Published in final edited form as:

Transplant Rev (Orlando). 2009 January ; 23(1): 19–24. doi:10.1016/j.trre.2008.08.006.

Mixed chimerism to induce tolerance: lessons learned from nonhuman primates

Toru Murakami, A. Benedict Cosimi, and Tatsuo Kawai*

Harvard Medical School, Department of Surgery, Transplantation Unit, Massachusetts General Hospital, MA 02114, USA

Abstract

The mixed chimerism approach has been demonstrated to be an effective means of inducing allograft tolerance. Based on our rodent studies on mixed chimerism, we previously developed a clinically relevant nonmyeloablative preparative regimen that permits the induction of mixed chimerism and renal allograft tolerance following donor bone marrow transplantation in major histocompatibility complex fully mismatched cynomolgus monkeys. This approach has been successfully extended to HLA matched or mismatched kidney transplant recipients. In the manuscript, we summarize some of the important conclusions made in our laboratories regarding induction of mixed chimerism and allograft tolerance in a nonhuman primate model.

1. Introduction

We recently reported successful induction of tolerance in 4 human recipients of HLA-mismatched kidney transplants through the mixed chimerism approach [1]. This human protocol was based on a conditioning regimen originally developed in mice [2] and successfully extended to nonhuman primates (NHPs) in our laboratories. Over the course of this decade-long translational research effort, we have observed significant biologic differences among rodents, monkeys, and humans. This variability is attributed to their genetic and developmental differences, which can affect innate and acquired immunologic functions as well as metabolic responses to various medications. Elucidating these biologic differences between species has proved pivotal to the successful extension of the basic observations in mice to NHP and humans. In this manuscript, we summarize some of the important conclusions as well as the areas that remain to be clarified in our primate model, especially regarding induction of mixed chimerism and tolerance of vascularized organs.

1.1. Transient hematopoietic chimerism and renal allograft tolerance

Since Owen's [3] seminal finding in the freemartin cow, it has been known that the state of mixed hematopoietic chimerism can result in tolerance of other tissue allografts [4]. It was then demonstrated in mice that major histocompatibility complex (MHC) fully mismatched skin allograft tolerance is achieved after induction of stable mixed chimerism by donor bone marrow transplantation (DBMT) using myeloablative conditioning [5]. In the attempt to make this approach more applicable to clinical organ transplantation, a nonmyeloablative regimen was then evaluated in mice using total body irradiation (TBI), a potent anti-T-cell monoclonal antibody (mAb) and thymic irradiation (TI). With this regimen, mice

consistently developed stable mixed chimerism and skin allograft tolerance from MHC fully mismatched donors [2]. In 1992, we launched a series of experiments in monkeys seeking to extend these results to a clinically relevant model.

The first monkey regimen evaluated included anti-CD3 mAb (6g12) [6], TBI 3 Gy (day -6), and TI 7 Gy (day -1). The initial recipients treated with this regimen failed to develop chimerism and rejected their kidney allografts by day 15. Because significant residual T cells had been detected in the lymph nodes of these monkeys, we replaced 6g12 with a horse antithymocyte globulin (ATGAM) and cyclosporine A (CyA). We had initially attempted to monitor the development of chimerism using a polyclonal serum against rhesus monkey MHC. Because of the high background of these agents, it was difficult to detect donor chimeric cells. We therefore have since used several mAbs to HLA class I, which has been known to cross-react with cynomolgus monkey MHC class I. We typically select a donor that is positive with an antibody to HLA and a recipient negative with it, so that chimerism can be detected by flow cytometric analysis. In the initial monkey treated with this revised regimen, multilineage chimerism was first detected on day 7. The percentage of donor cells continued to increase until day 20, at which point, it was more than 90% in myeloid and 8% in lymphoid lineages. However, donor chimerism then started to decline and became undetectable by day 30. Since chimerism was no longer detectable, we anticipated that the renal allograft would be rejected soon after the discontinuation of CyA. However, to our surprise, the monkey did not reject his allograft. His kidney function remained stable for years with no histopathologic evidence of rejection (Fig. 1A). At 1 year after the kidney transplant, we performed skin transplantation from the original kidney and marrow donor and also from 2 third-party monkeys. The renal allograft recipient accepted the skin only from the kidney donor, with the 2 other skin allografts being completely rejected by day 10 (Fig. 1B), confirming the donor specificity of the ongoing hyporesponsiveness. To reduce the morbidity and mortality of the regimen, we further modified the protocol by fractionating the TBI to 1.5 Gy $\times$ 2 (on days -6 and -5). With this modification (the standard regimen, Fig. 2), 11 of 13 recipients developed transient chimerism and 8 of 13 acquired tolerance with the longest survival exceeding 14 years (standard, Fig. 3A).

