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NEW CONCEPTS OF IMMUNE MODULATION IN XENOTRANSPLANTATION

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Abstract

The shortage of human organs for transplantation has focused research on the possibility of transplanting pig organs into humans. Many factors contribute to the failure of a pig organ graft in a primate. A rapid innate immune response (natural anti-pig antibody, complement activation, and an innate cellular response, e.g., neutrophils, monocytes, macrophages, NK cells) is followed by an adaptive immune response, although T cell infiltration of the graft has rarely been reported. Other factors (e.g., coagulation dysregulation, inflammation) appear to play a significantly greater role than in allotransplantation. The immune responses to a pig xenograft cannot therefore be controlled simply by suppression of T cell activity.

Before xenotransplantation can be introduced successfully into the clinic, the problems of the innate, coagulopathic, and inflammatory responses will have to be overcome, most likely by the transplantation of organs from genetically-engineered pigs. Many of the genetic manipulations aimed at protecting against these responses also reduce the adaptive response. The T cell and elicited antibody responses can be prevented by the biologic and/or pharmacologic agents currently available, in particular, by costimulation blockade-based regimens. The exogenous immunosuppressive regimen may be significantly reduced by the presence of a graft from a pig transgenic for a mutant (human) class II transactivator gene, resulting in downregulation of SLA class II expression, or from a pig with 'local' vascular endothelial cell expression of an immunosuppressive gene, e.g., CTLA4-Ig. The immunomodulatory efficacy of regulatory T cells or mesenchymal stromal cells has been demonstrated *in vitro*, but not yet *in vivo*.

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Keywords

Co-stimulation blockade; immunosuppression; nonhuman primate; pig; xenotransplantation

Introduction

There is a critical and increasing shortage of organs for the purposes of transplantation. In the USA alone approximately 110,000 patients are on the waiting list for an organ of one sort or another, and yet during the current year only approximately 30,000 organs will become available from deceased human donors. Almost 20 patients waiting for a human organ die each day, i.e., almost 7,000 per year. If islet transplantation becomes clinically more successful, as it slowly is, then the situation will be much more serious. There are an estimated 1.5 million type 1 diabetic patients taking insulin each day in the USA.

Xenotransplantation, i.e., the transplantation of organs, tissues, and cells across species, particularly using genetically-engineered pigs as a source for clinical transplantation, offers the potential to resolve this shortage (1-3). In recent years, considerable progress has been made in overcoming the major immunologic and pathophysiologic barriers, and the field is steadily drawing closer to a time when clinical trials would be justified.

Primate response to a pig organ xenograft

Many factors contribute to the failure of a pig organ graft in a primate. These include a rapid innate immune response, characterized by natural anti-pig antibody binding to the vascular endothelium of the graft with activation of the complement cascade (4,5). In addition, an innate cellular response, consisting mainly of neutrophils, monocytes, macrophages, and natural killer [NK] cells, is involved (6). If the initial antibody-mediated complement activation, which results in hyperacute rejection, can be prevented, then the innate cellular response contributes to the development of a delayed form of rejection known variously as acute humoral xenograft rejection, acute vascular rejection, or delayed xenograft rejection (6,7).

The innate response is followed by an adaptive immune response that may be stronger than the adaptive response to an allograft, although this point remains controversial (8-10). Histopathological features of classical acute cellular rejection (i.e., T cell infiltration of the graft), however, have rarely been reported in a pig organ graft, which may be because acute cellular rejection is preceded by acute humoral xenograft rejection or because it has been successfully prevented by the relatively intensive immunosuppressive therapy that nonhuman primate (NHP) recipients of pig grafts have received in studies performed thus far.

In allogeneic systems, the T cell response elicited through the direct pathway is critical in inducing acute rejection, whereas indirect allorecognition may predominate later as a factor in the development of chronic rejection. Primate xenogeneic recognition is through the direct and indirect pathways, and so in pig-to-primate xenotransplantation both pathways are

involved in rejection (11-14). It has been suggested that both direct and indirect xenoresponses are stronger than in their counterparts in the alloresponse (11).

Pig stimulator cells directly activate human T cells (8,10), reflecting productive interaction between human T cell receptors and swine leukocyte antigen (SLA) class I and class II peptide complexes (14). Two donor cell types are likely to be the major stimulators of direct T cell recognition - (i) the migratory passenger leukocytes known as antigen-presenting cells, including dendritic cells (15-17), and (ii) SLA class I and class II-positive vascular endothelial cells (ECs) (18,19). Although pig antigen-presenting cells are transient components in the graft, pig vascular ECs are usually permanently present.

In contrast to human aortic ECs, pig aortic ECs constitutively express costimulatory molecules (e.g., CD80/86, CD40) (20-22). This is likely an important factor contributing to rejection of pig xenografts, and thus relatively intensive immunosuppressive therapy has been required to date. This may result in impaired immunity, which is associated with susceptibility to infection (23). Human T cells may proliferate against various peptides (not only SLA class I and II) derived from pig cells through the indirect pathway (11,12,24).

