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OBM Transplant. Author manuscript; available in PMC 2020 September 16.

Published in final edited form as:

OBM Transplant. 2019 ; 3(3): . doi:10.21926/obm.transplant.1903080.

Induction of Tolerance Towards Solid Organ Allografts Using Hematopoietic Cell Transplantation in Large Animal Models

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Abstract

Background—The application of hematopoietic cell transplantation for induction of immune tolerance has been limited by toxicities associated with conditioning regimens and to graft-versus-host disease (GVHD). Decades of animal studies have culminated into sufficient control of these two problems, making immune tolerance a viable alternative to life-long application of immunosuppressive drugs to prevent allograft rejection.

Methods—Studies in mice have paved the way for the application of HCT with limited toxicity in large animal models. Resultant studies in the pig, dog, and ultimately the nonhuman primate have led to appropriate methods for achieving nonmyeloablative irradiation protocols, dose, and timing of post-grafting immunosuppressive drugs, monoclonal antibody therapy, and biologicals for costimulatory molecule blockade. The genetics field has been extensively evaluated in appreciation of the ultimate need to obtain organs from MHC-mismatched unrelated donors.

Results—Nonmyeloablative conditioning regimens have been shown to be successful in inducing immune tolerance across all three animal models. Postgrafting immunosuppression is also important in assuring sustained donor hematopoiesis for tolerance. Donor chimerism need not be permanent to establish stable engraftment of donor organs, thereby essentially eliminating the risk of GVHD. Using nonmyeloablative HCT with monoclonal antibody immunosuppression, the kidney has been successfully transplanted in MHC-mismatched nonhuman primates.

Conclusions—Nonmyeloablative HCT for the establishment of temporary mixed chimerism has led to the establishment of stable tolerance against solid organ allografts in large animal models. The kidney, considered a tolerogenic organ, has been successfully transplanted in the clinic. Other

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SS Graves, Ph.D., researched and prepared the manuscript

DW Mathes, MD, reviewed and offered constructive criticisms of the manuscript.

R Storb, MD, reviewed and offered direction and constructive criticisms of the manuscript.

Competing Interests

The authors have declared that no competing interests exist.

Additional Materials (if any): None.

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organs such as heart, lung, and vascularized composite allografts (face and hands), remain distant possibilities. Further study in large animal models will be required to improve tolerance against these organs before success can be attained in the clinic.

Keywords

Tolerance; hematopoietic cell transplantation; preclinical; large animal model; organ transplantation

1. Introduction

Conventionally, the gold standard for acceptance of solid organ transplantation has been through pharmacological application of immunosuppressive agents. Although such agents have been remarkably successful for short-term allograft survival, long-term survival has been elusive. The downside to long-term application of immunosuppressive drugs is increased risk for infections [1], malignancy [2], and ultimately chronic rejection of the organ graft [3, 4]. Thus, it has long been the goal of transplantation biologists to develop alternative methods that render the patient's immune system specifically tolerant towards the allograft without requiring life-long administration of immunosuppressive drugs. Immunological tolerance through hematopoietic cell transplantation (HCT) has proven to be an effective solution to this problem both in preclinical animal models and the clinic [5, 6].

The concept of immune tolerance was first developed in the mid-1940s when Ray Owen published on the observation that dizygotic cattle twins of different sires expressed both sets of paternal blood group antigens as a result of in utero sharing of the same placenta [7]. This phenomenon, later known as hematopoietic chimerism, was based on the principle that hematopoietic cells of one organism cannot be rejected by another thus resulting in a state of immune tolerance. Burnett's tolerance hypothesis published in 1949 stated that immunologic self and non-self-discrimination occurred prenatally [8]. Based on Burnett's hypothesis, in 1953 Medawar and colleagues reported on studies using inbred strains of mice in which stable mixed hematopoietic chimerism was established in neonates of CBA mice injected with adult cells from A-strain mice on day 15–16 of pregnancy [9]. In adulthood, the transplanted mice were tolerant to hematopoietic cells and skin grafts from the donor and mice of the same donor strain. These observations led to the awarding of the Nobel Prize to both of Burnet and Medawar. Later in 1955, Main and Prehn reported that mice given myeloablative total body irradiation (TBI) and donor marrow achieved full hematopoietic cell chimerism and accepted skin grafts from hematopoietic cell donors without the need of immunosuppressive drugs [10]. Early studies in the clinical setting revealed that patients given HCT for the treatment of acute leukemia also accepted kidneys from the marrow donors several years later without requiring immunosuppression following the development of acute renal failure [11].

