Induced Prostanoid Synthesis Regulates the Balance between Th1- and Th2-Producing Inflammatory Cytokines in the Thymus of Diet-Restricted Mice

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Multiple external and internal factors have been reported to induce thymic involution. Involution involves dramatic reduction in size and function of the thymus, leading to various immunodeficiency-related disorders. Therefore, clarifying and manipulating molecular mechanisms governing thymic involution are clinically important, although only a few studies have dealt with this issue. In the present study, we investigated the molecular mechanisms underlying thymic involution using a murine acute diet-restriction model. Gene expression analyses indicated that the expression of T helper 1 (Th1)-producing cytokines, namely interferon-γ and interleukin (IL)-2, was down-regulated, while that of Th2-producing IL-5, IL-6, IL-10 and IL-13 was up-regulated, suggesting that acute diet-restriction regulates the polarization of naïve T cells to a Th2-like phenotype during thymic involution. mRNAs for prostanoid biosynthetic enzymes were up-regulated by acute diet-restriction. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses detected the increased production of prostanoids, particularly prostaglandin D2 and thromboxane B2, a metabolite of thromboxane A2, in the diet-restricted thymus. Administration of non-steroidal anti-inflammatory drugs, namely aspirin and etodolac, to inhibit prostanoid synthesis suppressed the biased expression of Th1- and Th2-cytokines as well as molecular markers of Th1 and Th2 cells in the diet-restricted thymus, without affecting the reduction of thymus size. In vitro stimulation of thymocytes with phorbol myristate acetate (PMA)/ionomycin confirmed the polarization of thymocytes from diet-restricted mice toward Th2 cells. These results indicated that the induced production of prostanoids during diet-restriction-induced thymic involution is involved in the polarization of naïve T cells in the thymus.

Key words thymic involution; diet-restriction; prostanoid; helper T cell; inflammatory cytokine; polarization

INTRODUCTION

The thymus is a primary lymphoid organ of mammals and plays a critical role in immune responses. It generates functionally immature antigen-specific T cells, which then migrate to the peripheral lymphoid tissue to mediate protection against invading microbes.1 As compared to other major organs, the thymus is highly dynamic. This indicates a capability of thymus to undergo multiple rounds of almost complete involution followed by rapid restoration. The process of thymic involution results in decreased thymopoiesis and migration of naïve T cells to the periphery, and contributes to immunosenescence and degeneration of the immune system.2,3 These changes are believed to significantly contribute towards the clinical features of immunosenescence-related disorders, including cancer and infectious diseases.4,5

Numerous factors induce thymic involution, including starvation, irradiation and immunosuppressive therapies.1,5 The thymic involution process has been reported to be associated with a reduction in tissue mass and thymic cellularity, and loss of tissue structure and abnormal architecture, thus leading to a decline in naïve T cell output.4,6 In fact, starvation or diet-restriction-induced thymic involution has been designated as “the barometer of malnutrition” as it exerts a pronounced effect on thymus.7 The involution of thymus by diet-restriction is thought to involve the rise of plasma levels of glucocorticoids and inflammatory cytokines. Diet-restriction attenuates inflammatory responses in mice.9 With regards to the inflammatory process, the levels of glucocorticoids and inflammatory cytokines are elevated, which then contribute to thymic involution.9 However, the signaling mechanisms involved in diet-restriction-induced thymic involution, or the induction of inflammatory cytokines associated with thymic involution, remain unclear.

Thymic progenitor cells differentiate into naïve T cells in the thymus, which progressively differentiate into unactivated helper T cell type 0 (Th0) cells. These are released to the periphery, followed by terminal differentiation into helper T cell types 1 (Th1) and 2 (Th2) upon immunological stimulation.10 Each Th0 cell is not completely fate-determined in the thymus, but tend to differentiate into either Th1 or Th2 cell. This plastic deviation of naïve T cells into Th1- or Th2-like cells, called polarization, affects cellular immune responses during T cell receptor (TCR) engagement.11 Although the polarization state is crucial for the determination of effectiveness of cellular immune responses, it is totally unknown whether the polarization of naïve T cells in the thymus is changed during thymic involution. Notably, Th1- and Th2-producing cytokines

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enhance the polarization of naïve T cells to have Th1-like or Th2-like characters, respectively, to form a positive feedback loop.

Prostanoids, including prostaglandins (PGs), prostaacyclin (PGI2), and thromboxane (Tx), are one of the major groups of lipid mediators, and are involved in numerous physiological reactions including inflammation and cellular differentiation. Prostanoids are produced from arachidonic acid in membrane phospholipids via the cyclooxygenase (COX) pathway. A Ca2+ influx across the plasma membrane upon stimulation of the cell leads to the activation of phospholipase A2 (PLA2), which cleaves arachidonic acid from membrane phospholipids. Released arachidonic acids are then metabolized to prostanoid or lipoxin molecules by the COX- or lipoxigenase-pathways, respectively. Three isoenzymes of COX, COX1–3, have been identified so far. COX1 mainly functions as a physiologic housekeeper and induces prostanoid synthesis during homeostasis of normal body functions, while COX2 is involved in prostanoid synthesis associated with inflammation.12) COX3, an mRNA splicing variant of COX1, has been reported to function in homeostatic and inflammatory responses in the nervous system.13) Reduced biosynthesis of prostanoids through inhibition of COX activity is the primary mechanism of the anti-inflammatory effect of nonsteroidal anti-inflammatory drugs (NSAIDs).14) In the thymus, prostanoids, in combination with other signaling molecules, particularly cytokines, play essential roles to regulate T cell differentiation, proliferation, and function. Prostanoids and cytokines in the thymus are important for the processes involved in normal T cell development.14) In contrast, the change in production of prostanoids during thymic involution and their functional roles for thymic involution have not yet been clarified. We here hypothesized that changes in prostanoid synthesis are involved in thymic involution.

