

Melatonin and Androgen Receptor Expression Interplay Modulates Cell-Mediated Immunity in Tropical Rodent *Funambulus pennanti*: An *In-Vivo* and *In-Vitro* Study

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Abstract

An inverse relation exists between melatonin and androgen in most of the seasonally breeding rodents, but the regulation of their receptors in modulation of immune function has never been reported. The present study accessed the expression pattern of melatonin receptor types (mt1R & mt2R), immune parameters (lymphoid organs weight, leucocyte count, delayed type hypersensitivity and lymphocyte proliferation) in spleen and thymus whereas androgen receptor (AR) expression in thymus of *Funambulus pennanti* during reproductively active phase. *In-vivo* melatonin treatment (Mel) and castration (Cx) significantly increased mt1R expression, immune parameters in spleen and thymus but decreased AR expression in thymus only when compared with sham control (Con) squirrels as AR expression was not observed in spleen. Mel alone or in combination with testosterone (T) to Cx squirrels significantly increased mt1R expression, immune parameters in spleen and thymus but decreased AR expression in thymus. T alone in Cx squirrels significantly decreased mt1R expression, immune parameters in spleen and thymus but increased thymic AR expression significantly. *In-vitro* thymocyte culture supported our *in-vivo* findings. Mel significantly increased mt1R expression, lymphocyte proliferation, IL-2 secretion but decreased AR expression. T alone significantly decreased aforementioned three parameters but increased AR expression. Combined treatment of Mel and T bring back all parameters to control level. Though we found high mt2R expression, but no significant change has been observed. Thus, present study suggests a clear-cut trade-off relation between mt1R and AR expression that might be acting as an important mediator in seasonal adjustment of immune function in tropical rodents.

Introduction

Animals living in tropical and temperate climate have evolved seasonal reproductive strategies that ensure adaptation to annual changes in environment and minimize the energetic cost of their reproductive efforts [1, 2]. Environmental factors, i.e., daylength, temperature, food availability etc. can have pronounced effects on the neuro-endocrine system which in turn can alter reproduction and immunity. Immune function is generally compromised during specific energetically demanding times

such as, winter, breeding (including pregnancy and lactation), migration or moulting which is consistent with the hypothesis that immune function is energetically optimized [1].

Animals have evolved mechanisms to cope with energetic trade-offs by reallocating energy in an attempt to maximize lifetime reproductive success. Specifically, steroid hormones and melatonin appears to be highly involved in modulating trade-offs between reproduction and immune function for the healthy survival. Melatonin is thought to be a primary mediator of seasonality in the

reproduction and immune function [3, 4]. In general, melatonin has negative effect on the reproductive axis but its effects on the immune system are varied and depend on the specific arm of the immune system tested, the species studied and the timing of its delivery.

Melatonin acts through its membrane receptors and putative cytoplasmic and nuclear sites to mediate a variety of physiological responses [5]. In mammals, two distinct G-protein-coupled high affinity melatonin receptor subtypes have been identified (mt1, mt2) [6, 7]. Both of these high affinity melatonin receptors have been localized on circulating lymphocytes of rodents, chickens, humans [8, 9] and on thymocytes and splenocytes in humans, several rodents and bird species [10, 11]. *In vitro* melatonin administration enhances the proliferative ability of splenocytes from both female and male prairie voles (*Microtus ochogaster*) [12, 13] and in house mice, this enhancement in mice is reported to be attenuated with the addition of the mt2 receptor antagonist, luzindole [14].

In seasonally breeding rodents, circulatory sex steroid varies throughout the annual reproductive cycle, hence may influence seasonal patterns of immune function and diseases. The negative effect of gonadal steroids on lymphoid tissues was known since 1929 [15] even before the importance of thymus in immune function had been recognized. Consequently, receptors for oestrogen and androgens are reported on lymphoid tissues in rodents [16] suggesting that the gonadal steroid can act directly on immune organs and thereby influence immune function [17, 18]. Importantly, the immune system acts to modulate sex steroid production by acting at different levels of the hypothalamo–pituitary–gonadal (HPG) axis [19].

Till date receptor mediated modulation of immune function by melatonin and gonadal steroid has been least studied in any tropical rodent. Therefore, we made an attempt to explore the immunomodulatory action of melatonin and gonadal steroid, testosterone mediated through their receptor expression by both *in-vivo* and *in-vitro* study in lymphoid tissues of a seasonally breeding Indian tropical squirrel *F. pennanti* during reproductively active phase when circulatory melatonin is low.

Materials and methods

All the experiments on the animals were conducted in accordance with Institutional practice and within the framework of revised Animal (Specific Procedure) Act of 2007 of Govt. of India on animal welfare.

Animal procurement and maintenance. Experiment was performed during reproductively active phase (April–June; temp. approx. max. 40 °C and min. 37 °C, humidity approx. 45% and day length approx. 13:22 h). The squirrels were collected from the vicinity of Varanasi (Lat. 25°, 18' N; Long. 83°, 1'E) in the month of April. After 2 weeks of laboratory acclimatization, healthy young

adult male squirrels of average weight 100 ± 10 g were randomly selected and divided into six groups having seven squirrels in each. Squirrels were kept in the wire net cages (25' × 25' × 30' in size) during experiments and were maintained in a well-ventilated room exposed to ambient conditions. Squirrels were fed with soaked gram seeds, (*Cicer arietinum*) nuts, seasonal fruits/vegetables and water *ad libitum*. The experimental groups were divided as follows:

In-vivo study.

Experimental Groups

Group I	Sham-operated saline treated control (Con)
Group II	Sham operated melatonin treated (M)
Group III	Castrated (Cx)
Group IV	Castrated + melatonin treated (Cx + Mel)
Group V	Castrated + testosterone (Cx + T)
Group VI	Castrated + melatonin + testosterone (Cx + Mel + T)

Operations. Castration was performed surgically with sodium pentobarbital (1.75 mg/kg body wt.) anaesthesia. The scrotal sac region was disinfected, and the skin was cut (1 cm) to expose the testis. The spermatic vein was ligated, and each testis was removed carefully. The scrotal sac was then closed with 2–3 discontinuous stitches. Antibiotic powder (Neosporin powder; Glaxo SmithKline, Mumbai, India) was applied locally to avoid any infection. Animals were kept individually till they recovered from anaesthesia. For sham operation, the cut was made on scrotal sac and then stitched with 2–3 discontinuous stitches. No mortality and pre-or post-operational bleeding was observed during operation.

