Xenotransplantation and tolerance

Benjamin Samstein¹ and Jeffrey L. Platt^{2*}

¹*Transplantation Biology, and the Department of Surgery, and* ²*Transplantation Biology and the Departments of Surgery, Immunology and Pediatrics, Medical Sciences Building 2-66, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA*

The application of xenotransplantation faces daunting immunological hurdles, some of which might be overcome with the induction of tolerance. Porcine organs transplanted into primates are subject to several types of rejection responses. Hyperacute rejection mediated by naturally occurring xenoreactive antibodies and complement can be overcome without tolerance. Acute vascular rejection and cellular rejection, however, may present important opportunities for immunological tolerance, and humoral rejection might be approached by various mechanisms including (i) clonal deletion, (ii) anergy, (iii) immune deviation, (iv) induction of immunoregulatory or suppressor cells, or (v) veto cells. B-cell tolerance, useful for preventing humoral rejection, might be approached through clonal anergy. It remains to be determined, however, whether tolerance induction is required for xenotransplantation and by which means the various mechanisms of tolerance can be applied in the setting of xenotransplantation. Regardless, the study of tolerance will surely expand understanding of the physiology and pathophysiology of the immune system.

Keywords: xenotransplantation; tolerance; lymphocytes; immunosuppression

1. INTRODUCTION

Those in the field of transplantation have long sought the means to induce tolerance to foreign tissues to avoid the use of immunosuppressive agents and medical complications of immunosuppressive treatments. Interest in tolerance may be heightened in circumstances where the risks of immunosuppression approach the benefits of transplantation, examples of which include pancreas transplantation and xenotransplantation. In the case of pancreas transplantation, the risks might exceed the benefits because an alternative therapy is available. In the case of xenotransplantation, the risks might exceed the benefits because of the daunting immunological response that would need to be suppressed. This article will review the subject of tolerance in xenotransplantation, considering especially where tolerance might be most useful and what approaches might be suitable for application in xenotransplantation of porcine tissues and organs in humans.

The subject of tolerance is raised in conjunction with the clinical application of xenotransplantation for two reasons. First, the immune response to an unmodified xenograft is so profound and cataclysmic that the immunosuppression required to suppress the immune response would be devastating, and, thus, some believe successful xenotransplantation will not be possible without tolerance (Sachs & Sablinski 1995). Second, because in xenotransplantation animal donors would be available well before the transplant, the opportunity for conditioning of either the donor or the recipient in advance of transplantation exists, allowing for a lengthy tolerance induction regimen.

Tolerance is the specific immune non-responsiveness to an immunogenetic stimulus, such as a foreign tissue graft. Tolerance presupposes that the recipient is immunocompetent and that the immune response to the desired transplant is qualitatively different from the immune response to other foreign tissue or pathogens. However, because the nature of the immune response and identity of relevant immunogens is sometimes ill-defined and because the relevant test of immunocompetence—namely the ability to reject a third-party graft—cannot be easily undertaken in the clinical setting, prolonged survival of a graft in the absence of immunosuppression is often used. The operative definition has two shortcomings. First, it presupposes in the absence of immunosuppression that the recipient is immunocompetent. However, that assumption might well be faulty in some situations where the regimen to induce tolerance leads to ablation of mature T cells. For example, an individual who has undergone myeloablative conditioning might not have a completely normal immune system. Second, some transplants can fail because of physiological incompatibilities. Non-immune failure of a xenograft, if mistaken for immune-mediated rejection, would be thought to be a failure of the tolerizing regimen when, in fact, tolerance might have been induced if not for physiological incompatibilities such as complement regulatory protein incompatibilities.

2. NON-IMMUNE RESPONSE TO XENOGRAPTS

A foreign tissue graft, whether from an allogeneic or a xenogeneic source must undergo successful engraftment, be physiologically compatible with the recipient, and avoid immune destruction in order to function enduringly. Successful engraftment and physiological

*Author for correspondence (platt.jeffrey@mayo.edu).

Figure 1. The immunological response to xenotransplantation. The immune response to xenotransplantation can be classified according to whether the graft consists of isolated cells or free tissues, such as islets of Langerhans or of a primarily vascularized organ such as the kidney or heart. (a) Vascularized organ grafts are subject to hyperacute and acute vascular rejection caused by the action of anti-donor antibodies on donor endothelium. If hyperacute or acute vascular rejection are averted, the graft may undergo accommodation, a condition in which the graft appears to resist injury despite the return of anti-donor antibodies to the circulation and the presence of an intact complement system. A vascularized organ graft may also be subject to cellular rejection and chronic rejection more or less like the corresponding types of rejection observed in allografts. (b) Free tissue grafts are subject to failure caused by primary non-function, failure of neovascularization or failure of the microenvironment to support the survival and function of the foreign tissue. If the free tissue or isolated cells engraft, they are then subject to cellular or humoral rejection. (Adapted from Nagayasu & Platt (1998) with permission from Landes Bioscience and *Graft*.)

compatibility are non-immune factors that are important for xenograft survival but that do not necessarily correlate with the induction of immune tolerance. Cellular xenografts, for example, require the micro-environment of the recipient to provide necessary factors for growth and survival. While cellular xenografts are not subject to the vascular rejection syndromes to be described below, they require angiogenesis to occur within the graft. Organ grafts, in which the humoral components of blood are brought into direct contact with the vascular lining, are subject to injury due to physiological incompatibilities in the coagulation system between the donor and the recipient that might, in turn, lead to thrombosis or organ failure (Bach *et al.* 1996; Lawson & Platt 1996).

3. THE IMMUNE RESPONSE TO XENOGRAPTS

An organ transplanted from one species, such as the pig, into a distant species, such as a human, is subject to the types of rejection listed in figure 1. The types of rejection seen in xenotransplants are the same as those seen in allotransplants; however, the incidence and severity is much greater in xenotransplants. Although hyperacute rejection is the most severe of immune reactions, tolerance is not needed to avoid it. Tolerance might be useful in preventing acute vascular, cellular and chronic rejection.

Figure 1 shows the elements of the immune system that are potentially involved in the immune response to xenografts. Presumably, it is these elements that need to be regulated in order to establish tolerance to a xenograft.

These elements include complement, T lymphocytes, natural and elicited antibodies, and natural killer (NK) cells. An enormous body of literature is available for further details on the activation of each of these elements (see Cooper *et al.* (1997) for reviews).

