BPB Reports

Regular Article

Prostaglandin-Modulated Interaction of Thymic Progenitor Cells with Blood Vessels during Estradiol-Induced Thymic Involution

Nurhanani Razali,¶ Io Horikawa,¶ Hirofumi Hohjoh, Chihiro Yoshikawa, and Hiroshi Hasegawa*

Laboratory of Hygienic Sciences, Kobe Pharmaceutical University; 4-19-1 Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan
Received July 10, 2019; Accepted July 13, 2019

Thymic involution-associated disfunction of thymus is implicated in aging, microbial infection, pregnancy, improper nutrition, and etc., therefore it is clinically important especially in aged societies. Excess administration of estradiol to male mice is known to induce thymic involution and used as a mouse model of thymic involution, whereas the mechanisms of which have not been well understood. Here we examined the role of prostanoids in the estradiol-induced thymic involution in mice. The administration of 17β-estradiol for 7 consecutive days induced thymic involution. In the involute thymus, the expression of mRNAs for some synthetic enzymes of prostanoids, including *Ptgs1*, *Ptgs2*, *Ptgds*, *Hpgds*, *Ptges1*, and *Tbxas*, are upregulated. In order to examine the roles of prostanoids in the thymic involution, we treated the mice with an NSAID, etodolac, following 17β-estradiol-administration. The etodolac-treatment partially inhibited the estradiol-induced reduction of thymic size and disorganization of the boundary between thymic cortex and medulla, as indicated by keratin 5 expression as well as by localization of innate immune cells. CD34-positive thymic progenitor cells localized near the blood vessels in the estradiol-administered thymus, although they were more dispersed by the etodolac-treatment. The association of CD34-positive cells with blood vessels is known to be mediated by E- and P-selectins, whose expressions were also regulated by estradiol-administration in an etodolac-sensitive manner. These results indicated the role of prostanoids in the histological change of thymus during estradiol-induced thymic involution.

Key words thymus, estradiol, prostanoid, selectin, blood vessel, thymic progenitor cells

INTRODUCTION

Thymus is a primary immune organ to play a sole role in T cell development. The reduction in size and function of thymus, which is generally called as thymic atrophy or thymic involution, is clinically important, because the reduced T cell immunity results in increased risk of infectious diseases and cancer initiation. Thymic involution is induced by various physiological and pathophysiological factors, including aging, infections, pregnancy, and improper nutrition.[1,2] Excess administration of sexual hormones has also been known to cause thymic involution in mice. Excess estradiol treatment into male mice causes thymic involution, which is accompanied by the defect in T cell development.[3,4] Although this effect of estradiol is known for a long time, few studies have been performed to clarify the molecular mechanisms governing the estradiol-induced thymic involution.

Prostanoids, including prostaglandins, thromboxane, and prostacyclin, are versatile lipid autacoids functioning in most tissues in the body. They are well-known as inflammatory mediators, produced from arachidonic acid in membrane phospholipids upon the initiation of inflammation. Cyclooxygenases, COX1 and COX2, encoded by *Ptgs1* and *Ptgs2* gene, respectively, metabolize arachidonic acid to unstable prostaglandin H_2 (PGH_2). PGH_2 is immediately converted to mature prostanoids by the enzymes associating with COX proteins, namely lipocalin-type and hematopoietic PGD synthase (encoded by *Ptgds* and *Hpgds* genes, respectively) for PGD_2; mPGES-1 (encoded by *Ptges1* gene), mPGES-2 (*Ptges2* gene), and cPGES (*Ptges3* gene) for PGE_2; AKR1B3 and AKR1B7 for $PGF_2\alpha$; PGIS (*Ptgis* gene) for prostacyclin (PGI_2); and TXAS (*Tbxas* gene) for thromboxane A_2 (TxA_2). The produced prostanoids are important not only for the inflammatory reactions, but also for the maintenance of healthy body. In the thymus, PGE_2 plays a critical role in T cell development.[5] Prostacyclin (PGI_2) is important for Th17 development.[6] TxA_2, whose receptor is highly expressed in the thymus,[7] is responsible for apoptosis and abrogation of alloreactive thymocytes.[8] Despite the well-investigated homeostatic roles of prostanoids, the involvement of prostanoids in thymic involution has been still obscure. We recently found an upregulation of prostanoid synthesis during acute diet-restriction-induced thymic involution, which regulates the balance of Th1 and Th2 differentiation in the thymus (Razali *et al.*, manuscript submitted). Thus, induced prostanoids are important for the regulation of thymus functions during thymic involution.

In this manuscript, we examined the upregulation of prostanoid synthases during estradiol-induced thymic involution. We also found that CD34-positive thymic progenitor cells (TPCs) localize near the blood vessels in the estradiol-administered thymus, although they are dispersed in the vehicle-administered thymus. This different localization of TPCs is

*To whom correspondence should be addressed. e-mail: h-hase@kobepharma-u.ac.jp
¶ The first two authors equally contributed to this study.

accompanied by expressional change of E- and P-selectin molecules. These effects of estradiol were canceled by the treatment with etodolac, a non-steroidal anti-inflammatory drug (NSAID). Thus, prostanoids play an important role in the thymic involution, especially in the selectin-mediated localization of TPCs.

MATERIALS AND METHODS

Animals ICR male mice (8–12 weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and individually housed in a temperature- and humidity-controlled room (23 ± 2°C, 55% ± 5% relative humidity) on a 12-h light/dark cycle (lights on at 07:00). The mice had free access to food pellets and water. All experimental procedures were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023) and the Science Council of Japan's Guidelines for the Proper Conduct of Animal Experiments. In addition, the experimental protocols were approved by Kobe Pharmaceutical University Committee for Animal Care and Use.

17β-estradiol (2 μg/mouse) (Fujifilm Wako Pure Chemical Corp., Osaka, Japan) dissolved in Coconad RK (Kao Corp., Tokyo, Japan) was subcutaneously administered every morning for 7 consecutive days. For the experiments of etodolac treatment, 5 mg/kg b.w./day (1 mg/mL) (Fujifilm Wako Pure Chemical Corp.) was peritoneally injected, following the 17β-estradiol injection. Equal amount of saline was injected for the control. The number of mice used was indicated in figure legends.

