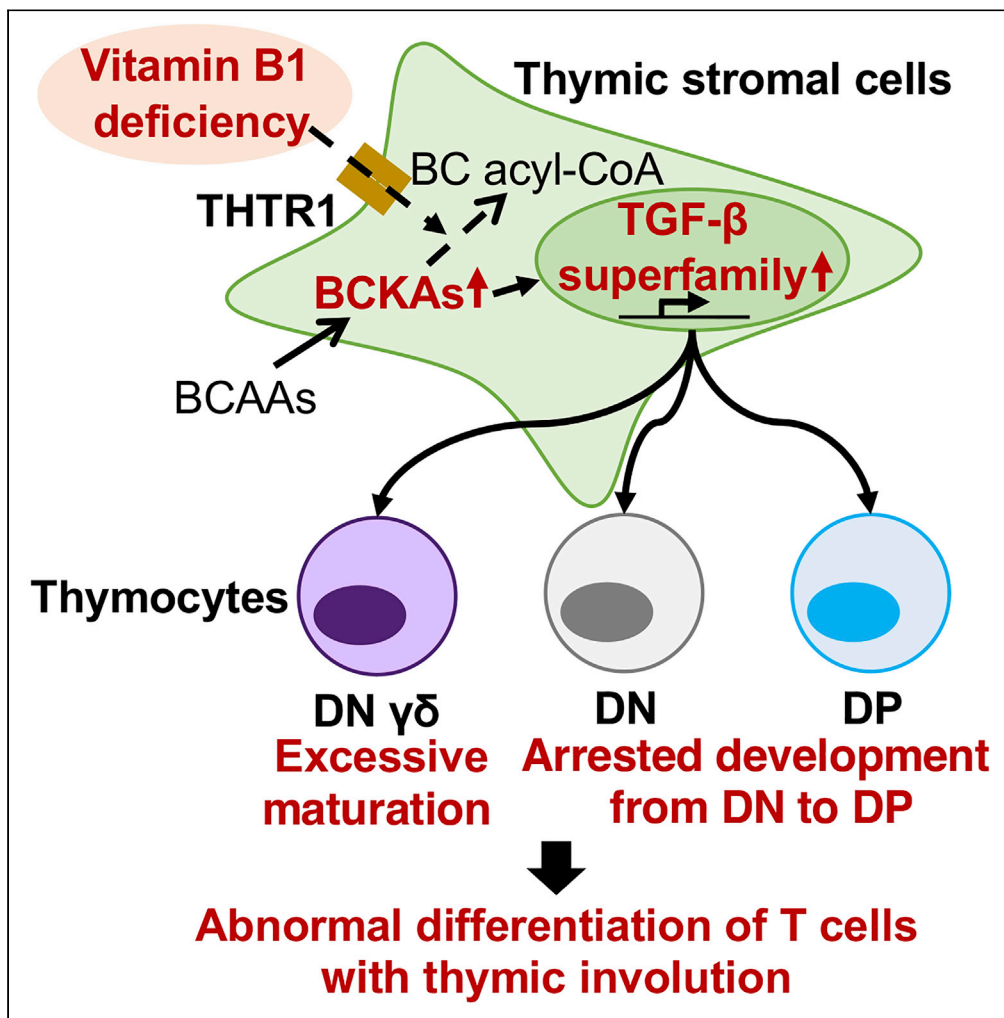Article

Vitamin B1 Supports the Differentiation of T Cells through TGF-β Superfamily Production in Thymic Stromal Cells

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HIGHLIGHTS

Vitamin B1 deficiency
induces excessive BCKA
production in the thymic
stromal cells

BCKAs increase the
production of TGF-β
superfamily from thymic
stromal cells

TGF-β superfamily
induces unbalanced
differentiation of
thymocytes

Vitamin B1 deficiency
induces thymic involution

Article

Vitamin B1 Supports the Differentiation of T Cells through TGF-β Superfamily Production in Thymic Stromal Cells

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SUMMARY

Homeostatic generation of T cells, which occurs in the thymus, is controlled at least in part by endogenous cytokines and ligands. In addition, nutritional factors are other key regulators for the homeostasis of host immunity, but whether and how nutrition affects the homeostatic generation of thymocytes remains to be established. Here, we showed that vitamin B1 deficiency resulted in a bias toward the maturation of γδ thymocytes accompanied by decreased differentiation into double-positive thymocytes during thymic involution. These events were mediated through the increased production of TGF-β superfamily members due to the accumulation of branched-chain α-keto acids in thymic stromal cells. These findings revealed essential roles of vitamin B1 in the appropriate differentiation of T cells through the metabolism of thymic stromal cells.

INTRODUCTION

The thymus is the primary lymphoid organ for generating T cells, a process that is regulated through the interaction of endogenous molecules with thymocytes (e.g., Notch and T cell receptor [TCR]) and stromal cells (e.g., Delta ligand and self-peptides on major histocompatibility complex [MHC]) (Hogquist and Jameson, 2014; Takahama, 2006). Initially, thymocytes lack cell surface expression of both CD4 and CD8 (i.e., double-negative [DN] cells) and develop from stage DN1 to DN4 as TCR expression increases due to the interaction between Notch on thymocytes and Delta ligand on thymic stromal cells (Takahama, 2006). The strength of the TCR signal from the MHC on thymic stromal cells with Delta-Notch interaction drives the transition to CD4$^+$CD8$^+$ double-positive (DP) thymocytes (Hogquist and Jameson, 2014; Takahama, 2006). Moreover, transforming growth factor (TGF)-β superfamily members, including TGF-β1, and Activin A from thymic stromal cells arrest the development of thymocytes by preventing their progression from the DN to the DP stage (Licona-Limón et al., 2009; Takahama, 2006) and induce the apoptosis of DP thymocytes (Szondy et al., 2003). Furthermore, a strong TCR signal and high TGF-β superfamily activity during the DN stage accelerate the development or maturation of γδ thymocytes (Hogquist and Jameson, 2014; Woolf et al., 2007).

T cells have metabolic heterogeneity in regard to their various functions and development stages (Bantug et al., 2018; Buck et al., 2015). Indeed, naive T cells are relatively dependent on bioenergetic catabolism, using oxidative phosphorylation and fatty acid oxidation for homeostatic maintenance (Bantug et al., 2018; Buck et al., 2015). In contrast, to meet metabolic demands, activated and proliferating T cells generally depend on anabolism characterized by high mechanistic target of rapamycin (mTOR) activity and fatty acid synthesis and catabolism through glycolysis and amino acid metabolism (Bantug et al., 2018; Buck et al., 2015). Recently, it was reported that the homeostatic production of thymocytes was regulated through the metabolic states of mTOR in both thymocytes (Yang et al., 2018) and thymic stromal cells (Wang et al., 2016).

Accumulating evidence suggests that nutritional factors are other key regulators of T cell development and differentiation. Indeed, malnutrition induces thymic involution (Savino et al., 2007), and several nutrients, including zinc and vitamin A, are necessary for the maintenance of thymus (Cunningham-Rundles et al., 2005). Regarding vitamin A, retinoic acid signaling in thymic epithelial cells (TEC) is required to maintain

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an appropriate developmental balance from medullary (m) TEC to cortical (c) TEC and thus support thymocyte development (Wendland et al., 2018).

Vitamin B1 (thiamine) is an essential nutrient for the central metabolism rooted in pyruvate, branched amino acids, and ribose 5-phosphate as well as the citric acid cycle (Dhir et al., 2019). We previously reported that naive B cells in Peyer patches rely metabolically on the citric acid cycle, which has a high requirement for vitamin B1, to produce ATP for energy (Kunisawa et al., 2015). In contrast, IgA-producing plasma cells in the intestinal lamina propria utilize the glycolytic pathway for ATP production and thus have a decreased requirement for vitamin B1 (Kunisawa et al., 2015). Indeed, mice maintained on a vitamin B1-deficient diet showed significant reduction of naive B cells in Peyer patches without remarkable changes in IgA-producing plasma cells in the intestinal lamina propria (Kunisawa et al., 2015). Given that Peyer patches are the site in naive B cells for the class switching of IgM to IgA, especially to intestinal antigens, maintaining mice on a vitamin B1-deficient diet resulted in impaired intestinal IgA responses against orally immunized vaccine antigens (Kunisawa et al., 2015).

In the current study, we explored the immunologic roles of vitamin B1 in T cell development in thymus and found that vitamin B1 was highly required for appropriate production of TGF-β superfamily members from thymic stromal cells. This process was controlled through the metabolism of branched-chain amino acids.

