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Immunological Challenges and Therapies in Xenotransplantation

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Xenotransplantation, or the transplantation of cells, tissues, or organs between different species, was proposed a long time ago as a possible solution to the worldwide shortage of human organs and tissues for transplantation. In this setting, the pig is currently seen as the most likely candidate species. In the last decade, progress in this field has been remarkable and includes a better insight into the immunological mechanisms underlying the rejection process. Several immunological hurdles nonetheless remain, such as the strong antibody-mediated and innate or adaptive cellular immune responses linked to coagulation derangements, precluding indefinite xenograft survival. This article reviews our current understanding of the immunological mechanisms involved in xenograft rejection and the potential strategies that may enable xenotransplantation to become a clinical reality in the not-too-distant future.

By "xenotransplantation," we conventionally refer to the transplantation of cells, tissues, or organs from one species to another. Current interest in xenotransplantation stems from the worldwide shortage of human organs, tissues, and cells for use in clinical transplantation. At least in theory, the imbalance between supply and demand could be wholly addressed if organs, tissues, or cells from other species could be transplanted into humans. The pig is currently considered the most appropriate candidate species because of its anatomical similarity, physiological compatibility, breeding characteristics, and for ethical reasons. Ongoing preclinical research in this field is consequently based on the use of pigs as donors and nonhuman primates as recipient species.

By now, we have gained a significant insight into the immunological processes underlying the rejection of porcine xenografts transplanted into primates. Considerable advances have also been made to elucidate the dysregulated coagulation occurring after porcine xenografts have been transplanted into primates. Despite the encouraging results obtained to date, especially in the field of cell xenotransplantation, several issues nonetheless remain to be addressed before any clinical application of xenotransplantation can proceed.

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This review summarizes current knowledge in this field, focusing exclusively on the immune mechanisms underlying the rejection of cardiac, renal, and islet xenografts, and on possible strategies to overcome these obstacles. The main emphasis is placed on the most clinically relevant pig-to-primate models. A comprehensive, accurate analysis of the coagulation derangements associated with xenotransplantation would be too lengthy for the format of this monograph; thus, for the sake of brevity, the reader is referred to other, excellent reviews recently published on the subject (Lin et al. 2009; Schmelzle et al. 2010; Cowan et al. 2011; Bulato et al. 2012).

MECHANISMS UNDERLYING XENOGRAFT REJECTION

Antibody-Mediated Xenograft Rejection

The rejection of a xenografted solid organ is characterized primarily by a picture compatible with a humorally driven immunological process. The humoral component of the immune response is a formidable barrier to short- and long-term organ survival. Hyperacute rejection (HAR) and acute humoral xenograft rejection (AHXR), also termed delayed xenograft rejection, are the main features of the humorally mediated xenograft rejection occurring when pig organs are transplanted into untreated primates.

HAR is a rapid, powerful process involving diffuse interstitial hemorrhage, edema, and thrombosis of the small vessels (Stevens and Platt 1992). This process is triggered by preexisting antibodies binding to xenograft antigens and prompting complement activation, graft endothelial cell activation and destruction, activation of the coagulation cascade, and graft rejection within minutes or hours. Preformed antipig antibodies are believed to be directed primarily against the terminal α3-galactose of the *N*-acetyllactosamine in glycoprotein and glycolipid carbohydrate chains (Galα3Galβ4Glc-Nac-R or αGal epitope) (Galili et al. 1988; Macher and Galili 2008). The synthesis of αGal is catalyzed by the α1-3 galactosyltransferase (α1-3 GalT), an enzyme expressed in nonprimate mammals, including the pig and in New World monkeys. The density of αGal epitopes in pig organs goes from 1 to 3×10^7 epitopes per cell, in endothelial and epithelial cells, respectively (Galili et al. 1988). These αGal epitopes have been identified in decellularized xenogeneic biological scaffolds, like the mammalian extracellular matrix used in surgical reconstructions, even after treatments to remove or mask antigenic epitopes (McPherson et al. 2000; Konakci et al. 2005; Stone et al. 2007). In human beings, and in Old World monkeys and apes, α1-3 GalT is inactive, the αGal epitope is not expressed, and there are high titers of anti-αGal antibodies due to exposure to similar epitopes expressed by bacteria hosted in the gut flora. A significant percentage of preformed total antibodies in human and nonhuman primates reacts with the αGal epitope (1%–8% of total IgM and 1–2.4% of total IgG) (Parker et al. 1994; McMorrow et al. 1997).

A detailed study to ascertain the nature and residence of cells producing anti-αGal antibodies found them located primarily in the spleen, in both naïve and immunized nonhuman primates, and to a lesser extent in the lymph nodes (LN) and bone morrow (BM) (Xu et al. 2006). Splenectomy does not seem to affect the number of cells secreting anti-αGal antibodies in the LN and BM, except in the case of sensitized animals, when exposure to αGal considerably increases their number, but only in the BM. The cells that produce anti-αGal IgM and IgG are the surface Ig-positive B cells and mature plasma cells, respectively. Six months after exposure to porcine tissues or hematopoietic cells, cells secreting anti-αGal antibodies mainly produce IgG and are found in the BM as long-lived mature plasma cells.