Several studies have investigated the relative contributions of the direct and indirect pathways in xenotransplantation (recently reviewed in 14 and 25). This topic is still debated, but what is known is that the T cell receptor repertoire, accessory molecule interactions, and cytokine production required for both direct and indirect pathways of recognition in the human response to pig antigens are functionally intact (8,25).

The immune response is complicated by factors that appear to play a significantly greater role in xenotransplantation than in allotransplantation. For example, there is major coagulation dysregulation between pig and primate, which contributes towards the development of thrombotic microangiopathy in the graft and/or a consumptive coagulopathy in the recipient (26-29). This dysregulation is believed to be at least partly initiated by the immune response, e.g., activation of the porcine ECs by recipient antibody and/or complement, and/or by direct interaction between recipient platelets and the graft ECs (30), but greatly enhanced by molecular incompatibilities between the coagulation-anticoagulation systems of the two species (31,32).

Factors involved in the coagulation cascade, such as thrombin, may increase the adaptive response (33) (Figure 1), and it may be for this reason that the adaptive response appears stronger than to an allograft, even though the T cell response itself (if unaffected by coagulation factors) may be comparable. Importantly, the mechanism by which this takes place has been demonstrated to be independent of upregulation of expression of SLA class II (Figure 2). Thrombin activation of pig ECs is associated with the upregulation of the costimulatory molecules CD80/86, which can amplify the T cell proliferative response to pig cells independent of the upregulation of SLA class II expression. It is therefore unlikely that complete blockade of SLA class II recognition will prevent T cell proliferation induced by thrombin.

There is also an inflammatory response to the graft, which may also contribute towards the xenoreactive immune response appearing stronger. There are data that indicate that there is

considerable ‘cross-talk’ between the innate and adaptive responses and between those responses and the factors responsible for coagulation dysfunction and inflammation (34,35). Together, these observations indicate that the immune response to a pig xenograft cannot be considered in isolation, and will not be controlled simply by suppression of T cell activity (as is generally possible in allotransplantation). Equal attention needs to be directed to the innate immune, coagulation, and inflammatory responses. (Of interest, a process by which an innate immune response is induced by the formation of thrombi inside blood vessels – in pathologic conditions unassociated with xenotransplantation - has recently been recognized and termed “immunothrombosis” [36].)

Suppression of the immune responses

The prevention or reduction of the innate immune response has been approached by the genetic engineering of the organ-source pig rather than by the administration of immunosuppressive agents, which are largely ineffective. In this respect, the transgenic expression of one or more human complement-regulatory proteins (hCRPs), e.g., CD46 (hMCP), CD55 (hDAF), CD59, contributes significant protection (37). Similarly, the introduction of pigs in which the gene for α1,3-galactosyltransferase has been deleted (GTKO pigs) (38-40), thus preventing the expression of the important galactose-α1,3-galactose (Gal) antigen, which is the major target for primate anti-pig antibodies (41,42), was a major advance (29,43). GTKO pigs expressing one or more hCRPs provide increased protection than either manipulation alone (44,45). Building on this genetic background, an increasing number of genetically-engineered pigs are becoming available for transplantation studies in NHPs (reviewed by Ekser 2012 [2]).

Although various techniques, e.g., plasmapheresis, extracorporeal immunoadsorption (46), the administration of natural or synthetic oligosaccharides (28,47,48) that prevent anti-Gal antibody binding to the graft, proved valuable in early studies, the value of these has largely been negated by the availability of GTKO pigs. Similarly, although cobra venom factor and other anti-complement agents were administered successfully (49,50), these are no longer necessary when the organ-source pig expresses one or more hCRP. Indeed, there are reasons to believe that cobra venom factor could be detrimental in some respects, as it can result in the release of the anaphylatoxin C5a, which contributes to innate and adaptive immune responses (51). Should an anti-complement agent prove necessary, e.g., if acute humoral xenograft rejection develops, then early evidence from allotransplantation suggests that the monoclonal antibody, eculizumab, might prove successful (52,53).

An unexpected bonus of these genetic manipulations is the observation that the absence of expression of Gal and the expression of a hCRP renders the T cell response to the graft weaker. Studies by Wilhite et al have demonstrated a significant reduction in the primate mixed lymphocyte reaction to pig ECs when Gal is absent or an hCRP is expressed (54,55) (Figure 3). Although there is a definite reduction in the human T cell proliferative response to GTKO/hCRP pig cells in vitro, it is difficult to quantify this effect in vivo in pig-to-NHP transplantation models as so many other factors, e.g., coagulation dysregulation, affect the outcome. In an immunocompetent rodent model, however, GTKO adipose-derived mesenchymal stromal cells (MSCs) induced a weaker anti-pig IgG antibody response than

did wild-type pig MSCs, and there was reduced cellular infiltration (lymphocytes, macrophages) in and around the GTKO MSCs (56). Any manipulation of the pig that results in even a small reduction in the human T cell response is clearly welcome.