RNA Preparation and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) RNA purification and qRT-PCR were performed as described previously.[9] Briefly, mice were sacrificed under anesthesia and their thymus was harvested for RNA purification. Other tissues, including heart, kidney, spleen, testis, and liver, were also collected for weight measurement. Left lobe of the harvested thymus was immediately immersed in Sepasol® (Nacalai Tesque, Inc., Kyoto, Japan) and total RNA was purified according to the manufacturer's instruction. Right lobe of the thymus was used for histological analyses. Complementary DNA was synthesized using ReverTra Ace® reagent (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instruction. The expression of mRNA was quantified using a CFX Connect™ Real-Time PCR Detection System with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc., CA, USA). The reaction condition was as follow: initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 30 s. Relative quantification was performed using the ΔΔCT method and the data were then normalized to the average value of vehicle-administered samples. The statistical significance of differences between groups was analyzed using Tukey-Kramer method. The primer sequences used in this study are listed in Table 1.

Histological Analyses For the histological analyses, the right lobe of thymus was fixed in 4% paraformaldehyde in PBS at 4°C overnight. They were cryoprotected in 30% sucrose in PBS at 4°C overnight and then embedded in optimal cutting temperature (OCT) compound (Sakura Finetek Japan, Tokyo, Japan). 20-μm sections were prepared on a cryostat (Three Medical GmbH, Mainz, Germany).

Table 1. Primer Sequences Used for qRT-PCR

Gene name	Protein product	Primers
Ptgs1	COX-1	5'-*GAAGCCTTACACCTCTTTCC*-3'
		5'-*GGGTAGAACTCTAAAGCATCG*-3'
Ptgs2	COX-2	5'-*AGTACCGCAAACGCTTCTCC*-3'
		5'-*CATCGATGTCACTGTAGAGG*-3'
Ptgds	L-PGDS	5'-*TGAAGGACGAGCTGAAGGAG*-3'
		5'-*TGACTGACTTCTCTCACCTG*-3'
Hpgds	H-PGDS	5'-*ATCAAGCACCTCGCCTTCTG*-3'
		5'-*GATATCCCAGTAGAAGTCTGC*-3'
Ptges1	mPGES-1	5'-*GGATGCGCTGAAACGTGGAG*-3'
		5'-*AGGAAAGGATAGATTGTCTCC*-3'
Ptges2	mPGES-2	5'-*ATGAAGCAGCCAACAAGTGG*-3'
		5'-*ACTCGCAGCACACCATACAC*-3'
Ptges3	cPGES	5'-*TCACATGGGTGGTGATGAGG*-3'
		5'-*ATCCAGGCGATGACAACAGC*-3'
Akr1b3	AKR1B3	5'-*AGCTACAACAGGAACTGGAG*-3'
		5'-*ACAGGTGCAAGCCACTTGAG*-3'
Akr1b7	AKR1B7	5'-*CGACTTCCAGTTGAGTGAGG*-3'
		5'-*AGGATAGTCCTCTTCAGTCC*-3'
Ptgis	PGIS	5'-*GAGCTGAAGCACACGGTCTG*-3'
		5'-*GAGTGTCTCATTGAGCACAC*-3'
Tbxas1	TXAS	5'-*AGAGACCCTGAGGATGTACC*-3'
		5'-*ATACGTTGTCCCAGCACCTC*-3'
Sele	E-selectin	5'-*CGTCCTCTGGAGAGTGGAGT*-3'
		5'-*TGTCGTGTTCCATGGGTAGC*-3'
Sell	L-selectin	5'-*ATTTCTCATTTGGCTGGCAAGG*-3'
		5'-*TTCACGGGAGGACTTGACG*-3'
Selp	P-selectin	5'-*GGGCTTCAGGACAATGGACA* -3'
		5'-*TGGAAGGTGCAGGTTGATCC*-3'
Rplp2	(internal control)	5'-*TACTAGACAGCGTGGGCATC*-3'
		5'-*CAACACCCTGAGCGATGACA*-3'

Giemsa staining was performed with a standard protocol. In brief, the tissue sections were washed in dH_2O and soaked in Giemsa staining solution (Nacalai Tesque, Inc.) at room temperature for 60 min. The signal was developed by a brief wash of the tissue sections in acetic acid solution (3 drops of glacial acetic acid in 100 mL dH_2O). They were dehydrated and examined under a Biozero BZ-8000 microscope (KEYENCE, Tokyo, Japan).

Immunohistochemistry was performed as described by Hasegawa *et al.* with minor modifications.[10] The tissue sections were washed with PBS and incubated in citrate buffer (pH 6.0) at 95°C for 40 min for antigen retrieval. They were then cooled to room temperature and washed in PBS three times. Endogenous peroxidases were inactivated by incubating the sections in 0.3% hydrogen peroxide in methanol at room temperature for 15 min. After three washes in PBS, the sections were blocked with a blocking solution (1.5% goat serum in PBS) and then incubated with rabbit antibody against Keratin 5 (Genetex, Inc., Irvine, CA) or rabbit antibody against CD34 (Abcam plc, Cambridge, UK) at 4°C overnight. The sections were then washed with high-salt PBS containing Tween-20 (0.5 M NaCl, 10 mM phosphate buffer [pH 7.4], 0.1% Tween-20) and incubated with horseradish peroxidase-conjugated donkey antibody against rabbit immunoglobulin G (Thermo Fisher Scientific, MA, USA) diluted in low-salt PBS containing Tween-20 (10 mM phosphate-buffer [pH 7.4], 0.05% Tween-20) at room temperature for 2 h. Peroxydase-positive signal was developed with ImmPACT DAB substrate (Vector laboratories, Burlingame, CA). Finally, the sec-

tions were counterstained with methyl green and examined under a Biozero BZ-8000 microscope.

Statistical Analyses Results were expressed as means ± standard deviation. The data were statistically analyzed using EZR software for Windows.[11] A Student's t-test was used for comparisons of means between two groups. One-way analysis of variance followed by Tukey-Kramer method was used to compare means among three groups. Statistical significance was evaluated by p value less than 0.05.

RESULTS

Induced Prostanoid Synthases during Estradiol-Induced Thymic Involution Administration of 17β-estradiol did not affect the total body weight (Fig. 1A), whereas the weight of thymus of estradiol-administered mice was nearly half of the vehicle-treated mice (Fig. 1B) as described previously.[3,4] The weights of heart, spleen, and kidney were, in contrast, not affected by the administration of estradiol (Fig. 1C-E).

