Evidence for Kidney Rejection after Combined Bone Marrow and Renal Transplantation Despite Ongoing Whole-blood Chimerism in Rhesus Macaques

Swetha K Ramakrishnan, Emory University
Andrew Page, Emory University
Alton B Farris III, Emory University
Karnail Singh, Emory University
Frank Leopardi, Emory University
Kelly Hamby, Emory University
Sharon Sen, Emory University
Aneesah Polnett, Emory University
Taylor Deane, Emory University
Mingqing Song, Emory University

Only first 10 authors above; see publication for full author list.

**Journal Title:** American Journal of Transplantation  
**Volume:** Volume 12, Number 7  
**Publisher:** Wiley-Blackwell | 2012-07, Pages 1755-1764  
**Type of Work:** Article | Post-print: After Peer Review  
**Publisher DOI:** 10.1111/j.1600-6143.2012.04045.x  
**Permanent URL:** http://pid.emory.edu/ark:/25593/f54cq

Final published version:  

**Copyright information:**  
© Copyright 2012 The American Society of Transplantation and the American Society of Transplant Surgeons  

Accessed January 23, 2020 2:05 PM EST
Evidence for Kidney Rejection after Combined Bone Marrow and Renal Transplantation Despite Ongoing Whole-blood Chimerism in Rhesus Macaques

Swetha K Ramakrishnan1,#, Andrew Page1,#, Alton B. Farris III1,2, Karnail Singh1, Frank Leopardi1, Kelly Hamby1, Sharon Sen1, Aneesah Polnett, Taylor Deane1, Mingqing Song1, Linda Stempera1, Elizabeth Strobert3, Allan D. Kirk1, Christian P. Larsen1, and Leslie S. Kean*1,4

1The Emory Transplant Center, Department of Surgery, Emory University School of Medicine, Atlanta, GA 30322
2Department of Pathology, Emory University School of Medicine, Atlanta GA 30322
3The Yerkes National Primate Research Center, Emory University, Atlanta, GA 30322
4Aflac Cancer Center and Blood Disorders Service, Department of Pediatrics and The Emory Transplant Center, Department of Surgery, Emory University School of Medicine, Atlanta, GA 30322

Abstract

Although there is evidence linking hematopoietic chimerism-induction and solid organ transplant tolerance, the mechanistic requirements for chimerism-induced tolerance are not clearly elucidated. To address this, we used an MHC-defined primate model to determine the impact of impermanent, T cell-poor, mixed-chimerism on renal allograft survival. We compared two cohorts: one receiving a bone marrow + renal transplant (“BMT/renal”) and one receiving only a renal transplant. Both cohorts received maintenance immunosuppression with CD28/CD40-directed costimulation blockade and sirolimus. As previously demonstrated, this transplant strategy consistently induced compartmentalized donor chimerism, (significant whole-blood chimerism, lacking T cell chimerism). This chimerism was not sufficient to prolong renal allograft acceptance: the BMT/renal mean survival time (MST, 76 days) was not significantly different than the renal transplant alone MST (85 days, p= 0.46), with histopathology documenting T-cell mediated rejection. Flow cytometric analysis revealed significant enrichment for CD28-/CD95+ CD4+ and CD8+ Tem cells in the rejected kidney, suggesting a link between CD28-negative Tem and costimulation blockade-resistant rejection. These results suggest that in some settings, transient T cell-poor chimerism is not sufficient to induce tolerance to a concurrently placed renal allograft and that the presence of this chimerism per se is not an independent biomarker to identify tolerance.

Keywords

Costimulation Blockade; Non-human Primate; Mixed Chimerism; Renal Transplant; Biomarker

*Corresponding Author Contact Information: Leslie S. Kean, 101 Woodruff Circle, NE, Room 5203, Emory University School of Medicine, Atlanta, GA 30322, Department Fax: 404-727-3660 Phone: 404-727-5265 Leslie.Kean@emory.edu.
#Co-first authors

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

Current immunosuppression strategies are inadequate to guarantee indefinite allograft survival, and are associated with significant toxicities. Thus, there is a critical need to develop strategies that can induce immune tolerance post-transplant. Clinically, the link between bone marrow replacement and tolerance to solid organ transplants is strong: there are numerous examples of bone marrow transplant (BMT) patients who demonstrate immune tolerance to subsequently placed solid organ allografts from the same donor. (1-7) However, complete replacement of a transplant recipient’s bone marrow (using currently available techniques) carries with it significant risks, that prohibit wide application in solid organ transplantation (SOT). In mice, partial replacement with donor bone marrow has been successfully deployed with minimally myeloablative strategies leading to stable, multilineage mixed-chimerism, and this less-toxic approach has induced robust immune tolerance to solid organ allografts. (8-15) Mechanistic investigations have revealed that chimerism promotes central thymic deletion of donor-and recipient-reactive T cells, (8, 9, 15) protecting against both chronic rejection and graft-versus-host disease (GvHD).

