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Evolving approaches of hematopoietic stem cell-based therapies to induce tolerance to organ transplants: the long road to tolerance

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Abstract

The immunoregulatory properties of hematopoietic stem cells (HSC) have been recognized for over 60 years, beginning when Owen (1945) reported that genetically disparate freemartin cattle sharing a common placenta were red blood cell chimeras, and subsequently when Billingham, Brent, and Medawar (1953) demonstrated that murine neonatal chimeras prepared by infusion of donor-derived hematopoietic cells exhibited donor-specific tolerance to skin allografts. Various approaches using HSC in organ transplantation have gradually brought the dream of inducing donor-specific tolerance in organ transplant recipients closer to reality. Several hurdles needed to be overcome, especially avoiding graft-versus-host disease, the toxicity of ablative conditioning and eliminating the need for close donor/recipient matching. For wide acceptance, HSC therapy must be safe and reproducible in mismatched donor/recipient combinations. Discoveries in other disciplines have often unexpectedly and synergistically contributed to progress. This review presents an historic perspective of the quest for tolerance in organ transplantation, highlighting current clinical approaches.

Keywords

chimerism; tolerance; immunosuppression; hematopoietic stem cells; facilitating cells; kidney transplantation

Introduction

Transplantation of solid organs and hematopoietic stem cells (HSC) has become a clinical reality over the past 60 years. Progress in surgery, immunology, radiation oncology, and drug development has contributed to this success, with involvement of numerous other basic science and clinical disciplines. Many critical discoveries were made first in animals, then translated successfully to the clinic. The two fields of clinical transplantation (bone marrow

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Conflict of Interest

Dr. Suzanne Ildstad has equity interest in Regenerex, LLC, a start-up biotech company. David Tollerud is an officer of Regenerex LLC. The company has not been capitalized. The other authors declare that they have no competing interests.

and solid organ) have common origins, but have subsequently evolved separately. Each, however, has propelled the other forward in a complementary fashion, benefitting from a number of advances. One example is the development of more effective immune-based nonspecific immunosuppressive agents. For hematopoietic stem cell transplantation (HSCT), the use of newer and more mechanistically focused immunosuppressive agents has resulted in reduced toxicity of conditioning¹ and provided prophylaxis and treatment for graft-versus-host disease (GVHD). In solid organ transplantation, they have allowed the replacement of vital organs that have been attacked by end-state-disease processes to become a clinically accepted reality. Similarly, the discovery of HLA antigens had an impact on GVHD-prevention as well as outcomes in solid organ transplants.² The focus of this review is to highlight the historical journey toward the induction of immune tolerance from bench to bedside and the consequent progress at reconvergence of HSCT and solid organ transplantation.

Ever since Billingham et al. reported in 1953 that neonatally-induced hematopoietic chimerism is associated with donor-specific tolerance to skin “homografts,”³ the induction of donor-specific tolerance has been aggressively pursued. Their neonatal chimera studies built on findings of Owen in 1945 that genetically disparate freemartin cattle that shared a common placenta are red blood cell chimeras.⁴ The fact that this chimerism persisted into adulthood suggested that a tolerant state had been induced. Over the ensuing years, a major motivating factor behind the pursuit of tolerance for solid organ transplantation has been the substantial toxicity of conventional immunosuppressive agents, with the need for life-long administration. Toxic effects include hypertension, cardiovascular disease, diabetes and other metabolic complications, osteoporosis, opportunistic infection, renal compromise, and an increased rate of malignancy.⁵ Transplant recipients are faced with the challenge of managing a schedule of taking 15–25 pills per day. In addition, the cost of maintaining a transplanted organ is substantial, ranging from \$15,000 to \$25,000 per year. Finally, despite perfect patient compliance, which is difficult at best, and the ability to pay for the medications, there is a fixed rate of graft loss due to chronic rejection and the inherent toxicity of the drugs themselves, with only 50% of deceased donor kidney transplants surviving for 10 years.⁶ Although the new generation of immunosuppressive agents has effectively prevented early acute rejection, little progress has been made in improving deteriorating graft survival curves beyond 5 years. In renal transplantation, efforts to minimize immunosuppression to avoid the associated toxicities have had limited success, with fewer than 1% of subjects able to successfully discontinue immunosuppression and maintain stable graft function.^{7–10} Similarly, although a few more liver transplant recipients can be successfully tapered from immunosuppression, at present this can be achieved only after several years, and only candidates with stable function and no prior rejection episodes on immunosuppressive drug monotherapy were eligible for planned weaning.^{7–9} Moreover, there is no reliable objective biomarker to predict which subjects will be successfully weaned vs. those which will experience rejection during the weaning process. It is currently unknown whether the transplant recipients who fail tapering of immunosuppression will experience reduced graft survival, as even reversible rejection in renal transplants has been correlated with impaired long-term allograft survival.^{11;12} Collectively, these challenges underscore the need for effective and safe approaches to induce donor-specific tolerance in organ transplant recipients. This comprehensive review will provide an historic perspective of the past 60-year quest to achieve what has been commonly termed the Holy Grail of transplantation:¹³ the induction of donor-specific transplantation tolerance.

