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Review article

Transplant research in nonhuman primates to evaluate clinically relevant immune strategies in organ transplantation

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ABSTRACT

Research in transplant immunology using non-human primate (NHP) species to evaluate immunologic strategies to prevent rejection and prolong allograft survival has yielded results that have translated successfully into human organ transplant patient management. Other therapies have not proceeded to human translation due to failure in NHP testing, arguably sparing humans the futility and risk of such testing. The NHP transplant models are ethically necessary for drug development in this field and provide the closest analogue to human transplant patients available. The refinement of this resource with respect to colony MHC typing, reagent and assay development, and availability to the research community has greatly enhanced knowledge about transplant immunology and drug development.

Contents

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1. Introduction

As research into transplantation and immune tolerance induction progresses, novel therapies and treatment approaches must be proven in the preclinical setting prior to use in patients. Because of their close homology with humans, nonhuman primates (NHPs) have played an important role in advancing the field of transplantation and have provided insights and breakthroughs that would not have been possible with *in vitro* or lower animal models alone [1,2].

The importance of non-human primates for transplantation research was reflected in a communication from an expert panel consisting of members of the National Institutes of Health (NIH) and the Food and Drug Administration (FDA) in 1998. They identified NHP transplantation tolerance research as essential to provide "…critical data on safety, toxicity. and potential efficacy that could not be obtained ethically in human clinical trials." [3] Six external scientific advisory panels over the intervening years have concurred that this preclinical research program is a critical and high-priority area of transplantation immunology research that merits continued support.

Others have reviewed the general topic of NHPs as models of human immunology and in transplantation more specifically [1,2,4–6]. This review examines the impact of NHP transplantation tolerance research over the past 19 years focusing on the: 1) contributions of NHP research to human transplantation; 2) predictive value of translating knowledge from NHP to humans with respect to efficacy and safety; and 3) knowledge gained from NHP transplantation models that has been applied more generally, for instance to autoimmunity or cancer immunology. Although work has touched on many topics, we have organized the lessons learned from NHP tolerance research into six major themes that encompass most of the efforts to date: 1) chimerism; 2) immune cell depletion; 3) costimulation blockade; 4) regulatory cell therapies; 5) control of antibody-mediated rejection; and 6) consequences of immunosuppression.

When reviewing these major themes, lessons from kidney, heart, lung, and islet allotransplant models will be presented. This review will not discuss xenotransplant in NHP. Following these sections we address the unique aspects of these translational models and their limitations, the development of the genetics of NHP models, and reagent development.

2. Surgical transplant procedures in non-human primates

2.1. Kidney transplantation

Kidney transplantation in rhesus macaques (Macaca mulatta) is performed in animals weighing between 3 and 8 kilograms. For a donor nephrectomy, a midline laparotomy is performed and abdominal viscera are retracted to expose the left-sided retroperitoneal structures. The donor animal's left kidney is preferentially procured because of the longer renal vein. The left ureter is mobilized and divided near the bladder. Prior to ligation of the renal vessels, both donor and recipient are given intravenous heparin. The donor's left renal artery and vein are ligated and divided separately. The donor organ is immediately flushed with ice cold University of Wisconsin (UW) solution. The organ is then implanted in the recipient via a midline incision and dissecting the infrarenal aorta and inferior vena cava. The donor renal vein is anastomosed to the recipient inferior vena cava and the donor renal artery to recipient aorta. The ureter is reimplanted using a modified Politano-Leadbetter technique. Finally, the recipient's native kidneys are removed in order to ensure that the transplanted kidney is fully life-sustaining. In most cases, the recipient's left kidney is removed for transplantation into the donor as a "swapping" kidney transplant.

2.2. Islet transplantation

Rhesus monkeys (Macaca mulatta) and cynomolgus monkeys (Macaca fascicularis) are the most commonly used nonhuman primate species in preclinical research on transplantation of allogeneic islets. Donor-recipient pairs are ABO-compatible and increasingly MHC defined to ensure MHC-disparity. Donors are often older and larger (rhesus monkeys weighing 10-20 kg and cynomolgus monkeys weighing 5-10 kg) than recipients (rhesus monkeys weighing 3-5 kg and cynomolgus monkeys weighing 2-6 kg) to provide an islet mass sufficient to reverse diabetes (≥10,000 islet equivalents/kg recipient body weight). Donors undergo pancreatectomy under general anesthesia, islets are liberated from the tissue using tissue-dissociating enzymes and subsequently separated from non-islet tissue on density gradients. Freshly isolated or short-term cultured islets are transplanted intraportally into streptozotocin-diabetic recipients via either a small midline abdominal incision and infusion into a branch of the superior mesenteric vein under general anesthesia or infusion into the portal vein in the awake recipient through an implanted portal venous catheter. Occasionally, islet are transplanted to other implantation sites. The anti-rejection prophylaxis is determined by the study protocol and recipients are monitored for one year or until graft loss. Posttransplant metabolic monitoring includes daily blood or capillary glucose measurements, weekly C-peptide determinations, and monthly or bi-monthly metabolic testing using standardized mixed meals or intravenous challenge with glucose or arginine. As determined by the protocol, exogenous insulin is administered to maintain near-normoglycemia.

2.3. Heterotopic heart transplantation

Cynomolgus monkeys weighing 3 to 7 kg are selected for compatible ABO blood types. Animals are anesthetized and intubated using intramuscular (I.M.) Ketamine (10mg/kg) and maintained with light Halothane anesthesia and intermittent I.V. and/or I.M. Ketamine plus Valium (0.8mg/kg).

Donor heart procurement: A median sternotomy is performed and the ascending aorta, superior vena cava and inferior vena cava (IVC) controlled with 2-0 silk ties. A 20-gauge angiocath is inserted through a purse string suture (6-0 Prolene) into the proximal ascending aorta and attached the cardioplegia delivery system. The donor is heparinized. Cold UW cardioplegia is initiated, the vena cavae are tied off, the aorta is cross clamped and the proximal IVC incised for drainage. The heart is bathed in ice cold saline during cardioplegia. The heart is explanted after the pulmonary veins are ligated in situ.

Heart implantation: A midline abdominal incision is performed. The abdominal aorta and abdominal vena cava are isolated. Both vessels are

controlled using a single side-biting clamp. The recipient is not heparinized. The donor pulmonary artery is anastomosed end to side to the recipient's inferior vena cava using 7-0 Prolene. Then the ascending aorta of the donor is anastomosed to the recipient's abdominal aorta in the same manner. The clamp is removed and heart reperfused.

2.4. Orthotopic lung transplantation

Cynomolgus monkeys weighing 3 to 7 kg are selected for compatible ABO blood types. Animals are anesthetized and intubated using intramuscular (I.M.) Ketamine (10mg/kg), and maintained with light Halothane anesthesia and intermittent I.V. and/or I.M. Ketamine plus Valium (0.8mg/kg).

A single-lung allograft is chosen over a double-lung graft, because the remaining native lung will be life sustaining for the monkey, regardless of the status of the allograft. The left lung is chosen in preference to the right based on anatomic considerations, although this requirement is by no means absolute.

Donor Lung Procurement: The donor monkey is positioned supine and a median sternotomy is performed. The vena cavae and aorta are circumferentially dissected, and a large-bore cannula is inserted into the main pulmonary artery after heparinization (5mg/kg). After the administration of 500 micrograms of prostaglandin E2 (to promote pulmonary vasodilation in order to enhance subsequent distribution of the pneumoplegia), venous inflow is occluded, and the aorta is cross-clamped. Two liters of pneumoplegia solution (Perfadex; Vitrolife AB; Gothenburg, Germany) containing 500 micrograms of prostaglandin E2 per liter are flushed through the pulmonary circulation, with drainage provided by amputation of the left atrial appendage. Ventilation is maintained to prevent the development of atelectasis. Concurrently, the chest cavity is cooled with iced saline. The heart-lung block is then surgically excised and placed on iced saline. The left lung is then isolated from the heart-lung block, and the donor pulmonary artery, atrial cuff, and bronchus are prepared for implantation.