Initial investigations using HCT for the treatment of hematological diseases relied on supralethal doses of irradiation to ablate tumor cells and ensure donor cell engraftment without rejection [12]. However, lethal graft versus-host disease (GVHD) was a common sequala following toxic conditioning regimens in animals and patients, making full donor

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chimerism for induction of tolerance as a replacement for immunosuppressive therapy impractical. The development of less toxic protocols using non-lethal doses of conditioning irradiation were critical for the advancement HCT for induction of tolerance in a non-malignancy setting. However, conditioning regimens using nonlethal doses of total body irradiation (TBI) would require additional intervention to assure successful donor hematopoietic cell engraftment. Various approaches to the problem have been reported including T-cell depletion studies in mice [13], application of postgrafting immunosuppression [14–16] or use of costimulatory molecule blockade [17] in canines. Another approach was total lymph node irradiation (TLI), in which only the thoracic and abdominal lymph nodes and spleen were irradiated [18, 19]. Advances such as these reduced the incidence of GVHD seen with lethal doses of TBI conditioning regimens and enabled the possibility of using HCT safely to induce tolerance to transplanted organ allografts. One early study on mixed murine chimerism by Warner et al [20] established mixed hematopoietic chimerism but skin grafts from the hematopoietic cell donors were rejected, a phenomenon known as split-tolerance [21]. Steinmueller and colleagues established complete hematopoietic chimerism and showed rejection of donor skin grafts but not of heart grafts [22]. However, in twin marmosets Porter and Gengozian studied natural chimerism and found indefinite survival of twin skin grafts [23].

It has been long recognized that there is a spectrum of acceptance of allograft acceptance, with skin at one end being difficult to accept and liver or kidney at the other being more resistant to rejection [24]. Both liver and kidney are considered tolerizing organs [25]. Operational tolerance, the survival of solid organ allografts without maintenance immunosuppression, can be seen in 20–30% of liver transplantation patients after withdrawal of immunosuppressive agents [25]. The liver has several cellular and humoral mechanisms to avert rejection at its disposal. These include release of soluble MHC-1 antigens, the presence of tolerogenic dendritic cells, production of IL-10 by liver sinusoidal endothelial cells and Kupffer cells, and hepatocyte expression of PDL-1 in response to IL-10 [26]. Skin is considered difficult to accept immunologically due to skin-specific antigens that escape hematological tolerance and Langerhans cells and monocyte-derived dendritic cells that are highly efficient at antigen presentation [24, 27].

The most effective means of achieving tolerance towards organ allografts in large animal transplantation models depends on the establishment of mixed multilineage hematopoietic cell chimerism [28–33]. Two types of chimerism have been described [34]. Microchimerism occurs following migration of passenger leukocytes from the transplanted organ into the periphery of the recipient but fail to engraft. In a study in which patients receiving a liver transplant and immunosuppression without HCT, a significant correlation was found between microchimerism and absence of allograft rejection [35]. The mechanisms by which microchimerism prevents allograft rejection have not been fully defined. Macrochimerism occurs following transplantation of the organ donor’s hematopoietic cells which engraft in hematopoietic compartments and produce multiple lineages of donor immune system. Macrochimerism can occur transiently or long-term as either full donor or mixed donor/recipient chimerism.

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Attaining durable mixed chimerism in the fully MHC-mismatched HCT setting has been difficult to obtain in nonhuman primates and in the clinic. Notable success stories are the recent reports on transient mixed and full donor chimerism leading to long-term kidney allograft tolerance in the clinic [36, 37]. These results have developed through several years of study using tolerance induction through non-myeloablative HCT in both small and large animal models. Three important observations are responsible for this success. First, multilineage hematopoietic cell chimerism is required. Second, peripheral regulatory mechanisms play critical role. Third, organ-specific tolerance has been observed for kidney and lung transplantation but not for the heart [38]. In this review we focus on the contributions that swine, canine, and nonhuman primate models have played towards the ultimate culmination of transient and long-term mixed hematopoietic chimerism and organ-specific tolerance in mankind.

2. Studies in mice and rats

Studies of hematopoietic macro- and micro-chimerism in mice have contributed significantly to the understanding of the mechanism of immune tolerance of hematopoietic cell transplantation in solid organ transplants [6]. These studies highlight the use of non-myeloablative protocols to achieve stable mixed hematopoietic chimerism of long duration across major and minor histocompatibility barriers [39–42]. Various protocols for establishing long-term hematopoietic tolerance were first attempted in murine models and later translated to large animal studies. Prolonged macrochimerism in mice leads to a robust form of tolerance that enables skin and solid organ grafts from the hematopoietic cell donor [43]. Another conditioning regimen described in mice used non-lethal irradiation, anti-CD4 and anti-CD8 depletion methods, followed by injection of MHC disparate marrow. The protocol resulted in long term chimerism without GVHD and the acceptance of donor-specific skin grafts but not third-party grafts [13]. In mice, the use of T cell-depleting antibodies with targeted irradiation of the thymus and nonmyeloablative TBI results in stable chimerism [13]. However, in nonhuman primates this approach leads to transient chimerism, suggesting tolerance in NHP and most likely in man is a more complex process than in murine models.

Central deletional tolerance as an important mechanism to eliminate host alloreactive cells has been shown to be an effective means of maintaining tolerance in mice [44, 45]. However, other studies on tolerance induction indicate the importance of peripheral CD4+ T regulatory cells for accepting and maintaining solid organ allografts [46, 47]. It has been concluded that both central deletional tolerogenic and peripheral regulatory mechanisms are required for optimal tolerance to skin grafts following induction of hematopoietic cell transplantation [47].

3. Reasons for Studies in Large Animal Models

Although mice have significantly contributed to the understanding of tolerance and its application to solid organ transplantation, there are significant differences between mice and larger transplantation relevant models such as the pig, dog, and nonhuman primate. Large animal models are more closely aligned with humans based on factors such as out breeding

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and genetic diversity, housing in non-gnotobiotic conditions, and life-span [48]. In addition, large animal models are amenable to the surgical procedures required for transplanting solid organs and vascularized composite tissue allografts (VCA). VCA are comprised of skin, muscle, vasculature and connective tissue and, in some cases, bone and marrow that must be anastomosed to appropriate vasculature and are amenable to multiple collections of biopsies for histopathological analysis [49].

