The Utility of Animal Models in Developing Immunosuppressive Agents

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Abstract

The immune system comprises an integrated network of cellular interactions. Some responses are predictable, while others are stochastic. While *in vitro* the outcome of stimulating a single type of cell may be stereotyped and reproducible, *in vivo* this is often not the case. This phenomenon often merits the use of animal models in predicting the impact of immunosuppressant drugs. A heavy burden of responsibility lies on the shoulders of the investigator when using animal models to study immunosuppressive agents. The principles of the three R's: refine (less suffering,), reduce (lower animal numbers) and replace (alternative *in vitro* assays) must be applied, as described elsewhere in this issue. Well designed animal model experiments have allowed us to develop all the immunosuppressive agents currently available for treating autoimmune disease and transplant recipients. In this review, we examine the common animal models used in developing immunosuppressive agents, focusing on drugs used in transplant surgery. Autoimmune disease, such as multiple sclerosis, is covered elsewhere in this issue. We look at the utility and limitations of small and large animal models in measuring potency and toxicity of immunosuppressive therapies.

1. Introduction

Immune responses are generally split into adaptive and innate responses (Bartl et al., 2003; Hale, 2006; Land, 2007). In practise, the two systems interact a great deal. Cells of the innate family include dendritic cells, monocyte/macrophages and natural killer (NK) cells. They have conserved receptors which typically bind similarly conserved epitopes on alloantigens e.g. lipopolysaccharide which enable a response to so-called "danger signals" (Kovarik and Siegrist, 2001). In evolution terms, the innate system is older and important for many taxa including plants and invertebrates. The adaptive system is thought to have evolved more recently, in jawed fish 500 million years ago(Rast and Litman, 1994). T cell receptors and antibody are only present in jawed vertebrates. This naturally has implications for choice of animal model in simulating immunosuppression for humans.

Adaptive responses, driven by B and T lymphocytes, are specific to the particular proteins of the foreign substance, or alloantigen (Cannon et al., 2004). T cells mature in the thymus (hence their name), where they learn to distinguish between self and non-self, mediated through recognition of epitopes by the T cell receptor; formed by genetic rearrangements between V,D and J segments, making it the most heterogeneous protein in the body (Honjo et al., 1981; Roth, 2000; Sollbach et al., 1994). T cells govern the immune response, providing activation signals to other cells and directly lysing cells with perforin and granzymes. B cells on the other hand are responsible for the production of antibodies and typically require T cell help for initial activation. Similar to T cells, B cells undergo V,D,J recombination of their immunoglobulin

receptor during somatic hypermutation to increase the affinity of the antibody they will ultimately produce for cognate antigen.

Immunosuppression is used therapeutically for management of conditions where the immune system becomes pathological instead of physiological. This occurs when there is undesired autoimmunity (to self) or alloimmunity (to foreign protein/carbohydrate/lipid). Autoimmune diseases include Lupus, Rheumatoid arthritis, Goodpasture's syndrome, Type 1 diabetes and Pernicious anaemia. Pathological alloimmunity is principally the domain of organ and cell transplant. Alloimmunity to transplants is unique in involving both indirect (T cells recognising antigen presented by self adaptor proteins, like in autoimmunity) and direct recognition (directly binding to foreign adaptor proteins without involvement of self antigen presenting cells) (Hale, 2006). Therapy is broadly similar for both auto- and allo-immunity, and involves drugs and biologics which dampen innate and adaptive immunity. Most immunosuppressive agents target adaptive immunity, with the exception of steroids which also inhibits innate responses (Taylor et al., 2005).

Animal models have been used extensively in the development of immunosuppressive drugs. Some models mirror specific autoimmune diseases, such as the extrinsic allergic encephalomyelitis mouse model for multiple sclerosis (Robinson et al., 2014), detailed elsewhere in this issue. Animal models have been used to simulate all types of human transplant, common examples being heart and skin transplants (Chong et al., 2013). Complementing the *in vivo* models, various *in vitro* techniques are available to elucidate the contribution individual cell types make to the overall immune response. These include routine analysis of blood levels of antibodies and antigens using enzyme-linked immunosorbent assay (ELISA). More specific investigations can include the analysis of mixed lymphocyte reaction (MLR), which examines the proliferation rates of T cells to alloantigen. Alternatively, flow cytometry can be employed to measure cell surface or internal expression levels of various markers of immune cell activation and maturation.