Lung implantation: The monkey is then positioned for a left anterolateral thoracotomy. The left chest is entered over the fifth rib, and the hilar structures of the native left lung are isolated. The right pulmonary artery is dissected free and surrounded with an implantable balloon occluder for use in post-transplant assessment of lung function. When the donor lung is ready for implantation, the recipient is heparinized (5mg/kg) and the native left lung is excised. The donor lung is then implanted, anastomosing the bronchus, the left atrial cuff, and the pulmonary artery in that order. After re-inflation of the donor graft, the lung is reperfused. The bronchial anastomosis is the wrapped with either a pedicle of pericardial fat or with an intercostal muscle flap to ensure bronchial anastomotic healing and integrity, in the face of high-level immunosuppression. The chest is then closed over two closed suction drains, which are removed postoperatively.

3. Chimerism and tolerance

Dr. Ben Cosimi at Massachusetts General Hospital (MGH) led one of the early transplant research groups using NHPs to model human biology. His group attempted to replicate in NHPs an observation first made in rodents, namely that a nonmyeloablative conditioning regimen and donor bone marrow infusion could induce mixed chimerism and tolerance of major histocompatibility complex (MHC)-mismatched renal allografts [7,8]. However, the regimen that induced mixed chimerism in mice failed to induce even transient mixed chimerism in their NHP model, presumably due to survival of residual recipient T cells in the lymph nodes and spleen. A one-month course of cyclosporine and splenectomy were therefore added to the protocol. The final protocol included total body irradiation (TBI, 1.5 Gy x 2), thymic irradiation (7 Gy), and anti–T cell antibody (horse anti-thymocyte globulin, ATG) followed by combined donor bone marrow (BM) and kidney transplantation, splenectomy and cyclosporine, which resulted in 11 of 13 recipients developing multi-lineage chimerism, and 9 surviving long-term with normal renal function [9]. Unlike the stable mixed chimerism seen in mouse experiments, chimerism in the macaques became undetectable within months of donor BM transplant (BMT) [8–10]. Nonetheless the donor kidney continued to function rejection-free, suggesting that peripheral, rather than central, tolerance mechanisms were largely responsible. To avoid splenectomy and improve the consistency of tolerance induction, the regimen was later modified by removing splenectomy and replacing it with a course of anti-CD154 monoclonal antibody (mAb). As with the earlier protocol, most NHP recipients developed transient chimerism and about 60% exhibited long-term allograft survival without the need for ongoing immunosuppressive drugs [11].

The MGH group, subsequently led by Dr. Kawai, investigated the mechanisms of tolerance in NHP renal allograft long-term survivors after short-term chimerism. They showed that tolerance could be abrogated years later by low-dose IL-2 infusions ($0.6–3.0 \times 10^6$ IU/m^2/day) for more than one week, possibly due to insufficient depletion of donor-reactive T memory (Tm) cells by the conditioning regimen [12–15]. Another lesson learned in this NHP model was that tolerance by transient mixed chimerism was organ-specific: cardiac allografts using the same regimen did not achieve tolerance [16] while lung allografts did [17].

The multi-day pretransplant conditioning regimen used by the MGH group does not lend itself to the setting of deceased donor transplantation due to the unpredictable timing of transplantation, nor to tolerance induction in previously transplanted living donor recipients. As a result, later efforts focused on developing a "delayed tolerance" protocol in which the renal transplant is performed under conventional immunosuppression followed by the conditioning regimen and BMT several months later [18–20]. However, alloreactive CD8+ Tm appeared to prevent induction of even transient chimerism. With the addition of a CD8-depleting mAb to the regimen and the resulting delayed homeostatic recovery of CD8+ T cells, 11 out of 13 NHPs developed transient mixed chimerism, and 70% demonstrated prolonged renal allograft function off immunosuppression [18]. Interestingly, despite induction of chimerism, the delayed tolerance protocol uniformly failed if applied one month after renal transplant when rejection was associated with significantly elevated serum levels of the inflammatory cytokine IL-6. However, the regimen could be successfully applied at 4 months after the renal transplant when the inflammatory response had subsided [19]. Given the lack of anti-CD154 approval for use in humans, the regimen was altered, substituting belatacept for anti-CD154, with 4/5 treated NHPs achieving transient chimerism and 3/5 achieving long-term renal allograft survival without continuous immunosuppression [21].

The MGH group attempted to extend the approach of transient mixed chimerism for tolerance induction to islet allografts. Unlike previous experiments involving kidney allografts, tolerance to islet allografts could be achieved with combined transplantation of MHC-mismatched islets and BM, despite the development of transient chimerism [22]. In contrast, a case report of a NHP previously made tolerant to a renal allograft through same-donor BM transplantation under nonmyeloablative conditioning demonstrated long-term, immunosuppression-free survival of a same-donor islet allograft transplanted 1000 days after the kidney-BM transplant [23]. The importance of the contribution of the allograft to tolerance induction in this preclinical model was confirmed in subsequent NHP studies, in which tolerance was induced to a composite islet-kidney graft transplanted 20-22 (n=3) or 208 (n=1) days after previous hematopoietic stem cell transplantation under nonmyeloablative conditioning [24]. Composite islet-kidney grafts were created by performing a donor partial pancreatectomy, harvesting the islet cells, and injecting them beneath the donor's kidney capsule at least three months before allogeneic islet-kidney transplantation.

Tolerance has been achieved in NHP [8,9,11] and now in human (see below) [25–27] kidney allotransplantation by combining

nonmyeloablative conditioning with donor bone marrow transplantation, resulting in transient donor chimerism. The mixed chimerism-approach that succeeded in NHP renal allograft recipients failed to induce tolerance in allogeneic heart transplant recipients despite the achievement of similar levels of chimerism [16]. The reasons for the different outcomes in different transplanted organs are unclear. However, it has become apparent that some organs, such as the kidney and the liver, are tolerance-prone while others, such as the heart and to some extent the lung, are tolerance-resistant [28]. The inherently tolerogenic nature of the kidney has been demonstrated by cotransplantation of heart and kidney allografts from the same donor. By cotransplanting kidneys, long-term stable tolerance of heart allografts has been achieved in NHPs, whereas the hearts would have been acutely rejected if transplanted alone [29,30]. Animals in these experiments were the first NHPs to demonstrate tolerance of cardiac allografts, demonstrating the possibility of achieving long-term tolerance of heart allografts in a consistent manner, an essential step forward in the effort to implement tolerance protocols for human heart transplant recipients. The necessity of donor kidney cotransplantation was also observed in attempts to induce tolerance of allogeneic islets through mixed chimerism conditioning [24]. The mechanism by which a renal allograft is able to confer tolerance upon a cotransplanted heart or islet allografts is unknown but probably results from the actions of cells or cell products intrinsic to the kidney (e.g. renal tubular epithelial cells [31], plasmacytoid dendritic cells [32], or erythropoietin [33]) which are especially effective at activating and expanding recipient regulatory T cells (Tregs) [28]. A better mechanistic understanding could lead to new approaches for developing tolerance in tolerance-resistant organs and tissues without the need for renal cotransplantation.

4. First demonstration of lung allograft tolerance

The “delayed tolerance” concept was recently combined with anti-IL-6 -receptor mAb (tocilizumab) therapy and applied to cynomolgus recipients of MHC disparate orthotopic lung allografts. One animal developed transient mixed chimerism and displayed long-term allograft survival but developed chronic rejection associated with obliterative bronchiolitis. Most importantly, two monkeys displayed permanent mixed chimerism, donor-specific T and B cell unresponsiveness and accepted permanently lung allografts without signs of chronic rejection. By incorporating tocilizumab into a mixed chimerism protocol, long term tolerance of NHP lung allografts has been achieved for the first time [17].