Porcine organs transplanted into immunocompetent primates are subject to very rapid failure due to hyperacute rejection. The first step in the pathogenesis of hyperacute rejection is the binding to graft endothelium of xenoreactive natural antibodies, so named because they recognize the cells of foreign species and because they are present in the circulation without a known history of exposure to the corresponding antigen (Boyden 1964; Platt *et al.* 1990). The natural antibodies in humans which bind to pig cells are predominantly directed against Gal α 1,3Gal, a saccharide expressed on the cells of lower mammals but not on the cells of humans, apes and Old World monkeys (Galili *et al.* 1988). The antibodies, which recognize Gal α 1,3Gal represent 85–95% of natural xenoreactive antibodies (Sandrin *et al.* 1993). The binding of xenoreactive antibodies to the graft leads to complement activation (Platt *et al.* 1991). Activated complement causes endothelial cells to retract, leading to exposure of the underlying matrix (Saadi & Platt 1995). This disruption of the endothelial monolayer brings about the loss of vascular integrity and may stimulate the adhesion and activation of platelets leading to the typical pathology seen in hyperacute rejection. Activation of complement is amplified because the complement regulatory proteins that limit complement activation are species specific and thus porcine complement regulatory proteins fail to

control primate complement proteins. Hyperacute rejection is effectively prevented by the removal of xeno-reactive antibodies from the recipient (Cooper *et al.* 1988; Lin *et al.* 1997), complement inhibition (Leventhal *et al.* 1993a; Magee *et al.* 1995; Pruitt *et al.* 1994) or the expression of recipient compatible complement regulatory proteins in the donor (McCurry *et al.* 1995; Pasternak *et al.* 1985). Thus, preventing hyperacute rejection does not require the induction of tolerance.

When hyperacute rejection is averted, xenografts become subject to acute vascular rejection (Leventhal *et al.* 1993b; Magee *et al.* 1995). Acute vascular rejection is currently the principal obstacle to the use of porcine organ xenografts in human subjects. Of the factors thought to initiate acute vascular rejection in xenografts, xenoreactive antibodies are now widely seen as playing a critical role in the pathogenesis of acute vascular rejection.

The evidence that antibodies, and the B lymphocytes that make them, are a critical factor in the pathogenesis of acute vascular rejection is strong. The onset of acute vascular xenograft rejection coincides with a striking increase in the synthesis of xenoreactive antibodies following exposure to porcine tissues (Cotterell *et al.* 1995). Reduction of antibody has prevented acute vascular rejection in a pig-to-baboon model (Lin *et al.* 1998a). Acute vascular rejection can be induced or hastened by the administration of xenoreactive antibodies (Perper & Najarian 1967). Humoral rejection may also stimulate T-cell mediated rejection by activating endothelium leading to upregulation of inflammatory cytokines, chemokines and increased expression of co-stimulatory molecules (Ihrcke *et al.* 1993).

Humoral rejection of xenografts can be mediated by both natural antibodies and elicited antibodies. The human natural anti-Gal α 1,3Gal antibodies are part of a larger pool of antibodies formed by the self-renewing population of T-independent B-1 B cells. While most natural xenoreactive antibodies recognize Gal α 1,3Gal, some xenoreactive antibodies may recognize other epitopes (Zhu 2000). While great effort is being made to produce porcine donors that do not express Gal α 1,3Gal, organs that do not express Gal α 1,3Gal might undergo humoral rejection due to other antigens recognized by natural and elicited antibodies.

Elicited antibodies may contribute to the pathogenesis of acute vascular rejection. Clearly, the elicited humoral response can play no role in the hyperacute rejection of xenografts by unsensitized recipients, as there is insufficient time for a response to occur. However, an elicited response could contribute to rejection of xenografts that are not rejected hyperacutely. In non-primate models of xenotransplantation (so-called 'concordant' models), where natural antibodies are not present, elicited antibodies can cause rapid vascular rejection. While it is clear that development of genetically altered porcine donors with decreased expression of Gal α 1,3Gal antigens could eliminate the reaction of natural antibodies with the graft, elicited antibodies might still be able to bind to the graft and their role is still unclear. Anti-porcine antibodies directed against antigens other than Gal α 1,3Gal have been identified within 20 days of infused porcine peripheral blood progenitor cells (Buhler *et al.* 2000). With

the persistent exposure to xenogeneic antigens that would accompany prolonged survival of a xenograft, it can be anticipated that more conventional elicited responses will be seen. Because of the greater disparity between primate and porcine proteins, it is likely that elicited antibodies will recognize porcine histocompatibility antigens (swine leucocyte antigen, SLA) and non-histocompatibility antigens, whereas in clinical transplantation at this time allogeneic antibodies are directed predominantly against human leucocyte antigen (HLA).

If hyperacute rejection and acute vascular rejection were prevented, xenografts would be subject to cellular rejection. Cellular rejection is the most common form of rejection seen in allografts, but it has not been well characterized in xenografts. Cellular rejection of allografts is characterized by interstitial infiltrate of T cells and macrophages (Porter 1992). Infiltration of T cells, macrophages and also NK cells may characterize cellular rejection of xenografts. Acute rejection is thought primarily to be a T-cell mediated process and thus the mainstays of therapy in the clinical setting of transplantation—calcineurin inhibitors—are directed against T cells.

The cellular immune response to xenotransplants may be more intense than the cellular response to allotransplants because the repertoire of T cells directed against xenografts may be more diverse, and because humoral and inflammatory reactions evoked by foreign tissue may amplify cellular immune responses (Platt 1998). These mechanisms, it will be seen, may have implications for the approaches selected for induction of tolerance to xenotransplantation.

The repertoire of T cells directed against a xenograft may be highly diverse. One mechanism underlying diversity of the xenoreactive T-cell repertoire may relate to the means of antigen presentation. Murray *et al.* (1994) showed that human T-cell populations are directly activated by swine endothelial cells. Limiting dilution analysis suggested that human T cells responding to porcine antigen-presenting cells (APCs) have a higher frequency than human T cells responding to allogeneic APCs (Murray *et al.* 1994). A second mechanism underlying diversity of the xenoreactive T-cell repertoire may be the diversity of antigen peptides in xenografts. In direct antigen presentation, intact major histocompatibility antigens on the surface of antigen-presenting cells of the donor (stimulator) stimulate T cells. This mechanism can be studied in mixed lymphocyte reactions. A second mechanism underlying diversity of the xenoreactive T-cell repertoire may be the diversity of antigen peptides in xenografts. In indirect presentation, foreign antigens are taken up by responder APCs and presented to the recipient T cells in the context of responder MHC. Yamada *et al.* (1995) showed that both the indirect and the direct pathway are functional in human–porcine interactions and that the strength of xenogeneic mixed leucocyte culture reactions is of the same order as allogeneic mixed leucocyte culture reactions. Both CD4⁺ and CD8⁺ T cells are capable of recognizing porcine xenoantigens (Olack *et al.* 2000; Shishido *et al.* 1997).

The humoral immune response to xenografts might be expected to amplify the cell-mediated immune response through several mechanisms. Antibody binding to

endothelial cells leads to endothelial cell activation, which is associated with the elaboration of cytokines, chemokines and adhesion molecules that mediate the infiltration of T cells into the graft (Ihrcke *et al.* 1993). NK cells and Fc-receptor bearing cells, such as macrophages, are activated upon contact with immunoglobulins attached to cell surfaces and secrete inflammatory cytokines that may recruit T cells to sites where antibodies have bound. Humoral immunity can further enhance T-cell mediated immunity because B cells are effective APCs.