2. Rodent models for immunosuppression

The development of immunosuppressive drugs largely parallels the development of organ transplantation, as it was only with the availability of these agents that successful human therapeutic transplantation became possible. Prior to development of potent immunosuppressive drugs, only transplantation between identical twins was possible, as in the first successful renal transplant between humans in 1954 by the Nobel laureate Joseph Murray (Calne, 1976). In the 1950s, experiments in dogs facilitated the development of 6-mercaptopurine and later its derivative azathioprine, important for short-term survival of renal allografts. Both of these are purine analogues, acting as competitive inhibitors of DNA synthesis. Co-administration with cortisone and other steroids gave better outcomes. In the 1980s, cyclosporine was licensed as the first calcineurin inhibitor, and improved transplant survival

dramatically. In the 1990s, this was largely superseded by the calcineurin inhibitor tacrolimus, also known as FK506. Subsequently, azathioprine was largely replaced by mycophenolate mofetil, which is also a DNA synthesis inhibitor, but acts by inhibiting the enzyme inosine monophosphate dehydrogenase, important for purine synthesis. Modern transplant maintenance immunosuppressive regimens largely use triple therapy with tacrolimus, mycophenolate mofetil and prednisone.

In the early days of transplantation the dog and pig model were most commonly used because it was technically easier to perform the larger vascular anastomoses. With the development of microsurgical techniques in the 1960s, rodents became the preferred model because of simplicity, favourable public and animal protective agencies opinion, and reduced costs (Chong et al., 2013). The Rat genome was sequenced in 2004 (Gibbs et al., 2004). Results revealed that the rat genome contains 2.75 billion base pairs. This compares with 2.9 billion in the human genome and 2.6 billion in the mouse. Humans have 23 pairs of chromosomes, compared with 21 in rats and 20 in mice. In spite of this the three species contain overall a very similar number of genes, and most disease-associated genes are highly conserved between the species.

Mice have proven invaluable in the assessment of immunosuppressive agents (Chong et al., 2013). The widespread availability of genetically modified mice, both transgenic and knockout, has made them invaluable assets in experimental models. Unlike rats, embryonic stem cells have been isolated and genetically manipulated in mice. In rats, eggs are sensitive to activation and do not tolerate genetic modification well, although this has been resolved within the last decade with cloning techniques allowing production of genetically modified rats (Doorschodt et al., 2014). Interestingly, studies on the rat genome sequencing have demonstrated that immune system-related genes have the highest rate of evolutionary change. This diversification of lymphocyte genes may mean that it is more difficult to extrapolate results of animal models such as the rat to humans. Interestingly, genes involved in detoxification show important differences between humans and rodents. Cytochrome P450 (CYP450) is important in metabolism of calcineurin inhibitors like cyclosporine, among other immunosuppressive drugs. The CYP450 subfamily member CYP2J has a single gene in humans, but four in rats and eight in mice (Uno et al., 2009). It can be seen that although rodents bear similarities to humans and are vital to examine and test agents, there are important constraints in applicability due these pharmacodynamic and pharmacokinetic differences.

The use of *in vivo* models facilitates the use of various measurements to determine immunosuppressive efficacy of drug and others agents. Take alcohol for example – if administered to a mouse orally for a week, numbers of T and B cells in the thymus and spleen diminish (Lopez et al., 1994; Saad and Jerrells, 1991). If these cells are isolated and stimulated *in vitro* with a mixed lymphocyte reaction MLR, cellular proliferation is reduced. Steroids when given to rabbits give similar results to humans. If dexamethasone is administered, neutrophilia is seen within a day. This is accompanied with lymphopenia. Lymphocyte numbers in the bone marrow are increased. Both lymphocyte and neutrophil function is suppressed, as measured by concanavalin-A proliferation and reactive oxygen species production respectively (Ulich et al.,

1988). Understanding of the complex effects that these agents have on the body could not be easily understood with in vitro models alone.

2.1. Organ Specific Rodent Transplant Models

Transplant models in rodents have been invaluable in understanding the human immune response, as well as aiding in development of immunosuppressive agents. Various models are available each with their own utility (Russell and Monaco, 1964). Lung and intestine transplants are more readily rejected than heart, kidney and liver (Bribriesco et al., 2013). Skin transplants, the most commonly used non-vascularised organ, are very readily rejected. Liver transplants are often spontaneously accepted and become tolerant even in the absence of exogenous immunosuppression (Calne, 1976; Jahr and Wolff, 1989; Pons et al., 2011).