The adaptive immune response can also be reduced by specific manipulation of the organ-source pig. Initial efforts to express an immunosuppressive agent, such as CTLA4-Ig, in the pig proved unsuccessful in that, although significantly reducing the primate T cell response (22), ubiquitous expression of this agent rendered the pig immune-incompetent and susceptible to infectious complications (57). The efficiency of this approach, however, was illustrated by the fact that, in pigs with ubiquitous expression of CTLA4-Ig, the blood levels of soluble CTLA4-Ig were up to 16-fold higher than the therapeutic levels required by systemic administration of this agent (57).

More recent techniques are allowing this or similar agents to be expressed only in specific target cells of the pig, e.g., the beta cells of the islets (using an insulin promoter) (58,59), vascular ECs (using an endothelium-specific Tie2 enhancer) (60,61), or neuronal cells (using a neuron-specific enolase promoter) (62,63).

There is some concern that, if the pig graft is producing an immunosuppressive agent even in a limited way, this could render the recipient immune-incompetent, particularly as the level of immunosuppression cannot easily be reduced. Nevertheless, the advantage of providing some endogenous ‘local’ immunosuppression to the graft is obvious, and a combination of endogenous and exogenous approaches may prove optimal.

A second approach that has been tested both *in vitro* and *in vivo* is rendering the pig transgenic for a mutant human class II transactivator gene, resulting in downregulation of SLA class II expression (CIITA-DN pigs) (64-66) (Figure 4). The primate T cell response to CIITA-DN pig cells/tissues, particularly when these cells have been activated, is significantly reduced (65,66).

As human T cells may proliferate against non-SLA pig proteins presented through the indirect pathway, strategies (e.g., costimulation blockade) directed to the prevention of sensitization to pig antigens presented by host antigen-presenting cells will almost certainly be required even when organs from genetically-engineered pigs have been transplanted.

The potency of NK cells in xenograft rejection remains uncertain, but the introduction of transgenes for HLA-E and/or G into the organ-source pig may negate any effect these cells might have (67-72). The mechanism of inhibition by these two HLA class I molecules is different, and therefore expression of both is likely to prove advantageous (71). Depletion or inhibition of other innate immune cells, e.g., neutrophils, monocytes, and macrophages, may be more difficult. Furthermore, these cells may be involved in leukocyte-platelet aggregation, indicating a state of platelet activation which results in the development of thrombotic microangiopathy (6,73).

Genetic modification of the pig may also minimize or prevent coagulation dysregulation. In this respect, pigs are currently available that express human thrombomodulin (TBM), human endothelial protein C receptor (EPCR), as well as CD39 and tissue factor pathway inhibitor.

Expression of the human transgenes helps control the known molecular incompatibilities between pig and primate that contribute to this dysfunction (31,32). Transgenic expression of more than one of these human genes will probably be necessary to completely prevent the development of thrombotic microangiopathy in the graft or consumptive coagulopathy in the recipient. It may be necessary, however, to provide additional systemic therapy in the form of an anti-platelet or anti-thrombin agent.

Control or reduction of the inflammatory response is also most likely to be controlled by genetic manipulation of the pig. Expression of TBM, EPCR, and/or CD39 is anticipated to reduce the inflammatory response in addition to coagulation dysfunction (74). Furthermore, pigs are now available that express hemeoxygenase-1 (HO-1) (75), although this gene has only very recently been expressed in pigs that are also protected from the innate response, e.g., on a GTKO/CD46 background, and so its role in controlling inflammation as well as its effect on coagulation has not yet been well-defined. In addition, hemeoxygenase-1 expression may reduce the adaptive response through T cell apoptosis (75). Other anti-inflammatory genes that might prove valuable include A20 (76,77).

Just as there may be a need for exogenous immunosuppression and/or anti-thrombotic therapy, there may also be a need for the administration of anti-inflammatory agents. In this respect, in addition to corticosteroids, there is evidence that high-dose statin therapy not only reduces the inflammatory response and platelet activation (78), but also downregulates the primate cellular response to pig antigens (79). Interleukin-6 receptor (IL-6R) blockade may have a role to play in suppressing the inflammatory response to a xenograft, and intriguing studies in non-xenotransplantation models by Tracey and his colleagues suggest that chronic vagal nerve stimulation or the administration of a parasympathomimetic agent might suppress this response (80).

Specific suppression of the adaptive immune response

‘Rejection’, or more accurately the mechanism of graft failure, of a pig xenograft is therefore complex. However, for the purposes of this review, subsequent attention will be concentrated on the adaptive response and, particularly, on what biologic and pharmacologic agents are likely to play an important role in its control. Pig liver (81) and lung (82) transplantation into NHPs result in rapid graft loss through complex mechanisms that are unrelated to the adaptive response, and in pig islet transplantation the problem of the instant blood-mediated inflammatory reaction has to be overcome (3). These topics deserve in depth consideration in another context, but in this review attention will be directed to immunosuppressive regimens that have been utilized in pig heart and kidney xenotransplantation models.

