Primate Models in Organ Transplantation

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Large animal models have long served as the proving grounds for advances in transplantation, bridging the gap between inbred mouse experimentation and human clinical trials. Although a variety of species have been and continue to be used, the emergence of highly targeted biologic- and antibody-based therapies has required models to have a high degree of homology with humans. Thus, the nonhuman primate has become the model of choice in many settings. This article will provide an overview of nonhuman primate models of transplantation. Issues of primate genetics and care will be introduced, and a brief overview of technical aspects for various transplant models will be discussed. Finally, several prominent immunosuppressive and tolerance strategies used in primates will be reviewed.

Animal experimentation has long provided a rational basis for the translation of treatments and techniques from the bench to the bedside. In general, all first-in-human trials require preparative animal experimentation to allow patients to make truly informed decisions about their participation. Properly designed animal studies in relevant species provide the necessary background experience with a novel approach to reasonably anticipate the efficacy or, at the very least, safety of a planned intervention. As such, they serve as a foundation on which human trials can be ethically designed, particularly in fields such as immunology, in which the complexity of the interactions involved has prevented the development of any sufficiently predictive *in vitro* model. Although animal models are far superior to *in vitro* models in projecting the potential of an approach, it must be recognized that they do not mimic clinical transplantation precisely, and thus cannot be expected to forecast the ultimate experience in humans.

The mouse model has formed the backbone of medical research and development for many years owing to the relative ease of breeding and genetic manipulation of the animals at a comparatively low cost. For immunology research, the mouse immune system offers sufficient homology for pathway determination and mechanistic studies, and indeed represents the ideal platform for this type of endeavor. In contrast, the large animal models (dog, pig, and primate) are significantly more expensive and, with the exception of inbred miniature swine (Sachs 1992; Mezrich et al. 2003), exhibit increased genetic diversity, making definitive mechanistic studies much more difficult, if not impossible. However, this complexity makes large animals suited to preclinical studies, in which the addition of often-unanticipated variables allows for the examination of practicality, safety, and generalized efficacy. In general, mice define pathways, and large animal models help establish whether a particular pathway's effect is suffi-

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ciently robust to emerge as dominant in the midst of the numerous uncontrolled variables typical of heterogeneous human populations.

In specific regard to transplantation immunology, mice have several potential drawbacks. Laboratory mice bred in clean environments and studied between 4 and 8 weeks old have a largely naïve immune system (Blattman et al. 2002), a fact likely responsible for the success of therapies, including methods of tolerance induction, in mice, and their subsequent failure when translated to large animals (Kirk 2003; Sachs 2003), or mice exposed to pathogens (Adams et al. 2003). Additionally, mice do not constitutively express class II antigens on vascular endothelium, unlike other large animal models, which may explain the importance of class II matching in the large animals models (Pescovitz et al. 1984; Choo et al. 1997). Furthermore, the efficacy of any regimen may also be dependent on the strain of mice used (Williams et al. 2000). The relative genetic diversity and immunologic experience of large animals helps to avoid many of these shortcomings, and, indeed, experimentation in a large animal model, most frequently primates (for reasons discussed below), has become a de facto requirement before initiation of human trials in transplantation (Sachs 2003; 't Hart et al. 2004).

The complexities of the immune response often cause therapies to fail in transition to large animals, or to humans. This is most often owing to one or a few critical differences between species rather than a failure of the concept. Interspecies differences in drug pharmacokinetics may lead to apparent failure of a regimen that may have been successful if adjustments for distribution or metabolism had been considered. Furthermore, modern biologic and antibody-based therapies may be profoundly altered by minor differences in molecular structure of the target molecule. The immunologic diversity of large animals can lead to significant differences in outcome via heterologous immune interactions, an experimental parameter that is challenging to quantify and control for. Finally, the practicalities of animal husbandry during treatment can only approximate the care human patients receive. Indwelling catheters and wound care in surgical models are often difficult. Mon-

itoring and vascular access is challenging in conscious animal models, often limiting the options for drug delivery and dosing schedules.

Another potential issue with large animal models is that of time, both in terms of the animal's age and survival of grafts. For practical reasons, adolescent animals are often used, and evidence suggests that these young animals, like young humans, have a predominately naïve immune system that will mature toward a memory phenotype as the animal ages (Nan et al. 1998a,b; Rodriguez-Carreno et al. 2002; Saalmüller et al. 2002). With regard to graft survival in animals, convention has established 100 days as indicative of acceptance in mouse models, whereas in larger animals, several years has been the norm. However, although this is practical in terms of the shorter life span and cost of maintaining the animals, no data support that graft survival should be interpreted proportionally to lifespan. Thus, data from these studies should be interpreted for what it is, and not extrapolated to indicate longer survival in longer-lived animals (Kirk 2003).