NK cells are large lymphocytes that lack T-cell receptors and are capable of mediating cytotoxicity. NK cells are capable of binding directly to and inducing morphological changes and activation of endothelium in porcine endothelium (Malyguine *et al.* 1996). NK cells have receptors for MHC class I molecules that, when stimulated, inhibit NK function (Lanier & Phillips 1996). Recognition of self class I inhibitory ligands is believed to be important in preventing the NK cell from killing normal autologous cells (Karlhofer *et al.* 1992). Because xenogeneic MHC molecules are not recognized by inhibitory receptors, NK cells might be expected to play a role in the rejection of xenografts. Consistent with this concept, NK cells do appear to play a role in resisting xenogeneic marrow engraftment (Sharabi *et al.* 1990) and in xenografts undergoing acute vascular rejection (Blakely *et al.* 1994). However, there is no clear evidence that NK cells contribute to solid organ xenograft rejection.

Finally, xenografts would probably be subject to chronic rejection, much like allografts, if the xenografts avoided acute vascular and cellular rejection. Chronic rejection of allografts occurs months after transplantation and is characterized by interstitial fibrosis and myointimal hypertrophy of arteries and arterioles (Porter 1992).

4. MECHANISMS OF TOLERANCE

As important as is the immune response to foreign antigens and pathogens, of equal importance is the regulation of immune responses to prevent reactivity that would damage healthy cells. Regulation of immunity in this way is a natural manifestation of tolerance, and recent years have brought new information about the mechanisms by which tolerance arises as a natural physiological response. The methods by which the induction of tolerance for therapeutic purposes is approached may reflect efforts to recapitulate 'natural tolerance'. A detailed review of these mechanisms is beyond the scope of our discussion, although pertinent aspects of the physiology of tolerance will be discussed here.

T-cell tolerance is thought to arise through several mechanisms. The mechanisms are often categorized as being peripheral or central. Peripheral tolerance refers to tolerance that develops after T cells have matured and left the thymus. Central tolerance refers to the events surrounding the deletion of T cells in the thymus and is primarily thought of as the clonal deletion or negative selection of self-reactive T cells. While negative selection in the thymus is an important mechanism of self-tolerance, it should be appreciated that the process by which T cells are selected for reactivity with MHC—so-called 'positive

Table 1. *Mechanisms of T-cell tolerance*

mechanism	central or peripheral
clonal deletion	central and peripheral
anergy	peripheral
immune deviation	peripheral
suppressor cells	central and peripheral
veto cells	peripheral

selection'—eliminates what would otherwise be a large number of potentially autoreactive cells. It should also be appreciated that a significant number of autoreactive cells exit the thymus to be controlled ultimately by peripheral mechanisms.

The mechanisms of T-cell tolerance include (i) clonal deletion, (ii) anergy, (iii) immune deviation, (iv) development of immunoregulatory cells or suppressor cells, and (v) development of veto cells (see table 1). Clonal deletion involves the elimination of cells, presumably by apoptosis. This elimination takes place primarily in the thymus but can occur in the periphery. Where the kinetics of T-cell survival has been studied, there has appeared to be a continuum between deletion and shortened time of lymphocyte survival. Clonal anergy refers to acquired non-responsiveness of lymphocytes to an adequate stimulus. A lymphocyte typically becomes anergic if it encounters antigen and the antigen receptor is stimulated, but further activating signals are not delivered to the cell. Immune deviation refers to a change in the differentiation of a population of T cells such that the cells elaborate different cytokines and that tolerance to antigens might result from the cytokine milieu. Suppressor T cells are T cells that respond to antigen—but instead of exhibiting effector properties, they inhibit the activation of effector lymphocytes. Although the mechanisms by which suppressor T cells function are not fully understood, a growing body of evidence suggests that suppressor T cells are evoked under conditions in which immune responses need to be limited to preserve homeostasis. The veto mechanism is mediated by CD8⁺ T cells that kill cells capable of specifically recognizing them (Vernabac *et al.* 1994).

B-cell tolerance primarily involves clonal deletion and clonal anergy (Townsend *et al.* 1999). Although B-cell tolerance is not generally segregated as central or peripheral, it is clear that the B-cell repertoire is shaped by events during B-cell development and by later events once B cells have matured and are present in the periphery. In the bone marrow, when multivalent membrane-bound antigen binds to immature B cells, those B cells are eliminated (Nemazee & Burki 1989). The natural B-cell repertoire is also shaped by competition in the peripheral follicular niches, where self-reactive B cells are unable to localize appropriately but become anergic and die (Cyster *et al.* 1994). An important mechanism of B-cell tolerance is brought into play when the B cell encounters antigens under conditions in which additional co-stimulation does not occur. The anergic state that ensues may inhibit antibody production, and it is one mechanism that is commonly used. B-cell tolerance may be facilitated by complement (Prodeus *et al.* 1998).

Complement enhances B-cell tolerance against soluble antigens (Carroll 1998). Deficiencies in C4 or CR2 (CD21–CD35) lead to loss of B-cell tolerance and autoimmune disease (Prodeus *et al.* 1998). How to make use of the role of complement in the establishment of B-cell tolerance is not clear.

5. APPROACHES TO TOLERANCE IN XENOTRANSPLANTATION

Approaches to tolerance induction include approaches aimed at the immune response against a single antigen and approaches aimed very broadly, for example, at foreign cells. Most approaches to induction of tolerance influence both T- and B-cell responses. This reflects the similarity of the stimuli for tolerance in B and T cells and the fact that T cells are needed to facilitate some B-cell responses. Approaches that have been explored for the induction of T-cell tolerance in xenotransplantation are (i) mixed chimerism, using bone marrow transplantation, (ii) thymic transplantation, (iii) induction of T-suppressor cells, (iv) co-stimulatory blockade, and (v) temporary T-cell depletion. These approaches will be discussed below.

Bone marrow transplantation for the induction of tolerance developed out of the work of Owen (1945) and then Billingham *et al.* (1953). Owen found that cattle that were fraternal twins were tolerant to the cells of the other twin and indeed those cells could be found in the circulation (Owen 1945). This state of tolerance was thought to arise from the exchange of haemopoietic cells during intra-uterine life before mature lymphocytes had developed. Billingham and colleagues found that tolerance could be deliberately induced by administering allogeneic marrow cells to the foetus and newborn (Billingham *et al.* 1953). Attempts to administer allogeneic bone marrow to mature individuals were less successful because the bone marrow would fail to engraft and because irradiation of the recipient to enable engraftment would give rise to immunodeficiency (due to the positive selection of T cells on a thymus that was incompatible with circulating APCs). To overcome this problem, Ildstad & Sachs (1984) found that mixing donor and recipient bone marrow would enable the engraftment of lymphocytes that could be selected on recipient thymus and have antigen presented by recipient APCs. This dual population of bone marrow cells is referred to as 'mixed chimerism'. The mixed chimerism approach aims to shape the immune repertoire of the recipient by positive selection and clonal deletion. Leucocytes in donor bone marrow migrate to the thymus and mediate the deletion of donor reactive T cells. Haemopoietic cells in the bone marrow can also mediate clonal deletion of B cells that express immunoglobulin recognizing donor antigen. Mixed chimerism would seem to require conditioning of the recipient by such means as whole-body irradiation to enable engraftment of foreign bone marrow cells (Ildstad & Sachs 1984).