Shortly after the reports by Owen and the Medawar group, Main and Prehn showed that radiation chimeras prepared with ablative conditioning were tolerant to donor skin grafts.^{14;15} Lorenz and Uphoff et al. had also observed that ablatively irradiated mice could be rescued from death by infusion of bone marrow cells or splenocytes.¹⁶ Although this

rescue was originally hypothesized to be due to humoral factors, Main and Prehn¹⁷ demonstrated that it was instead mediated by a cellular mechanism and that myeloablatively conditioned adult mice given allogeneic bone marrow were rescued from ablation and were also tolerant to donor skin grafts. Based on these findings, Rapaport and also Thomas validated similar outcomes in dogs, reporting induction of unresponsiveness to canine renal allografts by total body irradiation (TBI) and bone marrow transplantation.^{18;19} The fact that hematopoietic chimerism induces tolerance to organ and tissue transplants has been confirmed in virtually all species tested to date.

One major hurdle that had to be addressed in translating tolerance to solid organ transplant recipients to the clinic was how to avoid the toxicity of ablative conditioning. While ablative conditioning was acceptable for the use of HSCT in hematologic malignancy, and had evolved in the field of bone marrow transplantation as standard of care,¹⁹ the risk was unacceptable when applied to nonmalignant disorders such as solid organ transplantation. Strategies to establish chimerism and tolerance with reduced conditioning that could be justified in organ transplant recipients were therefore pursued. Monaco et al. reported significant graft prolongation with lymphodepletion using anti-lymphocyte serum (ALS) plus donor bone marrow cell infusion, first in mice and subsequently in a similar conditioning and bone marrow protocol in kidney transplant recipients.^{20;21} This was followed by the further attempts of Barber et al.²² and by the extensive studies of Starzl et al. using infusion of donor bone marrow cells into unconditioned recipients of solid organ allografts. Although chimerism was not established, a significant reduction in chronic rejection was observed.^{23;24} Similar beneficial effects of pre-transplant whole blood transfusions were also noted.^{25;26}

Meanwhile, Ildstad and Sachs extended the model of neonatal tolerance to immunocompetent adult mouse recipients by establishing mixed chimerism.²⁷ Notably, administration of T cell-depleted (TCD) syngeneic plus TCD allogeneic or xenogeneic rat bone marrow cells into ablated mouse recipients resulted in mixed hematopoietic chimerism and robust donor-specific tolerance to skin grafts. The tolerance associated with durable chimerism extended even across species barriers. One critical observation was that animals with 1% donor chimerism were just as tolerant as ones with 100% donor, suggesting that complete replacement of the recipient hematopoietic system with that of the donor was not a prerequisite to tolerance induction. This observation continued to reinforce interest in development of nonmyeloablative or reduced-intensity conditioning approaches to establish chimerism.²⁸⁻³¹

The fact that one could establish chimerism and tolerance with nonmyeloablative conditioning has substantially reduced the risk:benefit ratio as it related to tolerance efforts in solid organ transplantation. This was a paradigm-shift for bone marrow transplantation, a field which had previously evolved to ablatively treat hematologic malignancies.¹⁹ Successful clinical application of tolerance-inducing strategies developed in animal models requires that recipient conditioning be relatively safe, relatively simple to perform, and that it can be successfully performed in mismatched donor/recipient combinations and/or outbred recipients. A spectrum of less intense approaches for conditioning has been tested in the context of donor bone marrow cell infusion, including standard immunosuppression with ALS or other lymphodepleting agents to establish microchimerism,^{22;24} or using total lymphoid irradiation,³²⁻³⁵ or low-dose TBI to establish macrochimerism.^{36;37} These studies confirmed that HSC and their progeny have important immunomodulatory features. Although the microchimerism approach did not result in immunosuppression-free donor-specific tolerance in organ transplantation recipients, a significant reduction of chronic rejection was observed.^{22;24;38;39} The mechanism of this effect has been attributed to reciprocal clonal exhaustion,²⁴ although the long held theory that participation of donor or