These results in monkeys emphasized that renal allograft tolerance can be induced in primates even with transient chimerism, in contrast to the results in mice where stable mixed chimerism is essential to induce skin allograft tolerance across full MHC barriers. In murine recipients with stable mixed chimerism, thymic deletion of donor-reactive T cells has been demonstrated as the major mechanism of tolerance [7]. However, in our monkeys, peripheral tolerance mechanisms must also be involved in maintaining the renal allograft tolerance after disappearance of mixed chimerism.

1.2. Splenectomy and CD154 blockade

As depicted in Fig. 1, splenectomy (Spx) was a component of the “standard” regimen. We subsequently tested the conditioning regimen without Spx. However, all of these recipients developed alloantibodies and rejected their allografts (no Spx, Fig. 3A). By adding a short course of CD154 blockade in place of Spx (the modified regimen), all 8 recipients consistently developed chimerism, and 7 of 8 survived long term. However, half of these recipients eventually developed chronic humoral rejection, which indicated that CD154 blockade was not sufficient to block B-cell activation (no Spx, +aCD154, Fig. 3A). The importance of Spx at the time of DBMT was also demonstrated in another approach, the delayed kidney transplant model. In this protocol, recipients first received conditioning and DBMT with or without Spx, followed by kidney transplantation (KTx) 22 to 120 days later without further immunosuppression [8]. If Spx was performed simultaneously with DBMT, the subsequently placed kidney allografts survived long-term (2 acquired tolerance) (DBMT with Spx, Fig. 3B). In contrast, if splenectomy was not performed at the time of DBMT, all

recipients developed antibody-mediated rejection of the kidney within 12 days of transplantation (DBMT without Spx, Fig. 3B).

1.3. Natural killer cell function and induction of chimerism

With ATGAM, T-cell deletion is only moderate and peripheral T-cell recovery ($>500/\text{mm}^3$) occurs within 20 days. Therefore, we evaluated replacement of ATGAM with LOCD2b, a rat IgG2b antihuman CD2 mAb [9]. Sequential monitoring of peripheral blood mononuclear cells revealed significantly greater T-cell depletion in the LOCD2b-treated animals compared with those receiving ATGAM. Nevertheless, all recipients ($n = 10$) treated with LOCD2b failed to develop chimerism, and only one recipient survived beyond 100 days. Further evaluation indicated that natural killer (NK) cells ($\text{CD}16^+\text{CD}8^+$) had been significantly more depleted in the ATGAM group and that NK function remained abrogated longer after ATGAM than LOCD2b treatment (3 weeks vs <5 days with LOCD2b). We also found that only 40% of NK cells are positive for CD2 in cynomolgus monkeys. Therefore, it is possible that these CD2-negative NK cells, which were not depleted by the LOCD2b treatment, inhibited the donor bone marrow cell engraftment. The validity of this hypothesis is supported by our clinical observations. In humans, 80% to 90% of NK cells express CD2 [10], and MEDI-507, another anti-CD2 mAb, which has been used in the clinical protocol, depleted NK cells and consistently induced mixed chimerism in all patients treated to date [1].