The early experience in pig-to-NHP heart and kidney transplantation models was comprehensively reviewed by Lambrigts et al in 1998 (83) and will not be repeated here. In summary, almost all studies before 1998 utilized conventional biologic (e.g., anti-thymocyte globulin [ATG]) and/or pharmacologic (e.g., cyclophosphamide, cyclosporine, tacrolimus, corticosteroids) agents. Heterotopic heart graft survival extended to 62 days, with life-supporting kidney graft survival extending to 35 days (83). Acute humoral xenograft

rejection was a major cause of graft failure, but, as a result of the necessity for intensive immunosuppressive therapy, many experiments were terminated when irreversible complications of this therapy developed, most commonly infection.

Approaches to exogenous immunomodulatory therapy

More recently, there has been a trend towards costimulation blockade-based regimens, initially introduced in xenotransplantation in 2000 by Buhler et al (84) in a hematopoietic stem cell transplantation model when it was observed that a cyclosporine-based regimen did not prevent an elicited anti-pig antibody response. The suppression of this response is clearly essential if a pig organ xenograft is to survive, and is a good indicator of the efficacy of any immunosuppressive regimen.

Subsequent experience in heterotopic and orthotopic heart transplantation models, and in the life-supporting kidney transplantation model, using either costimulation- or conventional pharmacologic-based regimens, indicates graft survival has increased (2,85). Survival after heterotopic heart transplantation now extends to 8 months (86,87) and after life-supporting kidney transplantation to almost 3 months (88,89). However, this is not yet to the extent that clinical trials can be contemplated. With either immunosuppressive approach, the causes of graft failure are generally no longer rejection, but thrombotic microangiopathy and/or consumptive coagulopathy (6,27-29). It is important to note that there may be significant differences in the primate response to a pig heart than to a pig kidney (90). These may account for the discrepancy in graft survival and for the predominance of thrombotic microangiopathy after heart transplantation and the more rapid onset of consumptive coagulopathy after kidney transplantation.

Despite the efficacy of anti-CD154mAb in xenotransplantation models, its thrombogenicity has necessitated efforts to develop costimulation blockade regimens that do not include this agent. At our own center, we have been able to replace it with a combination of an anti-CD40mAb and LEA29Y (belatacept) (Ekser B and Iwase H, unpublished).

Cell therapy approaches

Although encouraging *in vitro* data have been reported, the immunomodulatory efficacy of regulatory T cells (Tregs) (10,91,92) or MSCs (55,93,94) has yet to be demonstrated in large animal models of xenotransplantation. Tregs have been demonstrated to suppress the xenoreactive effector T cell response *in vitro*, as have MSCs.

Importantly, Tregs have been reported to have some effect in suppressing the innate immune response in allotransplantation. Muller et al (14,95) have reviewed the potential role of Tregs in xenotransplantation, and have drawn attention to the increasing evidence for the ability of Tregs to suppress xenogeneic T cell responses, as well as NK and B cell activation. They suggest that it will be important to use immunosuppressive drugs that allow Treg expansion while controlling T effector cell proliferation; targeting the CD154:CD40 pathway is beneficial in this respect (although this will need to be through an anti-CD40mAb unless the thrombogenic effect of anti-CD154mAb can be prevented), as is the administration of rapamycin (96). They also emphasize the tolerogenic environment

promoted by IL-10, and speculate that secretion of recombinant IL-10 by genetically-modified pigs could contribute to tolerance induction. The selective recruitment of Tregs to the xenograft mediated by transgenic expression of human cytokines, e.g., CCL17, CCL22, on pig ECs may also help overcome cellular rejection.

The over-expression of programmed cell death ligand 1 (PD-L1) in porcine ECs inhibits the proliferation of T effector cells, augments IL-10 secretion, and allows expansion of $CD73^+$ Tregs in vitro (97). Muller et al have suggested that these studies, together with those reported by Plege (98), indicate that PD-1/PD-ligand interactions may promote the suppressive function of Tregs in xenotransplantation (14).

Survival and function of MSCs across species barriers have been extensively reported (reviewed by Li et al [94]). The immunomodulatory, anti-inflammatory, and/or regenerative potential of pig MSCs is being explored (55,93) as the ready availability of adipose-derived MSCs from large pigs would minimize the logistics of providing unlimited numbers of these cells. Initial *in vitro* studies indicate that MSCs from genetically-engineered pigs are no more immunogenic than human MSCs, and yet have a comparable immunomodulatory effect on the human T cell response (55,93).

Although Tregs and/or MSCs may augment biologic and/or pharmacologic immunosuppression, we are not optimistic that either will entirely replace more standard therapy in the near future.