recipient regulatory networks was also increasingly considered.^{40;41} These pioneering studies demonstrated the safety of the HSC-based approach and opened the door for clinical translation. The remainder of this review will focus on clinical protocols actively underway, reviewing outcomes first in unconditioned recipients and subsequently in nonmyeloablatively conditioned recipients.

HSCT and immunoregulation in the HLA identical Northwestern Renal Transplant Trial

It had been generally accepted since an early report by investigators at Duke University in 1972 that in HLA identical recipients, complete immunosuppression withdrawal was not routinely possible.⁴² Nonetheless, van de Wetering et al. in 2009 reported successful withdrawal of anti-proliferative immunosuppressive therapy in a group of long-term HLA-identical renal transplant recipients without previous rejection, leaving them on maintenance low-dose methylprednisolone (5 mg therapy daily).⁴³ In a recent long-term follow-up of these subjects, no acute or chronic rejection has been seen clinically. Protocol biopsies have not been performed (van de Wetering, personal communication).

The current Northwestern University study proposed that immunoregulatory mechanisms might be amplified in subjects with HLA-identical and only minor histocompatibility antigen (MHA) donor/recipient disparities in the context of tolerance-promoting HSC and the temporary use of immunosuppressive agents to the point of withdrawal. Such regulatory mechanisms had been tested in inbred MHC identical MHA-disparate mice nearly 40 years previously by Medawar. Pre-treatment of MHC antigen identical recipients with nonviable donor spleen cell extracts as induction therapy resulted in permanent survival of skin allografts.⁴⁴ Tolerance was also achieved in MHC-matched minor antigen disparate mice transplanted with large but not small donor skin allografts (also without viable donor marrow treatment).⁴⁵ It was then demonstrated that recipient strain “suppressor cells” in the form of splenocytes would adoptively transfer this graft acceptance to secondary recipients.⁴¹ Based on these considerations, a clinical trial of planned slow weaning of immunosuppression in unconditioned HLA-identical living donor renal transplant recipients was initiated at Northwestern University in 2008. The rationale did not include the need for permanent chimerism, merely the expectation that a prolonged immunoregulatory environment could be provided by donor HSC infusions without myeloablation, but with the use of alemtuzumab, a strongly lymphodepleting anti-CD52 pan-lymphocytic monoclonal antibody hypothesized to promote a tolerogenic immunoregulatory state. It is hypothesized that the lymphodepletion induced by anti-CD52 promotes an immunoregulatory state with immune reconstitution from homeostatic proliferation of residual lymphocytes.⁴⁶ Four donor CD34-selected HSC infusions are performed postoperatively during the first 9 months after renal transplantation. Cell dose ranged from 0.5 to 4×10^6 cells/kg recipient body weight per dose. Conventional immunosuppression is tapered after conversion to sirolimus monotherapy. Twenty HLA identical sibling renal transplant donor/recipient pairs have been performed using this approach. The first ten subjects are summarized in Table 1. This has resulted in only transient microchimerism that disappeared after one year (Table 1). Immunosuppression is totally withdrawn by 24 months in subjects with stable renal function and normal protocol biopsies. Increased numbers of CD4⁺/CD25^{high}/FoxP3⁺ regulatory T cells (T_{reg}) were present in the peripheral blood of these subjects during lymphoid reconstitution, suggesting induction of a long-lasting immunoregulatory state (Table 1). The percentage T_{reg} ranged from 0.8% to 4% of total CD4 count pre-transplant. The numbers in the table represent the fold change from this baseline in each subject. This correlated with donor-specific functional T_{reg} effects also seen in an *in vitro* lymphoproliferation assay.⁴⁷ Five of the first ten recipients followed for longer than 3 years have had immunosuppression successfully withdrawn for between 14 and 24 months. Protocol biopsies one year after withdrawal are devoid of rejection (Table 1). Two others had Banff-1A rejection on protocol