1.4. Costimulatory blockade and mixed chimerism

Because of the pivotal role of the CD40L (CD154)-CD40 pathway in T- and B-cell responses, it was expected that agents reacting with those molecules could be important for tolerance induction. In fact, based upon the efficacy of anti-CD154 mAb for induction of allograft tolerance in rodent models [11], it was anticipated that this agent would quickly be used clinically. Prolonged allograft survival without maintenance immunosuppression after CD154 blockade was subsequently reported in renal, heart, and islet allograft models in NHP [12–14]. Unfortunately, these favorable results were not reproduced in the initial clinical trials, where acute rejection of the kidney allografts was observed [15]. This antibody was also tested clinically for autoimmune diseases, but all trials were halted when an unusually high incidence of thromboembolic complications was noted to be associated with anti-CD154 mAb treatment. We had also noted a high incidence of thromboembolic complications in our cynomolgus monkey renal allograft recipients treated with anti-CD154 mAb [16]. Most of these complications were observed immediately (within 6 hours) after transplantation, presenting primarily in the renal allograft. Complete autopsies, however, revealed that extra renal sites were also involved, including the superior mesenteric artery, femoral artery, and, in one recipient, the right atrium, resulting in a Budd-Chiari-like syndrome. With the addition of perioperative heparin (100 IU/kg for 2 days), the incidence of thrombosis was decreased, but only to 20%. We therefore tested a combination of heparin and intraoperative prostaglandin E1 (PGE1) (0.3–0.5 $\mu\text{g}/\text{kg}$ per hour) in the next 24 nonsplenectomized and 2 splenectomized renal allograft recipients. Addition of PGE1 failed to decrease the incidence of thrombosis. However, after the addition of ketorolac (0.5 mg/kg, intramuscularly on day –1 and intravenously on day 0 to the heparin and PGE1 regimen), we observed no thromboembolism. Unfortunately, this regimen led to a troubling bleeding tendency in some recipients, leading us to change the prophylactic regimen to ketorolac alone (0.5 mg/kg on days –1 and 0). No adverse effect on renal function was observed in recipients treated with this dose of ketorolac. With this current regimen, no thromboembolic complications have been observed in 25 consecutive kidney transplant recipients treated with anti-CD154 mAb.

As described above, we had evaluated the effect of substituting CD154 blockade for Spx in induction of chimerism and tolerance [17]. With CD154 blockade, induction of chimerism was significantly improved and monkeys survived long term (Fig. 3A). Nevertheless, late chronic rejection was observed in 3 of 8 recipients, indicating further modifications of the regimen would be necessary.

2. Cyclophosphamide and TBI

In our initial clinical protocol, cyclophosphamide (CP) was used in place of TBI because of a concern of secondary malignancies after TBI and favorable results of clinical trials using nonmyeloablative CP-based regimens for treatment of refractory malignancies [18]. Therefore, in the attempt to develop a more clinically similar NHP model, we evaluated CP in place of TBI in our monkey conditioning regimen. The initially treated NHPs revealed significant resistance to CP treatment. Although a total of 120 mg/kg (60×2 mg/kg) of CP consistently permitted chimerism in humans [1], 200 mg/kg (50×4 mg/kg) of CP with additional CD154 blockade was required in cynomolgus monkeys. Despite successful chimerism induction, none of the monkey recipients treated with CP achieved long-term renal allograft survival, in striking contrast to the TBI-treated animals. The leading causes of failure in NHP after CP treatment were posttransplant B-cell lymphoma and acute rejection (manuscript submitted). Among various parameters observed after conditioning, we noted a significant difference in B-cell depletion between the CP- and TBI-treated groups. In recipients treated with CP, significantly higher levels of CD20⁺ B cells remained in the peripheral blood, which might have contributed to the high incidence of humoral rejection and posttransplant lymphoproliferative disease. Our studies revealed several advantages of TBI over CP in both efficacy and safety for induction of mixed chimerism and renal allograft tolerance in NHP. These observations suggest that low-dose TBI should probably be explored in the clinical setting.