RESULTS

Vitamin B1 Supports the Homeostatic Development of T Cells in the Thymus

First, we evaluated whether dietary vitamin B1 affects T cell development in the thymus. Macroscopic analysis revealed that mice maintained on vitamin B1-deficient chow demonstrated remarkable decreases in the volume and weight of thymus and the total number of thymus cells, namely, thymic involution, compared with mice maintained on control chow (Figures 1A and 1B). Accordingly, the amount of vitamin B1 in thymus gradually decreased during the 3 weeks after dietary vitamin B1 was discontinued (Figure 1C). Flow cytometric analysis indicated that the proportion of DP thymocytes was decreased preferentially (Figure 1D). In contrast, vitamin B1 deficiency did not affect the overall proportion of TCRγδ$^+$ cells among DN thymocytes (Figure 1E), but the proportion of cells that transitioned from immature, CD44$^-$CD24$^+$ cells to the mature, CD44$^+$CD24$^-$ subset of TCRγδ$^+$ DN thymocytes was increased (Figure 1F).

Given that increased expression of RUNX3 reportedly induces the maturation of DN TCRγδ$^+$ thymocytes in thymus (Woolf et al., 2007), we assessed *Runx3* mRNA expression in DN thymocytes by using qRT-PCR analysis. *Runx3* mRNA expression levels in DN thymocytes were upregulated in vitamin B1-deficient mice compared with the control group (Figure 1G). These results suggest that vitamin B1 is required to control RUNX3 expression and thus maintain appropriate generation of DP and DN TCRγδ$^+$ thymocytes.

High Requirement for Vitamin B1 in Thymic Stromal Cells

We investigated whether vitamin B1 affects thymocytes directly. When purified DN thymocytes were cocultured with the bone marrow-derived OP9–DL1 cell line as stromal cells, they developed to single-positive and DP thymocytes (Lai et al., 2010). A similar pattern emerged when cultures were treated with oxythiamine, a vitamin B1 inhibitor (Figure 2A). Therefore, these results suggested that vitamin B1 inhibition did not directly affect thymocyte development *in vitro*, unlike *in vivo* vitamin B1 deficiency. Moreover, because we hypothesized that OP9–DL1 cells incubated with oxythiamine might not sufficiently recapitulate the *in vivo* thymic stromal environment in vitamin B1-deficient mice, we assessed the mRNA expression levels of thiamine transporter 1 (THTR1) (Dutta et al., 1999) in the OP9–DL1 cells and thymic stromal cells. Indeed, the levels of *Thtr1* mRNA were significantly higher in thymic stromal cells than in OP9–DL1 cells (Figure S1). We then used immunohistochemistry to examine thiamine transporter 1 (THTR1) levels and showed that a population of EpCAM$^+$ stromal cells expressed higher levels of THTR1 than did CD45$^+$ thymocytes (Figure 2B). Given these findings, we then adopted a fetal thymic organ culture (FTOC) system to better mimic the thymic environment *in vivo*. Although treatment with oxythiamine did not significantly alter the proportion of DP thymocytes that developed from DN1–3 thymocytes (Figure S2), the proportion of CD24$^-$, mature DN TCRγδ$^+$ thymocytes increased (Figure 2C). This result indicates that the thymic environment created by stromal cells requires vitamin B1 for the appropriate control of thymocyte development.
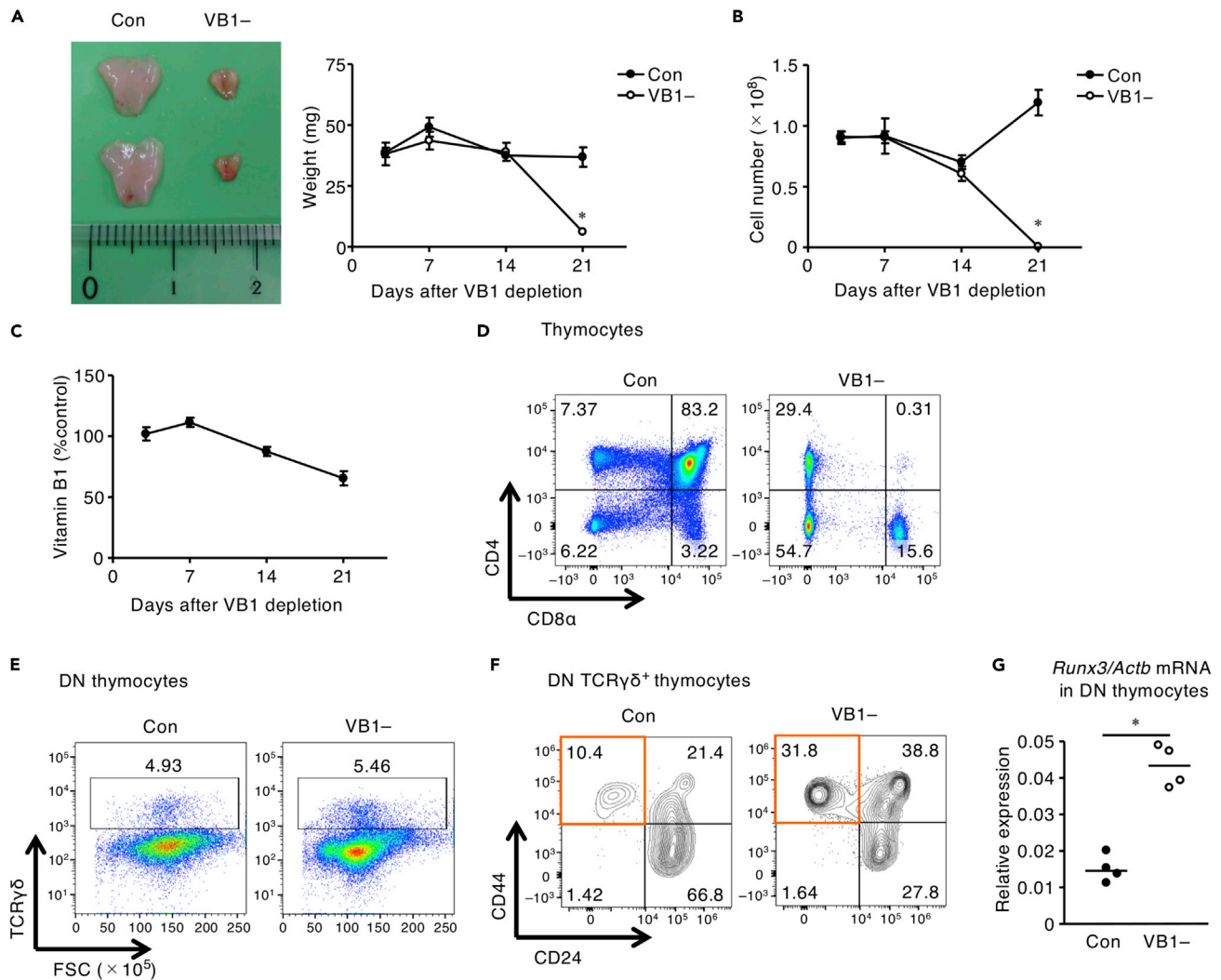
Figure 1. Vitamin B1 Is Required to Inhibit Increases in Mature TCRγδ⁺ DN Thymocytes

(A–F) (A) Macroscopic analysis of thymus on day 21, thymus weight; (B) total number of thymocytes at days 3, 7, 14, and 21 of feeding mice a vitamin B1-deficient diet (VB1–) or control diet (Con); and (C) thymic vitamin B1 concentration at days 3, 7, 14, and 21 of feeding mice a vitamin B1-deficient diet (VB1–) relative to control diet. FACS plots of (D) CD4 and CD8α on live thymocytes gated on 7-AAD⁻, (E) of TCRγδ⁺ cells among DN thymocytes, and (F) of CD44 and CD24 among DN– TCRγδ⁺ thymocytes after 3 weeks of VB1– or Con diet. Scale, 1 cm. Horizontal lines indicate median values. p values were obtained by using the Mann-Whitney U-test (*p < 0.05). The data shown are reproducible and are representative of two to five independent experiments.

(G) The levels of *Runx3* mRNA in DN and DP thymocytes sorted from the VB1– and Con groups are shown. Horizontal lines indicate median values. p values were obtained by using the Mann-Whitney U-test (*p < 0.05).