biopsy one year after immunosuppressive withdrawal without deterioration of renal function. They were restarted on standard immunosuppressive therapy. Two had recurrent disease and immunosuppression was never withdrawn, and one additional recipient could not be withdrawn because the protocol biopsy at 24 months post-operatively was not quiescent. This patient had a PRA (Panel Reactive HLA Antibodies) of 29% pre-operatively, suggesting sensitization. At the time of this publication, approximately 50% of subjects have been withdrawn from immunosuppressive agents for > 1 year. Subjects range from 2 to 52 months post-transplant. None of the 20 subjects has had deterioration from the optimum renal function that occurred in the early post-transplant period. To our knowledge, the studies described herein are the only ones attempted in the literature. This is in contrast with a much lower success rate in HLA-mismatched subjects weaned earlier after transplant.¹⁰

HSCT in conditioned HLA-identical related kidney recipients: the Stanford Study

In 1977, Slavin et al. reported that donor-specific transplantation tolerance could be achieved for skin allografts using fractionated total lymphoid irradiation (TLI) followed by infusion of donor bone marrow cells in adult mice.^{31,32,34} This study was conceived as a result of a clinical protocol using TLI at that center developed for radiotherapy of Hodgkin's Disease.⁴⁸ The Slavin/Strober approach established durable chimerism in mismatched mice and was described by the authors to be clinically relevant because the conditioning was seen to be relatively safe in humans. It was subsequently reported that the TLI could be used successfully in larger outbred species to establish chimerism and tolerance.^{49,50} The findings from subsequent extensive preclinical investigations were successfully translated to the clinic starting in 2005. A total of 16 HLA-identical living donor sibling kidney transplant recipients were conditioned with fractionated TLI (800 or 1200 cGy total) followed by infusion of CD34⁺ selected donor bone marrow cells supplemented with 1×10^6 CD3⁺ T cells/kg.³⁵ Conditioning consisted of rabbit anti-thymocyte globulin, methylprednisolone, the fractionated TLI, and conventional immunosuppression (Figure 2B). Recipients were initially maintained on cyclosporine and mycophenolate mofetil per standard of care. In the early subjects, the cyclosporine was tapered starting at 3 months and discontinued at 6 months if stable chimerism and no GVHD occurred.³³ More recently, weaning of immunosuppression was performed if there was no clinical evidence of rejection and if a protocol biopsy was normal, irrespective of whether chimerism had persisted.³⁵ Three of the 16 subjects experienced rejection during tapering of immunosuppression and were restarted on conventional immunosuppression. One subject experienced recurrent disease. The remaining subjects were completely weaned from immunosuppression and have maintained stable renal function. Of note, protocol biopsies were not performed. The hospital stay was not prolonged by the conditioning. This study demonstrated the safety and feasibility of the TLI-based conditioning in renal transplant recipients. An added benefit is that because conditioning and infusion of the HSCT product were performed after kidney graft placement, it could theoretically be applied to recipients of deceased donor organs, which comprise the substantial majority of transplant recipients.

One major limitation to this approach is the requirement for HLA-related and matched donors, since almost all deceased donor organ transplants are unrelated and unmatched. In fact, a similar TLI-ALG conditioning regimen was tested by the group prior to the HLA-identical study in HLA-mismatched recipients from 2000–2004.³² In these mismatched recipients, three of the four transplanted developed whole blood macrochimerism, with the highest level 16% donor. However, chimerism was lost in all subjects within 3 months. Hence, one of the more stringent requirements for successful translation of stem cell-based therapies to the clinic, the ability to successfully perform the procedure in mismatched recipients, has yet to be met.

The Massachusetts General Hospital (MGH) kidney/HSCT study in conditioned related haploidentical kidney/HSCT recipients

Extensive mechanistically-based preclinical studies in mice and nonhuman primates resulted in the MGH clinical combined kidney/HSCT tolerance protocol. In studies using mice it was observed that low dose irradiation plus *in vivo* administration of T cell lymphodepleting agents almost completely eliminated peripheral but not thymic T cells.²⁸ The addition of 700 cGy of thymic irradiation to the conditioning resulted in durable chimerism and tolerance induction.²⁸ Translation of this approach to a nonhuman primate model did not achieve durable chimerism but resulted in operational tolerance.⁵¹ It was hypothesized that while chimerism was required to induce tolerance, it was not required to maintain it. Instead, immune regulation was hypothesized to maintain the tolerant state following the loss of chimerism. Donor antigen from the kidney allograft was believed to directly contribute to the regulatory mechanism.⁵¹ These preclinical studies were subsequently formulated into a clinical trial.