Hence, we investigated the polarization of thymic lymphocytes during diet-restriction-induced thymic involution by observing the expression of inflammatory cytokines and Th1/Th2-marker genes. The diet-restriction-induced thymic involution was accompanied by prostanoid production, which was responsible for the change of naïve T cell polarization. In vitro culture experiment of thymocytes indicated that the thymocytes from diet-restricted mice tended to differentiate into Th2 cells upon differentiating stimulation. The results indicated that induced prostanoid synthesis in the involuting thymus affects naïve T cell polarization so as to have Th2-like characters.

MATERIALS AND METHODS

Animals and Experimental Procedures Male ICR mice (8-weeks old) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were housed in a temperature-controlled room in the Animal Facility at Kobe Pharmaceutical University with a 12-h light/dark cycle. They had access to food pellets and water ad libitum. All experimental procedures were performed and conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan following approval by the Kobe Pharmaceutical University Committee for Animal Care and Use.

Diet-Restriction Treatment and NSAID-Injection Mice were housed in individual cages and allowed to acclimatize for at least 1 week. Diet-restriction was performed by taking the mice into new cages without food pellets and housed for 48 h. Water was freely accessible to the mice during diet-restriction. For NSAID-treatment experiments, mice were grouped as Group A, treated with ad libitum diet and intraperitoneal (i.p.) injection of saline every 24 h; B, with diet-restriction for 48 h and i.p. injection of saline every 24 h; C, with diet-restriction for 48 h and i.p. injection of 5 mg/kg body weight/d aspirin (1 mg/mL in saline) every 24 h; D, with diet-restriction for 48 h and i.p. injection of 5 mg/kg body weight/d etodolac (1 mg/mL in saline) every 24 h. All tested mice were weighed before and after treatment.

Tissue Collection Mice were anesthetized and transcardially perfused with phosphate buffered saline (PBS). Thymus, heart, and kidney were collected and weighed. Thymus images were captured using a digital camera (Hozan Tool Ind. Co., Ltd., Osaka, Japan) attached to an inverted microscope (Kenis Ltd., Osaka, Japan).

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analyses Following the thymus tissue collection, thymus was rapidly frozen on dry ice and stored at −80°C. Frozen thymus was homogenized in methanol with beads homogenizer. Samples were extracted by solid phase extraction using Oasis hydrophilic-lipophilic balance (HLB) extraction cartridges (Waters, Milford, MA, U.S.A.) with deuterium-labeled internal standard (PGE2-d9). For measuring prostanoids, LC-MS/MS-based analyses were performed using an HPLC system Nexera X2 (Shimadzu, Kyoto, Japan) with a linear ion trap triple quadruple mass spectrometer QTRAP 5500 (SCIEX, Framingham, MA, U.S.A.). A reverse-phase column (Kinetex C18, 1.7 μm, 150 × 2.1 mm, Phenomenex, Torrance, CA, U.S.A.) was used for chromatographic separation. Samples were eluted with mobile phase A (0.1% formic acid in water) and B (acetonitrile). The flow rate was 0.3 mL/min. The gradient of mobile phase B concentration was programmed as 30% (0–1 min)–80% (5–6 min)–100% (8–9.5 min)–30% (9.51–12 min). MS/MS analysis were conducted in negative ion mode, and prostanoids were quantified by multiple reaction monitoring (MRM). MRM transitions of PGE2, PGD2, PGF2α, 6-keto PGF2α, and TxB2 were 351 > 271 m/z, 351 > 271 m/z, 353 > 193 m/z, 369 > 163 m/z, and 369 > 169 m/z, respectively. Quantification was performed using standard curve for each compound and the calculated values were corrected by recovery rates of internal standard.

In Vitro Stimulation of Thymic Cells In vitro culture of thymic cells was performed according to previous manuscript.15) Briefly, thymic lobes were collected from the anesthetized mice. They were grinded on cell strainers (70 μm, Falcon) with RPMI-1640 medium (Nacalai Tesque) to obtain thymic cells. The cells were then centrifuged and suspended in RPMI-1640 medium containing 10% fetal bovine serum. They were seeded in 12 well plates at 1.0 × 10⁷ cells/well and stimulated with 25 ng/mL phorbol myristate acetate (PMA) and 1 μg/mL ionomycin. After culturing at 37°C, 5% CO2 for 3 h, the cells were collected in 1.5 mL tubes and centrifuged at 4°C to analyze mRNA expression.

RNA Extraction and RT-Quantitative PCR Analyses RNA extraction and RT-qPCR analysis were performed as described in our previous manuscript.16) Briefly, tissues were initially homogenized in ice-cold Sepasol-RNA I super G with a potter homogenizer. Total RNA was extracted and
purified according to the manufacturer’s instructions. The concentrations of extracted RNA were determined by using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, U.S.A.). cDNA was synthesized using ReverTra Ace reagent (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer’s instructions. The expression of target genes was determined using a CFG Connect real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). PCR amplification was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories) with primer pairs as listed in Table 1. PCR was performed with the following thermocycling parameters; 1 min of initial DNA polymerase activation and DNA denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, and primer annealing at 60°C for 30 s. Melt curves of the real-time PCR products were analyzed from 65°C to 95°C. Differences in gene expression, expressed as fold-change, were calculated using the ∆∆Ct method, where Rplp2 was used as a reference gene for normalizing expression.

