

**Thymic involution with age:  
regression of the adrenal reticularis by peripheral T cells**

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## Abbreviations

Adx:	adrenalectomy
DAB:	3,3'-diaminobenzidine
DAPI:	4',6-diamino-2-phenylindole
DEX:	dexamethasone
DHEA:	dehydroepiandrosterone
DP:	double positive
ELISA:	enzyme-linked immunosorbent assay
FITC:	fluorescence
GC:	glucocorticoid
HE:	hematoxylin and eosin
HPA:	hypothalamus-pituitary-adrenal
i.p.	intraperitoneally
MEM:	minimum essential medium
MHC:	major histocompatibility complex
MLs:	Minor lymphocyte stimulating
OCT:	optimal cutting temperature
PBS:	phosphate-buffered saline
PE:	phycoerythrin
s.c.	subcutaneously
SD:	standard deviation
SP:	single positive
TCR:	T cell receptor
ZF:	zona fasciculata
ZG:	zona glomerulosa
ZR:	zona reticularis

## **Abstract**

T cell dependent immune functions gradually decline with age. The thymus that produces T cells involutes after puberty, resulting in declines of thymic functions with age. It is thought that changes in the production of immunoregulatory hormones, including glucocorticoid (GC) that induces thymocyte death, result in thymic involution with age. However, it is possible that these hormones are not enough for this process because the amount of GC in the sera at a normal physiological state rarely increases with age. Recently, mice with abnormalities in the generation of a normal T cell repertoire are reported to not show thymic involution after puberty. Furthermore, the profile of thymic involution is inversely correlated with an increase in peripheral T cells. These results may indicate that a lack of functional, mature T cells in the periphery does not cause age-related thymic involution, suggesting that, during ontogeny, the gradual generation of peripheral T cells that encounter various environmental antigens can be activated and cause the post-puberty involution of the thymus. Based on this idea, I studied involvement of peripheral T cells in thymic involution after puberty. I found that the prevention of T cell generation delayed the initiation of thymic involution. On the other hand, the activation of T cells by anti-CD3 antibodies increased the serum concentration of GC and thymic atrophy, which was completely prevented by adrenalectomy. In the adrenals of growing mice, the activity of the zona fasciculata, which produces GC, increased and plateaued by the weaning period; however, the zona reticularis (ZR), which produces dehydroepiandrosterone (DHEA) that has

anti-GC actions, started to decline just before puberty. These changes reflect the amounts of blood-circulating hormones. Thymic involution was significantly retarded by DHEA administration. The involution of ZR with age was not observed in athymic nude mice. However, the repopulation of T cells resulted in its atrophy. Thymic atrophy after immune stimulation was preceded continuously by the infiltration of activated T cells into the ZR, by the expression of the major histocompatibility complex (MHC) class II on ZR cells, and by the loss of ZR cells by apoptosis. Thus, T cells are involved in thymic involution through an increase in GC activity due to ZR cell-killing.

## Introduction

The immune functions gradually decline with age and mainly occur in T cell dependent immune functions. The functioning of the thymus that produces T cells declines with age after puberty and are associated with the progressive decline of T cell functions. As this process is closely related to the age-dependent generation of several immune disorders (Fig. 1) and ultimately determines life span, an immunological theory for aging has been proposed (1, 2). < Fig.

The commonly observed process of thymic atrophy with age has been thought to be genetically “programmed” in mammals, and the following possible mechanisms have been proposed (3, 4): intrinsic defects in the physiology and cell-signaling of all thymus-comprising cells (5-7); an imbalanced production of certain cytokines by stromal cells within and outside of the thymus that support themselves and/or thymocyte progenitors (8-10); and the generation of immunoregulatory hormones, including growth hormones (11), sex steroids (12), and glucocorticoids (GCs) (13). Among these immunoregulatory hormones, it is thought that growth hormones are not adequate for factors of thymic involution after puberty because the serum level of growth hormones that positively influence the thymic functions peaks for the neonate and gradual decline with age. Sex steroids and GCs, which cause thymic atrophy and increased serum concentration in young adults, may be a promising candidate for the “program” conductor. Although the removal of sex steroids by castration in male mice was shown to delay thymic involution, its effects are transient.

Furthermore, the amount of GC in the sera at a normal physiological state rarely increases with age in several species of mammals (14-16). Thus, it is possible that these hormones are not enough for this process.

Alternatively, factors that prevent the age-related decrease of thymic function or those that cure or retard thymic involution are important issues to address. In this regard, mice with abnormalities in the generation of a normal T cell repertoire are reported to not show thymic involution after puberty; this has been shown for T cell receptor (TCR) transgenic mice that have monoclonal T cells that react to limited kinds of determinants (17) and also in mice that are major histocompatibility complex (MHC) deficient and do not generate mature T cells (18). These results strongly suggest that a lack of functional, mature T cells in the periphery does not cause age-related thymic involution, suggesting that, during ontogeny, the gradual generation of peripheral T cells that encounter various environmental antigens can be activated and cause the post-puberty involution of the thymus.

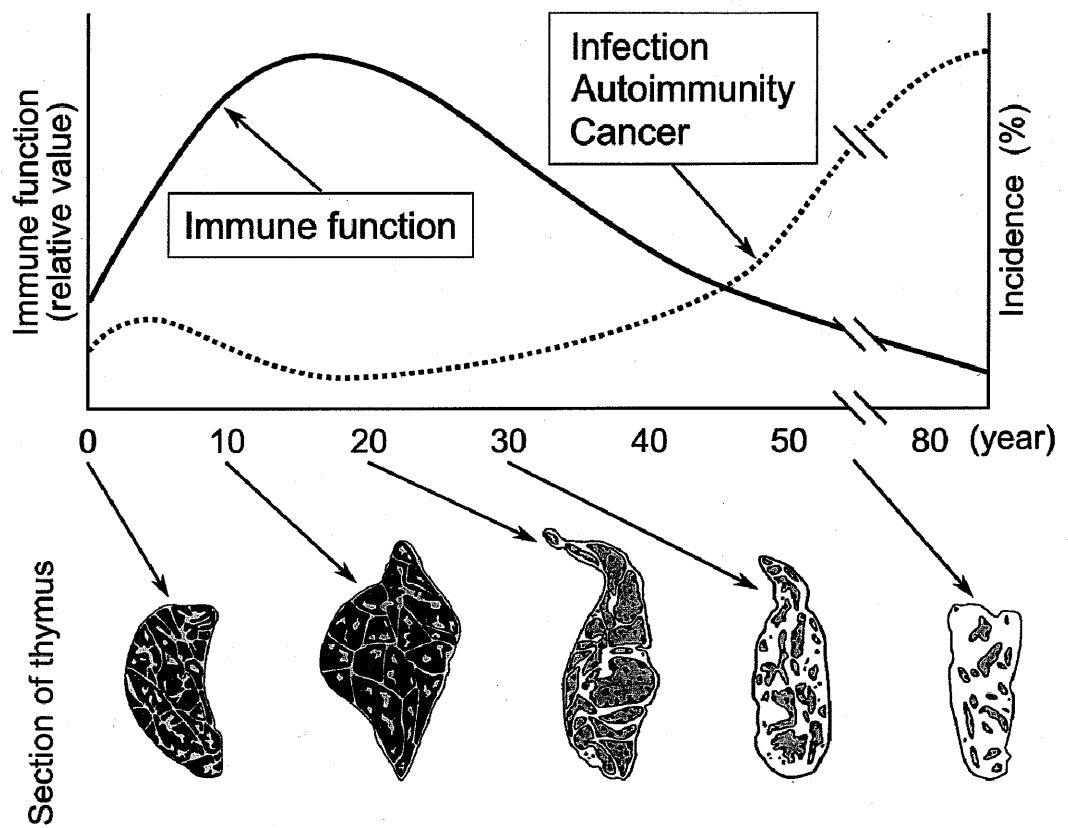
Based on this idea, the removal of peripheral T cells should prevent thymic involution after puberty, or their delayed appearance may cause the thymus to regress later. Yoshida previously treated newborn mice with anti-CD3 monoclonal antibodies to prevent the generation of mature T cells (19). In these mice, the onset of thymic involution was progressively delayed as treatment continued. Therefore, thymic involution was controlled by peripheral T cells that were likely responding to environmental antigens through the hypothalamus-pituitary-adrenal (HPA) axis because thymic involution after administration of anti-CD3 antibodies

into adult mice, which served as a mimic of antigenic stimulation, was completely prevented by adrenalectomy. Thymic involution was also significantly prevented by treatment with metyrapone, an inhibitor of GC synthesis (19), and was indispensably dependent on the GC receptor (20). GC is a significant factor in thymic involution, although this process is not completely dependent on this factor because the amount of GC in the sera does not adequately increase with age (14-16).

After careful examination of the dynamism of the adrenal cortex, I conclude that thymic involution after puberty appears in the age-related decline of dehydroepiandrosterone (DHEA) with anti-GC activity due to the overlooked phenomena of T cell-mediated loss of DHEA production. Therefore, thymic involution after puberty is due to the age-related decline of DHEA, which is due to an effect of peripheral T cells, and the program of thymic involution is induced by immune activation throughout an individual's life.



**Fig. 1.** Impact of the decline in the immune functions due to the age-related thymic involution. The immune functions (solid line) gradually decline with age and occur in T cell dependent immune responses. The progressive decline of T cell functions are closely related to increase in susceptibilities to infection, autoimmune disease, and cancer (dotted line). It is thought that the cause of the age-related decline of T cell functions is thymic involution after puberty. In human, thymus sections stained with H&E show regression for lymphoid tissue that contain a cortex (black) and a medulla (gray) with age. Subsequently, thymus tissue is gradually replaced by fat tissue (white) (21).