3. Extension of the conditioning regimen to other organ transplant recipients

Initial studies attempting to extend our protocol to nonkidney allografts (heart, lung, and islet) have been performed in cynomolgus monkeys. In a heterotopic cardiac allograft model, 3 of 5 recipients have developed multi-lineage chimerism, and allograft survival in these recipients was prolonged to 138, 428, and 509 days. Although in vitro assays demonstrated donor-specific hyporesponsiveness, long-term heart allograft recipients eventually developed humoral and cellular immunity against the donor and rejected the grafts, resulting in failure of induction of full tolerance [19].

As an initial attempt to extend this approach to cellular transplants, we performed islet transplantation in our NHP model. Diabetes was induced by streptozocin in a kidney transplant recipient whose allograft kidney function had been stable for more than 2 years after immunosuppression was withdrawn. Islets obtained by partial pancreatectomy of the original bone marrow and kidney donor were then transplanted without immunosuppression. By 2 weeks after the initial islet transplantation, exogenous insulin administration became unnecessary with fasting blood glucose levels stabilizing at less than 100 mg/dL. Islet biopsies taken on days 34 and 154 from an aliquot that had been placed beneath the kidney capsule revealed no histologic evidence of rejection and demonstrated intense insulin staining. Nevertheless, the recipient gradually became insulin dependent, necessitating killing of the donor for a second islet transplant, performed on day 189. After the second islet transplantation, the fasting blood glucose again stabilized at less than 80 mg/dL with increased levels of C peptide. The recipient then remained insulin free for another 100 days. Because of worsening hyperglycemia, the animal was killed on day 384 after the islet cell

transplantation. The autopsy demonstrated viable islets with strong insulin staining both in the liver (Fig. 4) and under the kidney capsule (not shown) with no histologic evidence of rejection. On the other hand, all of our trials of mixed chimerism and isolated islet allografts without kidney allografts have been unsuccessful despite successful induction of chimerism. Studies attempting to induce tolerance in lung transplant recipients are also underway, but tolerance induction has not been achieved so far despite induction of mixed chimerism. Islet or lung transplants may be particularly vulnerable to inflammatory responses or rejection by memory T cells, and organ-specific modifications of the regimen will be necessary to achieve tolerance of these organs.

4. Delayed tolerance induction and memory T cells

Our current NHP and clinical regimen requires treatment of subjects beginning 6 days before organ transplantation, which limits its applicability to only living donor allograft recipients. In our initial attempt to modify our protocol for deceased donor transplantation studies, we compressed administration of all treatments to the 24-hour perioperative transplant period. However, such simple compression of the regimen not only failed to induce chimerism but also led to unacceptable toxicity (unpublished data). Therefore, we have more recently developed the “delayed tolerance” protocol. In this NHP model, KTx is performed first with conventional immunosuppressive therapy, followed by the nonmyeloablative conditioning and DBMT several months later (Fig. 5). If this approach proved to be feasible, any stable recipient of either living donor or deceased donor KTx could be a potential candidate, if either fresh (living donor) or cryopreserved (deceased donor) donor bone marrow cells are available. To date, we have concluded that it is more difficult to induce mixed chimerism and allograft tolerance using the “delayed tolerance” protocol than in simultaneous kidney and bone marrow transplantation. This could be explained by specific activation of memory T cells by the initially transplanted organ, even without clinical evidence of rejection. Extensive flow cytometric analysis analyses of T-cell subsets in our NHP recipients revealed that a substantial number of memory T cells remained after the conditioning regimen, in spite of effective depletion of naïve T cells. To further analyze subsets of these residual memory T cells, we used CD95 and CD28 to define memory T cells, based on the studies by Pitcher et al [20] in rhesus monkeys. These analyses revealed that CD4 central memory cells were resistant to the conditioning regimen. Both CD8 effector memory T cells (T_{EM}) and CD8 central memory T cells were initially depleted effectively, but rapid postconditioning expansion of CD8 T_{EM} was observed after day 5, whereas CD8 central memory T cell counts remained unchanged. We therefore added to the conditioning regimen a humanized anti-CD8 mAb, cM-T807, which effectively inhibited expansion of CD8 T_{EM} . This has resulted in improved chimerism and tolerance induction in approximately 40% of the NHP studied. The antibody cM-T807 is a strong depleting antibody, which effectively depletes CD8⁺ cells from the peripheral blood and lymph nodes [21]. These results may indicate that a powerful depleting antibody can be a tool to overcome homeostatic proliferation of memory T cells. These initial studies provided “proof of principle” that the mixed chimerism approach can induce tolerance even several months after organ transplantation by additional intervention against CD8 memory T cells [22].