**Statistical Analyses** Results are expressed as means ± standard deviation. The data were statistically analyzed using SPSS software, version 21.0 (IBM Corp., Armonk, NY, U.S.A.) and EZR software for Windows. Student’s t-test was used for comparisons of means between groups of mice with diet-restriction treatment compared to mice provided with diet. One-way ANOVA and Tukey–Kramer test were used to compare means among groups.

**RESULTS**

### Expression of Inflammatory Cytokines in the Involuting Thymus after Diet-Restriction Treatment

Previous studies have reported decreased thymus weight relative to body weight after diet-restriction treatment. Diet-restriction treatment for 48h resulted in approximately 18% reduction in body weight (Fig. 1A). This treatment reduced the thymus weight, normalized to whole body weight, by approximately 15% (Figs. 1B–D). On the other hand, the weights of heart and kidney relative to body weight were not significantly affected by diet-restriction treatment (Fig. 1D). These results indicated that the diet-restriction treatment induced thymic involution.

We then examined the polarization of naïve T cells in the thymus during thymic involution. Th1 cells mainly produce inflammatory cytokines interferon (IFN)-γ and interleukin (IL)-2, while Th2 cells are characterized to release IL-4, IL-5, and IL-10.19) The expression of mRNAs for Il-4 and Il-2 was significantly down-regulated by 0.71- and 0.77-fold, respectively (Fig. 2). In contrast, Il-5 and Il-10 mRNA expression was significantly up-regulated by 1.61- and 3.36-fold, respectively, in the thymus of diet-restricted mice compared to the thymus of control mice. These results indicated that diet-restriction treatment deviates naïve T cells to Th2-like cells. Notably, the expression of Il-4 mRNA was not significantly affected by the diet-restriction treatment.

### Expression of the Enzymes Involved in Prostanoid Synthesis in the Thymus of Diet-Restricted Mice

Prostanoids at inflamed sites regulate cytokine productions. On the other hand, prostanoids are involved in T cell development in the thymus during thymic involution. Th1 cells mainly produce prostanoids, while Th2 cells are characterized to release IL-4, IL-5, and IL-10. Therefore, prostanoids are involved in T cell development in the thymus during thymic involution.

#### Table 1. Primer Sequences for the Genes Used for RT-qPCR Analyses

| Gene  | Forward primer | Reverse primer | Gene ID |
|-------|----------------|----------------|--------|
| Rplp2 | 5'-TACTAGACAGCGGTGGTAC-3' | 5'-CAACACTCTGAGCAGTACA-3' | 67186 |
| Ifn-g | 5'-TCAAGGTCATGAGATGGGAGA-3' | 5'-TGCTCTGAGGATTTCATG-3' | 15978 |
| Il-2  | 5'-TTGGTCCCTGCTGGAAC-3' | 5'-GGAGCCTAGAGCAGCTCA-3' | 16189 |
| Il-4  | 5'-ACGGACAGCTGTCGGAAGG-3' | 5'-TCCCTGCGCCACCTTCCCC-3' | 16191 |
| Il-5  | 5'-CTCTGGGGATCTGGGAAT-3' | 5'-CCAGTGGTGATGACCATC-3' | 16193 |
| Il-6  | 5'-CTACACTGCCACCCCTTCCG-3' | 5'-GGGATAAATCCCTAAGCATCG-3' | 19924 |
| Cox1  | 5'-GAAGGTCACACCTCTTCCC-3' | 5'-CATCGAGCTGACTGAGG-3' | 19925 |
| Cox2  | 5'-AGTACCGGAGCAGCTTCCC-3' | 5'-TGACTGACTTCTCACCCTG-3' | 19215 |
| Ptgds | 5'-TGAAGGACCGAGCTAGAGG-3' | 5'-GATACCCGCTAGAGAAGTCG-3' | 54846 |
| Hpgds | 5'-ATCAAGCGCCTCGCTCTTGG-3' | 5'-AGGAAAGAGATGATGTTCC3' | 64292 |
| Ptgds-1 | 5'-GGATGCGGTGGTACAGAGG-3' | 5'-ACTCGCAAGCCACATACAC-3' | 96979 |
| Ptgds-2 | 5'-ATCACATGGGTTGATGAGG-3' | 5'-ATTCACGCGAGTACACAG-3' | 56351 |
| Akr1b3 | 5'-AGCTACACAGAGGAATGAGG-3' | 5'-ACAGTGGCAGAGCAGCATG-3' | 11677 |
| Akr1b7 | 5'-GCACCTACAGGCTGAGG-3' | 5'-ACAGTGGCAGAGCAGCATG-3' | 11997 |
| Ptgds | 5'-GAGGTCACACCTCTTCCC-3' | 5'-AGTACCGACTTCTCACCCTG-3' | 19223 |
| Tbxas1 | 5'-GAGGCAGCTGCCAGATGACT-3' | 5'-ATACCTGGTCTCCACGACCTC-3' | 21391 |
| Gata3 | 5'-CCCTATCAAGCCGGAGCAGG-3' | 5'-CCCATTAGGGTCTCCTCC-3' | 14462 |
| Tbx21 | 5'-GTGTCAGGAGGATTGAAGTC-3' | 5'-ACCAGGAGAAGCAGAAGTGG-3' | 57765 |
| Sta1 | 5'-CTACAGGTCACTTAAAGACG-3' | 5'-ACTGACATTCAAGCAGGAC-3' | 20846 |
| Sta3 | 5'-CAGAGTTCAACGACCTGACC-3' | 5'-GGTACACTCCAGTCTCGAG-3' | 20848 |
| Sta4 | 5'-GCAATTTCTACTCTACTG-3' | 5'-TGCAGAAGTTGGCCAGAGTG-3' | 20849 |
| Sta5 | 5'-CAAGAAGCAGAGGGCTGTTTC-3' | 5'-GCTCTCAAAACTTGGTGG-3' | 20850 |
| Sta6 | 5'-CTCCTCCCGTCTTACGACT-3' | 5'-AGTAGGAGCAAGAGGAGTC-3' | 20852 |
Prostaglandin endoperoxide synthases, known as COX1 and 2, produce the unstable intermittent, prostaglandin H$_2$ (PGH$_2$), which is further metabolized into each prostanoid by specific enzymes. Lipocalin prostaglandin D synthase (L-PGDS) encoded by the Ptgds gene, and hematopoietic prostaglandin D synthase (H-PGDS) encoded by Hpgds, produce prostaglandin D$_2$ (PGD$_2$) from PGH$_2$. Three prostaglandin E synthases, m-PGES1, m-PGES2 and c-PGES (encoded by Ptges1, Ptges2 and Ptges3, respectively), produce prostaglandin E$_2$ (PGE$_2$). Prostacyclin (PGI$_2$) is produced by prostaglandin I$_2$ synthase, PGIS (encoded by Ptgis). Aldo-keto reductase family 1, member B3 (Akrlb3 gene product) and member B7 (Akrlb7 gene product) produce prostaglandin F$_2$α (PGF$_2$α), and thromboxane A synthase 1 (Tbxas1 gene product) produces thromboxane A$_2$ (TxA$_2$).

