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Cultured Thymus Tissue Implantation Promotes Donor-specific Tolerance to Allogeneic Heart Transplants

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Summary: We promoted donor-specific tolerance to cardiac allografts via clinically relevant cultured thymus tissue implantation (CTTI) as used in athymic infants.

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**Abbreviations**

Ab: Antibody  
Ag: Antigen  
APC: Antigen Presenting Cell  
CK: cytokeratin  
CTTI: Cultured Thymus Tissue Implantation  
DC: Dendritic Cell  
DSA: Donor-specific Antibody  
H&E: Hematoxylin and Eosin  
IP: Intraperitoneal  
ISHLT: International Society for Heart & Lung Transplantation  
mAb: monoclonal antibody  
MHC: Major Histocompatibility Complex  
MST: mean survival time  
PBMC: peripheral blood mononuclear cells  
POD: Post-Operative Day  
Tfh: T follicular helper
Abstract

Eighty-six infants born without a thymus have been treated with allogeneic cultured thymus tissue implantation (CTTI). These infants, who lack T cells and are profoundly immunodeficient at birth, after CTTI from an unmatched donor develop genetically-recipient T cells that are tolerant to both their own major histocompatibility antigens and those of the donor. We tested use of CTTI with the goal of inducing tolerance to unmatched heart transplants in immunocompetent rats. We thymectomized and T cell depleted Lewis rats. The rats were then given Lewis x Dark Agouti (LWxDA) CTTI under the kidney capsule and vascularized DA heart transplants in the abdomen. Cyclosporine was administered for 4 months. The control group did not receive CTTI. Recipients with CTTI showed repopulation of naïve and recent thymic emigrant CD4 T cells; controls had none. Recipients of CTTI did not reject DA cardiac allografts. Control animals did not reject DA grafts, due to lack of functional T cells. To confirm donor-specific unresponsiveness, MHC-mismatched Brown Norway (BN) hearts were transplanted 6 months after the initial DA heart transplant. LW rats with (LWxDA) CTTI rejected the third-party BN hearts (mean survival time 10d; n=5). Controls did not (n=5). CTTI recipients produced antibody against third party BN donor but not against the DA thymus donor demonstrating humoral donor-specific tolerance. Taken together, F1(LWxDA) CTTI given to Lewis rats resulted in specific tolerance to the allogeneic DA MHC expressed in the donor thymus with resulting long-term survival of DA heart transplants after withdrawal of all immunosuppression.
**Introduction**

Ray Owen first observed immune tolerance to other individual’s blood in Freemartin calves (1), and later Peter Medawar was the first to induce transplant tolerance by injecting neonatal mice with adult donor tissue/cell suspension (2). These examples of tolerance induction were related to the immature nature of the neonatal immune system (3, 4). Later, the central role of T cells in transplant rejection was identified (5, 6), and deletion of developing alloreactive T cells in the host thymus was thought to be the primary mechanism of tolerance induction (7, 8). There were many attempts to promote tolerance to allogeneic organs by introducing thymus composite tissues or vascularized thymus in which the thymus expressed donor MHC in miniature swine (9-12). The thymoheart was formed by injecting finely minced thymus tissue of the donor type into the atrial appendages of miniature swine. Those hearts were able to survive in an allogeneic recipient for up to approximately 194 days (10). A similar procedure was performed for the thymokidney (9). Tolerance was observed in the miniature swine that received a thymokidney and were followed for up to 60 days. Another study transplanted allogeneic vascularized donor thymic lobes and donor hearts at the same time (12). Allograft survival in the miniature swine was followed up to 301 days when the last animal was sacrificed. Despite success for up to 300 days, data have not been published showing the long-term success needed for human transplantation.

Experimental allogeneic cultured thymus tissue implantation used in 86 athymic infants with complete DiGeorge anomaly resulted in development of naïve T cells in the 61 survivors (71%) (13, 14). Almost 30% of the infants died from preexisting conditions and infections. Studies of tolerance induction in survivors after CTTI from 1993 to 2018 using mixed lymphocyte reactions showed development of tolerance to the alloantigens of the thymus donor in the athymic infants (14). One example of tolerance induction to a solid organ occurred in an infant with complete DiGeorge anomaly who had profound hypoparathyroidism. The infant was treated with CTTI plus parental parathyroid. The CTTI expressed the Class II antigens of the parent that were not inherited by the infant (15). The CTTI did not express the 3 mismatched parental Class I alleles. The parental parathyroid functioned for 10 years with not immune suppression until the patient was given a measles/mumps/rubella (MMR) vaccine. The parental parathyroid was rejected within 2 weeks and the patient had to return to calcium replacement. The mechanism for the loss of parathyroid function was likely rejection of the parental parathyroid by recipient CD8 T cells, one third of which are inherently alloreactive and would have recognized the parental unmatched Class I antigens as foreign. If the CTTI had expressed the uninherited Class I antigens, the CD8 T cells developing in the thymus would have been deleted if they reacted against the uninherited Class I antigens, and the patient would almost certainly still have parathyroid function.