A full review of the ethical principles surrounding animal experimentation is beyond the scope of this article. However, it is worth noting that no animal experiment can be ethical unless it has a reasonable chance of answering a worthwhile question. Specific guidance for ethical treatment of animal subjects can be found in the *Guide for the Care and Use of Laboratory Animals* (National Research Council 2011) and in the publications of the American Association for Laboratory Animal Care (www.aaalac.org/resources). In general, all animal experiments should embrace the principles of reduction, replacement, and refinement. Animal models should be reserved for when no reasonable alternative exists, and any procedure should be maximally refined for the benefit of the animal's welfare. Any experiment should be designed to minimize the number of animals used without endangering the ability of the experiment to answer the question at hand. An experiment that fails to answer the question posed using fewer animals is less ethical than an experiment that succeeds using more animals, and is virtually useless.

The choice of large animal model can often be daunting. Dogs, pigs, and nonhuman primates have all been used extensively in transplantation research, and each has sufficient anatomic and physiologic similarity to humans to be useful. In this article we will focus on primates, as that model has become the predominant model used owing to the need for highly conserved targets when testing biologic and antibody-based therapies. However, primates are not a model of convenience, and should be considered only when a therapy is being anticipated for translation to humans, and data are required to facilitate this transition in a safe and ethically viable fashion.

PRIMATE MODELS OF TRANSPLANTATION

Primate studies in transplantation have used a variety of species, including baboons, macaques, and, rarely, chimpanzees. Chimpanzees offer the highest degree of homology with humans, even serving (historically) as graft donors for humans (Reemtsma 1969); however, their endangered status and evolutionary stature have raised questions regarding the ethics of their use and led to increased restrictions, rendering them a historical model only. Indeed, the National Institutes of Health (NIH) has now eliminated the chimpanzee as a funded model. Baboons have commonly been used in xenotransplantation studies, as their relatively large size better accommodates the porcine organs. Macaques (*cynomolgus*, rhesus, and pigtail) are the most widely used of the nonhuman primates (NHPs) owing to their small size, defined major histocompatibility complex (MHC), and relative ease of care. Macaques are sufficiently homologous with humans such that most molecular targets will cross-react, with the notable exception of CD3. The remainder of this article will focus predominantly on the rhesus macaque. Numerous applications are described to provide the reader with a general knowledge of the procedures performed using NHPs. For specific methodological detail, the reader is referred to the primary literature (Kirk et al. 1997; Pierson et al. 1999; Hausen et al. 2000a; Elster et al. 2001a; Adams et al. 2002; Cendales et al. 2005).

Genetics in Primate Transplantation

In human studies of transplantation, the MHC matching of donor–recipient pairs is often known. Although the effect of MHC disparity on outcome varies by organ, generally, better-matched pairs have better results. The same can be said of primates; however, until recently, MHC typing of primates was difficult, based on incomplete mapping of the region, and frequently omitted. Although NHPs cannot be readily inbred to the same extent as mice, or even dogs or pigs, they are not truly outbred either. Most monkeys used in research are from dedicated colonies with limited interbreeding. The genetic disparity of potential transplant pairs was established using relatively limited techniques, usually mixed lymphocyte reactions and analysis of the DR region. Failure to adequately control for this experimental variable introduces a significant confounding factor in the interpretation of results.

Recent studies of the rhesus macaque paired with technological advances have begun to allow for easier assessment of MHC matching. The rhesus MHC is located on chromosome 4 and, owing to significant duplication of class I A and B genes (known as mamu-A and mamu-B) and, to a lesser extent, class II duplication, is significantly larger than the human MHC. Owing to the duplication of these genes, individual animals express up to four mamu-A genes and 14 mamu-B genes per chromosome (Kean et al. 2012). Furthermore, these genes are expressed in a hierarchical pattern rather than the co-dominant expression seen in humans (Otting et al. 2005). The combination of these two facts makes the rhesus MHC extremely complex, particularly for heterozygotes, and renders allele-specific DNA-based typing ineffective at predicting the expression of MHC molecules. Recently, several DNA microsatellite probes were identified that allowed for the determination of autosomal and sex chromosome inheritance between generations of macaques. This allowed for accurate pedigrees to be developed of rhesus breeding groups and determination of inheritance of complete MHC haplotypes (Andrade et al. 2004; Penedo et al. 2005). The de-

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velopment of massively parallel pyrosequencing allowed for comprehensive analysis of MHC expression (Wiseman et al. 2009) and confirmed the extreme complexity of the rhesus MHC. Pyrosequencing can be used to determine the complete MHC type for any individual animal. By combining these two technologies, investigators can design experiments with knowledge of the MHC disparity between potential transplant pairs, greatly increasing the validity of the results. To do so, however, requires substantial coordination with the primate breeding facility, and advanced planning to achieve the desired degree of match or mismatch while still minimizing the number of animals used.