Vitamin B1 Controls the Production of TGF-β Superfamily Members in Thymic Stromal Cells

Both TGF-βs and Activin A (also known as Inhba), which belongs to the TGF-β superfamily, reportedly block the developmental transition of thymocytes from the DN to DP stage (Licona-Limón et al., 2009; Takahama et al., 1994) and induce the apoptosis of DP thymocytes (Szondy et al., 2003). Moreover, RUNX3 transcription is upregulated by TGF-β superfamily members through the phosphorylation of smad2 and smad3 (Klunker et al., 2009; Reis et al., 2013). Prompted by these reports and our current findings, we evaluated the effect of vitamin B1 deficiency on the production of TGF-β superfamily members in thymic stromal cells. Compared with the control group, thymic stromal cells from the vitamin B1-deficient condition had increased levels of *Tgfb2*, *Gdf10*, and *Inhba* mRNA (Figure 3A); mRNA transcript levels did not differ between the control and vitamin B1-deficient groups for any other TGF-β superfamily member (Figure S3). In addition, flow cytometric analysis consistently demonstrated that vitamin B1 deficiency increased the proportion of TCRγδ⁺ thymocytes expressing phosphorylated smad2 and smad3; these signaling
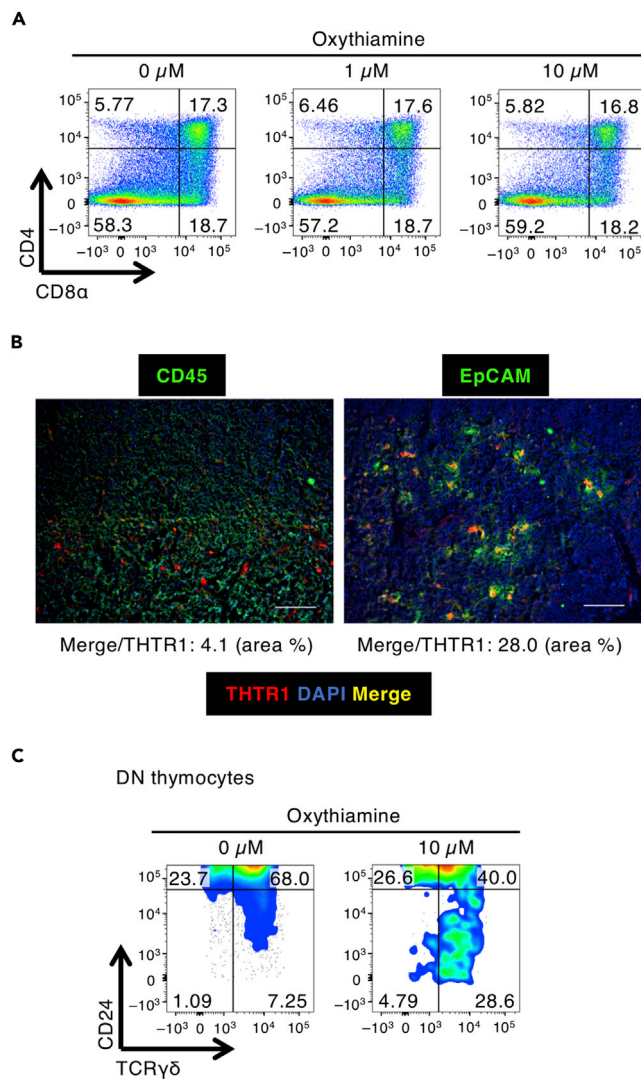
Figure 2. The Requirement for Vitamin B1 Is Higher in Thymic Stromal Cells Than in Thymocytes

(A) FACS plots of developing thymocytes sorted from DN1–3 to DP are shown after incubation for 7 days in the presence of oxythiamine and OP9–DL1 cells.

(B) Immunohistochemistry of murine thymus was performed by using monoclonal antibodies (mAbs) to either CD45 or EpCAM and THTR1. Scale bar, 100 μm. Merge area (yellow) was quantified as the color threshold of Merge (Y: 100, U: 0, and V: 120) and THTR1 (R: 100, G: 0, and B: 0) by using ImageJ.

(C) FACS plots of CD24$^-$ cells among DN TCRγδ$^+$ thymocytes from sorted DN1–3 cells reconstituted in fetal thymic organ culture and incubated in the presence of oxythiamine. The data shown are reproducible and representative of five independent experiments.

molecules are induced by the TGF-β superfamily (Figure 3B). These results suggest that vitamin B1 modulates the expression of *Tgfb2*, *Gdf10*, and *Inhba* in thymic stromal cells, thus maintaining the homeostatic generation of DP and TCRγδ$^+$ thymocytes through the phosphorylation of smad2 and smad3.

Vitamin B1 Promotes the Metabolism of Branched-Chain α-Keto Acids in Thymic Stromal Cells

Vitamin B1 is well known as a necessary coenzyme for catalyzing the conversion of pyruvate to acetyl-CoA, of α-ketoglutarate to succinyl-CoA, of branched-chain α-keto acids (BCKAs) to branched-chain acyl-CoA, and of ribose 5-phosphate to glyceraldehyde 3-phosphate (Manoli and Venditti, 2016; Whitfield et al., 2018). Therefore, we wondered what metabolic changes might occur in thymic stromal cells. Metabolomic analysis using ion chromatography with Fourier transform mass spectrometry (IC-MS) showed that
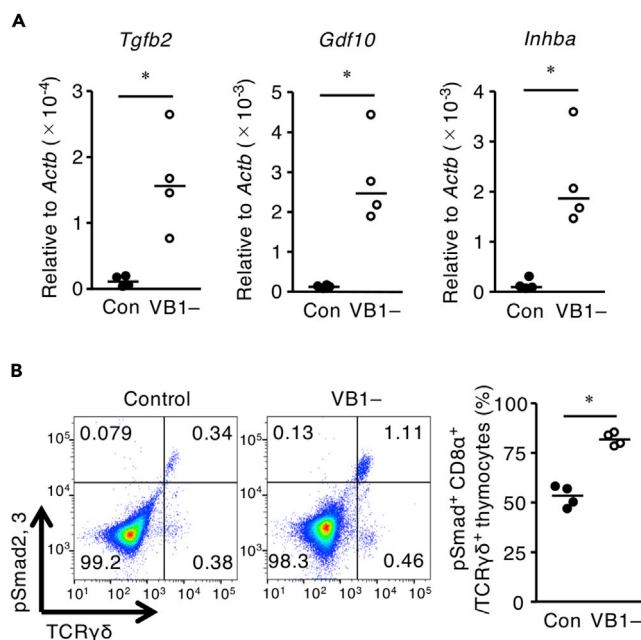
Figure 3. Thymic Stromal Cells Require Vitamin B1 to Inhibit Excessive Production of the TGF-β Superfamily *In Vivo*

(A) The levels of *Gdf10*, *Inhba*, and *Tgfb2* mRNAs in thymic stromal cells after 3 weeks of feeding mice a vitamin B1-deficient diet (VB1–) or control diet (Con). Horizontal lines indicate median values. p values were obtained by using the Mann-Whitney *U*-test (*p < 0.05). The data shown are reproducible and representative of two independent experiments.

(B) FACS plots showing the ratios of cells positive for phosphorylated smad2, smad3$^+$, and CD8α$^+$ among TCRγδ$^+$ thymocytes from VB1– and Con mice. Horizontal lines indicate median values. p values were obtained by using the Mann-Whitney *U*-test (*p < 0.05).

ketoleucine and ketoisoleucine, which are BCKAs that are generated as intermediate metabolites from branched-chain amino acids (BCAAs), were increased in the supernatant of thymic stromal cells that had been treated with oxythiamine; however, metabolites associated with the citric acid cycle and glycolysis remained unchanged (Figures 4 and S4).

BCKAs Induced Excessive Production of TGF-β Superfamily Members

We investigated whether the BCKAs that accumulated in thymic stromal cells due to vitamin B1 insufficiency could increase the quantities of mRNAs encoding TGF-β superfamily members. Culturing thymic stromal cells in media containing ketoleucine and ketoisoleucine increased the mRNA expression of *Inhba*, *Tgfb2*, and *Gdf10* (Figures 5 and S5). These results suggest that vitamin B1-mediated metabolism of BCAAs, especially BCKAs, controls the production of TGF-β superfamily members in thymic stromal cells.

Activin A, TGF-β, and GDF10 act as ligands for the TGF-β superfamily receptors ALK4 and ActRIIA (to Activin A and GDF10), ALK5 (to TGF-β), and others (Heldin and Moustakas, 2016), whereas K02288 inhibits various TGF-β superfamily receptors (e.g., ALK2, ALK1, ALK6, ALK3, ActRIIA, ALK4, and ALK5) (Sanvitale et al., 2013). These activities allowed us to use FTOC to evaluate the effect of K02288 on the oxythiamine-induced maturation of DN γδ thymocytes. Treatment with K02288 canceled the oxythiamine-induced excessive maturation of DN γδ thymocytes in FTOCs (Figure 6). These results suggest that impaired thymocyte differentiation in the vitamin B1-deficient condition was mediated by TGF-β superfamily members.