The skin transplant was the first model developed and used by Sir Peter Medawar in the 1940s and 50s in seminal experiments which led to the understanding of self and non-self(Brent et al., 1976). This was the only model to study transplant immunology until the 1960s. The skin transplant model is technically straightforward. It involves a small square of full thickness skin to be excised from the donor animal and deep fat is dissected off. This is placed on a similar sized defect on the recipient abdominal wall and secured with glue or sutures. The graft is protected for 3-5 days with a dressing. This procedure will induce a rapid tempo of T cell mediated rejection within 7-12 days for mice different at the major histocompatibility complex (MHC) locus.

The first rodent vascularized organ transplants were performed in the rat but now virtually all organ transplants performed in the rat are also performed in mice. Although technically more challenging (due to very small diameter of vessels), mouse models of transplantation have several advantages. First, many congenic, transgenic, and knockout strains are available (see Table 1). There are specific mouse strains to dissect specific cell population or the effect of the absence of this particular population. Second, there are more reagents including monoclonal antibodies available for mice. Third, due to small body weight, mice need only about one tenth the amount of the drugs rats consume. The disadvantage of the mouse models is mainly technical, but it has been shown that spontaneous acceptance of both liver and kidney graft occurs more frequently in all mouse strains compared to rats (Qian S, Demeteris A, Murase N, Rao A, Fung J, Starzl T. Murine liver allograft transplantation: tolerance and donor cell chimerism. Hepatology 1994; 19: 916-924).

Table 1 Mice strains useful in Immunologic Experiments

Scid	Absence of functional T cells and B cells
RAG-1	Absence of functional T cells and B cells
Nude	Deficiency of T cell function
129/Sv	Deficiency of B cell function

Xid	Deficiency of B cell function
Beige	Decreased NK activity
DC-less	Absence of dendritic cells
MHC II deficient	lack of MHC class II
MHC I / II deficient	lack of MHC class I and II

Adapted from Cosgrove et al(Cosgrove et al., 1991)

Heart transplants are the most popular vascularised allograft performed. The heart is transplanted in a heterotopic position, with the veins of the heart tied off. The donor aorta is anastomosed to recipient aorta and donor pulmonary artery anastomosed to recipient inferior vena cava. This makes a non-physiological model of circulation, and the heart is subject to a moderate level of chronic ischaemia, so histological changes do not exactly mirror those seen in humans (Chong et al., 2013). Graft function is monitored by palpation of heart beating, or by using electrocardiogram (Martins, 2008).

Kidney transplants in the mouse are generally performed in nephrectomised animals so that it is a life sustaining procedure. The kidney is revascularised and ureter connected to bladder. Graft function is monitored by analysis of serum creatinine, however this is technically difficult. In some MHC mismatched strain combinations e.g. BALB/c to C57BL/6, up to 40% of recipients become tolerant to the renal allograft in the absence of immunosuppression, in marked contrast to humans where this never occurs (Kingsley et al., 2007). Again, this highlights the important differences between rodents and humans, limiting the translational reliability of these models.

Liver transplant in rats and mice are technically very challenging and success is directly related to very short anhepatic phase (<20min). There are different techniques, with cuff and without cuff to perform the vascular anastomoses. The graft can be arterialized or not to simplify technique, however, arterialized grafts have better outcomes and long-term survival rates. The biliary reconstruction is always performed with stenting. Graft function is monitored with AST, ALT, AP, bilirubin, albumin.

2.2. Rodent Models of Rejection

The advent of DNA recombination technologies has allowed us to produce to genetically modified murine models of antibody-mediated rejection. Recombinase Activating Gene (RAG) knockout mice lack the gene necessary for production of T cell and B cell receptors, so that they

are B/T cell deficient. Anti-donor MHC antibodies injected into RAG knockout mice can precipitate hyperacute (at time of transplant) or acute rejection of allografts. This is a weak model for hyperacute rejection, with the tempo and magnitude of rejection much less than that seen in humans. This is thought to be the result of excess complement regulatory proteins in mice, and can be overcome by the addition of exogenous complement. Anti-donor MHC antibody has also been injected to allograft recipient mice in models of chronic rejection(Bedi et al., 2010). In these models, the degree of HLA mis-match between donor and recipient is less. These studies have shown that complement fixation is not always necessary, and that other effector functions such as Natural Killer (NK) cell binding to antibody is adequate to drive chronic rejection (Villard, 2011).