## **Materials and methods**

### ***Animals***

BALB/c CrSlc, BALB/c Slc-*nu/nu*, and BALB/c Slc-*nu/+* mice were purchased from Japan SLC (Shizuoka), and maintained and mated in my laboratory. All animal experiments were performed based on the Guidance for Animal Experiments of Niigata University.

### ***Antibodies and reagents***

Anti-CD3 antibodies obtained by ammonium sulfate-cutting at 45% saturation of the culture-supernatant from the 145-2C11 hybridoma were further purified using a DEAE cellulose column and were dissolved in phosphate-buffered saline (PBS); part of this solution was intraperitoneally (i.p.) injected into mice at a dose based on body weight (b.w.) equivalence (typically 5.5 µg/g of b.w.). This dose is suitable for causing thymic atrophy when injected into adult mice (19). Dexamethasone (DEX; Wako, Osaka) was dissolved in 100% ethanol and diluted to 5 mg/ml in PBS, part of which was i.p. injected into each mouse at a dose of 300 µg. DHEA (Sigma chemical, St. Louis, MO, USA) was dissolved in 100% ethanol and diluted to 8 mg/ml in PBS, part of which was either subcutaneously (s.c.) injected into each mouse at a dose of 1.6 mg (22) or was served as drinking water at a dose of 100 µg/ml (23). Antibodies used for flow cytometry or immunohistochemistry included the following: as a blocking solution, normal goat IgG (Rockland, Gilbertsville, PA, USA); as primary antibodies,

phycoerythrin (PE)-labeled rat anti-mouse CD4 (PharMingen, San Diego, USA), biotin-labeled rat anti-mouse CD8 (PharMingen), anti-human CD3 $\epsilon$  (DAKO, Glostrup, Denmark) and rat anti-mouse MHC class II (eBioscience, San Diego, USA); as secondary antibodies, fluorescence (FITC)-streptavidin (Vector Laboratories, Burlingame, CA, USA), FITC-labeled goat anti-rabbit IgG (Bethyl Laboratories, Montgomery, TX, USA) and PE-labeled goat anti-rat IgG (Beckman Coulter, Fullerton, CA, USA).

#### ***Cell suspension and spleen cell transfer***

Single cell suspensions of spleens from the BALB/c-*nu*/+ mice were aseptically prepared by mincing with minimum essential medium (MEM) on a 200-mesh stainless-steel screen. Cell viability was determined by trypan blue-dye exclusion,  $5 \times 10^7$  cells suspended in 50  $\mu$ l and  $8 \times 10^7$  cells suspended in 100  $\mu$ l were i.p. transferred into newborn BALB/c-*nu*/*nu* mice at 7 and 14 days of age.

#### ***Flow cytometric analysis***

Approximately  $1 \times 10^6$  cells from the thymus and spleen were stained with antibodies by direct or indirect methods. Stained cells were analyzed using an EPICS-XL flow cytometer and EXPO 32 software (Beckman Coulter, Miami, USA). Dead cells were gated out by forward and sideward scattering and by propidium iodide staining based on a previous report (19).

### ***Castration***

The castration of 3-week-old BALB/c male mice was performed under anesthesia through a scrotal incision. Sham-operations for control mice were performed using the same procedure but without removing the testes. The castrated and sham-operated mice were killed at the age of 6 weeks.

### ***Adrenalectomy***

Adrenalectomy was performed bilaterally at 6 weeks of age, as described previously (19). The mice were anesthetized, and their abdomens were opened. The adrenal gland was removed with a small pair of ring forceps, and the opening was closed by suture clips. Sham-adrenalectomies were performed in the same way, except with the removal of adrenal glands. After complete recovery from anesthetization, mice were given drinking water containing 0.9% NaCl.

### ***Treatment with DHEA***

To see GC antagonistic activity of DHEA, 5-week-old mice were treated s.c. with DHEA or its vehicle every 2 days. On the second day, mice were given DEX by i.p. injection at the same time. DEX-treated mice were killed 24 h after injection. Control mice were injected s.c. with 10% ethanol using the same procedure. To see the effect on age-related thymic involution, mice were given DHEA in 0.05% ethanol-containing drinking water starting when weaned at 4 weeks of age and were tested at 8 weeks of age. Control mice were served water with an equivalent concentration of ethanol.

### ***Histological examination***

The thymi and adrenal glands were removed and fixed in Bouin's fluid. After fixation for 24 h, thymus weights were measured using an electronic balance. Tissues were embedded in paraffin, and 4- $\mu$ m-thick sections were cut and stained with hematoxylin and eosin (HE) and were then examined by light microscopy. The width of the adrenal cortex on the median sections was measured at a magnification of  $\times 200$  and calculated as an average value of multiple different fields for five sections from each mouse. The volume of zona fasciculata (ZF) and zona reticularis (ZR) were calculated by cubic measurement using  $(ZF + ZR)^3 - ZR^3$  to determine ZF activity and  $ZR^3$  to determine ZR activity. ZF/ZR ratio was determined from the volumes of the ZF and ZR. If necessary, cell density in the ZR was counted and calculated for an average value of 0.01-mm<sup>2</sup> cross-sections.

### ***Immunohistochemistry***

Immunohistochemical staining in paraffin-embedded sections was performed with a Vectastain avidin-biotin peroxidase complex (Elite ABC) kit (Vector Laboratories). Sections were deparaffinized, hydrated, and autoclaved in a 10 mM citrate buffer (pH 7.0) at 121°C for 20 min for heat-induced antigen retrieval. The sections were treated for 30 min in methanol containing 0.3% hydrogen peroxide to block endogenous activities. Non-specific binding sites were blocked with normal goat serum for 30 min. The slides were incubated overnight with rabbit anti-human CD3ε at 4°C, and the biotinylated secondary antibody solution and ABC reagent were each applied for an hour. Peroxidase enzyme activity was detected using 3,3'-diaminobenzidine (DAB) with nickel enhancement (0.2% DAB and 0.05% nickel ammonium sulfate in 50 mM Tris buffer, pH 7.6). The sections were then counterstained with hematoxylin.

Frozen sections (5 μm) of adrenal glands embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Tokyo) were treated with normal goat IgG for 30 min to prevent non-specific staining. The slides were then incubated with rabbit anti-human CD3ε, PE-labeled rat anti-mouse CD4, or rat anti-mouse MHC class II for an hour at room temperature. Then, the sections were incubated with FITC-labeled goat anti-rabbit IgG or PE-labeled goat anti-rat IgG for 30 min. Nuclear DNA was stained with 4',6-diamino-2-phenylindole (DAPI; Sigma-Aldrich) and were then examined using fluorescence microscopy.

### ***Serum hormone measurements***

Serum samples for hormone measurements were obtained from jugular veins of mice between 0900 and 1100 hours. Sera were stored at -80°C until analysis. Concentrations of hormones in the sera were measured by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. The ELISA kit for corticosterone was purchased from Cayman Chemical (Ann Arbor, MI, USA). The ELISA kit for DHEA was obtained from Assay Designs (MI, USA). All samples were assayed in duplicate. The absorbance (OD<sub>415</sub>) was measured using a micro plate reader (Tosoh, Tokyo). Serum hormone concentration (ng/ml) was determined after extrapolation from a standard curve prepared with known concentrations of hormone. Detection limits for corticosterone and DHEA were 24 and 2.9 pg/ml, respectively.

### ***Statistical analysis***

Data are expressed as the mean  $\pm$  standard deviation (SD). Differences were determined by the Student's *t* test. A *p* value < 0.05 was considered significant.



## Results

### *Prevention of peripheral T cell generation delays thymic involution*

I first investigated the general nature of thymus during the aging process. As shown in Fig. 2, cell numbers in the thymus increased early in life, reached a plateau at 4-5 weeks of age, followed by a few weeks of decline toward the base-line level. These changes were accompanied with changes in cell components of the thymus. The numbers of the CD4 and CD8 double positive (DP) immature thymocytes and CD4 or CD8 single positive (SP) mature thymocytes were significantly reduced in aged mice ( $p < 0.01$ ). Alternatively, non-lymphocytes and stromal cells with a CD4 and CD8 double negative phenotype were less sensitive to the effects of aging and were increased relative to their original proportion (Fig. 2b, inset). These phenomena were sharply reflected by thymus histological analysis, particularly the profound decline in cellularity, especially in the cortex where T cells are generated (Fig. 2a, inset).