Induction of tolerance to a pig xenograft

As with allotransplantation, the ultimate goal is to induce a state of immunological tolerance to the pig graft. In xenotransplantation, this goal has been vigorously explored by Sachs' group, both through the induction of hematopoietic cell chimerism (99) and thymus transplantation (88,89). It is likely that further genetic manipulations to the pig (to control the innate, coagulopathic, and inflammatory responses) will be required before this goal can be successfully achieved. As outlined above, the in vivo or in vitro expansion and maintenance of Tregs may play a role.

Conclusions

1. Before xenotransplantation can be introduced successfully into the clinic, the problems of the innate, coagulopathic, and inflammatory responses will have to be overcome, most likely by the transplantation of organs from specifically genetically-engineered pigs. Many of the genetic manipulations aimed at protecting against these responses will also reduce the adaptive response.
2. The T cell and elicited antibody response can be prevented by the biologic and/or pharmacologic agents currently available. In particular, costimulation blockade-based regimens (that do not include an anti-CD154mAb), augmented by low-dose pharmacologic therapy, have shown encouraging efficacy.

3. The exogenous immunosuppressive regimen may be significantly reduced by the presence of a graft from a CIITA-DN pig or a pig with 'local' expression of an immunosuppressive gene, e.g., CTLA4-Ig or LEA29Y (belatacept).

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ABBREVIATIONS

CIITA-DN	MHC class II transactivator dominant-negative mutant
ECs	endothelial cells
EPCR	endothelial protein C receptor
GTKO	α1,3-galactosyltransferase gene-knockout
hCRP	human complement-regulatory protein
MSCs	mesenchymal stromal cells
NHP	nonhuman primate
TBM	thrombomodulin
Tregs	regulatory T cells