The humoral response to sugars is primarily T-cell independent, although evidence from several lines of research suggests that natural and elicited anti-αGal humoral response is primarily T-cell dependent. First, a significantly reduced natural and elicited anti-αGal IgM were observed in an α1-3 GalT- and TCR-β-deficient mouse model lacking any αGal epitope expression and functional αβ T cells, by comparison with mice with normal αβ T cells (TCR-β^+) (Cretin et al. 2002). Second, blocking

the CD40/CD154 pathway's interaction between B and T cells with an anti-CD154 agent inhibits the elicited anti-αGal IgM (Cretin et al. 2002) and IgG (Tanemura et al. 2000) response. Third, immunization with T-cell-independent agents expressing αGal epitopes, such as an αGal multivalent polyacrylamide polymer (Cretin et al. 2002) or glycolipids (Tanemura et al. 2000), induces a mild increase in IgM antibodies and no IgG. There is consequently evidence of a limited amount of anti-αGal IgM production occurring via T-cell-independent mechanisms (Tanemura et al. 2000; Cretin et al. 2002). The demonstration that immunizing αGalT-KO mice with pig cell membranes elicits anti-αGal IgM and IgG, induces an expansion of the anti-αGal B cell clones, and causes a strong in vitro T-cell stimulation even after αGal expression has been suppressed leads to the hypothesis that xenopeptides bearing αGal epitopes are internalized, processed by B cells, and presented to helper T cells. The resulting activation of helper T lymphocytes enables the B cells to complete their activation process, ultimately resulting in proliferation, isotype switching, and the generation of plasma cells and high-affinity anti-αGal antibodies (Tanemura et al. 2000). Finally, there are reports of follicular dendritic cells expressing complement receptors 1 and 2 being involved in the presentation of immune complexes to αGal-reactive B cells and being needed for antigen-specific anti-αGal response (Shimizu et al. 2007).

At this stage, thanks to the many approaches pursued with a view to removing preexisting anti-αGal antibodies and controlling their effector functions, HAR of a solid organ xenograft has become a rare event. Solid organ xenografts are nonetheless still rejected within days or months as a result of AHXR, even when the donor has been genetically engineered and expresses no αGal epitope (GalT-KO pigs). After the transplantation of GalT-KO kidneys, AHXR is characterized histologically by a thrombotic microangiopathic glomerulopathy with increasing IgM, IgG, C4d, and C5b-9 deposition in the glomeruli, with thrombi forming inside the injured glomeruli, loss of capillaries, and endothelial cell death (Shimizu et al. 2012). A similar picture can be seen after heart xenotransplantation (Shimizu et al. 2008), in which case, AHXR is characterized by antibody and complement deposition on the capillary walls, multiple microthrombi in the capillaries, myocardial ischemia, and necrosis. The pathogenesis of AHXR is assumed to be multifactorial, but preformed and induced antibodies directed against the endothelium are believed to be the primary factors triggering AHXR, resulting in endothelial activation and orienting the anticoagulative properties of the endothelium toward a procoagulative phenotype favoring thrombosis (Crikis et al. 2006).

The damage seen in the AHXR process, and in the thrombotic microangiopathy (TM) occurring even when porcine GalT-KO organs are used, gives the impression that αGal epitopes may still be expressed by GalT-KO organs, or else that other antibodies not directed against αGal are involved. It has now been shown, in fact, that another enzyme called iGb_3 synthase leads to αGal epitope production in GalT-KO animals (Sharma et al. 2003), although this has not been confirmed by other investigators (Diswall et al. 2010, 2011; Fang et al. 2012; Puga Yung et al. 2012; Tahiri et al. 2013). In any case, the levels of both IgM and IgG anti-αGal antibodies remain stable after GalT-KO xenografts have been transplanted into nonhuman primates (Chen et al. 2005; Kuwaki et al. 2005), suggesting that—with the coverage afforded by current immunosuppression—any remaining αGal epitopes are poorly immunogenic and may not be responsible for any graft damage. In this light, much attention has been paid to the influence of antibodies against non-αGal epitopes on humoral rejection. High levels of natural and elicited IgM and IgG against non-αGal are detected in xenografted primates and associated with the onset of AHXR, even when the potentially detrimental role of αGal antibodies is averted by absorption (Lam et al. 2004; Chen et al. 2006).

In healthy humans, albeit with some interindividual variability, it has been reported that 13% of IgM and 36% of IgG binding to pig endothelial cells are directed against non-αGal epitopes, and these antibodies can cause cell

damage via complement fixation and antibody-dependent cell-mediated cytotoxicity (ADCC) (Baumann et al. 2007). Little is known about the kinetics of the anti-αGal and anti-non-αGal antibody response elicited in humans. When mouse fibroblasts were injected into humans as part of a gene therapy approach, there was a very rapid and sustained anti-αGal antibody response, with a 100-fold increase in antibody titers that dropped back within the second month, possibly as a consequence of the immunizing fibroblasts being eliminated. As for non-αGal antibodies, the immune response elicited took longer and never reached the same levels as the anti-αGal antibodies. In another study, pig ligaments lacking any αGal epitopes implanted into humans elicited an anti-non-αGal antibody response directed against both carbohydrate and protein structures (Breimer 2011; Galili 2012). Two months after the ligaments were implanted, there was a rise in non-αGal IgG that peaked at 6 mo and decreased thereafter (coinciding with the pig tissue being gradually replaced by recipient collagen), returning to pretransplant levels within 2 yr. Like the anti-αGal antibody response, the natural and elicited response against anti-non-αGal also appears to be T-cell dependent. This hypothesis is supported by the fact that there was no evidence of any elicited anti-non-αGal antibody response after blockade of the CD40–CD154 activation pathway and no subsequent T-cell help to B lymphocytes (Buhler et al. 2000). Similarly to what happens following the xenografting of wild-type organs, humoral response to GalT-KO pigs is not polyclonal—it is restricted. It is encoded, in this case, by V3-21 germline progenitors in the V_{H^3} family (Kiernan et al. 2008), an observation that may have therapeutic implications.

