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The Rejuvenating Effects of Leuprolide-Acetate on the Aged Baboon's Thymus

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Abstract

Background—We have previously demonstrated that the juvenile thymus plays an essential role in tolerance induced by both renal transplantation and a short course of calcineurin inhibitors. Aged thymi have a decreased ability to induce tolerance. Luteinizing Hormone-Releasing Hormone (LHRH) is known to pharmacologically rejuvenate the thymus in rodents. In order to develop a clinically applicable regimen of transplantation tolerance in adults, we sought to determine if thymic rejuvenation would occur with LHRH agonism in non-human primates.

Methods and Results—Thymic rejuvenation was evaluated by magnetic resonance imaging (MRI), histology, as well as in-vitro cellular and molecular tests. Four aged male hamadryas baboons underwent subcutaneous injection of a 3-month depot of Lupron (11.25mg; LI) and were followed for 3 months. Thymi increased volumetrically by MRI. After LI, thymic cellularity markedly increased within the cortical and medullary thymus. Additionally, a significant increase in the CD4⁺/CD45RA^{hi} population in the peripheral blood occurred for 50 days after LI, and flow cytometry of thymic tissue revealed a large increase in the percentage of CD4⁺/CD8⁺ cells. TREC assay corroborated enhancement in thymic function.

Conclusion—These data indicate that LI is associated with thymic rejuvenation in baboons, and further confirm that extrinsic factors play an important role in thymic rejuvenation in a non-human primate model.

Keywords

LHRH agonist; Thymic rejuvenation; Baboons

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1. INTRODUCTION

Tolerance remains an important goal of transplantation biology. Our laboratory has previously shown that transplantation of a class-I disparate or fully MHC-disparate kidney followed by 12-days of either high-dose CyA or FK506 facilitated allogeneic tolerance induction in MGH miniature swine (1–4). However, surgical manipulation of the thymus or surgical thymectomy prior to transplantation interfered with tolerance induction (5,6). Conversely, thymectomy following transplantation does not lead to tolerance abrogation (5,6). These data suggest that tolerance induction in this model is thymus-dependent, but maintenance of tolerance is not (5–8). Clinically, this euthymic restriction suggests that tolerance induction protocols may only be possible for children and adolescents. If the adult thymus could be rejuvenated, it would be possible to extend the potential clinical applicability of such protocols.

In small animal models, orchiectomy leading to decreased testosterone (surgical castration) has a trophic effect on the thymus (9). By suppressing testosterone levels via chemical castration, using a 28-day course of leuprolide acetate (Lupron, LHRH agonist), investigators also observed rejuvenation of the aged rodent thymus (9). Conversely, pregnancy (which leads to increased estrogen) has a pro-involutory effect on the thymus, and this effect is thought to modulate maternal immune responses (10). Other laboratories have also shown that estrogen alone can induce thymic involution (11). Although the mechanisms remain elusive, a decrease in thymic LHRH binding sites with increasing age has been correlated with thymic involution (12). In large animal studies, we have reported that when aged thymi were transplanted into juvenile swine, these thymi were morphologically rejuvenated and restored function was observed (13). These data suggest that (A) the immunologic environment of the juvenile animal is different from that of the adult, and (B) extrinsic factors in juvenile animals play an essential role in the rejuvenation of aged thymi. In our subsequent study performed using MGH swine model, we demonstrated that a subcutaneous injection of Lupron led to histologic thymic rejuvenation (14).

Although miniature swine have proven to be an excellent large animal, pre-clinical model with respect to many parameters of transplantation biology (15,16), studies in non-human primates are helpful to confirm clinical applicability. Here, we attempted to rejuvenate the thymi of aged, non-human primates using Lupron. In this study we showed that the LHRH agonist rejuvenated aged, involuted thymi by 3 months using radiologic, histologic, cellular and molecular examinations.

2. Objective and Hypothesis

We hypothesized that LHRH agonists might reverse the negative effects that aging has on the thymus. We investigated whether LHRH agonist injection would lead to thymic rejuvenation as assessed by imaging, as well as cellular and molecular assays in aged baboons.