Despite a large number of successful approaches for the induction of tolerance in rodent models, ultimately many of these protocols were unsuccessful when translated to nonhuman primates or the clinic. Various protocols meeting this discrepancy, including those using calcineurin inhibitors, anti-lymphocyte serum, anti-CD25, and donor stem cell transplantation, were successful in mice but ultimately failed in large animal models for induction of tolerance [50]. Reasons for this discrepancy may be that previous antigen exposure results in allo-specific memory T and B cells which are present in large animals and humans but are nearly absent in mouse colonies [51–53]. Furthermore, expression of class II MHC antigens is present on endothelial cells of large animals and humans, but they are absent on the endothelial cells of rodents [54]. For these reasons, protocols developed in rodent models should be vetted in a large animal model before undergoing clinical trials.

4. Studies in Swine

Swine, and miniature swine in particular, have been useful large animal models with which to study HCT-induced tolerance induction for solid organ transplantation [55–58]. As in all HCT models, the characterization of the major histocompatibility complex in miniature swine, known as the miniature swine leukocyte antigen (mSLA) complex, is required in order to assess the level of identity and transplantation success. The genome of mSLA contains a class I gene family comprised of 7 members and two subfamily genes, one of which encodes for classical transplantation antigens [59]. In a study assessing the factors affecting renal allografts performed in inbred miniature swine without donor HCT, it was found all MHC-mismatched kidney transplants were rejected within a mean of 12 days, while most MSLA-matched renal grafts were accepted long-term. Skin graft survival was shown to be extended in animals that accepted their renal allografts compared to skin grafts performed across the same MHC barriers of normal pigs [60].

4.1 Skin grafts: swine

The initial studies on the successful establishment of hematopoietic chimerism and tissue tolerance between MHC-mismatched mice using fractionated TLI performed by Slavin et al. [18] were subsequently attempted in pigs. The Massachusetts General Hospital (MGH) group developed a modified nonmyeloablative HCT protocol for the induction of stable mixed hematopoietic chimerism enabling skin transplantation in miniature pigs [28]. The protocol consisted of administering two doses of 150 cGy TBI combined with 7 Gy thymic irradiation, followed by host T-cell depletion with an anti-porcine CD3 immunotoxin conjugate. Infusion of MHC-matched mobilized stem cells or marrow resulted in stable multilineage mixed lymphohematopoietic cell chimerism without clinical evidence of GVHD. Long-term acceptance of donor skin grafts and rejection of third-party skin

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indicated successful donor-specific tolerance had been achieved. An important component of the protocol was the requirement of successful host T-cell depletion. T-cell depletion has been shown to be more difficult to achieve in large animals compared to mice [61, 62].

4.2 Kidney grafts: swine

Successful establishment of mixed hematopoietic chimerism between MHC-disparate individuals resulting in specific tolerance towards organ grafts from the marrow donor is a crucial goal for successful translation to the clinic. This approach was tested for induction of tolerance to kidney allografts in swine given MHC-haploidentical marrow transplants after fractionated total lymphoid irradiation totaling either 3,250 or 2,800 cGy. No permanent chimerism was observed except in one animal. Transplantation of marrow matched kidneys was performed at the time of marrow infusion. No postgrafting immunosuppression was given. However, acute renal failure was observed in most of the animals on days 4–6 after grafting [63]. In a later study, pigs were rendered tolerant to class II mismatched MHC by infusing class II MHC-mismatched marrow followed by a short course of CSP. Recipients receiving a kidney matched to their respective marrow donor showed normal serum creatinine levels for periods greater than 200 days after transplant. Thus, addition of BMT and CSP therapy was found to induce specific tolerance to single haplotype mismatched class II kidneys obtained from matched marrow donors [64]. Pigs conditioned with two doses of TBI (1150 cGy total) plus cyclophosphamide, received MHC-mismatched marrow 5 months or more before kidney transplantation from respective marrow-matched donors. Four of five animals accepted the kidney grafts more than 200 days after transplant. Recipients of MHC-mismatched kidney transplants without marrow transplants were quickly rejected within 7 days, again indicating donor-specific tolerance can be obtained by marrow transplantation [65]. The question whether high levels of stable long-term mixed chimerism are necessary for tolerance in allograft acceptance was evaluated in miniature swine given MHC-haploidentical mobilized stem cell transplants after nonmyeloablative conditioning [66]. Animals received CSP after transplantation immunosuppression. Tolerance was tested by giving donor-matched kidney allografts to recipients after completion of CSP therapy. The presence of donor-derived colony forming units (CFU) in the marrow was found to have a high correlation with tolerance induction as indicated by renal allograft acceptance. In addition, thymic and peripheral blood chimerism was in step with donor CFU in the bone marrow and kidney graft tolerance. The results suggest in vitro analysis of donor chimerism may be used to predict allograft acceptance in the clinical setting.