Changes in Peripheral T Lymphocytes

We next investigated the effect of vitamin B1 deficiency on the number of peripheral T lymphocytes in the gut, the site of numerous TCRγδ$^+$ T lymphocytes, under immunologically naive conditions. Flow cytometric analysis revealed that vitamin B1 deficiency increased the proportion of CD8αα TCRγδ$^+$ T lymphocytes in the small intestine (Figure 7A). A similar increase occurred in the CD8αα subpopulation of TCRβ$^+$ T
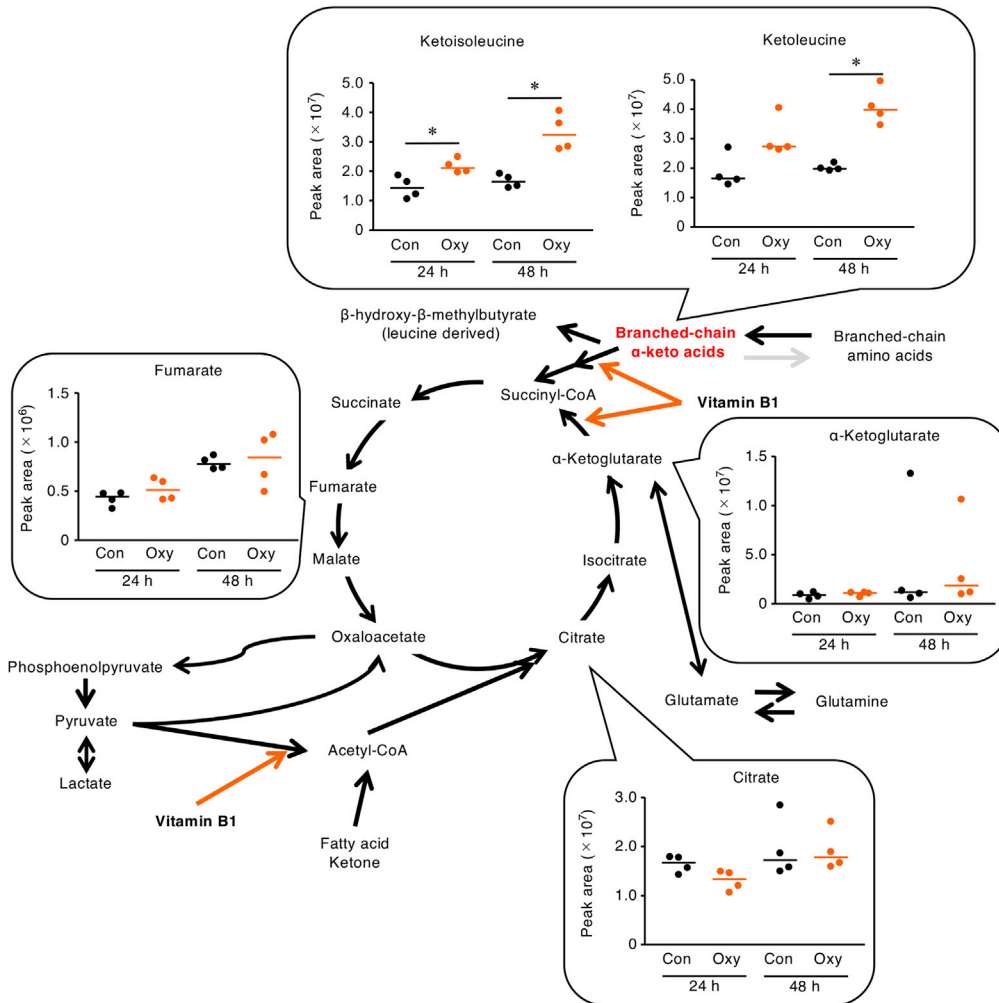
Figure 4. Vitamin B1 Is Required for Appropriate Metabolism of Branched-Chain α-Keto Acids in Thymic Stroma In Vitro

We used IC-MS to analyze citric-acid cycle metabolites in supernatants from murine thymic stromal cells that had been incubated with oxythiamine (Oxy) for 24 or 48 h. Horizontal lines indicate median values. Con, no-treatment control. *p < 0.05 (Mann-Whitney U-test).

lymphocytes, an unconventional T lymphocyte subset derived from DN thymocytes (Konkel et al., 2011; Pobezinsky et al., 2012) (Figure 7B), whereas vitamin B1 deficiency decreased the number of CD4 TCRβ[+] T lymphocytes in the small intestine (Figure 7C). In addition, we found that the numbers of CD4 and CD8α IFN-γ[+] TCRβ[+] T lymphocytes, which are the main functional subsets in spleen (Saxena et al., 2012), were reduced in the spleens of vitamin B1-deficient mice (Figure S7). These results suggest that vitamin B1 is required for the appropriate differentiation and function of T lymphocytes in peripheral tissues.

DISCUSSION

Here we showed that vitamin B1 modulates the metabolism of BCKAs and consequent production of TGF-β superfamily members in thymic stromal cells; these activities are required to maintain the appropriate differentiation of DP and γδ thymocytes (Figure 8). These findings extend current knowledge by revealing the novel role of vitamin B1 as an essential micronutrient for homeostatic development of immune cells.

Our current study showed that thymic stromal cells—but not CD45^{+} thymocytes—express THTR1 (Figure 2). Notably, THTR1 was not expressed in all thymic stromal cells (Figure 2B). In addition to cTEC and mTEC, recent studies using single-cell RNA sequencing have indicated that EpCAM[+] thymic stromal cells
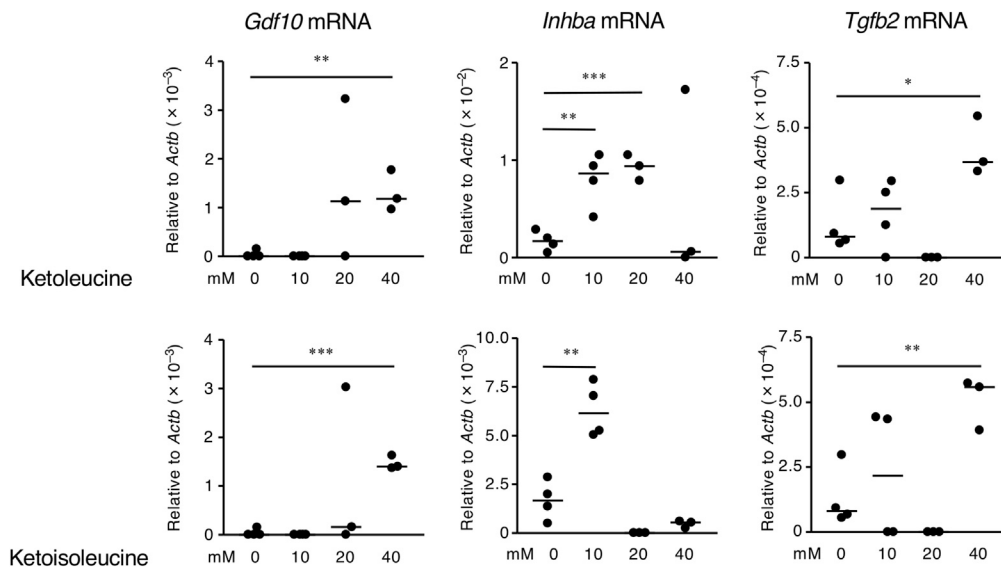
Figure 5. Branched-Chain α-Keto Acids Induce Excessive Production of the TGF-β Superfamily in Thymic Stromal Cells

Murine thymic stromal cells were incubated for 48 h with either ketoleucine or ketoisoleucine, which are branched-chain α-keto acids. The levels of *Gdf10*, *Inhba*, and *Tgfb2* mRNAs in the thymic stromal cells were determined by using qRT-PCR. p values were obtained by using the two-tailed unpaired Student's *t* test (*p < 0.05, **p < 0.01, ***p < 0.001). The data shown are reproducible and representative of two independent experiments.

comprise several functionally heterogeneous subsets, including tuft cells (Bornstein et al., 2018; Inglesfield et al., 2019; Miller et al., 2018). We found that some EpCAM⁺ thymic stromal cells preferentially express THTR1, to support appropriate development of thymocytes (Takahama, 2006).

