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Role of bone marrow maturity, insulin-like growth factor 1 receptor and forkhead box protein N1 in thymic involution and rejuvenation

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Abstract

Thymic involution is associated with age-related changes of the immune system. Utilizing our innovative technique of transplantation of a thymus as isolated vascularized graft in MHC-inbred miniature swine, we have previously demonstrated that aged thymi are rejuvenated after transplantation into juvenile swine. Here we have studied the role of insulin-like growth factor (IGF) and forkhead-box protein-N1 (FOXN1) as well as bone marrow (BM) in thymic rejuvenation and involution. We examined thymic rejuvenation and involution by means of histology and flow cytometry. Thymic function was assessed by the ability to induce tolerance of

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The authors declare no conflicts of interest.

Animals were cared for according to the guidelines of the Massachusetts General Hospital (MGH) Institutional Animal Care and Use Committee.

Authors contributions:

Masayuki Tasaki:	Primarily performed research in Groups 1, 2 and 3, analyzed the data and wrote the manuscript
Vincenzo Villani:	Primarily performed research in Group 4, analyzed the data and wrote the manuscript
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Taylor Cormack and Shannon Moran:	Assisted with the research (<i>in vivo</i> and <i>in vitro</i> studies)
David H. Sachs:	Participated in data analysis and writing of the manuscript
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allogeneic kidneys. Aged thymi were rejuvenated in a juvenile environment, and successfully induced organ tolerance, while juvenile thymi in aged recipients involuted and had a limited ability to induce tolerance. However, juvenile BM inhibited the involution process of juvenile thymi in aged recipients. An elevated expression of both FOXP1 and IGF1 receptors (IGF-1R) was observed in juvenile thymi and rejuvenated thymi. Juvenile BM plays a role in promoting the local thymic milieu as indicated by its ability to inhibit thymic involution in aged animals. The expression of FOXP1 and IGF-1R was noted to increase under conditions that stimulated rejuvenation, suggesting that these factors are involved in thymic recovery.

INTRODUCTION

The role of thymus in transplantation (Tx) tolerance has been studied extensively in this laboratory utilizing major histocompatibility complex (MHC)-defined inbred miniature swine (1–5). We have reported that the presence of a juvenile host thymus is an essential requirement for the induction of tolerance of class I-mismatched renal allografts (1). We have also demonstrated that thymic structure and function decline with age, resulting in an inability to induce tolerance (2).

To further study the mechanisms of thymus-dependent tolerance and thymic aging, we have developed a procedure for the transplantation of thymi as vascularized thymic lobes (VTL) in Massachusetts General Hospital (MGH) miniature swine (6). Unlike thymic tissue, which requires re-vascularization in recipients following transplantation, the VTL technique enables transfer of donor thymi into recipients without ischemic damage. In addition, we have utilized MHC-defined lines of miniature swine as a unique large animal model with breeding characteristics that make possible studies of transplantation across reproducible genetic combinations (7, 8). Use of these animals has allowed us to transfer MHC-matched thymi and/or bone marrow (BM) to “age mismatched” recipients in order to study the processes of thymic rejuvenation and involution as well as to study the role of BM maturity in tolerance induction (9).

We have previously reported that aged, involuted VTL grafts underwent rejuvenation, both histologically and functionally, after transplantation into MHC-matched juvenile recipients, indicating that the host environment (i.e. factors extrinsic to the thymus) was responsible for this phenomenon (10). In this study, we first confirmed our previous findings and then extended the studies to (i) determine whether juvenile thymi involuted when they were transferred to aged hosts, (ii) determine whether involuted thymi lost their ability to induce transplant tolerance, and (iii) examine identifiable factors involved in thymic rejuvenation/involution.

Materials and Methods

Animals and Experimental Groups

10 recipients and 15 donors (10 for thymic grafts, three for kidney grafts and two for bone marrow grafts) of MHC-defined inbred MGH-miniature swine were used. The immunogenetic characteristics of this herd and of the intra-MHC recombinant haplotypes

have been described previously (7, 8). Juvenile swine (2–3 months of age) and aged swine (of more than 18 months of age) were used. Recipients for thymic Tx were divided into four groups (Fig 1 and Table 1): juvenile recipients and aged thymic donors (Group 1), aged recipients and juvenile thymic donors (Group 2), aged recipients and aged thymic donors (Group 3), and aged recipients of juvenile BM and thymus (Group 4). Vascularized thymic Tx were performed in either MHC-matched or class I MHC-mismatched combinations in Groups 1–3. In Group 4, the donors and recipients were MHC-matched. Animals were cared for according to the guidelines of the MGH Institutional Animal Care and Use Committee.

Surgical Procedures

a) Complete thymectomy in recipients—We have previously demonstrated that the presence of the host thymus interferes with the acceptance of an MHC-mismatched donor thymus (9). Therefore, except for the MHC-matched animals in group 1, all the recipients underwent thymectomy three weeks prior to thymus Tx as previously described (9,10).

b) Vascularized thymic lobe (VTL) transplantation—The procedure of VTL Tx has been previously reported (6). Briefly, cervical thymic lobes were carefully harvested from the donor, perfused with cold Euro-Collins solution, and transplanted into the retroperitoneum of recipients, anastomosing the thymic vessels to the abdominal aorta and the inferior vena cava. This technique led to reperfusion of the VTL grafts with blood immediately upon unclamping. Recipients were treated with either 12 days (in MHC-matched pairs) or 28 days (in class I-mismatched pairs) of FK506. Blood levels of FK506 were adjusted to achieve a trough level between 30 and 40 ng/ml in the first 12 days, and tapered to 20ng/ml thereafter in animals that were treated with 28 days of FK 506.