Several other findings in recipients of CTTI should be mentioned. Recipients of CTTI were able to control viral infections such as Epstein–Barr virus that would have been fatal prior to CTTI (16). In addition, these infants, who had essentially no naïve T cells prior to CTTI, developed naïve T cells approximately 6 months after CTTI (13, 17). Biopsies of the transplanted cultured thymus tissue have demonstrated thymopoiesis on immunohistochemistry (18).
Flow cytometry and spectratyping have shown development of a diverse T cell repertoire. Based on the human data showing tolerance to unmatched thymus MHC antigens, we evaluated CTTI, with the same methods used clinically, for its ability to induce donor-specific tolerance in a rat heart transplantation model. Our studies show that transplanting unmatched hearts along with donor CTTI expressing the heart donor’s MHC class I and class II antigens induces tolerance to the antigens of the donor heart while preserving alloreactivity toward other MHC antigens.
Results

In vitro thymic cultures, in vivo engraftment

In order to perform allogeneic CTTI in a rat model with procedures used for the treatment of athymic infants with complete DiGeorge anomaly, thymus was harvested from 3-day-old F1 (Lewis x Dark Agouti, LWxDA) rat pups. Each postnatal thymus was cut into 4 pieces and cultured for 5 to 7 days in thymus organ medium (Fig. S1; see also methods). Histologic analysis (Fig. S2) showed reduced Ki67⁺, CD3⁺ cells in the thymus after culture, similar to the change seen after culture of thymus tissue used for patients (18). As in cultured human thymus, the network of thymic epithelial cells (TEC) was preserved in the rat cultured thymus tissue based on cytokeratin (CK) staining (Fig. S2). The thymus, after being in culture for 5 to 7 days, was transplanted under the kidney capsule of a Lewis rat. As shown in Fig. 1A, recipients were thymectomized and treated with anti-CD5 mAb (OX19; 1 mg, IP) every 5 days for three doses. The recipients also received cyclosporine (CsA), approximately 2.5 mg/kg/day after thymus transplantation using osmotic pumps. CsA was discontinued 4 months after thymus transplantation when the test group had naïve T cells over 10%. At 6 to 7 months, the 3rd party BN heart was transplanted in the neck. The thymus graft and all hearts were evaluated at necropsy 8 months after CTTI when the test group rejected the cervical BN heart. As predicted, recipient-derived LW T cells, not expressing DA MHC, appeared in the peripheral blood of thymus transplant recipients (Fig. S3, panel B). We evaluated T cell and its subpopulations as well as non-T cell populations (B cell and NK cell) via flow cytometry (Fig 1B and Fig S4). Gating strategies for each subpopulation is shown in supplemental figure 5. We predicted that the LW T cells would be tolerant to the DA antigen of the DA heart because the LW T cells had developed in an LWxDA thymus (Fig. 1A). Total circulating CD3 T cell numbers were not significantly different in between groups prior to transplantation. As expected, LW recipients with CTTI showed significantly increased numbers of circulating CD4 and CD8 T cells compared to control animals without CTTI after transplantation (Fig. 1B). Furthermore, animals with CTTI showed significantly increased repopulation of naïve (CD62L⁺CD45RC⁻) CD4 and CD8 T cells as well as recent thymic emigrant (RTE, CD90⁻CD45RC⁻) T cells in the peripheral blood while control groups without thymus transplantation showed low level of circulating naïve CD4 and CD8 T cells and did not show circulating RTE CD4 and CD8 T cells (Fig. 1B). In addition, immunohistologic analysis of transplanted cultured thymus tissue explanted on day 180 showed normal thymus histology, viable T cells (CD3), T cell proliferation (Ki67), and a lacy pattern of CK with Hassall body formation (arrow) on TECs from the CTTI (Fig. 1C). These observations confirm the viability and function (thymopoiesis) of the transplanted thymus in the animals receiving allogeneic heart transplantation. Taken together, rats that received cardiac allografts with CTTI demonstrated thymopoiesis with naïve T cell development.