Perhaps not surprisingly, the translation of mixed-chimerism-induction from mice to outbred, pathogen-exposed non-human primates (NHP) and to patients has proven challenging, both after MHC-matched and MHC-mismatched transplantation. (16-19) This is to be expected: the clinical BMT experience has demonstrated the immunologic instability of mixed-chimerism, especially in the setting of low T cell chimerism: evidence suggests that with current immunosuppression strategies, this usually results in transplant rejection, or, more rarely, converts to full donor chimerism. (20-22) While Strober and colleagues have recently reported compelling results using total lymphoid irradiation and ATG, documenting stable, multilineage mixed-chimerism and renal allograft acceptance in several HLA-matched recipients, (23) it is not yet clear whether this regimen will be as successful with HLA-mismatched transplants, (24) in which durable mixed chimerism-induction without GvHD will be more challenging to produce.

While the strategy described in Scandling et al., (23) has emphasized the same components of chimerism shown to be critical in murine studies (chimerism that is stable and multilineage), selected NHP and clinical studies have demonstrated immune tolerance to renal allografts in the setting of transient hematopoietic chimerism. (17, 25, 26) Kawai et al., have described NHP experiments in which renal transplants were accepted in approximately half of highly MHC-matched cynomolgus macaque recipients that had been treated with a regimen including splenectomy or anti-CD154 treatment, TBI, thymic irradiation, ATG and donor bone marrow; whereas no acceptance of the renal allograft occurred in the absence of donor bone marrow. (17, 25, 26) These results suggest that with some preparative regimens and immunosuppressive strategies, (and potentially requiring highly MHC-matched transplant pairs (27), avoidance of acute renal allograft rejection (but not cardiac or lung allograft rejection (16, 28) can occur without stable hematopoietic chimerism. Several patients have now been treated with a related (but non-identical) combined bone marrow and renal transplant regimen, and some have been successfully weaned from immunosuppression. (29-32) However, a cytokine release syndrome (occurring during autologous hematopoietic reconstitution) has also occurred, (33), suggesting that there may be clinical risks to early rejection of donor chimerism and re-establishment of recipient hematopoiesis.

Our group has previously reported the creation of high-level, long-lived (but impermanent) bone marrow chimerism in rhesus macaques, (18, 19, 34) who were treated with a non-myeloablative, busulfan + costimulation blockade/sirolimus-based regimen that is similar to the one that has been shown to induce tolerance in mice. (8, 12) These studies have
documented that (1) in NHP, busulfan + costimulation blockade/sirolimus induces high-level whole-blood and bone marrow chimerism; (2) that whole-blood chimerism correlates closely with myeloid (neutrophil) chimerism; and (3) that this transplant regimen results in compartmentalized chimerism, with little-to-no T cell chimerism. (18, 19, 34) In our previous studies, we have shown that the duration of chimerism is directly related to the degree of MHC matching between donor and recipient, (19) and that, although rare, stable mixed-chimerism and tolerance to fully MHC-matched donor bone marrow can occur. (19) In contrast, with MHC-mismatched transplants, we have shown that although high level, T cell-poor chimerism can be routinely created, the donor bone marrow is promptly rejected upon cessation of immunosuppression, and this rejection is associated with a skewing of the recipient T cell compartment towards a CD95+ memory phenotype. (18, 19, 34)

Given that the compartmentalized chimerism has been unstable, and that recipient-derived, donor-reactive T cells are clearly present and poised to reject the donor bone marrow, (18, 19, 34) we hypothesized that this type of chimerism would not be protective of a concurrently placed renal allograft. In this report, we document that in our system, chimerism per se was not sufficient to prolong renal allograft rejection, and that, even in the setting of ongoing immunosuppression and ongoing compartmentalized chimerism, renal allograft rejection can occur. These results have important clinical implications for chimerism-based tolerance-induction and suggest that for reproducible tolerance to become a clinical reality, increased T cell chimerism may be an important goal.