Drugs and treatments. Commercial testosterone (Aquaviron; Nicholas Pharma, Mumbai, India) was purchased and diluted in normal saline (0.9%) upto desired concentration. Following 24 h of castration Aquaviron was injected intramuscularly during morning hours (10:00–11:00 h ~ 10 h after sunrise) at a dose of 1 mg/100 g body wt. Melatonin (Sigma-Aldrich Chemicals, St. Louis, MO, USA) was first dissolved in few drops of ethanol and then diluted with normal saline upto desired concentration and injected (25 µg/100 g body wt.) during evening hours (between 17:30–18:00 h ~ 1 h before sunset). The sham-operated control received ethanolic saline. All the injections were continued for 30 days. Experiment was performed in two sets. Second set of squirrels was subjected for delayed type hypersensitivity (DTH) response study.

Sample collection. After 24 h of last injection, squirrels were weighed and sacrificed by decapitation during night time between (8:00–10:00 pm). Blood was collected in heparinized tubes and processed for total leucocyte (TLC) and % lymphocyte count (LC) and centrifuged at 254 g for 20 min at 4 °C. Plasma was kept at –20 °C till the radioimmunoassay of testosterone was performed. Spleen

and thymus was dissected out on ice, weighed and processed for the assay of blastogenic response.

Delayed type hypersensitivity (DTH). After 30 days of treatment, DTH was induced by application of the antigen oxazolone (Sigma, St. Louis, MO, USA) to the ear pinna of squirrel following initial immunization by applying 100 μ l of oxazolone [5% (wt/vol)] on shaved abdominal area for two consecutive days. On day 6, ear thickness was measured with a Vernier calliper to determine baseline thickness, and oxazolone immune response was challenged by applying 50 μ l of oxazolone [0.5% (wt/vol) in 4:1, acetone: olive oil] to the skin of the dorsal surface of the right pinna. Left ear was treated with vehicle alone. Ear swellings were measured after every 24 h for the consecutive 3 days to record maximum DTH response. On second day, maximum response was observed and it is being shown in the graph. All measurements were made on the same relative region of the ear pinna of the experimental animals. Percent thickness was calculated by comparing differences between treated and non-treated ear pinna [20].

$$\frac{\text{Oxazolone treated ear pinna thickness}}{\text{Vehicle treated ear pinna thickness}} \times 100$$

Total leucocyte and percent lymphocyte count (TLC & % LC). Total leucocyte and lymphocyte (no./mm³) from peripheral blood were counted as described earlier and published elsewhere [21, 22].

Blastogenic response (in terms of % stimulation ratio). Tissue culture medium RPMI-1640 and all other chemical were purchased from Sigma Chemicals, USA. The culture medium was supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% foetal calf serum. Spleen and thymus were dissected out and processed for preparation of single cell suspension. The cell suspension were suspended in ice-cold RPMI-1640 culture medium, and the erythrocytes were lysed by hypotonic shock using equal volume of cold ammonium chloride tris-buffer (tris hydroxymethylene aminomethane; SRL, Mumbai, India); 0.5% tris buffer and 0.84% NH₄Cl mixed in 1:10 ratio; pH 7.2). The cell suspension was washed in culture medium. Two millilitres of cell suspension (1 \times 10⁶ cells/ml) was placed in duplicate culture tubes and kept at 37 °C in a 5% CO₂ incubator for 72 h. Blastogenic response was measured in terms of [³H] thymidine (specific activity 8.9 Ci/mM; BARC, India) uptake against stimulation by Concanavalin A (Con A; T cell mitogen; Sigma) of the splenocytes and thymocytes [20, 23].

$$\%SR = \frac{\text{CPM with Con A}}{\text{CPM without Con A}} \times 100$$

Radioimmunoassay. Radioimmunoassay of testosterone was performed according to the manufacturer's instruction (Immunotech, Mersielles Cedex, France). The intra and inter assay variation was 14.8% and 15%, respectively.

The sensitivity was in between 0.025 and 20 ng/ml, and recovery percentage was between 91 and 117.

Validation of melatonin (mt1R & mt2R) and androgen receptor (AR) antibody. Validation for melatonin receptor antibodies in this species has been performed as reported and published elsewhere [24]. mt1 and mt2R antibody was purchased from Santa Cruz, Biotech (mt1R; R-18, sc-13186 & mt2R; T-18, sc-13177; Santa Cruz Biotech, California, USA). Antibodies against melatonin receptor subtypes (mt1R and mt2R) were validated for the use in the spleen and thymus of Indian palm squirrel with the use of these antibodies in the rat brain as positive control. Immunoblot analysis for both mt1R and mt2R in spleen and thymus showed a single band between 35 and 40 kDa, corresponding to similar band obtained in rat brain used as positive control [24].

Similarly, AR antibody was purchased from Santa Cruz, Biotech (AR, N-20; Santa-Cruz Biotech) and validated for use in the thymus of *F. pennanti* with immunoblot analysis performed in rat prostate as positive control. Immunoblot analysis for AR in thymus of squirrel showed a single band between 100 and 120 kDa corresponding to similar band obtained in rat prostate used as positive control (data not shown). No AR expression was observed in spleen tissue.

Western blot analysis. Western blot was performed as published elsewhere [24]. Spleen and thymus was homogenized and lysed in lysis buffer [RIPA buffer containing aprotinin, sodium orthovanadate and phenylmethylsulfonyl fluoride (PMSF)] and quantified by Bradford method [25]. Aliquots containing 60 μ g protein for melatonin receptor subtypes (mt1R and mt2R) and 100 μ g for AR were resolved with 12% (for Mel receptor) and 10% (for AR) SDS-polyacrylamide gel electrophoresis respectively then followed by electrotransfer to nitrocellulose membrane (Bioscience, Keene NH, USA). Immunodetection was carried out by using melatonin receptor antibodies (mt1R, R-18, & mt2R, T-18, diluted 1:200; Santa Cruz Biotech), AR antibody (AR, N-20, diluted 1:250; Santa Cruz Biotech) and β -actin antibody (A-2228, diluted 1:1000; Sigma-Aldrich Chemicals) all were diluted in phosphate buffer saline (PBS; 0.1 M NaH₂PO₄, Na₂HPO₄, NaCl; pH 7.4) containing 5% skimmed milk and 0.1% Tween-20 followed by horseradish peroxidase-conjugated secondary antibody (donkey anti-goat IgG, for melatonin receptor subtypes, donkey anti-rabbit IgG for AR and donkey anti-mouse IgG for β -actin, diluted 1:10,000), which were further, detected using Super Signal West Pico Chemiluminescent Substrate (# 34080; Thermo Scientific, Rockford, IL, USA). Bands were quantified by measurement of optical density using Scion Image Analysis Software (Scion Corporation, MD, USA). Values were expressed as the ratio of the density of the specific signal to β -actin signal [24].