In order to examine whether production of prostanoids is affected during the estradiol-induced thymic involution, the expressions of synthetic enzymes of prostanoids were evaluated by qRT-PCR. Among 11 synthetic enzymes of prostanoids, *Ptges1* and *Tbxas* were significantly upregulated in response to the treatment of estradiol (Fig. 2). *Ptgs1*, *Ptgs2*, *Ptgds*, and *Hpgds* were slightly upregulated, although the difference was not statistically significant. *Ptgis* was also highly upregulated in some samples, whereas the expression level of *Ptgis* was diverse among samples, indicating the regulation of *Ptgis* depends on individual differences or it is affected by any other signaling factor(s). *Ptges2*, *Ptges3*, *Akr1b3*, and *Akr1b7* were not affected by the administration of estradiol. These results indicate that synthesis of some specific prostanoids was increased during the estradiol-induced thymic involution.

Effect of Etodolac on the Estradiol-Induced Thymic Involution In order to examine the role of increased prostanoid synthesis in the estradiol-induced thymic involution, we treated the estradiol-administered mice with an NSAID, etodolac. Saline was used for vehicle treatment as a control. Administration of etodolac by itself did not affect the size and tissue morphology of the thymus (Razali *et al.*, unpublished observation). Treatment with etodolac partially suppressed the reduction in size of the thymus induced by estradiol (Fig. 3A and B). Thus, prostanoid induction is involved in the estradiol-induced thymic involution, at least in part.

Previous studies have indicated that boundary between cortex and medulla was disorganized during thymic involution.[12,13] Therefore, we examined whether prostanoids are involved in this disorganization of corticomedullary boundary. The corticomedullary boundary was clearly observed in the vehicle-administered with saline-treated group (Fig. 4A). This boundary was not apparent during the estradiol-induced thymic involution. The treatment with etodolac recuperated this disorganization of corticomedullary boundary, suggesting

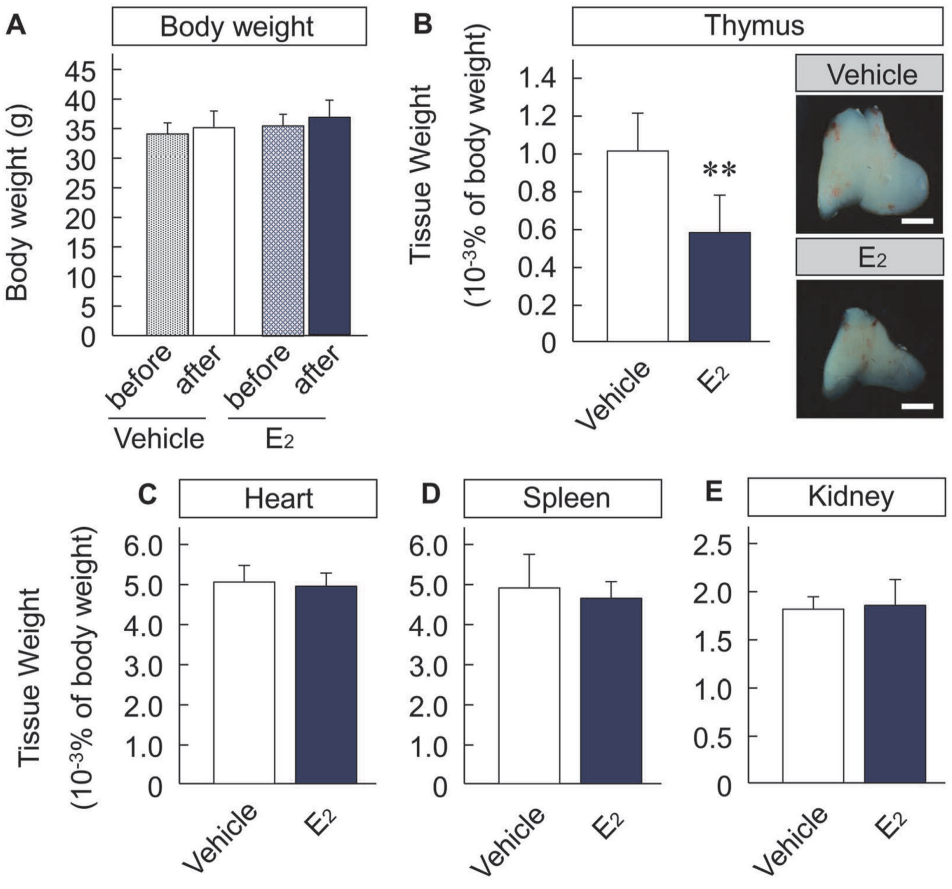

Fig. 1. Thymic Involution Induced by the Administration of 17β-Estradiol (E_2)

A: Body weight of the mice administered with vehicle (n=6) or E_2 (n=7) before and after the administration. B: The weight of thymus relative to body weight. Scale bars indicate 1 mm. C-E: The weights of heart (C), spleen (D), and kidney (E) relative to body weight. Error bars indicate standard deviation. $^{**}p < 0.01$.