A total of 10 subjects were transplanted with unmodified GCSF-mobilized HSCT from related haploidentical donors beginning in 2002.^{52–54} All subjects exhibited low levels of donor chimerism in the peripheral blood for up to 21 days as assessed by flow cytometric analysis. The third subject enrolled developed irreversible humoral rejection resulting in loss of the renal allograft.^{51;52} As a result, anti-CD20 mAb treatment was added to the recipient conditioning regimen to target B cells and humoral immunity. A second subject developed acute cellular rejection 7 weeks after immunosuppression had been discontinued and coincident with a viral infection, with subsequent graft loss.¹³ A third subject of the 10 developed the expected “engraftment syndrome” observed in 9 of 10 recipients and renal function did not normalize.⁵³ After discontinuation of cyclosporine with the presumptive diagnosis of calcineurin inhibitor induced microangiopathy, the graft was lost. Therefore, 3 of 10 subjects experienced graft loss.⁵³ A fourth subject has developed evidence of chronic rejection manifested by C4d deposition on biopsy as well as histologic abnormalities.⁵¹ Anti-Class II antibodies have also developed in this subject. Nonetheless, there has been persistently normal renal function in the remaining six subjects without immunosuppression for up to 8 years.

However, there remained several unexplained side effects experienced by subjects to be dealt with in the study. Transient “engraftment syndrome” manifested by acute renal dysfunction, edema, capillary leak syndrome, and fluid retention starting post-operative day 10–12 occurred in 9 of the 10 subjects. An accompanying significant rise in creatinine combined with the histologic abnormalities observed in some of the subjects leads to the question of whether this represents atypical rejection. The subjects who developed engraftment syndrome were presumptively treated for rejection. Notably, all subjects exhibited donor-specific tolerance in MLR assays despite the varying outcomes.^{51;52;55}

Donor HSCT plus low-intensity conditioning with 200 cGy/fludarabine/cyclophosphamide conditioning in mismatched related and unrelated living donor kidney recipients: the Northwestern University/Institute for Cellular Therapeutics, University of Louisville study

This clinical protocol was predicated on two observations made initially in small animal studies: the nonmyeloablative conditioning approach and the engineering of the donor HSC product to reduce the risk of GVHD (Figure 4). Using a dose-titration of TBI in a mouse model for HSCT, it was determined that if immune-based immunosuppression was added to the conditioning, the TBI dose could be significantly reduced.^{29;30} The addition of cyclophosphamide 2 to 3 days post-transplant to the regimen allowed an additional reduction in the minimum TBI dose required to establish durable chimerism.³⁰ The cyclophosphamide mechanistically targets alloreactive donor and recipient cells and spares

the HSC. This was validated successfully in a dog model⁵⁶ and then utilized in humans in pioneering work by Fuchs et al.⁵⁷ Subjects with hematologic malignancies and co-morbidities that precluded ablative conditioning were enrolled and managed as outpatients. Subjects were conditioned with fludarabine (30 mg/m²/day, days -6 to -2), cyclophosphamide (50 mg/kg/dose, day -6, -5 and day +3 and/or +4) plus 200 cGy TBI day -1 followed by transplantation of unmanipulated G-CSF mobilized HSCT on day 0. The safety of the approach has now been confirmed in a large number of subjects and a significant reduction in transplant-related morbidity and mortality has occurred. These landmark studies have significantly impacted the field of HSCT. Graft failure occurred in only 13% of subjects and the cumulative incidence of grade II-IV acute GVHD was substantially reduced to 43% in haploidentical recipients with 10% grade III-IV and 10% chronic GVHD.^{58,59} Although this level of GVHD was an acceptable risk and benefit ratio in the setting of hematologic malignancy, it would be unacceptable in kidney transplant recipients. As discussed below, the current use of this refined low-intensity conditioning regimen together with an engineered HSCT product has all but eliminated GVHD in recipients of mismatched kidney/HSCT.