Some mouse heart transplant models have shown the importance of regulatory B cells, which can down-regulate an immune response in an IL-10 dependent fashion (Kwun et al., 2012). These models have used induction with anti-T cell Ig and mucin 1 (TIM-1) in order to boost numbers of regulatory B cells. The process of accommodation has also been better understood through murine models. Accommodation occurs in human ABO blood group incompatible kidney transplants where the kidney survives in spite of alloantibody against it. The graft ameliorates antibody damage through the up-regulation of a number of protective regulatory proteins such as Bcl, haemoxygenase-1 (HO-1) and decay accelerating factor (DAF). Complement is activated to some extent on the graft, as evidenced by C4d deposition. This is seen in mouse xenotransplant models, for example in rat to mouse heart transplants, where cobra venom factor is used in induction to prevent early complement activation.

Mouse models have been used in developing tolerance strategies, where the allograft survives in the absence of ongoing immunosuppression (Brent et al., 1976; Kingsley et al., 2007). In some strain combinations, kidney transplants are accepted spontaneously, in the absence of any medication, for example transplantation from A/J (H-2a) to C57BL/6 (H-2b) murine genetic backgrounds. This is very different from the scenario in humans, where spontaneous tolerance has never been seen. Two other approaches to tolerance in murine models are mixed chimerism or co-stimulation blockade(Sachs et al., 2011). Mixed chimerism is the only approach to date which has been extrapolated to humans. Myeloablation is followed by donor bone marrow infusion around the time of organ transplant, and can reliably lead to donor specific tolerance. Co-stimulation with cytotoxic lymphocyte antigen-4 (CTLA-4) or anti-CD154 also gives tolerance in the mouse model (Najafian and Sayegh, 2000; Verbinnen et al., 2010; Yamada and Sayegh, 2002). This has not been applied to humans. Indeed, anti-CD154 gives thromboembolic side-effects in humans, because of expression of CD154 on platelets in humans unlike mice (Kirk et al., 2001).

3. Large animal models

The first recorded experimental large animal transplants took place from 1900-1910. Alexis Carrel who pioneered vascular anastomosis and performed renal and heart transplants on dogs in the absence of immunosuppression, experiments that led him to win the Nobel prize in

Medicine in 1912. Kidneys were transplanted from rabbits to humans, but failed within a couple of days (Calne, 1976).

Although rodent models of immunosuppression have provided useful data, large animal models mirror much more closely the events in humans. Laboratory mice are typically bred in specific pathogen free conditions, so their immune systems are relatively naive and have not had alloantigen exposure sufficient to develop significant immunologic memory(Chong et al., 2013). Rodents are also often transplanted very young, in the first few months of life, which further limits how well experiments can be extrapolated to humans. This contrasts with research primates, for example, which are captured in the wild having had exposure to environmental pathogens which allow generation of immunological memory. Although experiments on outbred rodents bred in non-specific pathogen free circumstances are useful, large animal models bear more similarities to humans (Dehoux and Gianello, 2007).

Almost all immunosuppressive agents are tested in large animals prior to phase one human trial for both safety and efficacy. In general, mechanistic studies for proof of concept are carried out in vitro and in rodents. Because of ethical concerns and expense, large animal studies are usually limited to proving effectiveness and lack of toxicity. Much important information about pharmacologic agents is gleaned from rodent studies, but there remain important constraints in the applicability to humans. Drug absorption is greatly different between large animals and humans(Anderson and Kirk, 2013; Dehoux and Gianello, 2007). Drug distribution also differs between species. Differences in target protein tertiary structure between species have implications for drug action, in particular for biologic antibody based therapies. Although large animals are more immunologically similar to humans than rodents, there have been several examples of important differences, again chiefly where tolerance is more easily achieved in large animals than in humans. Large animal studies are used for final confirmation of safety and efficacy after extensive drug development studies in rodent models.