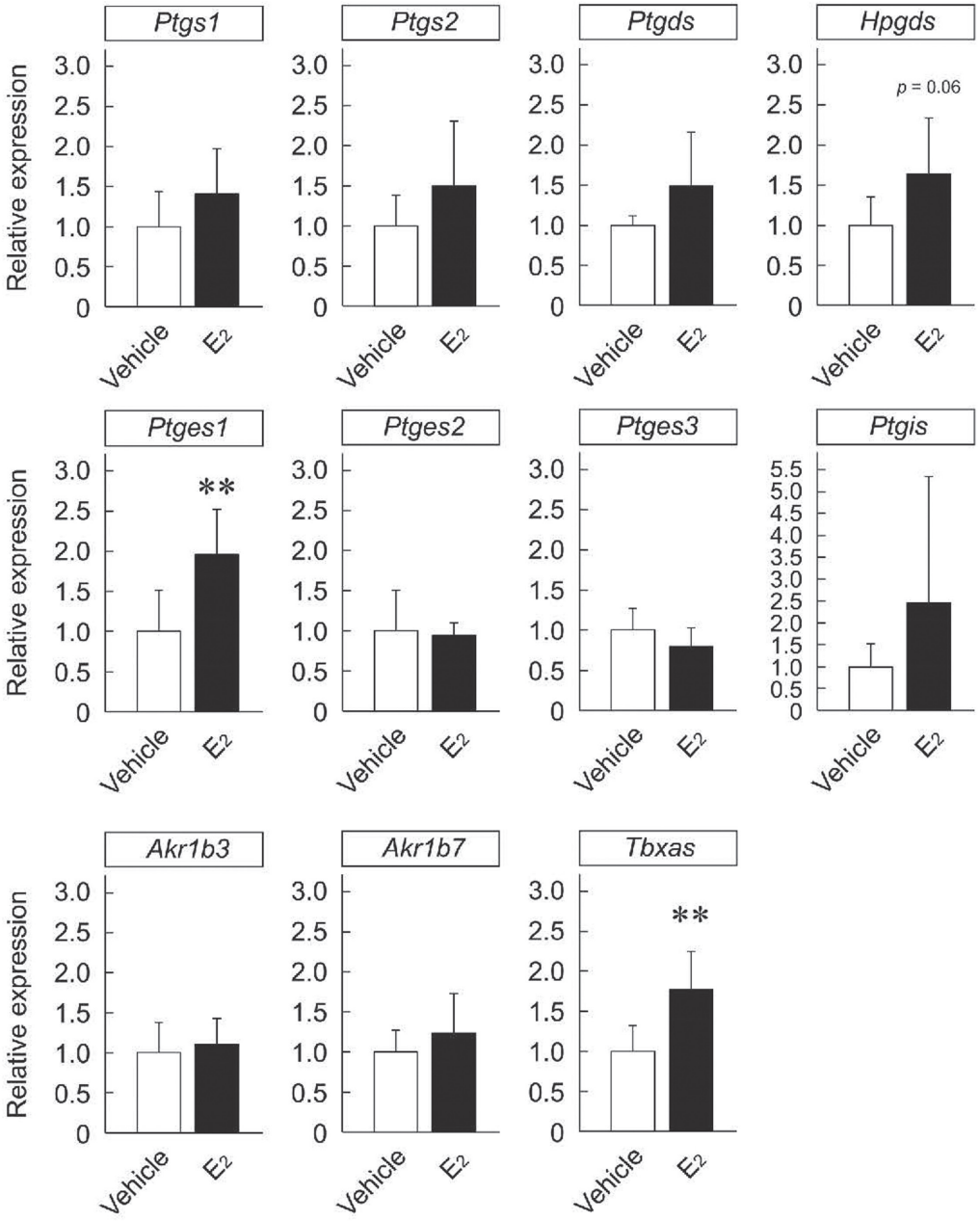

Fig. 2. Increased Expression of Prostanoid Synthases during the Estradiol-Induced Thymic Involution

The mRNA expression in the thymus of vehicle- (n=6) or E_2- (n=7) administered mice was examined by qRT-PCR. Error bars indicate standard deviation. $^{**}p < 0.01$, compared to vehicle-treated samples by Student's T-test.

that prostanoid is involved in the regulation of corticomedullary boundary. This boundary formation is required to restrict innate immune cells in the thymic medulla. In the vehicle and saline-treated thymus, eosin-positive innate immune cells, likely to be neutrophils and eosinophils, are restricted in the medulla and few cells were observed in the cortex (Fig. 4A, red arrowhead). In the involute thymus with the administration of estradiol, many eosin-positive cells were observed in the cortex, which was again recuperated by the treatment with etodolac. These results suggest that prostanoid is required for the functional integrity of the corticomedullary boundary.

The corticomedullary boundary is formed by the specific types of thymic epithelial cells, namely cortical and medullary thymic epithelial cells (cTECs and mTECs).[14,15] Immunostaining with antibody against keratin 5, a marker for cTECs, showed that keratin 5-positive signals were restricted in the cortex, whereas it is extended to the medulla in the thymus treated with estradiol (Fig. 4B). The treatment with etodolac suppressed the extension of the keratin 5 signals to the medulla, although some keratin 5-positive signals were still observed in the medulla (Fig. 4B). There results supported the notion that prostanoids play an important role in the disorganization of boundary between cortex and medulla during estradiol-induced thymic involution.

Relationship between TPCs and Blood Vessels, Which is Modulated by Prostanoids Blood vessels function not only as a tube for transporting oxygen, nutrients, and waste products in the body, but also as a signaling center for organizing changes of the body.[16–18] In order to examine the change in blood vessel patterns, we immunostained the thymus sections by anti-CD31 and anti-CD34 antibodies. The number of

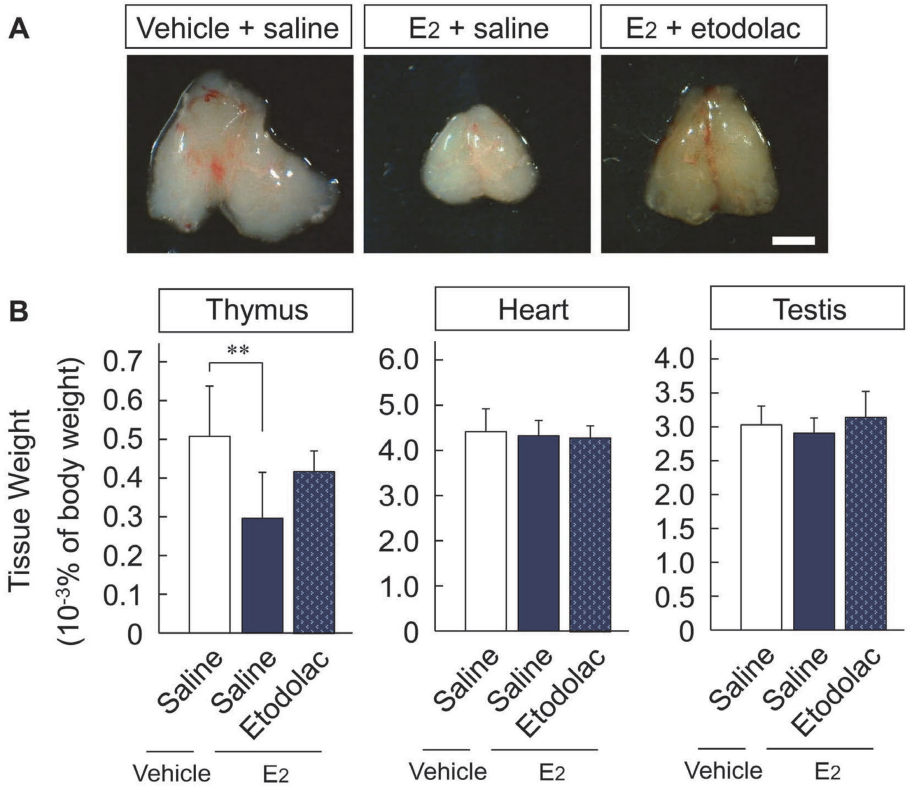

Fig. 3. Effect of Etodolac on the Estradiol-Induced Thymic Involution

A: Representative overview images of the thymus treated with vehicle/saline, E_2/saline, or E_2/etodolac as described in Materials and Methods. Scale bar indicates 1 mm. B: Average weight of the tissues relative to body weight at the end of the treatment (n=6, 7, and 5, respectively). Error bars indicate standard deviation. $^{**}p < 0.01$, compared by Tukey-Kramer method.

