Review

Achievement of Cellular Immunity and Discordant Xenogeneic Tolerance in Mice by Porcine Thymus Grafts

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Specific cellular immune tolerance may be essential for successful xenotransplantation in humans. Thymectomized (ATX), T and NK cell-depleted immunocompetent mice grafted with xenogeneic fetal pig thymic and liver tissue (FP THY/LIV) result in efficient mouse thymopoiesis and peripheral repopulation of functional mouse $CD4^+$ T cell. Very importantly, the reconstituted mouse T cells are specifically tolerant to pig donor antigens. Studies demonstrated that porcine MHCs mediated positive and negative selection of mouse thymocytes in FP THY grafts, whereas mouse MHCs were involved in negative selection in grafts. Therefore, T cell tolerance to xenogeneic donor antigens could be induced by grafting donor thymus tissue. Xenogeneic thymic replacement might have a potential role in the reconstitution of cellular immunity in patients with AIDS or other immunodeficiencies caused by thymus dysfunction. *Cellular & Molecular Immunology*. 2004;1(3): 173-179.

Key Words: xenotransplantation, thymus, tolerance, T cells

Introduction

Xenotransplantation has attracted a marked attention and interest in the past decade, because it may be one of the possible approaches to solve the severe organ shortage of human donors that greatly limits today's advancement in clinical transplantation. Miniature swine, because of their size and physiologic similarities to humans, their excellent breeding characteristics, and their potential for genetic manipulation, have been widely considered as the most likely xenograft donors to humans (1). However, the immune response to xenogeneic organs tends to be much stronger than that toward allografts, and the unacceptable high levels and long lasting treatment of immunosuppression would likely be necessary to avoid xenograft rejection (2, 3). Therefore, it is much practicable to induce specific tolerance to donor antigens by "re-education" of recipients' immune system, which would make successful discordant xenogeneic porcine organ transplantation to humans possible without chronic immunosuppressive therapy, and allows the maintenance of host immunocompetence (3).

We have recently observed that efficient mouse thymopoiesis and peripheral mouse $CD4^+$ T cell repopulation occurs when xenogeneic fetal pig thymic and liver tissue (FP THY/LIV) is grafted to thymectomized (ATX), T and NK cell-depleted immunocompetent mice (4). Very importantly, the reconstituted mouse T cells are specifically tolerant to pig donors, as indicated by nonresponsiveness to host antigens in mixed lymphocyte reaction (MLR) assays, and the long-term acceptance of donor MHC-matched pig skin grafts (4-6). Thus, our studies have provided the first demonstration that grafting of donor thymus tissue provides a novel approach to discordant xenogeneic tolerance induction, and may also have applicability to the treatment of AIDS and other immunodeficiency diseases associated with thymic dysfunction.

Introduction of the pig thymus/liver-grafted, T and NK cell-depleted ATX mouse model

Studies utilizing either mice expressing a transgenic TCR specific for a viral peptide, or MHC-mismatched thymic stroma and bone marrow-derived cells have demonstrated that thymic stroma can anergize and delete developing T cells, leading to tolerance induction. On the other hand, FP THY grafts in immunodeficient SCID mice survived long term (>4 months). We therefore evaluated the capacity of pig thymic tissue to support mouse T cell maturation and to

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Abbreviations: ATX, thymectomized; HAS, heat stable antigen; KLH, keyhole limpet hemocyanin; MLR, mixed lymphocyte reaction; FP THY/LIV, fetal pig thymus and liver; Mtv, mammary tumor virus; HEL, hen egg-white lysozyme; NP THY/SPL, neonatal pig thymus and spleen; TI, thymic irradiation; WBI, whole body irradiation.

toleranize murine T cells in either immunodeficient BALB/c nude mice or in T and NK cell-depleted ATX immunocompetent C57BL/10 (B10) or C57BL/6 (B6) mice.

The protocol for the FP THY/LIV-grafted, T and NK cell-depleted ATX mouse model was illustrated in Figure 1. Generally, eight to twelve week old euthymic or ATX B10 or B6 mice received i.p. injections of mAbs GK1.5 (rat anti-mouse CD4), 2.43 (rat anti-mouse CD8), 30-H12 (rat anti-mouse Thy1.2), and PK136 (mouse anti-mouse NK1.1) in depleting doses on days -6, -1, +7, and +14. On day 0, 3 Gy of whole body irradiation (WBI) was administered to recipients, and one second trimester (gestational day 48-75, estimated by observed estrus or mating and confirmed by ultrasound examination of the fetuses) miniature swine fetal thymic and liver fragment, each about 1 mm^3 in size, was transplanted under the kidney capsule via a midline laparotomy incision. After the abdomen was closed in two layers, 1×10^8 fetal pig liver cells (FLC) were injected i.p.(7). In a variation on this protocol, BALB/c nude mice were depleted of NK cells with an i.p. injection of 50 μl of rabbit anti-mouse asialo-GM1 serum on day -2, and received 3 Gy WBI and FP THY/LIV grafts and i.p. injection of 1×10^8 FLCs on day 0 (5, 8).