3. Materials and methods

Animals

Male baboons (*Papio hamadryas*) were purchased from Mannheimer Foundation, Homestead, FL. All animals were housed at the Transplantation Biology Research Center, Massachusetts General Hospital (MGH), Boston, MA. All animal care and procedures were performed in accordance with the guidelines of the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health. The study protocol was approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Thymic Biopsy and histology

Thymic biopsies were performed through a partial sternotomy. The chest was opened, the thymus was inspected, and a wedge biopsy was obtained. Biopsy samples were read by board certified pathologists. Thymic tissues were fixed in 10% buffered formalin and embedded in paraffin for light microscopy. Tissues were stained with hematoxylin and eosin (H&E). To detect CD3+ T cells, cytokeratin+ thymic epithelial cells, and FoxP3+ regulatory T cells in thymus, 10%-buffered, formalin fixed, and paraffin-embedded tissue sections were stained with (a) polyclonal rabbit anti-human CD3 antibody (A0452: DAKO, Glostrup, Denmark), (b) monoclonal mouse anti-human cytokeratin antibody (AE1/AE3, DAKO), and (c) polyclonal rabbit anti-human FoxP3 antibody (ab10563, abcam) using the standard avidin-biotin-peroxidase complex technique. In order to optimize the detection of cytokeratin and FoxP3, sections were microwaved for 10 minutes in 0.01 M sodium citrate (pH 6.0) before incubation with the primary antibody. For CD3, tissue sections were incubated with 0.1% pepsin/0.01N HCL for 30 minutes before incubation with primary antibody.

Leuprolide acetate administration

After sedation, Leuprolide acetate (Lupron, Norwood Abbey Ltd., Melbourne, Australia) 11.25 mg (a 3-month depot dose) was given as a single subcutaneous injection in the right flank.

Magnetic Resonance Imaging (MRI)

Thymi were imaged prior to administration of Lupron to establish baseline morphometric parameters. Additionally, animals were imaged at monthly intervals, for three months following Lupron. Animals were sedated with a continuous infusion of propofol (injection dose: at 2.5mg/kg for the induction and at 200ug/kg/min as maintenance) and underwent MRI. Standard T1 and T2 weighted axial and sagittal images were obtained using standard chest sequencing and 1.5 Tesla magnet. Images were interpreted by senior radiology staff and thymic area was calculated by measuring the thymus' largest dimensions (width and length). The area (width $\times$ depth, cm²) was calculated for comparison of images. Thymus areas per recipient body weight were compared using a single tailed Student's t-test.

Cell Isolation

Peripheral blood mononuclear cells (PBMC) were obtained from blood through centrifugation of a Histopaque gradient. Absolute numbers of cells were determined by multiplying the percentage of specific cell-populations as identified on flow cytometry by the total leukocyte count measured at the same time point. Relative increases in cell counts were determined by dividing cell-population values (at a given time point) by the initial cell-population value at time zero.

Flow cytometry

T cell populations in both the peripheral blood and thymic biopsies were assessed pre- and post-LI. Key populations of interest including recent thymic emigrants (defined as CD4⁺/CD45RA^{hi}) were followed serially in each animal following administration of Lupron.

DNA extraction

For DNA isolation from leukocytes, the buffy coat was collected and cells were washed in Hanks' buffered salt solution (Invitrogen). DNA was extracted from the PBMC using a DNeasy kit (Qiagen). The DNA concentrations were determined by nanodrop and Hoechst dye spectrophotometers. All samples were measured twice and fell within a 5% margin of error. Cells from tissues were obtained from biopsy samples. The tissue was homogenized mechanically and DNA was isolated with the DNeasy tissue isolation kit (Qiagen).

T cell receptor excision circle analysis (TREC)

For quantifying (single joint TREC) sjTREC molecules, a standard curve of sjTREC molecules was established using a cloned plasmid (17). A known number of plasmid molecules were serially diluted 10-fold to obtain a standard curve. Standards and samples were run in triplicate, and each experiment was performed twice.