4.3 VCA: swine

Using the nonmyeloablative HCT model for tolerance induction in swine developed by Huang et al. [28], investigators at MGH reported on a study using a similar approach to induce tolerance to a heterotopic limb transplant in miniature swine in which donors and recipients were selected based on at least one or two MHC haplotype differences [67]. No conditioning with irradiation was used; however, T-cell depletion was accomplished with anti-CD3 immunotoxin conjugate. Mixed chimerism was established with either donor marrow or mobilized peripheral blood stem cells. Although macrochimerism was present in recipients of mobilized stem cells, none was observed in pigs receiving donor marrow.

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Despite this, the musculoskeletal portion of limb transplants, but not the skin, survived in recipients of both stem cell sources, a condition known as split tolerance. Animals receiving cytokine mobilized stem cells developed clinical GVHD, while those receiving marrow did not. These studies are important, as they indicate long-term mixed chimerism is not required for graft acceptance, and though not completely successful, they formed the basis for the development of a tolerance protocol for the acceptance of elements of reconstructive surgery.

In another approach to achieving VCA tolerance, investigators achieved multilineage macrochimerism in swine through in utero injection of fetuses with T cell-depleted marrow from fully major MHC mismatch donors [68]. Control VCAs in swine not rendered tolerant to donor antigens were rejected within 21 days. Chimeric animals accepted VCAs and did not produce alloreactive antibodies nor demonstrate alloreactivity against the donor in vitro. Although not clinically translatable, the study does provide evidence that tolerance across a full MHC barrier can lead to long term acceptance of a VCA graft without chronic immunosuppression. Clarification of the importance of MHC antigen matching was revealed in swine given nonmyeloablative conditioning and mismatched for MHC class I or class II antigens [69]. Whereas class II mismatched hematopoietic stem cell and VCA transplants resulted in stable VCA engraftment, recipients of class I mismatched tissues were rejected due to donor CD8+ lymphocyte infiltration into the skin.

5. Studies in Canines

Early interest in the immunological response of the host to bone marrow transplantation led to the development of a tightly controlled dog breeding program in Cooperstown, NY [70]. Marrow transplantation between some of the dogs in this colony produced occasional long-term graft survivors that were likely due to histocompatibility antigen matching but not erythrocyte antigens. Later studies showed that leukocyte-specific antisera, produced by cross-immunizing littermates with buffy coat cells, could be used to select MHC compatible donors and recipient dogs for transplantation studies that delineated superior graft acceptance in both related and unrelated littermates [71, 72]. In the early 1970s, canine leukocyte specific antigens were identified in litters of mongrels and dogs of various breeds following generation of a collection of lymphocytotoxic antisera [71]. The antisera were used to identify donor and recipient MHC identical transplant pairs through in vitro assays that could be used to perform transplantation of hematopoietic cells, kidneys, liver, and hearts, to demonstrate the importance of donor-recipient compatibility for these antigens. Elucidation of the MHC of dogs gave rise to the nomenclature dog leukocyte antigen (DLA) complex. The eventual typing of highly polymorphic canine class I and class II DLA genes allowed for accurate matching of donor and recipient pairs for HCT and solid organ transplantation studies on par with those of the human transplantation condition [73–75].

The practicality of using hematopoietic cell chimerism to safely induce tolerance for solid organ transplantation (SOT) was achieved following the demonstration that chimerism could be safely established using nonmyeloablative conditioning regimen of 2 Gy TBI, followed by infusion of DLA-identical marrow and a short course of postgrafting immunosuppression with CSP and MMF [16, 76]. Nonmyeloablative HCT not only extended HCT for the treatment of hematological disorders in older patients, protocols of this nature opened the

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door to the possibilities of tolerance induction for SOT with reduced toxicity and lower probability of GVHD.

5.1 Skin grafts, canines

As in murine models, performance of full-thickness skin grafts onto mixed chimeric dogs is a reliable and rigorous test for donor immune tolerance. Skin may be transplanted as a full-thickness graft or as part of a vascularized composite allograft (VCA) in which donor and recipient blood vessels are anastomosed, thereby greatly reducing hypoxia within the graft and improving graft survival. Skin graft rejection studies from marrow and solid organ donors as well as third party dogs are often used to demonstrate donor specific tolerance [77–80]. Yunosov and colleagues showed that dogs with stable mixed hematopoietic cell chimerism could be successfully transplanted with skin grafts at a median of 92 weeks after HCT [80]. Loss of donor chimerism correlated with skin graft rejection. However, donor specific tolerance to DLA-identical skin grafts from marrow donors was found to be complete or partial since donor derived skin grafts in recipients with mixed donor chimerism developed an inflammatory reaction without skin graft loss, suggesting a condition of partial donor specific tolerance.

5.2 Kidney grafts: canines

Using leukocyte specific antisera for selection of DLA-identical pairs of transplantation, Rapaport and colleagues [81] showed that kidney transplants from leukocyte group-compatible donors into recipients in the absence of mixed chimerism had a mean survival time of 25.5 days, while kidneys transplanted from leukocyte incompatible donors had a mean survival time of 13.1 days. With the successful development of donor chimerism through marrow transplantation, DLA-identical kidney transplantation into mixed chimeric recipients was examined [78]. Initially, chimeric hosts were established following supralethal dose of total body irradiation in dogs receiving marrow from littermates as well as non-littermates. Within 43 to 120 days after marrow transplantation, chimeric recipients underwent bilateral nephrectomy and transplantation from their respective marrow donors. Long-term acceptance of renal and skin allografts was achieved in littermate chimeric recipients of both littermate and non-littermate donors, while skin allografts from DLA-incompatible donors were summarily rejected within a mean of 14.7 days [82]. These results indicated that specific donor antigen tolerance could be achieved in dogs with mixed hematopoietic chimerism following a myeloablative conditioning regimen.