c) Thymus biopsy—VTL biopsies were carried out on the day of transplantation, 2 months, 4 months and 6 months after VTL transplantation. Approximately $1 \times 0.5 \times 0.5$ cm of thymic tissue was biopsied for histology (formalin-fixed and frozen samples) and flow cytometric analysis (6). The sections of naïve thymus used for morphometric analysis were taken from the recipients who did not undergo host thymectomy. Tissue samples were fixed in 1% formaldehyde solution (Denison Pharmaceuticals, Pawtucket, RI), embedded in paraffin, and subsequently sectioned. Tissues were stained using hematoxylin/eosin (H&E).

e) Bone marrow (BM) transplantation—BM Tx in miniature swine has been described elsewhere (11–13). Briefly, the donor animal was anesthetized and exsanguinated. The BM was surgically harvested from the humeri, tibiae and vertebral bodies. BM fragments were then finely minced and suspended in processing medium consisting of $1 \times$ Dulbecco's Modified Eagle's Medium (GIBCO BRL, Gaithersburg, MD), supplemented with 5% fetal bovine serum, 1 mM glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, and 50 µg/mL gentamicin. The cells suspension was subsequently filtered twice and the cell pellet was resuspended in Roswell Park Memorial Institute (RPMI) medium and infused intravenously to the recipient animal, using a filtered blood transfusion set ($3 - 4.3 \times 10^8$ cells/kg).

f) Kidney transplantation and catheter placement—The kidney Tx procedure has been reported previously (1, 9). Both native kidneys were removed on the day of kidney Tx.

Two semi-permanent indwelling Hickman Silastic central venous catheters were placed in the external jugular veins to facilitate drug administration and frequent blood draws (1, 9). Renal function was evaluated with daily measurements of serum creatinine. Kidney biopsy specimens were examined and graded by a senior pathologist (14).

Evaluation of thymic rejuvenation/involution

a) Monoclonal antibodies (mAbs) used for phenotypic characterization of cell populations in thymic tissue and peripheral blood lymphocytes—Thymocyte development was assessed by immunohistochemistry and Fluorescence-activated cell sorter (FACS) analysis using the murine anti-swine mAbs 898H2-6-15 (mouse IgG2a, anti-swine CD3), 74-12-4 (IgG2b, anti-swine CD4), 76-2-11 (IgG2a, anti-swine CD8), 76-7-4 (IgG2a, anti-swine CD1), 231.3B2 (IgG1, anti-swine CD25), 1038H-10-9 (anti-porcine allelic antigen (PAA)), 2.12.3a (IgM, class Id), 16.7.E4.2 (IgM, class Ic) (15).

b) Preparation of thymocytes and peripheral blood lymphocytes (PBLs)—Biopsied tissue from thymic grafts (100–200 mg) was finely minced in Hank's balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA) buffer; the cell suspension was then filtered through a 200 μ m nylon mesh. For separation of peripheral blood lymphocytes (PBLs), freshly heparinized whole blood was diluted 1:2 with HBSS and the mononuclear cells were obtained by gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, Natick, MA) (6).

c) Flow cytometry—FACS analysis of PBLs and thymocytes as well as anti-donor antibody development was performed using a Becton-Dickinson FACScaliber (San Jose, CA) with CellQuest FACStation software (Becton Dickinson, San Jose, CA) as previously reported (6). Phenotypic analysis of cells was performed by three-color staining using fluorochrome-conjugated murine anti-swine mAbs, which were the same as those used for immunohistochemistry (see below). Development of elicited anti-donor antibodies was assessed by using PBMCs from SLA-matched donors incubated with decomplexed recipient sera with anti-pig IgG or IgM conjugated to FITC.

d) Morphometric analysis—Morphometric studies were performed on thymic biopsy samples using the NIH Image software (Scion Image, Frederick, MD). Biopsy samples with at least 15 lobules were stained with H&E and the areas of cortex and medulla were measured to obtain a ratio of the cortical area to the medullary area (C/M ratio) in the cross-section of the thymus. As we reported previously (10), the C/M ratio is an indicator of the degree of rejuvenation of the thymus.

e) Immunohistochemistry—Formalin-fixed paraffin embedded samples and frozen samples were used for immunohistochemistry. Anti-Forkhead box protein N1 (FOXN1) antibody (Santa Cruz: sc-23566), anti-insulin growth factor 1 receptor (IGF-1R) antibody (Abcam: ab90657), anti-pan cytokeratin (Dako), and anti-cytokeratin 14 (CK14) antibody (Santa Cruz: sc-53253) were used for immunohistochemistry, which was performed as previously described (14). The number of FOXN1 positive cells in the thymus or thymic

grafts was determined by counting the number of positive cells in at least five randomly chosen high power fields (x400).

f) Enzyme-linked immunosorbent assay (ELISA)—Serum Insulin like Growth Factor 1 (IGF-1) level was investigated with the Porcine IGF-1 ELISA kit (Hözel Diagnostika, Germany: E07I0009) following the manufacturer's instructions.

In Vitro Cellular Assays

a) Cell-mediated lymphocytotoxicity (CML) assays—CML assays were set up before class I-mismatched VTL Tx and kidney Tx, and following transplants. The methodology has been previously described (1).

b) CD25-depleted CML assays—To test whether anti-donor cytotoxic T lymphocyte activity was restored by removal of CD25-positive cells from responders, CD25-depleted CMLs were set up with PBLs from a long-term acceptor. CD25-positive cells were depleted by magnetic cell separation (MACS; Miltenyi Biotec, Auburn, CA). CD25-negative cells were used as responders and cultured with stimulators in the same manner as in a primary CML. Completion of CD25 depletion was confirmed by FACS analysis (10).