Materials and Methods

Experimental Animals

This study utilized rhesus macaques, obtained from the Yerkes National Primate Research Center or the NIAID-sponsored Rhesus macaque colony (Yemassee, SC). All animals were treated in accordance with IACUC regulations. For this study, donor and recipient pairs were chosen that were matched for one MHC haplotype based on MHC microsatellite typing. (19, 35)

Bone marrow transplant (BMT) protocol

Bone marrow was harvested from the femurs, humeri and vertebrae as previously described (18), after which the marrow was filtered using an MH2150 Bone Marrow Collection Kit from Bio Access Inc. (Baltimore, MD).

Renal Transplantation was performed as previously described. (36) In animals receiving combined BMT and renal transplantation, the bone marrow was infused intravenously 6 hours after renal transplantation.

Immunomodulation

All BMT recipient animals were prepared with a single dose of intravenous busulfan (9.5mg/kg, Otsuka Pharmaceuticals, Rockville, MD). Animals not receiving a BMT did not receive busulfan. All recipients were also given two peri-transplant doses of basiliximab (0.3mg/kg on days -1 and 3 relative to transplant). Maintenance immunosuppression consisted of the 3A8 monoclonal antibody (obtained from the Non-human Primate Reagent Resource, Cambridge MA), CTLA4Ig (Bristol Myers Squibb, Pennington, NJ) and sirolimus (LC laboratories, Woburn MA). CTLA4Ig was dosed at 20mg/kg on day -6, -1, 0, 3, 5, 9, 16, and every 14 days thereafter, until day of death. 3A8 was dosed as follows: 20mg/kg/dose × 2 doses on day -6 and day -1, 10mg/kg/dose × four doses on days 0, 3, 5,9, and then maintenance dosing at 5mg/kg/dose twice weekly thereafter, continuing until day of death.
Sirolimus was given intramuscularly with dosing adjusted to reach a trough value targeted at 5-15 ng/dL.

Creatinine-based diagnosis of renal transplant rejection

For this study, the day of renal transplant rejection was designated as the first day that the serum creatinine reached 2.5 mg/dL (without a subsequent decline), in recipients that subsequently showed evidence of histologic allograft rejection on terminal analysis. It should be noted that occasionally, the creatinine transiently increased to levels greater than 2.5 mg/dL, but subsequently declined (see R5 and R9, Figure 1F,G). Since these levels rapidly corrected with supportive care measures, they did not trigger a diagnosis of rejection.

Histologic Assessment

Renal tissue fixed in 10% formalin was processed in paraffin blocks for both hematoxylin/eosin staining as well as for immunohistochemistry. Hematoxylin/eosin-stained sections were assigned a score and diagnosis based on the Banff pathology criteria (37) by a pathologist (A.B.F) who was blinded to the experimental treatment cohorts. Tissue sections were also labeled with antigen-specific primary antibodies, counterstained with hematoxylin, and then visualized using the LSAB+ labeled Streptavidin-Biotin kit (Dako; Carpenteria, Ca). Primary antibodies used for T cell, B cell, and macrophage staining were anti-human CD3, CD79α, and CD68 (Dako; Glostrup, Denmark). The percent of parenchymal inflammatory cells composed of each cell type was determined by visual approximation of the proportion of inflammatory cells staining with each antigen.

Chimerism determination

Chimerism was measured using real-time PCR (38, 39) or DNA microsatellite-based quantification as previously described. (18, 19, 40) T or B cell chimerism was determined by sorting CD3+/CD20− T cells or CD3−/CD20+ B cells using a FACSaria cell sorter (Becton Dickinson, San Diego, CA) prior to molecular analysis.

CMV monitoring

The number of CMV copies/mL of whole-blood was determined as previously described. (18, 19, 40)

T cell purification from explanted renal allografts

Explanted kidneys were perfused with 50mLs of PBS ex vivo. The renal tissue was then manually disrupted using the blunt end of a syringe in ice-cold PBS, followed by centrifugation at 1500 RPM at 4°C for five minutes. RBC were then lysed with High Yield Lyse solution (Invitrogen) followed by recentrifugation at 1500 RPM at 4°C for five minutes. The cell pellet was then stained for flow cytometry.