Specificity protein 1 (Sp1) increases THTR1 in proximal tubular epithelial cells (Larkin et al., 2012), and p63 downregulates the activity of Sp1 in human nasal epithelial cells (Kaneko et al., 2017). The expression level of p63 in TECs is negatively correlated with that of FoxN1, one of the main differential transcription factors in TECs (Burnley et al., 2013). Therefore, the THTR1 content of TECs might be orchestrated through FoxN1-induced downregulation of p63 and thus upregulation of Sp1. Furthermore, compared with other cTECs and mTECs, the MHC class II^high cTECs among EpCAM⁺ thymic stromal cells show greater expression of *FoxN1* mRNA (Nowell et al., 2011; O'Neill et al., 2016); this result implies that FoxN1-initiated control of THTR1 expression might be upregulated in MHC class II^high cTECs.

We found that vitamin B1 insufficiency increased BCKA levels and the production of some TGF-β superfamily members, including *Inhba*, *Gdf10*, and *Tgfb2*, in thymic stromal cells (Figures 3 and 5). Ketoleucine increased intracellular Ca²⁺ levels in rat cerebral cortex *in vitro* (Funchal et al., 2005). Moreover, increased levels of intracellular Ca²⁺, which induces TGF-β in the 3T3TβRII cell line (Xiao et al., 2008), activated p38 MAPK and ERK1/2 in bone marrow macrophages *in vitro* (Zhou et al., 2010). Why *Inhba*, *Gdf10*, and *Tgfb2* mRNAs specifically are increased in thymic stromal cells remains unknown at this point. However, our current findings suggest that BCKAs, including ketoleucine and ketoisoleucine, may increase intracellular Ca²⁺ in thymic stromal cells, thus inducing MAPK- and ERK-mediated mRNA expression of TGF-β superfamily members, including *Inhba*, *Gdf10*, and *Tgfb2*.

We showed that dietary deficiency of vitamin B1 decreased the number of DP thymocytes and increased the numbers of mature γδ thymocytes in mice; these changes were associated with the increased expression of *Runx3* mRNA and the phosphorylation of smad2 and smad3 in thymocytes (Figures 1 and 3). In support of our current findings, previous studies showed that various TGF-β superfamily members, including Activin A, GDF10, and TGF-β2, induce the phosphorylation of Smad2 and Smad3 (Heldin and Moustakas, 2016) and upregulate the expression of RUNX3 (Jin et al., 2004; Reis et al., 2013) and that TGF-β2 induces the RUNX3-mediated maturation of γδ thymocytes (Woolf et al., 2007). In addition, TGF-β and Activin A
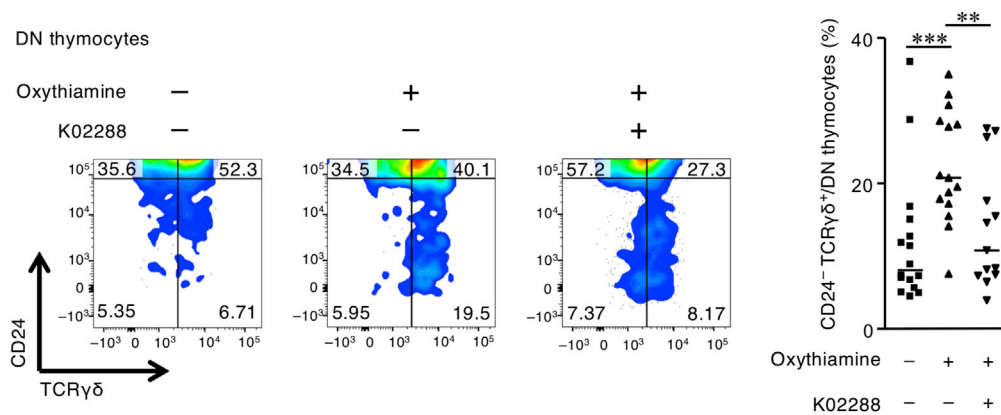
Figure 6. Vitamin B1 Inhibition Induces Excessive Maturation of γδ Thymocytes through TGF-β Superfamily Signaling in Fetal Thymic Organ Cultures

FACS plot of CD24⁻ TCRγδ⁺ cells among DN developing thymocytes from sorted DN1–3 cells reconstituted in fetal thymic organ cultures incubated in the presence of oxythiamine, the TGF-β superfamily inhibitor K02288, or both agents. Horizontal lines indicate median values. p values were obtained by using the Mann-Whitney *U*-test (***p < 0.001, **p < 0.01). The data shown are pooled from three independent experiments, which yielded reproducible data.

block thymocyte progression from the DN stage to the DP stage and induce the apoptosis of DP thymocytes (Licona-Limón et al., 2009; Szondy et al., 2003; Takahama et al., 1994).

Consistent with the *in vivo* results, treatment with oxythiamine increased the proportion of CD24⁻ mature DN γδ thymocytes (Figure 2C) but did not significantly alter the proportion of DP thymocytes that developed from DN1–3 thymocytes (Figure S2). As an explanation of this apparent discrepancy, previous studies showed that fetal TECs had a greater rate of cell growth than those from adult thymus (Cowan et al., 2019) and that increased cell growth activity was associated with low dependency on BCAA catabolism in cardiomyocytes (Shao et al., 2018). Thus, under vitamin B1-deficient conditions, BCKA accumulation likely was lower in FTOCs than in adult thymus. In support of this notion, exogenous addition of BCKAs reduced the proportion of DP thymocytes in FTOCs (Figure S6). Furthermore, given that TGF-β receptors (i.e., Tgfβr1, Tgfβr2) were more highly expressed in TCRγδ⁺ thymocytes than in DP thymocytes (Do et al., 2010), DN γδ thymocytes might have greater sensitivity to the oxythiamine-induced production of BCKAs and TGF-β superfamily ligands.

In line with the immunologic phenotypes in the thymus, vitamin B1 deficiency decreased the population of DP thymocyte-derived CD4 TCRβ⁺ T lymphocytes in the gut (Figure 6). In contrast, we noted increases in the numbers of TCRγδ⁺ T lymphocytes and CD8αα TCRβ⁺ T lymphocytes (Figure 6), which developed from DN thymocytes through TGF-β-mediated RUNX3 expression (Konkel et al., 2011; Pobezinsky et al., 2012). Moreover, we showed that vitamin B1 deficiency led to a decrease in IFN-γ⁺ TCRβ⁺ T lymphocytes (Figure S7). Given that pyruvate dehydrogenase (PDH) induces the production of acetyl-CoA to induce the transcription of IFN-γ transcription (Peng et al., 2016) and that vitamin B1 is an essential co-enzyme for PDH (Kunisawa et al., 2015), vitamin B1 deficiency plausibly impaired PDH activity and consequently inhibited IFN-γ production.

Together, our current findings indicate that vitamin B1 is required for the appropriate differentiation of thymocytes, especially the development of DN cells into DP or γδ thymocytes. This regulation is mediated through control of the production of TGF-β superfamily members including Activin A, GDF10, and TGF-β2; this regulation is achieved by promoting the metabolism of BCKAs in thymic stromal cells. These findings provide new evidence of vitamin B1-mediated interaction between stromal and immune cells for the appropriate development of thymocytes.