Statistical Analyses

The statistical significance of differences in data was evaluated using the nonparametric Mann–Whitney test using the SPSS software (IBM Corporation, Armonk, NY). $P < 0.05$ was considered to indicate statistically significant difference.

Results

The recipient/donor combinations utilized and a brief summary of results are presented in Table 1.

Additional studies on the rejuvenation of aged thymic grafts after transplantation into juvenile recipients across a class I mismatched barrier (Group 1)

We have previously demonstrated that aged thymi rejuvenated after they were transplanted as VTL into juvenile thymecomized hosts across a class I-mismatched barrier (10). This result was confirmed in swine #20847, which was thymectomized three weeks prior to receiving a class I-mismatched VTL with 28 days of FK506. Following VTL transplantation, the C/M ratio (Fig 2a), CD4/CD8 double positive (DP) cells and CD1 single positive (SP) cells of the thymic graft increased while the ratio of CD3 SP cells decreased (Fig 2b), indicative of thymic rejuvenation. CD4/CD8 DP and CD1 SP cells of the thymic graft started decreasing 4 months after VTL transplantation due to the growing of the recipients (Fig 2b).

The immunological status of #20847 was examined by CML response. Standard CML assays showed donor-specific unresponsiveness by two months after VTL Tx (Fig 2c). We further assessed T regulatory cells in this animal; anti-donor specific unresponsiveness was

not restored by depletion of CD25-positive cells (Fig 2d, red dashed line), suggesting that a regulatory mechanism is not responsible for the induction of tolerance in this model.

Immunohistologic examination indicated that donor cells with dendritic-like morphology (Fig 2e, yellow), as well as donor stromal cells, persisted (Fig 2e, green) in the rejuvenated thymic graft. In addition, recipient-type cells with dendritic morphology had migrated into this area (Fig 2e, red).

Thymectomy was not required for acceptance of aged VTL in juvenile recipients across MHC-matched barriers (Group 1)

We previously demonstrated that host thymectomy was required for acceptance of VTL grafts across MHC mismatched barriers (9). In this study, we examined if host thymectomy was required for acceptance of aged VTL grafts in juvenile hosts across an MHC-matched, but minor-mismatched, barrier. Two juvenile recipients, #20786 and #20888, received VTL grafts from MHC-matched, minor antigens-mismatched aged donors, with 12 days of FK506. Both thymic grafts rejuvenated (Fig 3a and b), in a manner similar to that observed in class I mismatched VTLs in euthymic animals (Group 1).

Rejuvenated host-type thymic grafts facilitated the induction of tolerance of class I-mismatched kidneys in thymectomized recipients (Group 1)

We have previously demonstrated that rejuvenated allogeneic thymi have the ability of inducing tolerance of kidneys across allogeneic barriers (9). In this study, we tested whether a rejuvenated MHC-matched thymic graft possesses the ability to enable the induction of tolerance as does a naïve thymus. We have previously reported that juvenile miniature swine uniformly accept class-I mismatched kidneys with a 12 day-course of high dose CyA, while thymectomized juvenile animals do not (1). In order to assess the function of rejuvenated thymic grafts, two juvenile thymectomized animals (#21191 and #21106) received SLA-matched rejuvenated aged VTL grafts from Group 1 animals (#20786 and #20888). These animals (#21191 and #21106) also received class I-mismatched kidneys on the same day and were treated with 12 days of CyA.

The class I-mismatched kidneys were accepted without any rise in serum creatinine after cessation of CyA (Fig 3c) and these recipients become specifically unresponsive to donor within two months following the Tx (Fig 3d). Kidney biopsies showed only minimal cell infiltrate without endothelialitis (Fig 3e and f).

Faster involution of juvenile thymi in aged recipients (Group 2)

We have previously demonstrated that juvenile VTLs induce tolerance in thymectomized juvenile hosts across fully allogeneic barriers (9). In addition, our current data described above (9) indicated that rejuvenated thymic grafts restored the capacity to induce tolerance in thymectomized juvenile hosts across a two-haplotype, class I-mismatched barrier. In order to test whether (i) juvenile thymi involute at a faster rate in aged hosts, and (ii) juvenile thymi in aged hosts can induce tolerance, three aged recipients received juvenile VTLs with 28 days of FK506 across a two-haplotype, class I-mismatched barrier. A naïve juvenile pig

was treated with FK506 for 28 days as a control for the effects of FK506 on thymus involution.

Representative data are shown in Fig 4. Histologically, the C/M ratios of the thymi decreased at rate that was markedly faster (Fig 4a and c) compared to the control (Fig 4e). C/M ratios decreased from 8.0, 4.5, and 6.9 on day 0 to 0.2 (day 114), 0.7 (day 118) and 0.8 (day 124) in animals #19167, #19978 and #20607, respectively. In contrast, in the control animal which received the same 28-day course of FK506 as the recipients in Group 2, the C/M ratio at day 0 was 7.5 and was 5.8 on day 170, which is comparable to the C/M data obtained in 53 naïve animals of different ages reported in a previous study (10) (Fig 4e).

FACS phenotypic analysis was consistent with the histologic findings described above. Juvenile thymic grafts in aged animals showed a decrease in both CD4/CD8 DP and CD1 SP cells, and an increase in CD3 SP cells (Fig 4b and d). In contrast, although a transient decrease in CD4/CD8 DP cells was seen at 2 months in the control likely due to the FK506, the levels of CD1 and CD3 SP cells remained stable throughout the experimental period and the CD4/CD8 DP cells recovered pre-treatment levels at four months (Fig 4f). These results indicate that juvenile thymi undergo accelerated involution in aged hosts.