Longitudinal flow cytometric analysis of T cell phenotype

Multicolor flow cytometry panels were used to identify naïve, central memory and effector memory CD4+ and CD8+ T cells by their differential expression of CD28 and CD95 as previously described. (18, 19, 34) As has been previously documented, the mean fluorescence intensity (MFI) of CD95 was slightly higher for CD8+ memory cells than for CD4+ memory cells, (41) and thus was gated accordingly. For the analysis shown in Figure 4, uniform CD4 and CD8 gates were used when comparing the T cell memory phenotypes in the blood and the kidney. Antibody clones and sources were: From BD Biosciences (San Jose CA): CD3, Clone SP34-2; CD8, Clone: RPA-T8; CD40, Clone 5C3. From eBioscience (San Diego CA): CD4, Clone OKT4; CD20, Clone: 2H7; CD28, Clone: CD28.2; CD95,
Clone: DX2. All flow cytometric analysis was performed using Flojo flow cytometry analysis software (Treestar, Ashland, OR).

Statistical Analysis

Statistical analysis was performed using the Prism Software statistical package (GraphPad, La Jolla CA). For the terminal analysis of T cell subpopulations in the blood and kidneys, the percentage data was log-transformed prior to paired T test analysis.

Results

Combined BMT/renal transplant in rhesus macaques

We identified a cohort of 10 pairs of rhesus macaques for these studies (Table 1). The animals in each pair were related to each other as full or half-siblings, and were one MHC-haplotype matched by DNA microsatellite analysis. (35, 42) The ten pairs were divided into two cohorts such that animals in Cohort #1 received a combined BMT/renal transplant, and animals in Cohort #2 received a renal transplant alone. The animals were transplanted using our previously published costimulation blockade-based immunosuppression strategy (which included CD28/CD40 costimulation blockade and sirolimus). (34) We have previously demonstrated that when combined with pre-transplant preparation with a non-myeloablative dose of busulfan, this strategy leads to high levels of whole-blood chimerism after hematopoietic stem cell transplant, and that this mixed-chimerism is stable for the length of immunosuppression. Importantly, our previous studies have also documented that this chimerism is compartmentalized (being concentrated in the myeloid lineage, with very little T cell chimerism), and is associated with rejection of the donor bone marrow after cessation of immunosuppression. (18, 19, 34) Thus, although this chimerism is longer-lived than most other published chimerism-induction protocols in NHP, (17, 25, 26) it is still transient in nature.

Induction of compartmentalized donor chimerism in the BMT/renal transplant cohort

Figure 1A shows that, as expected from previous studies (18, 19, 34) the animals in the BMT/renal cohort all developed significant whole-blood donor chimerism (average peak chimerism of 55.4 +/- 8.3%), and that, with the exception of one animal (R3), this chimerism was stable for the length of the experiment. As shown in Figure 1B and Table 1, and also as expected from our previous studies, (18, 19, 34) despite high levels of whole-blood chimerism (35 +/- 10%), these recipients developed very little T cell (0.6 +/- 0.6 %) or B cell (8 +/- 6%) chimerism in the peripheral blood, and very little thymic chimerism (4 +/- 3%), when measured during the terminal analysis. While blood draw limits precluded the analysis of T cell chimerism prior to necropsy in R1-R3, for two animals (R4 and R5), we were able to measure T cell chimerism at two time-points (during ongoing renal transplant function and at the terminal analysis). As shown in Figure 1C, neither R4 nor R5 demonstrated T chimerism at either time-point.

Animal R3 was an outlier in this study, and rapidly lost donor chimerism. As shown in Figure 1D, animal R3 developed high-level CMV viremia very early after transplant (peak viral load = 255,672 copies/mL, Figure 1D). Early CMV reactivation is well documented to increase the risk of bone marrow non-engraftment; thus, it is likely that this was the mechanism of failure of R3’s bone marrow allograft. While one other animal in the BMT/renal cohort (R2, Figure 1D) and a single animal in the renal alone transplant cohort (R7, Figure 1E) also developed transient post-transplant CMV, neither had viral loads as high as R3, and both animals were able to engraft the transplanted bone marrow and/or kidney.
Renal allograft rejection occurred despite chimerism-induction in the BMT/renal cohort