Because of the limited amount of thymic tissue available from fetuses, extension of this approach to the use of neonatal pig thymic tissue would be more practicable for future application in pig-to-primate experiments or even in humans. Furthermore, neonatal pig donors have the advantage over fetal donors of being able to be kept alive after thymic tissue donation, thus allowing subsequent evaluation of tolerance to donor-specific organ grafts (9). For neonatal pig thymus/spleen (NP THY/ SPL)-grafted ATX B6 mouse model, recipient mice were conditioned as same as that in FP THY/LIV-grafted ATX B6 mice described above. Instead of FP THY/LIV tissue, one miniature swine neonatal pig (less than 24 hours after birth) thymic and spleen fragment, each about 1 mm^3 in size, was transplanted under the kidney capsule (10).

Repopulation of phenotypically and functionally mature mouse $CD4^+$ T cells in T and NK cell-depleted, ATX mice by grafting porcine thymus

The thymus is the central lymphoid organ for T cell development, and it provides a specialized micro-encironment allowing TCR gene rearrangement, positive and negative selection, and maturation of T cells (11). In the FP THY/LIV-grafted mouse model, mouse $CD4^+$ T cells recovered to normal levels by 8 weeks in control FP LIV-grafted euthymic B10 mice treated with the standard regimen described above (4). In contrast, simultaneously similarly treated ATX B10 mice grafted with FP LIV alone demonstrated very low levels of peripheral $CD4^+$ T cells at any time points, due to the absence of thymus. However, mouse $CD4^+$ T cells showed significant repopulation in the periphery of T and NK cell-depleted ATX B10 mice grafted with FP THY/LIV by 8 weeks post-implantation(4). The high levels of mouse $CD4^+$ T cells in periphery blood lymphocytes (PBL) of FP THY/LIV-grafted ATX B10 mice for at least 30 weeks post grafting, although they had declined somewhat from their peak points (4). The efficient peripheral repopulation of mouse $CD4^+$ T cells in T and NK cell-depleted ATX B10.A(4R), ATX B10.A, ATX BALB/c, ATX MHC class II deficient mice (IIKO) or BALB/c nude mice by grafting FP THY was also observed (8, 12-14).

Using NP THY grafts in NK cell-depleted BALB/c nude mice or in T and NK cell-depleted ATX B6 mice allowed peripheral repopulation of mouse $CD4^+$ T cells, but with markedly lower efficiency than that is observed with fetal pig thymus grafting (9, 10). This difference may be due to the limited growth potential of neonatal pig thymic grafts. However, more recent studies suggest that grafting more pieces of NP THY tissue and other sites of NP THY grafting, such as in the mediastinum and the mesentery, can improve the efficiency of peripheral mouse $CD4^+$ T cell repopulation. These results suggest that other factors besides the age of the tissue donor may affect the