In allogeneic models in primates, long-lasting donor-specific tolerance has been achieved through mixed chimerism (Kawai *et al.* 1995). Mixed chimerism has been achieved in rat-to-mouse models, allowing prolonged skin graft survival, although the donor-specific skin grafts

were chronically rejected (Ildstad *et al.* 1992; Sharabi *et al.* 1990). In such rat–mouse chimeras, the donor T-cell repertoire can be shown to lack certain receptor families, suggesting evidence for clonal deletion as the mechanism of tolerance (Tomita *et al.* 1994). Also consistent with clonal deletion is the presence of rat cells with dendritic morphology in the thymuses of chimeric mice (Sykes *et al.* 1997). Mixed xenogeneic chimerism has been much more difficult to achieve in primates. After transplanting porcine bone marrow into cynomolgus monkeys, Sablinski *et al.* (1999) observed 2% bone marrow chimerism. Unfortunately, these monkeys had no apparent B-cell tolerance, as anti-pig IgM and IgG were found to be at pretransplant levels soon after transplantation (Sablinski *et al.* 1999).

The major disadvantage of tolerance induction through bone marrow transplantation is the severity of the conditioning regimen. Such a regimen could compromise immune responses for extended periods. One additional potential disadvantage to the use of mixed chimerism for xenogeneic tolerance is that the recipient might only be made tolerant to those antigens expressed by bone marrow cells. In xenotransplantation it is possible that tissue specific antigens, not expressed in the bone marrow, might engender an immune response. Thus, adequate tolerance could well be established to antigens expressed in haemopoietic cells, but the xenograft could still succumb to rejection.

Another approach for the induction of tolerance for xenotransplantation involves transplantation of xenogeneic thymus plus haemopoietic cells that may induce central tolerance by the deletion of donor-reactive cells. Zhao *et al.* (1996) reported the permanent porcine skin graft survival in thymectomized mice, depleted of T and NK cells, which received foetal porcine thymus/liver grafts. Murine T cells that developed in these mice proliferated normally and were capable of clearing pathogenic infections (Zhao *et al.* 1997). Recently, Yamada *et al.* (2000) demonstrated that transplantation of allogeneic thymus would allow tolerance to MHC class I-mismatched kidneys in a large animal model.

Peripheral tolerance by induction of anergy or suppression is emerging as a useful way to control the immune response to transplantation antigens. The advantage of peripheral tolerance is that it does not require conditioning of the recipient. It may also in some cases allow tolerance to be induced to a greater diversity of antigens than central tolerance. The means of inducing peripheral tolerance are numerous. Here we shall discuss the generation of suppressor and the blockade of co-stimulation of T cells.

Immunoregulatory or suppressor cells inhibit the activation of other immune cells. Immunoregulatory cells of a number of different phenotypes have been identified (see table 2). Ciubotariu *et al.* (1998) has identified xenospecific suppressor T cells in CD8⁺CD28[−] phenotype. These cells appear to prevent co-stimulation by APCs, thus limiting the immune response (Li *et al.* 1999). Suppressor cells expressing CD4⁺ have also recently been identified (Fowell & Mason 1993; Sakaguchi *et al.* 1995; Suri-Payer *et al.* 1998). Some suppressor cells may express CD25⁺, the α -component of the interleukin (IL) 2 receptor. Suppressor cells seem capable of providing

Table 2. *Immunoregulatory cells*

phenotype	function	activity in transplant	reference
CD8 ⁺ CD28 ⁻	inhibit CD4 ⁺ T helper cell proliferation	unknown	Li <i>et al.</i> (1999)
CD4 ⁺ CD25 ⁺	unknown	unknown	Takahashi <i>et al.</i> (2000); Thornton & Shevach (1998)
CD4 ⁺ CD45RB ^{low}	unknown	prevent allograft rejection by CD4 ⁺ CD45RB ^{high} cells	Davies <i>et al.</i> (1999)
CD3 ⁺ DN	unknown	prolonged skin allograft survival	Zhang <i>et al.</i> (2000)
NK1.1 ⁺ TCR α ⁺ cells	unknown	unknown	Sonoda <i>et al.</i> (1999)

Table 3. *Examples of co-stimulatory pathways*

T cell	APC	functions	reference
CD40L	CD40	generation of 1 and 2 Ab responses; synergy with CD28–CD80/86	Armitage <i>et al.</i> (1992); Foy <i>et al.</i> (1993); Hollenbaugh <i>et al.</i> (1992); Roy <i>et al.</i> (1993)
CD28	CD80, CD86	increased IL-2, IL-4, IFN- γ , TNF- α ; initiation of clonal expansion	Lanier <i>et al.</i> (1995); Linsley <i>et al.</i> (1991a, 1994)
CTLA-4	CD80, CD86	downregulation of T-cell function	Brunet <i>et al.</i> (1987); Linsley <i>et al.</i> (1991b, 1994); Parsons <i>et al.</i> (1996)
CD30L 4-1BB	CD30 4-1BBL	initiation of T-cell proliferation, IL-2 production	Romagnani (1995); Smith <i>et al.</i> (1993) Hurtado <i>et al.</i> (1995); Kim <i>et al.</i> (1998)

protection against autoimmune disease and offer protection against rejection (Davies *et al.* 1999). CD25⁺CD4⁺ T cells appear to function by the elaboration of the immunosuppressive cytokine, transforming growth factor β (TGF- β), and the expression of the negative regulator of T-cell activation, CTLA-4 (Read *et al.* 2000; Takahashi *et al.* 2000). If suppressor cells exist in humans to the same degree as rodents, efforts to expand these populations *in vitro* might prove useful for the induction of tolerance.

Another means by which peripheral tolerance has been induced is by blocking co-stimulation. The concept underlying this approach is based on the two-signal theory of T-cell activation. T cells require both a primary signal, the engagement of their T-cell receptor with MHC-bearing peptide, as well as a second signal from specialized APCs. A number of important co-stimulatory pathways have now been identified. Examples of co-stimulatory pathways are listed in table 3. Two important pathways are the B7–CD28 pathway and the CD40 and CD40L pathway (Boussiotis *et al.* 1993; Jenkins *et al.* 1991; Schwartz 1992). CD28 and CD40 ligand are found on the cell surface of T cells, and therapeutic approaches have attempted to block their action by the use of monoclonal antibodies such as CTLA-4-Ig and anti-CD40L. Elwood *et al.* (1998) used a combined CD40 and CD28 blockade in pig skin xenografts to mice and demonstrated prolonged graft survival. Although Elwood did not specifically demonstrate that xenoreactive T cells had become anergized, co-stimulatory blockade *in vitro* has been shown to induce anergy in T cells (Tan *et al.* 1993). Lehnert *et al.* (2000) have demonstrated that a short course of CD40 and CD28 pathway blockade leads to

indefinite pancreatic islet survival in a concordant rat-to-mouse model.

