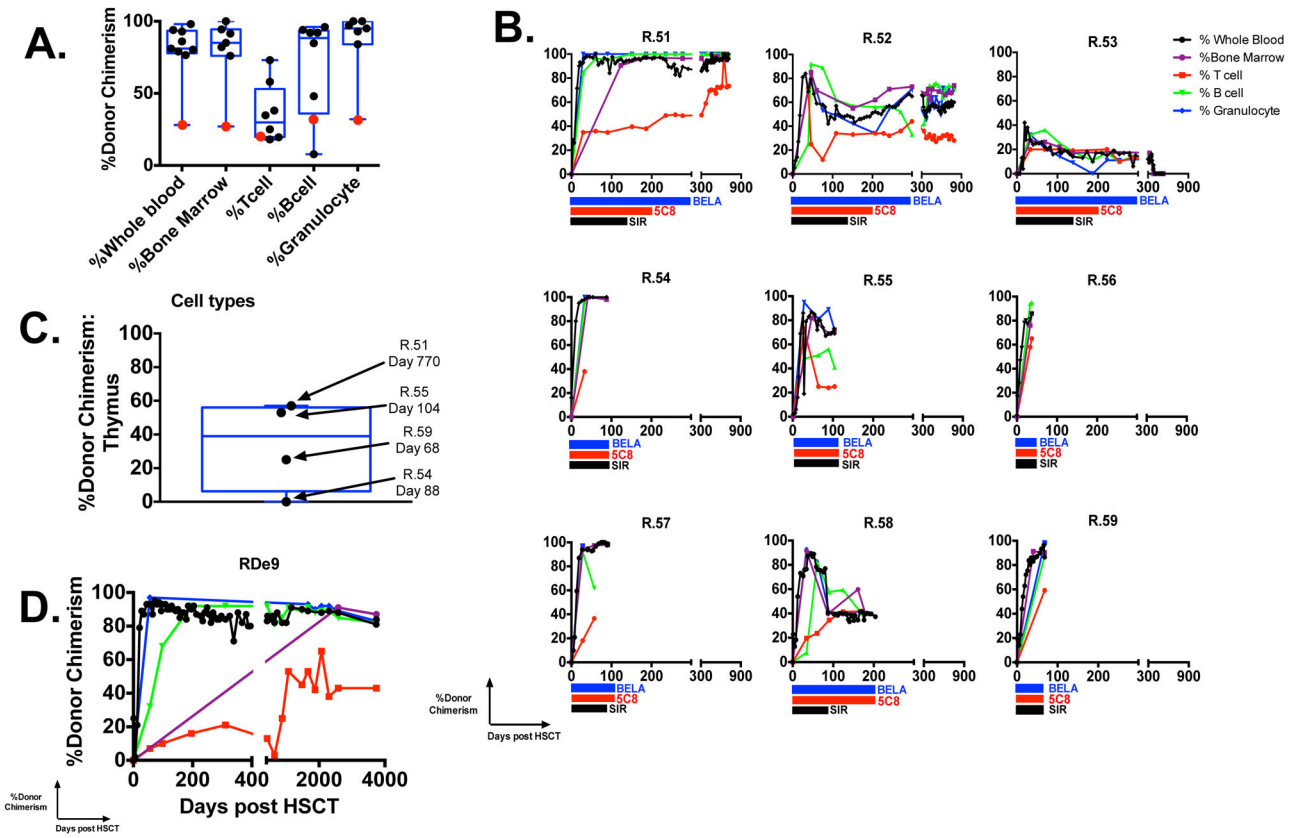

Figure 2. Multilineage mixed chimerism-induction after nonmyeloablative HSCT in MHC-matched transplant pairs

(A) Whole blood, bone marrow, T cell, B cell, and granulocyte chimerism of recipient animals at day 30 post-transplant shown as individual points. R.53 is shown as a red point, all other recipients shown as black points. Mean and standard deviation are shown as blue boxes and whiskers.

(B) Longitudinal analysis of whole blood chimerism, bone marrow chimerism, T cell chimerism, B cell chimerism, and granulocyte chimerism for all transplant recipients. Color-coded bars below each graph indicate duration of immunosuppression (black bar: Sirolimus, “Sir”; red bar: anti-CD154, “5C8”; blue bar: Belatacept, “Bela”).