As shown in Figure 1 and Table 1, although the BMT/renal transplant recipients R1, R2, R4 and R5 all developed sustained whole-blood chimerism for the length of the study (Figure 1A), they all rejected the donor kidney despite the presence of this chimerism. Thus, as shown in Figure 1F, each animal ultimately developed a rising serum creatinine, which was accompanied by renal allograft rejection as measured by histologic examination (Table 1 and Figure 2). In contrast, in our previously-published cohort of animals receiving a BMT alone (using the same costimulation blockade/sirolimus immunosuppression strategy (34) normal creatinine values were maintained for the duration of the transplant, with the average serum creatinine at the time of BMT rejection being 0.7 +/- 0.2 mg/dL (data not shown). As shown in Table 1 and Figure 2, in this study, histologic and immunohistochemical examination of the transplanted kidneys by a pathologist specializing in renal transplant pathology demonstrated T cell mediated rejection (TCMR) by Banff criteria, with a preponderance of CD3+ T cell infiltration (75-90% of the cellular infiltrates) and less pronounced CD79+ B cell (5-20%) and CD68+ macrophage (5-10%) infiltration. Figure 2 shows representative immunohistochemistry from a normal kidney (insets), and from the terminal analysis of recipient R5, which documents these cellular infiltrates. R4 alone demonstrated Grade III Interstitial Fibrosis and Tubular Atrophy (IFTA) in addition to superimposed borderline TCMR (Table 1). Consistent with this histologic analysis, with the exception of animal R4, none of the 10 recipients developed significant donor-specific antibody (not shown). Only R4 demonstrated anti-donor antibody, which was present by day 13 post-transplant, and which persisted for the remainder of the transplant course (not shown).

Figure 1G and Table 1 demonstrate the longitudinal analysis of serum creatinine and the histologic analysis of the rejected kidneys in the “renal transplant alone” cohort. As shown in the Figure and Table, these animals also demonstrated predominant TCMR at the time of necropsy, with a preponderance of CD3+ T cell infiltration (75-90% of the cellular infiltrates) and less pronounced CD79+ B cell (5-20%) and CD68+ macrophage (5%) infiltration. All animals were sacrificed in the setting of a rising creatinine, except for animal R9 who was sacrificed after a post-biopsy hemorrhagic complication. His creatinine was low (1.5) prior to the biopsy. Thus, data for R9 was death-censored in the subsequent survival analysis.

Figure 3 shows the survival analysis comparing the day of transplant rejection (defined as the day that a rising serum creatinine reached 2.5 mg/dL in animals with subsequent necropsy-proven kidney rejection) in the two experimental cohorts. The mean survival time (MST) for the BMT/renal cohort was 76±18 days and for the renal alone cohort was 85±21 days. R3, the single recipient who demonstrated early rejection of donor bone marrow, was not included in the survival analysis of the BMT/renal cohort, given the fact that there was not ongoing donor chimerism at the time of renal transplant rejection. As shown in the Figure, log-rank testing revealed no statistically significant difference in the survival of the BMT/renal cohort compared to the renal alone cohort (p = 0.46).

A kidney-specific signature of rejection identified by flow-cytometric analysis

Our previous studies have documented that animals undergoing rejection of donor bone marrow demonstrate a shift in the peripheral blood of both CD4+ and CD8+ cells towards a CD95+ memory phenotype. (19, 34) The current study allowed us to determine whether the CD95+ rejection phenotype was site-specific, or whether it would be present in the peripheral blood in animals that were only undergoing renal transplant rejection. As shown in the representative example in Figure 4A, and the summary data shown in Figure 4B, both CD4+ and CD8+ T cells isolated from the rejecting kidneys were highly skewed towards a
CD95+ memory phenotype, which was not present in the peripheral blood at the time of kidney rejection. As shown in these Figures, the CD8+ cells in the rejecting kidney consisted predominantly CD28-/CD95+ Tem cells. Interestingly, while there were quantitatively more Tcm and Tn CD4+ T cells than Tem CD4+ T cells in the rejecting kidney, the relative enrichment for the CD28-/CD95+ CD4+ Tem cells in the rejected kidney compared to the peripheral blood was much more striking than for the Tcm population. Thus, the CD28-negative CD4+ Tem were enriched by an average of 12.2-fold (p = 0.0003) in the rejected kidney compared to the peripheral blood, whereas CD28-positive CD4+ Tcm were enriched by 2.2 fold (p= 0.03). This enrichment for both CD4+ and CD8+ CD28-negative cells infiltrating the rejecting kidney may have mechanistic significance, given that fact that these transplant recipients were receiving CD28-directed CTLA4Ig-containing costimulation blockade-based immunosuppression at the time of rejection, and the CD28-negative cells would be expected to be more resistant to this blockade.