4. RESULTS

Lupron was physiologically active in baboons

A subcutaneous injection of Lupron 3-month depot (LI) was given over the right flank in 4 aged hamadryas baboons in aged baboons (Table 1. Mean body weight 13.8kg, mean age at 44.5 months old). Lupron led to a transient rise in testosterone levels, following a similar pattern to that observed following Lupron therapy in human males, indicating that the agent was active in baboons and affected the same hormonal pathways (18,19). Serum testosterone then decreased below the level of chemical castration (50 µg/mL) within the first two weeks, where levels remained for 3 months. Testosterone increased thereafter (Fig. 1a), to supranormal levels.

Lupron led to increases in thymus volume and enhancement on MRI

To assess whether MRI was an appropriate tool for the imaging of the baboon thymus, we performed MRIs on a juvenile baboon (6.2 kg body weight, age 21 months old) and an aged baboon (17.8 kg, 42 months old) prior to studying experimental baboons with LI. The juvenile thymus was homogeneous on MRI, and relatively larger per unit body weight.

Glands of juvenile animals were less fibrotic when compared with the aged baboon, which were more lobulated (Fig. 1 b, c, d, and e), as is demonstrated by brightness on T2 weighted imaging. Having confirmed that MRI was capable of distinguishing the juvenile from the aged thymus, all 4 experimental aged baboons underwent MRI pre-Lupron injection, and monthly thereafter. In order to assess thymic volume objectively, a morphometric study was performed. We observed variability in thymic volume between baboons, however after receiving Lupron, regardless of initial size, Lupron uniformly led to increased thymic volume. Minimal change in volume was observed on 1 or 2 month MRI, however at 3 months, moderate enlargement of the thymus was observed on depth-controlled sagittal images (Table 1). Animal follow up was six months.

The thymus was grossly rejuvenated after Lupron

All baboons underwent pre-Lupron injection biopsy of the thymus to gauge thymic histology. Then, following the 3-month MRI, all animals underwent repeat biopsy. On observation at the time of initial biopsy thymi appeared atrophic with marked fatty involution and fibrosis (Fig. 1 d), however, by 3-months post-injection, thymi had visibly enlarged (Fig 1 e, Table 1). As shown in table 1, we observed an increase in mean thymic area (per LI-recipient body weight) from 0.64 cm²/kg to 0.98 cm²/kg (an increase of 60.4%) by 3 months following LI.

Flow cytometric evidence of thymic rejuvenation after Lupron

Flow cytometry performed on thymic tissues biopsied before LI and again at 3 months after LI demonstrated marked increases in various cell types (Table 2). Notably, there were large increases in both CD3+ and CD4+/CD8+ cell populations, consistent with thymic function as well as more modest increases in CD4+/CD45RAhi cells (recent thymic emigrants).

Histologic evidence of thymic rejuvenation after Lupron

In the juvenile thymus, we observed more prominent cortical and medullary tissue as evidenced by CD3+ mature T cells and cytokeratin staining of the thymic epithelial network in thymic medulla (Figure 2 a, b, and c). Abundant FoxP3+ cells were present in medulla of juvenile animals (Figure 2d). In contrast, the size of the aged thymus was decreased and the cortical tissues were markedly thinner (Figure 2e), indicating atrophy. The aged thymus demonstrated fewer CD3+ T cells (Figure 2f) and less cytokeratin+ thymic epithelial tissue (Figure 2g). Notably, there were few visible FoxP3+ cells (Figure 2h). By 6 months after LI, the previously atrophic thymus was noted to be rejuvenated. A fuller cortex and medulla were again visible on H&E staining (Figure 2i). Immature thymocytes lacking CD3 aggregated in a thickened cortex (Figure 2j). In the medulla, CD3+ T cells and cytokeratin+ thymic epithelial tissue were observed (Figure 2j, k, l). A comparison of figures 2d, h, and l show that that the density of FoxP3 positive cells (brown stain) was far lower in the aged thymus, whereas the rejuvenated thymus appears very similar to the juvenile thymus.