Involuting thymic grafts are less able to induce tolerance

The immune status of the Group 2 animals was evaluated by *in vitro* assays (Fig 5a–c). All of the recipients showed donor-specific unresponsiveness in CML two months after VTL Tx (#19167, #19978 and #20607), however, two of these animals later recovered their anti-donor responses (Fig 5a: #19978 and #20607). None of the animals developed anti-donor IgG antibody following VTL transplantation (Fig 5b). The anti-donor response of #19167 was not restored by CD25 depletion of the responder cells in a CML assay (Fig 5c), while #19978 had anti-donor response after CD25 depletion (Fig 5d). If juvenile thymic graft were accepted like #19167, regulatory mechanisms were less involved in the maintenance of tolerance similar to Group 1. Histological examination of #19167 thymic biopsy on day 170 showed that morphologically donor-type DC-like cells (yellow cells indicated with arrows) remained in the thymic graft (Fig 5e).

To assess *in vivo* the function of the involuting thymi, three animals in Group 2 received kidney grafts MHC-matched to the VTL donor without any immunosuppression, more than 4 months after VTL Tx. Swine #19167, the animal which maintained *in vitro* unresponsiveness, accepted the donor MHC-matched kidney without a rejection crisis (Fig 6a red line and Fig 6b left panel). In contrast, the other two animals (#19978, #20607) rejected kidney grafts acutely on days 53 and 15, respectively (Fig 6a, black and blue lines, and Fig 6b right panel). CML assays showed that #19167 maintained donor-specific unresponsiveness (Fig 6c, left), while the other animals had increased anti-donor responses at the time of rejection (Fig 6c, right). These results indicate that juvenile thymi are able to induce tolerance in the peritransplant period, however this tolerant state fades away rapidly with the quick involution of the thymus.

Involuted thymi from aged donors into aged recipients (Group 3)

In Group 3 (#20248 and #20438), aged VTLs were transplanted into aged recipients as a control. One animal received an MHC-matched VTL and the other received a class I-mismatched VTL, both were treated with 28 days of FK506.

The MHC-matched VTL of #20438 maintained its involuted profile (10) (Fig 7a). However, the class I-mismatched VTL of #20248 was found to be fibrotic on day 58 (Fig 7b) and the animal showed anti-donor responsiveness in vitro (Fig 7c). The C/M ratio could not be measured in this sample due to the destruction of the thymic structure. These findings suggest that involuted thymi in aged hosts are not undergoing rejuvenation and were therefore unable to induce tolerance.

Role of BM in thymic involution (Group 4)

The results above demonstrate that juvenile thymi involute within two months when they are transplanted into aged hosts. In order to determine whether BM has a role in this process, two aged animals were lethally irradiated and reconstituted with BM from juvenile animals. To minimize the potential for graft versus host disease (GvHD) effects, we used MHC-matched donor recipient pairs and the VTLs were transplanted after confirmation of donor-type hematopoietic cell reconstitution (3–5 weeks after MHC-matched BM Tx) (Fig. 8A).

Both aged recipients (#19639 and #21179) of juvenile BM and juvenile thymi showed a reduced rate of involution in the transplanted thymi (Fig 8B–a and b). The C/M ratios were 6.9 and 7.4 at day 0 in #19639 and #21179, respectively; at later times these values were 4.1 (POD 120) and 4.2 (POD 92). The reduction of the C/M ratios with time was more gradual when compared to the animals in Group 2 (Fig 4). Phenotypic analysis of the thymi showed that the levels of CD4/CD8 DP as well as CD1 and CD3 single positive thymocytes remained relatively stable for 4 months post-transplant (Fig 8B–c). In contrast, in Group 2 animals (aged recipients of juvenile thymi, without BM treatment), the CD4/CD8 DP and CD1 SP thymocytes decreased while the CD3 SP increased, indicating thymic involution. This observation suggested that the addition of juvenile BM in the Group 4 animals had slowed the onset of thymic involution.

The role of intra-thymic and systemic factors in thymic involution/rejuvenation

In order to better understand intra-thymic events in this model, we evaluated the expression of FOXP1 and IGF-1, two molecules that have been reported to be involved in age-associated thymic involution in rodent models (16, 17).

(1) FOXP1 expression in the thymus correlated with thymic involution/rejuvenation—We confirmed that FOXP1 is expressed in the juvenile naïve thymus of MGH miniature swine (Fig 9A–a) and, as it would be expected, that the level of expression was markedly decreased in aged naïve thymi (Fig 9A–b). Expression of FOXP1 increased in the rejuvenated aged VTLs in Group 1 (Fig 9A–c and d). In contrast, FOXP1 positive cells markedly decreased in the juvenile VTLs in Group 2 (Fig 9A–e and f) that were undergoing involution. The VTL grafts from Group 4 maintained thymic structure and had a density of FOXP1-positive cells (Fig 9A–g and h) comparable to that observed in naïve juvenile thymi.

The average of FOXP1 positive cells in rejuvenated VTLs in Group 1 was similar to that of juvenile naïve thymi; in contrast, the number of FOXP1 positive cells was significantly higher than that of aged naïve thymi and involuted VTLs in Group 2 (Fig 9B). The VTL grafts from Group 4 demonstrated a similar level of FOXP1 positive cells to that of juvenile naïve thymi and rejuvenated VTLs in Group 1 (Fig 9B).

(2) Serum levels of IGF-1 and thymic IGF-1 receptor expression suggested that thymic IGF-1 receptor positive cells in BM may play an important role in the prevention of thymic involution—Serum IGF-1 levels were measured in naïve pigs of varying ages (n=60). Serum IGF-1 levels were higher in juvenile animals and decreased with age (Fig 10a). The average serum IGF-1 level in juvenile pigs (1–3 month old) was significantly higher than that of aged pigs (20–26 months old): 45.1 ng/dL vs. 3.6 ng/dL, respectively (p<0.05).