(C) Thymic chimerism at time of necropsy for 4 recipients for which thymic tissue was obtainable at necropsy (R.51, R.54, R.55, R59). Individual chimerism values shown as black points. Mean and standard deviation are shown as blue boxes and whiskers.

(D) Long-term follow up of whole blood chimerism, bone marrow chimerism, T cell chimerism, B cell chimerism, and granulocyte chimerism for a previously reported (36) recipient (‘RDe9’) with stable multilineage mixed-chimerism.

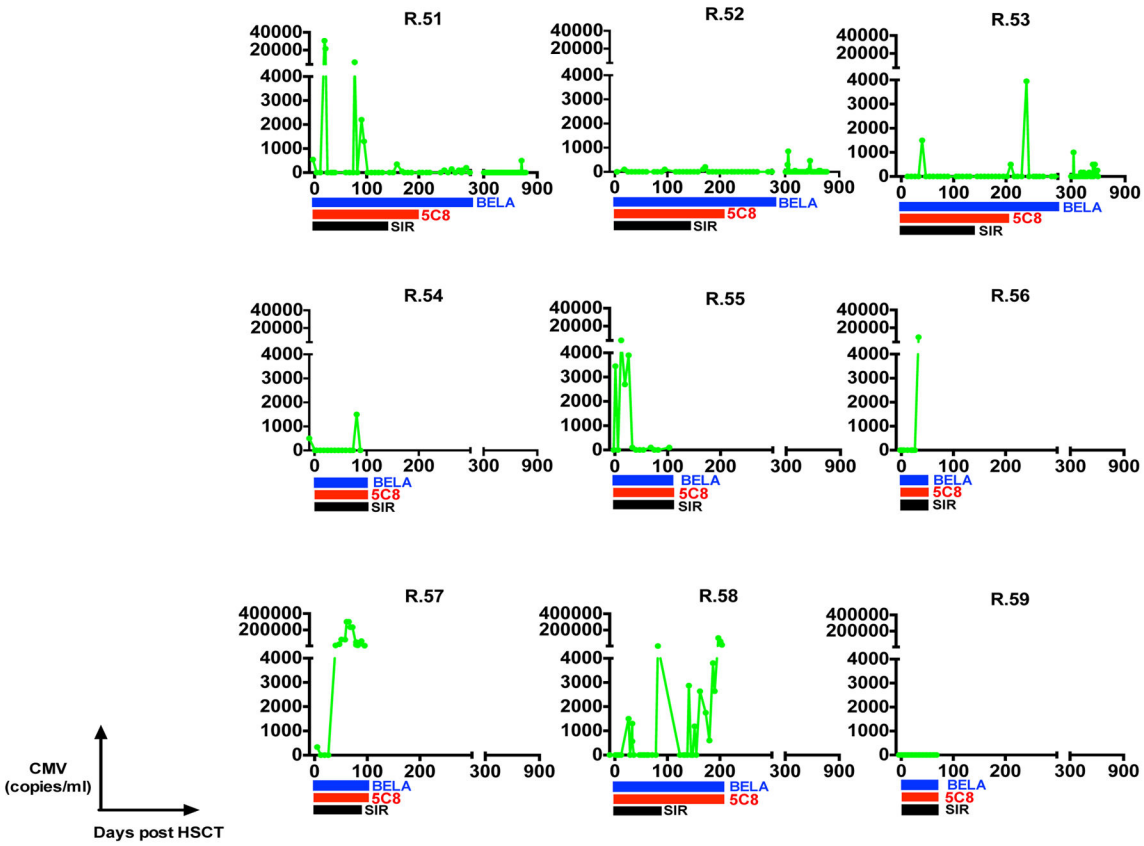

Figure 3. Longitudinal Analysis of CMV Viral Load

CMV viral load is shown for all transplanted animals. Color-coded bars below each graph indicate duration of immunosuppression (black bar: Sirolimus, “Sir”; red bar: anti-CD154, “5C8”; blue bar: Belatacept, “Bela”).