No graft rejection with or without CTTI in thymectomized recipients

It was expected that T cells reactive to the DA donor would not develop since the T cells developed in CTTI expressing DA as well as LW. We evaluated the DA heart for evidence of rejection. As shown in Fig. 2A, LW rats with DA heart transplants without any immunosuppressive treatment rejected the DA heart grafts within 10 days (the DA control). However, even after developing RTE (CD90⁻CD45RC⁻) T cells, LW recipients with CTTI did not reject (no cessation of beating) the DA cardiac allografts (n=8). Unexpectedly, LW control animals without CTTI
also did not reject the DA cardiac graft (n=9). Both groups showed good beating quality for the entire study period (day 180). Since continuous graft beating does not necessarily imply absence of rejection, we sacrificed two recipients two months after cessation of immunosuppression (prior to 3rd party BN cervical heart transplantation) to confirm that there was no rejection. The explanted cardiac allografts (DA hearts) from both animals showed minimal mononuclear cell infiltration ([Fig. 2B](#fig2b)) with no signs of rejection by 2004 International Society for Heart &Lung Transplantation (ISHLT) grading ([Fig. 2C](#fig2c)). Based on the reconstitution of naïve T cells after CTTI, we believe that animals with CTTI lost their donor-reactive T cell repertoire while animals without CTTI did not fully reconstitute their T cell populations (general hyporesponsiveness).

Alloreactivity against third-party vascularized heart transplantation

In order to confirm the donor-specific unresponsiveness (tolerance) versus general hyporesponsiveness, we performed additional fully MHC mismatched BN heart transplantation in both groups of animals at 6 to 7 months (day 180 to 210) after DA heart transplantation ([Fig. 1A](#fig1a)). As shown in [Fig. 3A](#fig3a), LW rats with CTTI rapidly rejected (cessation of graft beating) the third-party BN heart (n=5, median survival time (MST) =10±1.0 days). However, the control LW animals without CTTI did not reject the third-party hearts ([Fig. 3A](#fig3a)) (n=6, MST≥38.5±8.9 days), possibly due to the lack of any allo reactive T cells. In accordance with the rejection of 3rd party heart by animals with CTTI and the lack of rejection of the third party heart in animals without CTTI ([Fig. 3A and 3B](#fig3ab)), histological analysis ([Fig. 3C](#fig3c)) confirmed that animals with CTTI showed increased mononuclear cell infiltration in the heart allograft ([Fig. 3C, 3rd panel](#fig3c_3rd_panel)) while animals without CTTI showed a pristine BN heart allograft ([Fig. 3C, 4th panel](#fig3c_4th_panel)). It is also notable that the BN hearts in the recipients with CTTI were greatly enlarged ([Fig. 3C, 3rd panel, Fig. S6 panel w/CTTI](#fig3c_3rd_panel_fig_s6_panel_w_ctti)) while the DA hearts were smaller than the native hearts ([Fig. S6 panel w/o CTTI](#fig_s6_panel_w_o_ctti)). BN hearts from recipients without CTTI did not show any increase in size ([Fig. S6](#fig_s6)). Histological analysis (ISHLT grading) of explanted BN hearts from rats with CTTI ([Fig. 3C, 3rd panel](#fig3c_3rd_panel)) showed grade 3R rejection with significantly increased inflammatory cell infiltration compared to syngeneic controls ([Fig. 3C first panel](#fig3c_first_panel)) or rats without CTTI ([Fig. 3C, 4th panel](#fig3c_4th_panel)).

Selective T cell infiltration in third-party hearts but not in DA hearts that shared the DA MHC of the CTTI

We used two conventional ways to define graft rejection in this rat heart transplantation model: heart beating/cessation measurements and the ISHLT human grading system. The former is insensitive with respect to low-grade rejection, while the latter is insensitive with respect to high-grade rejection. Therefore, we evaluated inflammatory cell infiltration in DA hearts from 3 rats at day 180 and in BN hearts at the time of sacrifice at 7 to 8 months in 5 rats. As shown in [Fig. 4A](#fig4a), rats that were treated with T cell depletion, CTTI, and four months of CsA did not show an increased level of inflammatory cell infiltration in the DA hearts after T cell repopulation. On the other hand, the animals given CTTI showed massive inflammatory cell infiltration in the third-party cardiac allograft (BN heart) ([Fig. 4B](#fig4b)). Rats without CTTI showed no infiltrates in the BN heart ([Fig. 4B](#fig4b)). Finally, we evaluated T cell infiltration with immunohistochemistry and confirmed a selective T cell infiltration in the BN ([Fig. 4C, 3rd panel](#fig4c_3rd_panel)), but not DA ([Figure 4C, 2nd panel](#fig4c_2nd_panel)), hearts of the animals with CTTI and a lack of T cell infiltration in both
hearts of animals without CTTI (Fig. S7). These data confirm that the T cell infiltration occurs only in the third-party BN graft, but not in the graft sharing MHC (DA) with the transplanted thymus, possibly due to lack of T cell repertoire (by negative selection) against (DA heart) donor antigens.