Engineering the HSC graft: the facilitating cell (FC)

The first description of FC was made in mice almost 20 years. Experiments using rare events cell sorting identified a novel CD8⁺/TCR⁻ bone marrow-derived cell that was necessary and sufficient to enable engraftment of purified HSC in ablated allogeneic recipients.⁶⁰ Although once considered controversial, the work was reproduced by Weissman et al.⁶¹ and much has been done to subsequently define critical FC subpopulations and their mechanism of action. The CD8⁺/TCR⁻ FC population is heterogeneous.^{60,61} Plasmacytoid precursor dendritic cells (p-preDC) comprise a major component of FC.⁶² Removal of p-pre-DC FC cripples FC function, but p-preDC and p-preDC FC do not replace FC total in their facilitating function. Therefore, p-pre DC FC are critical but not sufficient for FC function. FC produce physiologically relevant levels of TNF α when co-cultured with HSC,⁶³ and FC induce antigen-specific T_{reg} *in vivo*⁶⁴ and *in vitro*.⁶⁵ Removal of p-preDC FC abrogates the induction of T_{reg} cells *in vivo*.⁶⁴ FC express a unique cell surface receptor complexed with CD3 ϵ .⁶⁶

After discovery of FC, and prior to embarking on clinical trials, we confirmed that a similar CD8⁺/TCR⁻ FC population exists in humans⁶⁷ (and manuscript in preparation). The existence of FC in humans has also been confirmed independently.⁶⁸ An approach to bioengineer human marrow to remove GVHD-inducing immune cells and preserve FC, HSC, and progenitors was developed, validated, and submitted to FDA in 1996, followed by the first clinical trial utilizing the FC manufacturing technology in subjects with hematologic malignancy who did not have a suitably matched donor.⁶⁹ Specifically, a ferromagnetic approach using monoclonal antibodies to remove donor mature GVHD-inducing immune cells and retain FC, HSC, and progenitors was utilized. In 54 ablatively conditioned related and unrelated mismatched donor transplants, we confirmed that we could establish engraftment without GVHD. This success opened the door to clinical trials to induce tolerance in organ transplant recipients.

In 2009, a phase 2 clinical trial of combined living donor kidney and HSCT was initiated in a collaboration between the Institute for Cellular Therapeutics (ICT) at the University of Louisville and Northwestern University.⁶⁷ To date, 15 HLA-mismatched related and unrelated subjects have been enrolled and transplanted.⁷⁰ Conditioning consisted of 3 doses of fludarabine (30 mg/kg/dose) days -4, -3, -2; two doses of cyclophosphamide (50 mg/kg/dose) days -3 and +3; and 200 cGy TBI on day -1 relative to the renal transplant (day 0) (Figure 2). Hemodialysis was performed 6-8 hours after the administration of fludarabine and cyclophosphamide to avoid toxicities of these agents prior to transplantation.

Tacrolimus (target trough concentrations 8–12 ng/ml) and mycophenolate mofetil (MMF) (1 gm orally twice daily if recipient weight < 80 kg, 1.25 gm twice daily if weight ≥ 80 kg) were started on day –3 and continued as maintenance immunosuppression post-transplant. Kidney transplantation was performed without antibody induction or oral corticosteroid therapy. The bioengineered FDA-regulated heavily manipulated HSC product enriched for FC (FCRx) was infused intravenously on the day following living donor kidney transplant. Patients were discharged on post-operative day 2 and thereafter managed as outpatients. All but one patient demonstrated peripheral blood macrochimerism post-transplantation. Engraftment failure occurred in one highly sensitized (PRA > 54%) transplant recipient. Chimerism was lost in 3 patients at 2, 3, and 6 months post-transplantation. Two of those subjects had received either a reduced cell dose and/or incomplete conditioning; the other had a PRA >20%. In 11 of the remaining 11 subjects, who received full conditioning and the target cell dose, high levels of chimerism resulted. All subjects demonstrated donor-specific hyporesponsiveness and were weaned from full dose immunosuppression. Complete immunosuppression withdrawal at one year post-transplant was successful in all of the patients with durable chimerism. There has been no GVHD or engraftment syndrome. Transplant loss due to renal artery thrombosis with subsequent successful re-transplantation occurred in one chimeric patient who developed sepsis following an atypical viral infection at month 3 following transplantation.