In-vitro study. Thymocyte culture was performed with four groups as follows:

Group I	Control (Con)
Group II	Melatonin treated (M)
Group III	Testosterone treated (T)
Group IV	Melatonin + testosterone (M+T)

MTT assay for thymocyte proliferation. Cell-mediated immune function was assessed by measuring thymocyte proliferation in response to the T-cell mitogen, concanavalin-A (Con-A), using a colorimetric assay based on the reduction of tetrazolium salt 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [26]. Thymus of squirrels were removed in sterile condition and single-cell suspensions were prepared by mincing it and grinding the mince between sterile frosted glass slides. Erythrocytes were lysed by hypotonic shock using equal volume of cold ammonium chloride tris buffer (tris hydroxymethylene aminomethane; SRL); 0.5% tris buffer and 0.84% NH₄Cl mixed in 1:10 ratio; pH 7.2). This single-cell suspension along with ice-cold culture medium (RPMI-1640: Hepes supplemented with 1% penicillin (5000 U/ml) streptomycin (100 µg/ml), 1% L-glutamine (2 mM/ml), 0.1% 2-mercaptoethanol (5 × 10⁻² M/ml), and 10% heat-inactivated foetal calf serum) was washed three times, and thymocyte counts and viability were determined with a haemocytometer and trypan blue exclusion. Viable cells (which exceeded 95%) were adjusted to 1 × 10⁷ cells/ml by dilution with culture medium, and 50-µl aliquots of each cell suspension were added to the wells of sterile flat-bottom 96-well culture plates. Concanavalin-A (Sigma-Aldrich) was added to the culture medium at the concentration of 10 µg/ml. A volume of 50 µl of each mitogen concentration was added to the wells of the plate that would contain the thymus cell suspensions to yield a final volume of 100 µl/well (each in duplicate) for those wells to which melatonin/testosterone or both was not added. Melatonin and testosterone (Sigma-Aldrich) was dissolved in absolute ethanol and diluted in RPMI-1640 medium to a standard stock solution. One hundred microlitres of melatonin (500 pg/ml) [14], testosterone (3 ng/ml) [27] individually or 50 µl each when added together was added to half of the wells that would contain the thymus cell suspensions to yield a final volume of 200 µl/well (each in duplicate). Con-A, melatonin and testosterone were added to the plates prior to the addition of cells. Plates were incubated at 37 °C with 5% CO₂ for 69 h prior to addition of 10 µl of MTT (5 mg/ml in phosphate-buffered saline; SRL, Bombay, India) per well. Plates were then incubated at 37 °C with 5% CO₂ for an additional 4 h. At 72 h, 160 µl of acid propanol (0.04 mol/l HCL in isopropanol) was

added to each culture and the optical density (OD) of each well was determined with a microplate reader (ELx-800; Biotek Instruments, Winooski, VT, USA) equipped with a 570-nm wavelength filter. Mean OD values for each set of duplicates were used in subsequent statistical analyses. Response was calculated as percent stimulation index representing the ratio of absorbance of mitogen-stimulated cultures to control cultures.

Western blot analysis-in-vitro. Thymus of squirrels was removed in sterile condition and thymocytes were collected by routine method as explained earlier. Cells were suspended in RPMI-1640 medium at a concentration of 1 × 10⁷ cell/ml. The 100 µl of suspension, 100 µl of melatonin (500 pg/ml)/testosterone (3 ng/ml) or both and rest RPMI-1640 medium was added to each well of 24-well culture plate, respectively to make the final volume of 1 ml. The cultures were centrifuged (706 g for 10 min.) after incubation at 37 °C, 5% CO₂ for 48 h. The cell suspension following 48-h incubation at 37 °C of all the groups were taken and centrifuged at 2500 rpm to collect cell pellet. Cell pellets were washed in phosphate-buffered saline (0.1 M; pH 7.4) by centrifuging them at 2500 rpm for 10 min. A homogenate was made with lysis buffer (1% NP40 (W/V), 0.1% SDS, 0.1% Aprotinin, 20 µl of 50 mM sodium orthovanadate, 1% PMSF in PBS) and mixed well, centrifuged at 12,000 g for 10 min at 4 °C. The protein content of the supernatant was determined using Bradford method [25]. A concentration of 25 µg of protein for mt1R and 40 µg for AR was run on a 12% and 10% gel, respectively. Gel with pre-stained marker (Fermentas, MD, USA) was electrotransferred (Fastblot; Biometra, Goettingen, Germany) on to nitrocellulose membrane followed by immunodetection by anti mt1R, anti mt2R antibody (mt1R;R-18; sc13186;mt2R; T-18; sc-13177; dilution, 1:200; Santa-Cruz Biotech) and anti AR (AR; N-20, dilution, 1:200; Santa-Cruz Biotech). Rest methodology and immunodetection was performed in a similar way as explained earlier. Bands were quantified by measurement of optical density using Scion Image Analysis Software (Scion Corporation, MD, USA). Values were expressed as the ratio of the density of the specific signal to β-actin signal.

IL-2 production and analysis in culture supernatant. IL-2 activity was estimated by the activated squirrel thymocyte proliferation. Thymus of squirrels was removed in sterile condition. Thymocytes were collected by routine method as explained earlier. Then the cells were suspended in RPMI-1640 medium at a concentration of 1 × 10⁷ cell/ml. The 100 µl of suspension, 100 µl of Con-A with final concentration of 100 µg/ml, and 800 µl RPMI-1640 medium were added to each well of 24-well culture plate, respectively, final volume 1 ml. The cultures were centrifuged (500 × g, 10 min.) after incubation at 37 °C, 5% CO₂ for

48 h. The supernatants were collected and preserved at -20°C for IL-2 estimation.