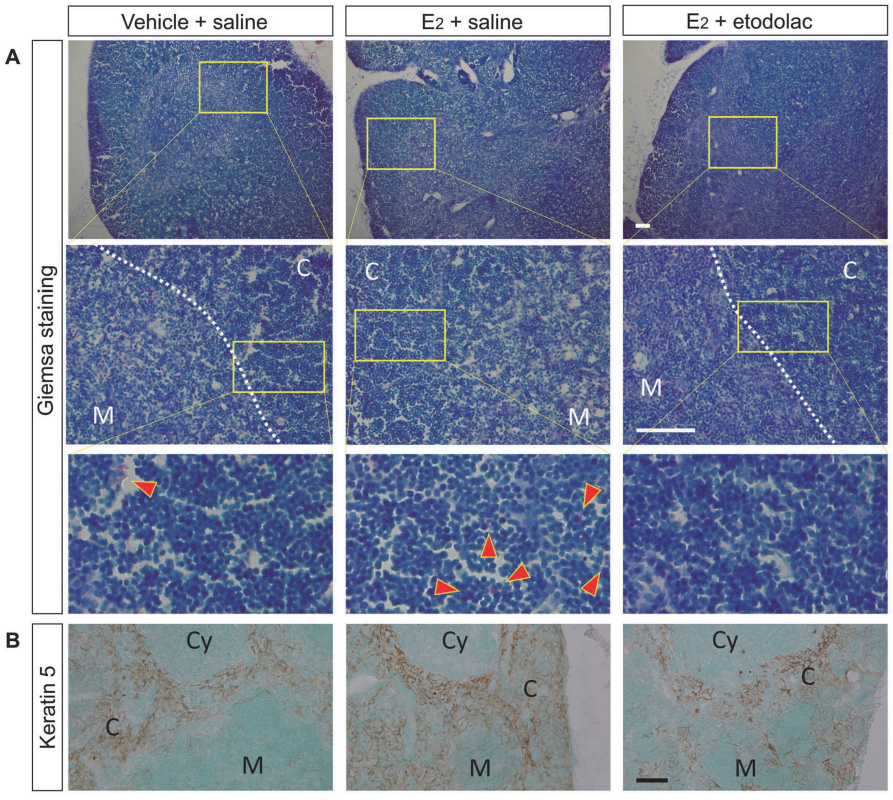

Fig. 4. Histological Characters of the Thymus from Mice Treated with Vehicle/Saline, E_2/Saline, or E_2/Etodolac.

A: Giemsa-stained sections. The areas surrounded with yellow boxes are enlarged in the below panels. The boundaries between cortex and medulla are indicated by white dotted lines. Red arrowheads indicate eosin-positive cells in the cortex. C; cortex, M; medulla. B: Localization of keratin 5. Tissue sections were stained with anti-keratin 5 antibody and the signals were developed with DAB (brown). Counter-staining was performed with methyl green (green). C; cortex, Cy; thymic cyst, M; medulla. Scale bars indicate 100 µm.

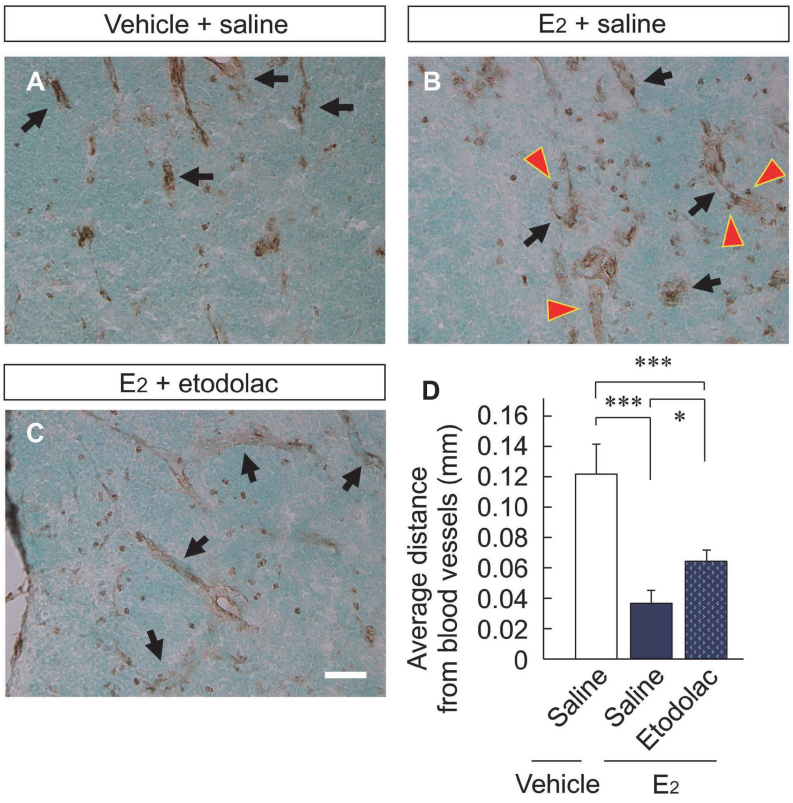

Fig. 5. Localization of CD34-Positive TPCs

A-C: Tissue sections of the thymus of vehicle/saline- (n=4), E_2/saline- (n=4), and E_2/etodolac- (n=4) treated mice were stained with anti-CD34 antibody and the positive signals were developed with DAB (brown). Counter-staining was performed with methyl green (green). D: Average distance between CD34-positive TPCs and blood vessels. Error bars indicate standard deviation. $*p < 0.05$, $***p < 0.001$, compared by Tukey-Kramer method.

CD31-positive blood vessels were slightly increased by the administration of estradiol, although the difference was not statistically significant (data not shown). In contrast to anti-CD31 antibody, which is blood vessel-specific, anti-CD34 antibody stains thymic progenitor cells (TPCs), in addition to blood vessel endothelial cells (Fig. 5). The number of CD34-positive blood vessels were also increased without statistical significance (data not shown). The small rounded CD34-positive TPCs were dispersed in the vehicle and saline-treated thymus (Fig. 5A), whereas they were accumulated near the blood vessels in the estradiol-treated involute thymus (Fig. 5B). Quantitative analysis revealed statistically significant reduction of the distance between TPCs and blood vessels, supporting this observation (Fig. 5D). The treatment with etodolac partially increased the distance (Fig. 5C and D). These results indicated that the interaction of CD34-positive TPCs with blood vessel endothelial cells is somehow enhanced during the estradiol-induced thymic involution in a prostanoid-dependent manner.