We have modified our primary end point for tapering and discontinuing immunosuppression from donor-specific hyporesponsiveness to durable T cell and whole blood chimerism after two subjects with only transient chimerism demonstrated subclinical rejection on protocol biopsy in spite of donor-specific hyporesponsiveness in MLR and CML assays.⁷⁰ These data suggest that low intensity conditioning plus FC-enriched HSC can safely achieve durable chimerism and successful immunosuppression withdrawal in mismatched allograft recipients without GVHD or engraftment syndrome. Nonspecific sensitization appears to represent an obstacle to successful induction of chimerism and tolerance. The presence of sustained T cell chimerism serves as a more robust tolerance biomarker than donor-specific hyporeactivity *in vitro*.

Biomarkers of tolerance: Is chimerism the new standard?

The mixed lymphocyte reaction (MLR) proliferative and cell mediated lympholysis (CML) assays have been considered a gold standard test for tolerance. It is interesting that some of the transiently chimeric subjects treated in the MGH study, the Stanford study, and the Northwestern/ICT study exhibited donor-specific hypoactivity *in vitro* in MLR and CML yet simultaneously experienced subclinical and even clinical rejection episodes. In the Northwestern University/ICT trial, two subjects who had transient chimerism and donor-specific hyporesponsiveness *in vitro* for up to 18 months demonstrated subclinical Banff1A rejection on protocol biopsy which resolved with anti-rejection therapy. Similarly, although all of the MGH subjects were reported to exhibit donor-specific hyporesponsiveness, three developed rejection and a fourth has C4d deposition on biopsy. Therefore, it appears that durable chimerism is the most robust biomarker for tolerance. The metric of T cell chimerism in HSCT has long been considered a predictor of durable engraftment.⁷¹

The future: delayed tolerance

The majority of organ transplants in most countries are from deceased donors. One final challenge to be addressed to make tolerance widely available is the fact that the HSCT product should be infused or cryopreserved within 48 hours of harvest. Because the conditioning takes approximately 5 days, an approach for delayed tolerance would be critical. Moreover, recipients of hearts, lungs, and livers are quite ill at the time of transplant; it would be beneficial if the patient could recover from the organ transplant and

the conditioning and HSCT performed in a delayed fashion. Such an approach would also benefit individuals who have already had a transplant from a living donor who is willing and able to donate HSCT. The fact that the product for the Northwestern University/ICT and Stanford studies was cryopreserved and still engrafted brings this one step closer to reality. A clinical protocol to address the living donor situation is approved at Northwestern (FDA IND 14900; clinicaltrials.gov) and is ready to begin enrollment. For deceased donors the HSCT source would be vertebral columns, which have been validated in a number of the clinical protocols reviewed in this manuscript. Hence, the timing is right.

Implications for other disorders

The HSC produces the entire immune system of the donor. Durable chimerism can therefore be used to provide a sustained source of normal red blood cells, platelets, white blood cells, and enzyme produced by HSC-derived cells if they are defective (Figure 3). One additional potential transformational application is the use of HSCT to reverse autoimmunity in type 1 diabetes,⁷² systemic lupus erythematosus, rheumatoid arthritis,⁷³ scleroderma,⁷⁴ and other autoimmune disorders.⁷⁵ Collectively, autoimmune disorders affect approximately 4% of the population worldwide. At present, only disease-modifying therapies are available. A simple and safe approach to perform HSCT in mismatched and unmatched recipients may provide hope for subjects living with numerous disorders who have no alternative therapy (such as metachromatic leukodystrophy and numerous other inherited metabolic disorders).

In summary, the field has reached a point where donor-specific tolerance is clearly within reach. This has been the result of contributions made by numerous investigators in work spanning over 60 years. The most important beneficiaries will be the patients who may no longer need to suffer the severe consequences of immunosuppressive therapy. We are grateful to those subjects who, over the decades, have bravely enrolled in clinical trials that have brought us to the dawn of a new era in transplantation.

Study Highlights for Original Research

It has been known for over 60 years that hematopoietic chimerism induces tolerance to organ transplants. However, a number of obstacles prevented the clinical application of this biologic therapy until recently. To be widely available, cell-based therapy must be simple to perform, safe, and be successful in mismatched donor: recipient pairs. Such an achievement could have a profound impact on treatment of autoimmune disorders, hemoglobinopathies, metabolic disorders, and type 1 diabetes, reaching far beyond organ transplantation.

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Figure 1. Free Martin cattle are genetically disparate cattle twins who share a common placenta, allowing exchange of hematopoietic-derived cells.