5. Human translation of NHP chimerism studies

The MGH group sucessfully translated work in their NHP kidney model to the clinic, initially performing HLA-mismatched living donor kidney transplants [25,26,34]. Rather than using irradiation and ATG, patients were treated with cyclophosphamide and anti-CD2 mAb to deplete lymphocytes based on experience with HLA-identical kidney/BM transplantation for refractory myeloma with renal failure [35]. All 10 enrolled patients developed transient chimerism, but antibody-mediated rejection (AMR) was observed in two of the first three subjects, prompting the investigators to add rituximab and early post-transplant steroids. Additionally, three patients experienced early acute kidney injury (AKI), presumed to occur secondary to cyclophosphamide, which was associated with loss of chimerism. When the drug was replaced with TBI in two patients, AKI did not occur. Ultimately seven of the 10 were weaned off immunosuppressive drugs and maintained good graft function for 4 or more years. Four of the seven have remained off immunosuppression (4.5-11.4 years) while the other three resumed taking immunosuppressive drugs due to chronic rejection or recurrence of original disease [26].

Several other groups have developed their own mixed chimerism NHP models for transplant tolerance. Dr. Leslie Kean’s group has focused on translating their successful results in a mouse model to achieve and maintain multilineage mixed chimerism in the macaque. Their regimen used donor BM or leukophereisis-derived hemataopietic stem cells and included a reduced-intensity busulfan-based conditioning regimen combined with immunosuppression using anti-CD154 or anti-CD40, belatacept, and sirolimus. This regimen resulted in persistent myeloid but not lymphoid chimerism, and while greater MHC-matching predicted longer survival, all recipients eventually rejected their grafts. These results support mouse data showing the necessity of T cell engraftment for allograft tolerance [36]. In MHC-matched pairs, the addition of low dose TBI (200-300cGy) enabled the development of durable, multilineage mixed chimerism with significant T cell chimerism, which is associated with tolerance induction; however, while on immunosuppression infectious complications (predominantly CMV reactivation) provided evidence of compromised anti-viral immunity and prevented six out of nine animals from reaching the protocol endpoint. Notably, for those animals successfully weaned from immunosuppression, a direct correlation between donor allograft survival and percent T-cell chimerism was observed [37]. Kaufman and Strober saw promising results in their rhesus model using a posttransplant tomotherapy-based total lymphoid irradiation (TLI) and ATG conditioning regimen coupled with mobilized peripheral blood CD34+ HSCs plus CD3+ T cells from the kidney donor (personal communication). This model is built on previous rodent and clinical work showing that TLI conditioning, as compared to TBI, is less apt to induce graft-versus-host -disease (GVHD) and may be safely applied early posttransplant [37,38]. Indeed, the approach successfully induced tolerance in human recipients of living, HLA-identical kidney transplants, with 16 of 22 HLA matched patients successfully withdrawn from immunosuppression for up to 5 years without rejection episodes or kidney disease recurrence. Extending this approach to HLA-mismatched donor/recipient pairs has proven more challenging [39]. In this regard, results from their unpublished NHP studies have informed the design and implementation of a human clinical trial in a three antigen mismatched kidney transplant protocol. Future work in this model aims to take advantage of the posttransplant conditioning regimen to extend the approach to deceased donor organs.

Clinical translation of the mixed chimerism approach continues to be guided and refined by NHP data. Perhaps the major lesson from these studies with regard to chimerism, is that although mice do not develop tolerance without stable mixed chimerism, such protocols in NHP and humans that produce only transient chimerism have the potential for long-lasting tolerance. The murine immunosuppressive regimen was necessarily altered in humans to address the problem of immunologic memory, and the NHP intermediate facilitated the translation of this conceptual approach. Despite a measure of clinical success in kidney transplantation, efforts are underway to establish consistent, preferably multilineage, chimerism and improve the durability of chimerism achieved, while reducing the risk of GVHD and maintaining functional viral immunity. Additional research is required to modifiy the models to allow for use of deceased donor organs and to extend the approach to other organs and cells. The NHP model has highlighted the need for a better understanding of the mechanisms involved in mediating tolerance so these pathways can be targeted in a way that will improve chimerism without sacrificing safety.

6. Immune cell depletion and tolerance

Although immune cell depletion, principally T cell depletion using polyclonal anti-thymocyte globulin (ATG), has long been used in solid organ transplantation to either treat rejection or as induction therapy [40,41], NHP experiments suggested the possibility of achieving peripheral donor-specific tolerance by T cell depletion at the time of transplantation [42,43]. Intended for treatment of T cell malignancies, Neville’s development of a CD3 immunotoxin enabled the depletion of peripheral blood T cells as well as lymph node T cells by 2–3 logs [44]. When

CD3 immunotoxin was applied to an NHP renal allograft model, most treated recipients remained rejection-free for over 100 days, and many developed tolerance to subsequent donor skin grafts but rejected third-party grafts [42].

Subsequent work sought to extend this observation by interrogating the necessary extent of T cell depletion, the influence of co-administration of conventional immunosuppressants on tolerance induction, and how to more reliably induce tolerance by T cell depletion. These efforts in NHPs led to the realization that such tolerance was "metastable" and might be broken later by various immune perturbations, including a skin graft [45,46]. The demonstration of NHP tolerance by peripheral depletion provided the foundation for future clinical trials. When it became apparent that development of an analogous human CD3-IT would be difficult, Calne [47] and then U.S. groups [48] adopted alemtuzumab (Campath-1H), a monoclonal antibody that binds CD52 on mature lymphocytes for depletion. Kirk's NIH study tested high-dose alemtuzumab in seven consecutive renal transplant recipients and found similar levels of lymphocyte depletion as the CD3-IT in NHPs; however, all human recipients developed atypical early rejection, characterized by a monocytic infiltrate, that responded to steroids and subsequent sirolimus monotherapy [48]. Unfortunately the utility of the NHP model to study alemtuzumab-based regimens is limited by the fact that alemtuzumab has a lower affinity for NHP CD52, necessitating relatively high doses, and the fact that CD52 is expressed on erythrocytes of macaques other than Indonesian-origin cynomolgus macaques, meaning that alemutuzumab treatement in most NHPs would result in lethal hemolytic anemia [49]. Thus, comparing the NHP data to an analogous human trial reveals similar though not identical results: while monkeys remained free of acute rejection, humans developed an atypical morphology of rejection that was rescued with additional immunosuppression. Nonetheless, the NHP alemtuzumab studies in Indonesian-origin cynomolgus macaques suggest the resistance of Tm cells to depletion and/or homeostatic expansion of effector Tm post-depeletion – a phenomenon also seen in patients receiving alemtuzumab induction therapy – might impede establishment of long-term tolerance [49,50] (reviewed in [51]).

This experience suggested that even compared to closely related NHP species, human immune responses may require more sophisticated manipulations to induce long-lasting tolerance. The basis for this greater resistance to tolerance may be related to a larger repertoire of immune cells or to more extensive immunologic exposure and heterologous responses in humans compared to captive species [52,53]. Although alemtuzumab treatment has not led to tolerance, it has enabled reduction in maintenance immunosuppression and is now a clinically used induction antibody in clinical renal transplantation, used in about 10% of patients by off-label use in the United States [54].