The age-dependent loss of thymocytes is inversely correlated with the age-dependent increase in peripheral T cells. When comparing the two lines of open circles in Figs. 3a and b, the loss of thymocytes with age becomes apparent, which may be the result of an increased generation of peripheral T cells. Next, I tested this possibility by preventing the generation of peripheral T cells by treating neonates with anti-CD3 monoclonal antibodies to prevent the development of both CD4 and CD8 SP T cells in the thymus (Fig. 3c) and to retard their generation in the periphery (Fig. 3a). Although this treatment will cause "septic" shock when mice are older than

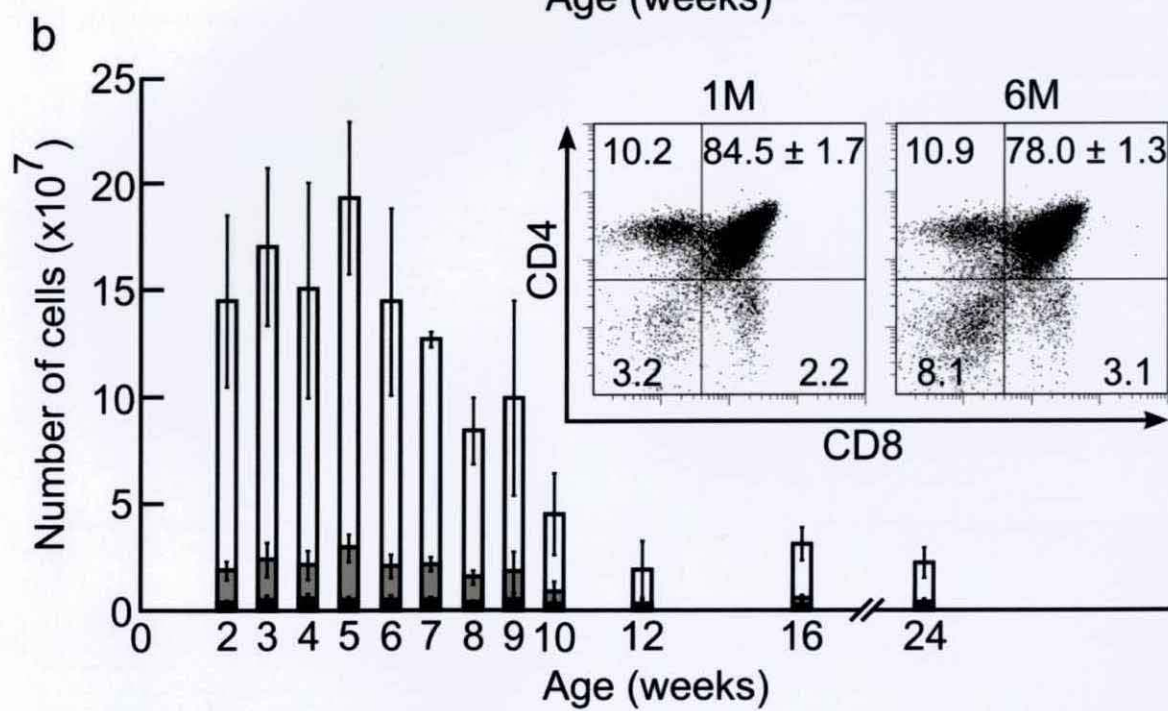
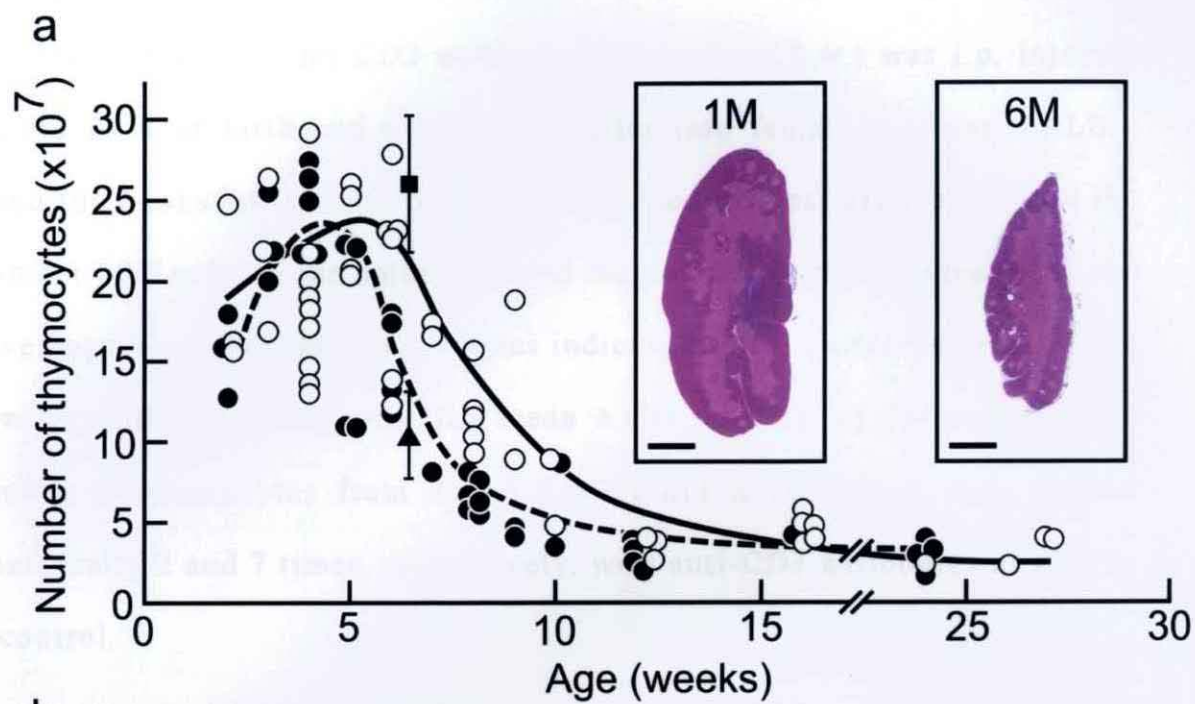
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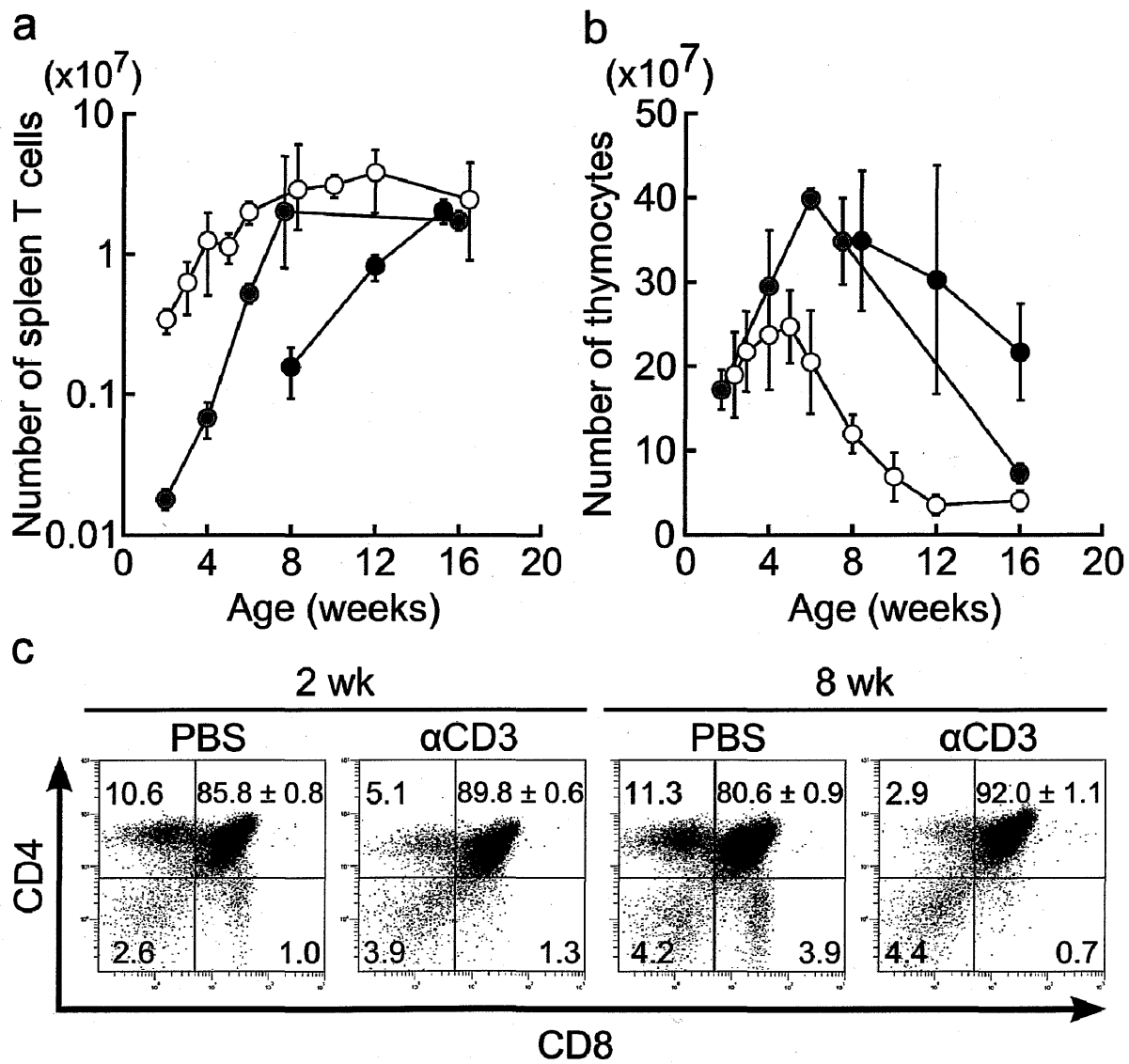
2 weeks, this does not occur in newborns because both the amount of mature T cells and maturity on the HPA axis are insignificant (19). Indeed, 2-week-old mice that have been treated with antibodies weekly after birth showed no significant reduction in thymocytes number ( $17.2 \pm 2.3 \times 10^7$  cells, n=2) compared with age-matched control mice ( $18.9 \pm 5.1 \times 10^7$  cells, n=3).