Nonmyeloablative or reduced intensity conditioning regimens were developed with cyclophosphamide conditioning [76] or low-dose TBI followed by cyclosporine (CSP) and mycophenolate mofetil (MMF) [16, 83], which established a state of mixed donor and host hematopoietic chimerism with tolerance to DLA of both the donor and recipient but without the toxicity of lethal doses of TBI. As a result, nonmyeloablative conditioning regimens for HCT became the standard for inducing tolerance for kidney transplantation in dogs [31, 33, 84–86].

Using the nonmyeloablative HCT model of 2 Gy TBI before and a short course of CSP and MMF after DLA-identical marrow transplantation, Kuhr and colleagues reported stable

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long-term peripheral blood lymphocyte and granulocyte chimerism in recipients [33]. Cross-over renal transplants from the marrow donor into the respective recipient following bilateral native nephrectomy was done within 8–17 months after HCT. A 5-year follow-up revealed donor renal allografts were functional in all recipients with no evidence of histological acute or chronic rejection while donors rejected the chimeric kidney grafts within 24 days. Tolerance was split between kidney and skin grafts with 2 of 4 animals showing rejection of delayed donor skin grafts [84]. Tolerance induction through the non-myeloablative conditioning regimen of HCT also extended to dogs receiving marrow grafts from two DLA-identical littermates resulting in trichimerism and immune tolerance towards a kidney graft from one of the HCT donors [85].

The question as to whether stable hematopoietic chimerism was required for tolerance to the kidney graft was answered in a separate study in which recipient dogs of DLA-identical marrow and kidney grafts underwent a second low-dose TBI conditioning and infusion of cryopreserved autologous G-CSF-mobilized PBMC after tolerance was established. The dogs rejected donor hematopoietic chimerism without rejecting the kidney grafts for periods greater than one year. Returning hematopoiesis to 100% host after kidney transplantation suggests GVHD due to donor chimerism can be avoided and that TBI of 100 cGy, which generally is sufficient for establishing short-term mixed chimerism, may be sufficient for tolerance induction for kidney transplantation [86].

Results of studies of tolerance induction and kidney transplantation in more clinically relevant conditions of DLA-haploidentical or mismatched recipients has been encouraging. Niemeyer and colleagues reported that following nonmyeloablative conditioning (200 cGy) TBI and with marrow transplantation followed by immunosuppression with CSP and MMF, DLA-haploidentical dogs were tolerant to kidney transplants for periods greater than 1-year post-transplant [31]. Dogs not receiving marrow transplants rejected the donor kidney. A study using a similar nonmyeloablative protocol and looking at marrow and kidney transplantation from DLA-identical or DLA-haploidentical dogs showed long-term renal allograft survival in both groups of dogs. However, renal allograft inflammation was present to a greater extent in the DLA-haploidentical group compared to the DLA-identical transplant group [79]. Studies using DLA-mismatched mongrel dogs given anti-thymocyte serum or anti-lymphocyte serum with donor marrow and temporary application of immunosuppressive therapy revealed that recipient dogs remained tolerant to their donor kidney allografts long-term [87, 88]. However, in a separate study, mongrel dogs conditioned with 1800 R to 3500 R TLI of differing widths of field followed by infusion of unrelated donor marrow failed to produce consistently high levels of mixed chimerism in 45 dogs [89]. Renal allografts given the following day were all rejected except for two dogs with highest percentage of donor chimerism.

5.3 Heart and lung grafts: canines

Reports of cardiac and lung transplantation in radiation and marrow transplanted chimeric dogs are limited but nonetheless show promise. A study reported by Rapaport and colleagues revealed that allogeneic unresponsiveness to the heart could be established in dogs treated with supralethal total body irradiation and marrow transplants from DLA-

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identical donors [90]. Orthotopic heart transplantation from marrow donors into 9 mixed chimeric recipients was performed 5–6 months later. Six of the 9 dogs died from procedural complications, one dog died from heart failure, and 2 dogs remained healthy for 545 and 547 days after surgery. The results are in line with the hypothesis that hematopoietic chimerism can lead to tolerance of DLA-identical heart transplantation. In unrelated mongrel dogs, heart and kidney transplants showed prolonged survival advantage following total lymphoid irradiation and marrow transplantation without immunosuppression [91]. In this investigation, the heart allografts survived longer than the kidney. However, a later study by Strober and colleagues revealed that cardiac allografts in unrelated mongrel dogs were accepted long-term following TLI and ATG in 40% of the animals. Curiously, in this study addition of marrow did not confer survival advantage to the cardiac allografts [92]. A similar study showed that marrow did not induce tolerance in this model was reported by Raaf et al. using kidney transplants instead of cardiac transplants in dogs conditioned with TLI [89]. The authors concluded that TLI was not sufficiently immunosuppressive in this model to condition animals for uniform marrow engraftment.