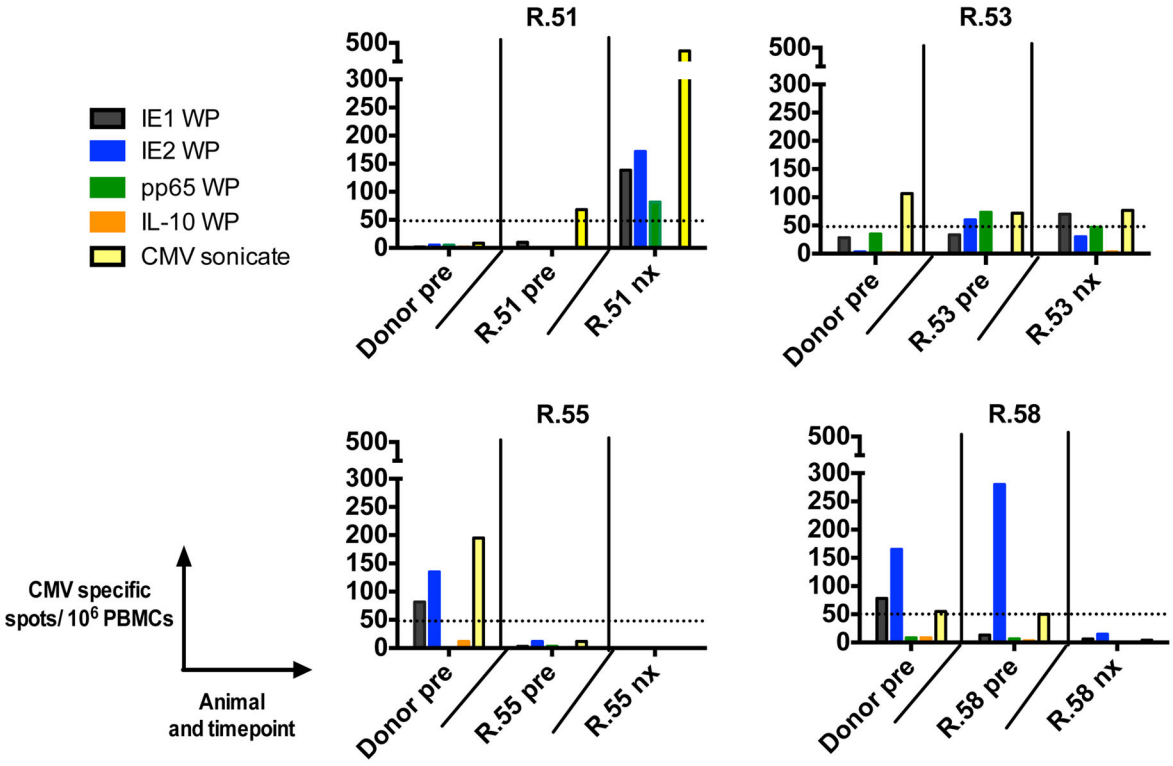

Figure 4.

CMV-specific T lymphocyte responses as measured by IFN-γ ELISPOT: Responses to whole peptide (WP) pools of rhesus CMV IE1 (Black), IE2 (Blue), pp65 (Green), and IL-10 (Orange) and rhesus CMV Sonicate (Yellow) are shown as spot-forming cells (SFC) per 1×10^6 lymphocytes. Results for four evaluable transplant recipients (R.51, R.53, R55, R.58), and their corresponding donors are shown. The threshold for a positive signal with this assay is 50 SFC/1×10^6 lymphocytes (indicated with the dashed line).