T-cell depletion, especially when combined with brief immunosuppression, has been effective in primate allograft models (Thomas *et al.* 2000). Rhesus monkeys treated with two postoperative doses of anti-CD3 linked to diphtheria toxin, combined with 15 days of deoxyspergualin, resulted in long-term tolerance (Thomas *et al.* 2000). The underlying mechanism of this protocol has been worked out. Although not yet tested in xenotransplantation, this model has significant potential in the clinical arena.

6. B-CELL TOLERANCE

The humoral response to xenotransplantation is probably the greatest hurdle to clinical application. Blocking T helper cell activation, either through tolerance or immunosuppression, is a powerful mechanism for inhibiting the humoral response. This hurdle reflects, in part, the resistance of humoral responses to immunosuppressive therapy and, in part, the considerable sensitivity of blood vessels to humoral mediated immunity. Recent advances in gene targeting and nuclear transfer have made the production of pigs without Gal α 1,3Gal possible (McCreath *et al.* 2000; Polejaeva *et al.* 2000). Without a large number of xenoreactive natural antibodies to Gal α 1,3Gal, controlling the T-cell response might be sufficient to control B-cell responses as they are in clinical allotransplantation. Efforts to induce humoral tolerance have involved bone marrow transplantation, the inhibition of co-stimulation, induction of anergy and deletion of activated B cells.

Among the first approaches to induction of B-cell tolerance was the deletion of activated B cells. This was accomplished by the intentional administration of antigen followed by administration of a cytotoxic agent (Dietrich & Dukor 1967; Schwartz *et al.* 1958). Deletion of activated B cells has been accomplished for soluble protein antigens such as factor VIII (Lian *et al.* 1989). Deletion of activated B cells is generally attempted in association with plasma exchange or plasmapheresis (Mollison *et al.* 1993).

Another approach, which appears to give rise to deletion of B cells, is administration of bone marrow bearing antigens of interest. Bone marrow transplantation affects especially the survival of B-cell precursors. Transplantation of murine bone marrow cells expressing Gal α 1,3Gal suppresses synthesis of anti-Gal α 1,3Gal antibodies (Ohdan *et al.* 1999). Though effective in mice, it is more difficult to achieve engraftment with xenogeneic bone marrow and, thus far, has not resulted in the deletion of B cells bearing receptors for Gal α 1,3Gal.

One way to avoid the hurdle to transplanting bone marrow between disparate species, is to express transplant antigens on autologous bone marrow and administer the autologous marrow as a transplant. Bracy *et al.* (1998) used a retroviral vector to express α 1,3-galactosyltransferase in the bone marrow of α 1,3-galactosyltransferase knockout mice (Bracy *et al.* 1998). Once given the transduced marrow, the mice ceased producing anti-Gal α 1,3Gal antibodies for 51 weeks after bone marrow transplantation (Bracy *et al.* 1998). Attempts have been made to introduce SLA class II genes into baboon marrow cells to decrease the elicited antibodies that presumably would be directed in large part against SLA. Currently, this approach has difficulties because of the inability in primates to achieve long-lasting *in vivo* expression of genes introduced by vectors (Ierino *et al.* 1999). While this approach might ultimately prove successful for specific genes, since porcine organs are likely to yield many antigenic peptides, clonal deletion of B cells specific for individual genes may fail. Organ-specific peptides that might elicit antibodies would probably not be tolerized by even a mixed chimerism approach and thus could trigger humoral-based rejection.

B-cell functions can be inhibited and perhaps tolerance induced by blocking co-stimulation. Since the CD40–CD40L pathway is thought to be important for class switching and the production of specific IgGs, blockade of this pathway might be useful. Buhler *et al.* (2000) showed that by using cyclosporine and anti-CD40L it is possible to prevent the development of elicited antibodies during mixed chimerism induction.

Other methods for inducing B-cell tolerance include administration of brief courses of immunosuppression such as leflunomide, a potent B-cell inhibitor, and the administration of donor antigen without co-stimulation. In a T-cell independent rodent model, temporary NK cell depletion, administration of leflunomide, together with donor antigen is allowed for long-term xenograft survival (Lin *et al.* 1998b). Xenograft recipients did not develop xenoreactive IgM antibodies (Lin *et al.* 1998b). Because the model was T-cell independent, one of the most potent mechanisms for stimulating B cells was missing. Administration of antigen arrays has been shown to eliminate

high affinity IgG antibody for long periods of time from peripheral circulation (Symer *et al.* 1995). Gal α 1,3Gal oligosaccharide given intravenously prior to transplant has minimally prolonged graft survival but has not resulted in tolerance (Cooper *et al.* 1993).

7. CONCLUSION

Donor-specific tolerance has been sought for decades as a means of eliminating the use of immunosuppression in patients with transplants. Xenotransplantation with its multiple immune hurdles provides an attractive potential application for the induction of tolerance. While controlling humoral and cellular responses is necessary for the introduction of clinical xenotransplantation, it is not clear if tolerance will be required for xenotransplantation. It may be that current and future immunosuppressive regimens that globally suppress the immune system will function sufficiently to prevent rejection of xenografts. Regardless of application, the study of tolerance continues to provide an excellent way to explore the functioning and control of the immune system.

Work by the authors is supported by grants from the National Institutes of Health. The authors wish to thank Kim Barber for her assistance with the preparation of the manuscript.