As shown in Fig. 3c, the longer the continuous treatment of the mice with anti-CD3 antibodies, the longer the suppression of mature T cell development lasted; these dynamic events were closely related to a greater delay in thymic involution (Fig. 3b). Surprisingly, mice that were 12 weeks of age and had been treated with anti-CD3 had an equivalent number of splenic T cells as 4-week-old mice (see closed circles in Fig. 3b).

**Fig. 2.** Age-related changes in the thymi of BALB/c mice. a) Number of thymocytes from female (open circles) and male (closed circles) mice of various ages plotted against age. Closed squares and triangles with a SD bar indicate the number of thymocytes in castrated and sham-operated male mice. HE-stained sections shown are from thymi of 1- and 6-month-old female mice. Scale bars 1 mm. b) Kinetic profiles of the number of CD4 or CD8 single positive (SP, shaded bars), CD4 and CD8 double positive (DP, open bars) and CD4 and CD8 double negative cells (closed bars). Flow cytometric profiles shown are representative of thymocyte subpopulations of 1-month-old (n=8) and 6-month-old (n=4) female mice in reference to CD4- and CD8-expressing cells.



**Fig. 3.** Delay of thymic involution by preventing the generation of peripheral T cells. Anti-CD3 antibody (5.5  $\mu\text{g/g}$  of b.w.) was i.p. injected within 24 h of birth and weekly thereafter into female newborn BALB/c mice for 2 (shaded circles) or 6 weeks (closed circles) after birth, and the number of T cells in the spleen (a) and the number of thymocytes (b) at the given age are indicated. Open circles indicate control untreated group. Each symbol with a bar indicates the mean  $\pm$  SD (n=2-5). c) Flow cytometric profile of thymocytes from 2 and 8-week-old mice, which were treated continually 2 and 7 times, respectively, with anti-CD3 antibodies or PBS as a control.



### ***Involvement of adrenal gland in thymic involution by T cell activation***

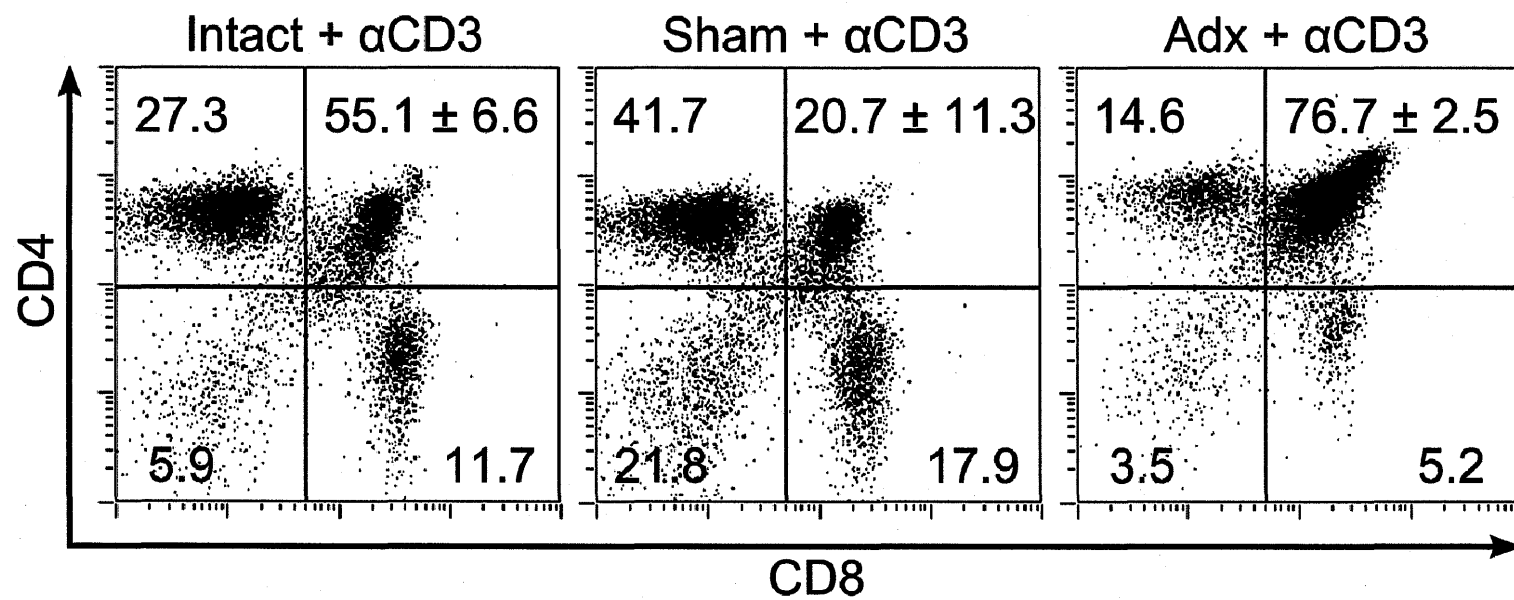
The next question was how the development of mature T cells was related with thymic involution. Because the T cell repertoire generated in growing animals recognizes various kinds of environmental antigens, and because such T cells should be activated when they encounter TCR-corresponding antigens, I modeled T cell stimulation with conventional antigens by inoculating with anti-CD3 monoclonal antibodies.

This treatment resulted in thymic involution 30 h after injection, with a drop in thymus weight from  $75.3 \pm 8.9$  mg (n=5) to  $33.4 \pm 6.3$  mg (n=9) and an 80% reduction in thymocyte number, with a severe loss of immature DP thymocytes ( $82.2 \pm 1.1$  to  $55.1 \pm 6.6$  %, Fig. 4) and strong atrophy in the cortex, as observed in the histological sections of aged thymus (data not shown). At this time, the serum concentration of GC from mice receiving anti-CD3 treatment was still increased relative to the controls (769 ng/ml compared to 131 ng/ml). These cytohistological figures closely resembled those of thymi from animals treated with DEX, an artificial GC chemical, as shown in a previous report (19). The involvement of GC was anticipated, and an adrenalectomy completely prevented the destruction of immature thymocytes (Fig. 4). Anti-CD3 treatment reduced thymus weight to  $29.4 \pm 11.6$  mg (n=6) in the sham-operated mice versus  $63.3 \pm 3.3$  mg (n=3) in the adrenalectomized mice ( $p < 0.01$ ). Even though the results of this experiment would indicate that GC is involved in thymic atrophy, other research has found no correlation between GC concentration and age (14-16). Therefore, I carefully surveyed the developmental histology profiles of the adrenal glands.

< Fig.

**Fig. 4.** Adrenalectomy prevents anti-CD3-activated T cell-mediated thymic atrophy. Adrenalectomized (n=3), sham-operated (n=6), or intact (n=8) mice received i.p. anti-CD3 antibodies at 6 weeks of age and were killed 30 h later; flow cytometric analysis was used to determine the CD4 and CD8 distribution in thymocytes (in cooperation with Ken Gotoh). Figures with profiles represent the cell percentage.





***Decline in DHEA activity due to adrenal reticularis regression after ablactation results in increase of GC activity after puberty***

The adrenal gland consists of the medulla and cortex, the latter being further categorized cytohistologically into the following three layers: zona glomerulosa (ZG), ZF, and ZR (Fig. 5a), which each produce mineral corticoids, GCs, and DHEA, respectively. The cellularities of each zone change in growing mice and the width of each zone are easily visualized in the histological sections. However, the size of the adrenal gland minimally changes for half a year after puberty, but the ratio of the width of ZF to ZR increases significantly during this time. The ZF, which may increase, reaches a plateau around puberty, while the ZR decreases significantly after ablactation, which occurred before puberty in this analysis (Figs. 5b and c). In addition, the number of ZR cells at 16 weeks ( $18 \pm 5$  cells in a given area of the section) was significantly reduced when compared with mice aged 4 weeks ( $53 \pm 4$  cells,  $p < 0.01$ ); the age-dependent change in the ZR area indicates changes in the number of ZR cells because the cell density did not differ.

< Fig. 5

Because the amounts of GC and DHEA in the sera are determined by the corresponding volumes of ZF and ZR, respectively, cubic measurement for each zone width is a more suitable estimate of the hormone production activity. The inset in Fig. 5c indicates the kinetic profiles for the relative volume of ZF and ZR in comparison with the volume at 4 weeks. The plateau GC production activity in the ZF clearly lasts 10 weeks and slightly reduces thereafter. Alternatively, the DHEA production activity in the ZR sharply peaks at 4 weeks of age and quickly drops before puberty.