With regard to the specificity of anti-non-αGal antibodies, recent studies have reported that they are directed against carbohydrates (that are distinct from αGal, although they are structurally related) or proteins. When Yeh et al. (2010) analyzed naïve human or immunized baboon sera, reactivity against a panel of selected synthetic non-αGal saccharides showed that human and baboon sera contain antisaccharide antibodies directed against several specific entities including αGal-penta, α-LacNAc, Forssman antigen, P1, Pk, and Neu5Gc. There was only a minimal presence of anti-β-LacNAc antibodies, and this is a very important finding because GalT-KO animals express large amounts of this carbohydrate, appearing de novo as a consequence of αGal epitope removal. Such studies were also able to show that pigs do not express the Forssman antigen (a glycolipid against which humans have several antibodies), as shown by the presence of the high levels of anti-Forssman antibodies and the absence of Forssman antigens in pig tissues. Unexpectedly, the same study found that baboons immunized with GalT-KO pig cells had no significantly increased IgM and IgG binding to αGal-tri, or any of the saccharides in the panel considered, a finding that contrasts with the report from Diswall et al. (2010), who identified a different pattern of glycolipid expression in organs from GalT-KO pigs. The P_1 antigen (not seen in wild-type kidneys) was detected in the GalT-KO line, for instance. Similarly, the X_2 antigen was found expressed in the heart from the GalT-KO line, but not in the wild-type line analyzed. These antigens should not be seen as novel antigens appearing ex novo in the GalT-KO line, however, because the glycosyltransferases involved (namely, α1-4 galactosyltransferase and β_1, 3GalNacT) occur naturally in the wild-type line. Deletion of the α1-3 GalT enzyme may, however, have diverted the sugar metabolism toward an increased or de novo expression of these glycolipids in some tissues.

Another set of particularly important target epitopes includes the glycans carrying *N*-glycolylneuraminic acid (Neu5GC), or the so-called Hanganutziu–Deicher antigen, which is abundantly present in many mammals, including pigs and monkeys, but not in humans (Varki 2010). Healthy humans consequently have a highly variable polyclonal antibody profile against Neu5GC. Natural anti-Neu5GC antibodies are predominantly IgG (Padler-Karavani et al. 2008), but may be IgM and IgA too, albeit to a lesser extent. Anti-Neu5GC can be induced in humans after exposure to porcine tissues (Blixt et al. 2009; Scobie et al. 2013). Nonhuman

primates express Neu5GC antigens, however, and are unable to elicit a humoral response against this epitope, making their use in relevant preclinical studies unfeasible; hence the development of surrogate rodent models, such as double-KO mice that lack both αGal and Neu5GC epitopes as a consequence of GalT and cytidine monophospho-*N*-acetylneuraminic acid hydrolase (CMAH) inactivation (Basnet et al. 2010). Extensive investigations have shown that anti-Neu5GC antibodies can induce complement-mediated cytotoxicity, albeit to a lesser extent than anti-αGal antibodies (Basnet et al. 2010), suggesting that anti-Neu5GC antibodies may be important in eliciting AHXR of GalT-KO organs. This hypothesis is further supported by the recent finding that GalT-KO pigs have an increased Neu5GC production (Park et al. 2011, 2012).

On the matter of xenogeneic proteins, two recent reports have identified several membrane proteins that are recognized by primates rejecting porcine cardiac xenografts. In particular, most recipients' elicited antixenograft repertoire included antibodies directed against fibronectin, several stress response and inflammation proteins, and also proteins involved in key endothelial cell functions (Byrne et al. 2008, 2011). These included proteins involved in regulating inflammation (e.g., annexin A2), hemostasis (e.g., CD9 and endothelial cell protein C receptor), or the complement cascade (e.g., CD46 and CD59), all potentially important functions in the context of AHXR. In theory at least, such elicited antibodies could block important cell functions, and the researchers speculated that it may be necessary to substitute these key porcine proteins with their human counterpart, instead of eliminating them, if porcine endothelial function needs to remain intact. A recent proteomic analysis identified several immunoreactive membrane proteins on GalT-KO pig liver endothelial cells recognized by natural human IgM and IgG (Burlak et al. 2012), and the investigators suggested that more than 800 different proteins may be recognized by preexisting IgG and/or IgM in human sera. However, they were unable to establish unequivocally whether antibodies bind to different proteins or to a limited number of carbohydrate epitopes expressed by a large number of different proteins.

Many groups have shown that the swine major histocompatibility complex (SLA) is recognized by anti-HLA antibodies in the sera of sensitized patients (Naziruddin et al. 1998; Diaz Varela et al. 2003), and that these antibodies may be cytotoxic to pig cells (Taylor et al. 1998; Mulder et al. 2010). The cross-reactivity between human and pig anti-MHC antibodies may be due to MHC epitopes conserved between the two species (Mulder et al. 2010). For a future clinical application of xenotransplantation, it may therefore be essential to select the most appropriate, matched donor pig haplotype.

Cell-Mediated Xenograft Rejection

The Role of T Lymphocytes

Although natural and elicited antibody responses have so far been considered the main barrier to successful xenotransplantation, the involvement of T cells in xenograft rejection has yet to be fully clarified. T cells contribute to the induction of antidonor antibodies, but it has not yet been unequivocally shown whether T cells alone are capable of mediating xenograft rejection.