Most NHP renal allograft recipients with long-term survival following depletion by CD3-immunotoxin developed alloantibody and subsequently progressed to chronic antibody-mediated rejection. Despite acceptance of donor skin and rejection of third-party skin grafts, tolerance was "split," in that it did not include B cell tolerance [55]. Notably, in an Immune Tolerance Network–sponsored clinical trial, kidney transplant recipients treated with alemtuzumab also developed anti-donor antibody in 50% of cases while on sirolimus maintenance monotherapy [56]. Thus the NHP model accurately predicted the human outcome, with both species showing a propensity for B cell activation leading to alloantibody following T cell depletion. In 2009, Bloom et al. reported that B cell activation factor (BAFF) was elevated following T cell depletion in humans, identifying a possible molecular mechanism underpinning the observed B cell activation [57]. Additional work sought to elucidate the roles of such B cell cytokines in driving B cell activation in the setting of T cell depletion. The NHP model demonstrated that following T cell depletion with CD3-immunotoxin, BAFF blockade with atacicept (which targets both BAFF and APRIL) was able to prevent B cell activation and alloantibody generation during treatment [58]. These observations supported the rationale for a subsequent clinical trial in renal transplantation in which alemtuzumab induction is followed by belatacept and sirolimus maintenance therapy. In this case, belatacept may prevent the development of de novo alloantibody [59].

Monkeys treated with T and B cell depletional induction and rapamycin monotherapy for maintenance enjoyed long-term islet allograft survival (>1,500 days in one NHP) associated with a predominance of immature and transitional B cells in the peripheral blood [60]. This regimen did not induce tolerance as donor-specific alloantibodies developed after discontinuation of rapamycin at day 200 posttransplant. A clinical trial designed to test the efficacy of islet allotransplantation in restoring insulin independence in type 1 diabetic islet recipients treated with anti-thymocyte globulin and rituximab for induction combined with rapamycin for maintenance immunosuppression (i.e. the regimen proven effective in monkeys) was terminated because of lack of efficacy in the clinical setting (ClinicalTrials.gov Identifier: NCT00468442).

7. Cardiac transplant applications of depletion

An effective treatment to prevent cardiac allograft vasculopathy (CAV), a major cause of mortality and morbidity following heart transplantation, is not available. Hopes rose when compelling data in NHPs demonstrated that depletion of CD20+ B cells with rituximab reduced CAV [61]. Rituximab showed effective B cell depletion in peripheral blood, secondary lymphoid organs, and the cardiac allograft. Combination therapy consisting of CD20+ B cell depletion and the calcineurin inhibitor cyclosporine A (CsA) resulted in improved median primary graft survival compared to treatment with either anti-CD20 or cyclosporine alone. Within the group of animals treated with both anti-CD20 and CsA, efficient B cell depletion substantially inhibited DSA production and markedly reduce the CAV severity score. These findings suggested that preemptive CD20+ B cell depletion in combination with a conventional immunosuppressive drug could mitigate chronic rejection. These encouraging findings led to the CTOT-11 study (ClinicalTrials.gov NCT01278745) which asked whether early treatment with rituximab to deplete B cells and diminish DSA production would attenuate CAV in human heart allotransplant recipients treated with conventional immunosuppression. The disappointing results showed that, compared to the placebo group, patients treated with rituximab demonstrated an increase, rather than a decrease, in intimal thickness at 12 months [62]. However, rituximab was not associated with increased rejection or development of anti-HLA antibodies. Longer term follow up is required to understand the effects on graft function and patient survival.

8. Costimulation blockade

In 1996, murine experiments by Larsen et al. showed that CD28 blockade using CTLA4Ig combined with CD40 ligand blockade prevented rejection of allogenic skin grafts [63]. A number of other investigators confirmed this observation in murine solid organ transplant models, suggesting that manipulation of these pathways could lead to immune tolerance while avoiding the risks of immune cell depletion [64] and toxicities associated with calcineurin inhibitors (reviewed in 21). In the transplant setting, murine models are notoriously easier to tolerize compared to humans [65]. Kirk et al. were the first to apply costimulation blockade to a NHP renal transplant model, showing prolonged allograft survival and suppression of *in vitro* T cell responses in MHC mismatched donor–recipient pairs [66]. This work spurred further efforts to plan clinical introduction of costimulation blockade in human transplantation, specifically to better understand the merits of using CTLA4Ig or anti-CD154 in combination with more conventional FDA-approved immunosuppressive drugs such as calcineurin inhibitors, mycophenolic acid, and steroids in order to pave the way for clinical translation [67].

9. CD40-CD154-directed costimulation blockade: on-target potency derailed by unexpected toxicity

Due to encouraging results with anti-CD154, a clinical trial was initiated at the NIH, University of Wisconsin, and University of California, San Francisco in renal transplant patients under the auspices of Biogen, Inc. Unexpected thrombotic complications led to early trial termination [68]. Further investigation revealed that expression of CD154 on platelets led to platelet aggregation when exposed to the anti-CD154 fusion protein [69,70]. Subsequently at least two other commercially developed anti-CD154 agents modified with the goal of avoiding thrombotic side effects were found efficacious in preventing rejection [71,72], but failed to progress in clinical development, in part due to concerns about their association with thrombosis. Twenty years later Bristol Myers Squibb (BMS) developed an anti-CD154 without thrombotic tendences by engineering a domain antibody lacking Fc binding activity. This drug effectively prevented NHP renal allograft rejection [73]. Unfortunately, despite efficacy in a human phase II clinical trial, further clinical development was halted. In contrast, CD154's receptor, CD40, has been targeted in experimental NHP islet, heart, and kidney transplant applications with promising results and currently at least one anti-CD40 antibody is in clinical trials for human transplant application [74,75]. The NIH-sponsored NHP Reagent Resource program has played a critical role in making a rhesus version of anti-CD40 (2C10R3) available to investigators.

The extraordinary efficacy of monotherapy with anti-CD154 in prolonging renal allograft survival in rhesus monkeys [66] was confirmed in islet allotransplant models in monkeys [76] and baboons [77]. In striking contrast to other strategies studied in monkeys, such as calcineurin inhibitor-based regimens [78], transplantation of viable allogeneic islets in adequate numbers under anti-CD154 monotherapy consistently allowed for islet engraftment and long-term insulin independence [76]. Because of the importance of the CD40:CD154 costimulatory pathway and the thromboembolic complications associated with anti-CD154 antibodies in renal transplant pilot clinical trials [79], subsequent studies examined the potency of antagonistic anti-CD40 monoclonal antibodies in islet allotransplantation in monkeys. Several antibodies (2C10R4, 3A8, ASKP1240) proved remarkably efficacious in this model without evidence of thromboembolism or B cell depletion [80–82], thereby providing a strong rationale for continued development of CD40-specific biologics for clinical translation.

To realize the full potential of biologics targeting the CD40:CD154 costimulatory pathway, adjuvant strategies for use with these biologics have been investigated. Studies in mouse models showed that activation induced cell death facilitated by CD154 blockade is potentiated by rapamycin [83] and that pre-transplant infusion of donor antigen infused under the cover of CD154 blockade prolongs rejection-free allograft survival [84]. Prompted by these studies, Kirk's group explored the tolerogenic potential of pre-transplant donor-specific transfusion in combination with a short course of anti-CD154 and rapamycin. This regimen improved survival in the exceptionally rigorous model of skin allotransplantation in monkeys [85] and led to operational renal allograft tolerance in three of five monkeys including donor-specific skin graft acceptance in the two animals tested [72]. Likewise, this regimen facilitated months of allograft function after cessation of immunosuppression in an NHP model of islet allotransplantation [86].