Discussion

In this study, we have used a rigorous, MHC-defined NHP model of both mixed-chimerism and renal transplantation to determine the impact of impermanent hematopoietic chimerism on renal allograft survival. These studies have benefited from a new NIH resource: the NIAID-sponsored MHC-defined primate colony. (19, 34, 42, 43) By studying transplant cohorts with uniform degrees of MHC disparity, we were able to eliminate a critical confounder that has historically plagued NHP transplant studies. We believe that this technical advance has considerably strengthened our ability to draw mechanistic interpretations from our experimental results.

In these studies, we created donor mixed-chimerism through an established regimen which combines busulfan-based pre-transplant recipient conditioning and costimulation blockade/sirolimus-based maintenance immunosuppression. (18, 19, 34) We have previously shown, and also document in this study, that this regimen reliably leads to significant donor chimerism as measured in the whole-blood and bone marrow, and that this chimerism is stable for the duration of immunosuppression. (18, 19, 34) However, this regimen creates compartmentalized chimerism, which is T- and B-cell poor. (18, 19, 34) Likely tied to the compartmentalized nature of the chimerism, our previous work has documented that the mixed-chimerism created by this regimen is impermanent, and upon the withdrawal of immunosuppression, rejection invariably ensues in the setting of donor-recipient MHC mismatch.

In the current transplant series, we compared experimental outcomes between a cohort of recipients that received a combined BMT/renal transplant with a cohort receiving a renal transplant alone. Our results document rejection of the renal allograft in both cohorts despite ongoing immunosuppression (and despite ongoing whole-blood chimerism), with renal transplant survival in the BMT/renal cohort that was not statistically significantly different than in the cohort that received the renal transplant alone (Figure 3). These results indicate that the induction of whole-blood chimerism itself was not sufficient to produce immune tolerance to a transplanted kidney, or even to prolong allograft acceptance.

Several characteristics of the mixed-chimerism that was created in this study may have contributed to the lack of chimerism stability, and subsequently to renal allograft rejection. First, as we have documented previously, pre-transplant preparation with busulfan alone produces compartmentalized chimerism: while significant whole-blood chimerism routinely develops post-transplant, very little, or no T cell chimerism is induced. Experience in clinical bone marrow transplantation supports the instability of compartmentalized, T cell-
poor chimerism. (20-22) While the exact mechanisms responsible for this instability are not known, it is possible that lymphocyte chimerism may directly promote stable donor engraftment through a cytotoxic effect on recipient APCs and recipient T cells, leading to the deletion of the donor-reactive T cell repertoire, and that T cell engraftment in the recipient thymus can promote central deletional tolerance. (44, 45) Donor T cell chimerism may also exert its salutary effect through regulation, with an as-yet-undefined threshold of donor-derived Tregs required to maintain tolerance to the allograft. In the setting of very low or absent T cell chimerism, neither of these mechanisms would be active, significantly increasing the risk of bone marrow rejection.

Our previous results have suggested that in the setting of compartmentalized donor chimerism, the combination of costimulation blockade and sirolimus is inadequate to fully tolerize the recipient T cell compartment. (18, 19, 34) Thus, although the rejection of donor bone marrow can be efficiently inhibited during ongoing immunosuppression, after immunosuppression withdrawal, sufficient donor-reactive T cells are present to rapidly reject the BMT. The observations from the present study suggest that there is a cohort of peripheral blood T cells which, while incapable of rejecting marrow-resident hematopoietic stem cells during ongoing immunosuppression, can nonetheless traffic to the allograft and reject the donor kidney. The observation of significant enrichment for CD28-negative T cells in the rejected kidneys (and the absence of this enrichment in the peripheral blood) suggests that the mechanism controlling costimulation blockade-resistant breakthrough rejection may involve the CD28-negative Tem subpopulation. These putatively costimulation blockade-resistant T cells (36) may have been at high risk for activation in the setting of ongoing exposure to donor hematopoietic antigens, leading, ultimately to the rejection of the concurrently placed renal allograft.