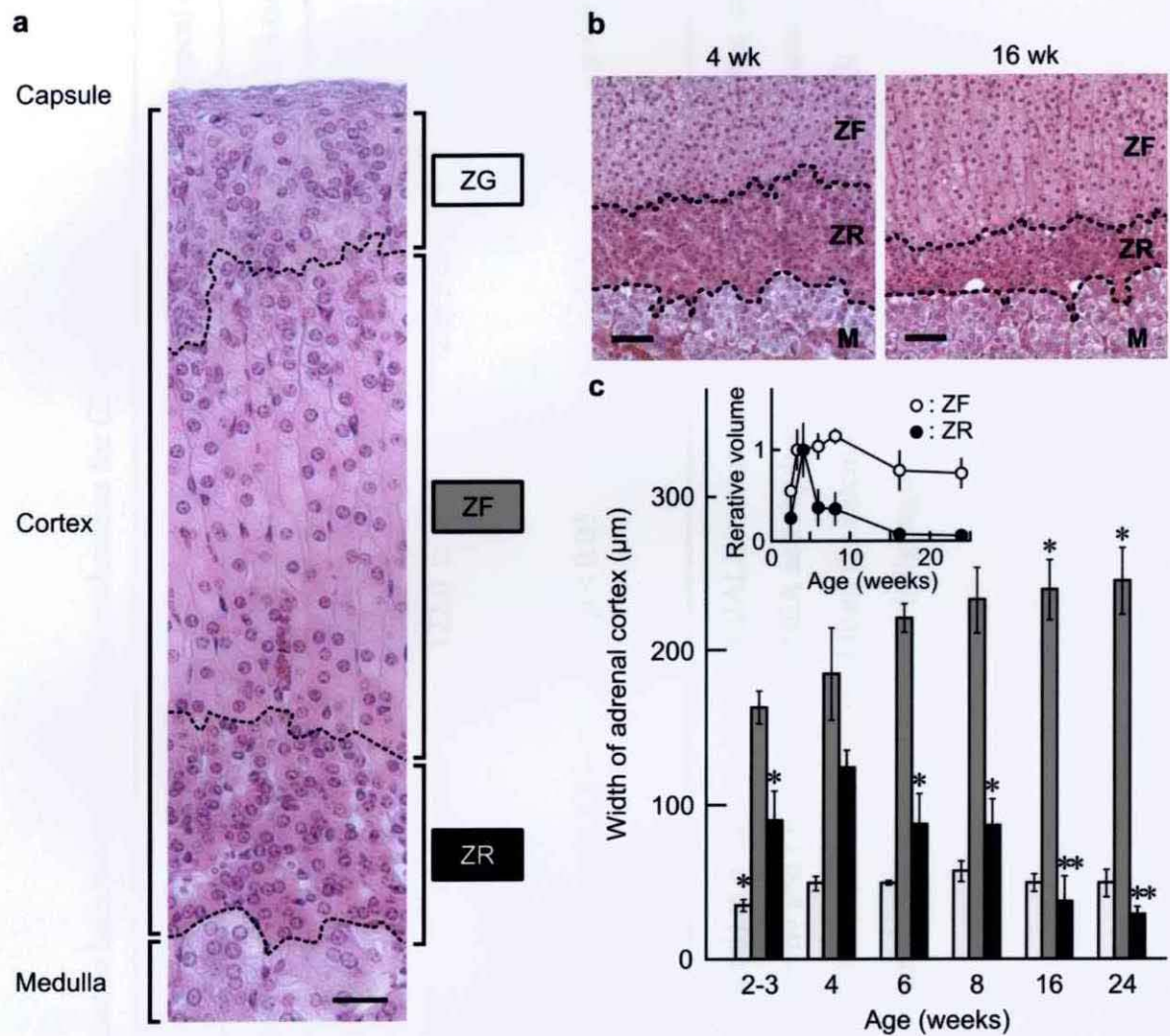
Next, I determined the dynamics in the histologically defined activities of ZF and ZR (i.e., histological activity) and the dynamics in the serum concentrations of their products, GC and DHEA (i.e., humoral activity), respectively, in mice either 6 or 16 weeks of age, in order to show that the histological activities are correlated with the serum concentration of their respective products. As indicated in Table 1, ZR decreased significantly over the 10-week period. The age-related changes in DHEA and the ZR were so striking that the GC/DHEA and ZF/ZR ratios were increased by factors of 2.41 (122/48) and 8.16 (432/53), respectively. < Table

Although I examined the serum concentrations of GC and DHEA by ELISA assay, the histological assay more accurately reflected the "age-related" physiology of the mice because the individual deviation from the mean in the histological data was smaller than that of the individual deviation that occurred when measuring humoral activities (Table 1). Therefore, histology is more accurate at estimated age-related physiologic events, and thus it was used in place of hormone serum concentration measurements.

**Fig. 5.** Age-related histological changes in the adrenal cortex of BALB/c mice. Sections of adrenal glands from BALB/c mice of various ages were stained with HE, and the width of each zone of their sections was measured.

a) Typical HE-stained adrenal gland, showing the zona glomerulosa (ZG), zona fasciculata (ZF), zona reticularis (ZR), and medulla (M). b) ZR area of HE-stained sections of adrenal gland at 4 and 16 weeks. Scale bars 50  $\mu\text{m}$ .

c) Changes in the width of each zone of the adrenal cortex from 3 to 24 weeks. Open, shaded, and closed bars indicate ZG, ZF, and ZR, respectively. All data are presented as the mean  $\pm$  SD of several mice (n=2-6). \*  $p < 0.05$ , \*\*  $p < 0.01$ , in comparison with 4-week-old mice. Inset: kinetic profiles of the volume of ZF (open circles) and ZR (closed circles); volume was calculated by cubic measurement using  $(\text{ZF} + \text{ZR})^3 - \text{ZR}^3$  for ZF activity and  $\text{ZR}^3$  for ZR activity.



**Table 1.** Correspondence between humoral and histological evaluations for GC and DHEA

	Humoral evaluation			Histological evaluation		
	GC (ng/ml)	DHEA (ng/ml)	GC/DHEA ratio	ZF (μm)	ZR (μm)	ZF/ZR ratio
6 wk	131.1 ± 83.9	2.7 ± 2.4	48.0 ± 30.7	221.1 ± 21.0	87.1 ± 17.1	52.9 ± 30.6
16wk	89.9 ± 48.9	0.7 ± 0.7	122.0 ± 63.7	239.1 ± 21.9	37.0 ± 5.1	431.7 ± 114.6
Comparison: <i>p</i> value	N.S.	<i>p</i> < 0.05	<i>p</i> < 0.05	N.S.	<i>p</i> < 0.01	<i>p</i> < 0.01

Serum GC and DHEA levels (ng/ml) in 6- and 16-week-old BALB/c mice were measured by ELISA assay, and GC/DHEA ratios were calculated. ZF widths for GC activity and ZR widths for DHEA activity (μm) on the paraffin sections stained with HE were histologically measured. The ZF/ZR ratios were estimated from the calculated volumes of the ZF and ZR (cf. Materials and Methods). All data are presented as the mean ± SD of several mice (n=4-8/group).

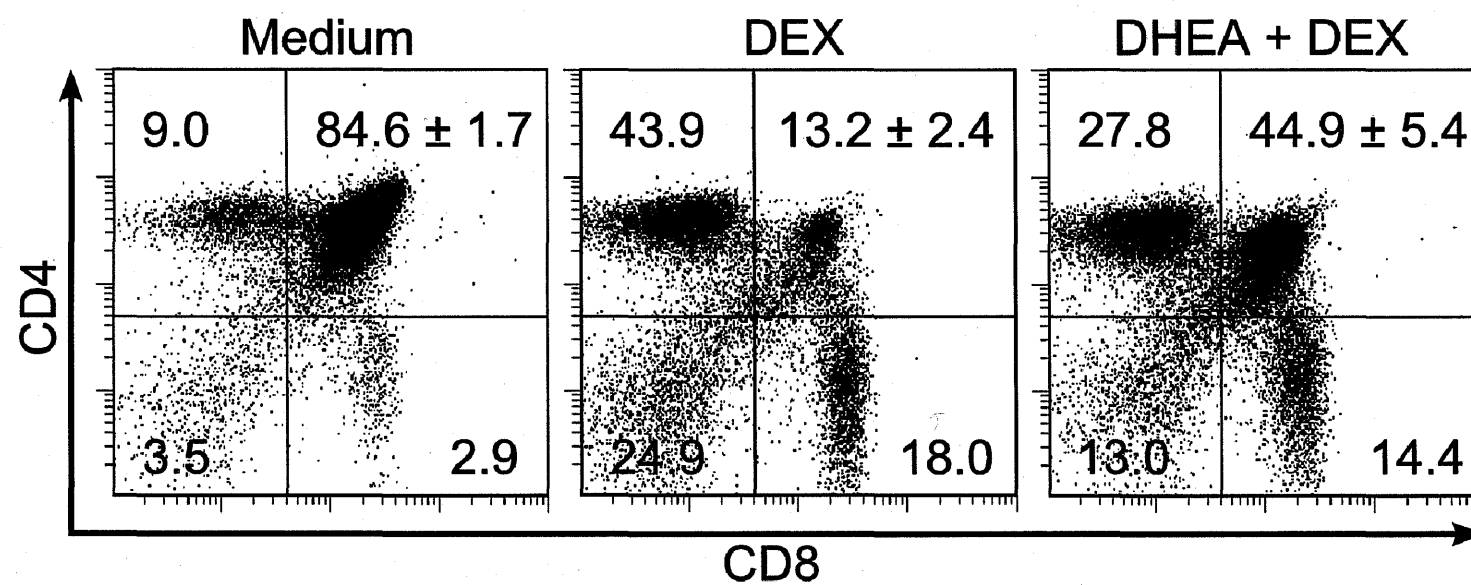
### ***Anti-GC effects of DHEA in thymic involution***

Because DHEA antagonizes GC function (22, 24, 25), the severe age-related decline of the ZR may result in an increase of GC activity itself, advancing thymic involution. To verify this, I tested the ability of DHEA to antagonize GC-induced thymic involution in mice that received DHEA 1 day before injection of DEX. As shown in Fig. 6, the proportion of DP thymocytes was significantly increased by DHEA administration ( $p < 0.01$ ), preventing thymic atrophy caused by DEX injection. The absolute number of CD4 and CD8 double positive or target thymocytes of DEX-treatment was significantly ( $p < 0.05$ ) increased by DHEA-treatment from  $2 \times 10^6$  (n=3) to  $16 \times 10^6$  (n=6).

< Fig.