Notably, IGF-1 receptor (IGF-1R) was highly expressed in juvenile naïve thymus compared to aged naïve thymus (Fig 10b and c). IGF-1Rs were mainly expressed in the medulla. Some IGF-1R positive cells also expressed cytokeratin 14 (CK14), suggesting that these were thymic epithelial cells. IGF-1R expression increased in the Group 1 thymic grafts after transplantation (Fig 10d) and decreased in the thymic grafts undergoing involution after Tx into aged pigs (Group 2, Fig 10e). Notably, IGF-1R expression was maintained in the juvenile thymic grafts that were transplanted into aged hosts reconstituted with juvenile BM cells (Fig 10f).

Discussion

In this study, we have demonstrated that (i) rejuvenation of thymus leads to a restored of ability to induce tolerance; (ii) involution of juvenile thymi occurs at a faster rate in aged animals than in juvenile animals and that these grafts lose the ability to induce tolerance; (iii) juvenile bone marrow in an aged recipient is sufficient to prolong maintenance of the juvenile structure of a transplanted juvenile graft; and (iv) thymic expression of both FOXP1 and IGF-1R correlate with in the process of rejuvenation/involution.

These studies confirm our previous findings demonstrating rejuvenation of aged thymi when transplanted into juvenile hosts (10). Here, we have demonstrated that if rejuvenation of the thymus is achieved, the rejuvenated organ can be transplanted simultaneously with an allogeneic kidney graft and facilitate tolerance induction (Group 1). These observations could have clinical relevance as an advanced technology for the induction of tolerance. Thus, for example, if one were able to isolate the factors necessary to rejuvenate the thymus, then *ex vivo* culture and bioengineering technology might be used to transplant a tolerance-facilitating thymus with juvenile characteristics, along with the allogeneic kidney.

Our results with thymus transplantation as well as the rejuvenating effect of luteinizing hormone–releasing hormone (LH-RH) agonist administration (18) indicate that extrinsic factors in the host environment, rather than intrinsic ones in the local thymic milieu, play an essential role in thymic rejuvenation. In this study, we further addressed mechanisms of thymic involution, focusing in particular on the role of bone marrow age. Although juvenile

VTLs rapidly involuted in aged animals (Group 2), the addition of juvenile BM to this protocol resulted in prolongation of the maintenance of juvenile thymic structures (Group 4). These data suggest that the juvenile BM either provides extrinsic factors that inhibit thymus involution or stimulates production of factors that promote the preservation of the juvenile thymic architecture.

Several possible mechanisms for age-associated thymic involution have been suggested. First, IGF-1 has been implicated as a positive thymic regulator based on the early observation that age-related decline in thymic function parallels a decline in IGF-1 plasma concentration (16). Consistent with this observation are the fact that administration of exogenous IGF-1 resulted in increased thymic mass and restored thymic function in mice (19) and the fact that IGF-1-Rs are expressed on thymic epithelial cells (TECs) (20, 21). Conversely, recent studies have demonstrated that FOXN1 is involved in age-associated thymic involution (17). FOXN1 is expressed on TECs, which form the thymic stroma (22). Functional maturation of TECs requires expression of FOXN1, which may also play a role in the cross-talk between TECs and the developing thymocytes (22). In addition, it has been shown that FOXN1 is expressed on BM cells (23) as well as BM-derived TECs (24). The expression of FOXN1 in BM cells does not change with age, however, it does play an important role in the maintenance of T-cell lineage through the development of T cell progenitor cells (23).

T cell commitment, differentiation and repertoire selection are mediated by the epithelial component of the thymic stroma and TECs are important to self tolerance (25). In the present study, a well-developed TECs network was present in the rejuvenated thymic grafts in Group1 similarly to what observed in naïve young thymi, as shown by immunohistochemistry (Supplemental information). In contrast, the TEC network was involuted in involuted thymic grafts in Group 2 similarly to what observed in naïve aged thymi (Supplemental information). Deletion of FOXN1 in the postnatal thymus has been shown to cause thymic atrophy and severe deterioration of the TEC network (26). These data suggest that restored TEC network accompanied by high expression of FOXN1 in the rejuvenated thymic grafts in Group 1 could have led to tolerance induction in our model. In contrast, the loss of TECs network and low FOXN1 expression in involuted thymic grafts in Group 2 probably resulted in a failure to induce tolerance.

The present study showed that both plasma levels of IGF-1 and the number of cells expressing IGF-1R and FOXN1 in the thymus correlated with age-associated thymic involution. The number of cells expressing IGF-1R and FOXN1 observed in naïve aged thymi were significantly lower, compared to juvenile animals. Notably, the relative number of cells expressing both IGF-1R and FOXN1 in rejuvenated VTLs was similar to that seen in naïve juvenile thymi. These results suggest that the IGF-1R and/or FOXN1-positive cells were derived from the juvenile BM or that cells from juvenile BM stimulated thymic cells to upregulate expression of IGF-1R as well as FOXN1.

In conclusion, thymic rejuvenation and involution were mainly dependent on extrinsic factors such as BM age, upregulation of IGF-1R, as well as FOXN1 expression in thymi.