Perioperative Care

The perioperative and follow-up care of NHPs can only approximate what is available for humans. Routine care and treatment of any complications should be performed in consultation with veterinary staff and be anticipated within the animal use protocols approved by the Institutional Animal Care and Use Committee (IACUC). The animal's clinical status should be monitored frequently, as changes in activity or appetite are often early signs of rejection, infection, or technical complications. Animals receiving nephrotoxic immunosuppression or those that receive renal grafts should be monitored for urine output. Laboratory values should be checked routinely postoperatively and with any clinical changes. Medications can be delivered by a number of routes; however, care should be taken when planning experiments to avoid overly frequent sedation for intravenous infusions. Medications requiring daily dosing should be given by mouth or by subcutaneous or intramuscular injection, if possible, unless specialized cages allowing for indwelling catheters in conscious animals are available. Anorexic or malnourished animals can receive supplemental nutrition via orogastric tube while sedated, and should be referred to veterinary staff for dietary modifications. Clinical medications analogous to the standard medications used for human transplantation, such as antibiotics or analgesics, are generally available through veter-

inary protocols, and although many require dose adjustments, most human agents work well in rhesus macaques. Finally, animals receiving immunosuppression should be monitored for the development of infections, and prophylaxis can be considered. Polymerase chain reaction (PCR)-based assays for rhesus macaque homologs of CMV and EBV, rhCMV (Yue and Barry 2008) and LCV (Rao et al. 2000; Rivallier et al. 2004), respectively, are available for monitoring. Regular monitoring of transplanted animals can provide insight into the extent of immunosuppression, and perhaps, indicate when a regimen may excessively compromise protective immunity (Lo et al. 2013; Lowe et al. 2013).

Renal Transplantation

Renal transplantation in primates mimics the intra-abdominal procedure used in small children. Following systemic heparinization of both the donor and recipient, the donor organ is excised and the vessels are anastomosed to the recipient's infrarenal aorta and vena cava (Fig. 1). Typically this is performed in a left-to-right fashion owing to the longer length of the left renal vessels. The donor ureter is tunneled through the retroperitoneum and a primary ureteroneocystostomy is formed, typically on the posterior wall of the bladder using a modified Leadbetter-Politano approach (Politano and Leadbetter 1958; Kayler et al. 2010). The recipient's native kidney(s) are removed following implantation (Kirk et al. 1997; Knechtle et al. 1997). When possible, the animals may be paired in a domino fashion, allowing each individual to act as a donor and recipient, reducing the number of animals needed and preventing the need for a simultaneous bilateral retroperitoneal dissection and native nephrectomy, which is particularly stressful for the animals. In general, renal transplantation is well tolerated by the animals and complications are limited, with most issues related to the ureter (Song et al. 2010).

Skin Transplantation

Like renal transplantation, skin transplantation in primates is relatively straightforward, and

Figure 1. Primate renal transplantation. (A) A retroperitoneal pocket is created starting medial to the native ureter and moving laterally. (B) The donor vessels are anastomosed to the recipient vena cava and aorta. (C) Following reperfusion, the donor ureter is tunneled through the retroperitoneum to a posterior cystotomy and anchored within the bladder.

offers a rigorous model in which to test immunosuppressive or tolerance strategies (Elster et al. 2001b). Donor grafts are typically taken from the abdominal wall and implanted on the back of the recipient to reduce the likelihood of the animal picking at the graft. It is important to remove cutaneous fat from the skin graft before transplantation, and similarly important not to remove subcutaneous tissue and microvasculature from the recipient site. To further protect

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the grafts during healing, soft bolsters should be used to hold the graft against the underlying tissue (Fig. 2). These can be removed after approximately 1 week. The animals also need to wear protective jackets to protect the grafts and bandages from damage during grooming. The animals generally need to acclimatize to wearing the jacket for a period of time preoperatively, and this may take several weeks before the primate is comfortable with the jacket.