In response to the diet-restriction treatment, all prostanoid synthases, with the exception of Akr1b3 and Ptges2, were up-regulated (Fig. 3). These results indicated that prostanoid synthesis might be up-regulated in response to diet-restriction treatment in the thymus of mice.

Production of Prostanoids in the Thymus of Diet-Restricted Mice

To confirm the production of each prostanoid species, LC-MS/MS analysis was performed. In this analysis, PGE$_2$, PGI$_2$, and PGF$_2$α are detected as intact active molecules, whereas PGH$_2$ and TxA$_2$ cannot be detected because of their short half-lives. The productions of PGH$_2$ and TxA$_2$ in the samples are estimated from the contents of 6-keto PGF$_1$α and
Tissue extract of the thymus of diet-restricted (n = 5) and diet-provided (n = 5) mice were assayed for prostanoid production using LC-MS/MS technology as described in Materials and Methods section. Error bars indicate standard deviation. p-Values from Student’s t-test were indicated.

The amounts of PGD2 and TxB2 in the thymus of diet-restricted mice were slightly higher than those in the thymus of control diet-provided mice (p = 0.057 and 0.061, respectively) (Fig. 4). In contrast, the contents of PGE2, PGF2α, and 6-keto PGF1α in the thymus were not apparently changed by the diet-restriction treatment. These results indicated that PGD2 and TxA2 production was specifically up-regulated during diet-restriction-induced thymic involution, despite the increased mRNA expression of almost all prostanoid synthases (Fig. 3).

Effect of NSAIDs in the Reduction of Thymus Size during Diet-Restriction-Induced Thymic Involution With regards to the induced production of prostanoids by diet-restriction, NSAIDs were administered in order to evaluate the contribution of prostanoids to thymic involution. NSAID-treatment itself did not affect the expression of prostanoid synthases in the thymus in a diet-provided condition, with the exception of Thxasl, whose expression was down-regulated in aspirin-injected mice (Supplementary Fig. 1). Figure 5A shows the body weight change of the mice provided with diet (Group A), diet-restricted with i.p. injections of saline (Group B), aspirin (Group C) and etodolac (Group D), before and after 48h. There was no change in the control group of mice provided with diet and injection of saline for 48h. The diet-restricted mice with saline injections (Group B) significantly lost body weight by approximately 18%. This result was similar to the observation shown in Fig. 1A. In mice injected with aspirin (Group C), the diet-restriction treatment also significantly reduced the average body weight by 14% after 48h. Furthermore, in mice injected with etodolac (Group D), diet-restriction treatment significantly reduced body weight by 14%. Thus, the levels of reduction in body weight were not significantly affected by the administration of NSAIDs.

Following the reduction of body weight, we examined the relative weight of thymus, heart and kidney after 48h of diet-restriction treatment. The weight of thymus relative to body weight in the diet-restricted mice injected with saline (Group B) was 31% lower than control mice with diet (Group A) (Fig. 5B). Similar reduction of thymus size was observed in diet-restricted mice injected with aspirin (Group C) and etodolac (Group D), by 20 and 46%, respectively (Fig. 5B). Thus, the administration of NSAIDs did not affect the reduction in thymus size induced by diet-restriction. The relative weights of heart and kidney were not significantly affected by diet-restriction treatment, with the exception of kidney in Group D (Figs. 5C, D). This reduction of relative kidney size may indicate etodolac toxicity under the diet-restricted condition, which will be examined in the following studies.