These factors may ultimately contribute to the preservation of donor TECs which play a major role in achieving thymic dependent transplantation tolerance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BM	Bone marrow
CyA	Cyclosporine A
CML	Cell-Mediated Lymphocytotoxicity
MHC	Major histocompatibility complex
MGH	Massachusetts General Hospital
POD	Post-operative day
Tx	Transplantation
VTL	vascularized thymic lobe

References

1. Yamada K, Gianello PR, Ierino FL, Lorf T, Shimizu A, Meehan S, Colvin RB, Sachs DH. Role of the thymus in transplantation tolerance in miniature swine. I. Requirement of the thymus for rapid and stable induction of tolerance to class I-mismatched renal allografts. *J Exp Med*. 1997; 186:497–506. [PubMed: 9254648]
2. Yamada K, Ierino FL, Gianello PR, Fishbein J, Lorf T, Shimizu A, Colvin RB, Sachs DH. Role of the thymus in transplantation tolerance miniature swine. II. Effect of steroids and age on the induction of tolerance to class I mismatched renal allografts. *Transplantation*. 1999; 67:458–467. [PubMed: 10030295]
3. Yamada K, Ierino FL, Gianello PR, Shimizu A, Colvin RB, Sachs DH. Role of the thymus in transplantation tolerance in miniature swine. III. Surgical manipulation of the thymus interferes with stable induction of tolerance to class I-mismatched renal allografts. *Transplantation*. 1999; 67:1112–1119. [PubMed: 10232560]
4. Vagefi PA, Ierino FL, Gianello PR, Shimizu A, Kamano C, Sachs DH, Yamada K. Role of the thymus in transplantation tolerance in miniature Swine: IV. The thymus is required during the induction phase, but not the maintenance phase, of renal allograft tolerance. *Transplantation*. 2004; 77:979–985. [PubMed: 15087757]
5. Yamada K, Choo JK, Allen JS, Erhorn AE, Menard MT, Mawulawde K, Slisz JK, Aretz T, Shimizu A, Sachs DH, Madsen JC. The effect of thymectomy on tolerance induction and cardiac allograft vasculopathy in a miniature swine heart/kidney transplantation model. *Transplantation*. 1999; 68:485–491. [PubMed: 10480404]

6. LaMattina JC, Kumagai N, Barth RN, Yamamoto S, Kitamura H, Moran SG, Mezrich JD, Sachs DH, Yamada K. Vascularized thymic lobe transplantation in miniature swine: I. Vascularized thymic lobe allografts support thymopoiesis. *Transplantation*. 2002; 73:826–831. [PubMed: 11907438]
7. Sachs DH. MHC homozygous miniature swine. In: Swindle, MM, Moody, DC., Phillips, LD., editors. *Swine as models in biomedical research*. Ames, IA: Iowa State University Press; 1992. p. 3-15.
8. Sachs DH, Leight G, Cone J, Schwarz S, Stuart L, Rosenberg S. Transplantation in miniature swine: I. Fixation of the major histocompatibility complex. *Transplantation*. 1976; 22:559–567. [PubMed: 137560]
9. Kamano C, Vagefi PA, Kumagai N, Yamamoto S, Barth RN, LaMattina JC, Moran SG, Sachs DH, Yamada K. Vascularized thymic lobe transplantation in miniature swine: thymopoiesis and tolerance induction across fully MHC-mismatched barriers. *Proc Natl Acad Sci U S A*. 2004; 101:3827–3832. [PubMed: 15007168]
10. Nobori S, Samelson-Jones E, Shimizu A, Hisashi Y, Yamamoto S, Kamano C, Teranishi K, Vagefi PA, Nuhn M, Okumi M, Wong B, Houser S, Sachs DH, Yamada K. Long-term acceptance of fully allogeneic cardiac grafts by cotransplantation of vascularized thymus in miniature swine. *Transplantation*. 2006; 81:26–35. [PubMed: 16421473]
11. Pennington LR, Sakamoto K, Popitz-Bergez FA, Pescovitz MD, McDonough MA, MacVittie TJ, Gress RE, Sachs DH. Bone marrow transplantation in miniature swine. I. Development of the model. *Transplantation*. 1988; 45:21–26. [PubMed: 3122383]
12. Smith CV, Suzuki T, Guzzetta PC, Nakajima K, Sundt TM, Mixon A, Spitzer TR, Eckhaus M, Sachs DH. Bone marrow transplantation in miniature swine: IV. Development of myeloablative regimens that allow engraftment across major histocompatibility barriers. *Transplantation*. 1993; 56:541–549. [PubMed: 8212147]
13. Huang CA, Fuchimoto Y, Scheier-Dolberg R, Murphy MC, Neville DMJ, Sachs DH. Stable mixed chimerism and tolerance using a nonmyeloablative preparative regimen in a large-animal model. *J Clin Invest*. 2000; 105:173–181. [PubMed: 10642595]
14. Shimizu A, Yamada K, Meehan SM, Sachs DH, Colvin RB. Acceptance reaction: intragraft events associated with tolerance to renal allografts in miniature swine. *J Am Soc Nephrol*. 2000; 11:2371–2380. [PubMed: 11095661]
15. Yamada K, Shimizu A, Utsugi R, Ierino FL, Gargollo P, Haller GW, Colvin RB, Sachs DH. Thymic transplantation in miniature swine. II. Induction of tolerance by transplantation of composite thymokidneys to thymectomized recipients. *Journal of Immunology*. 2000; 164:3079–3086.
16. Kelley KW, Meier WA, Minshall C, Schacher DH, Liu Q, VanHoy R, Burgess W, Dantzer R. Insulin growth factor-I inhibits apoptosis in hematopoietic progenitor cells. Implications in thymic aging. *Ann N Y Acad Sci*. 1998; 840:518–524. [PubMed: 9629278]
17. Ortman CL, Dittmar KA, Witte PL, Le PT. Molecular characterization of the mouse involuted thymus: aberrations in expression of transcription regulators in thymocyte and epithelial compartments. *Int Immunol*. Jul. 2002; 14:813–822.
18. Hirakata A, Okumi M, Griesemer AD, Shimizu A, Nobori S, Tena A, Moran S, Arn S, Boyd RL, Sachs DH, Yamada K. Reversal of age-related thymic involution by an LHRH agonist in miniature swine. *Transpl Immunol*. 2010; 24:76–81. [PubMed: 20692342]
19. Chu YW, Schmitz S, Choudhury B, Telford W, Kapoor V, Garfield S, Howe D, Gress RE. Exogenous insulin-like growth factor 1 enhances thymopoiesis predominantly through thymic epithelial cell expansion. *Blood*. 2008; 112:2836–2846. [PubMed: 18658030]
20. Kooijman R, Scholtens LE, Rijkers GT, Zegers BJ. Type I insulin-like growth factor receptor expression in different developmental stages of human thymocytes. *J Endocrinol*. 1995; 147:203–209. [PubMed: 7490549]
21. de Mello-Coelho V, Villa-Verde DM, Dardenne M, Savino W. Pituitary hormones modulate cell-cell interactions between thymocytes and thymic epithelial cells. *J Neuroimmunol*. 1997; 76:39–49. [PubMed: 9184631]