The interaction between TPCs and blood vessel endothelial cells is controlled by selectin molecules.[19] Therefore, we examined the expression of E-, L-, and P-selectins in the estradiol-treated involute thymus. The expression of E- and P-selectins was significantly upregulated during the estradiol-induced thymic involution, although L-selectin expression was not affected (Fig. 6). The upregulation of E- and P-selectins was suppressed by the treatment with etodolac at least in part. Thus, the expression of E- and P-selectins is regulated by prostanoids during thymic involution.

DISCUSSION

In this study, we examined the role of prostanoids in the estradiol-induced thymic involution. T cell-immune deficiency associated with thymic atrophy or involution is clinically important in the aged society, because of the increased risks of tumor initiation and infections. Our study aimed to reveal the fundamental mechanisms of thymic involution to contribute to maintenance of T cell immunity during life.

Upregulation of Prostanoid Synthesis in the Thymic Involution Prostanoids have been known to play important roles in T cell development.[20] Previous studies have reported that the production of prostanoids is affected by some environmental stimulation in the thymus. Nanoparticle TiO_2 exposure increased the expression of COX-2.[21] PGE_2 production in monocytes was reported to be induced by CD83 antigen.[5] Thus, with regard to the pronounced changes of prostanoids in thymus, we have examined the expression of prostanoid synthases in the thymus during the thymic involution caused by acute diet-restriction (Razali *et al.*, manuscript submitted) and excess estradiol-administration (present study). Both stimulations induced the expression of prostanoid synthases, suggesting that the production of prostanoids in the thymus are regulated by various stimuli to control T cell development. The level of induced expression of COX-1 and COX-2 by the estradiol-administration, as well as of other selected prostanoid synthases, however, was faint and not statistically significant (Fig. 2). This may be due to the high production level of prostanoids in the thymus at steady state. The expression

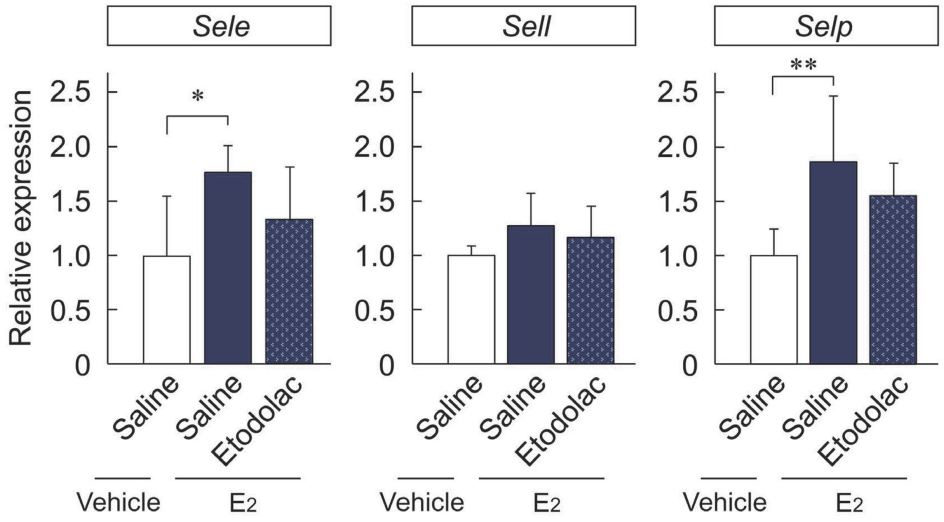

Fig. 6. Increased Expression of E- and P-Selectins During the Estradiol-Induced Thymic Involution

The mRNA expression in the thymus of vehicle/saline- (n=6), E_2/saline- (n=5), and E_2/etodolac- (n=5) treated mice was examined by qRT-PCR. Error bars indicate standard deviation. *$p < 0.05$, **$p < 0.01$, compared by Tukey-Kramer method.

level of COX-2 in the thymus is higher, compared to those in spleen, skin, lung and kidney.[22] PGE_2 is important for normal T cell development to regulate the differentiation of naïve T cells to Th1, Th17, and Treg lineages.[23–25] mPGES-1 is involved in the regulation of Th1 and Th17 responses.[26] Although estrogen has been known to block T cell development in the earliest stage of double negative thymocytes, that is, CD44+;CD25- cells to CD44+;CD25+ cells,[3] the effect of excess estradiol-administration on the T cell lineage determination has not been elucidated. Therefore, it would be interesting to know how the induced prostanoid synthesis controls T cell lineages during the estradiol-induced thymic involution.

It is still unclear which estrogen receptor is responsible for the estradiol-induced thymic involution. ERα and ERβ are described to be expressed in both thymocytes and thymic stromal cells.[27] Previous studies have implicated the involvement of ERα, whereas other manuscripts suggested the involvement of ERβ.[28,29] Our preliminary results revealed no expressional change of ERα and β by the estradiol-administration. Interestingly, the expression of progesterone receptor and androgen receptor in the thymus is upregulated in the estradiol-induced involute thymus (Hasegawa, unpublished observation). Progesterone signaling is known to be required for the pregnancy-related thymic involution.[30,31] Testosterone has also been known to exert thymic involution[32,33] and our preliminary experiments also showed similar results (Yoshikawa and Hasegawa, unpublished observation). Prostanoids modulate the expression of progesterone receptor.[34,35] As for androgen receptor, PGE_2 has been shown to upregulate androgen receptor expression through EP2 receptor-mediated cAMP signaling.[36] EP3 receptor signaling, in contrast, suppresses the expression of androgen receptor.[37] Thus, estradiol exerts thymic involution through the expressional change of progesterone and/or testosterone receptor expression, which could be regulated by prostanoids.

Interaction of TPCs with Blood Vessels Recent studies have indicated that blood vessels were dynamically remodeled in adult body, not only by the disease progression, including tumor formation and inflammation, but also by healthy physiological stimulation, such as practical training and pregnancy. So far, the blood vessel remodeling during thymic involution has not been described. We recently found that the new blood vessel formation has been observed during diet-restriction induced thymic involution (Razali *et al.*, manuscript in preparation). This blood vessel remodeling was also observed during estradiol-induced thymic involution (data not shown), although the induction by estradiol-administration was weaker compared to that induced by diet-restriction. Thus, blood vessel remodeling may be a mediator of thymic involution-related immune deficiency.