T-cell antixenograft immune response was studied first in vitro (Yamada et al. 1995; Dorling et al. 1996), and it was found that T-cell responses against pig xenografts could be at least as strong as in the allotransplantation setting (Yamada et al. 1995; Lin et al. 2008). These studies showed that, as in the allogeneic response, human T cells are able to recognize porcine MHC molecules via direct and indirect recognition pathways (Yamada et al. 1995; Dorling et al. 1996). Direct T-cell xenoresponses accounted for $>75\%$ of the xenodirected lymphocyte response observed in mixed lymphocyte reaction (MLR) studies (Yamada et al. 1995; Tahara et al. 2010) and were mediated mainly by $CD4^+$ cells directed primarily against SLA-DR, but also against SLA-DQ molecules (Yamada et al. 1995; Dorling et al. 1996). In contrast, the same researchers reported little to no direct recognition of SLA class I antigens by $CD8^+$ human T cells. The strong direct T-cell reaction is

not due to a higher frequency of human xenoreactive $CD4^+$ T cells than in the allogeneic counterpart, and there was no evidence of any differences in $CD8^+$ T-cell content between allo- and xenorelated direct reactions (Tahara et al. 2010). These studies also suggest that the $CD4^+$ helper function is needed for xenoreactive $CD8^+$ T cells to proliferate (Tahara et al. 2010). Indirect antipig T-cell responses appear to be mediated primarily by $CD4^+$ T cells (Dorling et al. 1996). The indirect antixenograft response is stronger than in the allogeneic counterpart, possibly because of the larger number of xenogeneic peptides presented by human antigen-presenting cells (Dorling et al. 1996).

As in allotransplantation, costimulatory signals are needed to fully activate the antixenograft T-cell responses via both direct and indirect pathways. In particular, CD40 and CD80/CD86 on antigen-presenting cells must interact with CD154 (CD40 L) and CD28 or cytotoxic T-lymphocyte antigen-4 (CTLA-4) on T cells. Extensive studies conducted by Rogers et al. (2003) have shown that porcine CD40, CD80, and CD86 are independently capable of costimulating human $CD4^+$ cells efficiently, albeit via different kinetics. In particular, porcine endothelial cells constitutively express CD80/CD86 (Koshika et al. 2011) and SLA class I, and possibly class II molecules as well (Choo et al. 1997). Porcine endothelial cells also trigger a direct, MHC-restricted $CD8^+$ T- and $CD4^+$ T-cell activation (Yamada et al. 1995; Dorling and Lechler 1998; Kim et al. 2010; Koshika et al. 2011; Wilhite et al. 2012). High levels of cytotoxicity have also been detected, mediated mainly by $CD4^+$ T cells, but also by $CD8^+$ T cells.

Analyzing in vitro lymphocyte proliferation induced by wild-type or GalT-KO endothelial cell shows that αGal epitope expression on the endothelium is associated with a greater proliferation of $CD4^+$ and $CD8^+$ T cells (Lin et al. 2008; Wilhite et al. 2012), suggesting an as-yet-unidentified role of αGal epitopes in sustaining T cell response. The absence of αGal is also associated with a significantly reduced secretion of INF-γ, TNF-α, IL-17A by $CD4^+$ T cells, and of INF-γ, granzyme-B, and the chemokine IP-10 by $CD8^+$ T cells (Wilhite et al. 2012), partially confirming earlier findings reported by Saethre et al. (2008), who showed that exposure to $Gal^{+/+}$ endothelial cells was associated with a significant release of human INF-γ, human and porcine proinflammatory IL-6 and IL-8, and several human β chemokines, whereas this picture was not seen after exposure to $Gal^{-/-}$ cells (Saethre et al. 2008). Unlike the report from Wilhite et al. (2012), however, the T-cell-recruiting α-chemokine IP-10 in the Saethre study was induced in cells lacking the αGal epitopes. Complement inhibition with Compstatin (a C3 inhibitor) or C5aR (a C5a antagonist) was able to abolish the production of human cytokines and chemokines, with the exception of the α-chemokine IP-10. Following exposure to human whole blood, $Gal^{+/+}$ (but not $Gal^{-/-}$) endothelial cells release porcine IL-6 and IL-8, a phenomenon that could be abolished by Compstatin. Taken together, these findings show the important role of the αGal epitope in the production of proinflammatory cytokines by human blood cells, as well as the central role of complement in mediating human immune effector functions and porcine cell activation. Recent evidence of a complement-induced T-cell and APC activation should also be borne in mind and warrants further analysis (Kwan et al. 2012).

Different cell infiltration patterns have been described after pig-to-primate solid organ xenotransplantation. In most cases, $CD8^+$ T cells with monocytes/macrophages, B cells, and some NK cells were the predominant cells detected in the graft at euthanasia (Ashton-Chess et al. 2003; Cozzi et al. 2003; Shimizu et al. 2012). $CD4^+$ T cells were only reported in a limited number of cases (Davila et al. 2006; Hisashi et al. 2008).

Taken together, in vitro and in vivo data convincingly point to the existence of a vigorous antixenograft T-cell immune response. Still, many investigators in the field share the impression that, with current immunosuppression, this cell-mediated immune response is less destructive than the response induced by activation of the humoral immune system, although the contribution of the T cells in the elicited antibody production should not be underestimated.

It is in the setting of nonvascularized xenografts such as porcine pancreatic islet xenotransplantation that T cells have been found to play the most significant part. Early studies on mice showed that fetal pig islets transplanted under the kidney capsule were rapidly rejected in normal but not in athymic (Karlsson-Parra et al. 1996) or TCR-deficient mice (Benda et al. 1998). The T cells infiltrating the graft were mainly $CD4^+$ cells, and no $CD8^+$ cells were detected. These studies also suggested that the xenogeneic islet rejection was a T-cell-dependent process, possibly via the stimulation of the effector activity of macrophages (Karlsson-Parra et al. 1996).

Returning to the clinically most relevant pig-to-primate models, porcine islets infused through the portal vein were lost *en masse* immediately after coming into contact with primate blood (Bennet et al. 2000) owing to the so-called instant blood-mediated inflammatory reaction (IBMIR), which is characterized by macroscopic coagulation, rapid platelet consumption, leukocyte infiltration, and the deposition of complement components (Goto et al. 2008; van der Windt et al. 2009). It has also been shown that islets from neonatal (Cardona et al. 2006) or adult pigs (Kirchhof et al. 2004) infused intraportally in nonimmunosuppressed primates and engrafted in the liver are destroyed within 3–5 d with a marked infiltration of $CD4^+$ and $CD8^+$ cells and macrophages (Kirchhof et al. 2004; Cardona et al. 2006).