Islet Transplantation

Nonhuman primates have been used extensively in the study of pancreatic islet transplantation. Following a period of training the animals for frequent glucose monitoring, the animals can be rendered diabetic by pancreatectomy or injection of streptozotocin, and diabetes is confirmed by the absence of native c-peptide pro-

Figure 2. NHP skin grafts. In the *upper* panel, grafts have been sewn in place and anchor sutures placed. The sutures are left long so that they can be used to affix cotton bolsters over the grafts to stabilize the grafts for approximately 1 week. In the *lower* panel, the grafts are shown after having healed to the underlying tissue. Note that the allografts are undergoing early rejection and appear erythematous when compared with the autografts.

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duction. Islet cells from the donor pancreas are isolated in the same manner in which human islets are isolated and then infused into the portal circulation by gravity (Adams et al. 2002; Badell et al. 2012a), although other sites for islet implantation are being investigated (Cantarelli and Piemonti 2011). Daily fasting and postprandial blood sugars can be easily monitored postoperatively to assess engraftment and survival of the islet cells. Histological implantation, rejection, and function all closely approximate human islet transplantation.

Heart and Lung Transplantation

The use of NHPs in studies of allogenic heart and lung transplantation is less common; however, examples do exist of their use (Hausen et al. 2000a,b; Azimzadeh et al. 2006). More frequently, primates are used as recipients of heart or lung grafts in studies of xenotransplantation, which will be discussed briefly below and more extensively in other portions of this text. Heart transplantation in primates is typically performed in a heterotopic fashion with the graft placed in the abdominal cavity (Pierson et al. 1999). The graft can then be followed by palpation, ultrasound, or even implanted EKG electrodes (Li et al. 2011). Larger NHPs, such as the baboon, or the cynomolgus macaque are often used in these procedures for technical reasons.

Vascularized Composite Allografts

Advances in immunosuppression and microsurgical technique has allowed for the transplantation of multiple tissues as a functional unit, such as hand and face transplantation. Multiple primate models of these vascularized composite allografts have been developed. Models involving either a portion of or whole hand transplantation in primates (Stark et al. 1987; Hovius et al. 1992) potentially negatively affect the function of the surviving recipient. Cendales et al. (2005) developed a model in which only a small portion of the radial forearm is transplanted, allowing for preserved function in both the donor and recipient (Fig. 3). A heterotopic model of facial transplantation has also been developed by a

group at the University of Maryland involving part of the mandible, overlying soft tissue, skin, and vasculature (Silverman et al. 2008; Barth et al. 2009).

Xenotransplantation

Xenotransplantation is covered more extensively in another article in this collection, so we will mention it here only briefly. Studies of xenotransplantation in primates frequently use swine as the organ donor, owing to the ease of breeding and genetic manipulation, such as knockouts of the Gal antigen (Lai et al. 2002; Phelps et al. 2003; Kolber-Simonds et al. 2004; Nottle et al. 2007). Baboons are typically used for solid organ xenotransplant experiments, as their larger size better accommodates the porcine organs. Macaques are often used as recipients of xenoislet transplants. When planning these experiments, it is worth noting that cynomolgus macaques have lower resting blood glucose levels than humans (Casu et al. 2008). Although choosing an ideal donor age has provided some controversy in these experiments, both adult and neonatal islets have been successfully transplanted (Cardona et al. 2006, 2007; Hering and Walawalkar 2009; Thompson et al. 2011).

STANDARD IMMUNOSUPPRESSION

Virtually all clinically used standard immunosuppressants have been found to be successful in prolonging graft survival in primate models, particularly when adjustments for distribution and metabolism have been considered. To focus on the relatively newer immunosuppressants and biologic therapies, we will mention the conventional immunosuppressants, which have been reviewed extensively elsewhere (Kirk 2003), only briefly.

Both azathioprine and mycophenolate have been successfully used in primate models of transplantation, as have calcineurin inhibitors. The development of tacrolimus is worth particular mention, as it was tested in a canine model first with promising results but significant toxicity. Only after follow-up studies in primates that showed its safety was the drug translated

Figure 3. Radial forearm vascularized composite allotransplantation (VCA) containing bone, muscle, tendon, nerve, vasculature, subcutaneous fat, and skin. (From Cendales et al. 2005; reprinted, with express permission, from the author.)

to human use (Todo et al. 1988). A cyclosporine-based regimen has even been shown to induce tolerance in a partially MHC-matched rhesus model (Jonker et al. 1998). Given their success and almost universal use in human transplantation, the calcineurin inhibitors remain a fre-

quently studied class of immunosuppressants, either in combination with new agents or as a control against which the new agent is tested. They are dosed at somewhat higher levels compared with humans dosing to achieve therapeutic targets. The mTOR inhibitor sirolimus has

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been frequently studied in primate models and been found to prolong graft survival alone (Weaver et al. 2009; Lo et al. 2013) or in combination with tacrolimus (Chen et al. 2000). However, it has been shown to have significant gastrointestinal toxicity in rhesus macaques (Montgomery et al. 2002a). Sirolimus has also been used in combination with T regulatory cell therapy to prolong renal graft survival in the rhesus model (Ma et al. 2011). Everolimus, another mTOR inhibitor, has also been successfully used in primate models of lung (Hausen et al. 2000b) and islet transplantation (Wijkstrom et al. 2004).