Limitations of the Study

This study demonstrated that vitamin B1 was necessary for the appropriate metabolic functions in thymic stromal cells for the homeostatic differentiation of T cells. Although we proposed that a specific subset of thymic stromal cells expressing high levels of THTR1 were responsible for it, we could not specify them due to the experimental restriction of anti-THTR1 antibody.
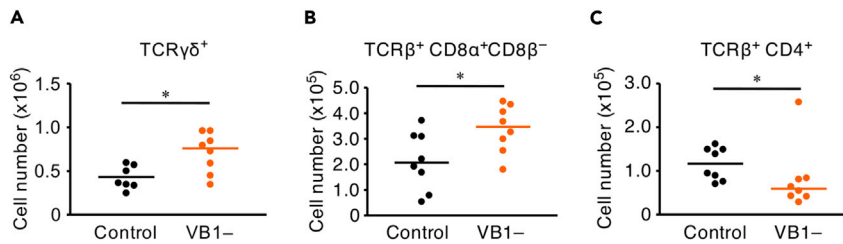
Figure 7. Vitamin B1 Suppresses the Excessive Production of Unconventional T Lymphocytes and Maintains Conventional CD4 T Lymphocytes in Small Intestine

(A–C) The total number of (A) TCRγδ$^+$, (B) CD8αα TCRβ$^+$, and (C) CD4 TCRβ$^+$ intestinal lymphocytes (i.e., lamina propria lymphocytes + intestinal epithelial lymphocytes) at 3 weeks after mice began a vitamin B1-deficient diet (VB1–) or control diet (Con). Horizontal lines indicate median values. p values were obtained by using the Mann-Whitney *U*-test (*p < 0.05). The data shown are pooled from three independent experiments, which yielded reproducible data.
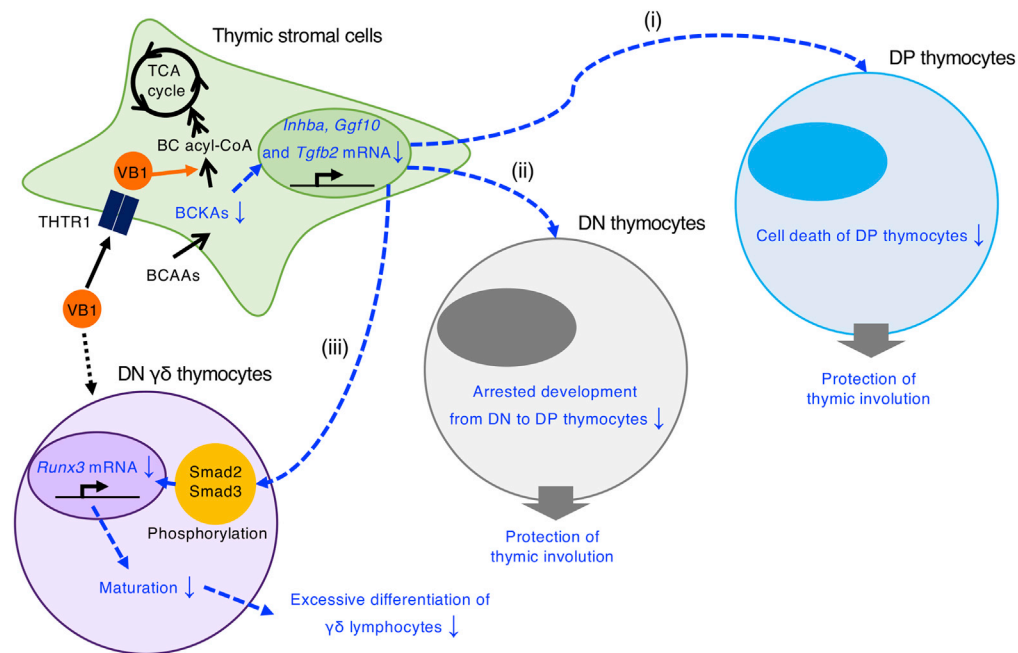
Resource Availability

Lead Contact

Further information and requests should be directed by the Lead Contact, Jun Kunisawa (kunisawa@nibiohn.go.jp).

Materials Availability

New unique reagents were not generated in this study.

Data and Code Availability

The data in this study are available from the corresponding author on request.

Figure 8. Hypothetical Scheme of the Mechanism Underlying Vitamin B1-Dependent Homeostatic Generation of Lymphocytes from Thymic Stromal Cells

Vitamin B1, which is transported through THTR1, is more highly required in thymic stromal cells than in thymocytes. Vitamin B1 suppresses overproduction of TGF-β superfamily members by promoting the metabolism of branched-chain α-keto acids in thymic stromal cells. Vitamin B1-dependent regulation of inappropriate TGF-β superfamily production (i) suppresses the apoptosis of DP thymocytes; (ii) protects against blockade of the DN to DP transition in thymocytes, which otherwise would cause spurious thymic involution; and (iii) prevents excess development of mature γδ thymocytes, leading to preferential differentiation of γδ lymphocytes.

AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest.

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Supplemental Information

Vitamin B1 Supports the Differentiation

of T Cells through TGF-β Superfamily

Production in Thymic Stromal Cells

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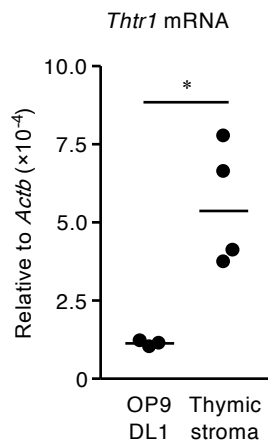
Thtr1 mRNA

Figure S1. The requirement for vitamin B1 is higher in thymic stromal cells than in OP9-DL1 cells, *Related to Figure 2.*
The levels of *Thtr1* mRNA in the OP9-DL1 cells (triplicate samples) and in thymic stromal cells (from 4 individual mice) are shown. *, $P < 0.05$ (two-tailed unpaired Student's *t*-test).

Thymocytes

Oxythiamine

0 μM

10 μM
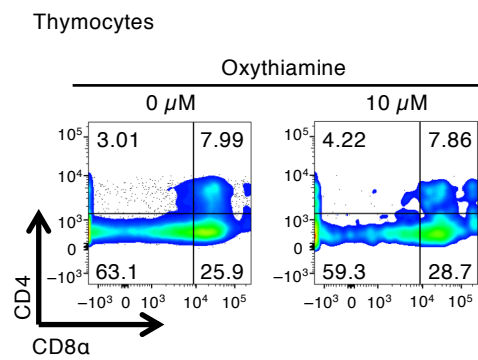
Figure S2. Vitamin B1 inhibition did not reduce DP thymocytes in fetal thymic organ cultures, *Related to Figure 2.*
FACS plots of CD4 and CD8α on developing thymocytes gated on 7-AAD$^-$ from sorted DN1-3 cells reconstituted in fetal thymic organ culture after incubation in the presence of oxythiamine are shown. The data shown are reproducible and representative of five independent experiments.

Figure S3. Thymic stromal cells require vitamin B1 to inhibit excessive production of TGF-β superfamily members *in vivo*, *Related to Figure 3.*

The levels of TGF-β superfamily mRNA in thymic stromal cells are shown at 3 weeks after initiation of a vitamin B1-deficient diet (VB1–) or control diet (Con; n = 4 per group). Horizontal lines indicate median values. *, *P* < 0.05 (Mann–Whitney *U*-test). The data shown are reproducible and representative of two independent experiments.

Figure S4. Glycolysis-related metabolic profiles in vitamin B1 inhibition of thymic stroma *in vitro,* *Related to Figure 4.*
We used IC-MS to investigate glycolysis-related metabolites in the supernatants from murine thymic stromal cells that had been incubated in the presence of oxythiamine (Oxy) or in its absence (control, Con) for 24 or 48 h. Horizontal lines indicate median values.
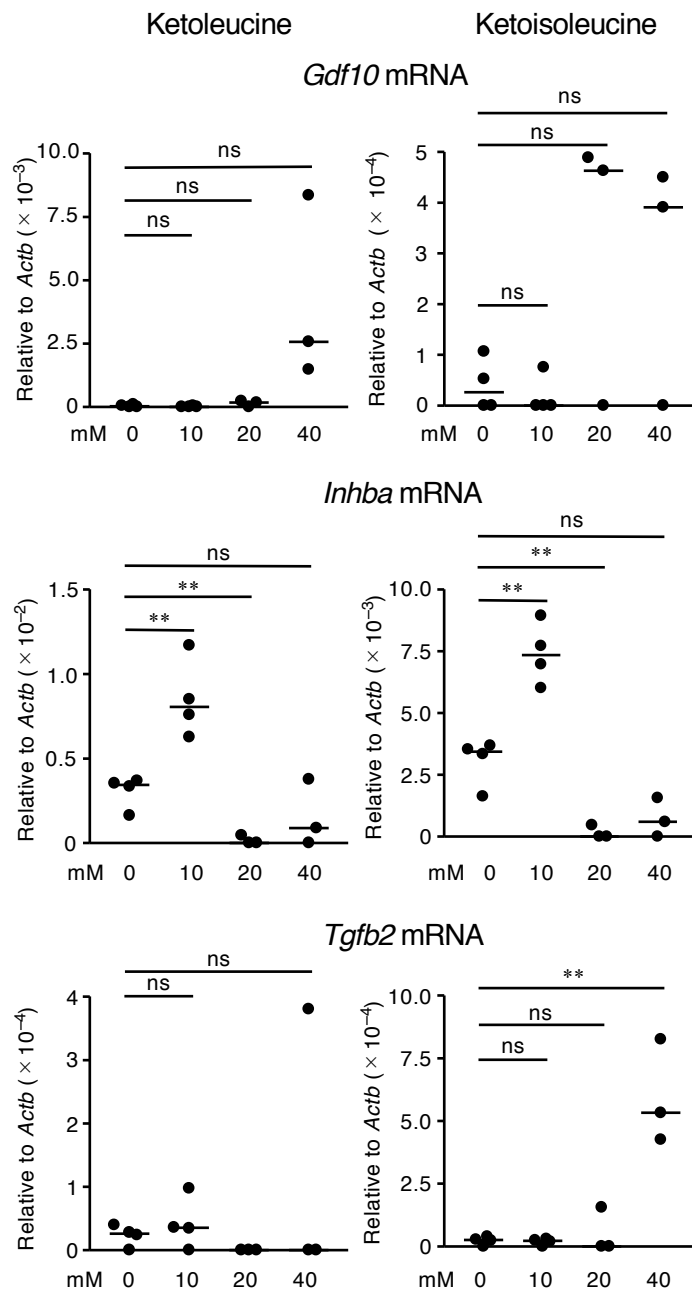
Figure S5. The effect of 12-h exposure to branched chain α-keto acids on production of the TGF-β superfamily in thymic stromal cells, *Related to Figure 5.*