When immunosuppressive therapy comprising basiliximab, FTY720, everolimus, and anti-CD154 was administered, adult islet survival was prolonged to up to 187 d (Hering et al. 2006). Despite the absence of any IgM or IgG and complement deposition, peri-/intragraft T-cell infiltration, both $CD4^+$ and $CD8^+$, and macrophages were apparent in the rejected grafts. The high levels of circulating indirectly activated donor-reactive T cells in rejecting recipients suggest a crucial role of such infiltrating cells in islet rejection, and their incomplete inhibition may have been the primary cause of graft rejection (Hering et al. 2006; Hering and Walawalkar 2009).

The Role of the Cells Involved in the Innate Immune Response

At least three types of cell—namely, neutrophils, natural killer (NK) cells, and macrophages—should be critically considered when analyzing the potential contribution to xenograft rejection of the cellular component of the innate immune system. For each of these, recruitment, adhesion, and *trans*-endothelial migration are mediated by different receptor–ligand interactions and finely regulated by chemotactic cytokines and chemokines released by host cells and the activated endothelium following xenotransplantation (Holgersson et al. 2002). Compatibility between human receptors and their porcine counterparts is therefore indispensable for the optimal migration and function of each of these cells.

Human naïve neutrophils have been found capable of directly recognizing and binding naïve xenogeneic endothelial cells more avidly than their allogeneic counterpart, irrespective of any presence of αGal, ICAM-1 (Sheikh et al. 2002), xenogeneic natural antibodies, or complement (Al-Mohanna et al. 1997; Cardozo et al. 2004). On the other hand, complement has been seen to increase neutrophil adhesion (Vercellotti et al. 1991). Human neutrophils activate porcine but not allogeneic endothelial cells (as shown by an increased P-selectin and VCAM-1 expression), making porcine endothelium more prone to human NK-driven cytotoxicity. In turn, porcine endothelium secretes an as-yet-unidentified soluble factor that is chemotactic for human neutrophils (Cardozo et al. 2004). The xenogeneic cell contact with porcine endothelial cell induces human neutrophil activation with an increased output of toxic reactive oxygen metabolites (ROM), irrespective of any presence of αGal epitopes, natural xenoantibodies, or complement (Al-Mohanna et al. 2005). Xenogeneic activation also prompts neutrophils to secrete proinflammatory cytokines, such as IL-1α/β, IL-6, and IL-8, involved in cellular and humoral adaptive immune responses and platelet activation, crucial players in the rejection process. As shown in some studies, polymorphonuclear neutrophils appear to

be the first line of cells infiltrating and possibly damaging (van der Windt et al. 2007) cell and solid-organ xenografts soon after transplantation (Kirchhof et al. 2004; Hisashi et al. 2008; Ezzelarab et al. 2009; Shimizu et al. 2012). As shown in the majority of reports, however, this infiltration appears to be replaced by T cells and macrophages (Kirchhof et al. 2004; Hisashi et al. 2008; Ezzelarab et al. 2009; Shimizu et al. 2012). Although in vitro studies suggest that neutrophils have the potential to damage porcine cells and tissues, there is only limited evidence of their detrimental role in vivo beyond HAR.

Other important players in the antixenograft innate immune response are the NK cells, a subset of lymphocytes capable of killing cells recognized as non-self, including tumor cells and virus-infected cells. Several lines of research have suggested that, following adhesion to porcine grafts, NK cells may have a detrimental role in pig-to-primate xenotransplantation. First, it has been shown in vitro that porcine cells are susceptible to direct cell-mediated damage and to ADCC mediated by naïve or activated human NK cells (Horvath-Arcidiacono et al. 2006; Schneider and Seebach 2008; Kennett et al. 2010; Sommaggio et al. 2012). The direct cytotoxic activity of NK cells, which is inhibited primarily by human MHC class I molecules through inhibitory NK receptors, is not blocked by SLA class I products. Ligands on porcine cells, one of which has recently been identified (Lilienfeld et al. 2006), can interact efficiently with the activating receptors NKp44 or NKG2D, resulting in direct human NK cell cytotoxicity (Forte et al. 2005). Interaction between the porcine costimulatory molecule CD86 and a variant form of CD28 on hNK cells may also be involved in direct NK cytotoxicity (Costa et al. 2002), whereas the expression of αGal residues on pig endothelium does not appear to have a major role, because the use of cells lacking αGal expression did not prevent direct NK-mediated cell lysis (Baumann et al. 2004; Horvath-Arcidiacono et al. 2006). Second, it has also been shown that, on activation by porcine endothelial cells, NK cells produce INF-γ, ultimately potentiating T-cell activity (Xu et al. 2002). Third, NK cells rapidly infiltrate porcine xenografts perfused ex vivo with human blood (Kirk et al. 1993; Inverardi and Pardi 1994). In explanted xenografts, on the other hand, NK cells were found in small numbers (Davila et al. 2006; Hisashi et al. 2008; Shimizu et al. 2012) or were not reported (Ashton-Chess et al. 2003; Cozzi et al. 2003; McGregor et al. 2005; Ezzelarab et al. 2009), except for one case in which the particular features of the model may have heavily influenced the outcome (Itescu et al. 1998). It is worth noting, however, that the in vivo studies conducted to date cannot definitely rule out the possibility of NK cells being directly implicated in the untimely failure of xenografts.