**Humoral response against donor antigens after thymus transplantation**

After native LW thymectomy followed by LWxDA CTTI, we hypothesized that there would be a lack of T cell help for cognate B cell and downstream humoral response against the DA donor antigen in recipients. Therefore, we evaluated anti-donor antibody responses to determine whether the allogeneic T cell unresponsiveness was associated with humoral tolerance against donor DA MHC. We tested serially collected recipient serum samples and performed flow crossmatch with PBMCs from DA and BN rats. As expected, unmanipulated LW rats that received DA or BN heart transplants without immunosuppression developed antibody against their donors (DA or BN, respectively) (Fig. 5A, first panel in each row). Animals with a syngeneic cardiac allograft did not produce antibody against either DA or BN MHC (Data not shown). Interestingly, similar to T cell hyporesponsiveness, no anti-DA Ab was detected in animals with or without CTTI (5A, 2nd and 3rd panels of top row and Fig. 5B). Anti-BN Ab was readily detected in animals with LWxDA CTTI but not in animals without CTTI, p<0.01 (5A, 2nd and 3rd panels of bottom row and Fig. 5C). Taken together, thymus co-transplantation resulted in specific tolerance to the allogeneic DA MHC expressed in the donor thymus, and thus long-term survival of the DA heart transplant via preventing development of both the donor-specific anti-DA T cell repertoire as well as preventing the donor (DA)-specific humoral response. Immunocompetence was demonstrated in these rats by the rapid rejection of third-party BN hearts as well as alloantibody response against BN donor cells.
Discussion

Achieving donor-specific immune tolerance remains the ultimate immunologic goal in transplantation. Most of the current approaches focus on controlling peripheral mature donor-reactive T cells by depletion (e.g. alemtuzumab, thymoglobulin, etc.) or suppression (e.g. calcineurin inhibitors, basiliximab, etc.) without targeting the production of alloreactive T cells in thymus. However, even with the dramatic advancement of immunosuppressive drugs and new immunomodulatory regimens, transplant tolerance has not yet been consistently achieved. The use of thymus tissue to promote or transfer immunologic tolerance such as by intrathymic injection of antigens has been investigated in many animal models (19-24). Over all, these studies have not been convincing due to the lack of histological evidence, proper controls, long-lasting efficacy, or translation into large animal models or humans. Our approach to achieve tolerance to donor antigens is to use donor CTTI to induce immune tolerance to allogeneic antigens. We hypothesize that tolerance to self is achieved because recipient dendritic cells induce apoptosis in thymocytes expressing T cell receptors that bind with high avidity/affinity to self-peptide:self-MHC on recipient dendritic cells (25-27). In addition, tolerance to donor is achieved because TEC (28) present donor antigens directly, donor-peptide:donor-MHC (29-33) or indirectly via recipient DC, donor-peptide:recipient-MHC to thymocytes. Thus, TECs are a key component promoting donor-specific tolerance by deletion of thymocytes that bind strongly to recipient or donor MHC during allogeneic thymus transplantation.