ELISA for IL-2. Sandwich ELISA was performed to quantify level of IL-2 in culture supernatants collected from all the four groups according to manufacturer's instruction (Immunotech). Intra assay variation was between 3.3% and 7.2% and inter assay variation was between 6.2% and 8.2%; sensitivity; 5 pg/ml and recovery was between 80% and 132%.

Statistical analysis. Statistical analysis of the data was performed with one-way ANOVA followed by Student Newman-Keuls multiple range test. The differences were considered significant when $P < 0.05$.

Results: *In-vivo* study

Body weight

Following castration and melatonin treatment, no significant effect on body weight was noted when compared to sham control squirrels. Melatonin and testosterone (Aquaviron) alone treatment to the castrated squirrels significantly ($P < 0.05$; $P < 0.01$ respectively) increased body weight, whereas combined treatment had no effect on body weight of castrated squirrels when compared with sham controls (Table 1). Significant increase ($P < 0.01$) in body weight was observed in groups treated with melatonin and testosterone alone or in combination when compared with castrated group.

Lymphoid organs weight

Significant ($P < 0.01$) increase in spleen weight was noted following melatonin treatment and castration when compared with sham-operated control group. Significant increase ($P < 0.01$) was observed when melatonin alone or in combination with testosterone given to squirrels compared to both castrated and sham control squirrels,

while the castrated squirrels treated with testosterone alone showed no significant change in spleen weight compared to sham control but showed significant decrease when compared with castrated squirrels (Table 1).

Thymus weight followed a similar pattern. Castrated group showed hypertrophy of thymus, testosterone treatment decreased thymus weight to the sham-operated control level. Melatonin in combination or alone significantly increased ($P < 0.05$) thymus weight more than testosterone treated and sham control group (Table 1).

Total leucocyte & % lymphocyte count

Significant increase in circulating total leucocyte and lymphocyte count was noted in melatonin-treated squirrels and castrated squirrels when compared with sham control group ($P < 0.01$) (Table 1), while testosterone treatment decreased the count to the control level. Melatonin alone or in combination with testosterone significantly increased the total leucocyte and % lymphocyte count in comparison with both sham-operated squirrels and castrated squirrels ($P < 0.01$) (Table 1).

Blastogenic response (% SR)

Percent stimulation ratio (% SR) denoting the change in cellular immunity (T-helper cells) in splenocytes and thymocytes to the T-cell mitogen concanavalin-A presented a significant increase in both splenocytes and thymocytes following melatonin treatment and castration when compared with sham control group ($P < 0.01$). Melatonin treatment alone or in combination increased % SR in both splenocytes and thymocytes significantly when compared with both sham-operated control and castrated squirrels ($P < 0.01$), while testosterone treatment alone reduced it to the level of sham-operated group significantly compared to castrated group (Fig. 1).

Table 1 Body weight (in grams), spleen & thymus weight (in g/100 g body weight), TLC (as cells/mm³), % LC, % DTH response (as % pinnae thickness), and plasma testosterone level (in ng/ml).

	Con	M	Cx	Cx+Mel	Cx+T	Cx+Mel+T
Body wt. (in grams)	97 ± 8.8	99 ± 5.2	92 ± 6	105 ± 3.6** ^a	115 ± 10.4** ^a	100 ± 4.48 ^a
Spleen wt. (g/100 g body wt.)	0.1702 ± 0.0098	0.205 ± 0.025**	0.2314 ± 0.010**	0.273 ± 0.010** ^a	0.1924 ± 0.010 ^a	0.252 ± 0.008** ^a
Thymus wt. (g/100 g body wt.)	0.147 ± 0.0088	0.194 ± 0.0099**	0.1962 ± 0.0130**	0.174 ± 0.0092** ^a	0.153 ± 0.0084 ^a	0.1874 ± 0.0171** ^a
TLC (cells/mm ³)	5790 ± 268	7690 ± 382**	7500 ± 300**	10030 ± 376** ^a	6600 ± 340 ^a	8650 ± 402.4** ^a
% LC	20.2 ± 2.24	30.5 ± 2.67**	27.8 ± 2.58**	48.4 ± 2.88** ^a	18.6 ± 2.08 ^a	37.8 ± 3.04** ^a
% DTH	135.2 ± 4.96	160 ± 2.89**	153 ± 2.8**	185.4 ± 5.28** ^a	142.2 ± 3.76 ^a	164.2 ± 4.16** ^a
Plasma Testosterone (ng/ml)	2.81 ± 0.210	2.37 ± 0.193*	0.054 ± 0.007	0.051 ± 0.005	3.20 ± 0.150** ^a	1.65 ± 0.094** ^a

TLC, total leucocyte; LC, lymphocyte count; DTH, delayed type hypersensitivity.

Values represent Mean ± SEM; N = 7; * $P < 0.05$; ** $P < 0.01$ Con versus M, Cx, Cx+Mel, Cx+ T, Cx+ Mel+ T; ^a $P < 0.01$ Cx versus Cx+Mel, Cx+ T, Cx+ Mel+ T.

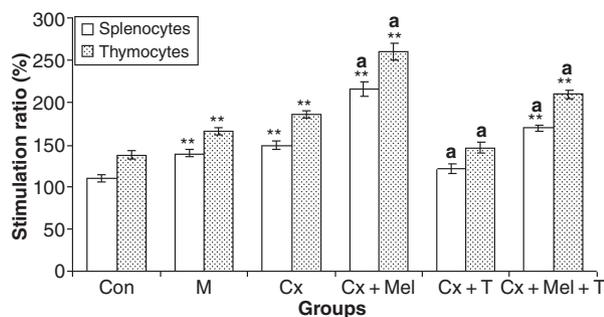


Figure 1 Histogram representing % stimulation ratio (% SR) in splenocytes and thymocytes of sham operated (Con), melatonin treated (M), castrated (Cx), castrated with melatonin (Cx+Mel), testosterone (Cx+T) and castrated with both melatonin and testosterone (Cx+Mel+T) treated *Funambulus pennanti*. Histogram represent Mean \pm SEM, N = 7. Vertical bar on histograms represents standard error. Significance of difference; ** $P < 0.01$, Con versus M, Cx, Cx+Mel, Cx+T & Cx+Mel+T; * $P < 0.01$, Cx versus Cx+Mel, Cx+T & Cx+Mel+T.