REFERENCES

- Armitage, R. J. (and 14 others) 1992 Molecular and biological characterization of a murine ligand for CD40. *Nature* **357**, 80–82.
- Bach, F. H., Winkler, H., Ferran, C., Hancock, W. W. & Robson, S. C. 1996 Delayed xenograft rejection. *Immunol. Today* **17**, 379–384.
- Billingham, R. E., Brent, L. & Medawar, P. B. 1953 Actively acquired tolerance of foreign cells. *Nature* **172**, 603.
- Blakely, M. L., Van Der Werf, W. J., Berndt, M. C., Dalmasso, A. P., Bach, F. H. & Hancock, W. W. 1994 Activation of intragraft endothelial and mononuclear cells during discordant xenograft rejection. *Transplantation* **58**, 1059–1066.
- Boussiotis, V. A., Freeman, G. J., Gray, G., Gribben, J. & Nadler, L. M. 1993 B7 but not intercellular adhesion molecule-1 costimulation prevents the induction of human alloantigen-specific tolerance. *J. Exp. Med.* **178**, 1753–1763.
- Boyd, S. V. 1964 Natural antibodies and the immune response. *Adv. Immunol.* **5**, 1–28.
- Bracy, J. L., Sachs, D. H. & Iacomini, J. 1998 Inhibition of xenoreactive natural antibody production by retroviral gene therapy. *Science* **281**, 1845–1847.
- Brunet, J.-F., Denizot, F., Luciani, M.-F., Roux-Dosseto, M., Suzan, M., Mattei, M.-G. & Golstein, P. 1987 A new member of the immunoglobulin superfamily—CTLA-4. *Nature* **328**, 267–270.
- Buhler, L. (and 16 others) 2000 High-dose porcine hematopoietic cell transplantation combined with CD40 ligand blockade in baboons prevents an induced anti-pig humoral response. *Transplantation* **69**, 2296–2304.
- Carroll, M. C. 1998 The lupus paradox. *Nature Genet.* **19**, 3–4.
- Ciubotariu, R., Colovai, A. I., Pennesi, G., Liu, Z., Smith, D., Berlocco, P., Cortesini, R. & Suci-Foca, N. 1998 Specific suppression of human CD4⁺ Th cell responses to pig MHC antigens by CD8⁺CD28-regulatory T cells. *J. Immunol.* **161**, 5193–5202.
- Cooper, D. K. C., Human, P. A., Lexer, G., Rose, A. G., Rees, J., Keraan, M. & Du Toit, E. 1988 Effects of cyclosporine and

- antibody adsorption on pig cardiac xenograft survival in the baboon. *J. Heart Transpl.* **7**, 238–246.
- Cooper, D. K. C. (and 11 others) 1993 Specific intravenous carbohydrate therapy: a new concept in inhibiting antibody-mediated rejection—experience with ABO-incompatible cardiac allografting in the baboon. *Transplantation* **56**, 769–777.
- Cooper, D. K. C., Kemp, E., Platt, J. L. & White, D. J. G. (eds) 1997 *Xenotransplantation: the transplantation of organs and tissues between species*, 2nd edn. Berlin: Springer.
- Cotterell, A. H., Collins, B. H., Parker, W., Harland, R. C. & Platt, J. L. 1995 The humoral immune response in humans following cross-perfusion of porcine organs. *Transplantation* **60**, 861–868.
- Cyster, J. G., Hartley, S. B. & Goodnow, C. C. 1994 Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. *Nature* **371**, 389–395.
- Davies, J. D., O'Connor, E., Hall, D., Krah, T., Trotter, J. & Sarvetnick, N. 1999 CD4⁺ CD45RB low-density cells from untreated mice prevent acute allograft rejection. *J. Immunol.* **163**, 5353–5357.
- Dietrich, F. M. & Dukor, P. 1967 The immune response to heterologous red cells in mice. III. Cyclophosphamide-induced tolerance to multispecies red cells. *Pathol. Microbiol.* **30**, 909–917.
- Elwood, E. T. (and 11 others) 1998 Prolonged acceptance of concordant and discordant xenografts with combined CD40 and CD28 pathway blockade. *Transplantation* **65**, 1422–1428.
- Fowell, D. & Mason, D. 1993 Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. characterization of the CD4⁺ T cell subset that inhibits this autoimmune potential. *J. Exp. Med.* **177**, 627–636.
- Foy, T. M., Shepherd, D. M., Durie, F. H., Aruffo, A., Ledbetter, J. A. & Noelle, R. J. 1993 *In vivo* CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. *J. Exp. Med.* **178**, 1567–1575.
- Galili, U., Shohet, S. B., Kobrin, E., Stults, C. L. M. & Macher, B. A. 1988 Man, apes, and old world monkeys differ from other mammals in the expression of α -galactosyl epitopes on nucleated cells. *J. Biol. Chem.* **263**, 17 755–17 762.
- Hollenbaugh, D., Grosmaire, L. S., Kullas, C. D., Chalupny, N. J., Braesch-Andersen, S., Noelle, R. J., Stamenkovic, I., Ledbetter, J. A. & Aruffo, A. 1992 The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. *EMBO J.* **11**, 4313–4321.
- Hurtado, J. C., Kim, S. H., Pollok, K. E., Lee, Z. H. & Kwon, B. S. 1995 Potential role of 4-1BB in T cell activation. Comparison with the costimulatory molecule CD28. *J. Immunol.* **155**, 3360–3367.
- Ierino, F. L. (and 21 others) 1999 Transfer of swine major histocompatibility complex class II genes into autologous bone marrow cells of baboons for the induction of tolerance across xenogeneic barriers. *Transplantation* **67**, 1119–1128.
- Ihrcke, N. S., Wrenshall, L. E., Lindman, B. J. & Platt, J. L. 1993 Role of heparan sulfate in immune system-blood vessel interactions. *Immunol. Today* **14**, 500–505.
- Ildstad, S. T. & Sachs, D. H. 1984 Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of allografts or xenografts. *Nature* **307**, 168–170.
- Ildstad, S. T., Vacchio, M. S., Markus, P. M., Hronakes, M. L., Wren, S. M. & Hodes, R. J. 1992 Cross-species transplantation tolerance: rat bone marrow-derived cells can contribute to the ligand for negative selection of mouse T cell receptor V β in chimeras tolerant to xenogeneic antigens (mouse + rat $\rightarrow$ mouse). *J. Exp. Med.* **175**, 147–155.
- Jenkins, M. K., Taylor, P. S., Norton, S. D. & Urdahl, K. B. 1991 CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol.* **147**, 2461–2466.
- Karlhofer, F. M., Ribaldo, R. K. & Yokoyama, W. M. 1992 MHC class I alloantigen specificity of Ly-49⁺ IL-2-activated natural killer cells. *Nature* **358**, 66–70.
- Kawai, T., Cosimi, A. B., Colvin, R. B., Powelson, J., Eason, J., Kozłowski, T., Sykes, M., Monroy, R., Tanaka, M. & Sachs, D. H. 1995 Mixed allogeneic chimerism and renal allograft tolerance in cynomolgus monkeys. *Transplantation* **59**, 256–262.
- Kim, Y. J., Kim, S. H., Mantel, P. & Kwon, B. S. 1998 Human 4-1BB regulates CD28 co-stimulation to promote Th1 cell responses. *Eur. J. Immunol.* **28**, 881–890.
- Lanier, L. L. & Phillips, J. H. 1996 Inhibitory MHC class I receptors on NK cells and T cells. *Immunol. Today* **17**, 86–91.
- Lanier, L. L., O'Fallon, S., Somoza, C., Phillips, J. H., Linsley, P. S., Okumura, K., Ito, D. & Azuma, M. 1995 CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. *J. Immunol.* **154**, 97–105.
- Lawson, J. H. & Platt, J. L. 1996 Molecular barriers to xenotransplantation. *Transplantation* **62**, 303–310.
- Lehnert, A. M., Yi, S., Burgess, J. S. & O'Connell, P. J. 2000 Pancreatic islet xenograft tolerance after short-term co-stimulation blockade is associated with increased CD4⁺ T cell apoptosis but not immune deviation. *Transplantation* **69**, 1176–1185.
- Leventhal, J. R., Dalmaso, A. P., Cromwell, J. W., Platt, J. L., Manivel, C. J., Bolman, R. M. & Matas, A. J. 1993a Prolongation of cardiac xenograft survival by depletion of complement. *Transplantation* **55**, 857–866.
- Leventhal, J. R., Matas, A. J., Sun, L. H., Reif, S., Bolman, R. M., Dalmaso, A. P. & Platt, J. L. 1993b The immunopathology of cardiac xenograft rejection in the guinea pig-to-rat model. *Transplantation* **56**, 1–8.
- Li, J., Liu, Z., Jiang, S., Cortesini, R., Lederman, S. & Suciufoca, N. 1999 T suppressor lymphocytes inhibit NF-kappa B-mediated transcription of CD86 gene in APC. *J. Immunol.* **163**, 6386–6392.
- Lian, E. C., Larcada, A. F. & Chiu, A. Y. 1989 Combination immunosuppressive therapy after factor VIII infusion for acquired factor VIII inhibitor. *Annals Int. Med.* **110**, 774–778.
- Lin, S. S. (and 13 others) 1997 The role of natural anti-Gal α 1,3Gal antibodies in hyperacute rejection of pig-to-baboon cardiac xenotransplants. *Transpl. Immunol.* **5**, 212–218.
- Lin, S. S. (and 13 others) 1998a The role of antibodies in acute vascular rejection of pig-to-baboon cardiac transplants. *J. Clin. Invest.* **101**, 1745–1756.
- Lin, Y., Goebles, J., Xia, G., Ji, P., Vandeputte, M. & Waer, M. 1998b Induction of specific transplantation tolerance across xenogeneic barriers in the T-independent immune compartment. *Nature Med.* **4**, 173–180.
- Linsley, P. S., Brady, W., Grosmaire, L., Aruffo, A., Damle, N. K. & Ledbetter, J. A. 1991a Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.* **173**, 721–730.
- Linsley, P. S., Brady, W., Urnes, M., Grosmaire, L. S., Damle, N. K. & Ledbetter, J. A. 1991b CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* **174**, 561–569.