Monocytes and differentiated macrophages are the third component of the innate immune system considered here. These phagocytic cells' involvement in xenograft rejection has been amply discussed, especially in islet xenotransplantation, although the reported degree and timing of infiltration have varied in the different models. After xenotransplantation, monocytes attracted by graft proinflammatory mediators migrate into the xenograft and infiltrate it by virtue of the cross-species interaction of adhesion molecules, including CD49d/CD29 and β_2-integrins (Hauzenberger et al. 2000; Schneider et al. 2009). Macrophage recruitment appears to be more intensive when xenogeneic rather than allogeneic cells are transplanted (Fox et al. 2001), and it seems to be T-cell independent. The role of αGal in increasing monocyte adhesion is still being debated because macrophage infiltration has been identified in adult porcine islet grafts known to express low levels of αGal epitopes, and in islets from GalT-KO donors (Thompson et al. 2011a). In xenotransplantation, macrophages exert their phagocytic action and modulate adaptive immunity by contributing to cell recruiting and antigen presentation. Upon contact with xenogeneic cells, macrophages have been seen to allow the recruitment of both $CD4^+$ and $CD8^+$ T cells. In turn, optimal macrophage activation requires a contribution from the $CD4^+$ T cells, possibly via the INF-γ pathway (Yi et al. 2002), ultimately resulting in graft rejection (Fox et al. 2001; Yi et al. 2003).

The macrophages' phagocytic activity is mediated by an ADCC mechanism or direct cell-to-cell contact (Jackson and Evans 2000). Like the case of NK cells, the "missing self" (Ljunggren and Karre 1990) has been suggested as the regulatory mechanism behind their phagocytic activity against xenografts. It has been shown, in fact, that the lack of any functional interaction between porcine CD47 (a membrane glycoprotein expressed ubiquitously on the cell surface of xenografts) with the human species-specific macrophage inhibitory receptor SIRPα makes porcine cells more susceptible to macrophage-mediated damage (Ide et al. 2007).

Macrophages activated by $CD4^+$ T cells have an important role in the destruction of pancreatic islet xenografts. In rodents, macrophages are reportedly the earliest infiltrating cell population after islet transplantation under the kidney capsule (Wallgren et al. 1995). Macrophages activated by $CD4^+$ T cells in islet-transplanted NOD-SCID have also been found to cause graft destruction within 8 d (Yi et al. 2003). After intraportal islet injection in nonhuman primates, T cells reportedly preceded the influx of macrophages (Kirchhof et al. 2004) into the graft. Together with T cells, macrophages might contribute to islet damage (Kirchhof et al. 2004; Cardona et al. 2006; Hering et al. 2006). They appear to have a pivotal role in cellular graft rejection, and they have also been identified soon after solid organ xenografting. They continue to be detectable up until the graft is rejected (Ashton-Chess et al. 2003; Cozzi et al. 2003; Hisashi et al. 2008; Ezzelarab et al. 2009; Shimizu et al. 2012), and they have been accused of contributing to the onset of graft failure and TM (Ezzelarab et al. 2009).

IMMUNE STRATEGIES TO EXTEND XENOGRAFT SURVIVAL

Preventing Humoral Rejection

Several strategies may be envisaged to deal with the detrimental role of the humoral antixenograft immune response, as briefly reviewed below.

Removing the Key Target Antigens

Removing the immunogenic epitopes recognized by preexisting or elicited antixenograft antibodies is an obvious approach that has been pursued successfully in the case of the αGal epitope (Dai et al. 2002; Lai et al. 2002; Phelps et al. 2003). The technology is available for preventing the expression of undesirable target antigens on pig cells (Hauschild et al. 2011).

At least two caveats should be borne in mind, however, when using such an approach. First, the number of epitopes potentially eliciting a humoral response to a xenograft is considerable, in theory at least (Burlak et al. 2012), making it unrealistic to try and delete them all. Second, deleting epitopes may sometimes result in an unpredictable de novo appearance of new target molecules that are recognized as foreign (Diswall et al. 2010). Therefore, apart from a handful of antigens (e.g., the αGal and possibly also the Neu5GC epitopes [Lutz et al. 2013], and a few others) whose deletion is considered crucial to help establish an advantageous immunological context, such as accommodation or tolerance, the application of this approach in xenotransplantation is probably of limited value.

Preventing the Activation of the Complement Cascade Triggered by Xenoreactive Antibodies

Several efforts have been made to eliminate the detrimental effect of the complement cascade's activation on the xenograft, including the use of specific soluble complement inhibitors, such as soluble complement receptor type 1 (Lam et al. 2005), or the cobra venom factor (Kobayashi et al. 1997), and the production of animals transgenic for human complement regulatory proteins (CRP), such as hCD55, hCD46, or hCD59 (Fodor et al. 1994; Cozzi and White 1995; McCurry et al. 1995; Cowan et al. 2000; Loveland et al. 2004; Menoret et al. 2004). This approach represents an elegant strategy for down-regulating local complement activation on the xenograft while preserving systemic complement activity as a first line of defense. The expression of hCRP by the xenograft cell sur-

face may have a beneficial role on T-cell immunity too because molecules such as hCD55 can negatively modulate T-cell expansion and function (Heeger et al. 2005; Kwan et al. 2012). Judging from the data obtained so far, however, pig xenografts expressing CRPs are not protected from the overwhelming complement activation occurring in AHXR, even if CRPs are expressed on a GalT-KO background. This underlines the existing limitation of the use of CRP transgenic organs (Le Bas-Bernardet et al. 2011; Tazelaar et al. 2011). Indeed, it may be necessary to implement a multistep inhibition of the complement cascade, associated with a tight regulation of the coagulation system involved in complement activation (Huber-Lang et al. 2006).