Ketoleucine and ketoisoleucine, which are branched chain α-keto acids, were each incubated for 12 h with murine thymic stromal cells. The levels of *Gdf10*, *Inhba*, and *Tgfb2* mRNA in the cells were determined by using qRT-PCR analysis (n = 3 or 4). *P* values were obtained by using the two-tailed unpaired Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ns, $P > 0.05$). The data shown are reproducible and representative of two independent experiments.
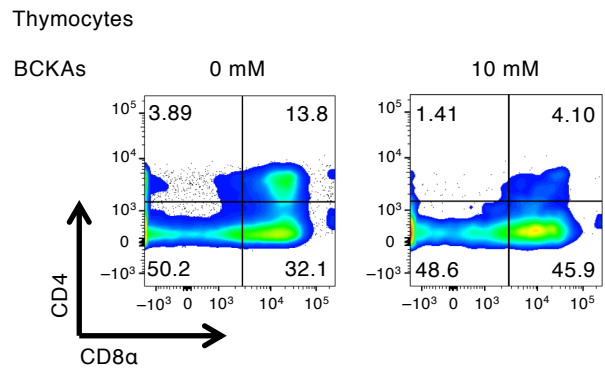
Thymocytes

Figure S6. Treatment with branched chain α-keto acids (BCKAs) decreased DP thymocytes in fetal thymic organ culture, *Related to Figure 6.*

FACS plot of DP thymocytes developed from sorted DN1-3 cells reconstituted in fetal thymic organ culture are shown after incubation in the absence or presence of ketoleucine and ketoisoleucine (10 mM each) for 24 h and then culturing for 9 days in fresh medium without ketoleucine and ketoisoleucine. The data shown are reproducible and representative of two independent experiments.
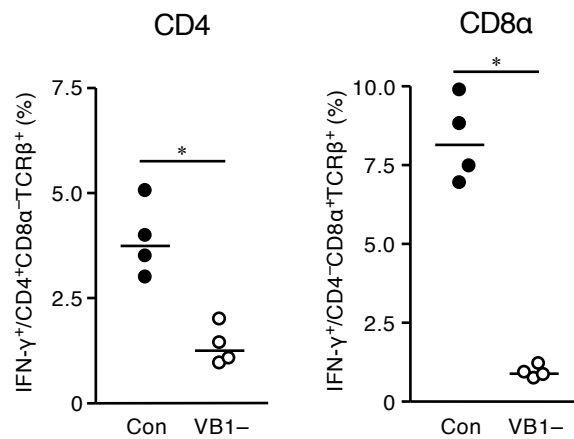
Figure S7. Vitamin B1 is necessary for IFN-γ production from peripheral T lymphocytes, *Related to Figure 7.*
The proportion of IFN-γ+ gated on CD4 or CD8αβ TCRβ+ T lymphocytes in spleen at 3 weeks after mice began to receive vitamin B1-deficient (VB1–) or control (Con) chow. Horizontal lines indicate median values. *P* values were obtained by using the Mann–Whitney U-test (*, *P* < 0.05). Data shown are reproducible and representative two independent experiments.

Transparent Methods

Mice

Female wild-type C57BL/6 mice (age, 7 weeks) were bought from Japan CLEA. Vitamin B1-deficient and control diets with chemically defined components were purchased from Oriental Yeast, as previously described (Kunisawa et al., 2015). All animals were kept under specific pathogen-free conditions in the experimental animal facilities of NIBIOHN. The experiments were approved by the Animal Care and Use Committees of the institute and were conducted according to their guidelines.

Assay for vitamin B1 concentration

Vitamin B1 concentration was measured by using VitaFast Vitamin B1 (r-Biopharm) according to a previous study (Kunisawa et al., 2015). Briefly, the vitamin B1 concentration in a water extract of thymus was assessed by vitamin B1-dependent growth of *Lactobacillus fermentum*.

Isolation of single cells

Lymphocytes were separated from small intestinal lamina propria and intestinal epithelium, as previously described (Kunisawa et al., 2015). Briefly, small intestinal tissue from which Peyer's patches had been removed was rinsed in RPMI 1640 medium and cut into small pieces; cells were dislodged by incubating tissue pieces for 15 min in RPMI 1640 medium containing 0.5 mM EDTA and 2% neonatal calf serum. The remaining tissue pieces then were

digested for 15 min in 1.0 mg/mL collagenase; this step was repeated. Solutions containing the cells obtained after EDTA exposure (containing intestinal epithelial lymphocytes) and those released after collagenase treatment (containing lymphocytes from the lamina propria) were passed through a 100-μm cell strainer and left them as separate cell populations, and then underwent centrifugation through a discontinuous Percoll (GE Healthcare) gradient; lymphocytes were isolated at the interface between the 40% and 75% Percoll layers.

For analysis of thymocytes only, thymic cells were dissociated by passage through a 100-μm cell strainer before centrifugation. For analysis of both thymocytes and stromal cells, these cells were separated from thymus as previously described with modifications (Miller et al., 2018; Seach et al., 2012). Briefly, thymus was cut into small pieces and stirred for 30 min at 37 ˚C in 0.3 mg/mL collagenase in RPMI 1640 medium containing 2% neonatal calf serum. The cells were dissociated further by passage through a 100-μm cell strainer and then subjected to centrifugation through a discontinuous Percoll gradient. Thymocytes were isolated at the interface between the 1.065 g/mL (middle) and 1.115 g/mL (bottom) Percoll layers. Thymic stromal cells were isolated at the interface between the 0 g/mL (top) and 1.065 g/mL (middle) Percoll layers.

Flow cytometry and cell sorting

Cells were treated with 7-AAD (Biolegend, catalog no. 420404) and subsequently with anti-CD16/CD32 (TruStain fcX; Biolegend, 101320) to block nonspecific binding; samples then were stained with the following monoclonal

antibodies (mAbs): APC-conjugated anti-mouse $\alpha4\beta7$ (Biolegend, 120607), CD25 (BD Biosciences, 557192), CD28 (Biolegend, 102109), and CD44 (Biolegend, 103012); APC-Cy7-conjugated anti-mouse CD25 (Biolegend, 102026), CD3ϵ (Biolegend, 557596), CD4 (Biolegend, 100526), and CD8α (Biolegend, 100714) mAbs; BV421-conjugated anti-mouse CD8α (Biolegend, 100738), TCRβ (Biolegend, 109230), and TCR$\gamma\delta$ (Biolegend, 118120); FITC-conjugated anti-mouse TCR$\gamma\delta$ (Biolegend, 118106); PE-conjugated anti-mouse CD4 (Biolegend, 100512), CD24 (Biolegend, 101807), and CD8β (BD Biosciences, 550796); PE-Cy7-conjugated anti-mouse CD4 (Biolegend, 100528) and CD45 (BD Biosciences, 552848); and PerCP-conjugated anti-mouse CD8α (Biolegend, 100732).

Intracellular staining followed the manufacturer's protocol with slight modifications. Briefly, cells were stained by using a Zombie-NIR Fixable Viability Kit (Biolegend) and then treated with anti-CD16/CD32 to block nonspecific binding, and stained with antibodies to cell-surface proteins as described above. Cells were then fixed and permeabilized by using a Foxp3 Staining Kit (eBioscience) or an intracellular staining kit (BD Biosciences). Subsequently, cells were treated with anti-CD16/CD32 to block nonspecific binding and then with PE-conjugated anti-mouse Smad2 (pS465/pS467)/Smad3 (pS423/pS425) (BD Biosciences, 562586) or AF647-conjugated anti-mouse IL-17A (BD Biosciences; 560184) and PE-conjugated anti-mouse IFN-γ (BioLegend, 505808). Stained cells underwent flow cytometry (FACS Aria, BD Biosciences; or MACSQuant, Miltenyi Biotec), and the data were analyzed by using FlowJo software (Tree Star).