Tolerance induction by CTTI is similar to tolerance induction via donor DC in hematopoietic stem cell transplantation (34, 35). A series of studies from Transplantation Biology Research Center (TBRC, Boston, MA) showed the crucial role of thymus in tolerance induction (36) and tested thymus transplantation with tolerance induction in a large animal model (9, 37, 38). In their series of studies, this group successfully used Class II matched/Class I mismatched donor (thymus and kidney or heart) as thymus composite tissues (thymokidney and thymoheart) with 12 days of CsA for transplant tolerance induction. This elegant concept of generating vascularized thymus prior to transplantation to induce tolerance, would be difficult to translate to the clinic without using xenotransplantation. The authors stated that non-vascularized thymus did not induce tolerance in their model. More precisely, however, non-vascularized thymus that was not cultured did not engraft long-term. We believe that the reason for lack of function of non-cultured thymus is that there is so much cell death in freshly harvested thymus. The dying thymocytes release deoxyadenosine which diffuses into neighboring thymocytes. The deoxyadenosine is then phosphorylated to deoxyadenosine monophosphate (dAMP) which is trapped in the cell. The dAMP is further phosphorylated into dATP which inhibits ribonucleotide reductase which prevents DNA synthesis (39, 40). Thymocytes die in this environment. The culture system used in this report likely prevents buildup of deoxyadenosine. In particular media is dripped on the thin thymus slices that are floating on sponges in a tissue culture dish. Every day the old medium is removed and new medium is dripped onto the tissue. The deoxyadenosine from the thymocytes is washed off the slices and thus does not lead to the toxic pathway described above.
The limitation of non-vascularized thymus transplantation can be overcome with a culture system as well as T cell depletion as shown in the present study. Experimental transplantation of allogeneic CTTI that retains TECs has been successfully applied to treat pediatric patients with complete DiGeorge anomaly who are born without thymus or thymus function (17, 41). Thymopoiesis has been documented by allograft biopsies and the presence of recipient naive T cells in the periphery (13, 18, 42). Studies of children treated with investigational CTTI show tolerance to donor MHC by mixed lymphocyte reactions (14). In addition, the infants with complete DiGeorge anomaly, after CTTI, are able to control infections such as Epstein Barr virus (16, 19). Based on these data in human infants, we hypothesize that implantation of allogeneic thymus expressing the MHC of a solid organ donor will result in donor-specific tolerance to both self and to the donor and also will retain functional T cells that will protect the recipient from infection.

In the present study, we largely adapted techniques from the clinical setting of CTTI. Rat thymus was cultured in a similar manner to that used for human thymus (42, 43). Cultured thymus tissue maintained the normal thymus structure but was partially thymocyte-depleted consistent with human data (Fig. S2). As shown in Fig. 1, panel C, and Fig. S1, panel D, the CTTI was well-engrafted under the kidney capsule with normal thymus structure. To test our tolerance hypothesis, we performed heterotopic DA heart transplantation together with F1(LWxDA) CTTI in thymectomized, T cell–depleted LW rats (Fig. 1A). The control group did not receive CTTI. Both groups of thymectomized LW rats developed circulating T cells after T cell depletion with anti-CD5 mAb followed by DA heart transplantation (Fig. 1B). In the control group, the return of circulating T cells was likely due to T cell repopulation from the periphery (memory T cells) whereas the group with CTTI also had, in addition, development of naive T cells in the thymus (Fig. 1B). The CTTI in LW rats that were given DA heart transplants showed normal thymus structure with thymopoiesis (Fig. 1C). There was no DA heart graft rejection in either group (with or without a F1(LWxDA) CTTI) when the heart beating was assessed by palpation (Fig. 2A). We hypothesized that the T cells in the control group, although present in the circulation, were not functional. To test this, we used a third-party BN heart transplant to assess if either group could reject the third-party graft. The third-party BN heart allografts were promptly rejected from animals with CTTI but were not rejected by animals without CTTI. Thus, only the animals with CTTI had specific tolerance to donor MHC with immunocompetence demonstrated by the ability to reject allogeneic BN hearts.

Currently, long-term graft survival is often hindered by donor-specific antibodies even with successful T cell control (44). We tested whether the donor-specific tolerance induced by cultured donor thymus tissue transplantation would have an impact on post-transplantation humoral responses. Our hypothesis was correct in that thymectomized, T cell–depleted LW rats given F1(LWxDA) CTTI did not develop DSA against the DA antigens in the abdominal DA heterotopic heart transplant (Fig. 5A, top row, middle panel and Fig. 5B) but did make DSA against the BN heart as detected at the time of graft rejection (Fig. 5A, bottom row, middle panel and Fig. 5C). This suggests that allogeneic, thymus–induced, donor-specific tolerance might control anti-donor humoral response as well. Lastly, it
is known that the major population of Foxp3+ Treg is generated in the thymus (45). Therefore, it is possible that Treg cells generated from CTTI could also provide additional regulation against donor Ag reactive T cells.

We believe that the present study provides proof-of-concept for donor thymus co-transplantation with solid organs for tolerance induction. The patient group that would most benefit from the procedure is infants needing heart transplants. Since postnatal thymic tissue is present and could be removed from the deceased donor infant, and the recipient thymus is routinely removed from infants undergoing heart transplantation, no additional procedure aside from CTTI would be needed to transfer this approach to the clinic. Overall, these results support the use of CTTI for the tolerance induction in organ transplantation, however, it remains to be tested as to how efficiently transplanted cultured thymus tissue will develop donor-specific tolerance in an immunocompetent large animal model with clinically relevant immunosuppressive regimens before moving into the clinic.
Materials and Methods