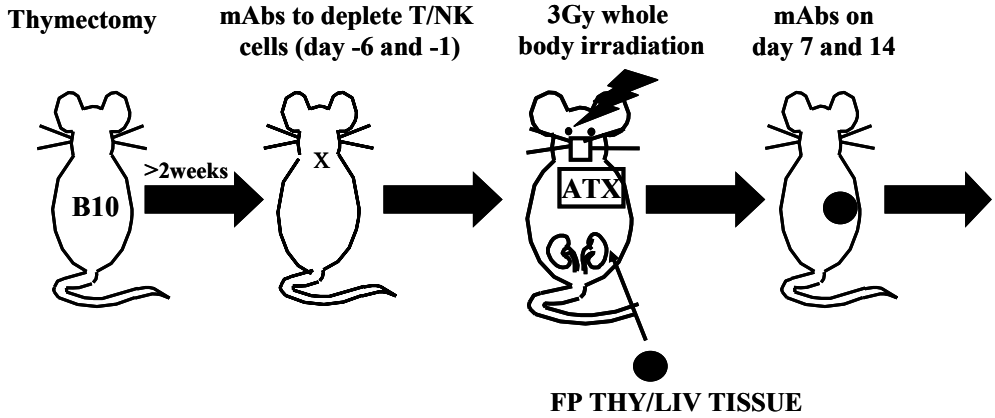

Figure 1. The protocol for FP THY- grafted, T/NK cell-depleted ATX mice. Eight to twelve weeks old euthymic B10 or B6 mice were thymectomized before Ab treatment. Injections of mAbs GK1.5, 2.43, 30-H12, and PK136 in depleting doses were performed on days -6, -1, +7, and +14. On day 0, 3 Gy of whole body irradiation (WBI) was administered to recipients, and one second trimester miniature swine fetal thymic fragment was transplanted under the kidney capsule.

efficiency of peripheral mouse $CD4^+$ T cell repopulation by porcine thymus grafting.

To address whether or not the peripheral repopulated mouse $CD4^+$ T cells in FP THY/LIV-grafted, T and NK cell-depleted ATX B10 mice were due to the emigration of mature $CD4^+$ T cells from FP THY grafts or the immune proliferative response and cell expansion of non-depleted residual $CD4^+$ T cells caused by xenogeneic pig anitgen stimulation. The naïve and memory phenotypies of $CD4^+$ T cells in this model were observed, because the activation of naïve T cells by antigens and their conversion to memory cells is associated with certain phenotypic alterations and the maintenance of the peripheral naïve $CD4^+$ T cell pool requires the presence of a functioning thymus (15). A high proportion of the repopulated mouse $CD4^+$ T cells in FP THY/LIV-grafted ATX B10 mice showed $CD44^{low}$ $CD45RB^{high}CD62L^{high}$ naïve phenotype as that in euthymic control mice, whereas most of the few mouse $CD4^+$ T cells in FP LIV-grafted ATX B10 mice expressed $CD44^{high}$ $CD45RB^{low}CD62L^{low}$ memory phenotype(4), providing evidence that FP THY grafts in T and NK cell-depleted ATX B10 mice are functional.

The direct evidence for the function of FP THY grafts able to support mouse thymopoiesis is that there are normal mouse thymocyte subpopulations (CD4 SP, CD8 SP, DN, and DP) in FP THY grafts (12). Furthermore, an ordered pattern of CD3, TCR, CD69, heat stable antigen (HSA), MHC class I, and Qa-2 expressions during thymocyte maturation in FP THY grafts as that in mouse thymi was observed (12), that is, mouse DP thymocytes in FP THY grafts express high levels of HSA and low levels of Qa-2 and TCR, whereas mature mouse CD4 SP or CD8 SP thymocytes express much low levels of HSA and up-regulated expression of TCR and Qa-2 as that in mouse thymi. In addition, the polycolonal Vβ family expressions on mouse CD4 SP cells in FP THY grafts and spleens of FP THY/LIV-grafted ATX B6 or ATX IIKO mice support the speculation that widely diverse T cell repertoire of mouse $CD4^+$ T cells shaping in FP THY grafts have been achieved (12), although much delicate molecular studies on the diversity of TCR repertoire should be performed.

The function of mouse T cells maturing in FP THY grafts was studied *in vivo* and *in vitro*. These reconstituted $CD4^+$ T cells express normal levels of activation markers (early activation marker, CD69, and IL-2 receptor α chain, CD25) and normal proliferative responses after stimulation with mitogen and alloantigens (4). Most of FP THY/LIV or NP THY/SPL-grafted BALB/c nude mice showed allogeneic responses in MLR assays and rejected allogeneic third party mouse skin grafts, whereas FP LIV-grafted BALB/c nude mice did not respond to allogeneic antigens in MLRs and accepted allogeneic skin grafts due to the deficiency of T cells (8). These reconstituted mouse $CD4^+$ T cells showed efficient proliferative responses to peptide antigen keyhole limpet hemocyanin (KLH) present by host mouse MHC following *in vivo* immunization, although responses of FP THY/LIV-grafted BALB/c nude mice to suboptimal KLH concentrations were somewhat lower than those of normal BALB/c mice (4). Most importantly, mouse T cells maturing in FP THY grafts are capable of protecting the mice from opportunity infection, as demonstrated by their ability to clear *Pneumocystis carinii* infection *in vivo* (4).