22. Su DM, Navarre S, Oh WJ, Condie BG, Manley NR. A domain of Foxn1 required for crosstalk-dependent thymic epithelial cell differentiation. *Nat Immunol.* 2003; 4:1128–1135. [PubMed: 14528302]
23. Zook EC, Zhang S, Gerstein RM, Witte PL, Le PT. Enhancing T lineage production in aged mice: a novel function of Foxn1 in the bone marrow niche. *J Immunol.* 2013; 191:5583–5593. [PubMed: 24184560]
24. Takaki T, Hosaka N, Miyake T, Cui W, Nishida T, Inaba M, Ikehara S. Presence of donor-derived thymic epithelial cells in [B6-->MRL/lpr] mice after allogeneic intra-bone marrow-bone marrow transplantation (IBM-BMT). *J Autoimmun.* 2008; 31:408–415. [PubMed: 19022623]
25. Manley NR, Richie ER, Blackburn CC, Condie BG, Sage J. Structure and function of the thymic microenvironment. *Front Biosci (Landmark Ed).* 2010; 16:2461–2477.
26. Chen L, Xiao S, Manley NR. Foxn1 is required to maintain the postnatal thymic microenvironment in a dosage sensitive manner. *Blood.* 2009; 113:567–574. [PubMed: 18978204]

Group 1 (aged to juvenile) **Group 2 (juvenile to aged)**

Group 3 (aged to aged)

Group 4 (juvenile to aged with juvenile BM)

Figure 1. Schematic diagram of experimental groups and swine MHC haplotypes in this model
The serial breeding of MGH swine has led to fixed, defined MHC classes.

Figure 2. Thymic transplantation from aged donors to juvenile recipients in class I-mismatched barrier (Group 1)

(a) The increasing of cortico-medullary (C/M) ratio in the thymic graft (day 0 and 119) and (b) the changing of FACS profile of the graft showed rejuvenation of the thymic graft in class I-mismatched thymic transplantation (#20847). (c) CML assay and (d) CML assay with CD25 depleted responders cells from #20847 at day 180 showed donor-specific unresponsiveness. (e) Immunofluorescence microscopy of the thymic graft of #20847 on day 180 (donor class I: green, pig class II: red). Donor stromal cells (green) were active and recipient dendritic cell were present in high numbers (red). Donor dendritic cells were also present (yellow).

Figure 3. Thymic transplantation from aged donors to juvenile recipients in a class I-mismatched barrier and retransplantation of the rejuvenated thymus

(a) The increasing of cortico-medullary (C/M) ratio in the thymic graft (day 0 and 108) and (b) the changing of FACS profile of the graft showed rejuvenation of the thymic graft in MHC-matched transplantation (#20786). (c) Serum creatinine level in #21106 and #21191 after kidney transplantation of rejuvenated thymic grafts transferred from #20888 and #20786, respectively. (d) CML assays after kidney transplantation demonstrated donor-specific unresponsiveness in both of #21106 and #21191. (e, f) No rejection showed in the kidney grafts of #21106 and #21191 on day 58 and 95, respectively.

Figure 4. Thymic transplantation from juvenile donors to aged recipients (Group 2)
Cortico-medullary (C/M) ratio in the thymic graft dramatically decreased in (a) #19167 and (c) #20607 compared to the control naïve thymus (e). CD4/CD8 DP and CD1 SP cells decreased and CD3 SP cells increased in the thymic grafts in (b) #19167 and (d) #20607. In contrast, control naïve thymus from an animal was treated with FK506 for 28 days had stable in CD4/CD8 DP, CD1 SP and CD3 SP cells (f). These results indicate that juvenile thymi undergo accelerated involution in aged hosts.

Figure 5. Immunological status of Group 2 animals (thymic transplantation from juvenile donors to aged recipients)

(a) CML assays after thymic transplantation of #19167, #19978 and #20607. Although #19167 had stable donor-specific unresponsiveness, #19978 and #20607 recovered their anti-donor responses. Dash line: anti-3rd party swine response, solid line: anti-donor response. (b) Anti-donor IgG antibody FACS after thymic Tx. MFI= mean fluorescence intensity. No IgG antibodies developed after Tx. (c) and (d) CML assays of #19167 and #19978 with CD25-depleted responder cells on day 170 and 160, respectively. Even though CD25 positive cells were depleted from responder cells, #19167 had stable donor-specific unresponsiveness. However, #19978 recovered anti-donor response. (e) Immunofluorescence microscopy of the thymic graft on day 170 (#19167). (Donor class I: green. Pig class II: red. Donor dendritic cells: yellow with allows).