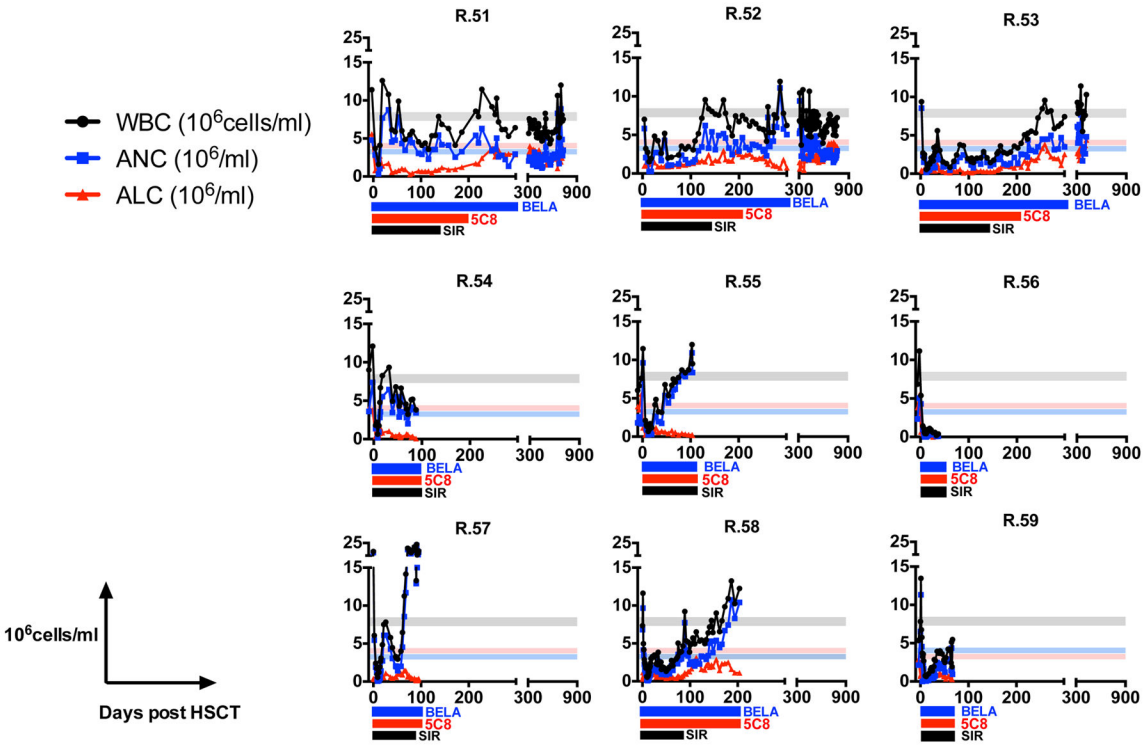

Figure 5.

Longitudinal analysis of hematologic reconstitution after transplant. Shown are the white blood cell count (WBC = black circles), absolute neutrophil count (ANC = blue squares), and absolute lymphocyte count (ALC = red triangles). Color-coded bars below each graph indicate duration of immunosuppression (black bar: Sirolimus, “Sir”; red bar: anti-CD154, “5C8”; blue bar: Belatacept, “Bela”). Normal values are shown in the graph (mean +/− SEM) for WBC (gray bar), ANC (red bar), ALC (blue bar).