Animal Models

Lewis (RT-1<sup>l</sup>) and BN (RT-1<sup>n</sup>) rats were purchased from Charles River. DA (RT-1<sup>nv</sup>) rats were purchased from Envigo. F1(LEW/DA; RT-1<sup>1av1</sup>) were bred by protocol staff at the Duke Breeding Core Division of Laboratory Animal Resources facility. Lewis recipients received thymectomies as previously described (46). Briefly, the submandibular glands and sternohyoid muscle were separated with blunt forceps to expose the overlying the trachea. A 1- to 1.5-cm incision was made in the sternal manubrium. A 7 cm alms-type retractor was used to retract the manubrium and the two halves of the sternohyoid muscles to expose the thymus. The thymus was grasped with blunt forceps and extracted. The cut ends of the sternum were closed with a single 3 to 4-0 silk suture. Two drops of 2.5mg/ml bupivacaine were applied on the incision and the outer layer of skin was closed with three or four 9-mm wound clips. All thymectomized rats were maintained on a diet containing Septra (PMI Nutrition International, LLC). To induce T cell depletion in vivo, 1mg anti-CD5 mAb (OX19; BioXCell, NH) was intraperitoneally administered on day 0, 5, and 10 after thymectomy and suppression with 0.25 mg/kg/d cyclosporine pump from day 0 (heart transplant & CTTI time point) to 4 months with respect to heart transplantation. All rats were used and maintained in accordance with the guidelines and compliance of the Duke Institutional Animal Research Ethics Committee.

In vitro thymus culture and CTTI

Thymus from three-day-old neonatal F1 (LEW/DA) rat pups were harvested steriley, cut into four pieces along the longitude natural seam, and transferred onto sterile nitrocellulose filters (MF-Millipore, Millipore Sigma) in a tissue culture dish with TOM medium. Thymus tissue was cultured in a CO<sub>2</sub> incubator with 5% CO<sub>2</sub> at 37° C for the desired length of time (5 to 7 days). The medium was changed daily. The thymus organ medium (TOM) was composed of HAMS F12 (Life Technologies) at 86.5%; Hepes (Life Technologies) at 25mM; L-Glutamine Life Technologies) at 2mM; Fetal Bovine Serum (Life Technologies) at 10%; and Pen-strep (Life Technologies) used at 1x. On the day of transplantation, the thymus pieces were rinsed with fresh medium and transplanted under the kidney capsule of a Lewis rat with one secure suture (10-0 monofilament). All manipulations took place under sterile conditions in a biological safety cabinet.

Abdominal and cervical heart transplantations

Full MHC mismatched DA (RT-1<sup>1av1</sup>) donor hearts were transplanted into thymectomized Lewis (RT-1<sup>l</sup>) recipients. Abdominal heart transplantation was performed using a modified technique of the methods described by Schmid et al. (47). Briefly, the donor heart was transplanted into the abdominal cavity of the recipient after a short period of cold ischemia in Euro-Collins solution. The donor pulmonary artery and aorta were anastomosed to the recipient inferior vena cava and descending aorta with an end-side fashion as the inflow and outflow vessels for circulation, using running 9/0 non-absorbable monofilament sutures. Cyclosporine A (CsA) was given via the osmotic pump (Model 2ML4, Alzet). The pump was loaded steriley and surgically inserted subcutaneously to mid-dorsal area of recipients. The osmotic pump was replaced every month for 4 months. For full MHC mismatched BN (RT-1<sup>n</sup>) third-party heart transplantation to the DA heart bearing Lewis recipients, we used cervical vascularized heart
transplantation described by Heron in a modified fashion (48). Briefly, the third-party heart was transplanted into the right side of cervical area via a longitudinal incision from submaxilla to the xiphoid. The donor pulmonary artery and external jugular vein were anastomosed end to end and the aorta was anastomosed to the right common carotid artery by cuffing technique. The grafts were monitored by daily palpation and later confirmed by laparotomy at the time of sacrifice. Animals were sacrificed on the day of rejection of the graft (cessation of beating) or a designated time point.