A bevy of newer immunosuppressants have also been tested in primate models. FTY720, which prevents the egress of lymphocytes out of lymph nodes, prolongs renal graft survival in macaques, either alone or in combination with other agents (Schuurman et al. 2002). Sotrastaurin, a selective protein kinase C inhibitor, also prolongs primate renal graft survival (Bigaud et al. 2012). It too was found to synergize with other agents, including the aforementioned FTY720 (Bigaud et al. 2006, 2012). The Janus kinase 3 inhibitor, tofacitinib (CP-690,550), was used in a cynomolgus renal transplant model and was shown to prolong graft survival and decrease circulating natural killer (NK) and T-cell populations, a result interpreted to be consistent with multiple cytokine blockade (Borie et al. 2005). Finally, a study of PG490-88, a prodrug form of triptolide, showed prolonged renal allograft survival in cynomolgus monkeys (Chen et al. 2006).

COSTIMULATION BLOCKADE

The blockade of costimulatory signals has repeatedly been used to induce tolerance in rodent models (Turka et al. 1992; Pearson et al. 1994; Larsen et al. 1996). However, the translation of these strategies to large animal models has been less successful. Two phenomena are believed to be primarily responsible for this discrepancy. First, more outbred animals will have a higher precursor frequency, and the alloreactive immune cells are capable of overcoming costimulation blockade by sheer numbers (Ford et al.

2007). Antigen recognition inefficiently generates some response in the absence of costimulation, and the high precursor frequency makes up for this inefficiency, allowing for an immunologically meaningful response (Viola and Lanzavecchia 1996; Germain 2001). Second, there are some cell populations, such as memory T cells, that appear to be less dependent on costimulation (London et al. 2000); thus, more experienced immune systems will be more apt to respond to antigen even during costimulatory blockade. In this review, we will focus on the CD28-B7 and CD40-CD154 pathways, which are the most extensively studied of the costimulatory pathways. However, as the role of costimulation has been increasingly studied, multiple receptor–ligand pairs have been discovered, and it is now generally accepted that multiple ancillary pathways exist to modulate the immune response (Ford and Larsen 2009; Pilat et al. 2011). Owing to the highly specific nature of the biologic agents used to block these signals, primate models have become the large animal model of choice for these studies (Kirk 1999).

Of existing costimulation blockade strategies, blockade of the CD28-B7 pathway has reached the furthest clinical development, with the FDA approval of belatacept for use in human kidney transplantation in July of 2011. Early work to study blockade of the CD28 pathway involved monoclonal antibodies against the B7 ligands CD80 and CD86. Three groups showed prolongation of renal allograft survival using the antibodies 1F1 and 3D1 either alone or in combination with other immunosuppressants (Hausen et al. 2001; Kirk et al. 2001; Montgomery et al. 2002b; Birsan et al. 2003). Another group used different clones, B7-24 and 1G10, in rhesus skin and renal transplant models with modest success (Ossevoort et al. 1998a,b). Despite these successes, further development of these antibodies was abandoned, as it was shown that both B7 molecules needed to be blocked to have efficacy (Kirk et al. 2001), and the B7-specific fusion protein approach accomplished this with a single agent, thus simplifying development plans.

A second strategy to block the CD28 pathway is via the soluble form of the CTLA4 recep-

tor, the fusion protein CTLA4-Ig, which binds both B7 ligands. Following success in several rodent models (Pearson et al. 1994; Akalin et al. 1996; Bolling et al. 1996), early studies in multiple primate models showed only modest prolongation of graft survival (Kirk et al. 1997; Levisetti et al. 1997; Krieger et al. 1998). Persistent interest in this pathway led to the development of belatacept, which differs from the base CTLA4-Ig protein by only two amino acids, but shows significantly higher inhibition of CD28 signaling (Larsen et al. 2005). Belatacept was shown to be effective as maintenance therapy in primate models of islet and renal transplantation (Cardona et al. 2006; Lo et al. 2013; Lowe et al. 2013), and subsequent clinical trials led to its successful translation to human use.