Effect of NSAIDs on the Expression of Inflammatory Cytokines and Th Subtype Markers during Diet-Restriction-Induced Thymic Involution In order to clarify the role of prostanoids in the diet-restriction-induced deviation of naïve T cell polarization, we examined the effects of NSAIDs on the expression of Th subtype-specific inflammatory cytokines, Ifn-g, Il-2 (for Th1 cells), Il-4, Il-5, Il-6, Il-10 and Il-13 (for Th2 cells). The administration of aspirin or etodolac by itself did not affect the expression of those inflammatory cytokines in thymus under the diet-provided condition (Supplementary Fig. 2). Next, we examined the expression of those cytokines under the diet-restricted condition. Similarly to Fig. 2, the diet-restriction treatment down-regulated the expression of Th1-producing cytokines and up-regulated that of Th2-producing cytokines (Fig. 6). Injection of aspirin (Group C) and etodolac (Group D) cancelled the down-regulation of Ifn-g and Il-2 by the diet-restriction treatment and, in contrast, up-regulated them (Fig. 6A). The enhanced expression of Il-5 in the diet-restricted thymus was also totally cancelled by the injection of aspirin and etodolac, and the level of its expression was even lower than in the thymus of diet-restricted mice injected with saline. The expression of Il-10 and Il-13 was significantly reduced in mice injected with aspirin and etodolac. The expression of Il-6 tended to be reduced by NSAIDs, although the difference was not statistically significant. Interestingly, the expression of Il-4 was significantly down-regulated in response to the injection of aspirin and etodolac, although the expression of Il-4 was not affected by the diet-restriction treatment by itself. These results indicated that the diet-restriction-induced biased polarization of naïve T cells depends on the enhanced production of prostanoids in the thymus.

To further evaluate the involvement of prostanoids in the polarization of Th1 and Th2 cells in thymus, we examined the expression of selected markers of Th1 cells namely T-box 21 (Tbx21, also known as T-box expressed in T-cells (T-bet)), signal transducer and activator of transcription (Stat) 1 and 4, and of Th2 cells, Stat3, Stat5, Stat6 and GATA binding protein 3 (Gata3). Diet-restriction down-regulated the expression of Th1 cells-expressing Tbx21, Stat1 and Stat4, and up-regulated Th2-marker, Stat3, Stat5 and Gata3 (Fig. 7), which supported the biased polarization of naïve T cells by diet-restriction. Aspirin and etodolac treatments themselves did not affect the expression of these Th1 and Th2 markers under the diet-provided condition (Supplementary Fig. 3). In contrast, NSAIDs effectively cancelled the deviated expression of Th1- and Th2-markers by diet-restriction (Fig. 7). Thus, the polarization of naïve T cells induced during diet-restriction-induced thymic involution depends on prostanoids. Interestingly, the expression of Stat6 was significantly down-regulated in response to administration of aspirin and etodolac under the diet-restricted
condition, although there was no significant change in the expression of Stat6 by diet-restriction itself.

Expression of Th1- and Th2-Inflammatory Cytokines and Th-Subtype Markers during Diet-Restriction-Induced Thymic Involution in in Vitro Stimulation of Thymic Cells

In vitro stimulation was generally used to induce the peripheral differentiation of T cells to Th1 and Th2 phenotype. Combinatorial treatment with protein kinase C-activating PMA and ionomycin, a calcium ionophore, is commonly used as a stimulant to induce differentiation of naïve T cells and production of a variety of cytokines. In order to clarify the functional consequence of biased polarization of thymic T cells by diet-restriction, we cultured thymocytes from control diet-provided and diet-restricted mice and stimulated with PMA/ionomycin. Consistent with the observations in previous manuscript, PMA/ionomycin treatment increased the expression of mRNA encoding Th1-producing cytokines, Ifn-g and Il-2, by about 5,000-fold in the control thymocytes from diet-provided mice (Fig. 8A). This treatment also increased the expression of mRNA encoding Th2-producing cytokines, Il-4, Il-5, Il-6, Il-10 and Il-13 (Fig. 8B). The induction of Th2-cytokines was 20 to 300-fold, which is much less than the induction of Th1-cytokines. The experiment with the thymocytes from diet-restricted mice showed less induction of Th1-producing cytokines and more production of Th2-cytokines (Fig. 8).

The expression of Th1- and Th2-subtype markers was also examined in the in vitro culture experiment (Fig. 9). Consistent with the expression of subtype-specific cytokines, Th1-markers, namely Tbx21 and Stat1, were highly up-regulated by the PMA/ionomycin-stimulation by 30- to 40-fold in the control diet-provided thymocytes (Fig. 9A). Another Th1-marker Stat4 was also up-regulated, although the induction level was lower than that of Tbx21 and Stat1. The induction of Th2-markers, Stat3, Stat5, Stat6 and Gata3 was also observed in the PMA/ionomycin-stimulated thymocytes of diet-provided mice, whose induction level was around 2- to 10-fold (Fig. 9B). Diet-restriction treatment greatly reduced the induction of Th1-expressing Tbx21, Stat1 and Stat4, and enhanced that of Th2-markers, Stat3, Stat5, Stat6 and Gata3 (Fig. 9). These results supported the biased polarization of naïve T cells by diet-restriction treatment indicated by the Th1- and Th2-producing cytokines (Fig. 8).

These observations of the biased in vitro differentiation of thymic cells consolidated the diet-restriction-induced biased polarization of naïve thymic T cells towards Th2-like phenotype.
DISCUSSION

Diet-Restriction-Induced Thymic Involution Involves the Polarization of Naïve T Cells with Regards to Inflammatory Cytokine Production

We first checked diet-restriction-induced thymic involution in our experimental system. As reported in previous manuscripts, acute diet-restriction for 48 h induced apparent reduction in thymus size relative to body weight (Fig. 1). Most previous researches have been performed using C57BL/6 or BALB/c mice.24,25) We utilized ICR mice in this study because previous studies reported that the negative exponential curve for thymus size represented aging-induced thymic involution is comparable between humans and ICR mice, indicating that ICR mice could be a good model of human thymic involution.26,27)

It has been reported that biological stresses, including diet-restriction, induce many types of inflammatory cytokines, which contribute to thymic involution.9,28) However, the functional roles of inflammatory cytokines in thymic involution remain obscure.