Figure 6. Kidney transplantation in Group 2

(a) Serum creatinine levels after kidney transplantation from MHC-matched donor. (b) Histology of the kidney grafts transplanted to #19167 (left) showed no rejection, but cell infiltration was seen in #19978 (right). (c) CML assays after kidney transplantation showed unresponsiveness in #19167 (left) and responsiveness in #19978 (right) against their donor cells.

Figure 7. Thymic graft histology and immunological status in Group 3
 (a) Histology of thymic grafts for #20438 (showing involution) and (b) #20248 (showing rejection). (c) CML showed anti-donor response after thymic Tx on day 58 for #20248.

Figure 8. Juvenile thymic grafts involuted at a slower rate in presence of juvenile bone marrow (Group 4)

A. Percentage of donor cells in PBMC following MHC matched BM Tx as assessed by FACS. Chimerisms were assessed using mAb specific for pig allelic antigen. Approximately 90% of lymphoid cells and more than 95% of myeloid cells in the peripheral blood of recipients were of donor origin by the time of VTL Tx. Green line: lymphocytes; Blue line: granulocytes; Red line: monocytes. **B.** Histologic and phenotypic analyses of VTL grafts. (a) Cortico-medullary (C/M) ratio in the thymic graft (days 0 and 120) of #19639. (b) Cortico-medullary (C/M) ratio in the thymic graft (days 0 and 92) of #21179. (c) FACS profile of the thymic graft in #19639. These data demonstrated that juvenile thymic grafts involuted at a slower rate compared to Group 2.

Figure 9. FOXN1 in the naïve thymus and graft thymus

A. Immunohistochemistry of FOXN1 in thymus.

Brown staining shows cells positive for FOXN1 in (a) juvenile naïve thymus (2 months old), (b) aged naïve thymus (31 months old), (c) thymic graft prior to thymic Tx in #20888 (20 months old) from Group 1, (d) rejuvenated thymic graft 170 days after thymic Tx in #20888 from Group 1, (e) thymic graft prior to thymic Tx in #19167 (3 months old) from Group 2, (f) involuted thymic grafts 170 days after thymic Tx in #19167 from Group 2, (g) thymic graft prior to thymic Tx in #21179 (5 months old) from Group 4, (h) thymic grafts 92 days after thymic Tx in #21179 from Group 4.

B. Semi-quantification of FOXN1 positive cells in thymus.

FOXN1 positive cells were counted in at least five randomly chosen high power fields (x400) in juvenile naïve thymi (n=3, average 3 months old), aged naïve thymi (n=3, average 27 months old), rejuvenated VTLs in Group1 (n=3), involuted VTLs in Group 2 (n=3), and VTLs with young BM in Group 4 (n=1). The counts of FOXN1 positive cells is shown as the average number ±SD.

Figure 10. Serum IGF-1 level and IGF-1R in naïve and graft thymi

(a) Serum IGF-1 level in naïve pigs of various ages. Immunofluorescence microscopy for IGF-1R (green) and cytokeratine 14 (red) in (b) naïve juvenile thymus of 3 months of age, (c) naïve adult thymus of 31 months of age, (d) rejuvenated thymic graft of #20847 on POD119, (e) involuted thymic graft of #19167 on POD 174, and (f) thymic graft on POD92 from #21179, an animal in Group 4 which received juvenile BM prior to thymus Tx.

Masayuki Tasaki:	Primarily performed research in Groups 1, 2 and 3, analyzed the data and wrote the manuscript
Vincenzo Villani:	Primarily performed research in Group 4, analyzed the data and wrote the manuscript
Akira Shimizu:	Responsible for pathologic diagnosis
Mitsuhiro Sekijima, Rei Yamada, Isabel Hanekamp, John Hanekamp and Akihiro Kawai:	Participated in the performance of the research, participated in data analysis
Taylor Cormack and Shannon Moran:	Assisted with the research (<i>in vivo</i> and <i>in vitro</i> studies)
David H. Sachs:	Participated in data analysis and writing of the manuscript
Kazuhiko Yamada:	Corresponding author. Primarily designed this research study, the performance of the research, data analysis, and writing of the paper

Table I

Recipient/donor combinations and briefly describes the results in each group of this study.

Group 1 (aged to juvenile)						
Recipient ID (MHC)	Host thymectomy	Mismatch of the thymic graft (MHC)	Thymic graft outcome	Secondary graft recipient (MHC)	Kidney graft mismatch	Results summary
20847 (KK)	Y	Class I (CC)	rejuvenated	N/A	N/A	Confirmed results from ref. 2
20786 (DD)	N	Matched (DD)	rejuvenated	21191 (DD)	Class I (GG)	Rejuvenation restored ability of thymus to induce tolerance
20888 (DD)	N	Matched (DD)	rejuvenated	21106 (DD)	Class I (GG)	

Group 2 (juvenile to aged)				
Recipient ID (MHC)	Host thymectomy	Mismatch of the thymic graft (MHC)	Thymic graft outcome	Results summary
19167 (JJ)	Y	Class I (CC)	Involved	Kidney accepted
19978 (LL)	Y	Class I (AA)	Involved	Kidney rejected day 45
20607 (KK)	Y	Class I (JJ)	Involved	Kidney rejected day 15

Group 3 (aged to aged)				
Recipient ID (MHC)	Host thymectomy	Mismatch of the thymic graft (MHC)	Thymic graft outcome	Results summary
20438 (DD)	Y	Matched (DD)	Involved	-
20248 (DD)	Y	Class I (HH)	Rejected	-

Group 4 (juvenile to aged with juvenile BM)				
BM donor mismatch	Recipient ID (MHC)	Host thymectomy	Mismatch of the thymic graft (MHC)	Results summary
Matched (DD)	19639 (DD)	Y	Matched (DD)	Juvenile BM prevented rapid thymus involution
Matched (DD)	21179 (DD)	Y	Matched (DD)	