Studies of tolerance aim to have animals accept allogeneic organs / tissues long term in the absence of ongoing drugs. Duration of studies for immunosuppressive agents in animal models varies considerably. In rodent models, tolerance is often defined as survival beyond 100 days, whereas in large animals and humans this usually is defined as several years. Tolerance studies are particularly prone to effects from the age of the animal. Younger animals are easier to tolerise as they have a more naive immune phenotype with less memory cells (Kawai et al., 1999; Kingsley et al., 2007).

3.1. Common large animal models

Pigs, dogs and non-human primates are the large animal species generally used in testing immunosuppressive agents. They all share the same components of the immune system that humans have. They are also anatomically very similar to humans. The tempo and histological findings of organ rejection are also very similar to humans (Dehoux and Gianello, 2007).

Early immunosuppressive experiments in the 1960s-1970s mostly used dogs, for example in the development of the anti-metabolites 6-mercaptopurine and azathioprine (Calne, 1976). Over time, ethical concerns over the use of domesticated animals for research have gradually reduced the use of dogs for research. Dogs are still used for some research on islet and bone marrow transplant. Pigs have largely superseded dogs due to fewer ethical concerns for an industrially farmed animal. Their fecundity also makes them much more practical and has enabled the development of inbred major histocompatibility complex characterised mini-pigs(Flechner, 1983). Non-human primates are certainly the most similar to humans, and are most often used for immediately pre-clinical research. For work with biologic immunosuppressive agents such as monoclonal antibodies, primates are essential as dogs and pigs are often too antigenically different from humans for reagents to bind effectively (Anderson and Kirk, 2013). However, although the use of primates is considered more appropriate because of the ability of antibodies to cross-react between species, it is not an exact science and there are exceptions such as an human anti-CD3 antibody which did not cross-react with macaque CD3 (Chatenoud, 2009). Furthermore, primates are very expensive to work with and raise much ethical concern from the public and scientists alike. Baboons, macaques (Rhesus, cynomolgus and pigtail) and chimpanzees are most commonly used. Baboons, being larger, are often used for xenotransplant experiments as there is space to fit pig organs into them. Chimpanzees are most similar to humans, as our closest living relatives (estimated 1-2% in nucleotide differences), but their endangered status makes their use ethically questionable (Anderson and Kirk, 2013).

As with smaller rodent species, a key aspect of variance in these large animals is differences in drug absorption. For example, although Sirolimus is fairly well absorbed in humans, it is poorly absorbed in monkeys. In monkeys, unabsorbed Sirolimus passes to the distal ileum, where it causes ulcerative lesions and severe diarrhea. Dogs also poorly absorb sirolimus, and are much more prone to tacrolimus toxicity. Pigs, in contrast, tolerate very high levels of calcineurin inhibitors, even to the extent of inducing tolerance in some transplant models (Dehoux and Gianello, 2007).

Dogs have been used in the development of most small molecule immunosuppressive agents, particularly azathioprine, cyclosporine, mycophenolate mofetil and tacrolimus. Tacrolimus was also extensively tested in macaques prior to human use because of toxicity concerns. Interestingly tolerance has never been achieved in dogs with small molecule immunosuppressives alone, unlike the situation for pigs and primates. This may be because of dogs being used older when they have more immunological memory, and dogs being perhaps more outbred than pigs/primates. Thus dogs are often regarded as more immunologically stringent than pigs and primates because it is difficult to achieve tolerance (Dehoux and Gianello, 2007).

Interestingly, cyclosporine or tacrolimus administration for a week at very high doses not tolerated in humans (trough level 60-80 ng/mL) can lead to tolerance of kidneys or livers in pigs. Indeed in around one fifth of pig transplants, a liver will become spontaneously tolerant in the absence of any immunosuppressive agent, albeit after a severe rejection episode (Canafax

and Ascher, 1983; Flechner, 1983). In primates, like pigs, tolerance can result sometimes from calcineurin inhibitor monotherapy (Anderson and Kirk, 2013; Kawai et al., 1999). Again, this points out the marked differences from humans, where there has never been tolerance following calcineurin inhibitor monotherapy in an allogeneic transplant.

Rapamycin is well tolerated in swine, with side effects of hyperlipidaemia and pneumonia. Monotherapy prolongs allograft survival to a similar extent to standard triple therapy. Primates also tolerate rapamycin well, and monotherapy prolongs renal transplant survival to 30 days (Taylor et al., 2005). In humans, rapamycin is poorly tolerated at the time of transplant because of wound dehiscence and seromas. This complication was not predicted in animal models.