Figure 2.

A) In the MGH protocol, recipients were conditioned with a combination of thymic irradiation, rituximab, cyclosporine, and anti-CD2 monoclonal antibody as shown. **B)** The Stanford protocol included conditioning with ATG in combination with 800–1200 cGy of fractionated total lymphoid irradiation. Cyropreserved CD34 selected G-CSF mobilized product supplemented with $1 \times 10^6 \text{ CD3}^+$ bone marrow cells per kg recipient body weight was infused day 14.

Conditions that may Benefit from or be Cured by a Bone Marrow/Facilitating Cell Transplant

Figure 3.
Conditions that will benefit from or be cured by a bone marrow/facilitating cell transplant.

Figure 4.
Schema of conditioning for NW/ICT protocol.

Patient Demographics and Clinical Courses in 10 of 20 Recipients in the Northwestern University HLA-Identical DHSC Protocol

Table 1

Pt#	Recipient		Donor			Native Disease	Peak PRA Pre-Tx	Mos Post-Tx	C _r ^b	Latest Urine Protein per 24 Hrs/Cells	Latest Tx Biopsy Results	Current daily IS Dose	Mos off IS	Maximum Microchimerism during Year 1 Post-Op ^{c,h}		% PBMC CD4 ⁺ CD25 ^{High} FoxP3 ⁺ T _{reg} ; Maximum change from Pre-op baseline
	Age ^a	Sex	Race	Age ^a	Sex	Race								% in Blood	% in Bone Marrow	
1	51	M	Cau	49	F	Cau	Polycystic	5%	54	1.2	Banff 1A – 36 mos	SRL 2 mg ^e	12 ^e	0.016	0.02	9X ^j
2	54	M	Cau	56	M	Cau	Multicystic	9%	52	1.1	Neg – 36 mos (Tol) ^{i,l}	0	28 ^l	0.10	NT ^j	9X ^j
3	46	F	Cau	45	M	Cau	IgA Neph	29%	46	0.7	Banff 1A-25 mos	MMF 1gm 2x ^e	1 ^e	0.10	NT ^j	14X
4	41	M	Hisp	39	F	Hisp	DM II	0%	45	1.6	Banff 1A – 36 mos	Prograf 2mg 2x - MMF 1gm 2x ^e	13 ^e	0.01	0.03	34X
5	39	M	Cau	33	F	Cau	IgA Neph	0%	40	1.4	Neg – 36 mos (Tol) ^{i,l}	0	16 ^l	2.30	NT ^j	32X
6	38	M	Cau	35	M	Cau	IgA Neph	0%	40	1.2	Neg – 36 mos (Tol) ^{i,l}	0	16 ^l	1.70	3.00	55X
7	20	M	Cau	22	F	Cau	Unk ^d	0%	39	1.3	?disease recurrence-18 mos	MMF 1gm 2x ^e	0 ^e	0.06	NT ^j	21X
8	24	M	Hisp	21	M	Hisp	Obstructive	11%	38	0.9	Neg – 36 mos (Tol) ^{i,l}	0	14 ^l	0.47	8.50	17X
9	39	M	Cau	30	M	Cau	IgA Neph	0%	34	1.4	Neg – 24 mos ^f	0	11	0.01	NT ^j	23X
10	45	M	AA	38	F	AA	Nephroscl	15%	29	2.1	FSGS – 24 mos	MMF 1gm 2x ^e	0 ^e	0.21	0.43	30X

^aAt transplantation

^bMost recent serum creatinine in mg/dl; all values still reflect post-op nadirs

^cPBMC and bone marrow microchimerism (up to 1% by STR analysis) lasted up to 1 year at most

^dNative kidney disease diagnosis was unknown (biopsies inconclusive), but possibly immune mediated, with the 18-month post-transplant biopsy showing dense deposit disease on electron microscopy

^eIS reinstated, or never withdrawn (see text), but with no change in renal function

^fNeg includes no acute or chronic rejection

^gThought to be present from native kidneys

^hNo chimerism seen in peripheral blood after Year 1 NT = not tested

ⁱPatient 1 and 2 did not have pre-transplant values. Therefore, this was determined from the mean of the other 9 patients

^jThought to have occurred *de novo* after transplantation
^kOperational tolerance – off immunosuppression for at least one year