**Flow cytometric analysis and monitoring DSA**

Peripheral blood was obtained from the cranial vena cava and stained with antibodies. To analyze naïve and recent thymic emigrants, we used the combination of anti-Rat CD3 APC (BD Biosciences); anti-Rat CD4 APC-Cy7 (Biolegend); anti-Rat CD8a V450 (BD); anti-Rat CD45 PE-Cy7 (BD); anti-Rat CD45RC – PE (BD); anti-Rat CD62L FITC (BD); and anti-Rat CD90 BV 510 (Biolegend). To assess percentages of T, B, and NK cells, we used the combination of anti-Rat TCR FITC (BD); anti-Rat CD4 APC-Cy7(Biolegend); anti-Rat CD8a V450 (BD); anti-Rat CD45 PE-Cy7 (BD); anti-Rat CD45RA PE (Invitrogen); anti-Rat NKR-P1A-APC (Invitrogen). For host vs donor discrimination, we use the combination of anti-Rat TCR APC (Biolegend), anti-Rat CD45 PE-Cy7 (BD); MHC Class I RT1Aa (Santa Cruz Biotechnology). We also used a secondary goat anti-mouse IgG (Invitrogen) for the non-conjugated MHC Class I RT1Aa. Donor-specific alloantibody (DSA) was assessed by flow crossmatch from serially collected recipient serum samples with DA donor or BN third party rats. FITC-conjugated pan-rat immunoglobulin antibody was added to the samples and incubated after washing. The T cells were stained with APC-conjugated anti-CD3. Samples were analyzed on a LSR fortessa (Beckman Coulter).

**Histology, Immunohistochemistry (IHC), and morphological analysis**

All cultured thymus and CTTI samples from under the kidney capsule were frozen in OCT (Optimal Cutting Compound; Tissue Tek). Control thymus tissue was obtained from newborn to 5-day-old rat pups. Four to five mm sections were stained for CD3 (polyclonal; Dako), Ki-67 (clone: SP6; Thermo), CK, (polyclonal; Invitrogen). IHC images were obtained using an Olympus Vanox AH-3 Microscope of the Olympus DP-70 Digital Camera System. The explanted hearts underwent serial sectioning (5µm) from the midventricular level to the base. H&E stains were performed for routine examination and grading of rejection. Graft infiltrating T cells were evaluated with polyclonal anti-CD3 (Dako) staining. Whole slides of grafts were scanned with an Aperio ScanScope XT (Aperio Technologies, Inc., Vista, CA).

**Statistical Analysis**

Experimental results were analyzed by a GraphPad Prism (GraphPad Software 7.0, San Diego, CA) and SAS version 9.4 (SAS Institute, Cary, NC). The log-rank test for differences in graft survival, student t-test for two group comparison and Turkey test was used for multiple group comparison. All the data were presented as mean ± SD. Values of $p$ which were less than 0.05 were considered as statistically significant.
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Contributions

J.K. designed experiments, performed surgical procedures, conducted in vitro experiments, analyzed, interpreted data and prepared the manuscript. J.L. designed experiments, cared for experimental animals, conducted in vitro experiments (including flow cytometry), and prepared the manuscript. D.C.R. participated in surgical procedures (thymectomy and phlebotomy) and cared for experimental animals. J.P participated in surgical procedures (heart transplantation). A.B.F. interpreted data (Pathologist). M.K. participated in analyzing data (Biostatistician). J.W.T. edited the manuscript. A.D.K. conceived of experimental design, and edited the manuscript. M.L.M. conceived of experimental design, interpreted data and prepared the manuscript.
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Figure 1

(A) Schematic outline of experimental design to show naïve T cell reconstitution, thymopoiesis, and donor-specific tolerance induced by CTTI. All Lewis (LW) rats were thymectomized and T cell depleted via anti-CD5 mAb prior to heart transplantation and CTTI. CTTI from F1(LWxDA) rats and hearts from DA rats were transplanted into thymectomized LW recipients. Cyclosporine (CsA) was given for four months after transplantation via osmotic pump. The third-party BN heart was transplanted into the neck 2 to 3 months after CsA discontinuation. Control rats experienced identical procedures except they did not receive CTTI.

(B) Circulating T cell repopulation after T cell depletion and thymus and heart transplantation. All animals showed dramatic reduction of circulating T cells after T cell depletion. Cardiac allograft recipients with CTTI (blue line) showed gradual repopulation of circulating T cells. Animals without CTTI also showed some degree of circulating T cells (red line). However, naïve
and recent thymic emigrants CD4 and CD8 T cells were significantly increased (p<0.01) in animals with CTTI while control animals showed no circulating naïve nor RTE CD4 and CD8 T cells. (C) Engrafted cultured thymus tissues under the renal capsule on day 180 in a recipient of cardiac allograft. Histology showed a distinct structure separate from renal tissue (Original magnification, x20). Engrafted cultured thymus tissue (right panel) showed a normal thymus structure (H&E), viable T cells (CD3), T cell proliferation (Ki67), and Hassall body formation (Black arrow) with a lacy pattern (Cytokeratin) on epithelial cells, confirming the viability of thymus with thymopoiesis. Original Magnification, × 200. Data are presented as means ± SD; n= 8-9 animals per group; student’s t-test, *P<0.05; **P< 0.01; ***P<0.001, ****P<0.0001; NS, not significant (p>0.05).