Naïve T cells are primed to be unactivated Th0 cells, which are activated by recognition of a peptide antigen–class II major histocompatibility complex (MHC) presented on antigen-presenting cells (APCs) through interaction with the TCR. After activation, Th0 cells begin to divide and give rise to a clone of effector cells specific for each antigen–class II MHC complex.29) These effector Th cells are CD4-positive and can be divided into three main subtypes, Th1, Th2, and Th17 cells, with distinct cytokine secretion-eliciting unique functional characteristics.30) Th1 cells secrete IFN-γ, IL-2, and tumor necrosis factor α (TNFα), controlling immunity against foreign pathogens.19) In contrast, Th2 cells produce various cytokines, including IL-4, IL-5, IL-6, IL-10, and IL-13, which are primarily involved in promoting humoral immunity, protecting against infection.31) Th17 cells produce predominantly

Fig. 6. Expression of Th1- and Th2-Producing Cytokines in the Thymus after Diet-Restriction Treatment and Injection of Saline or NSAIDs

The expression of mRNA for (A) Th1-producing cytokines (Ifn-g and Il-2) and (B) Th2-producing cytokines (Il-4, Il-5, Il-6, Il-10 and Il-13) in the thymus after diet-restriction treatment in mice injected with saline (blue bars), aspirin (yellow dotted bars) and etodolac (yellow lined bars) compared to that of control group mice provided with diet was examined by RT-qPCR analysis. The expression of all genes was normalized to that of Rplp2, and calculated as relative change. Error bars indicate standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001 (n = 5–6). (Color figure can be accessed in the online version.)
the inflammatory cytokine IL-17, and play an important role in protecting the host from invading pathogens, especially on environmental surfaces.32) These Th subtype-specific inflammatory cytokines also regulate the characteristics of T cells. Th1-producing IFN-γ and IL-2 polarize or progress differentiation into Th1 cells, whereas Th2-producing IL-5 and IL-10 enhance the polarization and differentiation into Th2 cells.33) Thus, these inflammatory cytokines function as autocrine polarization signals. In particular, those cytokines produced in the thymus are also involved in the polarization of T cells.34) Polarization of T cells plays a vital role dictating the function and fate of T cells. In the present study, diet-restriction treatment significantly decreased the expression of Th1 cytokines and increased the expression of Th2-produced cytokines, (Figs. 2, 6). Thus, diet-restriction prepares the cytokine environment to induce the polarization of naïve T cells toward Th2 cells during thymic involution. This biased polarization during thymic involution was evidenced by the expression of Th1- and Th2-specific transcription factors (Fig. 7). The biased polarization of Th0 cells in the thymus induced by diet-restriction would regulate the activation and migration of T cells to the site of infection or immune activation.39,35)

### Functional Consequences of Biased Polarization of Naïve T Cells Induced by Diet-Restriction

Naïve T cells emigrated from the thymus differentiate into functionally mature Th1 or Th2 cells upon stimulations, such as infection and immune reactions. The most commonly used experimental method of differentiation of naïve T cells upon the stimulation is the treatment with PMA and ionomycin, which induces the differentiation of naïve T cells toward both Th1 and Th2 cells.21,23) (Figs. 8, 9). Thymocytes from the mice with diet-restriction treatment failed to express Th1-producing cytokines and cell type markers and tended to express more Th2-producing cytokines and cell type markers (Figs. 8, 9). Thus, the naïve T cells in the thymus undergone the polarization toward Th2-like cells by diet-restriction treatment prefer differentiation toward Th2 cells upon the differentiating stimulation. It would be interesting to know whether the biased polarization during thymic involution affect pathophysiology of the diseases related to excess Th1 functions, including Crohn’s disease, rheu-

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**Fig. 7.** Expression of Th1- and Th2-Marker Genes in the Thymus after Diet-Restriction Treatment and Injection of Saline or NSAIDs

The expression of mRNA for (A) Th1-markers (Tbx21, Stat1 and Stat4) and (B) Th2-markers (Stat3, Stat5, Stat6 and Gata3) in the thymus by diet-restriction treatment in mice injected with saline (blue bars), aspirin (yellow dotted bars) and etodolac (yellow lined bars) compared to that of control group mice provided with diet was examined by RT-qPCR analysis. The expression of all genes was normalized to that of Rplp2, a housekeeping gene. Error bars indicate standard deviation. *p < 0.05, **p < 0.01, ***p < 0.001 (n = 5–6). (Color figure can be accessed in the online version.)
matoid arthritis and multiple sclerosis as well as bacteria/virus infection and exposure to external/intrinsic allergens.\textsuperscript{36–38)} Interestingly, the expression of \textit{Il-4} was highly induced in the stimulated thymocytes from control diet-provided mice and it was enhanced by the diet-restriction treatment. This conflicts with the unchanged expression of \textit{Il-4} \textit{in vivo} (Figs. 2, 6). Similarly to our results, previous examination of the thymus under obesity-induced thymic involution revealed unchanged expression of \textit{Il-4} mRNA, although IFN-\textgamma and IL-2 were affected.\textsuperscript{39)} IL-4 is one of the main Th2-producing cytokines that plays a crucial role in inflammatory events and promotes differentiation and proliferation of Th2 cells.\textsuperscript{40)} Recent studies have indicated that IL-4 in the thymus inhibits the commitment of early thymic progenitor cells to T cell lineage as well as the differentiation of CD8\textsuperscript{+} cytotoxic T cells.\textsuperscript{41,42)} IL-4 expression in the thymus may be strictly controlled to avoid its unpleasant effect on T cell development.