3.2. Costimulation blockade

In an adaptive immune response, initiation involves binding of a T cell receptor to its cognate specific alloantigen in the context of major histocompatibility complex (MHC). In humans, major histocompatibility complex is known as human lymphocyte antigen (HLA). Activation also requires the presence of costimulation from other accessory molecules on the surface of the T cell and antigen presenting cell. This requirement for costimulation is less stringent for memory cells. This partly explains why young naïve specific pathogen free rodents are more permissible to tolerance than older large animals which have many memory cells independent of costimulation (Moreau et al., 2013).

There are many costimulation molecules. The best characterised is CD28 on T cells which binds B7.1 (CD80) and B7.2 (CD86) on antigen presenting cells. This CD28 provides the so-called signal two for T cell activation; with signal one being the T cell receptor – MHC interaction with antigen presenting cells. CD28 ligation activates downstream GTPases and causes IL-2 production. For the B cell – T cell interaction, the best characterised co-stimulation interaction is between CD40 on B cells and CD40L on T cells (Verbinnen et al., 2010).

Cytotoxic lymphocyte antigen-4 immunoglobulin, (CTLA-4Ig) has been developed with activity against B7, as well as other immunosuppressive agents against CD40, CD40L and other costimulatory molecules (Najafian and Sayegh, 2000). These agents have been tested in rodents and non-human primates. Costimulation blockade has strong efficacy in rodent models, giving rise to tolerance for example in heterotopic heart transplant. In primates however, efficacy is less marked with prolongation of survival seen rather than tolerance. In non-human primate models, administration of CTLA4-IG prolongs kidney transplant survival from 5-8 days to 20-30 days (Najafian and Sayegh, 2000). CTLA4-Ig prevents the binding of B7.1(CD80) and B7.2(CD86) to CD28 and CTLA4. This is normally a stimulatory interaction, although inhibitory signals are also transduced by CTLA4. The reasons for reduced efficacy in primates are unclear, but it may be that the antibody has less affinity, or the need for costimulation is less with the more pronounced memory response. Efforts to improve the binding efficacy of CTLA4-Ig or to use separate antibodies to B7.1 and B7.2 have shown some albeit modest improvement in

survival. Combining the antibody with other therapies including basiliximab (anti-CD25) and giving the antibody repeatedly as a maintenance rather than induction agent, have likewise shown some improvement in survival.

The CD40-CD40L (CD154) costimulation interaction has also been the target for blocking strategies (Kirk et al., 2001). CD40L on T cells binds CD40 on antigen presenting cells (APCs), and seems to function more in activating the APCs rather than the T cell. Humanised antibody against CD154 has shown reasonable efficacy in primate models(Pierson et al., 1999). The dose used is generally higher than that for other antibody therapies, and may relate to the expression of CD40L on cells other than T cells, including platelets. Anti-CD154 has prolonged survival in renal, islet, heart and skin transplants in primates. When used as monotherapy with monthly injections for one year, transplant survival as long as five years has been seen, although there are typically changes of chronic rejection. There has been some debate about whether conventional immunosuppressive drugs are antagonistic with anti-CD154. Studies targeting CD40 itself have not shown as much efficacy as antibody against CD40L, suggesting the antibody has less avidity, or perhaps that CD40L has other functions than simply binding to CD40 (Yamada and Sayegh, 2002).

The limitations of animal studies were highlighted very tragically in the development of the biological therapeutic agent TGN1412 (Emanuel and Miller, 2007; Nada and Somberg, 2007). This is a humanized monoclonal antibody targeting the costimulation molecule CD28. It was aimed for use in rheumatoid arthritis and some forms of leukaemia. Initial studies in mouse and subsequent work in cynomolgus macaques gave promising results. Although an agonist anti-CD28 antibody, it caused significant immunosuppression. This was purported to be the result of preferential activation of regulatory T cells versus effector cells. Phase one clinical trials in humans were carried out in London on eight healthy male volunteers. Two were given placebo. The remaining six were given TGN1412 at 1/500 the dose given to macaques. In spite of the low dose, all six rapidly developed severe cytokine release syndrome and became profoundly unwell. All were admitted to intensive care and treated with steroids. One volunteer had all fingers and toes amputated. All survived, but studies showed a persistent leucopaenia and particularly a deficiency of regulatory T cells. One volunteer has early signs of a lymphoid malignancy.