To increase the tolerogenic potency of donor antigen, the Luo and Miller group induced apoptosis in donor leukocytes prior to their peritransplant infusion [87]. Intravenous infusions of ethylcarbodiimide (ECDI)-treated apoptotic donor splenocytes on days -7 and +1 relative to transplant on day 0 induce robust tolerance to fully MHC-mismatched islet allografts [87], and, when combined with short-term rapamycin, also to heart allografts in mice [88]. Mechanisms of tolerance induced by allo-antigen delivery via apoptotic donor leukocytes in these models involve clonal anergy of anti-donor $CD4^+$ T cells with direct specificity, clonal deletion of anti-donor $CD4^+$ T cells with indirect specificity, and regulation by $CD4^+$ Tregs and myeloid-derived suppressor cells (MDSC) [89–91]. The tolerogenic efficacy of apoptotic donor leukocytes has recently been investigated in two NHP islet transplant models. The MGH group demonstrated prolonged islet allograft survival for up to 133 days in NHPs treated with a single intraportal infusion of apoptotic donor leukocytes on day 0, a single dose of anti-thymocyte globulin, three doses of anti-IL-6R antibody, and a 30-day course of rapamycin [92]. Investigators at the University of Minnesota and Northwestern University examined the efficacy and immunobiology of peritransplant intravenous infusions of apoptotic donor leukocytes on days -7 and +1 under the cover of transient costimulatory blockade, rapamycin, and cytokine antagonists through day +21 posttransplant. This regimen induced long-term (≥1 year) operational tolerance to intraportal islet allografts in five of five non-sensitized, MHC class I-disparate and one MHC class II DRB allele-matched rhesus macaques. Operational tolerance in this model was associated with dominant regulation, which suppressed the proliferation and cytolytic effector function of donor-specific CD8+ effector memory T cells present in non-tolerant controls that received the identical immunotherapy but no negative vaccination with donor antigen.

Targeting the CD40/CD154 pathways in a NHP heart transplant recipients has succeeded in prolonging allograft survival but not inducing tolerance (reviewed in [93]). CD154 blockade with hu5C8 prolonged heart allograft survival and attenuated CAV when combined with selective CD28 blockade [94]. IDEC-131 also prolonged cardiac allograft survival but was less efficacious than hu5C8 [95].

10. CD28 blockade: human translation of a NHP success

Further development of CTLA4Ig continued with investigation of possible tolerance approaches in a NHP renal allograft model. However after multiple efforts, including combining costimulation blockade with cyclosporine, steroid, busulfan (immune cell depletion) and/or donor leukocyte infusion, the Emory group concluded that tolerance was not likely to be achieved using CTLA4Ig, especially as monotherapy, and efforts should instead focus on developing the agent as a continuous maintenance immunosuppressive drug. Furthermore, CTLA4Ig itself lacked adequate potency in blocking the interactions between CD28 and CD80/86 for NHP or human purposes [96]. In response to the potency issue, BMS engineered belatacept, a construct with higher affinity for CD80/86. After successful NHP trials of belatacept in renal transplantation, the drug entered human clinical trials and was approved in 2011 by the FDA as the first calcineurin inhibitor-free alternative immunosuppression for human renal transplantation use [97,98]. Belatacept results in higher glomerular filtration rate (GFR) and lower cardiovascular risk for renal transplant patients despite a higher rate of rejection compared to calcineurin-inhibitor based immunosuppression. Clinically the drug continues to lag behind tacrolimus in renal transplantation, however its use is increasing in the United States as clinicians observe the long-term data showing superior graft function and improved metabolic profile. Belatacept provides the best current example of a drug that depended directly and critically on a NHP solid organ transplant model for its development and successful transition to human clinical trials including FDA approval and commercial marketing. Although murine systems showed immunosuppressive properties and even tolerance in some regimens using CTLA4Ig, the analogous drug from BMS, abatacept, lacked adequate efficacy in NHP to progress to human application in solid organ transplantation. Because CTLA4Ig/belatacept binds the shared ligands of both CTLA-4 and CD28, it not only blocks activation of effector T cells via CD28, it also blocks the inhibitory signals downstream of CTLA-4, including those involved in Treg function. This may be related to its lack of tolerance induction in NHP. In other words, the NHP transplant model provided a critical and predictive link to human application that the murine model was unable to deliver.

Bluestone's group reported in 1997 the first studies of a CD28 antagonist in a preclinical islet allotransplant model. A two-week course of

CTLA4-Ig (abatacept) prolonged graft survival in cynomologus monkeys, which was associated with donor-specific hyporesponsiveness in MLR assays [99]. Post-transplant humoral responses to the graft were suppressed in all treated monkeys. In subsequent studies by the Emory group, induction with IL-2R antibody and maintenance with LEA29Y (belatacept) combined with rapamycin prevented islet allograft rejection in mismatched rhesus monkeys, and prevented priming of anti-donor T and B cell responses [100]. This calcineurin inhibitor- and steroid-free maintenance immunosuppression protocol was successfully translated to the clinical setting when combined with anti-thymocyte globulin for induction. All five type 1 diabetic patients achieved insulin independence after a single intraportal islet allotransplant and islet allograft function was maintained for several years [101,102].

11. Targeting T cell memory

Evidence suggests that in contrast to specific pathogen-free mice, antigenic exposure characterisic of humans and socially-housed NHPs results in immunologic memory responses, including allo-reactive responses, that impede induction of transplant tolerance (reviewed in 52 and 53). Indeed, Tm cell populations, which are less dependent on CD28 for activation, are thought to mediate the acute rejection noted in clinical trials of belatacept, a phenomenon referred to as costimulatory blockade resistant rejection (CoBRR). NHP studies predicted CD28-CoBRR and continue to focus on ways to address it (reviewed in [103]). Additionally, homeostatic repopulation following cell depletion with alemtuzumab or ATG results in expansion of effector Tm cells or both effector and central Tm cells, respectively (reviewed in [104]). Therefore, to prevent rejection and promote tolerance numerous studies have attempted to more specifically target Tm cells.

LFA-1 is an adhesion molecule with multiple functions preferentially expressed by Tm cells relative to naïve T cells. LFA-1 blockade as monotherapy had shown limited success in extending cardiac allograft survival in a NHP model [105] and was examined for its potential to augment belatacept-based therapies. When used as a short-term induction therapy in combination with belatacept or basiliximab and sirolimus in the NHP islet model [106], anti-LFA-1 antibodies significantly extended allograft survival. However the same group saw no improvement in renal allograft survival compared to belatacept alone when either anti-LFA-1 [107] or agents specifically tartegting the high-affinity actived form of LFA-1 [108] were added to the regimen. Of interest within the context of limited efficacy in renal transplantation in preclinical and clinical studies, a pilot islet allotransplant clinical trial in eight recipients with type 1 diabetes showed excellent immunosuppressive efficacy of maintenance therapy with the anti-LFA-1 antibody efalizumab combined with rapamycin in recipients given anti-thymocyte globulin for induction [101]. Development of efalizumab (anti-LFA-1) in transplantation stopped when the drug, licensed for treatment of psoriasis, was voluntarily withdrawn from the market after three out of approximately 40,000 patients (0.008%) developed progressive multifocal leukoencephalopathy, a rare but potentially fatal disease associated with the JC polyoma virus [109,110].

Thus, despite initial evidence in NHPs that adhesion molecule blockade with anti-LFA-1 might prevent rejection mediated by Tm cells [111], the model also suggested a limited efficacy of LFA blockade with respect to preventing renal transplant rejection [107,108]. This observation presaged the human clinical trial data in renal transplantation in which the drug failed to meet efficacy endpoints.

Similar to the observations summarized for LFA-1 blockade, evaluation of the ICOS–ICOS-ligand pathway revealed that a novel ICOS-Ig human Fc-fusion protein either alone or in combination with belatacept did not prolong NHP renal allograft survival. This agent has not progressed in human transplant trials given its limited efficacy in the NHP transplant model [112].

Recently, use of a humanized anti-OX40L antibody in combination with belatacept significantly prolonged renal allograft survival in the NHP compared to either agent given as a monotherapy [113]. OX40 is expressed predominantly on activated T cells, influences their effector function, and plays a critical role in the generation of T cell memory [114]. Although tolerance was not achieved with this protocol, the combined therapy was nondepleting, suppressed development of DSA, and did not appear to significantly compromise protective immunity. A human clinical trial using the antibody to treat asthma similarly noted no safety concerns [115], while an islet allograft study in mice indicated that OX40 blockade limited T effector responses without restricting Treg activity, promoting tolerance in the context of CD154 deficiency [116]. Taken together, these results suggest that inclusion of anti-OX40L in clinical transplantation protocols could prove beneficial.