**Fig. 6.** Dehydroepiandrosterone prevents dexamethasone-induced thymocyte death. Six-week-old BALB/c mice were treated with dexamethasone (DEX) after pretreatment with either dehydroepiandrosterone (DHEA) (1.6 mg/mouse) or 200  $\mu$ l of 10% ethanol as a control, were killed 24 hours later, and were analyzed by flow cytometry for the distribution of thymocyte subpopulations. Figures indicate percentage of cell numbers. Data were collected from 3 to 6 mice per group. The recovered cell number of CD4 and CD8 double positive cell analysis was  $60.1 \pm 16.1 \times 10^6$ ,  $2.4 \pm 1.0 \times 10^6$ , and  $16.4 \pm 5.5 \times 10^6$  for the medium-, DEX-, and DHEA+DEX-groups, respectively (DEX vs. DHEA + DEX;  $p < 0.01$ ).





### ***DHEA treatment retarded thymic involution in growing mice***

If age-related decline of DHEA result in GC activity, continuous administration of DHEA should delay thymic involution with age. To investigate the effect of DHEA for thymic involution after puberty, the 4-week-old mice that the DHEA production activity in the ZR peaks were treated with DHEA in their drinking water for 4 week. As a result, DHEA treatment significantly retarded thymic involution. Thus, the mean numbers of thymocytes at 8 weeks of age in the DHEA-treated group and in the control group were  $10.5 \pm 1.9 \times 10^7$  (n=8) and  $6.5 \pm 2.2 \times 10^7$  (n=6), respectively ( $p < 0.01$ ). Similarly, the numbers of thymocytes in male mice showed a tendency to increase by DHEA-treatment from  $10.7 \pm 0.1 \times 10^7$  (n=2) to  $14.4 \pm 2.6 \times 10^7$  (n=2). The numbers of peripheral T cells were not affected by DHEA treatment (data not shown).

### ***T cells are involved in the regression of zona reticularis***

The ZF/ZR ratio at 16 weeks ( $431.7 \pm 114.6$  in Table 1) was significantly reduced ( $226.7 \pm 140.4$ ,  $p < 0.05$ ) when T cell generation was inhibited by anti-CD3 treatment during the first 6 weeks of life; the ZF and ZR of these mice were  $271.6 \pm 32.1 \mu\text{m}$  and  $57.4 \pm 13.5 \mu\text{m}$ , respectively. This histological change was accompanied by a marked delay in thymic involution (Fig. 3).

To understand the involvement of T cells in the increase of the ZF/ZR ratio after puberty, histological examination of the adrenals from athymic nude (*nu/nu*) and *nu/+* mice were performed. As shown in Table 2 and Fig. 7, 16-week-old *nu/+* mice showed a larger ZF and a significantly reduced ZR when compared with 6-week-old *nu/+* mice. However, the age-matched *nu/nu* mice showed a significant increase in the ZF when compared with 6-week-old *nu/nu* mice, as observed in the *nu/+* mice. Surprisingly, 16-week-old *nu/nu* mice showed a slightly increased ZR when compared with 6-week-old *nu/nu* mice; the ZF/ZR ratio was the same as that of the 6-week-old mice. This may indicate that T cells are involved in ZR involution. Thus, I tested whether T cell transfer reduced the ZR in *nu/nu* mice. Indeed, T cell reconstitution in *nu/nu* mice significantly reduced the ZR without changes in ZF. The ZF/ZR ratio had also significantly increased to the same level as that of *nu/+* mice. This result indicates that the increase of ZF with age is not always dependent on T cells and that ZR regression is controlled by T cells.

**Table 2.** Demonstration of T cell involvement in age-related regression of adrenal zona reticularis

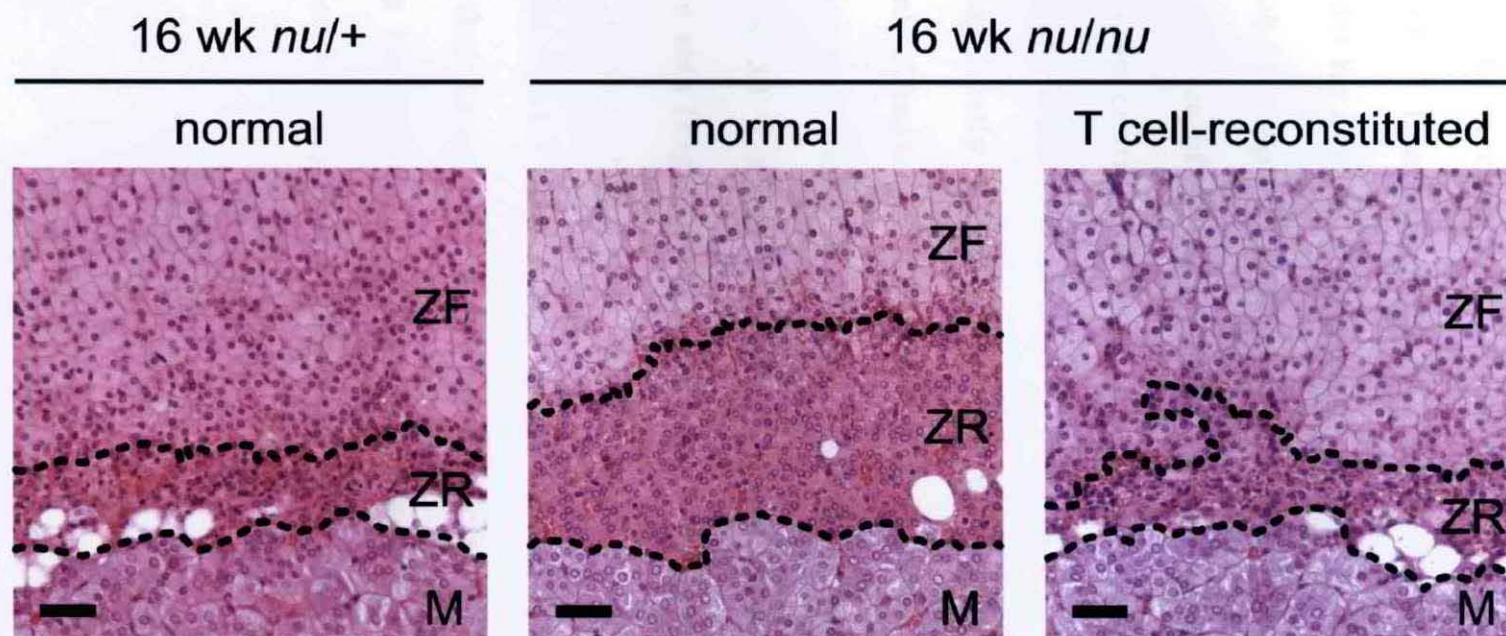
Mice	Age	ZF (μm)	ZR (μm)	ZF/ZR ratio
<i>nu/+</i>	6 wk	239.0 ± 15.1	87.5 ± 8.1	52.9 ± 14.9
	16 wk	304.7 ± 29.6	51.7 ± 11.0	400.1 ± 232.2
<i>nu/nu</i>	6 wk	201.9 ± 22.6	79.9 ± 11.8	46.8 ± 20.1
	16 wk	266.2 ± 30.2	95.5 ± 18.1	56.6 ± 21.6
	16 wk (T)	293.5 ± 34.1	54.8 ± 7.4	261.2 ± 54.6

Adrenal sections of 6- and 16-week-old BALB/c *nu/+* mice, age-matched BALB/c *nu/nu* mice, and 16-week-old BALB/c *nu/nu* mice reconstituted with T cells as neonates, indicated by 16 wk (T) (for details, see Materials and Methods), were stained with HE. The width of each zone of these sections was measured, and the ZF/ZR ratios were estimated from the volumes (cf. Materials and Methods).

All data are presented as the mean ± SD (n=3-7/group).

\*  $p < 0.05$ , \*\*  $p < 0.01$

**Fig. 7.** T cell-mediated regression of the adrenal zona reticularis. Adrenal sections of 16-week-old BALB/c-*nu*/+ mice, age-matched BALB/c-*nu*/*nu* mice, and BALB/c-*nu*/*nu* mice reconstituted with T cells as neonates were stained with HE to identify the ZG, ZF, ZR, and medulla (M). Scale bars 50  $\mu$ m. These figures are histological representatives of each group of mice (n=3-7), whose detailed data are shown in Table 2.



*Possible mechanisms for the regression of the zona reticularis by activated T cells*

Next, I explored the mechanisms by which peripheral T cells induced regression of the ZR. I treated 6-week-old BALB/c mice with anti-CD3 antibodies as a model for adrenal changes.

Thymocyte death by apoptosis began 20 h after treatment with anti-CD3 antibodies, where the size of the ZF increased slightly without any further increment (Fig. 8b). In contrast, the size of the ZR significantly dropped 40-60 h after antibody treatment ( $\alpha$ CD3 vs. PBS in Fig. 8c), which was when the thymus was mostly damaged (data not shown).