Thymic emigrants in the peripheral blood increased following Lupron Injection, but memory cells remained stable

We assessed recent thymic emigrants in the peripheral blood by flow-cytometric analysis. We observed increases in the percentage of CD4⁺/CD45RA^{hi} cells following Lupron injection (Fig. 3a, $p=NS$). Absolute numbers of the CD4⁺/CD45RA^{hi} population doubled by 50 days and this increase was statistically significant ($p=0.02$) (Fig 3b). This population, however, decreased to near-baseline levels by day 120. We also addressed change in memory (CD95⁺/CD28⁺) T cells. The CD95⁺/CD28⁺ double positive population increased by a marginal percentage during the course of Lupron, but this was not statistically significant ($p=NS$) (Fig. 3b).

Confirmation of rejuvenated thymic function using TREC analysis

In order to further determine if Lupron led to enhanced thymopoiesis, we calculated the TCR excision circle frequency (sjTREC) using both thymic biopsies and PBLs. Three-month post-LI thymic biopsy tissue had a 10-fold increase in TREC (copies per 100,000 cells) as compared to that prior to LI (Fig 3, top panel). We also observed a 2-fold increase in the TREC frequency in PBLs at 1 month post-LI. Additionally, there was a slight increase at 3-months post-LI (Fig. 4, bottom panel).

5. DISCUSSION and REFERENCES

The thymus is the origin of T cell immunity. The thymus shapes the repertoire of T cell reactivity and avoids autoimmunity through negative selection of autoreactive T cells, but is subject to involution and dysfunction over time (20). Moreover, the development of regulatory T (Treg) cells, which also occurs in the thymus as a result of clonal diversion, is essential for the maintenance of immune tolerance and homeostasis. TCR specificity to self antigens appears to be a primary determinant for Treg cell lineage commitment in the thymus (21). We have previously demonstrated that the juvenile thymus is required for the induction of tolerance (7,8). We have also reported that thymectomized hosts (7) and aged hosts (8) failed to achieve tolerance whereas juvenile euthymic hosts (4) or hosts with rejuvenated thymi (13) underwent successful induction of tolerance of allogeneic kidneys in MGH miniature swine. Since considerable previous data have indicated that the reason for this age dependence lies in the importance of a non-involved, functional thymus during the induction period, we reasoned that if pharmacologic rejuvenation of thymi successfully restores function of thymi, this rejuvenation could broaden the applicability of this thymus-dependent method of renal allograft tolerance induction. Our laboratory has shown previously that LHRH agonists successfully reversed the involution of thymic architecture in a swine model (14). The present investigation was performed to determine if the same LHRH effects would occur in a non-human primate model. Here, we sought to evaluate if involuted, aged thymi could be rejuvenated with LI in baboons by using clinically relevant tools such as MRI, histology, molecular TRECs and flow cytometric analyses. In summary, we have demonstrated that following Lupron administration 1) the thymus showed evidence of rejuvenation by MRI and histology at 3 months, 2) the frequency and absolute number of recent thymic emigrants in the peripheral blood, as assessed by flow cytometric detection of CD4⁺CD45RA^{hi} population, increased following treatment, and 3) these phenotypically

defined recent thymic emigrants were confirmed to result from increased thymopoiesis by marked elevation in the frequency of sjTRECs.

The large majority of the tolerance induction data from our laboratory were obtained using a miniature swine model. This model utilizes renal transplantation followed by a short course of Cyclosporine A. However, using an alternative swine model of tolerance induction utilizing a 28 day infusion of FK-506 also led to successful tolerance induction. Although our experience is not yet published, we have observed successful tolerance induction in juvenile non-human primates using this same 28-day infusion of FK-506 (Yamada et al., unpublished). Thus, a possible strategy for tolerance induction is to utilize androgen-based therapies such as Leuprolide acetate injections to reverse the effects of thymic involution in aged animals, thereby converting these aged animals from non-tolerance protocol candidates to animals that may potentially become tolerant.