- Linsley, P. S., Greene, J. L., Brady, W., Bajorath, J., Ledbetter, J. A. & Peach, R. 1994 Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity* **1**, 793–801.
- McCreath, K. J., Howcroft, J., Campbell, K. H. S., Colman, A., Schnieke, A. E. & Kind, A. J. 2000 Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature* **405**, 1066–1069.
- McCurry, K. R., Kooyman, D. L., Alvarado, C. G., Cotterell, A. H., Martin, M. J., Logan, J. S. & Platt, J. L. 1995 Human complement regulatory proteins protect swine-to-primate cardiac xenografts from humoral injury. *Nature Med.* **1**, 423–427.
- Magee, J. C., Collins, B. H., Harland, R. C., Lindman, B. J., Bollinger, R. R., Frank, M. M. & Platt, J. L. 1995 Immunoglobulin prevents complement mediated hyperacute rejection in swine-to-primate xenotransplantation. *J. Clin. Invest.* **96**, 2404–2412.
- Malyguine, A. M., Saadi, S., Platt, J. L. & Dawson, J. R. 1996 Human natural killer cells induce morphologic changes in porcine endothelial cell monolayers. *Transplantation* **61**, 161–164.
- Mollison, P. L., Engelfriet, C. P. & Contreras, M. 1993 *Blood transfusion in clinical medicine*. Oxford, UK: Blackwell Scientific Publications.
- Murray, A. G., Khodadoust, M. M., Pober, J. S. & Bothwell, A. L. M. 1994 Porcine aortic endothelial cells activate human T cells: direct presentation of MHC antigens and costimulation by ligands for human CD2 and CD28. *Immunity* **1**, 57–63.
- Nagayasu, T. & Platt, J. L. 1998 Progress in xenotransplantation. *Graft* **1**, 19–24.
- Nemazee, D. A. & Burki, K. 1989 Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature* **337**, 562–566.
- Ohdan, H., Yang, Y. G., Shimizu, A., Swenson, K. G. & Sykes, M. 1999 Mixed chimerism induced without lethal conditioning prevents T cell- and anti-Gal α 1,3Gal-mediated graft rejection. *J. Clin. Invest.* **104**, 281–290.
- Olack, B., Manna, P., Jaramillo, A., Steward, N., Swanson, C., Kaesberg, D., Poindexter, N., Howard, T. & Mohanakumar, T. 2000 Indirect recognition of porcine swine leukocyte Ag class I molecules expressed on islets by human CD4⁺ T lymphocytes. *J. Immunol.* **165**, 1294–1299.
- Owen, R. 1945 Immunogenetic consequences of vascular anastomoses between bovine twins. *Science* **102**, 400–401.
- Parsons, K. R., Young, J. R., Collins, B. A. & Howard, C. J. 1996 Cattle CTLA-4, CD28 and chicken CD28 bind CD86: MYPPPY is not conserved in cattle CD28. *Immunogenetics* **43**, 388–391.
- Pasternak, C. A., Alder, G. M., Bashford, C. L., Buckley, C. D., Micklem, K. J. & Patel, K. 1985 Cell damage by viruses, toxins and complement: common features of pore-formation and its inhibition by Ca²⁺. *Biochem. Soc. Symp.* **50**, 247–264.
- Perper, R. J. & Najarian, J. S. 1967 Experimental renal heterotransplantation. III. Passive transfer of transplantation immunity. *Transplantation* **5**, 514–533.
- Platt, J. L. 1998 New directions for organ transplantation. *Nature* **392**(Suppl.), 11–17.
- Platt, J. L., Turman, M. A., Noreen, H. J., Fischel, R. J., Bolman, R. M. & Bach, F. H. 1990 An ELISA assay for xenoreactive natural antibodies. *Transplantation* **49**, 1000–1001.
- Platt, J. L., Fischel, R. J., Matas, A. J., Reif, S. A., Bolman, R. M. & Bach, F. H. 1991 Immunopathology of hyperacute xenograft rejection in a swine-to-primate model. *Transplantation* **52**, 214–220.
- Polejaeva, I. A. (and 11 others) 2000 Cloned piglets produced by nuclear transfer from adult somatic cells. *Nature* **407**, 86–90.
- Porter, K. A. 1992. Renal transplantation. In *Pathology of the kidney*, vol. 3, 4th edn (ed. R. H. Heptinstall), pp. 1799–1933. Boston, MA: Little, Brown and Co.
- Prodeus, A., Goerg, S., Shen, L., Pozdnyakova, O. O., Chu, L., Alicot, E. M., Goodnow, C. C. & Carroll, M. C. 1998 A critical role for complement in maintenance of self-tolerance. *Immunity* **9**, 721–731.
- Pruitt, S. K. (and 11 others) 1994 The effect of soluble complement receptor type 1 on hyperacute rejection of porcine xenografts. *Transplantation* **57**, 363–370.
- Read, S., Malmstrom, V. & Powrie, F. 2000 Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25⁺CD4⁺ regulatory cells that control intestinal inflammation. *J. Exp. Med.* **192**, 295–302.
- Romagnani, S. 1995 Role for CD30 antigen in human T helper 2-type responses. *CIBA Found. Symp.* **195**, 55–62.
- Roy, M., Waldschmidt, T., Aruffo, A., Ledbetter, J. A. & Noelle, R. J. 1993 The regulation of the expression of gp39, the CD40 ligand, on normal and cloned CD4⁺ T cells. *J. Immunol.* **151**, 2497–2510.
- Saadi, S. & Platt, J. L. 1995 Transient perturbation of endothelial integrity induced by antibodies and complement. *J. Exp. Med.* **181**, 21–31.
- Sablinski, T. (and 11 others) 1999 Long-term discordant xenogeneic (porcine-to-primate) bone marrow engraftment in a monkey treated with porcine-specific growth factors. *Transplantation* **67**, 972–977.
- Sachs, D. H. & Sablinski, T. 1995 Tolerance across discordant xenogeneic barriers. *Xenotransplantation* **2**, 234–239.
- Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. 1995 Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α -chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* **155**, 1151–1164.
- Sandrin, M. S., Vaughan, H. A., Dabkowski, P. L. & McKenzie, I. F. C. 1993 Anti-pig IgM antibodies in human serum react predominantly with Gal α (1,3)Gal epitopes. *Proc. Natl Acad. Sci. USA* **90**, 11391–11395.
- Schwartz, R. H. 1992 Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* **71**, 1065–1068.
- Schwartz, R., Stack, J. & Dameshek, W. 1958 Effect of 6-mercaptopurine on antibody production. *Proc. Soc. Exp. Biol. Med.* **99**, 164–167.
- Sharabi, Y., Aksentjevich, I., Sundt, T. M., Sachs, D. H. & Sykes, M. 1990 Specific tolerance induction across a xenogeneic barrier: production of mixed rat/mouse lymphohematopoietic chimeras using a nonlethal preparative regimen. *J. Exp. Med.* **172**, 195–202.
- Shishido, S., Naziruddin, B., Howard, T. & Mohanakumar, T. 1997 Recognition of porcine major histocompatibility complex class I antigens by human CD8⁺ cytolytic T cell clones. *Transplantation* **64**, 340–346.
- Smith, C. A., Gruss, H. J., Davis, T., Anderson, D., Farrah, T., Baker, E., Sutherland, G. R., Brannan, C. I., Copeland, N. G. & Jenkins, N. A. 1993 CD30 antigen, a marker for Hodgkin's lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology to TNF. *Cell* **73**, 1349–1360.
- Sonoda, K. H., Exley, M., Snapper, S., Balk, S. P. & Stein-Streilein, J. 1999 CD1-reactive natural killer T cells are required for development of systemic tolerance through an immune-privileged site. *J. Exp. Med.* **190**, 1215–1226.
- Suri-Payer, E., Amar, A. Z., Thornton, A. M. & Shevach, E. M. 1998 CD4⁺CD25⁺ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J. Immunol.* **160**, 1212–1218.