Blockade of the CD28 pathway via targeting of the B7 ligands has the potential drawback of blockade of inhibitory signals through CTLA4, and significant interest exists in developing therapeutics that will simultaneously block CD28 signaling while leaving CTLA4 signaling intact. The use of full monoclonal antibodies directed against CD28 was set back by the disastrous results of the TGN1412 trial, in which an anti-CD28 antibody with superagonistic qualities caused life-threatening inflammatory responses in all six human volunteers who received the drug (Suntharalingam et al. 2006). Although several recommendations have been made for the design of future trials to avoid a repeat of the TGN141 trial (Stebbins et al. 2007; Findlay et al. 2010), there has been, understandably, extreme caution in developing antibodies against CD28. Since TGN1412, two trials using modified CD28 antibodies have been reported: the monovalent fusion antibody sc28AT has been shown to prolong primate graft survival (Poirier et al. 2010), and a pegylated monovalent Fab' antibody, FR104, has been promising in rodent models, but thus far only pharmacokinetic studies have been reported in primates (Poirier et al. 2012).

The CD40-CD154 pathway has also been extensively studied in primate models. The monoclonal antibody hu5C8, directed against CD154, has been shown to be efficacious in multiple primate transplant models involving several or-

gans (Kenyon et al. 1999a,b; Kirk et al. 1999; Pierson et al. 1999; Elster et al. 2001b; Xu et al. 2001). Similarly, two other antibodies against CD154, IDEC-131 and ABI793, have been shown to prolong graft survival in primate models (Pfeiffer et al. 2001; Pierson et al. 2001; Xu et al. 2001, 2003; Kanmaz et al. 2004; Schuler et al. 2004; Preston et al. 2005; Azimzadeh et al. 2006; Pearl et al. 2007). Somewhat fortuitously, hu5C8 was one of the first antibodies to be tested at the relatively high dose of 20 mg/kg. This was important, as it was later discovered that CD154 was present on multiple cell types throughout the body, offering large quantities of the ligand to compete for binding, and, in some cases, initiate rejection (Xu et al. 2006). It is possible that the efficacy of CD154 blockade would not have been appreciated had a lower dose been used. Unfortunately, however, the widespread distribution of CD154, particularly on platelets, has led to concerns regarding the thromboembolic potential of CD154-directed therapies, and this has limited the clinical development of these therapies (Kawai et al. 2000).

The success of the CD154 antibodies prompted continued investigation of the pathway, focusing on CD40. Multiple antibodies against CD40 have been tested, with efficacy on graft survival and effect on B-cell populations depending on the clone used: Chi220 (Pearson et al. 2002; Adams et al. 2005), 3A8 (Badell et al. 2012a,b; Page et al. 2012a), 4D11 (Imai et al. 2007; Aoyagi et al. 2009), ch5D12 (de Vos et al. 2004), or 2C10 (Lowe et al. 2012).

The combination of CD28-B7 and CD40-CD154 pathway blockade has been studied on multiple occasions. Depending on the agents used, the combination has only sometimes provided a graft survival advantage over either agent alone. However, even in cases in which no synergy was seen in terms of graft survival, there does appear to be an advantage of combination therapy in abating the humoral response (Ossevoort et al. 1998b; Montgomery et al. 2002b). Clearly, as our understanding of these and other costimulatory pathways improves, there will be plenty of opportunity to further develop strategies to take advantage of these molecules for immune modulation.

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ADHESION BLOCKADE

A number of surface molecules on immune cells mediate the interaction between these cells, their environment, and each other. Many of these adhesion molecules are important for cell trafficking and homing, whereas some have been found to stabilize the immune synapse, and even provide costimulatory signals. This section will focus on two prominent adhesion molecules in transplantation experiments.

The integrin LFA-1 is the most extensively studied of the adhesion molecules. It has been shown to be important in cell trafficking and homing (Hamann et al. 1988; Kavanaugh et al. 1991; Warnock et al. 1998), antigen presentation (Moy and Brian 1992; Pribila et al. 2004), and costimulation (Van Seventer et al. 1990; Nicolls and Gill 2006). The apparent importance of LFA-1 in multiple areas of the immune response prompted significant interest as a strategy for immunosuppression. Anti-LFA-1-based regimens have been successful in prolonging graft survival in primate models of skin (Berlin et al. 1992), cardiac (Poston et al. 2000), and islet transplantation (Badell et al. 2010). Translation of these strategies to human use has been challenging. Studies of the humanized anti-LFA-1 agent, efalizumab, in kidney (Vincenti et al. 2007) and islet (Turgeon et al. 2010) transplantation were promising. However, the drug, which was widely used for psoriasis, was found to be the cause of four cases of progressive multifocal leukoencephalopathy in that population, and was removed from the market. Any future anti-LFA-1-based regimens will likely need to be more selective in their blockade to avoid deleterious effects on protective immunity.