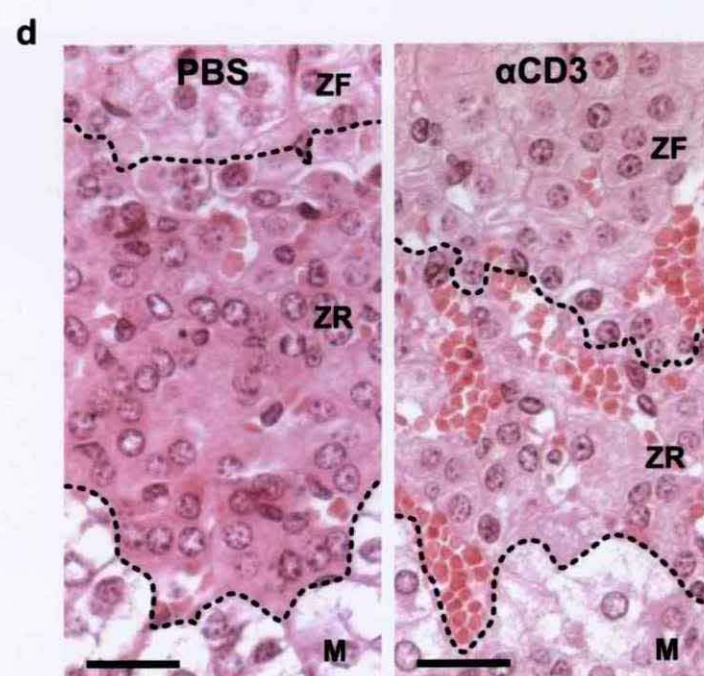
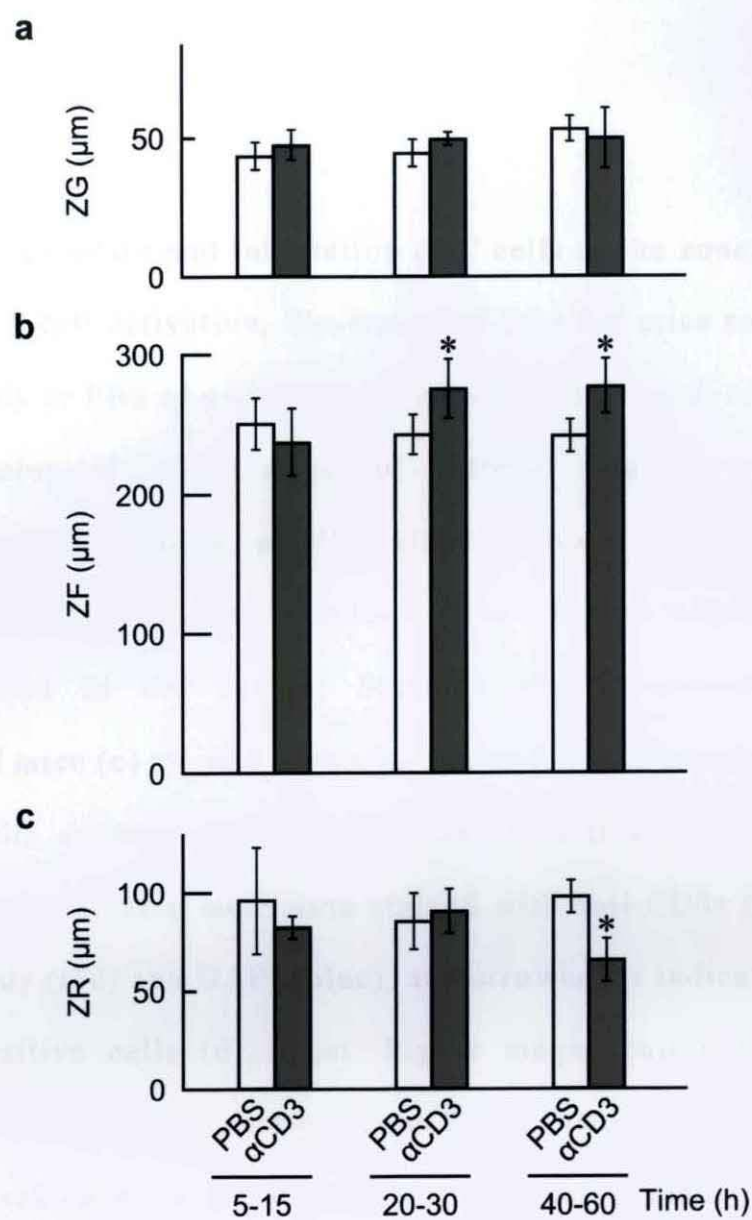
< Fig. 8

Cytohistological changes in the ZR, which occurred 30-60 h after antibody treatment, are shown in Fig. 9. Many apoptosis cells were observed in the ZR 30 h after treatment (Fig. 9a), and many T cells accumulated in this area (Fig. 9c). These T cells appeared activated, based on their large size and irregularly shaped surfaces (Fig. 9d, inset). Interestingly, the activated T cells were CD4 but not CD8 positive (Fig. 9d). This suggests that their target cells would be MHC class II positive; I found that only ZR cells became class II positive, in addition to the vascular endothelia and blood leukocytes that are also class II positive (Fig. 9f).

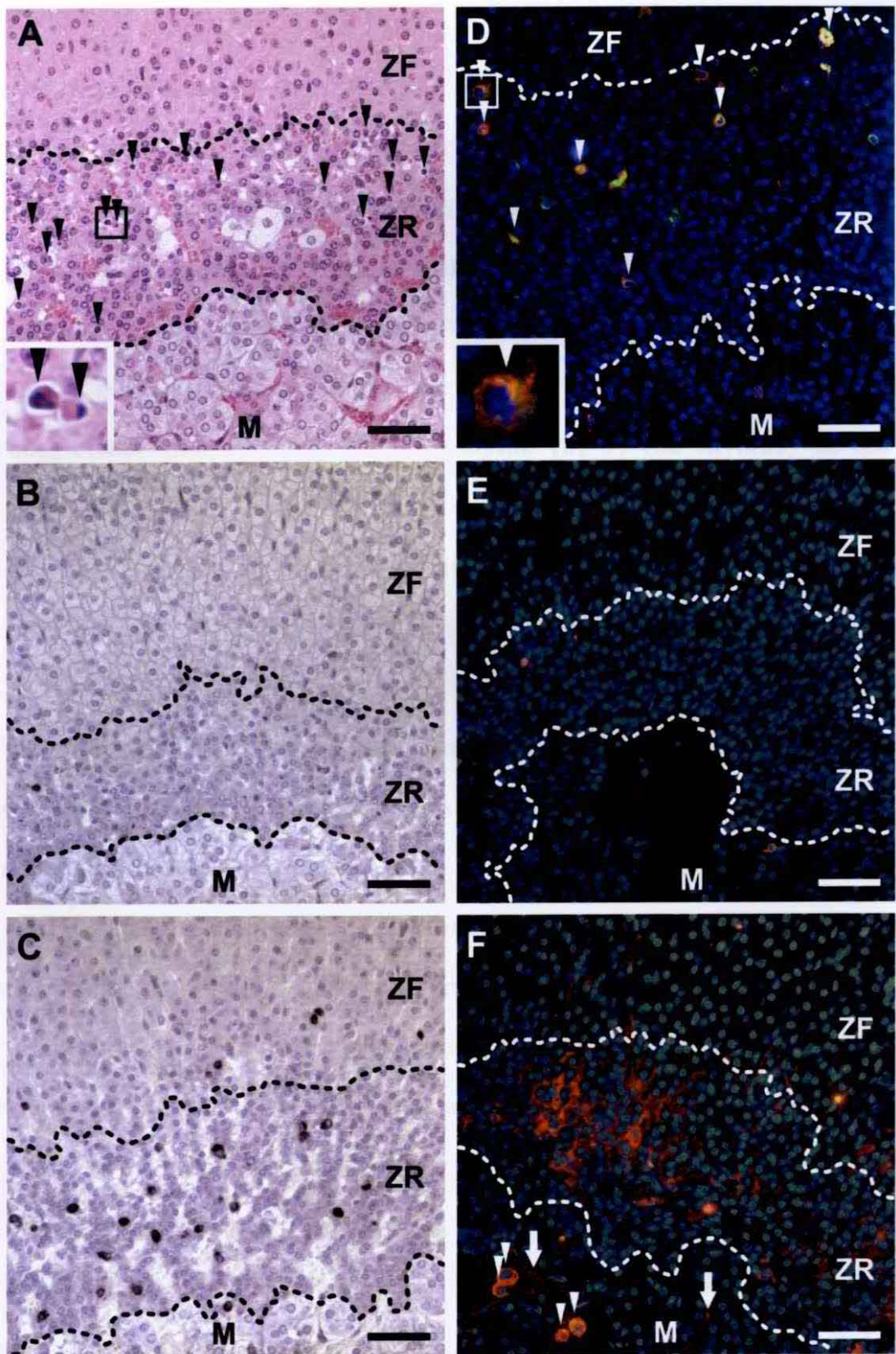
< Fig. 9

**Fig. 8.** Regression of zona reticularis by activation of peripheral T cells. Six-week-old BALB/c mice received i.p. anti-CD3 antibody or PBS as a control and were killed 5-60 h later for histological observation of the adrenal cortex by HE staining. All mice were divided into three groups based on their length of treatment: 5-15, 20-30, and 40-60 h. The widths of the zona glomerulosa (ZG) (a), zona fasciculata (ZF) (b), and zona reticularis (ZR) (c) of the adrenal cortex from sections of each mouse were measured. Open and closed bars indicate PBS-treated and anti-CD3-treated groups. All data are presented as the mean  $\pm$  SD (n=3-8 per group). Asterisk indicates  $p < 0.05$  compared to PBS control mice. d) Histological presentation of a reduced ZR by 40 h of treatment with anti-CD3 antibody (right) and PBS control (left). Scale bars 50  $\mu$ m.