Consistent with our results, a patient with complete DiGeorge syndrome receiving an HLA-mismatched allogeneic thymus graft showed recovery of $CD4^+$ T cells and normal Ab responses to tetanus toxoid and pneumococcal vaccine immunization, suggesting successful immune restoration (16, 17). It was most recently reported that nude mice reconstituted with an MHC-incompatible allogeneic or even xenogeneic rat thymus generate effector T cells which are restricted to the host MHC (18), although our experimental results do not support the idea that bone marrow-derived cells mediate positive selection (discussed below). Thus, xenogeneic thymus grafts may potentially offer an important adjunct for the treatment of immunodeficiencies due to the thymus failure.

Reconstitution of immunity will be critical for the survival of HIV-infected individuals once viral load is brought under control. It has been reported that AIDS caused by HIV infection is often associated with disruption of the thymic microenvironment , which may play a role in peripheral T cell reconstitution even at the age of 56 years (19, 20) . Recently, it was observed that excellent human thymopoiesis in FP THY grafts in fetal human liver/FP THY-grafted SCID mouse model occurred and these human T cells maturing in FP THY grafts were functional and tolerant to donor pig, as their respenses to allogeneic and xenogeneic but not to donor pig antigens in MLR assays (21). On the other hand, evidence suggests that human $CD4^+$ T cells may also play a dominant role in the rejection of porcine xenografts, and blocking the function of $CD4^+$ T cells might effectively prevent cellular rejection of porcine xenografts in humans. Therefore, it is possible that the rejection of porcine thymic grafts by the "late-stage" HIV-infected patients will be weak and easy to avoid without requiring strong immunosuppressive therapy. Thus, thymic grafting might be a useful adjunct to strategies to achieve immune restoration in HIV-infected patients, and xenogeneic thymus transplantation would be potentially useful if the xenogeneic donor is resistant to HIV infection.

MHC-restriction of T cells is generally believed to be determined by the MHC of the thymus (22). It would therefore be expected that mouse T cells maturing in FP THY grafts could not respond to peptide antigen KLH presented by host mouse MHC. However, other data support a role of intrathymic hemopoietic cells in imprinting T cells with MHC-restriction specificity. In addition, our studies (12, 13) in two different mouse lines of TCR-transgenic mice demonstrated that pig but not host mouse MHC mediates positive selection of TCR-transgenic mouse thymocytes with a known mouse MHC-restricting element in FP THY grafts, and mouse T cells with a transgenic TCR maturing in FP THY grafts respond to peptide antigen hen egg-white lysozyme (HEL) presented by host MHC, which will be discussed below. Thus, we speculate that the mouse T cell repertoire which is positively selected exclusively by porcine MHC elements in FP THY grafts has sufficient cross-reactivity with host mouse MHC/foreign peptide complexes to confer a high level of host mouse MHC-restricted immunocompetence. It has recently been reported that large numbers of polyclonal thymocytes can be positively selected by a single

MHC/peptide ligand, and that many of these maturing T cells react with the selecting MHC bound to other peptides, and also react frequently with allogeneic MHC molecules. Collectively, our studies and those involving allogeneic thymic grafting in humans suggest that the MHC restriction of T cells may be a relatively favored specificity but not an overly exclusive term.

In contrast to mouse $CD4^+$ T cells, efficient repopulation of mouse $CD8^+$ T cells in the periphery of FP THY/LIV-grafted, T and NK cell-depleted, ATX B10 or BALB/c nude mice has not been observed (4). However, mouse CD8 SP cells in FP THY grafts show a normal positive and negative selections, maturation pattern and cytolytic function (23), suggesting that mouse $CD8^+$ T cells can develop normally in FP THY grafts. We speculate that the poor repopulation of $CD8^+$ T cells in the periphery may be due to a failure to emigrate from the thymus to the periphery, and/or to low efficiency of maturation of this subset in FP THY grafts. Another possible explanation is the poor survival of mouse $CD8^+$ T cells maturing in FP THY grafts in the periphery. It has been reported that the peripheral $CD8^+$ T cell survival is dependent on the interaction of TCR and its selecting MHC class I. In our FP THY/LIV-grafted ATX B6 mouse model, mouse $CD8^+$ T cells might be positively selected by pig thymic epithelium in FP THY grafts, however, no pig class I^+ cells were detectable in the periphery by FCM. Thus, the peripheral survival efficiency of these $CD8^+$ T cells is somehow questionable.