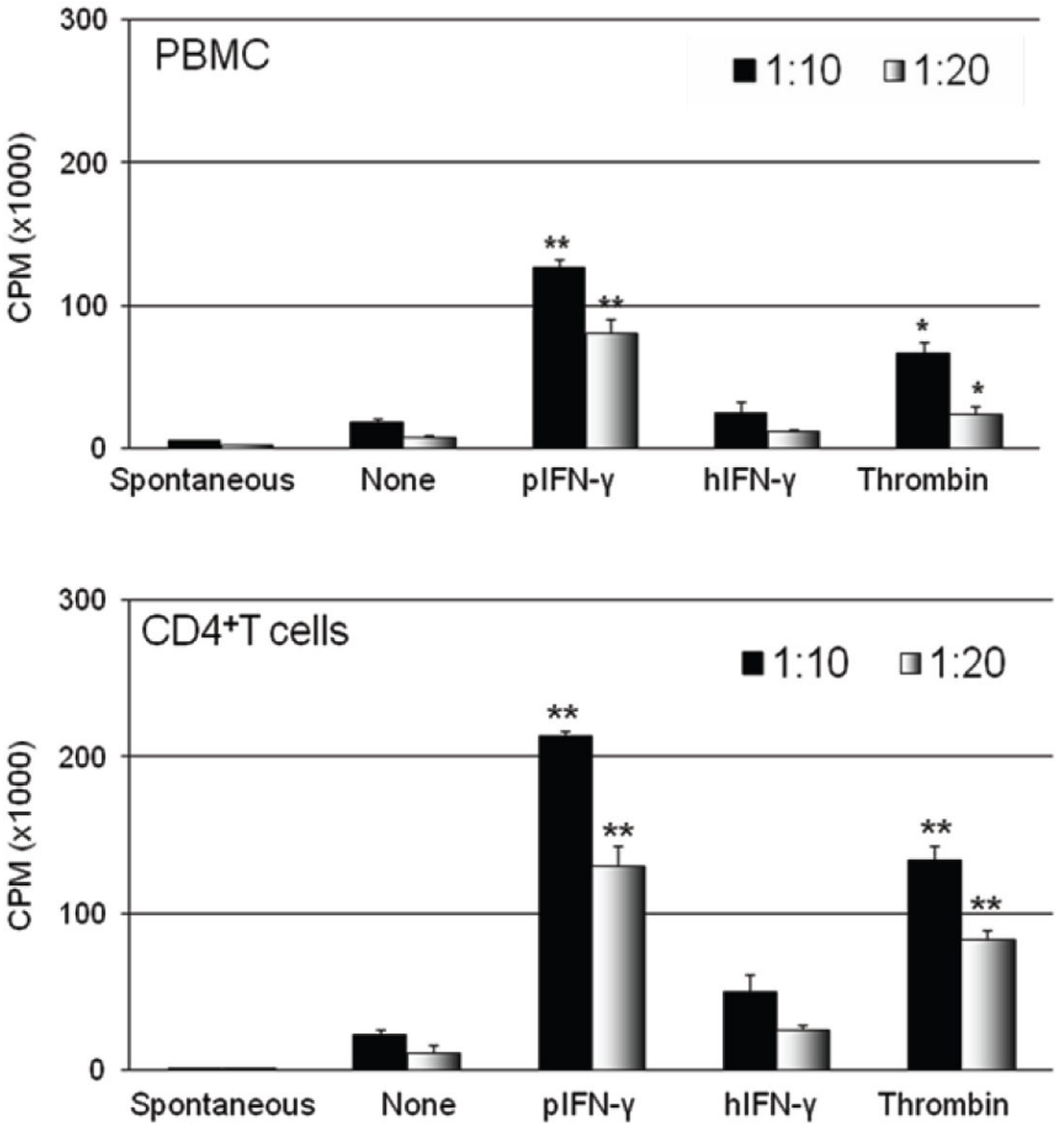

Figure 1. Increased human PBMC and CD4$^+$T cell proliferation in response to thrombin-activated porcine aortic endothelial cells (pAEC)

GTKO pAEC were activated using thrombin (40U/mL), pIFN-γ (40U/mL), or hIFN-γ (200U/mL), for 24h. The human PBMC (top) and CD4$^+$T cell (bottom) proliferative responses to pIFN-γ- and thrombin-activated GTKO pAEC were significantly higher than to non-activated pAEC. (Stimulator:responder ratios of 1:10 and 1:20.) Data are representative of three different experiments. (*$p<0.01$, **$p<0.001$ in comparison to non-activated pAEC). (Reproduced from Ezzelarab C, et al, *Xenotransplantation* 2012; 19:311-316 [33] with permission.)

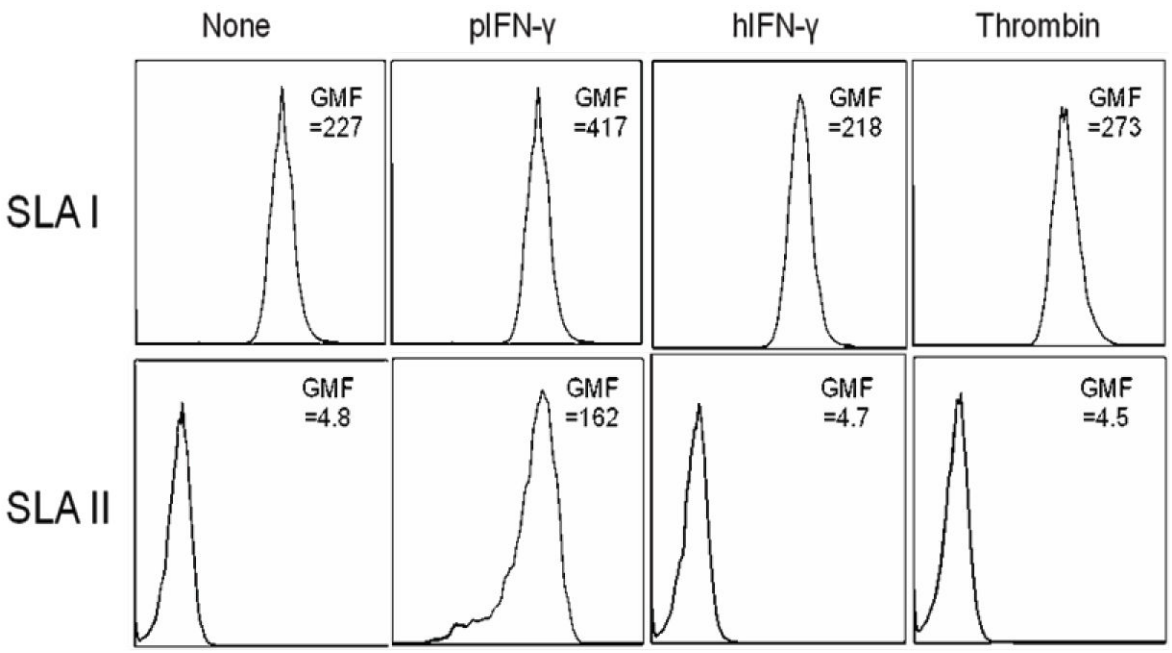

Figure 2. Thrombin does not upregulate SLA class I or II expression on GTKO porcine aortic endothelial cells (pAEC)

GTKO pAEC were activated using thrombin (40U/mL), pIFN-γ (40U/mL), or hIFN-γ (200U/mL) for 24h. SLA class II expression was upregulated only after pIFN-γ activation, but not after thrombin or hIFN-γ activation. There was no change in SLA class I expression after activation. Data are representative of three different experiments. (Reproduced from Ezzelarab C, et al, *Xenotransplantation* 2012; 19:311-316 [33] with permission).