Removing Xenoreactive Antibodies or Cells Producing Xenoreactive Antibodies

Removing or neutralizing xenoreactive antibodies with the aid of antigen-specific immunoadsorbants (Katopodis et al. 2002; Brandl et al. 2007), ex vivo organ perfusion, or nonspecific tools such as Sepharose beads conjugated with polyclonal antibodies against human Ig (Brenner et al. 2005) may help to protect grafts, at least temporarily, from offending antibodies. Strategies have also been attempted to delete antibody-producing cells using aspecific pharmacological immunosuppression (e.g., with cyclophosphamide) (Cozzi et al. 2000, 2003) or specifically targeting the B-cell lineage with anti-CD20 monoclonal antibodies (Vugmeyster et al. 2005; Mohiuddin et al. 2012), with ricin A–labeled target molecules (Tanemura et al. 2002), or even using the proteasome inhibitor bortezomib (Bauer et al. 2010). To date, however, findings indicate that none of these strategies is yet able to prolong xenograft survival indefinitely, suggesting that these approaches need to be further refined.

Inducing Accommodation and B-Cell Tolerance

Inducing accommodation—defined as long-term graft survival notwithstanding the continuing presence of xenoreactive antibodies and complement (Bach et al. 1991)—is a very appealing option for preventing antibody-mediated damage. Certain changes in the graft, such as the up-regulation of protective genes or changes in the antigens targeted by the humoral response after transplantation, may at least partially explain the mechanisms behind accommodation (Koch et al. 2004). Alternatively, changes in the recipient's antibody repertoire may underlie this fortunate situation. Be that as it may, accommodation is currently seen as a phenomenon requiring further investigation to better identify its potential in the primate xenotransplantation setting.

The induction of B-cell tolerance against xenogeneic antigens is another very attractive idea for preventing antibody-mediated damage. Inducing chimerism in the recipient has been considered indispensable to achieving tolerance. Elegant preliminary studies conducted in mice to induce a tolerance of B-cell clones producing anti-αGal antibodies have convincingly shown that inducing a mixed hematopoietic chimerism may lead to B-cell tolerance. This mixed hematopoietic chimerism was obtained by infusing allogeneic (Ohdan et al. 1999) αGal^+ T-cell-depleted bone marrow into αGal^- mice submitted to lethal whole-body irradiation (Yang et al. 1998) or to a nonmyeloablative regimen (Ohdan et al. 1999). The resulting chimerism was associated with an indefinite acceptance of αGal^+ grafts with no need for further immunosuppression (Ohdan et al. 1999). Tolerance early after transplantation was induced by anergy, requiring the persistence of αGal^+ cells, whereas clonal deletion or receptor editing was believed to be the mechanism involved in B-cell tolerization at subsequent stages (Kawahara et al. 2005). B-cell tolerance has also been achieved in relation to non-αGal epitopes (Aksentijevich et al. 1992). Using a microchimerism-based approach, Griesemer and colleagues recently succeeded in obtaining a specific humoral unresponsiveness to swine antigens in baboons. This is a promising strategy and its refinement may, at some stage, enable long-term xenograft acceptance in primates (Tseng et al. 2004; Griesemer et al. 2010).

Preventing Cell-Mediated Rejection

Several strategies have also been developed to block innate and adaptive cell-mediated xenograft rejection.

Systemic Immunosuppression

Many immunosuppressive approaches have been attempted to improve xenograft survival, including conventional and also more recently developed molecules or biologics. The introduction of anti-CD154 monoclonal antibodies has contributed to a significantly longer solid-organ and islet xenograft survival (Kuwaki et al. 2005; Yamada et al. 2005; Cardona et al. 2006; Hering et al. 2006), while preventing elicited antibody responses (Ezzelarab et al. 2012). These results have been severely penalized by thromboembolic events that have precluded further use of anti-CD154 antibodies and prompted researchers to explore other therapeutic options, such as anti-CD40 agents or CTLA-4Ig-based strategies in combination with immunosuppressive drugs (Ezzelarab et al. 2009; Thompson et al. 2011b). Using an anti-CD40 antibody associated with belatacept extended neonatal porcine islet survival (Thompson et al. 2011b), although further studies are needed to confirm this finding. On the other hand, Ezzelarab et al. (2012) found in an artery-patch xenotransplantation model that CTLA-4Ig therapy was probably not as efficacious as CD154/CD40 inhibitors in preventing cellular and humoral response (Dons et al. 2012).

Local Immunosuppression Using Specifically Engineered Source Animals

Genetic engineering of the xenograft has also been proposed as a means to inhibit rejection locally, while containing systemic immunosuppression and its side effects. Several approaches have been considered, and novel pig lines expressing molecules that may block the recipient's immune cells have been produced.

When a mutant form of the CIITA gene (an essential coactivator for the transcription of MHC class II genes) was introduced on a GalT-KO/CD46/CD55 background, SLA II molecules were down-regulated and $CD4^+$ T-cell proliferation was inhibited in MLR studies (Ayares et al. 2011). Several porcine and human CTLA-4Ig transgenic animals were obtained (Martin et al. 2005; Phelps et al. 2009; Koshika et al. 2011), and hCTLA-4Ig transgenic pigs expressing the transgene selectively in neurons enabled a long-term survival of neural precursors in mice (Martin et al. 2005) and primates (Aron Badin et al. 2009). Because the constitutive expression of pCTLA-4Ig resulted in severely immunosuppressed animals, pigs with an inducible pCTLA-4Ig expression have recently been obtained (Klymiuk et al. 2012). As for the NK cells, transgenic pigs expressing HLA-E (a molecule inhibiting NK cell adhesion and cytotoxicity) have also been produced (Weiss et al. 2009). Transgenic pigs expressing human CD47 have likewise recently been generated (Tena et al. 2011). Although the potential of these genetic modifications still remains to be tested in vivo, their association with other strategies may lead to a better graft survival.