Several markers such as CD62, CD44 as well as CD4 and CD45RA^{hi} have been used to assess thymic emigrants (22–24). Because we used the CD4⁺/CD45RA^{hi} population to assess recent thymic emigrants in the swine model (25), we elected to use the same parameter in this baboon model. In the present model, absolute numbers of the CD4⁺/CD45RA^{hi} population in PBL clearly increased after Lupron injection, suggesting that the thymus was productive of new T cells. TRECs, which are known to be an appropriate marker for thymic output (26) supported our flow-cytometric analysis by confirming active thymocyte development. Our histologic data clearly show a treatment effect with Lupron. Anti-CD1 mAbs were not available for immunohistochemistry as they did not cross react with baboon thymocytes. However, both CD3⁺ cells and cytokeratin staining demonstrated rejuvenated medullary and cortical thymus as well as the enhanced presence of Hassall bodies.

We have previously demonstrated that (A) the thymus plays an essential role in the induction of tolerance (7,8) and (B) that animals with a rejuvenated thymus (by transplantation of an aged thymus to a juvenile host) successfully underwent tolerance induction (13) in MGH miniature swine. Although we showed thymic rejuvenation through demonstration of active thymopoiesis following LI in this report, we have not yet determined if aged baboons with rejuvenated thymi may successfully undergo tolerance induction. A key question that remains to be addressed is whether 2–3 months duration of thymic rejuvenation by a single injection of a 3-month depot sufficiently rejuvenates the thymus such that it will support tolerance induction. In our swine model, we have previously demonstrated that a functional thymus is required for tolerance induction, but that the thymus is no longer required beyond 6 weeks after kidney transplantation (7,27). In the context of the present work, we hypothesize that the single 3-month regimen of Lupron may be sufficient to allow the rejuvenation of the aged thymus and thus aid in the induction of tolerance.

However, other peripheral factors in aged environments may interfere with induction of thymic dependent tolerance. We observed that the present LI regimen had no effect on central T-memory cells. Central memory T-cells, defined here as CD28⁺/CD95⁺, are known to represent a major hurdle to transplantation (28). In contrast to mice or inbred miniature

swine housed in an isolated environment, it is possible that non-human-primates and humans are exposed to foreign antigens over time (through infection) and via microbial mimicry that these events lead to the development of larger numbers of memory T cells (29,30). Memory T-cells, especially central memory T cells, potentially induce a heterologous immune response. Specifically, virally induced alloreactive memory may interfere with tolerance induction (28). Therefore, an additional future strategy, such as T cell depletion to eliminate memory T cells, may be needed along with a LI-based regimen in order to overcome the effect of memory T cells. Further studies to determine whether rejuvenation of thymii by LI facilitates for the induction of tolerance of allogeneic kidneys as well as in vitro studies to address the appropriate balance of recent thymic emigrants and memory T cells in tolerance induction protocols are ongoing in our laboratory.

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Abbreviations

CyA	Cyclosporine A
FSH	Follicle Stimulating Hormone
LHRH	Luteinizing Hormone-Releasing Hormone
MGH	Massachusetts General Hospital
MHC	Major Histocompatibility Complex
MRI	Magnetic Resonance Imaging
NHP	Non-human Primate
PBL	Peripheral Blood Lymphocyte
TREC	T cell Receptor Excision Circle
LI	Leuprolide Acetate, Lupron

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Highlights

- The study demonstrated the rejuvenating effects of Leuprolide-Acetate (Lupron) on the aged thymus in non-human primates
- Luteinizing Hormone-Releasing Hormone (LHRH) agonist injection led to thymic rejuvenation in aged baboons
- LHRH agonist injection led to increases in thymus volume and enhancement on MRI
- CD4⁺/CD45RA^{hi} population doubled by 50 days after administration of LHRH agonist
- TREC assay confirmed that baboon thymic rejuvenation occurs after LHRH agonist injection

Fig 1.