5. Future perspective

The next goals of our NHP studies include (1) clarification of the mechanisms of the allograft tolerance after transient mixed chimerism, (2) establishment of the delayed tolerance induction protocol by adding other modalities to control memory T-cell responses, (3) better control of B-cell immunity to prevent late onset chronic rejection, and (4) evaluation of antiinflammatory agents in the induction of tolerance.

Our recent studies in both NHP and humans are promising and provide hope that regimens for tolerance induction will be available as a routine treatment of various organ transplantation.

References

1. Kawai T, Cosimi AB, Spitzer TR, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med*. 2008; 358:353–61. [PubMed: 18216355]
2. Sharabi Y, Sachs DH. Mixed chimerism and permanent specific transplantation tolerance induced by a nonlethal preparative regimen. *J Exp Med*. 1989; 169:493–502. [PubMed: 2562984]
3. Owen RD. Immunogenetic consequences of vascular anastomoses between bovine twins. *Science*. 1945; 102:400–1. [PubMed: 17755278]
4. Anderson D, Billingham RE, Lampkin GH, Medawar P. Use of skin grafting to distinguish between monozygotic and dizygotic twins in cattle. *Heredity*. 1951; 5:379–97.
5. Ildstad ST, Sachs DH. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature*. 1984; 307:168–70. [PubMed: 6361574]
6. Kawai T, Wong J, MacLean J, Cosimi AB, Wee S. Characterization of a monoclonal antibody (6G12) recognizing the cynomolgus monkey CD3 antigen. *Transplant Proc*. 1994; 26:1845–6. [PubMed: 8066600]
7. Tomita Y, Khan A, Sykes M. Role of intrathymic clonal deletion and peripheral anergy in transplantation tolerance induced by bone marrow transplantation in mice conditioned with a nonmyeloablative regimen. *J Immunol*. 1994; 153:1087–98. [PubMed: 8027542]
8. Kawai T, Poncelet A, Sachs DH, et al. Long-term outcome and alloantibody production in a non-myeloablative regimen for induction of renal allograft tolerance. *Transplantation*. 1999; 68:1767–75. [PubMed: 10609955]
9. Kawai T, Wee SL, Bazin H, et al. Association of natural killer cell depletion with induction of mixed chimerism and allograft tolerance in non-human primates. *Transplantation*. 2000; 70:368–74. [PubMed: 10933165]
10. Ritz J, Schmidt RE, Michon J, Hercend T, Schlossman SF. Characterization of functional surface structures on human natural killer cells. *Adv Immunol*. 1988; 42:181–211. [PubMed: 3284289]
11. Sayegh MH, Turka LA. The role of T-cell costimulatory activation pathways in transplant rejection. *N Engl J Med*. 1998; 338:1813–21. [PubMed: 9632449]
12. Pierson RN III, Crowe JE Jr, Pfeiffer S, Atkinson J, Azimzadeh A, Miller GG. CD40-ligand in primate cardiac allograft and viral immunity. *Immunol Res*. 2001; 23:253–62. [PubMed: 11444390]
13. Kenyon NS, Chatzipetrou M, Masetti M, et al. Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154. *Proc Natl Acad Sci U S A*. 1999; 96:8132–7. [PubMed: 10393960]
14. Kirk AD, Burkly LC, Batty DS, et al. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat Med*. 1999; 5:686–93. [PubMed: 10371508]
15. Kirk AD, Knechtle SJ, Sollinger HW, Vincenti F, Stecher S, Nadeau K. Preliminary results of the use of humanized anti-CD154 in human renal allotransplantation. *Am J Transplant*. 2001; 1:1S91.
16. Kawai T, Andrews D, Colvin RB, Sachs DH, Cosimi AB. Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med*. 2000; 6:114.
17. Kawai T, Sogawa H, Boskovic S, et al. CD154 blockade for induction of mixed chimerism and prolonged renal allograft survival in nonhuman primates. *Am J Transplant*. 2004; 4:1391–8. [PubMed: 15307826]
18. Sykes M, Preffer F, McAfee S, et al. Mixed lymphohaemopoietic chimerism and graft-versus-lymphoma effects after non-myeloablative therapy and HLA-mismatched bone-marrow transplantation. *Lancet*. 1999; 353:1755–9. [PubMed: 10347989]
19. Kawai T, Cosimi AB, Wee SL, et al. Effect of mixed hematopoietic chimerism on cardiac allograft survival in cynomolgus monkeys. *Transplantation*. 2002; 73:1757–64. [PubMed: 12084998]