Figure 2

Figure 2. Long-term cardiac allograft survival regardless of thymus co-transplantation. (A) Graft survival of DA heart in recipients with or without CTTI. Kaplan-Meier survival curve showed significantly prolonged graft survival from animals with or without CTTI and syngeneic controls (LW heart into LW rat) as compared to LW rats with DA heart transplants without immunosuppression/thymectomy (DA control). (B) Representative scanned image of explanted DA heart graft at day 180 from animals with and without CTTI. Images were adapted from whole slide scan. (C) ISHLT grading showed a significant reduction of rejection in both recipients with CTTI and without CTTI compared to DA control with no immunosuppression (n = 3-4 per group). Turkey test, ***P<0.001; NS, not significant (p>0.05).
Figure 3. Donor-specific tolerance in animals with CTTI versus general hypo-responsiveness in animals without CTTI. (A) Graft survival of BN (cervical) heart in recipients with or without CTTI. Recipients with CTTI rejected the BN heart rapidly (MST=10±1.0 days) while recipients without CTTI did not reject the third-party BN hearts. The BN control (no immunosuppression/thymectomy) shows rejection of BN hearts by LW rats. The syngeneic control shows lack of rejection of LW hearts by LW rats. Kaplan-Meier survival curve showed significant differences in the graft survival. (B) ISHLT grading showed a severe rejection of BN heart in animals with CTTI while a significant reduction of rejection in recipient without CTTI compared to BN control with no immunosuppression (n = 3-5 per group). Turkey test, ***P<0.001; NS, not significant (p>0.05). (C) Representative scanned image of explanted BN heart graft at the time of rejection or designated time points post-transplantation. Both BN heart grafts from animals with CTTI (3rd panel) and allogeneic (2nd panel) showed severe mononuclear cell infiltration while BN heart grafts from animals without CTTI (4th panel) or animals with syngeneic CTTI (1st panel) showed no sign of rejection. Images were adapted from whole slide scan.
Figure 4

Figure 4. T cell infiltration (CD3 IHC) in LW, DA, and BN hearts. (A) Quantification of inflammatory cells in the primary abdominal DA cardiac allograft. Both DA grafts from animals with or without CTTI showed no significantly reduced inflammatory cell infiltration (at POD180) compared to DA control grafts (in LW rats with no immunosuppression) showing a high level of graft infiltration of immune cells. (B) Quantification of inflammatory cells in secondary cervical BN cardiac allografts. Both the BN control grafts (in LW rats with no immunosuppression) and BN grafts from recipients with CTTI showed significantly elevated inflammatory cell infiltration. The BN grafts from animals without CTTI showed significantly reduced inflammatory cell infiltration compared to BN control. (C) Heart allografts from DA and BN rats were harvested with native heart at the time of BN heart rejection. Grossly, native heart and DA heart (POD 196) did not show dramatic increase of T cells, while BN heart (POD14) showed a massive amount of T cells in recipients with CTTI. Images were adapted from whole slide scan. For panels A and B, 3-5 animals per group were analyzed; Turkey test, *P<0.05; **P< 0.01; ***P<0.001; NS, not significant (p>0.05).
Figure 5

**Figure 5. Donor-specific humoral tolerance with CTTI.** (A) Representative histogram plots for post-transplant donor-specific alloantibody (anti-DA and anti-BN antibodies) measured by T cell flow crossmatch. LW recipients with or without CTTI did not generate any antibodies against DA antigen (Fig. 5A, 2nd and 3rd plots, respectively, in top row) while animals with CTTI were able to generate antibody against BN antigen (Fig 5A, 2nd panel, bottom row). Serum samples from LW recipients of DA heart transplantation without immunosuppression (Fig 5A, 1st panel in top row) and from LW recipients of BN heart transplantation without immunosuppression (Fig 5A, 1st panel in bottom row) were used as positive controls (DA control and BN control) for anti-DA or anti-BN antibody, respectively. 

(B) Level of anti-DA antibody after primary DA heart transplantation. Serum samples from animals with CTTI and without CTTI after DA heart transplantation (POD 180) did not develop antibody against DA cells.

(C) Levels of anti-BN antibody after secondary BN heart transplantation. Animals with CTTI showed significantly elevated anti-BN Abs while animals without CTTI showed no antibodies against BN cells. Total 4-5 animals per group were analyzed; student’s t-test.