Predictably, the company developing TGN1412, TeGenero, became bankrupt shortly afterward. There was much criticism for the trial design, where all six volunteers received the drug within 20 minutes of each other, meaning it was too late to prevent drug administration to the other five when the first volunteer became unwell. Reasons for the idiosyncratic human reaction to the drug are unclear, in spite of the dose being $1/500^{th}$ that used in the primate study. Speculation is that the antibody was raised to human CD28, and therefore has a much higher avidity and potency in humans versus the macaques. Some suggested that effects on humans are more pronounced because of the abundance of memory cells when compared to laboratory animals which are raised in a more pathogen free environment.

Strategies using CTLA4-Ig together with anti-CD40L have shown synergism in some circumstances. Again, results have been much more impressive in rodents than in primates. In primates, survival of renal allografts up to 300 days has been seen, with only a short induction course of CTLA4-Ig and anti-CD40L. Lower levels of alloantibody have been seen with combination therapy. Some say though that if the agents are administered singly in optimal doses that little synergism exists (Verbinnen et al., 2010).

3.3. Cell Depletion

Cell depletional strategies began with the use of anti-CD4 and anti-CD8 antibodies. In dogs, these had a modest effect on survival of renal allografts, up to 35 days. When combined with total body irradiation to further deplete T cells, these antibodies could result in operational tolerance in baboons, prolonging survival beyond one year (Kawai et al., 1999).

Depletional studies have used the anti-CD3 diphtheria immunotoxin chimera (Chatenoud, 2009). This provides very potent T cell depletion, and kills the T cells unlike the human anti-CD3 OKT3 which simply causes internalization of CD3. Results using this immunotoxin have been very impressive, with operational tolerance in many primate transplant models. Survival over two years without histologic changes of chronic rejection has been observed in primate renal transplants.

In humans, T cell depletion strategies are potent as in primates, although tolerance has not been seen in the absence of mixed chimerism. Depletion at the time of transplant reduces the need for long term maintenance immunosuppression. Antibodies used in primates such as the anti-CD3 immunotoxin are not usable in humans because of the different protein structure, so that antibodies against the same epitope do not cross-react between species. In humans, alemtuzumab (anti-CD52) and anti-thymocyte globulin (ATG) have been the clinically used depletional antibody therapy (Kirk, 2003).

3.4. Tolerance strategies

Many attempts to derive tolerance to allotransplants have aimed to provide haemopoietic chimerism. This relates to studies in the 1950s of the importance of having donor and recipient haemopoietic cells mixed for achieving tolerance (Brent et al., 1976; Chong et al., 2013). Twin cattle were observed to have a mix of each other's red cells, and also to be tolerant of skin transplants from each other. This relates to a shared placenta in utero. Experiments in rodents demonstrated that injecting alloantigenic haemopoietic cells to a foetus results in subsequent tolerance to that alloantigen.

In large animal models, efforts to induce tolerance involve initial T cell depletion with anti-thymocyte globulin, followed by allogeneic bone marrow transplant around the time of organ transplant (Kawai et al., 1999). Some protocols have used myeloablative conditioning to make space prior to bone marrow infusion. This has used total body irradiation or cyclophosphamide. Results have been encouraging with evidence of tolerance in primate models and dogs. Interestingly the haemopoietic chimerism tends to be transient, lasting around a month, but the organ tolerance persists in spite of loss of donor haemopoietic cells. Even using polymerase chain reaction (PCR) to detect donor blood cells at the DNA level generally fails to demonstrate microchimerism beyond a month. These strategies have been applied clinically to humans with success. Trials have shown that myeloablative or non-myeloablative conditioning followed by bone marrow and renal transplant can lead to tolerance up to ten years, again in the absence of ongoing microchimerism (Sachs et al., 2014; Sachs et al., 2011).