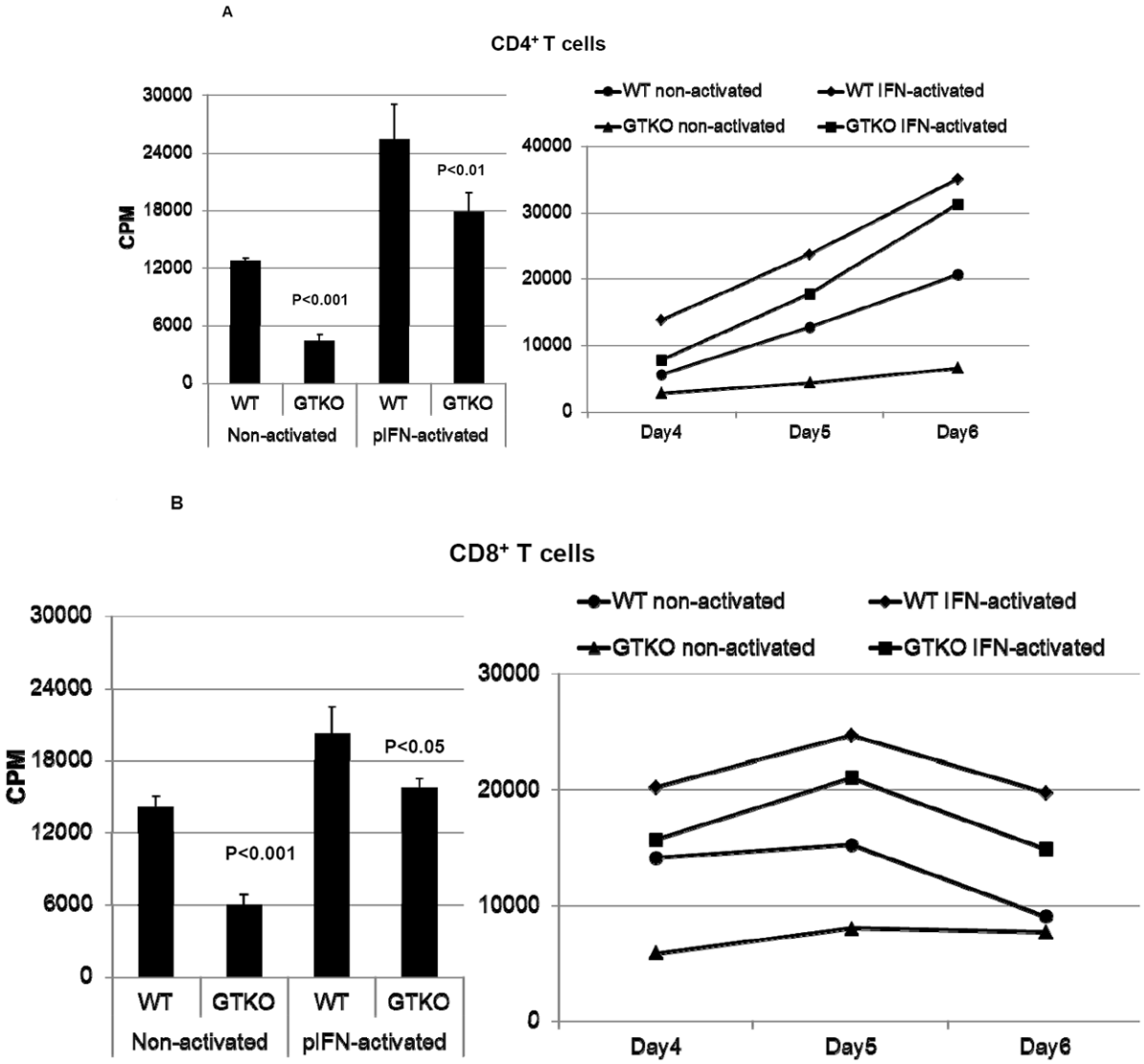

Figure 3. Proliferative response of human T cells to wild-type (WT) and GTKO porcine aortic endothelial cells (pAEC)

A: The proliferative response of human $CD4^+$T cells (n=3) to WT and GTKO pAEC before and after activation by pIFN-γ (left). The response was significantly less to GTKO pAEC before ($p<0.001$) and after ($p<0.01$) activation. Additionally, MLRs were harvested on 3 consecutive days (4, 5 and 6) where $CD4^+$T cell proliferation was consistently lower in response to GTKO pAEC (right).

B: The proliferative response of human $CD8^+$T cells (n=3) to WT and GTKO pAEC before and after activation by pIFN-γ (left). The response was significantly less to GTKO pAEC before ($p<0.001$) and after ($p<0.05$) activation. Additionally, MLRs were harvested on 3 consecutive days (4, 5 and 6) where $CD8^+$T cell proliferation was consistently lower in response to GTKO pAEC (right).

In both **A** and **B**, ^3H incorporation values are presented as CPM. Data represent the mean (+/- SEM) and are representative of three different experiments. (Reproduced from Wilhite T, et al, *Xenotransplantation* 2012; 19:56-63 [54] with permission).