Hormonal analysis

Radioimmunoassay of plasma testosterone presented non-significant change after melatonin treatment, while non-detectable level was noted in circulation of castrated group squirrels compared to control squirrels. No change in plasma testosterone level was noted in castrated group treated with melatonin alone but significant increase ($P < 0.01$) was recorded in groups of castrated squirrels treated with testosterone (Cx+T) alone compared to both sham control and castrated group. Testosterone treatment along with melatonin (Cx+Mel+T) to castrated squirrels significantly decreased ($P < 0.01$) circulatory testosterone level compared to sham control but increased it significantly ($P < 0.01$) when compared with castrated squirrels (Table 1).

Western blot analysis

To check the expression of melatonin receptor subtypes (mt1R & mt2R) and AR in lymphoid tissues, i.e., spleen and thymus of *F. pennanti* at translational level, we performed western blot analysis. Melatonin receptor (mt1R & mt2R) proteins as a single band between 35 and 40 kDa was detected that precisely corresponds to the predicted molecular mass of the receptor [7, 24, 28] and AR protein as a band between 100 and 120 kDa in thymus only as we detected no specific band of AR in spleen tissue.

Significant increase ($P < 0.01$) in mt1R expression (in terms of % band intensity) in spleen was observed following melatonin treatment and castration when compared with sham-operated squirrels. Castrated squirrels treated with melatonin alone showed significant increase in mt1R expression when compared with both sham control and castrated squirrels, while significant decrease ($P < 0.01$) in expression was recorded in castrated group treated with testosterone compared to sham control and castrated group. Combine treatment of both melatonin and testosterone caused significant decrease ($P < 0.01$) in expression of mt1 receptor when compared with castrated, while no significant change was observed in comparison with sham control group (Fig. 2).

Similarly in thymus, mt1 receptor showed same pattern as that of spleen with significant changes (Fig. 3). In both spleen and thymus, castration with melatonin treatment showed maximum mt1R expression in comparison with all other groups (Figs. 2 and 3). Although mt2R showed high expression when compared to mt1R in both spleen and thymus, no significant change has been recorded in its expression pattern (in terms of %

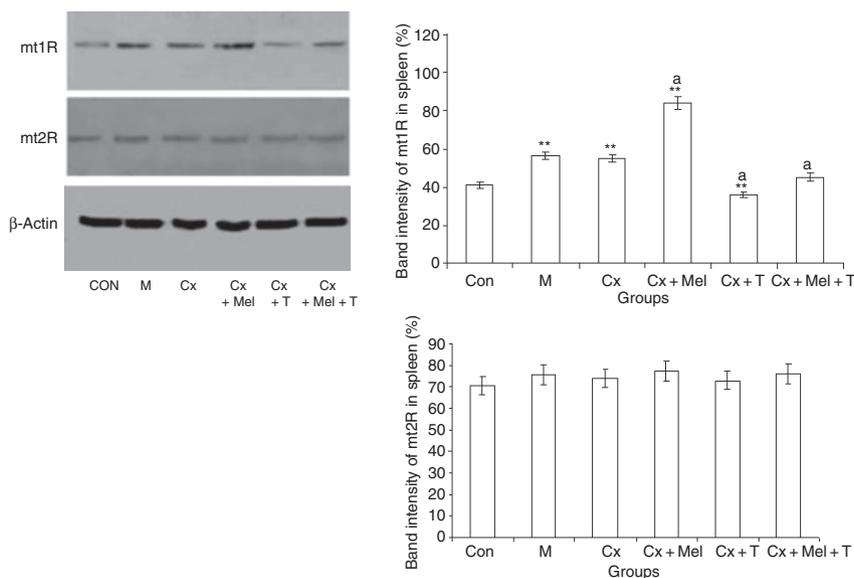


Figure 2 Western blot analysis of mt1 & mt2 receptor expression in spleen of sham operated (Con), melatonin treated (M), castrated (Cx), castrated with melatonin (Cx+Mel), testosterone (Cx+T) and castrated with both melatonin and testosterone (Cx+Mel+T) treated *Funambulus pennanti*. β -actin was used as loading control. Left panel shows the blots, while right and lower panel shows the % band intensity of mt1 & mt2 receptor respectively following Scion image analysis. Histogram represent Mean \pm SEM, N = 7. Vertical bar on histograms represents standard error. Significance of difference; ** $P < 0.01$, Con versus M, Cx, Cx+Mel, Cx+T & Cx+Mel+T; * $P < 0.01$, Cx versus Cx+Mel, Cx+T & Cx+Mel+T.

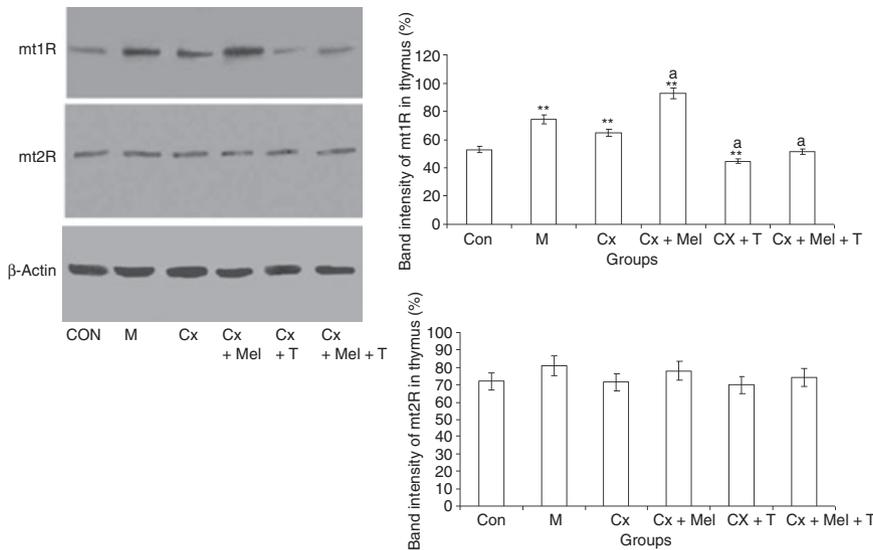


Figure 3 Western blot analysis of mt1 & mt2 receptor expression in thymus of sham operated (Con), melatonin treated (M), castrated (Cx), castrated with melatonin treatment (Cx+Mel), testosterone (Cx+T), and castrated with both melatonin and testosterone (Cx+Mel+T) treated *Funambulus pennanti*. β -actin was used as loading control. Left panel shows the blots, while right and lower panel shows % band intensity of mt1 & mt2 receptor respectively following Scion image analysis. Histogram represent Mean \pm SEM, N = 7. Vertical bar on histograms represents standard error. Significance of difference; ** $P < 0.01$, Con versus M, Cx, Cx+Mel, Cx+T & Cx+Mel+T; ^a $P < 0.01$, Cx versus Cx+Mel, Cx+T & Cx+Mel+T.

band intensity) in any group when compared with sham-operated control and castrated squirrels (Figs. 2 and 3).

