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# 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 more 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 diseases, such as multiple sclerosis, are 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.

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## 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. These autoimmune disorders are covered elsewhere is this issue. Pathological alloimmunity is principally the domain of organ and cell transplant, and will prove the main focus of this paper. Alloimmunity to transplants is unique in involving both

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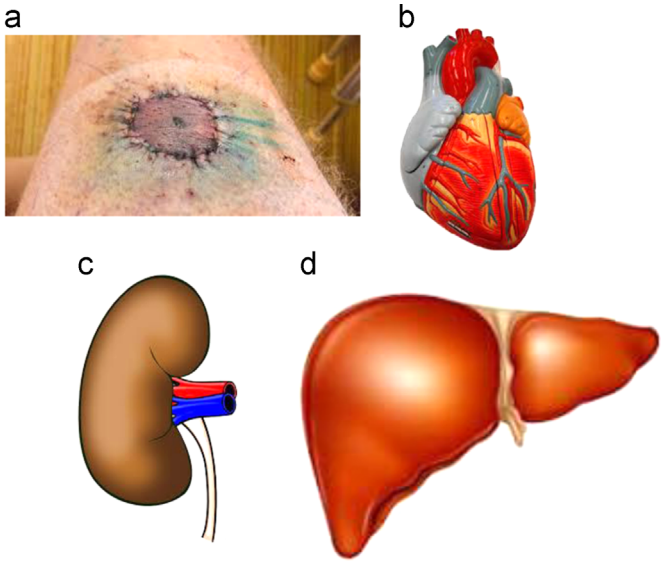


**Fig. 1.** The commonly used animal models for organ/tissue transplant. (a) Skin transplant – non-vascularised, but a very stringent model of rejection with rapid kinetics of rejection and often difficult to get long term survival/tolerance (b) Heart transplant – the most commonly performed and technically most straightforward vascularised transplant. Transplanted into heterotopic position in abdomen or neck. More straightforward than skin to gain tolerance, but more difficult than liver or kidney. (c) Kidney transplant – placed in abdomen. Recipient usually has native kidneys removed so that this is a physiological life sustaining model. (d) Liver transplant – orthotopic. Technically the most challenging, particularly in mice. Often spontaneously tolerant.

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 (Fig. 1) (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 1960 s, 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 to 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

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