Our results suggest that for chimerism to reproducibly induce robust post-transplant immune tolerance in NHP and in patients, it may be necessary to closely adhere to the lessons learned from murine models, and from clinical BMT, and thus pursue strategies that reproducibly lead to chimerism that is both multilineage and stable. While in murine models, this is possible through the use of pre-transplant conditioning with busulfan alone, in NHP and patients, the induction of T cell chimerism will likely require a more complex conditioning regimen, directed at creating T-cell as well as stem-cell space prior to transplant. In preliminary experiments, we have now used both pre-transplant irradiation, as well post-transplant lympho-ablation followed by donor lymphocyte infusions, which have each led to increased T cell space and T cell chimerism (Ramakrishnan et al., unpublished). Ongoing experiments are evaluating the impact of this increased T cell chimerism on tolerance-induction.

While transient, compartmentalized chimerism has thus far not carried a substantial risk of GvHD,(16, 18, 19, 25, 28) the pursuit of strategies designed for chimerism permanence, which include the induction of significant T cell chimerism, will require close attention to the attendant increased risk of GvHD, and will require strategies to effectively prevent this deadly complication of BMT. Our NHP studies have indicated that costimulation blockade-based immunosuppression can significantly protect from GvHD, even after high-risk MHC mismatched transplantation. (40) Thus, costimulation blockade-based therapies may be central to future strategies to create stable T cell mixed-chimerism, using pre-transplant conditioning which specifically creates space for donor T cell engraftment.

The results of the current study indicate that in some settings impermanent, T cell-poor chimerism may not be sufficient to induce immune tolerance to solid organ allografts. Strategies to create permanent, multilineage chimerism across MHC barriers in outbred NHP and in patients thus represent a critical area for future translational and clinical
investigation. The creation of the unique MHC-defined rhesus macaque colony by NIAID (19, 46) is expected to be a central resource for these experiments, and will allow NHP tolerance-induction studies to most rigorously recapitulate the immunologic barriers faced during clinical transplantation.

Acknowledgments

This work was supported by Yerkes National Primate Research Center Base Grant, #RR00165. ADK was supported by JDRF 1-2008-594 and NIH 1U01AI079223, and 5U19 AI051731. CPL was supported by NIH grant # 5U19 AI051731. LSK was supported by grant # 5K08 AI065822, 5U19 AI051731, 1R01 HL095791 and U24 RR018109, and by a Burroughs Wellcome Fund Career Award in the Biomedical Sciences. Some of the reagents used were produced by the Nonhuman Primate Reagent Resource supported by NIAID contract #HHSN27220090037C.

References


Figure 1.
Longitudinal analysis of bone marrow chimerism, serum CMV viral load and serum creatinine in the BMT/renal and renal alone transplant cohorts.
A: Longitudinal analysis of donor chimerism in BMT/renal transplant recipients R1-R5. The percentage of donor cells in the whole-blood was determined either through qPCR or microsatellite analysis of disparate MHC alleles (described in Methods).
C. Whole-blood and T cell chimerism for R4 and R5. Shown are the two measurements of T cell chimerism for R4 and R5, demonstrating that both during ongoing renal transplant function, and at the time of renal transplant rejection, no T cell chimerism was present.

D. CMV viral load in the BMT/renal cohort was determined by PCR as described in Methods and expressed as copies/mL of whole-blood.

E. CMV viral load in the Renal alone transplant cohort was determined by PCR as described in Methods and expressed as copies/mL of whole-blood.

F. Longitudinal analysis of the serum creatinine (determined by clinical chemistry analysis) is shown for the BMT/renal transplant recipients. A serum creatinine threshold of 2.5 mg/dL (shown as a horizontal black line in the figure) was designated as the day of transplant rejection in recipients with continually-rising creatinine and who subsequently had histologically-proven rejection on pathologic analysis.