AR expression (in terms of % band intensity) showed significant decrease ($P < 0.01$) in expression following melatonin treatment and castration when compared with sham control squirrels. Melatonin alone or in combination with testosterone showed significant decrease, in AR expression when compared with both sham control ($P < 0.01$) and castrated squirrels ($P < 0.05$). Squirrels treated with testosterone alone showed significant decrease in AR expression compared to sham control but increased significantly compared to castrated squirrels (Fig. 4). We did not find expression of AR in spleen tissue.

Delayed-type hypersensitivity

Significant increase ($P < 0.01$) in DTH was observed in squirrels following melatonin treatment and castration when compared with sham-operated squirrels. Melatonin administration alone or in combination with testosterone further increased DTH response significantly ($P < 0.01$) when compared with both sham control and castrated squirrels. Testosterone treatment had no significant effect in comparison with sham control, while significant decrease was observed when compared with castrated squirrels ($P < 0.01$) (Table 1).

Results: *In-vitro* study

Thymocyte proliferation

Thymocyte proliferation (in terms of % stimulation index) increased significantly ($P < 0.05$) following melatonin treatment, while testosterone decreased it significantly ($P < 0.05$). Combined treatment of melatonin and testosterone also increased the proliferation significantly ($P < 0.05$) when compared with control group (Fig. 5).

Western blot analysis

mt1R expression increased significantly ($P < 0.01$) following melatonin treatment and decreased significantly ($P < 0.01$) when testosterone was added to the thymocytes compared to control group. Addition of both melatonin and testosterone together bring the mt1R expression to the control level and showed no significant

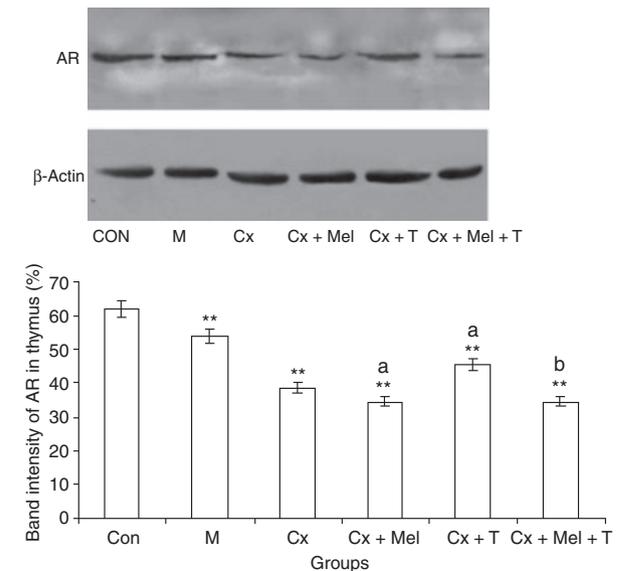


Figure 4 Western blot analysis of androgen receptor (AR) expression in thymus of sham operated (Con), melatonin treated (M), castrated (Cx), castrated with melatonin treatment (Cx+Mel), testosterone (Cx+T), and castrated with both melatonin and testosterone (Cx+Mel+T) treated *Funambulus pennanti*. β -actin was used as loading control. Lower panel shows the % band intensity of AR following Scion image analysis. Histogram represents Mean \pm SEM, N = 7. Vertical bar on histograms represents standard error. Significance of difference; ** $P < 0.01$, Con versus M, Cx, Cx+Mel, Cx+T, Cx+Mel+T; ^a $P < 0.01$ and ^b $P < 0.05$, Cx versus Cx+Mel, Cx+T & Cx+Mel+T.

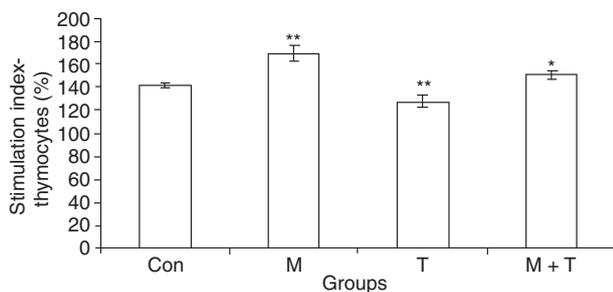


Figure 5 Histogram representing % stimulation ratio (% SR) in thymocytes of control (Con), melatonin treated (M), testosterone (T), and with both melatonin and testosterone treated (M+T) *Funambulus pennanti*. Histogram represents Mean \pm SEM, N = 7. Vertical bar on histograms represents standard error. Significance of difference; * $P < 0.05$; ** $P < 0.01$, Con versus M, T & M+T.

change compared to control group. Though mt2R expression recorded high in all the groups, but no significant change in expression was observed in any group when compared with control group (Fig. 6).

AR expression was decreased significantly ($P < 0.01$) following melatonin treatment, while testosterone increased its expression significantly ($P < 0.01$) when compared with control group. Both melatonin and testosterone together bring back the AR expression to the control level (Fig. 7).

IL-2 assay

Interleukin-2 in culture supernatant increased significantly ($P < 0.01$) following melatonin treatment, while testosterone decreased it significantly ($P < 0.05$). Both melatonin and testosterone again increased the IL-2 level significantly ($P < 0.05$) when compared with control group (Fig. 8).

Discussion

Testosterone generally suppresses immune function in mammals and birds [1, 29, 30] but is equally important for the healthy reproduction and survival. Our current knowledge regarding the effect of sex steroid hormones on immune function is relatively limited to that of mice, rats and humans [31, 32]; studies have never been extended to any tropical seasonal breeder where nature acts as a contraceptive and protects the animals from the prolonged exposure to testosterone that may negatively influence the physical ability and longevity. Our study provides some evidences for the involvement of melatonin and melatonin receptors (mt1R & mt2R) expression and its interplay with AR expression in lymphoid organs (spleen & thymus) that play an important role in mediating seasonal adjustment in immunity in tropical rodent *F. pennanti* [20].