20. Pitcher CJ, Hagen SI, Walker JM, et al. Development and homeostasis of T cell memory in rhesus macaque. *J Immunol.* 2002; 168:29–43. [PubMed: 11751943]
21. Schmitz JE, Simon MA, Kuroda MJ, et al. A nonhuman primate model for the selective elimination of CD8+ lymphocytes using a mouse-human chimeric monoclonal antibody. *Am J Pathol.* 1999; 154:1923–32. [PubMed: 10362819]
22. Koyama I, Nadazdin O, Boskovic S, et al. Depletion of CD8 memory T cells for induction of tolerance of a previously transplanted kidney allograft. *Am J Transplant.* 2007; 7:1055–61. [PubMed: 17286617]

Fig. 1.

Allograft biopsy taken at 10 years after KTx from a long-term survivor showing no signs of rejection (A). Skin graft performed on a long-term survivor without immunosuppression. Skin from kidney donor was accepted, whereas skin grafts (one fresh, one frozen) from third-party monkey were rejected by day 10. The photograph was taken 28 days after skin transplantation (B).

Fig. 2.

The standard regimen consisted of nonmyeloablative dose of TBI (1.5 Gy $\times$ 2) on day -6 and -5, local TI (7 Gy) on day -1, and peritransplant ATGAM (horse ATGAM). Combined kidney and bone marrow transplantation and Spx were performed on day 0, followed by a 1-month course of CyA administration.

Fig. 3.

With the standard regimen, 11 of 13 recipients developed transient mixed chimerism and 8 of 13 survived long-term without rejection. Without splenectomy (Spx), all recipients developed antibody-mediated rejection. Addition of anti-CD154 mAb was partially successful to eliminate the requirement of Spx (A). If Spx is performed simultaneously with DBMT, kidney grafts transplanted later were not rejected even without immunosuppression. In contrast, if Spx was not performed at that time of DBMT, all recipients quickly developed antibody-mediated rejection (B).

Fig. 4.
Transplanted islets with positive insulin staining in the liver were found at autopsy (day 380).

Fig. 5.

In the delayed tolerance protocol, KTx is performed on day 0 and the recipient is treated with conventional immunosuppression (tacrolimus, mycophenolate mofetil, steroids) for several months. The recipient receives a 6-day conditioning regimen with DBMT, followed by a 1-month course of tacrolimus. Addition of anti-CD8 mAb was necessary for successful induction of chimerism and tolerance.