**Roles of Prostanoids during Diet-Restriction-Induced Thymic Involution**

It has been reported that increased production of prostanoids is responsible for the regulation of balance between Th1- and Th2-producing inflammatory cytokines during inflammatory responses.\textsuperscript{43)} This led us to hypothesize that prostanoids might play important roles in modulating changes in the expression of those Th1- and Th2-produced cytokines during diet restriction-induced thymic involution.

We observed the significant induction of prostanoids during diet-restriction-induced thymic involution. Although the mRNAs encoding most prostanoid synthases were up-regulated by the diet-restriction, LC-MS/MS analyses detected the induction of only PGD\textsubscript{2} and TxA\textsubscript{2}. These results indicate that PGD\textsubscript{2} and TxA\textsubscript{2} contents in the thymus were increased during thymic involution at least. However, considering a very short half-lives as well as a possible diffusion of prostanoids through the blood and lymph vessels rich in the thymus, our results do not exclude the possible induction of other prostanoids.\textsuperscript{44)}

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Fig. 8. Expression of Th1- and Th2-Producing Cytokines in \textit{in Vitro}-Activated Cultured Thymocytes from Diet-Restricted Mice

The expression of mRNAs for (A) Th1-producing cytokines (\textit{Ifn-g} and \textit{Il-2}) and (B) Th2-producing cytokines (\textit{Il-4}, \textit{Il-5}, \textit{Il-6}, \textit{Il-10} and \textit{Il-13}) in the cultured thymocytes from diet-provided or diet-restricted mice, with or without the 3h-stimulation with PMA and ionomycin, was examined by RT-qPCR analysis. The expression of all genes was normalized to that of \textit{Rplp2}, and calculated as relative change. Error bars indicate standard deviation. \textit{**p}<0.01, ***p<0.001 (n=6).
Importantly, prostanoids are not responsible for the reduction of thymus size (Fig. 5). The mechanisms regulating thymus size during diet-restriction-induced thymic involution is still unclear. The most likely candidate is glucocorticoid, as acute diet-restriction or starvation elevates blood glucocorticoid level and glucocorticoid is involved in thymic involution.45–47) The mechanisms related to determination of thymus size wait future studies.

Prostanoids influence cytokine production to regulate the differentiation of naïve CD4+ T cells to Th1, Th2 and Th17 cell phenotypes.48) Cyclooxygenases and some specific prostaglandins generally facilitate Th2 phenotype, while suppress Th1 differentiation. Induced production of PGD2 during inflammation was reported to reduce the secretion of Th1-producing cytokines, IFN-γ, while increasing those of Th2 cytokines, namely IL-4, IL-5, IL-10 and IL-13.49) PGD2 exerts its function by activating two G-protein coupled receptors, D-type prostanoid receptor 1 (DP1) and 2 (DP2). DP2 is also referred to as chemoattractant receptor homologous-molecule expressed in Th2 cells (CRTH2).50) CRTH2 receptor activation is primarily linked to pro-inflammatory effects including initiation and potentiation of immune cell migration and Th2 cytokine production namely IL4, IL-5 and IL-13 to enhance Th2-mediated inflammatory response.50–52)

The action of TxA2 is mediated by a thromboxane-prostanoid (TP) receptor.53) TP receptor is expressed at high level in the thymus, most prominently in immature double positive (CD4+/CD8+) thymocytes, and stimulation of TP receptor on these cells promotes apoptosis prior to the positive and negative selections, suggesting that TP receptor might play a role in the course of maturing T lymphocyte selection.54,55) In the lipopolysaccharide (LPS)-induced thymic inflammation, enhanced production of TxA2 in the thymus stimulated the release of TNFα, causing apoptosis and loss of thymocytes.53) Enhanced production of TxA2 has been reported to positively correlate with increased lipid peroxidation and free radical

Fig. 9. Expression of Th1- and Th2-Marker Genes in in Vitro-Activated Cultured Thymocytes from Diet-Restricted Mice

The expression of mRNAs for (A) Th1-marker genes (Tbx21, Stat1 and Stat4) and Th2-marker genes (Stat3, Stat5, Stat6 and Gata3) in the cultured thymocytes from diet-provided or diet-restricted mice, with or without the 3h-stimulation with PMA and ionomycin, was examined by RT-qPCR analysis. The expression of all genes was normalized to that of Rplp2, and calculated as relative change. Error bars indicate standard deviation. **p < 0.01, ***p < 0.001 (n = 6).
generation and to mediate DNA fragmentation, programmed cell death and apoptosis, that finally induce thymic involution.\(^{56}\) Thus, TxA\(_2\) plays a central role in the regulation of apoptosis in the thymus through the oxidative stress signaling. On the other hand, specific action of TxA\(_2\) in modulating the differentiation or polarization of naïve T cells has not yet been known. Increased production of TxA\(_2\) may specifically induce apoptosis to eventually lead to thymic involution without affecting the polarization of naïve T cells in the thymus. However, it would be worth to note that TxB\(_2\), a stable TxA\(_2\) metabolite, activates CRTH2 receptor during inflammation.\(^{57}\) Therefore, it is possible that TxA\(_2\) acts on CRTH2 receptor to activate the signaling pathway by itself or to modulate the action of PGD\(_2\) to be involved in the polarization of naïve T cells during thymic involution.