The trade-off relationship between melatonin and gonadal steroids in seasonally breeding rodents has already been accepted [1, 2, 20, 21]. We propose that not only these two hormones but their receptors also respond differentially to different experimental conditions in a tropical rodent because of a cross-talk between circulating melatonin and testosterone and their receptors on lymphoid tissues (spleen and thymus). Melatonin is immunostimulatory in nature when injected *in-vivo* to this tropical rodent [22] but what specific relation exists between the two hormones at translational level has never been explored. Mostly melatonin acts directly through its two G-protein coupled membrane receptors on immune cells and is having an inverse relation with circulating gonadal steroids. Therefore, alteration of gonadal function indirectly may affect immune function.

Presence of melatonin membrane receptors on lymphocytes is known to regulate both humoral and cellular immunity [9, 33, 34]. We have recently reported locali-

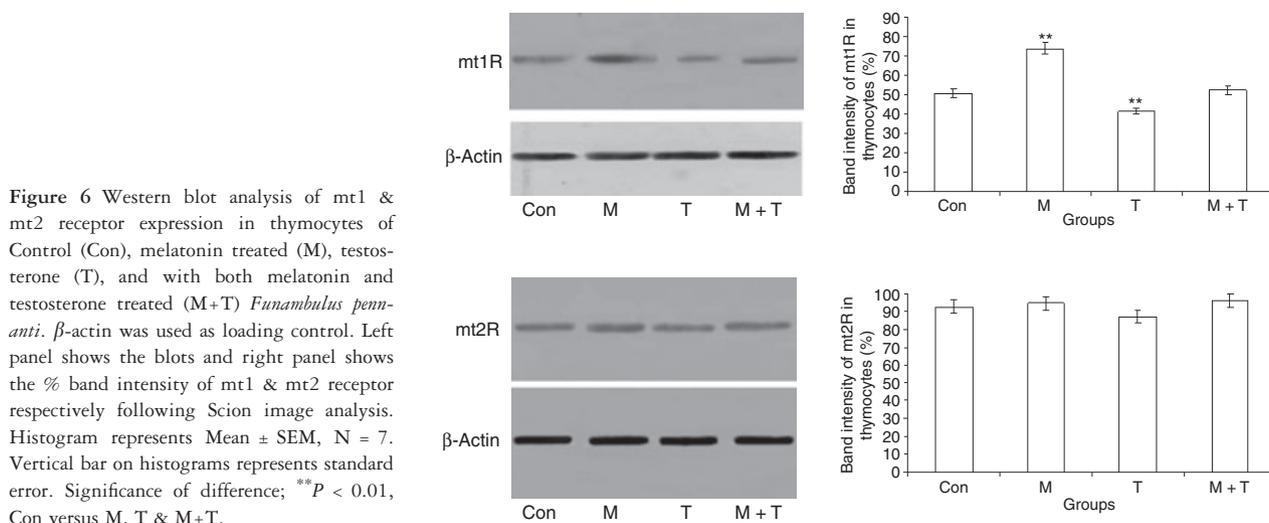


Figure 6 Western blot analysis of mt1 & mt2 receptor expression in thymocytes of Control (Con), melatonin treated (M), testosterone (T), and with both melatonin and testosterone treated (M+T) *Funambulus pennanti*. β -actin was used as loading control. Left panel shows the blots and right panel shows the % band intensity of mt1 & mt2 receptor respectively following Scion image analysis. Histogram represents Mean \pm SEM, N = 7. Vertical bar on histograms represents standard error. Significance of difference; ** $P < 0.01$, Con versus M, T & M+T.

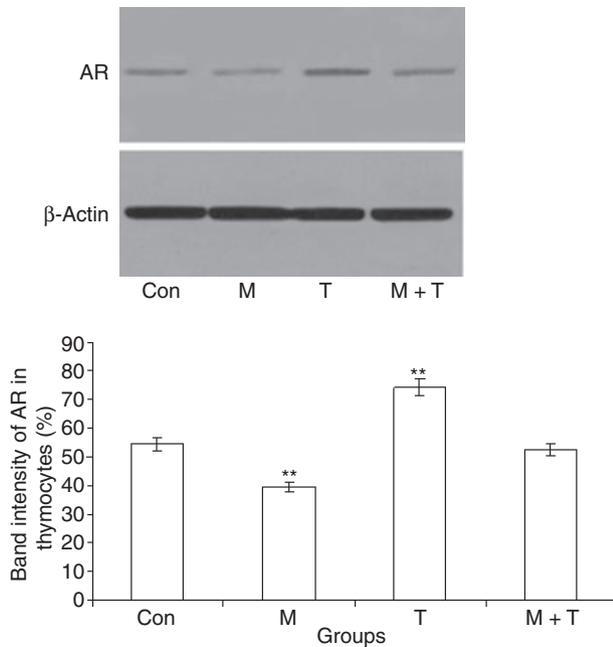


Figure 7 Western blot analysis of androgen receptor (AR) expression in thymocytes of Control (Con), melatonin treated (M), testosterone (T), and with both melatonin and testosterone treated (M+T) *Funambulus pennanti*. β -actin was used as loading control. Upper panel shows the blot and lower panel shows % band intensity of AR following Scion image analysis. Histogram represent Mean \pm SEM, N = 7. Vertical bar on histograms represents standard error. Significance of difference; ** $P < 0.01$, Con versus M, T & M+T.

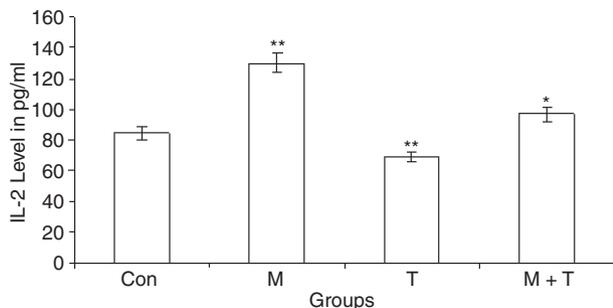


Figure 8 Histogram representing IL-2 level in culture medium of thymocytes of Control (Con), melatonin treated (M), testosterone treated (T), and with both melatonin and testosterone (M+T) treatment to *Funambulus pennanti*. Histogram represents Mean \pm SEM, N = 7. Vertical bar on histograms represent standard error. Significance of difference; * $P < 0.05$; ** $P < 0.01$, Con versus M, T & M+T.

zation of melatonin membrane receptors (mt1R & mt2R) in spleen and thymus of *F. pennanti* [24]. In the present study, we observed a significant increase in expression of mt1R following melatonin treatment to castrated squirrels, though castration alone increased cell mediating immunity in terms of percent stimulation ratio in splenocytes and thymocytes of squirrels. Testosterone

administration to castrated group reduced cell-mediated immunity significantly because of its immunosuppressive effect [1, 29, 30]. Further, the result of delayed-type hypersensitivity supported our view by increasing T-helper memory cells following castration and melatonin treatment in squirrels.