Recipient mouse T cells maturing in porcine thymus are specifically tolerant to pig antigens

After we observed that efficient reconstitution of cellular immunity and specific unresponsiveness of mouse T cells maturing in FP THY grafts to pig antigens in MLRs, tolerance to donors of FP THY/LIV-grafted, T and NK cell-depleted ATX immunocompetent mice was further determined by grafting with donor MHC-matched pig skin after efficient peripheral repopulation of mouse $CD4^+$ T cells. MHC-matched pig skin was accepted by FP THY/LIV-grafted, T and NK cell-depleted ATX B10 mice for more than 100 days without any evidence of rejection (6). In contrast, allogeneic BALB/c skin was rejected in a similar time as that observed for euthymic control mice. T and NK cell-depleted ATX B10 mice grafted only with FP LIV (i.e. with no thymus graft) either rejected or accepted both pig and mouse skin (6). In addition, immunodeficient BALB/c nude mice were not able to reject both allogeneic third party mouse skin and xenogeneic pig skin. However, many of NP THY/SPL-grafted BALB/c nude mice rejected third party allogeneic mouse skin and the following third party xenogeneic pig skin, whereas they accepted the skin grafts from the donor of thymus long term (10). Similar results were also observed in NP THY/SPL-grafted, T and NK cell-depleted ATX B6 mice (9). These data demonstrate that specific xenogeneic skin graft tolerance is achieved by grafting donor thymic tissue in immuno-deficient BALB/c nude, or in T and NK cell-depleted, ATX immunocompetent B10 or B6 mice in which cellular immunity has been reconstituted by porcine thymic grafting.

Intrathymic graft clonal deletion appears to be one of the mechanisms by which mouse T cells maturing in FP THY grafts become tolerant to both host and donor antigens. These studies establish the principle that central tolerance by thymic clonal deletion can be achieved across discordant xenogeneic barriers (6). On the other hand, our studies also support the role of regulator cells in discordant xenogeneic tolerance in this model. Thus, both central thymic clonal deletion, which may play a major role here, and pheripheral regulating tolerance were involved in the discordant xenogeneic tolerance in this pig-to-mouse model.

Recipient mouse T cell repertoire shaping in discordant xenogeneic FP THY grafts: evidence that intrathymic clonal deletion is mediated by both pig and mouse MHC class II molecules and that positive selection is mediated solely by pig MHC in FP THY grafts

T cell precursors in thymi undergo growth, differentiation, education, and finally become mature T cells in an ordered sequence that can be monitored by assaying a panel of cell surface markers, such as CD4 and CD8. The repertoire of mature T cells appears to be influenced by both positive and negative selection events, in which TCR-peptide/MHC interaction plays a critical role.

Positive selection of mouse thymocytes in FP THY grafts

"AND" TCR transgenic mice (TgN(TcrAND)53Hed) have been produced by Kaye et al. using TCR genes (Vα11Vβ3) derived from a T-cell clone that recognizes pigeon cytochrome C peptide 88-104 presented by $I\text{-}A^b$ or $I\text{-}E^k$. Mouse $CD4^+$ T cells bearing the transgenic TCR are positively selected by mouse MHC class II, $I\text{-}A^b$ or $I\text{-}E^k$, are not positively selected in $I\text{-}A^kI\text{-}E^-$ mice, and are negatively selected by $H\text{-}2^s$ (24). In this FP THY-grafted ATX "AND" mouse model, we observed that FP THY grafting could reconstitute transgenic "AND" CD4 cells (13). Furthermore, similar levels of intrathymic and peripheral mouse $CD4^+CD8^-$ cells expressing the "AND" transgenic TCR were reconstituted in FP THY-grafted ATX "AND" mice that had a positive selecting or non-selecting host MHC background. To exclude the possibility that the selection of mouse $CD4^+$ T cells with a mouse MHC-restricted TCR selected by pig MHC in "AND" mice is a special case and to prove its general valid, another non-related TCR transgenic3A9 mice (TgN(Tcr3A9)# Mmd), in which $CD4^+$ T cells with a transgenic TCR (Vα3Vβ8.2) recognize hen egg-white lysozyme (HEL) peptide 46-61 presented by $I\text{-}A^k$, were employed. Similarly significant reconstitution of peripheral mouse $CD4^+CD8^-$ cells expressing the transgenic TCR and comparable levels of CD4 SP thymocytes with a transgenic TCR in FP THY grafts was observed in FP THY-grafted ATX "3A9" mice regardless the host had a positive selecting or class II-deficient MHC background (14), and these mouse $CD4^+$ T cells with a transgenic TCR developing in FP THY grafts in a host mouse MHC class II-deficient background (TgN (Tcr3A9)#Mmd × IIKO F3) showed excellent responses to

peptide antigen HEL present by mouse MHC class II *in vitro* (14). This result, together with our observation that efficient peripheral repopulation of polyclonal mouse $CD4^+$ T cells in FP THY-grafted ATX IIKO mice (12), collectively suggests that pig MHC but not host mouse MHC mediates positive selection of mouse thymocytes in FP THY grafts.