**Fig. 9.** Adrenal cell death and infiltration of T cells in the zona reticularis after peripheral T cell activation. Six-week-old BALB/c mice received i.p. anti-CD3 antibody or PBS as a control and were killed 30 (a-d) or 60 h (e, f) later for histological observation of adrenal glands by HE or immunohistochemical staining. a) HE-stained sections indicate apoptotic figures (arrowheads) in the zona reticularis. Inset: higher magnification of the area enclosed in the square. Sections of PBS-control (b) and antibody-treated mice (c) stained with anti-human CD3 $\epsilon$  antibody. Note that CD3-bearing cells accumulated in ZR of antibody-treated mice. Frozen sections of antibody-treated mice were stained with anti-CD3 $\epsilon$  (green) and anti-CD4 antibody (red) and DAPI (blue), and arrowheads indicate CD3 and CD4 double positive cells (d). Inset: higher magnification of the area enclosed in the square. Frozen sections from PBS-control mice (e) and those from antibody-treated mice (f) stained with anti-MHC class II antibody (red) and DAPI (blue): arrowheads and arrows indicate MHC class II-positive blood leukocytes and vascular endothelia, respectively, in antibody-treated mice. Scale bars 50  $\mu$ m.



## Discussion

The current paradigm is that thymic involution after puberty is the major cause of age-associated immune dysfunction through alterations in T cell function, and the central cause of this thymic involution, which is thought to be intrinsically programmed in animals, remains unsolved, even though many hypotheses have been proposed. The age-related effects on thymic function are mainly observed in the age-related increases or decreases of cytokines, either inside (5, 7, 10) or outside (8, 9) the thymus, in the age-related loss of the supplement of T cell progenitors (26-28) or in the age-related dysfunction and atrophy under endocrinological effects (11, 29). However, the central events causing the above aging effects on thymic function remain to be understood.

Decades have passed since the pioneering work of Besedovsky et al. (30, 31), who demonstrated that immune responses stimulate and activate the neuroendocrinological axis of the hypothalamus-pituitary-adrenals, producing GC immunosuppressive factors to control the immune system. This means that the limited potency of immune activation generates poorer immune suppression. Indeed, mice with abnormal or restricted T cell repertoires (32) typically do not show age-related thymic involution (17, 18). On the contrary, mice with T cells that have a more general receptor repertoire experience thymic involution.

When the development of peripheral T cells was prevented by neonatal treatment with anti-CD3 antibodies, thymic involution was delayed until the late appearance of an adequate numbers of T cells, as demonstrated in my



report (Fig. 3b). The thymocytes which were generated following treatment with anti-CD3 antibody appeared normal by cytological and histological examination. Furthermore, when (BALB/c  $\times$  DBA/2) F1 or Mls-1<sup>a</sup> mice were used, the thymic T cell repertoire from mice that had been treated with anti-CD3 as neonates also seemed normal because the autoreactive V $\beta$ 6<sup>+</sup> T cells in the thymus were the same as those of untreated F1 mice (data not shown); the complete depletion of mature V $\beta$ 6<sup>+</sup> CD4- and CD8-SP thymocytes was observed in normal mice (33).

Contrary to the above loss-of-T cell experiments for delayed thymic involution, I attempted gain-of-T cell experiments by inoculating mice with a large amount of Thy1.2 semi-allogeneic T cells, but failed to demonstrate an effect; this was likely due to an unknown regulatory mechanism. The numbers of peripheral T cells were unchanged, even though Thy1.2 chimerism in Thy1.1 mice was successfully achieved (data not shown). Thus, I carried out T cell-reconstitution experiments using athymic nude mice in which no ZR regression was observed, even in aged mice. The exact involvement of T cells in the decline of ZR cellularity was clearly demonstrated, as shown in the age-matched *nu*/+ heterozygous mice in which the size of the ZR regressed with age (Fig. 7, Table 2).

The target of the peripheral T cells was adrenal ZR cells that produce DHEA (34), which has anti-GC activities (22, 24, 25), as was demonstrated by the prevention of thymic involution after the inoculation of DEX when DHEA was administered (Fig. 6). In addition, DHEA treatment of mice after ablactation retarded thymic involution. Therefore, the GC/DHEA ratio was clearly elevated after ablactation, resulting in stronger GC activity that led

to thymic involution after puberty.

The final issue raised in this manuscript is the potential mechanism by which peripheral T cells may mediate ZR cell loss. T cell activation by anti-CD3 antibodies leads to acute thymic involution caused by ZF GC production (34) as a stress response to immune activation (30, 31, 35). Although this idea may be questionable (3), T cell activation is a plausible mechanism for the development of chronic thymic involution through the aging process, based on the observation that the thymic involution seems to be closely associated with ZR regression following T cell activation (Fig. 10). The long-lasting effects of occasional T cell responses result in thymic involution after puberty. MHC class II molecules are first expressed on epithelial cells in the murine embryonic thymus following the initial migration of T cell progenitors into the thymus (36, 37). In the same manner, T cell activation throughout the life span of an organism may cause the expression of MHC class II antigens on ZR cells, as seen in Fig. 9; ZR cells in mice older than 3 months naturally express MHC class II antigens (data not shown). Contrary to murine ZR cells, those cells in humans constitutively express MHC class II antigens (38), and peripheral T cells instigate DHEA production (39); nevertheless, it is very interesting that DHEA expression is controlled by peripheral T cells in evolutionarily distant species.

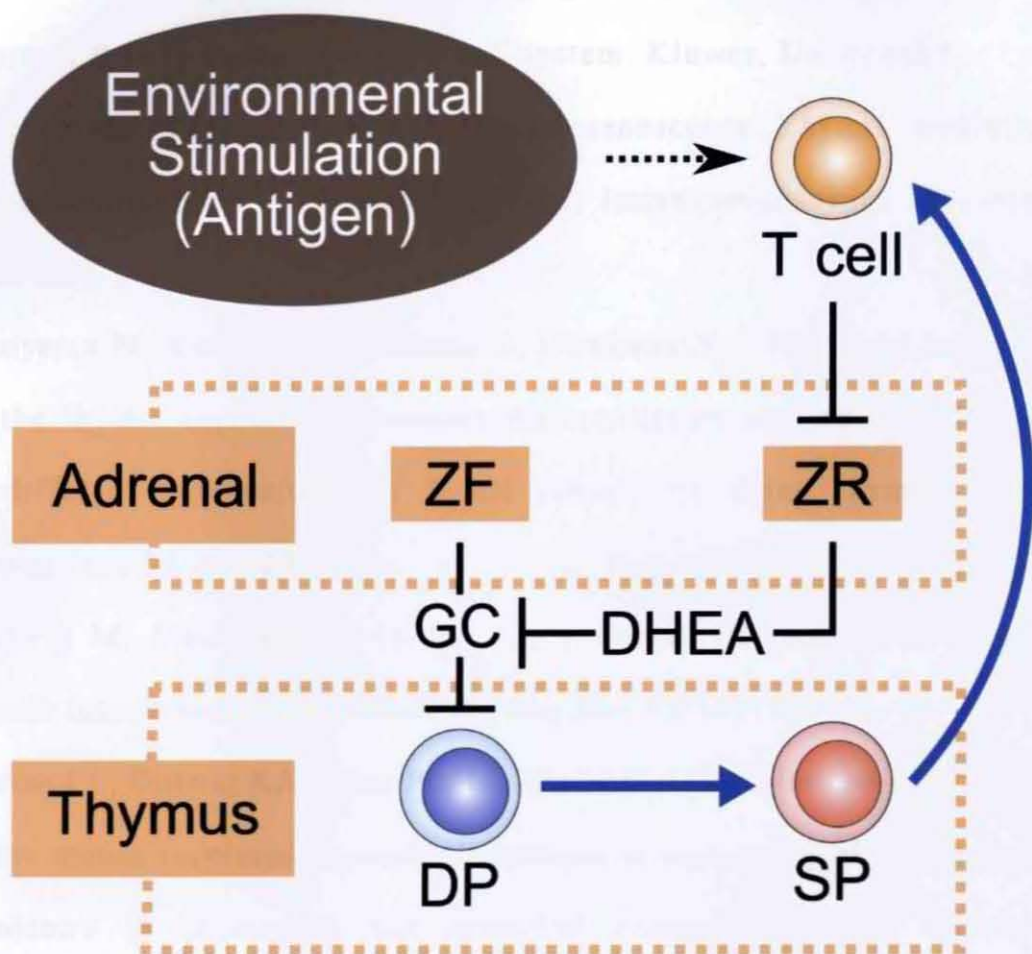
< Fig. 1

I conclude that the ZR cell loss by apoptosis was caused by CD4<sup>+</sup> T cells that were activated by environmental antigens, regardless of whether the antigens were of self- or non-self-origin. These T cells may not be specific for ZR cells because similar types of cells that are easily found in lung

capillaries (data not shown) are likely present in the capillaries during T cell blood circulation. These cells may induce class II antigen expression and the subsequent apoptosis of ZR cells. The series of panels in Fig. 9 clearly and accurately illustrate a scenario where class II-expressing ZR cells are susceptible to contact with T cells, although the exact mechanism of ZR cell killing by activated T cells remains to be determined.

**Fig. 10.** Proposed model for the thymic involution after puberty by peripheral T cells. As peripheral T cells from mature SP thymocytes are activated through the stimulation by environmental antigens, they cause the loss of ZR cells by apoptosis and reduce production of DHEA with anti-GC actions. Therefore, the GC/DHEA ratio would elevate after ablactation, resulting in an increased GC activity that easily leads to the thymic involution characterized by decrease of immature DP thymocytes after puberty.





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