G. Longitudinal analysis of the serum creatinine (determined by clinical chemistry analysis) is shown for the Renal alone transplant recipients. A serum creatinine threshold of 2.5 mg/dL (shown as a horizontal black line in the figure) was designated as the day of transplant rejection in recipients with continually-rising creatinine and who subsequently had histologically-proven rejection on pathologic analysis.
Figure 2.
Immunohistochemical analysis of transplant rejection. Shown are representative photomicrographs from transplant recipient R5, documenting infiltration of T cells, B cells/plasma cells and monocyte/macrophages into the rejecting kidney.
A. Routine histology (hematoxylin and eosin) of R5 shows foci of tubulitis [arrows].
B. A CD3 stain shows a dense T cell infiltrate with a focus of tubulitis [arrow]. Inset: normal kidney stained with anti-CD3.
D. A CD68 stain shows numerous monocyte/macrophages within injured renal tubules [red arrow] and also in the interstitial inflammatory infiltrate [purple arrow]. Inset: normal kidney stained with anti-CD68.
All images of the rejecting kidney are shown at the same magnification. Bar = 100 μm
Figure 3.
Renal allograft survival analysis comparing the BMT/Renal and the Renal alone transplant cohorts. Renal allograft survival analysis was performed using a log-rank test after designating the day of transplant rejection as the day that a continually rising serum creatinine reached 2.5 mg/dL in the setting of subsequent histologically-proven renal allograft rejection on terminal pathologic analysis. R3 was not included in this analysis given the fact that donor chimerism wasn’t present at the time of renal allograft rejection. R9 was death-censored (at day +94 post-transplant) given the fact that the death of this recipient was due to non-rejection causes. Statistical analysis showed that the survival curves were not statistically significantly different from each other (p = 0.46).
Figure 4.
Flow cytometric analysis reveals enrichment for CD28-/CD95+ Tem cells in the rejected kidney allografts.
A. Representative flow cytometry plots showing the CD28 and CD95 expression in both CD4+ and CD8+ T cells in the peripheral blood and the kidney at the time of terminal analysis for recipient R4.
B. The percent of CD4+ and CD8+ Tn, Tcm and Tem in the peripheral blood and the rejected kidney at the time of terminal analysis is shown. Red: Peripheral blood at the terminal analysis. Blue: Kidney at the terminal analysis. Adequate renal T cells were purified from R4, R6, R8 and R10 to perform the comparative analysis. The relative percentages of Tn, Tcm and Tem was determined for each of these recipients using FloJo flow cytometry analysis software and is depicted as the mean +/- SEM. The percentage data was log-transformed prior to paired T test analysis. *: p<0.05; **: p<0.01; ***: p<0.001.
<table>
<thead>
<tr>
<th>Recipient</th>
<th>Transplant Cohort</th>
<th>Bone Marrow Transplant: total nucleated cells/kg</th>
<th>% Whole Blood Chimerism (terminal analysis)</th>
<th>% T Cell Chimerism (terminal analysis)</th>
<th>Day Post-Transplant for Creatinine &gt;2.5 mg/dL</th>
<th>Banff Diagnosis</th>
<th>% of Cellular Infiltrate Expressing Indicated Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>BMT/Renal</td>
<td>6.3 x 10^8</td>
<td>5%</td>
<td>0%</td>
<td>0</td>
<td>TCMR1B</td>
<td>90%</td>
</tr>
<tr>
<td>R2</td>
<td>BMT/Renal</td>
<td>2.4 x 10^9</td>
<td>49%</td>
<td>0%</td>
<td>41</td>
<td>TCMR1A</td>
<td>85%</td>
</tr>
<tr>
<td>R3</td>
<td>BMT/Renal</td>
<td>1.9 x 10^9</td>
<td>3%</td>
<td>0%</td>
<td>34</td>
<td>TCMR3</td>
<td>90%</td>
</tr>
<tr>
<td>R4</td>
<td>BMT/Renal</td>
<td>1.5 x 10^9</td>
<td>59%</td>
<td>0%</td>
<td>51</td>
<td>TCMR3</td>
<td>80%</td>
</tr>
<tr>
<td>R5</td>
<td>BMT/Renal/Renal</td>
<td>1.26 x 10^9</td>
<td>44%</td>
<td>3%</td>
<td>98</td>
<td>TCMR1B</td>
<td>75%</td>
</tr>
<tr>
<td>R6</td>
<td>Renal Alone</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>114</td>
<td>Borderline TCMR</td>
<td>75%</td>
</tr>
<tr>
<td>R7</td>
<td>Renal Alone</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>121</td>
<td>Borderline TCMR</td>
<td>90%</td>
</tr>
<tr>
<td>R8</td>
<td>Renal Alone</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>55</td>
<td>Borderline TCMR</td>
<td>90%</td>
</tr>
<tr>
<td>R9</td>
<td>Renal Alone</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>20</td>
<td>TCMR1B</td>
<td>80%</td>
</tr>
<tr>
<td>R10</td>
<td>Renal Alone</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>15</td>
<td>TCMR1B</td>
<td>80%</td>
</tr>
</tbody>
</table>

*Sacrificed due to a biopsy-related complication*