4. Studies of toxicity: Cyclosporine as paradigm

Cyclosporine was first used clinically in the early 1980s (Canafax and Ascher, 1983; Flechner, 1983). It gave a massive improvement in survival of transplanted organs, and brought organ transplantation from experimental therapy to mainstream practise. Cyclosporine acts by binding cyclophilin in the cytoplasm of T cells. This complex acts to inhibit the phosphatase calcineurin, which in turn inhibits T cell proliferation (Robson, 2003). For the purposes of this article, it will be used as a prototype in describing the use of animal models in toxicity studies for immunosuppressive agents.

Perhaps the most important side effect of cyclosporine is deterioration in renal function (Whiting et al., 1985). This has been shown in rat single nephron puncture studies to be driven by increased vascular resistance, and reduced blood flow, in the glomerulus. The reduced blood flow is paralleled with a proportional or greater drop in glomerular filtration rate. This indicates that the vascular resistance is principally governed by constriction of the afferent arteriole which is the inflow vessel to the nephron, more so than constriction of the efferent arteriole. Urine volume, concentrating ability and osmolality are unaffected by cyclosporine. Studies in rats have revealed a series of electrolyte abnormalities precipitated by cyclosporine administration. These include low magnesium and bicarbonate, and high potassium. It seems cyclosporine inhibits loop of Henle reabsorption of magnesium and bicarbonate, as well as inhibiting distal tubule potassium and acid secretion. These electrolyte abnormalities resemble pre-renal azotaemia (dehydration)

The histological changes or damage to the kidney following cyclosporine administration have been extensively studied in the rat (Whiting et al., 1985). Changes are seen in both the vasculature and the tubules. Tubular ballooning (vacuolisation) and inclusion bodies are seen, and are reversible on discontinuation of cyclosporine. This represents dilated endoplasmic reticulum and lysosomes respectively, and is generally seen with high serum levels of cyclosporine. Interestingly, changes to the vasculature are only seen in humans and not in rats. In humans, cyclosporine leads to vacuolisation of the smooth muscle cells and endothelium of

arterioles. This can lead to endothelial necrosis, exposing the basement membrane to activate the clotting cascade with platelet adhesion. Ischaemia of the tubules ensues, which is typically seen as striped interstitial fibrosis on biopsy.

In humans, cyclosporine administration is associated with hypertension (Flechner, 1983). The precise mechanism is unclear. Certainly constriction of renal afferent arterioles activates the renin-angiotensin axis to cause peripheral vasoconstriction. There also seems to be a direct effect of cyclosporine making endothelium and smooth muscle cells more sensitive to vasopressin and angiotensin II, again leading to systemic hypertension. Interestingly this peripheral vasoconstriction is not seen in rats given cyclosporine, although rabbits have a similar response to humans.

The effect of cyclosporine on the rennin-angiotensin system varies between species. In rats there is a clear activation, but this seems more transient in humans with prolonged administration. In both species, there is a hypertrophy of the juxtaglomerular apparatus where renin is produced. The relative importance of the renin secretion driving the systemic hypertension or the other way around is not entirely clear.

Cyclosporine leads to impaired glucose tolerance and diabetes in humans (Taylor et al., 2005). This is more common in co-therapy with steroids. The cause is unclear, but in rats cyclosporine causes vacuolisation of pancreatic islets and leads to reduced insulin content and release from the pancreas. In humans this is less clear, and there is more evidence for insulin resistance rather than reduced insulin output.

On the liver, cyclosporine causes a cholestatic picture in both humans and rats. This seems to be mediated by inhibition of hepatocyte membrane bile salt transport proteins. There is no corresponding toxicity to hepatocytes, as evidenced by the serum biochemical picture of raised bilirubin without corresponding change in transaminases

5. Discussion

In summary, it can be seen that animal models provide a useful means of testing immunosuppressive agents prior to use in humans. Limitations of these studies are an important constraint, and not all animal data can be extrapolated to humans. In particular, differences in the immune system between humans and rodents relate to the older age of human transplant recipients and greater exposure to pathogens. This creates more memory cells and therefore less dependence on co-stimulation and greater resistance to tolerance in humans. In large animals, the memory cell differences with humans are less pronounced, given that the animals are not generally raised specific pathogen free. However, important differences exist particularly in drug absorption and in the effects of biologic agents such as antibody where

target protein epitopes may differ. Ethical concerns are naturally greater for large animal use over rodent use. In spite of limitations, animal models remain the most useful predictor of immunosuppressive agent utility, and currently seem a pre-requisite for human use of new agents.

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