Negative selection of mouse thymocytes in FP THY grafts

Intrathymic tolerance can be induced by a variety of mechanisms, including clonal deletion, anergy, and suppression. To determine whether clonal deletion was involved in tolerance induction in FP THY grafts, we analyzed Vβ usage among intrathymic and peripheral CD4 SP T cells. $V\beta11^+$ T cells are deleted intrathymically when the mammary tumor virus (Mtv)-8 and Mtv-9-associated superantigens, Dvb11-1 and Dvb11-2, respectively, are presented by MHC class II I-E molecules (25). $V\beta5.1/5.2^+$ T cells recognize Mtv-6 and Mtv-9-derived superantigens, also in association with I-E molecules. Although they have Mtv-8 and Mtv-9 in their genome, IIKO mixed C57BL/6J and 129/Sv genetic background and $H\text{-}2^b$ (B10 or B6) wild-type mice do not delete $V\beta5^+$ or $V\beta11^+$ T cells because they do not express MHC class II I-E molecules, which are required for the presentation of superantigens present in the B6/B10 genome. However, a significant reduction of $V\beta5.1/5.2^+$ and $V\beta11^+$ CD4 SP cells was observed in FP THY grafts and peripheral lymphoid tissues of FP THY/LIV-grafted ATX B10/B6 or IIKO mice. In contrast, the levels of $V\beta6^+$, $V\beta7^+$ and $V\beta8.1/8.2^+$ CD4 SP cells in FP THY grafts in wild-type and IIKO mice were not significantly reduced compared to the normal host strain, suggesting that inefficient positive selection cannot explain the decrease in percentages of CD4 SP cells using Vβs that recognize host superantigens (12). This study demonstrates that pig MHC participates in negative selection of mouse thymocytes in FP THY grafts. Since pig is the only source of class II MHC antigens in MHC class II-deficient mice, this deletion must be explained by the presentation of mouse-derived superantigens on porcine MHC molecules in the grafts. However, much more complete deletion of $V\beta5.1/5.2^+$ and $V\beta11^+$ $CD4^+$ cells was observed in BALB/c nude mice grafted with FP THY/LIV than in ATX B10 mice simultaneously grafted with FP THY/LIV (6). This result suggests that mouse host MHC also participates in negative selection, since BALB/c but not B10 mice express the I-E class II MHC molecules needed to present the superantigens that delete Vβ5 and Vβ11. This conclusion is further supported by the observation of host mouse MHC class II^+ cells in FP THY grafts detected by immunohistochemical staining (6). In addition, in the FP THY-grafted ATX "AND" mouse model, we observed similar efficient clonal deletion of mouse thymocytes (CD4/8 DP or CD4 SP cells) with the transgenic TCR which crossreacts with $H\text{-}2^s$ and can be negatively selected by $H\text{-}2^s$, in FP THY grafts and in mouse thymus in "AND" mice with a $H\text{-}2^s$ MHC background (26). We conclude that both pig and mouse MHC participate in negative selection of the developing mouse T cell repertoire in FP THY grafts.

Based on the serial experimental results mentioned above, we conclude: donor pig MHC mediates positive selection of mouse thymocytes, while both pig and mouse MHC participate in negative selection of mouse thymocytes in FP THY grafts. However, whether MHC molecules expressed on pig thymic epithelium or pig bone marrow derived antigen-presenting cells or both participates in negative selection of mouse thymocytes in FP THY grafts is not addressed so far.