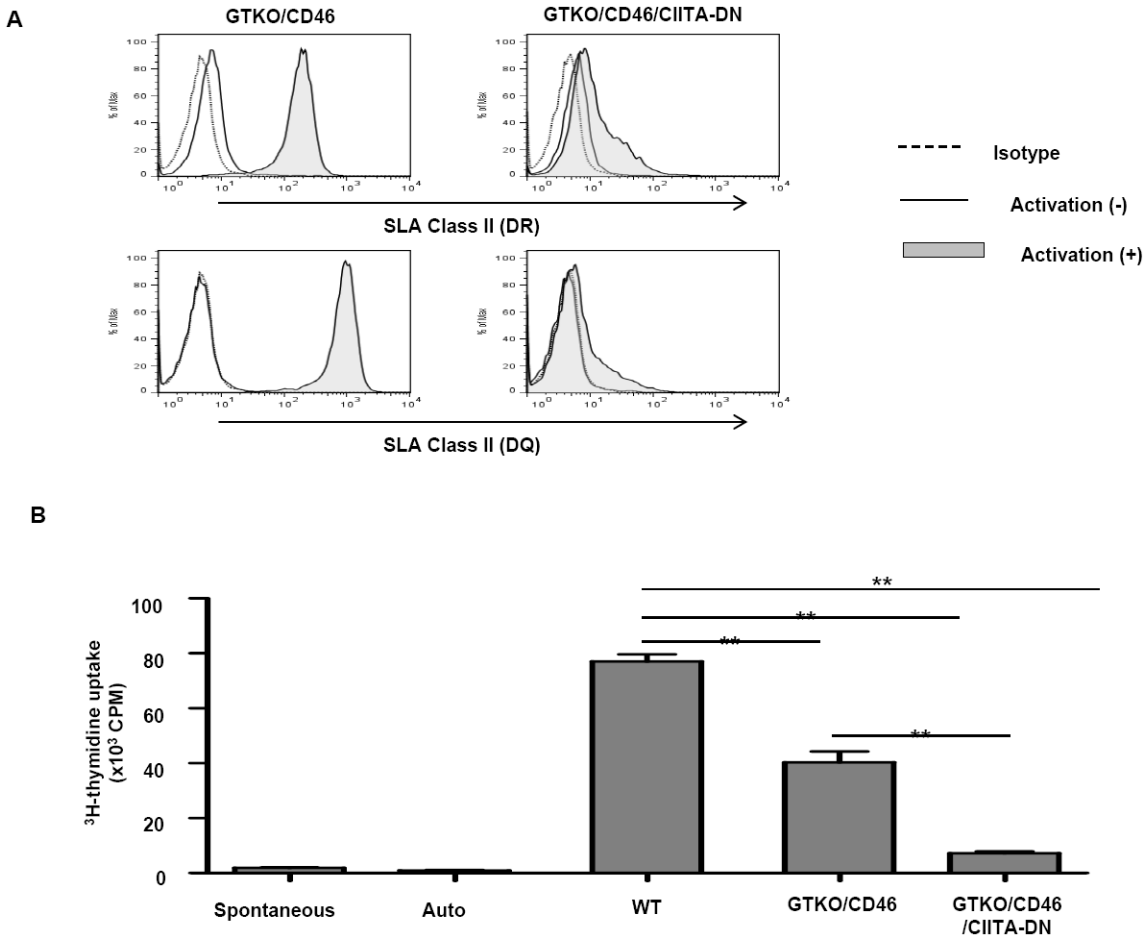

Figure 4.

(A) Significant down-regulation of SLA class II expression on aortic endothelial cells from GTKO/CD46/CIITA-DN pigs

The expression of SLA class II DR and DQ on GTKO/CD46/CIITA-DN porcine aortic endothelial cells (pAECs) was compared with those on GTKO/CD46 pAECs. pAECs were activated with pIFN-γ (50ng/mL) for 48h. The pAECs were stained with specific anti-SLA DR or DQ mAbs. Isotype control (dotted line), quiescent (solid line), and activated pAECs (gray filled).

Expression of SLA class II on quiescent pAECs was undetectable or minimal on both GTKO/CD46 and GTKO/CD46/CIITA-DN pAECs. However, expression of SLA class II on GTKO/CD46 pAECs was significantly up-regulated when the pAECs were activated with pIFN-γ for 48h. In contrast, up-regulation of expression of SLA class II on GTKO/CD46/CIITA-DN pAECs was minimal.

(B) Significant reduction of the $hCD4^+$T cell response to CIITA-DN cells

$hCD4^+$T cells were co-cultured with PBMCs from WT, GTKO/CD46, and GTKO/CD46/CIITA-DN pigs for 6 days. The responses of $hCD4^+$T cells were measured by ^{3}H-thymidine incorporation. As a negative control, $hCD4^+$T cells were cultured with medium only (spontaneous) or autologus PBMCs (auto). There was a significantly lower $hCD4^+$T cell

response to GTKO/CD46/CIITA-DN pig PBMCs than to either WT or GTKO/CD46 pig PBMCs (**p<0.01).