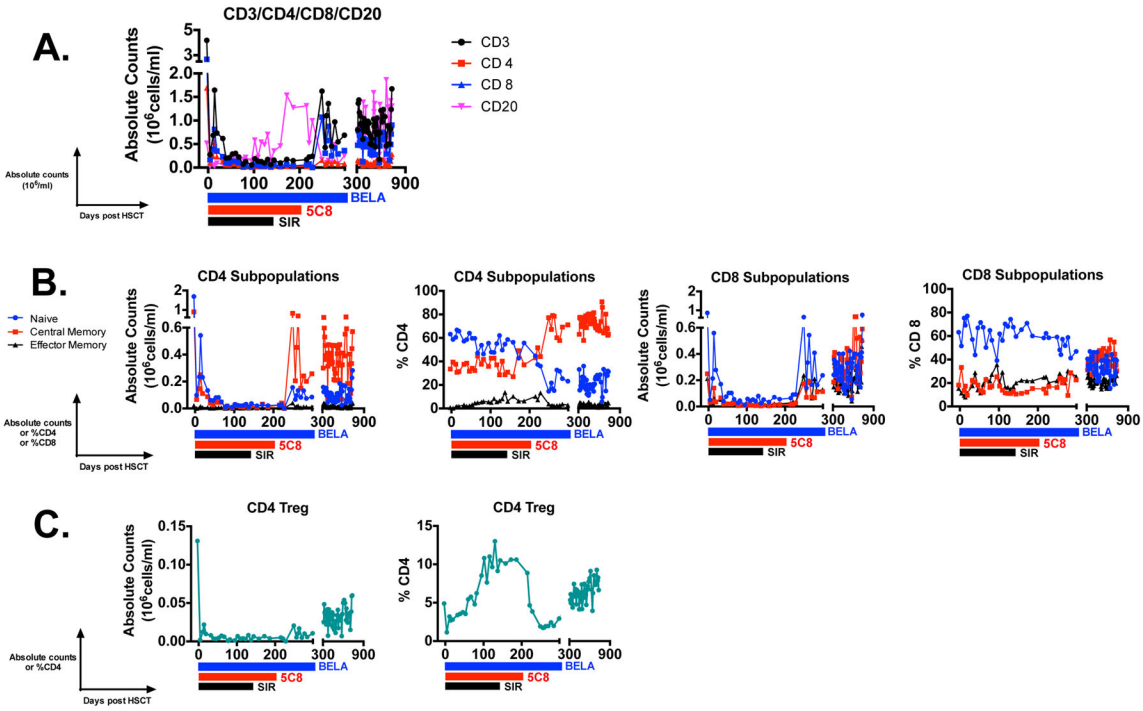

Figure 6. Immunophenotypic analysis of T cell subset balance after transplant in R.51

(A) Longitudinal analysis of R.51 showing the absolute number of CD3+/CD20− T cells, CD3−/CD20+ B cells, CD3+/CD20−/CD4+/CD8− T cells and CD3+/CD20−/CD4−/CD8+ T cells in R.51. Black circles: CD3+T cells. Pink upside triangles: CD20+ B cells. Red squares: CD4+ T cells. Blue triangles: CD8+ T cells. X-axis: Day post-transplant. Y-axis: Absolute cell counts. Color-coded bars below each graph indicate duration of immunosuppression (black bar: Sirolimus, “Sir”; red bar: anti-CD154, “5C8”; blue bar: Belatacept, “Bela”).

(B) Longitudinal analysis of R.51 showing absolute numbers and relative % of either CD4+ or CD8+ T cell subsets, defined as follows: Naive (CD28+/CD95−, blue circles), Central memory (CD28+/CD95+, red squares) and Effector memory (CD28−/CD95+, black triangles). X-axis: Day post-transplant. Y-axis: Absolute cell counts or % of CD4+ or CD8+ T cells. Color-coded bars below each graph indicate duration of immunosuppression (black bar: Sirolimus, “Sir”; red bar: anti-CD154, “5C8”; blue bar: Belatacept, “Bela”).

(C) Longitudinal analysis of R.51 showing absolute numbers and % of CD4+ T cells of CD4+/CD3+/FoxP3+/CD25+ Tregs. X- axis: Days post-transplant. Y-axis: absolute cell count or % Tregs of total CD4+ T cells. Color-coded bars below each graph indicate duration of immunosuppression (black bar: Sirolimus, “Sir”; red bar: anti-CD154, “5C8”; blue bar: Belatacept, “Bela”).