The increased blood vessels were observed to be more associated with CD34-positive TPCs (Fig. 5). Hematopoietic stem cells, which differentiate into T cells in the end, migrate into the thymus as CD34-negative thymus-settling progenitors. After the thymus-settling progenitors enter the thymus, they differentiate into TPCs.[38,39] TPCs, then, disperse into the cortex and undergoes further differentiation and selections to be specific T cell lineages. However, the regulation of dispersion of TPCs have not been understood very well. E- and P-selectins are expressed in blood vessel endothelial cells and required for T cell emigration.[40] P-selectin has been reported to control colonization of hematopoietic stem cells in the thymus of zebrafish, which may be comparable to TPCs in mammals.[41] In addition, P-selectin is implicated to play an important role in TPCs homing in mice.[19] However, the function of selectins in regulating TPCs in estradiol-induced thymic involution had remained elusive. As described above, estradiol-administration blocks double negative TPCs to be differentiated.[3] Another previous study reported that the estradiol-administration resulted in the preferential depletion of early progenitors and reduced proliferation of double negative 3 (DN3) and DN4 cell subsets.[4] Together with our results presented here, these negative effect of estradiol in TPC maintenance and differentiation may be due to the regulation of the localization of TPCs, which enter into the thymus through blood vessels and into the thymic stroma by detaching from blood vessel endothelial cells. In addition, our results, for the first time, indicated that E- and P-selectins are regulated during thymic involution. Hence, further studies are required to clarify the role of increased expression of E- and P-selectin

in the dispersion and differentiation capability of TPCs. Interestingly, sphingosine-1-phosphate, which is important for the expression of P-selectin, is upregulated during thymic involution.[42] Further studies will be performed to clarify the role of sphingosine signaling during thymic involution induced by estradiol and acute diet-restriction.

Acknowledgments This work was supported by Kaken-hi (25122703, 25430002 to Hasegawa). The authors thank Drs. Kimie Nakagawa and Bishnu Devi Maharjan in Laboratory of Hygienic Sciences, Kobe Pharmaceutical University, for their kind help in the maintenance of mice and warm support in the laboratory management.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Boehm T, Swann JB. Thymus involution and regeneration: two sides of the same coin? *Nat. Rev. Immunol.*, **13**, 831–838 (2013).
2) Lynch HE, Goldberg GL, Chidgey A, Van den Brink MRM, Boyd R, Sempowski GD. Thymic involution and immune reconstitution. *Trends Immunol.*, **30**, 366–373 (2009).
3) Rijhsinghani AG, Thompson K, Bhatia SK, Waldschmidt TJ. Estrogen blocks early T cell development in the thymus. *Am. J. Reprod. Immunol.*, **36**, 269–277 (1996).
4) Zoller AL, Kersh GJ. Estrogen induces thymic atrophy by eliminating early thymic progenitors and inhibiting proliferation of β-selected thymocytes. *J. Immunol.*, **176**, 7371–7378 (2006).
5) Chen L, Zhu Y, Zhang G, Gao C, Zhong W, Zhang X. CD83-stimulated monocytes suppress T-cell immune responses through production of prostaglandin E_2. *Proc. Natl. Acad. Sci. USA*, **108**, 18778–18783 (2011).
6) Jaffar Z, Ferrini ME, Shaw PK, FitzGerald GA, Roberts K. Prostaglandin I_2 promotes the development of IL-17–producing γδ T Cells that associate with the epithelium during allergic lung inflammation. *J. Immunol.*, **187**, 5380–5391 (2011).
7) Namba T, Sugimoto Y, Hirata M, Hayashi Y, Honda A, Watabe A, Negishi M, Ichikawa A, Narumiya S. Mouse thromboxane A_2 receptor: cDNA cloning, expression and northern blot analysis. *Biochem. Biophys. Res. Commun.*, **184**, 1197–1203 (1992).
8) Remuzzi G, Noris M, Benigni A, Imberti O, Sayegh MH, Perico N. Thromboxane A_2 receptor blocking abrogates donor-specific unresponsiveness to renal allografts induced by thymic recognition of major histocompatibility allopeptides. *J. Exp. Med.*, **180**, 1967–1972 (1994).
9) Hirono J, Sanaki H, Kitada K, Sada H, Suzuki A, Lie LK, Segi-Nishida E, Nakagawa K, Hasegawa H. Expression of tissue inhibitor of metalloproteinases and matrix metalloproteinases in the ischemic brain of photothrombosis model mice. *Neuroreport*, **29**, 174–180 (2018).
10) Hasegawa H, Noguchi J, Yamashita M, Okada R, Sugimoto R, Furuya M, Unoki T, Funakoshi Y, Baba T, Kanaho Y. Phosphatidylinositol 4-phosphate 5-kinase is indispensable for mouse spermatogenesis. *Biol. Reprod.*, **86, 136**, 1-12 (2012).
11) Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant.*, **48**, 452–458 (2013).
12) Pearse G. Histopathology of the thymus. *Toxicol. Pathol.*, **34**, 515–547 (2006).
13) Rezzani R, Nardo L, Favero G, Peroni M, Rodella LF. Thymus and aging: morphological, radiological, and functional overview. *Age (Dordr.)*, **36**, 313–351 (2014).
14) Abramson J, Anderson G. Thymic epithelial cells. *Annu. Rev. Immunol.*, **35**, 85–118 (2017).
15) Alexandropoulos K, Danzl NM. Thymic epithelial cells: antigen presenting cells that regulate T cell repertoire and tolerance development. *Immunol. Res.*, **54**, 177–190 (2012).
16) Danese S, Dejana E, Fiocchi C. Immune regulation by microvascular endothelial cells: directing innate and adaptive immunity, coagulation, and inflammation. *J. Immunol.*, **178**, 6017–6022 (2007).
17) Ramasamy SK, Kusumbe AP, Adams RH. Regulation of tissue morphogenesis by endothelial cell-derived signals. *Trends Cell Biol.*, **25**, 148–157 (2015).
18) van Hinsbergh VWM. Endothelium-role in regulation of coagulation and inflammation. *Semin. Immunopathol.*, **34**, 93–106 (2012).
19) Gossens K, Naus S, Corbel SY, Lin S, Rossi FMV, Kast J, Ziltener HJ. Thymic progenitor homing and lymphocyte homeostasis are linked via S1P-controlled expression of thymic P-selectin/CCL25. *J. Exp. Med.*, **206**, 761–778 (2009).
20) Rocca B, Spain LM, Puré E, Langenbach R, Patrono C, FitzGerald GA. Distinct roles of prostaglandin H synthases 1 and 2 in T-cell development. *J. Clin. Invest.*, **103**, 1469–1477 (1999).
21) Hong F, Zhou Y, Zhou Y, Wang L. Immunotoxic effects of thymus in mice following exposure to nanoparticulate TiO_2. *Environ. Toxicol.*, **32**, 2234–2243 (2017).
22) Kirkby NS, Chan MV, Zaiss AK, Garcia-Vaz E, Jiao J, Berglund LM, Verdu EF, Ahmetaj-Shala B, Wallace JL, Herschman HR. Systematic study of constitutive cyclooxygenase-2 expression: role of NF-κB and NFAT transcriptional pathways. *Proc. Natl. Acad. Sci. USA*, **113**, 434–439 (2016).
23) Boniface K, Bak-Jensen KS, Li Y, Blumenschein WM, McGeachy MJ, McClanahan TK, McKenzie BS, Kastelein RA, Cua DJ, de Waal Malefyt R. Prostaglandin E_2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *J. Exp. Med.*, **206**, 535–548 (2009).
24) Stobo JD, Kennedy MS, Goldyne ME. Prostaglandin E modulation of the mitogenic response of human T cells: differential response of T-cell subpopulations. *J. Clin. Invest.*, **64**, 1188–1195 (1979).
25) Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, Sugimoto Y, Narumiya S. Prostaglandin E_2–EP4 signaling promotes immune inflammation through TH1 cell differentiation and TH17 cell expansion. *Nat. Med.*, **15**, 633–640 (2009).
26) Maseda D, Johnson EM, Nyhoff LE, Baron B, Kojima F, Wilhelm AJ, Ward MR, Woodward JG, Brand DD, Crofford LJ. mPGES1-dependent Prostaglandin E_2 (PGE_2) controls antigen-specific Th17 and Th1 responses by regulating T autocrine and paracrine PGE_2 production. *J. Immunol.*, **200**, 725–736 (2018).
27) Lindberg MK, Weihua Z, Andersson N, Moverare S, Gao H, Vidal O, Erlandsson M, Windahl S, Andersson G, Lubahn DB. Estrogen receptor specificity for the effects of estrogen in ovariectomized mice. *J. Endocrinol.*, **174**, 167–178 (2002).
28) Li J, McMurray RW. Effects of estrogen receptor subtype-selective agonists on immune functions in ovariectomized mice. *Int. Immunopharmacol.*, **6**, 1413–1423 (2006).
29) Nohara K, Ao K, Miyamoto Y, Suzuki T, Imaizumi S, Tateishi Y, Omura S, Tohyama C, Kobayashi T. Arsenite-induced thymus atrophy is mediated by cell cycle arrest: a characteristic downregulation of E2F-related genes revealed by a microarray approach. *Toxicol. Sci.*, **101**, 226–238 (2007).
30) Laan M, Haljasorg U, Kisand K, Salumets A, Peterson P. Pregnancy-induced thymic involution is associated with suppression of chemokines essential for T-lymphoid progenitor homing. *Eur. J. Immunol.*, **46**, 2008–2017 (2016).
31) Tibbetts TA, DeMayo F, Rich S, Conneely OM, O'Malley BW. Progesterone receptors in the thymus are required for thymic involution during pregnancy and for normal fertility. *Proc. Natl. Acad. Sci. USA*, **96**, 12021–12026 (1999).
32) Mase Y, Oishi T. Effects of castration and testosterone treatment on the development and involution of the bursa of Fabricius and the thymus in the Japanese quail. *Gen. Comp. Endocrinol.*, **84**, 426–433 (1991).
33) Öner H, Ozan E. Effects of gonadal hormones on thymus gland after bilateral ovariectomy and orchidectomy in rats. *Arch. Androl.*, **48**, 115–126 (2002).
34) Madsen G, Zakar T, Ku CY, Sanborn BM, Smith R, Mesiano S. Prostaglandins differentially modulate progesterone receptor-A and -B expression in human myometrial cells: evidence for prostaglandin-induced functional progesterone withdrawal. *J. Clin. Endocrinol. Metab.*, **89**, 1010–1013 (2004).