Short-term lung allograft survival was observed in recipients of DLA-identical and DLA-mismatched lung allografts following 200 cGy TBI with a short course of immunosuppression consisting of CSP and MMF (86). No HCT was performed in these preliminary experiments. For DLA-mismatched allografts, 2 Gy TBI and monoclonal S5 (anti-CD44) was also administered [93]. With the addition of HCT, tolerance for long-term acceptance of orthotopic lung transplants was achieved [32, 93]. Here, Nash and colleagues reported that stable mixed chimeric dogs given heterotopic lung allografts from their respective DLA-identical marrow donors had significantly prolonged survival of their lung grafts over nonchimeric counterparts. Furthermore, there was a significant increase in CD3+ T cells with a Treg phenotype, expression of FoxP3+, IL10, and TGFB, in the peripheral blood and lungs with a decrease in copy number of cells of this phenotype in spleen and lymph nodes. These changes suggest mobilization of regulatory cells to the peripheral tissue including the graft to control graft rejection [32].

5.4 Vascularized composite allografts (VCA): Canines

VCA represents an appropriate model for transplantation of the face and hands in the clinic, and like solid organ transplantation, requires immune tolerance for long-term graft acceptance. VCA is composed of donor muscle, vasculature, connective tissue, and skin. Due to the high antigenicity of the skin, split tolerance has been observed [67, 94]. In the dog model, results have been more promising. Preliminary studies using DLA-identical littermates, showed five VCA transplants between DLA-identical donor and recipient dogs survived 15 to 30 days in the absence of marrow transplantation and postgrafting immunosuppression [49]. Long-term tolerance to a VCA can be accomplished with either VCA transplantation several months after or coincident with HCT in the canine DLA-identical transplantation model [95, 96]. In a more clinically relevant setting, we recently tested immune tolerance to DLA-haploidentical marrow or G-CSF-mobilized peripheral blood stem cells coincident with VCA transplantation [97]. Conditioning was done with 4.5 Gy TBI, and postgrafting immunosuppression was accomplished with a brief course of CSP and MMF (Figure 1). Dogs receiving G-CSF mobilized stem cells had a superior

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engraftment rate compared to dogs given marrow, but G-CSF-mobilized stem cells resulted in the incidence of GVHD and one dog rejecting the hematopoietic graft while retaining the VCA. These studies suggest that mixed chimerism is important in the early stages of VCA acceptance, but chimerism can be eliminated at a later point to reduce the risk of GVHD.

6. Rationale for Primate Studies

Phylogenetically, nonhuman primates (NHP) are the most stringent models in which to test mixed chimerism protocols for the induction of tolerance towards transplanted tissues in the clinical setting. There are several reasons for using NHP model versus other large animal models. Immunosuppressive drugs function similarly for both NHP and humans. Monoclonal antibodies commonly show specificity for human and NHP leukocytes and stem cells for both binding and function with similar antagonistic or agonistic results. Costimulatory molecules and their ligands, targeted during conditioning regimens prior to HCT, have also been validated in NHP and translated to the clinic [98, 99]. One notable exception is the anti-CD28 mAb, TGN 1412. Although safe in monkeys, TGN 1412 was proved disastrous in a study with human volunteers [100]. Kidney transplantation is the most likely organ to be successfully transplanted in conjunction with HCT-induced immune tolerance. Besides the liver, the bar for kidney tolerance is set lowest among the transplantable organs. Furthermore, successful kidney transplantation following HCT for hematologic disease has been previously demonstrated in human patients [12, 101, 102].

6.1 Primate studies: Kidney

Immune tolerance using nonmyeloablative conditioning regimens and HCT in nonhuman primates eventually translated to successful protocols for transplantation of kidney allografts in the clinical setting. The key to allograft tolerance was establishment of persistent mixed chimerism. This has been easily achieved in mice [13] but more difficult to obtain in nonhuman primates [103, 104]. Various approaches for achieving tolerance were tested in nonhuman primates using a variety of stringent conditioning regimens and postgrafting immunosuppression for acceptance of solid organ allografts.

Believing that persistent mixed chimerism was required for successful allograft transplantation, investigators at Emory University pursued a strategy for long-term mixed chimerism in rhesus macaques after non-myeloablative conditioning with a single dose of busulfan, mTor inhibition with sirolimus, and CD28/CD154 costimulatory molecule blockade. High levels of mixed chimerism were established transiently for a median of 145 days. Additional recipient treatment with CD8 depletion, donor lymphocyte infusion, inclusion of deoxyspergualin, and recipient thymectomy failed to prolong the period of mixed chimerism [105]. However, subsequent studies revealed that transient mixed chimerism following nonmyeloablative conditioning was sufficient for long-term kidney allograft survival [104, 106, 107].

The MGH group developed a basic nonmyeloablative protocol that successfully produced mixed chimerism and renal allograft tolerance in MHC-mismatched nonhuman primates. The initial protocol consisted of 300 rad midline tissue doses and TBI 700 rad thymic irradiation, followed by donor bone marrow infusion and kidney transplantation from MHC-

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mismatched donors. Monkeys treated with the basic protocol, rejected their allografts by day 15. Addition of postgrafting immunosuppression with CSP (30 days) resulted in monkeys developing multilineage chimerism and long-term kidney allograft acceptance [106]. In subsequent studies, splenectomy on Day 0 was included in the protocol to eliminate alloantibody formation [104] as well as the addition of anti-human thymocyte globulin to further suppress anti-donor T cell activity [104]. Later studies investigated costimulatory molecule blockade with anti-CD154 [98] or CTLA4-Ig (belatacept) [99] to replace splenectomy, improve mixed chimerism, and acceptance of the renal allograft. Heterologous immunologic memory, a significant barrier to HCT-induced tolerance when performed several weeks after kidney transplantation, was successfully reduced by a course of anti-CD8 mAb or ATG [108, 109]. Overall, these essential studies indicate that transient mixed chimerism is sufficient to induce long-term immune tolerance against a kidney allograft in the MHC-mismatched setting and provided sufficient proof of principle for translation to the clinic.