Using Mechanical Barriers

Encapsulating xenogeneic islets has been suggested as a strategy to prevent cell-mediated rejection and enable graft survival in the absence of immunosuppression. The crucial issues to tackle in this area include the biocompatibility of the encapsulating material, the mechanical stability of the capsules, and the selection of the implantation site (Dufrane et al. 2006b). Microencapsulation of pig islets in water-soluble polymers, such as alginate (Dufrane et al. 2006a), or macroencapsulation in subcutaneous devices permeable to glucose, insulin, and nutrients (but not to antibodies or the immune system's cellular components) have enabled islets to survive in primates for up to 6 mo without any immunosuppression (Dufrane et al. 2010). These findings suggest that, notwithstanding the currently limited life span of such devices, refining this approach may lead in the future to the successful clinical application of islet xenotransplantation.

T-Cell Tolerance and Tregsv

The feasibility of inducing T-cell tolerance has also been explored. Early studies on T-cell tolerance in mice showed that transplanting fetal or neonatal pig thymic tissue into thymectomized mice could induce a central tolerance of pig skin grafts (Zhao et al. 1996). This strategy was successfully applied to the transplantation of GalT-KO pig kidneys into baboons, resulting in a normal renal function for up to 83 d (Yamada et al. 2005; Griesemer et al. 2009). This result was achieved by transplanting thymic tissue under the donor renal capsule (thymokidney) in recipients exposed to a protocol that included thymectomy, splenectomy, T-cell depletion, and whole-body irradiation. Doing without whole-body irradiation enabled a mean survival of more than 50 d. Thymic grafts supported thymopoiesis, and there was evidence of $CD4^+CD3^-$ baboon cells adjacent to porcine thymic epithelial cells (Griesemer et al. 2009). Donor-specific unresponsiveness with respect to the normal responses to allogeneic third parties was also detected in CTL assays. As an alternative approach to enabling T-cell tolerance in xenotransplantation, thymic lobe (vascularized thymic lobe, VTL) transplantation from either αGal^+ (Yamamoto et al. 2005) or GalT-KO donors (Yamada et al. 2005) has also been proposed. This procedure enabled early thymopoiesis, reconstitution of the recipient's naïve T-cell population, and donor-specific unresponsiveness, albeit for a limited time (Yamamoto et al. 2005). These regimens to induce T-cell tolerance clearly require further fine-tuning, but the results achieved to date suggest that this approach may soon become a viable option for primates.

Inducing tolerance through regulatory T-cell-based therapies also has its appeal for both allo- and xenotransplantation. Regulatory T cells have been found to suppress T-cell-direct (Wu et al. 2008) and -indirect xenogeneic responses to T cells (Nishimura et al. 2010), B cells (Singh et al. 2012), macrophage activation (Fu et al. 2008), and-antigen-presenting cell functions (Cederbom et al. 2000; Fu et al. 2008). Several types of regulatory T cells (Tregs) have been described, and those naturally occurring in the immune system (nTregs) account for nearly 3%–10% of the peripheral $CD4^+$ T cells (Dons et al. 2010; Muller et al. 2011). Phenotypically, nTregs are characterized by the constitutive presence of CD4, CD25, the IL-2 receptor α-chain, and the intracellular expression of transcription factor Forkhead box P3 (Foxp3), and by little or no expression of CD127, the IL-2 receptor α-chain. The suppressor activity of nTregs depends on cell-to-cell contact via important costimulatory molecules, which include CTLA-4, membrane-bound TGF-β latency-associated peptide (LAP), soluble inducible costimulatory molecule (ICOS), galectin-1, CD39, CD73, and PD-1, or it is mediated by regulatory cytokines such as IL-10, TGF-β, or IL-35 (Muller et al. 2011, 2012). Notably, the expression of hPD-L1 (the ligand of activated T cells PD-1) by porcine endothelial cells stimulates the proliferation of human $Foxp3^+CD4^+$ T cells both in vitro and in vivo, inducing the expression of CD73 by Tregs and promoting the production of IL-10, and reducing the proinflammatory Th1 and Th17 cytokines. The in vivo infusion of $hPD\text{-}L1^+$ endothelial cells prolongs the survival of porcine skin grafts, favoring the expansion of $Foxp3^+CD4^+$ T cells (Ding et al. 2011). Much the same results have been obtained in mice by grafting porcine endothelial cells transfected with ICOS-Ig, a soluble form of ICOS (Hodgson et al. 2011), suggesting that Treg-based strategies may represent a novel path toward achieving tolerance in xenotransplantation.

CONCLUSIONS

To sum up, despite the challenges that remain, the potential health benefits afforded by xenotransplantation make it a fascinating area of research that deserves to be pursued. Important preclinical data have been generated in recent years, making the initiation of clinical trials a realistic option for the not-too-distant future. At this point, many researchers in this field would agree that genetic engineering of the donor species may be needed to improve current results. Such genetic engineering should selec-

tively address only major interspecies molecular discrepancies, like those involved in regulating the complement or coagulation cascades, eliminate highly immunogenic epitopes, or block major immune cell activation pathways. This should ultimately contribute to the development of the ideal milieu in which novel immunosuppressive drugs or innovative strategies designed to enable accommodation or tolerance will have the greatest chances of success in terms of achieving long-term xenograft survival. It has also become clear that, before xenotransplantation can enter the clinical arena, a multidisciplinary approach will be needed to comprehensively tackle the various issues relating to the use of xenografts to cure human diseases. The safety-related, ethical, and regulatory issues of xenotransplantation (which were not the object of this review) are already being addressed by highly specialized and dedicated investigators, and the multidisciplinary effort currently under way is expected to enable xenotransplantation to happen with a favorable risk/benefit ratio in the foreseeable future.

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