The Kirk group has continued to investigate ways to overcome belatacept-resistant rejection without resorting to calcineurin inhibitors [59]. Lo et al. reported that sirolimus combined with belatacept successfully prevented renal allograft rejection in all animals in a NHP model, skewed the T cell population to a naïve phenotype, and did not require depletional induction therapy [117]. However, Tregs declined twofold and treatment did not lead to tolerance as evidenced by rejection following drug withdrawal. This NHP protocol accurately predicted the utility of combined belatacept–sirolimus in human renal transplantation, as later reported by Kirk et al. using alemtuzumab induction, belatacept–sirolimus maintenance therapy, and complete avoidance of calcineurin-inhibitors and steroids **[59]**. In this 20-patient human protocol, most have remained rejection-free and were weaned to belatacept monotherapy, suggesting this approach should be investigated further as a lower-toxicity immunosuppressive protocol associated with excellent graft function and no graft loss.

Promising results in both a baboon renal transplant model and a cynomolgus macaque cardiac transplant model with sc28AT, a monovalent antagonist antibody to CD28, in combination with calcineurin inhibition [118] led to the development of a humanized version with a longer half-life, FR104, for further study. In the context of NHP renal transplantation, FR104 was effective in preventing rejection, promoting allograft survival, and inhibiting generation of DSA in CNI-free or CNI-low treatment regimens [119]. Furthermore in a similar model incorporating a 1-month course of low-dose tacrolimus, FR104 was superior to belatacept in preventing steroid-resistant acute rejection, possibly related to IL-21 modulation of T follicular helper cell recall responses [120]. Clinical trials in autoimmunity using FR104 and a similar agent, lulizumab, are currently underway (reviewed in [121]).

12. Regulatory cell therapies and tolerance

Because increased Tregs and regulatory dendritic cells (DCregs) are associated with tolerance and stable allograft survival, a rationale has emerged for clinical testing of regulatory immune cells generated and/or expanded ex vivo as cell-based therapy to reduce dependence on pharmacologic immunosuppression [122,123]. Preliminary work focused on methods to mobilize and characterize NHP regulatory cell populations [124–126]. Ezzelarab reported that short-term costimulation blockade and tapered rapamycin in combination with vitamin D3/IL10-conditioned, maturation-resistant, donor-derived DCregs infused seven days before transplant significantly prolonged NHP renal allograft survival [127]. This result was achieved without sensitizing the host and with selective attenuation of T memory responses to donor. In addition, expression of the T-box transcription factor Eomesodermin (Eomes), which is known to regulate Tm survival/persistence, inversely correlated with graft survival and may prove useful as a biomarker predictive of organ transplant outcomes [128]. Plans for phase I/II clinical trials of DCregs in kidney and liver transplantation are currently underway. Notably, when a similar approach was used with unpulsed, or donor antigen-pulsed host DCregs delivered 1 day pre-transplant – a protocol possible with deceased donors – protection was, respectively, absent or

modest but not significant. Differences in the amount of donor antigen delivered or the timing of infusion were cited as possible explainations for the disappointing results [129].

This NHP work was preceded by rodent work in cardiac transplantation models showing that 1) donor DCregs infused one week before transplantation, combined with perioperative lymphodepletion, resulted in permanent, donor-specific allograft survival in 50% of rats [130]; and 2) recipient DCregs were more effective than donor DCregs and significantly prolonged allograft survival when administered 1 day pre-transplant [131]. Although working in different organ models, these rodent results were either less pronounced or simply not observed in the NHP, respectively. This once again illustrates the benefit of proceeding logically from rodent to NHP transplant models before human application when possible, given the uncertainty of the outcome in higher, outbred species. As a further caution, when infusing Tregs in NHPs rather than DCregs, the same group noted that alloantibody responses and Tm cell responses were unexpectedly heightened by the Treg infusion and accelerated rejection of renal allografts [132]. Two prior murine transplant studies did not accurately predict this outcome [133,134], although conversion of Foxp3+ Tregs into effector cells had been described previously in lymphopenic mice [135] offering a possible explaination for the unfortunate outcome. One murine study did show rapid rejection of skin and heart allografts with infusion of allo-specific Tregs alone, as opposed to infusion of allo-specific Tregs combined with hematopoietic stem cells [136]. Given the inconclusive data in rodents, the results of this NHP study further underscore the importance of the NHP intermediate in revealing unanticipated limitations of an approach, sparing human injury.

The Sykes group tested Treg infusion in the mixed chimerism model as a means of extending donor chimerism without increasing toxicity of the regimen or risk of GVHD [137]. For this protocol a conditioning regimen of total body irradiation, thymic irradiation, and ATG preceded BMT, polyclonal host Treg infusions, and treatment with anti-CD154 and cyclosporine. Kidney transplants were done four months later, a time previously shown to result in rapid rejection using this protocol without Tregs [9]. While addition of Tregs was able to prolong a state of chimerism that included T cell chimerism, and promote tolerance in one animal compared to controls, CMV reactivation and antiviral treatment prevented sustained chimerism or caused severe complications in multiple animals, indicating substantial refinement of the protocol will be necessary before attempting clinical translation.

13. Tregs and Tmems in heart vs. kidney recipients

Infusion of regulatory immune cells has prolonged renal allograft survival in nonhuman primate transplant models [129,138]. However, in a heterotopic NHP heart transplant model, animals receiving adoptive Treg infusions did not see extended allograft survival compared to control animals not receiving infusions. In fact, administration of multiple Treg infusions decreased allograft survival. One explanation is the observed accelerated recovery of effector T cells, particularly effector memory CD8 T cells, B cells, and an accelerated and high-level production of donor-specific antibodies after Treg infusions [132]. These cautionary results raise important questions, including: 1) How does recipient conditioning and/or maintenance immunosuppression affect the infused Tregs? 2) Do infused Tregs retain their regulatory properties, or can they adopt effector cell properties? And 3) Are Tregs going to be more efficacious for kidney versus heart allografts? [139] Addressing these issues in NHP models will serve to improve the safety and efficacy of human Treg immunotherapy.

14. Mesenchymal cell therapy

Multipotent mesenchymal stem cells (MSC) have immunosuppressive and regenerative properties, and are currently under development as a cellular therapy for a variety of conditions and diseases [140]. Kenyon's group, using a NHP marginal islet mass and bone marrow transplant model to address islet engraftment and early loss, showed for the first time in a NHP model that intravenous infusion of either islet-donor or third-party marrow MSCs more than doubled islet engraftment compared to animals without MSC infusion and was accompanied by an increased percentage of Tregs in the circulation. Additional MSC infusions could reverse episodes of islet graft rejection. Furthermore, they provided evidence that the inflammatory response during rejection may enhance the efficacy of MSC suppression [141]. This study supports the incorporation of MSCs as an adjunct cellular therapy in islet transplantation.

15. Prevention and treatment of alloantibody as a barrier to tolerance

While most tolerance efforts in transplantation have focused on developing T cell tolerance, it has also become clear that alloantibody prevents tolerance and increases graft loss [142,143]. Managing the B cell response and preventing the development of alloantibody and plasma cells has become one of the most pressing needs in transplant biology and clinical transplantation. NHP models can address some of these issues. Similar to humans, anti-donor antibody in NHPs predicts chronic graft injury and, if not causative, is at least associated with graft loss [144,145]. Table 1 summarizes evidence supporting a role for AMR in graft injury in NHP experimental models.

B cell alloimmunity in the NHP model has been characterized pathologically showing a correlation of alloantibodies in NHP renal allografts with classic histologic features of AMR [152]. The temporal progression of chronic AMR was demonstrated to correspond with the human biology of antibody-mediated graft injury as defined by the 2005 Banff consensus [153]. Capillary C4d deposition and alloantibodies in transplanted grafts are essential in diagnosing AMR in humans [154,155]. Thus NHP models of AMR accurately parallel human pathology and offer not only a safe and effective way to evaluate human therapeutics targeting B cells and plasma cells, but also provide a means of probing mechanisms of humoral immunity relevant to human transplantation.