Following upon combined kidney and marrow transplantation success in NHP, investigators have successfully induced stable mixed hematopoietic cell chimerism and tolerance to human renal allografts using local thymic irradiation or TLI and donor marrow transplantation in the HLA-identical setting [101, 110, 111]. Breaching the HLA-mismatched barrier, although more difficult, has also been successful and allowed for more widespread application of immune tolerance induction for kidney transplantation.

There are three major clinical transplant centers located at Stanford, Northwestern, and MGH in Boston that are evaluating hematopoietic cell chimerism for induction of tolerance towards kidney transplantation [112]. Human leukocyte antigen (HLA)-mismatched protocols differ and are a result of a cumulation of both small and large animal transplantation preclinical studies and HLA-matched clinical studies. The goal of the Stanford group is to obtain long-term mixed chimerism without GVHD. Their protocol relies on fractionated dosing of total lymphoid irradiation and anti-thymocyte Gama globulin (ATG) for conditioning, administered immediately after kidney transplantation (day = 0). Column-purified CD34+ stem cells from granulocyte-colony stimulating factor (G-CSF)-mobilized peripheral blood supplemented with CD3+ cells are administered 11 days after kidney transplantation [113]. Using HLA-matched patients, the results were quite promising, with complete withdrawal of immunosuppressive drugs in 16 of 22 patients without rejection or episodes of kidney disease for up to 5 years. The results of achieving sustained macrochimerism in HLA-mismatched patients have been attained [36, 113]. Multilineage chimerism has been achieved in four HLA-mismatched patients given a CD34+ mobilized stem cells and kidney transplant after nonmyeloablative conditioning consisting of TLI and ATG. No GVHD was observed, and macrochimerism developed in 3 of 4 patients in this study [36].

The Northwest group has performed clinical studies on both HLA-identical and haploidentical kidney transplantation. In the haploidentical setting, conditioning included fludarabine, cyclophosphamide, and 200 cGy TBI. Chimerism was induced using a proprietary facilitator cell enriched product obtained from G-CSF mobilized blood mononuclear cells administered the day after kidney transplantation. Postgrafting

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immunosuppression was done using tacrolimus and MMF with taper after 6 months if a renal biopsy was clear. Stable donor chimerism was seen in 12 of 19 subjects [114].

The MGH group has taken a different approach for HLA-mismatched patients and demonstrating that transient chimerism is sufficient for maintaining tolerance to a kidney allograft. In one study, 10 patients received a kidney and marrow from haploidentical donors following thymic irradiation with 2 doses of cyclophosphamide given before transplantation. ATG with or without rituximab was given to deplete T and B cells respectively. Postgrafting immunosuppression was completed by 8 months after transplant once rejection was not apparent. All 10 patients had transient chimerism, with 4 patients achieving donor tolerance as defined as living immunosuppression free for periods in a range of 4.5 to 11.4 years [115].

The favorable outcome of these studies is remarkable, yet further development will be needed to ensure robust success in larger patient cohorts and translation to organs for successful long-term engraftment.

7. Conclusions

Nonmyeloablative hematopoietic cell transplantation is a realistic alternative to life-long application of immunosuppressive drug regimens for the induction of tolerance to solid organ allografts. The steps taken to reach this state of clinical acceptance could not have been accomplished without application of stepwise developmental studies in large animal preclinical models. This process has taken a course of decades of experimentation moving from success in identical to haploidentical MHC animal models. The kidney, perhaps one of the two most easily transplanted organs, the other being the liver, has now been successfully transplanted into human patients through carefully designed studies carried out in nonhuman primates.

Although the success seen in tolerance induction through hematopoietic cell chimerism for kidney allografts is very encouraging, other less readily accepted tissues such as the lung, heart, and especially the skin will likely require more extensive conditioning or posttransplant immunosuppression methods mediated through costimulatory molecule blockade, T-memory cell ablation, T-regulatory cell immunotherapy, or other suppressor cell manipulation.

Recent studies have shown that novel strategies for reducing the incidences of acute and chronic GVHD have been proven effective in both malignant and nonmalignant HCT. In vivo T-cell depletion with alemtuzumab [116, 117] has proven to be a promising approach for the prevention of acute and chronic GVHD. Anti-thymocyte globulin (ATG), introduced into the conditioning regimen for the prevention of graft rejection in patients with aplastic anemia in 1987 [118], has become an important component for prophylactic treatment for GVHD [119, 120]. Several studies have been reported using nonmyeloablative HCT and T cell receptor (TCRα/β) and CD19+ cell depletion protocols for the treatment of children with nonmalignant disorders such as Wiskott Aldrich Syndrome, hemolytic anemia, and hemoglobinopathies [121–125]. In these studies, HCT grafts were from HLA-matched

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related, unrelated, and haploidentical donors. Overall survival was high in patients treated with this T/B cell depletion conditioning regimen [122, 123]. However, GVHD, immune reconstitution, and morbidity and mortality remain an issue when using TCRαβ+/CD19-depleted grafts [123]. Nevertheless, a TCRαβ+/CD19 depletion protocol, especially when combined with conditioning the host with plerixafor and G-CSF prior to transplantation, may be a rational approach for solid organ transplantation [122].