- Sykes, M., Zhao, Y. & Yang, Y. G. 1997 Tolerance induction for xenotransplantation. *World J. Surg.* **21**, 932–938.
- Symer, D. E., Reim, J., Dintzis, R. Z., Voss Jr, E. W. & Dintzis, H. M. 1995 Durable elimination of high affinity, T cell-dependent antibodies by low molecular weight antigen arrays *in vivo*. *J. Immunol.* **155**, 5608–5616.
- Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W. & Sakaguchi, S. 2000 Immunologic self-tolerance maintained by CD25⁺CD4⁺ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* **192**, 303–309.
- Tan, P., Anasetti, C., Hansen, J. A., Melrose, J., Brunvand, M., Bradshaw, J., Ledbetter, J. A. & Linsley, P. S. 1993 Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. *J. Exp. Med.* **177**, 165–173.
- Thomas, J. M., Eckhoff, D. E., Contreras, J. L., Lobashevsky, A. L., Hubbard, W. J., Moore, J. K., Cook, W. J., Thomas, F. T. & Neville Jr, D. M. 2000 Durable donor-specific T and B cell tolerance in rhesus macaques induced with peritransplantation anti-CD3 immunotoxin and deoxyspergualin. *Transplantation* **69**, 2497–2503.
- Thornton, A. M. & Shevach, E. M. 1998 CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J. Exp. Med.* **188**, 287–296.
- Tomita, Y., Lee, L. A. & Sykes, M. 1994 Engraftment of rat bone marrow and its role in negative selection of murine T cells in mice conditioned with a modified nonmyeloablative regimen. *Xenotransplantation* **1**, 109–117.
- Townsend, S. E., Weintraub, B. C. & Goodnow, C. C. 1999 Growing up on the streets: why B-cell development differs from T-cell development. *Immunol. Today* **20**, 217–220.
- Vernabac, K. M., Carver, F. M., Haisch, C. E. & Thomas, J. M. 1994 A role for transforming growth factor- β in the veto mechanism in transplant tolerance. *Transplantation* **57**, 893–900.
- Yamada, K., Sachs, D. H. & DerSimonian, H. 1995 Human anti-porcine xenogeneic T cell response. Evidence for allelic specificity of mixed leukocyte reaction and for both direct and indirect pathways of recognition. *J. Immunol.* **155**, 5249–5256.
- Yamada, K., Shimizu, A., Utsugi, R., Ierino, F. L., Gargollo, P., Haller, G. W., Colvin, R. B. & Sachs, D. H. 2000 Thymic transplantation in miniature swine. II. Induction of tolerance by transplantation of composite thymokidneys to thymectomized recipients. *J. Immunol.* **164**, 3079–3086.
- Zhang, Z., Yand, L., Young, K. J., DuFemple, B. & Zhang, L. 2000 Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nature Med.* **6**, 782–789.
- Zhao, Y., Swenson, K., Sergio, J. J., Arn, J. S., Sachs, D. H. & Sykes, M. 1996 Skin graft tolerance across a discordant xenogeneic barrier. *Nature Med.* **2**, 1211–1216.
- Zhao, Y., Fishman, J. A., Sergio, J. J., Oliveros, J. L., Pearson, D. A., Szot, G. L., Wilkinson, R. A., Arn, J. S., Sachs, D. H. & Sykes, M. 1997 Immune restoration by fetal pig thymus grafts in T cell-depleted, thymectomized mice. *J. Immunol.* **158**, 1641–1649.
- Zhu, A. 2000 Binding of human natural antibodies to nonalphaGal xenoantigens on porcine erythrocytes. *Transplantation* **69**, 2422–2428.