The hypothalamo-pituitary-pineal-gonadal (HPPG) axis is known to modulate the immune system [35]. This reciprocal cross-talk allows the maintenance of both the immune system and endocrine systems within narrow limits and may contribute to interplay in regulation of the seasonal variation in two mega events, i.e. reproduction and immunity. During short photoperiod of winter, circulating androgen level decreases and duration of melatonin secretion increases in most of the seasonal breeding mammals; hence, short photoperiodic treatment could be equivalent to the functional castration that increased peripheral melatonin level and decreased testosterone [36], which enhanced the immune function in this rodent. Significant increase in melatonin receptor (mt1) expression on lymphocytes in spleen and thymus might be responsible for stimulatory effect, while AR expression in thymus might have only a regulatory effect in cellular immunity. Further, absence of AR on circulating lymphocytes suggests that the androgenic effects on lymphocytes may be indirect *via* aromatization of androgens to oestrogens and is a novel aspect to study in near future [37]. Melatonin receptors have been localized on spleen and thymus of rodents [10, 24, 38] which might have accounted for the enhanced cell proliferation and caused increase in spleen and thymus weight following melatonin treatment in castrated and melatonin-treated squirrels.

In order to be specific with action of melatonin and androgen through their receptors on lymphoid organs, we accessed quantitative expression of melatonin receptor subtypes (mt1R & mt2R) and AR. When compared to mt1R, the expression studies on mt2R has been less reported. We found a prominent expression of mt2 receptor in both spleen and thymus, while mt1 receptor expression significantly varied according to experimental conditions. Castration and melatonin treatment increased the expression of mt1 receptor when compared to sham control squirrels. Melatonin alone or in combination with testosterone significantly increased the expression of mt1R in both spleen and thymus, while testosterone treatment alone decreased it compared to castrated squirrels. Melatonin mt2 receptor showed relatively high expression in both spleen and thymus than that of mt1R but no significant change has been observed suggesting importance of mt1R in modulating cellular immunity in *F. pennanti*.

We have also checked AR expression in both lymphatic tissues spleen and thymus. We did not observe expression of AR in spleen but a significant change has

been noted in thymus. AR expression decreased significantly in thymus following castration and melatonin treatment. Testosterone alone or in combination with melatonin decreased expression when compared with sham control group. Thus, a decrease in AR expression complemented the increase in mt1R expression, which truly signifies their 'trade-off' relation at least in thymus. Thymus is a primary lymphoid organ, and unlike other rodents the *F. pennanti* showed functional thymus even at adult stage [21]; thus, this change in expression pattern of mt1R and AR in thymus highlights its role in mediating cell-mediated immunity in response to different seasonal and experimental conditions.

To strengthen further 'trade-off' hypothesis, we performed *in-vitro* study with thymocytes and checked the expression of melatonin and AR along with thymocyte proliferation and interleukin secretion (IL-2), a cytokine generally secreted by lymphocytes in response to melatonin (34). Melatonin treatment increased, while testosterone alone decreased the mt1R expression. Combined treatment of melatonin and testosterone decreased mt1R expression to the control level. Though mt2 receptor expression was high, but no significant change has been observed in any group when compared to control group. Addition of melatonin to the cell suspension decreased AR expression, while testosterone increased it significantly. Combined treatment of both the hormones decreased AR expression to the control level. mt1R increased expression (and decreased AR expression) might have increased the thymocyte proliferation as well as IL-2 secretion. Decreased mt1R and increased AR expression might have decreased the thymocyte proliferation and IL-2 level in thymocyte culture. This interplay between mt1R and AR expression pattern modulated the cell-mediated immunity in terms of thymocyte proliferation and interleukin secretion, which are well-known markers of cell-mediated immunity, thus suggesting a 'trade-off' relation in expression of AR and mt1 receptor in thymus being responsible for increased cell-mediated immunity following castration and melatonin treatment both *in-vivo* and *in-vitro*.

Melatonin has been proposed to regulate the immune system by affecting cytokine production of immunocompetent cells [39]. The observed increase in lymphocyte proliferation in spleen, thymus and IL-2 level could be attributed to the increased production of Th-1 cells in thymocytes following melatonin treatment. Our results also suggest that castration might have up-regulated mt1 receptor expression highlighting the prominent role of mt1R in mediating immune enhancement when there is less or no androgen in circulation (with low expression of AR). In order to assign more prominent role to mt1R in cell-mediated immunity, thymus could be the centre for the interplay between mt1R and AR at translational

level. It has been earlier reported that in mice mt2R modulates cellular immunity [40]; however, in tropical rodents, mt1R might be responsible for the regulation of cell-mediated immune function as it has presented strong immunoreactivity when compared to mt2R in both spleen and thymus [24]. Further, the role of mt2R could not be ruled out completely as it showed high and fluctuating expression (both *in-vivo* and *in-vitro*) in response to melatonin and testosterone treatment. For specific answer, some more study with selective melatonin receptor antagonist is needed for tropical rodents.

Finally, the HPG-thymic and HPG-splenic axis appears to play a significant role in the mechanism by which the sex hormones regulate the immune response and implies that important interaction exist between nervous system, endocrine system and the immune system. Our present study provides a clear picture of 'cross talk' between two important hormones, i. e. melatonin and testosterone along with their receptor expression pattern both *in-vivo* and *in-vitro* during different environmental and physiological conditions that would be helpful in unravelling the exact mechanism behind stimulatory effect of melatonin on immunity. The present study for the first time reports an inverse or 'trade-off' relation between melatonin receptor (mt1R) and AR expression in primary lymphoid tissue thymus, which could be the major regulating centre in mediating cellular immunity during different physiological and seasonal conditions in tropical rodents.

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