In conclusion, the large animal models are well-positioned to take on the tasks of investigating new approaches to establishing long-term tolerance towards solid organ transplants. It is possible that a combinatorial approach of using the appropriate posttransplant immunosuppressive drug regimen combined with costimulatory molecule blockade that leads to both central and peripheral tolerance may overcome the failures seen in humans [126]. Large animal models provide a fast, translatable, and relatively facile method to test a variety of protocols to meet the goal of improving successful organ-specific tolerance in the clinic.

8. Ethics Statement

It is expected that all studies reported in this review have passed all ethics examination by in- house IACUC and or ALAC review.

Acknowledgments

We thank Helen Crawford for her assistance with manuscript and figure preparation.

Fundings

This work was supported by grants P3O CA015074 and PO1 CA078902 from the National Cancer Institute and the National Institutes of Health, Bethesda, MD 078902

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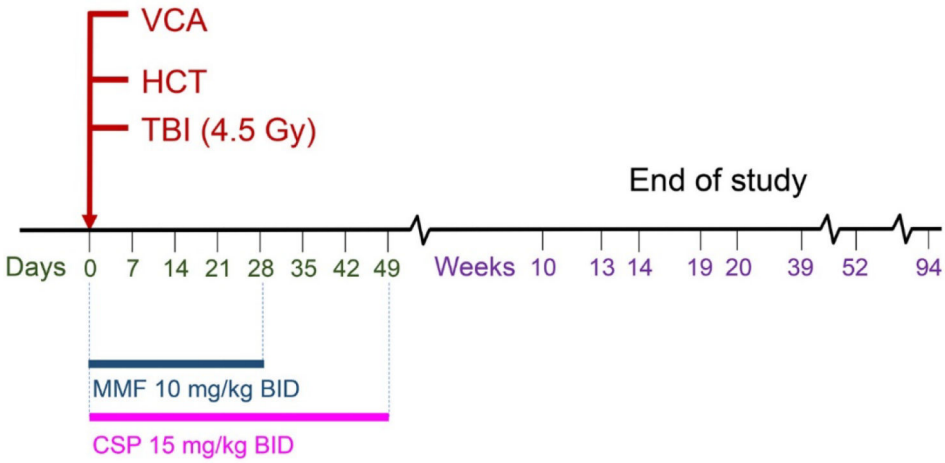

Figure 1. Protocol for induction of tolerance in DLA haploidentical VCA transplantation.

Eight dogs were transplanted with DLA-mismatched marrow and simultaneously given a VCA using a non-myeloablative transplantation protocol. Dogs were followed for tolerance to their donor grafts for the periods shown to the right of the time line break (10 through 94 weeks).

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Table 1

Illustrating the noteworthy accomplishments in the three large animals for immune tolerance to SOT

Animal model	MHC	Conditioning pre-transplant	Stem cells	Organ transplant	Conditioning post-transplant	Chimerism	Tolerance	Reference
Canine								
Kidney	Identical	1–2 Gy TBI	Marrow/G-PBMC day0	Kidney day = 0	CSP/MMF	Stable	Yes	Kuhr [33]
VCA	Haplo	4.5 Gy TBI	Marrow/G-PBMC day0	VCA, day = 0	CSP/MMF	Stable	marrow ± G-PBMC +	Chang [97]
Swine								
Kidney	Mismatch	Myeloablative	Marrow Day 0	Kidney, 5–12 months after HCT	None	Stable	yes	Guzzetta [65]
Kidney	Mismatch, Class II	4.5 Gy + CY		Kidney, 4–5 months after HCT	None	Stable	yes	Smith [64]
VCA	Mismatch		T-cell depl. Marrow day 0	VCA, 24 h after HCT	None	Stable G-mobilized Transient marrow	split	Hittiaratchy [67]
VCA	Mismatch	"Nonmyeloablative"	Marrow, Day 0	VCA, day 3 after HCT	None	Stable	Class II yes Class I no	Shanmugarajah [69]
NHP								
Kidney	Mismatch	ATG, TBI (300 Rads) TI (700 Rads) splenectomy	Marrow, day=0	Kidney, Day=0	CSP	Transient	yes	Kawai [115]
Kidney	Mismatch	3 Gy TBI + 7 Gy TI	Marrow	Kidney day 21–120	CSP	Stable mixed to Day 30–60	Yes	Kawai [104]

CSP = cyclosporine; CY = cyclophosphamide; Haplo = haploidentical; G-PBMC = granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood mononuclear cells; MHC = major histocompatibility complex; MMF = mycophenolate mofetil; T-cell depl = T-cell depleted; TBI = total body irradiation; TI = thymic irradiation; VCA = vascularized composite tissue allografts.

OBM Transplant. Author manuscript; available in PMC 2020 September 16.