The interaction of CD2 and LFA-3 primarily strengthens the interaction of T cells and antigen-presenting cells (APCs), but also, like LFA-1, is capable of transducing costimulatory signals directly (Bromberg 1993). Multiple agents to block this interaction have been developed with modest results in primate models (Kaplon et al. 1996; Dehoux et al. 2000). Weaver et al. (2009) used the fusion protein alefacept (LFA3-Ig) in addition to CTLA4-Ig in a rhesus renal transplant model with significant prolongation

of graft survival. However, a subsequent study by the same group, which replaced CTLA4-Ig with belatacept, showed no benefit with the addition of alefacept and an increase in viral infections in both renal and islet models (Lo et al. 2013; Lowe et al. 2013). Clearly, further experiments will be needed to clarify the role of CD2 blockade in the future.

Much like the exploding number of costimulatory receptor–ligand pairs that have been discovered over recent years, increasing numbers of adhesion molecules are being discovered and studied. As our understanding of these molecules improves, it is likely we may be able to exploit these pathways to modulate the alloimmune response. One such candidate is the integrin VLA-4, for which an agent, natalizumab, is already approved for use in multiple sclerosis, making it an ideal candidate for translation for use in transplantation.

DEPLETION

One strategy to deal with the aforementioned issue of relatively high precursor frequency and sensitization is to remove those cells from the body before transplantation. Thus, lymphocyte depletion is seen as an adjuvant to other therapies in the development of tolerance regimens. However, at present there are no readily available NHP-specific depletion agents; thus, human specific preparations are used. These have efficacy in NHPs, but they do not mimic the effects seen in humans. Thus, NHP models are not as satisfactory for studying depletion as they are for the more specific pathway blockade described above.

Lymphocyte depletion with polyclonal preparations was one of the first strategies developed. These preparations include both anti-lymphocytic antibodies as well as antibodies against a variety of other cell-surface molecules, particularly adhesion molecules, which may be of use in cases in which ischemic injury leads to activated endothelium providing an interface for leukocyte adhesion and migration (Hammer and Thein 2002). Rabbit antithymocyte globulin (ATG) must be given in much higher doses in primates than humans, likely owing to

decreased specificity for NHP epitopes, notably CD3 (Preville et al. 2001). ATG has been shown to prolong primate graft survival (Thomas et al. 1978); however, its dosing requirements, half-life, and specificity differ so significantly from that seen in humans (the species to which ATG is raised) that it is unlikely that ATG's behavior in NHPs is a satisfactory surrogate for its use in the clinic. ATG fails to induce tolerance in most models, and is thus used primarily as induction therapy followed by some form of maintenance therapy, a variety of which have been successful (Hirshberg et al. 2003; Liu et al. 2007). However, a study by Haanstra et al. (2006) found that ATG combined with costimulation blockade shortened the time to rejection, possibly owing to decreased intragraft inhibitory molecules. The lymphocyte repopulation following depletion with ATG relatives enriches memory populations (Pearl et al. 2007), potentially with lower costimulation requirements.

A variety of monoclonal antibodies have been developed to eliminate or incapacitate reactive effector T cells. Depletion with CD3 and CD4 antibodies conjugated to idarubicin allowed for operational tolerance in half of baboon kidney grafts when paired with TLI, but only when given preoperatively (Myburgh et al. 2001). However, use of a nondepleting antibody, OKT4A, only modestly prolonged graft survival (Cosimi et al. 1990; Wee et al. 1992; Mourad et al. 1998). Depletion based on another potential target, CD45RB, has been shown to prolong graft survival in cynomolgus macaques (Chen et al. 2007). B cells have also been targeted in depletion studies, with the agent rituximab showing prolongation of graft survival in primate islet (Liu et al. 2007) and cardiac models (Kelishadi et al. 2010).