16. NHP models of antibody-mediated rejection

Early T cell depletion with CD3 immunotoxin in combination with low-dose tacrolimus and alefacept (anti-LFA-3) reliably results in development of donor-specific antibody (DSA) within one month post kidney transplant in a NHP model of de novo AMR [149]. Using this model, Kwun reported that neutralizing BAFF/APRIL with atacicept prevented formation of DSA and development of AMR; however, when atacicept treatment was discontinued alloantibody and AMR progressed [58]. Using the same NHP de novo AMR model, Kim et al. reported that costimulation blockade with either belatacept or anti-CD40 mAb prevented alloantibody formation and decreased isotype switching and central Tm cells. Germinal centers (GCs) experienced a decrease in B cell clonal expansion, follicular helper T (Tfh) cells, and IL-21 production. Thus the addition of costimulation blockade suppressed Tfh cells, ultimately preventing AMR [150].

Having shown the benefit of costimulation blockade in preventing de novo alloantibody, efforts turned to reversing sensitization in a NHP model. Sensitization, meaning prior immunization to non-self MHC antigens, remains a significant unsolved issue in human heart, lung, and kidney transplantation. Therefore, a relevant NHP model of sensitization could prove useful for both understanding mechanisms of B cell activation/inactivation and for developing more effective therapies, including how to best disrupt plasma cell alloantibody production. In 2016 Burghuber first reported the essential features of this model [151], which uses skin grafting to sensitize monkeys followed by an observation of the kinetics of alloantibody production and decay. During the period of linear decay, monkeys were treated with

Table 1
Models of antibody-mediated rejection.

First author (ref)	Species	Organ	Treatment regimen
Haanstra [142]	Rhesus	Kidney	Anti-CD40 + Anti-CD86
Kawai [16]	Cynomolgus	Heart	TBI, TI, ATG, splenectomy, BMT
Smith [146]	Cynomolgus	Kidney + BMT	TBI, BMT, anti-CD154, splenectomy, anti-CD8
Torrealba [46]	Rhesus	Kidney	CD3-IT
Azimzedeh [147]	Cynomolgus	Heart	Anti-CD154 + BMT +/- ATG
Schroder [148]	Cynomolgus	Heart	Anti-CCR5 + CsA
Page [149]	Rhesus	Kidney	CD3-IT, anti-LFA3, Tac
Kim [150]	Rhesus	Kidney	(CD3-IT, anti-LFA3, Tac) plus belatacept or anti-CD40
Burghuber [151]	Rhesus	Kidney	bortezomib, anti-CD40, belatacept

novel agents to lower their alloantibody levels and target existing plasma cells and GCs, the locus of Tfh-mediated B cell activation. Subsequently Kwun made the seminal observation that proteasome inhibition alone, which resulted in plasma cell depletion, could activate a compensatory GC response, increasing circulating IgG+ B cells and increasing proliferating (Ki67+) B cells in the GC B cell follicles. Tfh were likewise expanded [156]. Building on this mechanistic observation, proteasome inhibition combined with costimulation blockade to block the GC response resulted in depletion of plasma cells, reduction of Tfh and B cell proliferation, lowering of alloantibody levels, and elimination of AMR-mediated injury of renal allografts [156].

The activation of the coagulation cascade by antibody and complement has prompted investigation of anti-complement strategies to prevent AMR in NHP models, as agents developed for human use often cannot be interrogated directly in rodent models and preclinical trials are mandated by ethical considerations [4,157].

17. Protective immunity in immunosuppressed solid organ transplant recipients

The impact of immunosuppressive regimens on protective immunity in terms of infection and malignancy requires critical evaluation and again demonstrates both similarities and differences between humans and NHP. A clinical report noted a decreased risk of cytomegalovirus (CMV) infection in transplant recipients receiving sirolimus as compared to other immunosuppressive agents [158]. In agreement with these clinical findings,Turner et al. reported on the advantages of sirolimus with respect to preservation of antiviral immunity and CD8 + T cell responses to vaccinia in rhesus macaques [159]. This work represented a pre-clinical test of the hypothesis that mTOR regulates CD8+ Tm differentiation and that rapamycin has immunostimulatory effects on the generation of memory CD8+ Tm cells [160]. Kean's group reported that CMV infection critically influences T cell reconstitution post-transplant, altering the T cell receptor repertoire [161]. Moreover the profound T cell depleting strategies used in NHP bone marrow chimerism protocols are known to pose additional risks of viral infection, including CMV (33) [162]. The use of anti-viral therapies has effectively prevented most CMV-related mortalities in human organ transplant patients, but CMV in NHP (a different, species-specific herpes virus) continues to be associated with mortality despite prophylactic and/or therapeutic anti-viral drug use. Severity of disease in NHP reflects the immunosuppressive potency of various drugs and drug combinations [163]. With respect to risk of malignancy, the rhesus macaque has demonstrated that broad immune targeting with combined CD3 depletion, calcineurin inhibition, anti-LFA1, and CD28 targeting may encourage lymphoma to develop [164], similar to observations made in humans who are heavily immunosuppressed [165].

18. Similarities and differences in NHP vs. human transplantation

With respect to triple drug immunosuppression (tacrolimus, mycophenolate mofetil, and steroids) that is the current standard for most human organ transplant immunosuppression, the same regimen effectively prevents renal allograft rejection in NHPs. However, this regimen does not prevent DSA and acute rejection in cynomolgous lung transplant recipients [166]. This may suggest that NHP lung transplantation represents a more stringent model immunologically than does lung transplantation in humans. Specifically, initial attempts to suppress lung allograft rejection using the combination of tacrolimus (FK; 0.2 mg/kg BID i.m. to maintain trough levels of 20-40 ng/ml), mycophenolate mofetil (MMF; 600 mg/square meter/dose by gavage), methylprednisone (MP; starting 2 mg/kg and tapering to a maintenance dose of 0.2 mg/kg i.m.) led to survival times between 21 and 97 days. However, the addition of anti-thymocyte globulin (ATG) induction therapy (50 mg/kg on days -2,-1,0) prevented ACR in two of three recipients, while the further addition of tocilizumab (10 mg/kg on days 0,7,14,21) prevented ACR and alloantibody formation in all recipients [166]. The need for supplementary immunosuppression to prevent ACR in NHP lung recipients as compared to humans may be explained by data showing that NHP lungs, in contrast to the human lungs, exist as highly inflamed organs, and/or that NHP recipients have a high precursor frequency of memory T cells reactive to allo-antigens [167].

When anti-thymocyte globulin (ATG) induction therapy was added to triple drug therapy, lung allograft survival improved but not consistently. By adding an additional four-day course of tocilizumab (Actemra®) a first-in-class humanized monoclonal antibody directed against the IL-6 receptor (IL-6R), consistent DSA-negative, long term survival was achieved in all recipients [166]. Of note, tocilizumab therapy was accompanied by a demonstrable increase in FoxP3+ Tregs [166]. IL-6 signaling blockade promoted DSA-free, long term survival and tolerance induction in NHP recipients of lung allografts. The first use of tocilizumab in human transplant recipients was recently reported. The drug was shown to be safe and effective not only in facilitating a reduction of alloantibody levels in difficult-to-desensitize kidney allograft recipients [168] but also in improving graft and patient survival in kidney transplant recipients with the most severe form of chronic AMR [169].

19. Understanding the genetics of NHP histocompatibility

There have been major technical advances over the past decade with respect to NHP MHC gene/allele discovery and the application of massively parallel pyrosequencing of cDNA-PCR amplicons to NHP MHC typing [170]. This methodology allows the complete MHC typing of any experimental animal, and if a sire, dam, and one to two offspring are typed, permits by inference the complete MHC typing for all subsequent progeny. These fundamental advances have enhanced the scientific rigor and usefulness of NHP transplant studies.