The role for suppressor cells in xenogeneic tolerance in the FP THY/LIV-grafted, ATX mouse model

Since the concept of suppressor T cells was first proposed over two decades ago, the phenomenon of T cell-mediated suppression is well established, especially in experimental models of transplantation tolerance and autoimmune disease. Either anergic, $CD45RB^{low}CD4^+$ or $CD25^+CD4^+$ T cells act as suppressor cells, and have been implicated in maintaining allogeneic and self-tolerance. However, the possible role of suppression in discordant xenogeneic tolerance is not clear. In our FP THY/LIV-grafted, T and NK cell-depleted ATX mouse model, ATX B10 mice grafted with FP LIV without a FP THY graft either rejected both pig and allogeneic mouse skin or accepted both grafts (due to their lack of $CD4^+$ T cell reconstitution), whereas ATX B10 mice grafted with FP THY/LIV specifically accepted pig skin and rejected allogeneic mouse skin grafts (6). These results suggest that tolerance induction includes an element of suppression, since residual host T cells in non-thymus grafted, T and NK cell-depleted ATX animals can apparently mediate pig skin graft rejection, but do not do so when large numbers of $CD4^+$ T cells recover from a grafted FP THY/LIV. We performed a preliminary study involving co-transfer of naïve BALB/c splenocytes with splenocytes from tolerant ATX BALB/c or BALB/c nude mice (grafted with FP THY/LIV) to syngeneic BALB/c nude mice as secondary recipients. The addition of tolerant spleen cells significantly delayed the ability of naïve T cells to reject pig skin grafts (Zhao and Sykes, unpublished data). In contrast, mouse skin allograft rejection was not delayed. These results suggest the existence of specific suppressor cells in FP THY/LIV-grafted ATX BALB/c mice. Furthermore, our recent studies in the adoptive transfer model suggest that mouse $CD4^+$ T cells maturing in FP THY grafts acts as a regulator cells in discordant xenogeneic tolerance in this FP THY/LIV-grafted mouse model. To further identify the phenotype of the regulatory $CD4^+$ T cells and the origin of these suppressor cells in this model is needed.

Conditioning regimen in ATX mice and the role of co-implantation of FP LIV or NP SPL for tolerance induction by grafting with porcine thymus

In the FP THY/LIV-grafted ATX B10 or B6 mouse model, ATX B10 or B6 recipient mice have been treated with four injections of a cocktail of mAbs (GK1.5, 2.43, 30-H12, and PK136) to deplete $CD4^+$, $CD8^+$ T cells, NK cells, and other $Thy1.2^+$ cell populations, respectively, before and after grafting of FP THY/LIV or NP THY/SPL (6). With this conditioning regimen, FP THY grafts survived, supported

efficient mouse thymopoiesis and peripheral mouse $CD4^+$ T cell repopulation, and induced specific pig skin graft tolerance in ATX immunocompetent mice. Our choice of this cocktail of mAbs in the pig to mouse model was originally based on the observation that each of these mAbs has been shown to be important in overcoming resistance to xenogeneic rat bone marrow engraftment in mice (27). However, the cell populations that resist different types of grafts from different donor species cannot be assumed to be identical, such as murine $CD8^+$ T cells are incapable of killing porcine targets, raising the question of whether or not depletion of these and other cell populations was necessary for the achievement of porcine thymic engraftment. Together with other efforts to simplify this protocol to make it more clinically practicable, we, therefore, performed a serial of experiments to address the following questions on the protocol to achieve skin graft tolerance in this porcine thymus-grafted ATX mouse model: 1) tolerance induction in euthymic immunocompetent mice; 2) the role of each of anti-CD4, anti-CD8, anti-THY1.2 and anti-NK mAbs currently used in the conditioning regimen; 3) the necessity of 3 Gy WBI of recipient mice; and 4) the importance of co-grafting of FP LIV or NP SPL with FP THY or NP THY, respectively, for tolerance induction in this model. The results were summarized briefly herein.

Our studies suggest that the essential requirement for mAb (GK1.5) to deplete host mouse $CD4^+$ cells to avoid the rejection of FP THY grafts in ATX immunocompetent B6 mice, and that host mouse non-$CD4^+$ cells, such as $CD8^+$, NK, natural killer T cells (NK/T), CD4 and CD8 double negative (DN) cells, and $TCR(\gamma/\delta)^+$ T cells, do not play a critical role in the acute, but not the chronic rejection of xenogeneic pig thymus grafts, at least when $CD4^+$ cells are not available. Because two of four FP THY grafts in $CD4^+$ cell-depleted, 3 Gy whole body-irradiated, ATX B6 mice, supporting efficient peripheral reconstitution of mouse $CD4^+$ T cells was eventually lost around 17 weeks post grafting, whereas four of four T and NK cell-depleted ATX B6 mice showed well-formed FP THY grafts, and three of three $CD8^+$ cell-depleted ATX B6 mice rejected FP THY grafts completely by 6 week post grafting (4 weeks post last mAb injection) (7). Accordingly, pig skin graft tolerance was not achieved in $CD4^+$ cell-depleted, 3 Gy whole body irradiated, FP THY/LIV-grafted ATX B6 mice, indicating the role of mouse non-$CD4^+$ cells in the rejection of xenogeneic pig skin grafts. However, excellent FP THY graft survival and donor MHC-matched pig skin graft tolerance in $CD4^+$ and $CD8^+$ cell-depleted (anti-CD4 and anti-CD8 mAbs only, no anti-Thy1.2 and anti-NK1.1 mAbs), 3Gy whole body-irradiated, FP THY/LIV-grafted ATX B6 mice was observed (28), further suggesting that mouse $CD8^+$ cells, with the help of $CD4^+$ cells, play some role in the rejection of discordant xenogeneic grafts, maybe especially in the chronic rejection phrase.