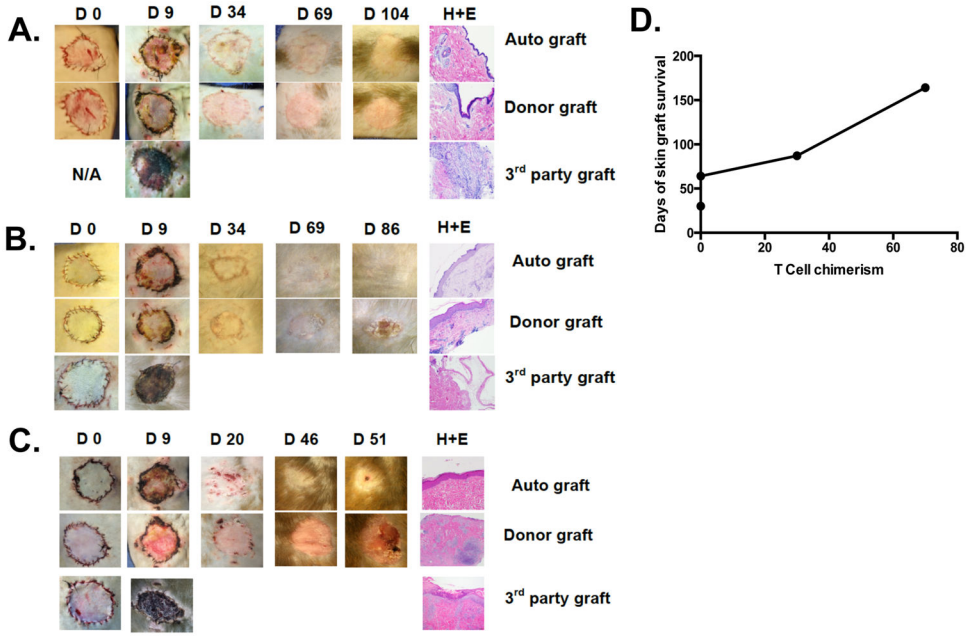

Figure 7. Skin grafting in long-term surviving transplant recipients, showing autologous skin grafts, donor skin grafts, and non-recipient, non-donor "third party" skin grafts

(A) R.51: Representative photographs of autologous, donor and third-party skin grafts at Days 0, 9, 34, 69 and 104 after graft placement. To the right of each graft, representative hemotoxylin and eosin (H&E) staining is shown documenting lymphocyte infiltration of the third party graft without infiltration of either the autologous or donor skin grafts.

(B) R.52: Representative photographs of autologous, donor and third-party skin grafts at Days 0, 9, 34, 69 and 86 after graft placement. Right column: Representative H&E staining of the autologous graft showing no lymphocytic infiltration, representative H&E staining of the donor graft showing lymphocytic infiltration and representative H&E staining of the third-party allograft with eschar formation post-rejection.

(C) R.53: Representative photographs of autologous, donor and third-party skin grafts at Days 0, 9, 20, 46, and 51 after graft placement. Right column: Representative H&E staining of the autologous graft showing no lymphocytic infiltration, representative H&E staining of the donor graft showing lymphocytic infiltration and representative H&E staining of the third-party allograft with eschar formation post-rejection.

(D) Graph showing the relationship between the % T cell chimerism (Y-axis) and donor skin graft acceptance (x axis). Data is from R.51, R.52 and R.53 from the current study and for a previously reported (36) recipient ('RQq9').

Table 1

Recipient #	Stem Cell Source	Total dose of TBI	TNC/kg	CD3/kg	CD34/kg	Indication for Experiment Termination	Infectious Disease Complications
R.51	PBSC	200cGy	1.4×10^9	9.5×10^7	1.5×10^6	End of experiment	CMV reactivation, Pneumonitis
R.52	PBSC	200cGy	1.3×10^9	6.0×10^7	2.6×10^6	End of experiment	CMV reactivation, Cellulitis, Gingivitis
R.53	PBSC	200cGy	2.9×10^8	2.6×10^6	7.4×10^5	End of experiment	CMV reactivation
R.54	PBSC	200cGy	6.0×10^8	2.1×10^7	3.1×10^6	Weight loss	CMV reactivation
R.55	PBSC	200cGy	4.3×10^8	2.2×10^7	1.1×10^6	Weight loss	CMV reactivation
R.56	PBSC	300cGy	8.4×10^8	3.2×10^7	1.2×10^6	Weight loss	CMV reactivation, Giardia enterocolitis
R.57	PBSC	300cGy	6.3×10^8	6.3×10^7	6.2×10^6	CMV colitis	High level CMV reactivation
R.58	PBSC	300cGy	3.0×10^9	1.2×10^8	6.4×10^6	CMV colitis	High level CMV reactivation
R.59	PBSC	300cGy	3.0×10^9	5.4×10^8	2.2×10^6	MDR E.coli end organ failure	Bacillus bacteremia, MDR E. coli bacteremia