A shows serum testosterone levels following LI. A brief increase in serum testosterone levels, followed by marked drop to undetectable levels were observed for 3 months. B-E show MRI imaging of baboon thymi. b) juvenile thymus imaged using MRI. The thymus is homogeneous in contrast to the thymus of an aged baboon as seen in panel c) the aged thymus is heterogenous and lobulated as it becomes replaced by bright (T2 weighted) fibrous tissue and fat. d) This is the thymus of B290 pre-Lupron injection. The thymus is then seen again on in panel e) and is appreciated to be volumetrically larger. Panels f) and g) show histologic findings (H&E, ×40 magnification) of thymi prior to LI (f) and 3 months post LI (g). Prior to LI administration, the aged thymus appears atrophic and hypocellular, with marked fatty involution, and fibrosis (f). A biopsy at 3 months post LI demonstrates the presence of normal thymic tissue with prominent cellular thymic cortex (g)

Fig. 2.

H&E (a, e, i), CD3 (b, f, j), cytokeratine (c, g, k) and FoxP3 (d, h, l) staining of thymi: a-d show representative findings of juvenile thymuses (sequential cut specimens from a 18 months of juvenile baboon), e-h show representative findings of aged thymuses (sequential cut specimens from an aged baboon) and i-l are sequential cut specimens of a re-juvenated thymus 6 months following LHRH administration.

Fig. 3.

a) Relative increase in the percentage of CD4/CD45RA^{high} double-positive population in the peripheral blood following LI, b) relative increase in the absolute number of CD4/CD45RA^{high} double-positive cells in the peripheral blood following LI, and c) change in the percentage of absolute CD95⁺/CD28⁺ double positive-population cell count in peripheral blood following LI. Recent thymic emigrants, defined as CD4⁺/CD45RA^{high} increased following LI whereas no change in memory T cells as observed.

Fig. 4. TREC analysis of thymi (top) and peripheral blood (bottom) following LI. TREC (copies/ 1×10^5 cells) was markedly increased both thymic tissue and peripheral blood following LI.

Table 1

Change in area of baboon thymus by MRI presented as cm². MRIs were interpreted by an attending radiologist at Massachusetts General Hospital. Following administration of leuprolide acetate, there was a modest increase in the area of baboon thymus. Increased area (cm²) is shown the right-most column.

Animal #	Area of Baboon Thymus (cm ²) on MRI		Body weight (kg)	Area/body weight ratio (cm ² /kg)		% Increase
	Pre-LI	Post-LI		Pre-LI	Post-LI	
B299	7.36	NA	6.2	1.187	NA	NA
B267	12.81	NA	17.8	0.720	NA	NA
B285	7.80	10.80	15.3	0.510	0.705	38.46
B290	5.95	12.04	13.6	0.438	0.885	102.35
B300	9.84	18.00	13.2	0.745	1.364	82.93
B301	10.45	12.32	13.0	0.804	0.948	17.89

B299 was a juvenile control animal; B267 was an aged control animal; B285, B290, B300 and B301 were experimental animals. **LI:** Lupron injection. MRIs were interpreted by an attending radiologist at Massachusetts General Hospital. Following administration of leuprolide acetate, there was a modest increase in the area of baboon thymus. Increased area (cm²) is shown the right-most column.

Table 2

Change in the percentage of various lymphocyte populations in thymic tissue as assessed by flow cytometry. Data was taken from 2 representative animals. CD3+ and CD4+/CD8+ populations increased by the greatest percentage following LI.

Cell Type (Thymic biopsies)	3-month Post-LI (%)	Pre-LI (%)	Difference (%)
CD3+	48.51	21.23	27.28
CD95+/CD28+	4.81	2.86	1.95
CD4+/CD8+	72.46	23.00	49.46
CD4+/CD45+RAhi	5.60	3.80	1.80

LI: Lupron injection