In addition, our current experiment indicated that 3 Gy WBI on recipient mice is not essential for pig skin graft tolerance induction in this model, as donor's mother skin grafts were accepted long term by FP THY/LIV-grafted, ATX B6 recipient mice which were treated with four injections of a cocktail of mAbs (GK1.5, 2.43, 30-H12, and PK136) alone without 3Gy WBI (28). Recently, pig skin graft tolerance was achieved by grafting FP THY in ATX B6 recipient mice which were conditioned with anti-CD4 and anti-CD8 mAbs alone, omitting the injection of anti-Thy1.2 and anti-NK1.1 mAbs, and 3Gy WBI simultaneously (9).

Both thymic epithelium and bone marrow derived APCs, such as dendritic cells in thymi mediate central clonal deletion or anergic tolerance, although bone marrow derived cells may be somewhat efficient for negative selection. In our pig to mouse model, the initial purposes for the co-implantation of FP LIV and administration of 10^8 FLC suspensions with FP THY grafts or the additional supply of NP SPL with NP THY are to sustain pig thymopoiesis and to improve the efficiency of tolerance induction, as the fetal pig liver is the major source of hematopoietic stem cells during the second trimester of fetal life, and hepatopoiesis shifts to the bone marrow and/or spleen at and beyond birth. However, our recent study showed similar efficiency of skin graft tolerance in T and NK cell-depleted ATX B6 mice either by grafting FP THY alone or by implanting FP THY/LIV/FLC (28), whereas lower efficiency of skin graft tolerance in T and NK cell-depleted ATX B6 mice induced by NP THY alone compared with its induction by NP THY/SPL (9). These results suggest that pig bone marrow derived cells may be involved in the thymic tolerance induction and that fetal pig thymus may have higher numbers of hematopoietic stem cells than that of neonatal pig thymus. Another possibility is that the tolerance inducing efficiency of FP THY, which may be mediated by pig thymic epithelium, is stronger than that of NP THY itself, due to its greater potential growth than that of NP THY.

Conclusions

Porcine thymus tissue grafted into T and NK cell-depleted ATX immunocompetent mice supports efficient mouse thymopoiesis and peripheral reconstitution of functional mouse cellular immunity, and induces specific pig skin graft tolerance across a highly separated species combination. Efficient peripheral reconstitution of mouse $CD8^+$ T cells was not observed in FP THY/LIV-grafted, T and NK cell-depleted ATX mice, whereas normal maturation pattern of mouse CD8 SP cells in FP THY grafts occured. The efficiency of FP THY supporting the peripheral mouse $CD4^+$ T cell repopulation and inducing tolerance is more efficient than that of NP THY. Our recent studies demonstrated that both central thymic clonal deletion and regulator cells were involved in the achievement of discordant xenogeneic tolerant statutes in this porcine thymus-grafted T and NK cell-depleted, ATX immunocompetent mouse model. Donor pig MHC solely mediates positive selection of mouse thymocytes, whereas both pig and mouse MHCs participate in the negative selection in FP THY grafts. Host mouse $CD4^+$ cells play a critical role for the rejection of FP THY grafts and the pre-depletion of mouse $CD4^+$ cells is essential for the FP THY graft survival and its subsequent supporting mouse thymopoiesis, but not sufficient for the skin graft tolerance induction.

It is clear that this study has the potential clinical applicability as a new approach to discordant xenogeneic tolerance induction and to the treatment of AIDS and other

immunodeficiency diseases associated with thymic dysfunction. To further understand the mechanisms for the reconstituted immune functions and tolerance induction in this model will increase our knowledge on the field of xenotransplantation immunology, especially on the T cell repertoire shaping in a discordant xenogeneic thymic environment. The related studies in a large animal model are essential before its clinical application.

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