The effects of PGE\(_2\) and PGI\(_2\) on the polarization of T cells are still unclear. There are many studies indicating that those prostanoids are involved in the differentiation to Th1.\(^{58-60}\) Zhou et al. reported that PGI\(_2\) inhibits increased IFN-\(\gamma\) production, as well as IL-4 and IL-10 production, in mouse splenocyte T cells during inflammation.\(^{61}\) PGI\(_2\) signaling also limits Th1 immune responses during inflammation, particularly with the suppression of \(\text{IFN}-\gamma\) and induction of \(\text{IL-10}\) expression from bone marrow-derived dendritic cells.\(^{62}\) Our results, demonstrating increased expression of \(\text{Pggs}\), together with the biased polarization of naïve T cells to Th2 cells, may indicate that PGI\(_2\) is involved in the polarization toward Th2 cells during diet-restriction-induced thymic involution. PGE\(_2\) also regulates the functions of helper T cells during inflammation by exerting its ability to selectively inhibit the production of the Th1 cytokines IFN-\(\gamma\) and IL-2,\(^{63}\) but not the Th2 cytokines IL-4 and IL-5, in mouse and human CD4\(^+\) T cells.\(^{64,65}\) Thus, our results suggested that the increased expression of prostanoid synthases, particularly m-PGES1 and m-PGES2 that catalyze the production of PGE\(_2\), would affect the polarization of naïve T cells into Th2-like phenotype through the production of prostanoids during thymic involution. Therefore, in addition to PGD\(_2\) and TxA\(_2\), PGI\(_2\) and PGE\(_2\) are also candidates to control naïve T cell polarization during diet-restriction-induced thymic involution. Further studies are required to clarify the functional importance of each prostanoid in the biased T cell polarization and resulting immune senescence during thymic involution.

NSAIDs do not act equally on COX1 and COX2. Aspirin, indomethacin, and ibuprofen preferentially inhibit COX1 rather than COX2, while etodolac is more potent against COX2.\(^{66}\) Here, the administration of aspirin and etodolac in diet-restricted mice increased the expression of Th1-producing cytokines in the thymus (Fig. 6). A previous study reported that aspirin enhances the proliferation of T cells by up-regulating mRNA expression and protein production of IFN-\(\gamma\), IL-2, and TNF\(_\alpha\) in Th1 cells by inhibiting COX activity on PGE\(_2\).\(^{67}\) Our results presented here, however, could not detect any effects of aspirin, or of etodolac, on the expression of prostanoid synthases (Supplementary Fig. 1). During diet-induced thymic involution, both aspirin and etodolac inhibited the biased polarization of naïve T cells, whereas aspirin was more potent than etodolac to inhibit the change of Th1- and Th2-cytokines and expression of \(\text{Ttx21}, \text{Stat1}\) and \(\text{Stat4}\) (Fig. 7). Although we have to consider the effectiveness of both NSAIDs in our experimental system, our results may indicate that the polarization of naïve T cells during thymic involution may be mainly mediated by COX1-producing prostanoids.

Taking our results altogether, with regards to the selective suppression of Th1-producing cytokines and increase of those by Th2, prostanoids play pivotal roles in regulating the Th1 and Th2 balance in thymic involution induced by diet-restriction.

**Intracellular Signaling Pathway Regulation under the Control of Prostanoids during Diet-Restriction-Induced Thymic Involution**

Prostanoids activate various intracellular signaling pathways. PGD\(_2\) binds to cell surface DP1 and CRTH2 receptors, which couple to Gs and Gi proteins, respectively. Especially, CRTH2 receptor is highly expressed in Th2 cells,\(^{50,68}\) indicating possible involvement in the biased polarization of naïve T cells during diet-restriction-induced thymic involution. CRTH2 inhibits adenylyl cyclase through the G\(\alpha\) protein to decrease the intracellular CAMP levels and activates phospholipase C\(\beta\), which generates inositol triphosphate (IP3), through G\(\beta\)\(\gamma\) complex, inducing the mobilization of Ca\(^{2+}\) from endoplasmic reticulum.\(^{69}\) TxA\(_2\) acts on TP receptor, which couples to Gq protein and increases intracellular Ca\(^{2+}\).\(^{70}\) Previous studies have indicated that the Ca\(^{2+}\) signaling is important for the differentiation of naïve T cells to Th2 cells.\(^{71,72}\) The increase in intracellular Ca\(^{2+}\) levels is also associated with Th2 cell activation, migration and chemotaxis and with increased expression of Th2-producing cytokines, IL-4, IL-5 and IL-13.\(^{73}\) cAMP, in contrast, directs naïve T cells to Th1 differentiation.\(^{58,59,74}\) Thus, the activation of CRTH2 by increased PGD\(_2\) during diet-restriction is likely to inhibit the polarization toward Th1 through the Gi-mediated inhibition of CAMP production and enhanced that toward Th2 through the activation of intracellular Ca\(^{2+}\) signaling. The TxA\(_2\)-TP-signal is unlikely to be involved in the polarization of naïve T cells as discussed above, although it induces Ca\(^{2+}\) mobilization through Gq protein. It will be interesting to know why naïve T cell polarization is controlled by the specific Ca\(^{2+}\) mobilization through G\(\beta\)\(\gamma\), but not by that through G\(\alpha\), and how the signals from PGD\(_2\)-activating G\(\beta\)\(\gamma\) and TxA\(_2\)-activating Gq crossstalk each other.

**CONCLUSION**

Acute diet-restriction induced prostanoid synthesis in the thymus, which down-regulated Th1-producing IFN-\(\gamma\) and IL-2, but up-regulated Th2-producing IL-4, IL-5, IL-6, IL-10 and IL-13. Such changes to the expression of cytokines deviate the Th1 and Th2 balance in thymic involution. Prostanoid synthesis may be a drug target to manipulate thymus functions in order to maintain cellular immunity under various biological stresses related to thymic involution.

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