35) Welsh TN, Hirst JJ, Palliser H, Zakar T. Progesterone receptor expression declines in the guinea pig uterus during functional progesterone withdrawal and in response to prostaglandins. *PLoS One*, **9**, e105253 (2014).
36) Kashiwagi E, Shiota M, Yokomizo A, Inokuchi J, Uchiumi T, Naito S. EP2 signaling mediates suppressive effects of celecoxib on androgen receptor expression and cell proliferation in prostate cancer. *Prostate Cancer Prostatic Dis.*, **17**, 10–17 (2014).
37) Kashiwagi E, Shiota M, Yokomizo A, Itsumi M, Inokuchi J, Uchiumi T, Naito S. Prostaglandin receptor EP3 mediates growth inhibitory effect of aspirin through androgen receptor and contributes to castration resistance in prostate cancer cells. *Endocr. Relat. Cancer*, **20**, 431–441 (2013).
38) Dzhagalov I, Phee H. How to find your way through the thymus: a practical guide for aspiring T cells. *Cell. Mol. Life Sci.*, **69**, 663–682 (2012).
39) Yang Q, Bell JJ, Bhandoola A. T-cell lineage determination. *Immunol. Rev.*, **238**, 12–22 (2010).
40) Auvinen K, Jalkanen S, Salmi M. Expression and function of endothelial selectins during human development. *Immunology*, **143**, 406–415 (2014).
41) Esain V, Kwan W, Carroll KJ, Cortes M, Li SY, Frechette GM, Sheward LMV, Nissim S, Goessling W, North TE. Cannabinoid Receptor-2 regulates embryonic hematopoietic stem cell development via prostaglandin E_2 and P-Selectin activity. *Stem Cells*, **33**, 2596–2612 (2015).
42) Kuchler L, Lisa KS, Giegerich AK, Knape T, Angioni C, Ferreirós N, Schmidt MV, Weigert A, Brüne B, von Knethen A. Elevated intrathymic sphingosine-1-phosphate promotes thymus involution during sepsis. *Mol. Immunol.*, **90**, 255–263 (2017).