Table 2

Two MHC-Haplotype Matched Pairs		MHC Microsatellites performed at California National Primate Research Center											Microsatellite for Chimerism Testing
Animal ID	**Relationship**	**222I18**	**MOG-CA**	**151L13**	**162B17A**	**162B17B**	**246K06**	**MICA**	**9P06**	**DRA-CA**	**D6S2876**	**D6S2741**	
D.51	Full sibling	167	121	301	250	309	283	200	189	120	203	271	**D13S765:** 236
D.51	Full sibling	168	123	309	240	293	285	200	191	132	195	269	
R.51	Full sibling	167	121	301	250	309	283	200	189	120	203	271	**D13S765:** 220
R.51	Full sibling	168	123	309	240	293	285	200	191	132	195	269	
D.52	Half sibling	173	127	305	252	309	285	200	175	126	215	279	**D8S1106:** 152
D.52	Half sibling	168	123	309	240	297	279	200	191	136	209	261	
R.52	Half sibling	173	127	305	252	309	285	200	175	126	215	279	**D8S1106:** 144
R.52	Half sibling	168	123	309	240	297	279	200	191	136	209	261	
D.53	Full sibling	167	125	319	242	281	275	194	185	128	0	287	**D13S765:** 220
D.53	Full sibling	167	121	301	250	309	281	194	187	112	219	269	
R.53	Full sibling	167	125	319	242	281	275	194	185	128	0	287	**D13S765:** 232
R.53	Full sibling	167	121	301	250	309	281	194	187	112	219	269	
D.54	Full sibling	173	127	305	252	309	283	200	175	126	215	279	**D7S513:** 207
D.54	Full sibling	168	123	309	240	297	279	200	191	136	209	261	
R.54	Full sibling	173	127	305	252	309	283	200	175	126	215	279	**D7S513:** 229
R.54	Full sibling	168	123	309	240	297	279	200	191	136	209	261	
D.56	Full sibling	167	125	319	242	281	275	194	185	128	0	287	**D3S1768:** 221
D.56	Full sibling	167	121	301	250	309	281	194	187	112	219	269	
R.56	Full sibling	167	125	319	242	281	275	194	185	128	0	287	**D3S1768:** 225
R.56	Full sibling	167	121	301	250	309	281	194	187	112	219	269	
D.57	Full sibling	173	127	305	242	311	277	191	185	134	210	281	**D8S1106:** 148
D.57	Full sibling	168	123	309	240	297	279	200	191	136	209	261	
R.57	Full sibling	173	127	305	242	311	277	191	185	134	210	281	**D8S1106:** 160
R.57	Full sibling	168	123	309	240	297	279	200	191	136	209	261	
D.58	Full sibling	173	121	299	240	295	279	200	175	128	219	277	**D13S765:** 224
D.58	Full sibling	173	127	305	242	293	281	200	175	136	209	277	

Two MHC-Haplotype Matched Pairs		MHC Microsatellites performed at California National Primate Research Center											Microsatellite for Chimerism Testing
Animal ID	**Relationship**	**222I18**	**MOG-CA**	**151L13**	**162B17A**	**162B17B**	**246K06**	**MICA**	**9P06**	**DRA-CA**	**D6S2876**	**D6S2741**	
R.58	Full sibling	173	121	299	240	295	279	200	175	128	219	277	**D13S765:** 232
R.58	Full sibling	173	127	305	242	293	281	200	175	136	209	277	
D.59	Full sibling	168	123	321	281	242	275	200	179	128	210	265	**D7S513:** 217
D.59	Full sibling	167	121	301	289	252	283	200	183	136	209	273	
R.59	Full sibling	168	123	321	281	242	275	200	179	128	210	265	**D7S513:** 189
R.59	Full sibling	167	121	301	289	252	283	200	183	136	209	273	
Two MHC-Haplotype Matched Pairs		**MHC Microsatellite performed at Wisconsin National Primate Research Center**											**Microsatellite for Chimerism Testing**
Animal ID	**Relationship**	**D6S2972**	**D6S2970**	**D6S2704**	**D6S2691**	**193435**	**MICA**	**D6S2782**	**D6S2892**	**DRA-CA**	**D6S2876**	**D6S27345**	
D.55	Full sibling	123.6	293.7	153.8	248.6	184.8	204.4	337.5	202.6	233.9	218.8	308.9	**D7S513:** 299
D.55	Full sibling	119.6	293.7	153.8	284.9	227.6	201.6	337.5	202.6	247.9	195.1	300.5	
R.55	Full sibling	123.6	293.7	153.8	248.7	184.8	204.4	337.6	202.6	234	218.8	309	**D7S513:** 193
R.55	Full sibling	119.6	293.7	153.8	285	227.7	201.6	337.6	202.6	248	215.1	300.5	