The most promising results in NHP lymphocyte depletion have been achieved using immunotoxin (IT), a fusion of a modified diphtheria toxin with a monoclonal antibody against CD3 (Neville et al. 1996; Ma et al. 1997). Knechtle has been able to show pretransplant depletion with IT promotes tolerance in renal transplant models (Knechtle et al. 1997, 1998a). Despite the ability of IT to reverse rejection in a rhesus renal transplant model (Knechtle et al. 1998b), it

has been somewhat less effective at prolonging graft survival when given at the time of transplant rather than beforehand (Armstrong et al. 1998; Jonker et al. 2002). The development of a humoral response leading to graft loss in these models using chemically linked IT, or the somewhat less effective recombinant IT (Ma et al. 1997; Kim et al. 2007), has been proposed as a useful model of chronic allograft rejection (Torrealba et al. 2003; Page et al. 2012b).

CHIMERISM

Chimerism has long been studied as a method of inducing allograft tolerance, dating to the studies of Owen (1945) in freemartin cattle, and later Medawar and colleagues (Billingham et al. 1956). Hematopoietic chimerism can be classified based on the relative number of donor cells in the general cell population. Infusion of hematopoietic stem cells into an unconditioned recipient leads to rapid rejection of the cells and failure to engraft. Microchimerism, in which <1% of circulating cells are of donor origin, and these are only detectable by very sensitive techniques such as PCR, is the most this model can produce. Establishment of mixed chimerism (>1% but <100%) or especially full chimerism (~100%), requires more intensely ablative conditioning of the recipient. The potential toxicity of these conditioning regimens has been a major barrier to clinical translation of chimerism-based approaches, given the relative safety of modern immunosuppression.

Donor-specific marrow infusions without ablative preconditioning have been studied in attempts to prolong primate allograft survival. However, it is worth noting that all transplant recipients are likely microchimeric to some degree (Starzl et al. 1992), and this does not lead to widespread tolerance. It is possible the establishment of microchimerism is thus simply a marker for sufficient immunosuppression (Wood and Sachs 1996).

Full chimerism, in which virtually all of the hematopoietic cells are of donor origin, is generally only achievable following intense conditioning in HLA identical siblings, if graft-versus-host-disease (GvHD) is to be avoided.

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However, there are multiple reports of full chimerism inducing donor-specific tolerance of subsequent renal grafts (Sayegh et al. 1991; Helg et al. 1994; Jacobsen et al. 1994) in humans. Full chimerism does have some drawbacks, however, both in the risk of GvHD and an apparent immune incompetence likely owing to MHC disparity between donor-derived immune cells and recipient-derived APCs (Sykes 2001).

Based on the studies of microchimerism, it is generally accepted that macrochimerism is necessary for durable tolerance to develop, and the drawbacks and impracticalities of full chimerism approaches have made mixed chimerism an area of intense study. A group in Boston has induced mixed chimerism in cynomolgus macaques using irradiation, bone marrow infusion, T-cell depletion, and splenectomy across MHC barriers without GvHD (Kawai et al. 1995; Kimikawa et al. 1997). These animals accepted renal allografts from their marrow donors and, in most cases, also did not produce donor-specific antibodies (Kawai et al. 1999). However, results in cardiac transplantation were somewhat less promising (Kawai et al. 2002), and this regimen also induced increased rates of posttransplant lymphoproliferative disorder (PTLD), likely related to lymphocryptovirus (LCV) in monkeys (Schmidtke et al. 2002). Further refinement of the regimen replaced splenectomy with CD154 blockade (Kawai et al. 2004), and these regimens formed the basis for the first human trial of mixed chimerism (Kawai et al. 2008).

Another group has attempted to induce mixed chimerism in rhesus macaques. In this model, busulfan is the primary conditioning agent, followed by basiliximab, combined costimulation blockade, and sirolimus (Kean et al. 2007). Although the regimen was successful in inducing long-lasting mixed chimerism, the marrow was found to be rapidly rejected once the maintenance immunosuppression was stopped (Larsen et al. 2010), and viral infections continued to be an issue (Kean et al. 2007). Replacing CD154 blockade with CD40 blockade did not prolong marrow graft acceptance beyond the cessation of immunosuppression (Page et al. 2012a). To date, the effect of these mixed chimerism regimens on solid organ

transplantation has not been reported. Despite the challenges of chimerism-based approaches to tolerance, they represent one of the few strategies that have been successfully translated to humans, and the promising results of preclinical studies warrant further research into the field.

SUMMARY

In this article, we have reviewed the use of primates in preclinical studies of transplantation, both in the technical aspects of working with primates, and also a number of strategies to prolong graft survival currently being studied. Some of these approaches have already found their way into the clinic, whereas others are still a far way off, in need of significant refinement before they can become a clinical reality. Primate models of transplantation offer an ideal arena in which to study these therapies and hone their application so they may be of the greatest benefit for our patients.

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