In concert with improved MHC typing methods, the support of breeding colonies of rhesus monkeys specifically for transplant research has ensured adequate access to required numbers of animals that are MHC-defined, outbred, and haplo-identical. NHP colonies have thus been used to develop a model of GVHD to translate findings from rodent models that test CTLA4Ig for in vivo prevention of GVHD. In turn, the encouraging findings of this NHP trial have led to the application of abatacept for the effective treatment of human GVHD [171]. An additional recent example of the utility of these NHP for study of GVHD is the work showing benefits of OX40L and mTOR blockade to control T cell activation and preserve Treg function [172]. MHC-defined colonies undergird NHP solid organ transplant studies investigating novel immunosuppression strategies by accounting for the degree of familial relatedness and MHC matching between donor–recipient pairs.

20. Reagent development for the study of NHP immunology

The National Institute of Allergy and Infectious Diseases (NIAID) and the NIH Office of Research Infrastructure Programs (ORIP) support the NHP Reagent Resource (http://www.nhpreagents.org/NHP/default.aspx), which develops, produces, and distributes immunologic reagents optimized for use in NHP research, including antibodies for NHP *in vitro* immunodiagnostics, and primatized immuno-modulating or immuno-depleting recombinant antibodies and fusion proteins for in vivo administration. These reagents have been valuable in elucidating the immunologic mechanisms responsible for transplant rejection and tolerance, and for protection against infectious disease. Several antibodies have been developed as proof-of-concept in NHPs for new therapeutic approaches in human transplantation medicine. Table 2 summarizes reagents available from the NHP Reagent Resource.

21. Additional questions addressed in NHP models

21.1. Effects of immunosuppression on tolerance biomarkers

Previous human studies have demonstrated that operationally tolerant renal allograft recipients have increased numbers of B cells in peripheral blood compared with patients who are stable on standard immunosuppression [173–175]. Importantly altered distribution of B cell subsets was observed as well, with tolerant patients having greatly increased proportions of naïve (IgD+CD27-) and transitional (IgD+CD27-CD24+CD38+) B cells, and reciprocally decreased proportions of memory (IgD-) B cell subsets. In one study, gene expression analysis revealed a "signature" of tolerance characterized by increased expression of three B cell specific genes, IGKV1D-13, IGLL1 and IGKV4-1 [176]. Follow-up data confirmed the reproducibility of the B cell analyses and the over-expression of IGKV1D-13 and IGLL1. However, tolerant patients generally were not distinguishable from healthy controls, i.e., the overexpression of naïve/transitional B cells and increased levels of IGKV1D-13 and IGLL1 were only observed in comparison with patients on immunosuppressive drug therapy. This has raised the question of whether the relevant signature is a "signature of drug therapy." Answering this question is critical to understanding whether or not a B cell derived signature can be a useful biomarker of tolerance, and one which can guide patient management. Unfortunately, this is not a question which can be studied directly in humans. However, this important question can be answered by comparing NHP recipients that are naïve or tolerant to animals on chronic immunosuppression but without grafts.

Table 2
Reagents for in vivo administration to nonhuman primates available from the NHP reagent resource.

Cell-depleting reagents
- Anti-CD4
- Anti-CD8α
- Anti-CD8β
- Anti-CD16
- Anti-CD20
- Anti-CD163[a]
- Anti-CD169[a]
- Anti-CD303[a]
- Anti-CD336
- CD3-immunotoxin[b]
- Anti-rhesus thymocyte globulin

Non-depleting reagents targeting cell receptors
- Anti-CD11a
- Anti-CD40
- Anti-CD45RB
- Anti-CD115[b]
- Anti-CD154
- Anti-CD279[b]
- Anti-α4β7 integrin
- TGFβ-Ig

Reagents targeting soluble mediators
- IFNγR-Ig
- IFNγR-Ig
- Anti-IL-10
- Anti-IL-15
- Anti-CCL20[a]

Table kindly provided by Dr. Keith Reimann
[a] In development with lead candidate antibody selected.
[b] Manufactured by Dr. Zhirui Wang, Massachusetts General Hospital.

21.2. Effects of brain death on tolerance induction

Based on recent advances in tolerance induction in human living-donor kidney allograft recipients [26,34], application of the same protocols to deceased-donor recipients was considered. However, this new initiative is supported by little experimental literature. The majority of small- and large-animal tolerance studies utilize healthy, living donors transplanted under optimal circumstances with short ischemic times. It is clear that brain death leads to hemodynamic instability, inflammation, neurohormonal changes, and immune activation in the donor which can result in organ damage, impaired graft survival, and ultimately, poorer recipient outcomes [177–180]. This is supported by results demonstrating ineffective tolerance induction in deceased-donor transplants with a protocol that was succesful in porcine recipients receiving lungs from healthy, living- donors [181]. Better understanding of the effects of brain death on tolerance induction in NHPs would inform and guide clinical protocols aimed at recipients of deceased donor organs.

22. NHP islet applications

While the lack of a NHP model with autoimmune diabetes precludes studies on the efficacy of strategies in preventing autoimmune recurrence, the NHP model has enabled the investigation of several other factors that determine islet allotransplant outcomes. These factors include innate immunity [182], islet engraftment in the absence of alloimmunity [183], bioengineered implantation sites [184], and non-immunologic mechanisms interfering with long-term islet allograft survival [185,186].

One of the significant challenges in islet transplantation is early loss and poor engraftment of the donor islets. Strom and Koulmanda demonstrated in a NHP marginal mass islet transplantation model, which allows for more robust assessment of islet loss and engraftment, that short-term peritransplant treatment with α -1 antitrypsin (AAT), an acute phase reactant, suppressed inflammation, prevented loss of transplanted islets, and enabled functional expansion of islet mass over time [186]. These findings have led to multiple clinical trials in new onset type 1 diabetes, one islet-kidney transplantation trial that is currently recruiting, and a planned islet transplant trial (ClinicalTrials.gov identifier: NCT01319331; NCT02005848; NCT01304537; NCT01661192). In addition, a Phase IV auto-islet transplant trial in patients undergoing total pancreatectomy is planned (ClinicalTrials.gov Identifier: NCT02947087).

23. Summary and conclusions

The development of NHP transplantation models has served a critical role in the clinical application of novel immunosuppressive drugs and strategies. Three prototypical examples summarized in this review are 1) chimerism as a tolerance strategy for solid organ transplantation; 2) T cell depletion with CD3 immunotoxin in the NHP as a precursor to alemtuzumab in humans, and 3) development of belatacept from a tolerance strategy with CTLA4-Ig in NHP through its transition to maintenance costimulation blockade in NHP and then humans. Therapies that have failed drug development milestones in NHP transplant models include LFA-1 blockade, chemokine blockade, sphingosine-1-phosphate agonists/antagonists, and IL-15 blockade. It can be argued that these failures were responsible for avoiding expensive, unnecessary, and

potentially harmful or futile human testing. The need for responsible use of outbred, large animal, non-human primates for such research derives from the fact that many of the candidate immunosuppressive agents target human receptors that cross-react with rhesus or cynomolgous receptors but not murine receptors. Furthermore, the NHP models have been far more predictive of human transplant immune responses than murine models. Limitations of the NHP models include 1) tolerance in humans appears to be rarer than in NHP transplantation, perhaps related to more diverse immune repertoire of humans; and 2) human toxicities, in particular impairment of protective immunity, may be difficult to precisely predict from NHP models due to differences in both host and infectious species. Nevertheless, NHP models have proven of enormous value as perhaps the closest biologic system for modeling human transplantation and thus transferring experimental risk from humans to another species. The mechanistic and genetic information learned from NHP transplant systems has been extensive and provided considerable new sophistication to our ability to ask discriminating questions about the effects of immunologic manipulations relevant to human organ and cell transplantation.

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