Reverse transcription–PCR analysis

Thymocytes and thymic stromal cells were obtained as described in the section titled *Isolation of Single Cells*. Sorted or collected cells were lysed in Sepasol-RNA I Super G (Nacalai Tesque), and total RNA was extracted according to the manufacturer's protocol. RNA was reverse-transcribed by using a SuperScript VILO cDNA Synthesis Kit (Invitrogen); cDNA underwent real-time reverse transcription–PCR amplification by using the Universal ProbeLibrary probe (Roche) and/or the SYBR Green system with primer sets specific for *Actb* (forward primer: 5′-aaggccaaccgtgaaaagat-3′, reverse primer: 5′-gtggtacgaccagaggcatac-3′; probe no. 56 or SYBR Green system), *Bmp2* (forward primer: 5′-agatctgtaccgcaggcact-3′, reverse primer: 5′-gttcctccacggcttcttc-3′; probe no. 20), *Bmp4* (forward primer: 5′-gaggagtttccatcacgaaga-3′, reverse primer: 5′-gctctgccgaggagatca-3′; probe no. 89), *Bmp7* (forward primer: 5′-cgagaccttccagatcacagt-3′, reverse primer: 5′-cagcaagaagaggtccgact-3′; probe no. 1), *Bmp8a* (forward primer: 5′-ctggtcatgagcttcgtcaa-3′, reverse primer: 5′-ccagtgtggctcctggtag-3′; probe no. 31), *Bmp8b* (forward primer: 5′-ctgtatgaactccaccaaccac-3′, reverse primer: 5′-ggggatgatatctggcttca-3′; probe no. 81), *Gdf1-variant1* (forward primer: 5′-ggagctactgcgcttacctg-3′, reverse primer: 5′-tgcctgacctccagtcataga-3′; probe no. 104), *Gdf1-variant2* (forward primer: 5′-ctccgctgactctcttgga-3′, reverse primer: 5′-aggtggtcgcaaaaacgat-3′; probe no. 79), *Gdf3* (forward primer: 5′-tgttcgtgggaacctgct-3′, reverse primer: 5′-gccatcttggaaaggtttctg-3′; probe no. 7), *Gdf5* (forward primer: 5′

-taacagcagcgtgaagttgg-3′ , reverse primer: 5′ -aggcactgatgtcaaacacg-3′ ;

probe no. 9), *Gdf6* (forward primer: 5′ -gctttgtagacagaggactggac-3′ , reverse

primer: 5′ -tgtggacacatcaaacaaatacttc-3′ ; probe no. 55), *Gdf7-variant1*

(forward primer: 5′ -gcttcacagaccaagcaactc-3′ , reverse primer: 5′

-ggatacgtcgaacaggaagc-3′ ; probe no. 70), *Gdf7-variant2* (forward primer: 5′

-tggtgccacaccacttca-3′ , reverse primer: 5′ -cgtcgaacaggaagctctg-3′ ; probe

no. 92), *Gdf9* (forward primer: 5′ -acccagcaaccaggtgac-3′ , reverse primer:

5′ -cgatttgagcaagtgttccat-3′ ; probe no. 62), *Gdf10* (forward primer: 5′

-gaagtacaaccgaagaggtgct-3′ , reverse primer: 5′ -ggcttttggtcgatcatttc-3′ ;

probe no. 52), *Gdf11* (forward primer: 5′ -gaatcgagatcaacgcctttt-3′ , reverse

primer: 5′ -gctccatgaaaggatgcag-3′ ; probe no. 17), *Gdf15-variant1* (forward

primer: 5′ -tcaaagacacactcaggacaca-3′ , reverse primer: 5′

-aggaacagcaggaacctcag-3′ ; probe no. 1), *Gdf15-variant2* (forward primer: 5′

-cctggtctggggatactgag-3′ , reverse primer: 5′ -ccatgtcgcttgtgtcctt-3′ ; probe

no. 98), *Inhba* (forward primer: 5′ -atcatcacctttgccgagtc-3′ , reverse primer:

5′ -tcactgccttccttggaaat-3′ ; probe no. 72), *Inhbb* (forward primer: 5′

-gatcatcagctttgcagagaca-3′ , reverse primer: 5′ -tgccttcattagagacgaagaa-3′ ;

probe no. 52), *Inhbc* (forward primer: 5′ -tcatcagctttgctgacaca-3′ , reverse

primer: 5′ -ttctaccagagaagtggaactcg-3′ ; probe no. 67), *Inhbe* (forward primer:

5′ -catcagctttgctaccatcataga-3′ , reverse primer: 5′

-aggtggtgggaccaaagag-3′ ; probe no. 11), *Mstn* (forward primer: 5′

-tggccatgatcttgctgtaa-3′ , reverse primer: 5′ -ccttgacttctaaaaagggattca-3′ ;

probe no. 2), *Nodal* (forward primer: 5′ -ccaaccatgcctacatcca-3′ , reverse

primer: 5′ -cacagcacgtggaaggaac-3′ ; probe no. 40), *Runx3* (forward primer:

5′ -gctctctcagcaccacgag-3′ , reverse primer: 5′ -tcaggtctgaggagccttg-3′ ;
probe no. 71), *Tgfb1* (forward primer: 5′ -tggagcaacatgtggaactc-3′ , reverse
primer: 5′ -gtcagcagccggttacca-3′ ; probe no. 72), *Tgfb2* (forward primer: 5′
-tggagttcagacactcaacaca-3′ , reverse primer: 5′ -aagcttcgggatttatggtgt-3′ ;
probe no. 73), *Tgfb3* (forward primer: 5′ -ccctggacaccaattactgc-3′ , reverse
primer: 5′ -tcaatataaaggggggcgtaca-3′ ; probe no. 25) and/or *Thtr1* (forward
primer: 5′ -cgacaagaacttgaccgaga-3′ , reverse primer: 5′
-aaggaacacgggaaacagc-3′ ; SYBR Green system).

Development of thymocytes in *vitro*

The protocol was established and modified according to previous experiments
(Kunisawa et al., 2015; Lai et al., 2010). Briefly OP9–DL1 stromal cells were
seeded into the wells of 6-well tissue culture plates (6 × 10^4/1.5 mL) and
incubated overnight in medium (complete [c]DMEM [high glucose; Nacalai
Tesque] with L-glutamine, 20% heat-inactivated fetal bovine serum [Gibco], 1%
penicillin and streptomycin [Nacalai Tesque], β-mercaptoethanol [50 mM; Gibco],
and 1% non-essential amino acids solution [Nacalai Tesque]) at 37ºC.
Single-cell suspensions of thymocytes were generated by tissue disruption
through a 100-μm nylon-mesh screen by using a syringe plunger. Sorted DN1–3
($CD25^+$ or $CD44^+$) thymocytes (12 × 10^3 cells/0.5 mL cDMEM) were seeded into
the wells of 6-well tissue culture plates containing a near-confluent monolayer (6
× 10^4) of OP9–DL1 stromal cells in the presence of 1 ng/mL IL-7 (Enzo Life
Sciences, ENZ-PRT138), 5 ng/mL Flt3L (Biolegend, 550706), and the
appropriate concentration of oxythiamine (Oxythiamine chloride hydrochloride,

Santa Cruz) and incubated for 7 days. Stained cells underwent flow cytometry (FACS Aria, BD Biosciences), and the data were analyzed by using FlowJo software (Tree Star).

Fetal thymic organ culture (FTOC)

The protocol was modified from that previously described (Hirano et al., 2015). Complete RPMI 1640 (Sigma–Aldrich) medium contained 10% fetal bovine serum, with L-glutamine, 10% heat-inactivated fetal bovine serum, 1% penicillin and streptomycin, β-mercaptoethanol (50 mM), sodium pyruvate (1 mM; Nacalai Tesque), 1% non-essential amino acids and HEPES buffer (10 mM; Nacalai Tesque). Fetal thymic lobes from day 15 embryos were placed on floating filter (GE Healthcare) and treated with 2-deoxyguanosine (Tokyo Chemical Industry) for 4 days. Cultures were then incubated in fresh medium without 2-deoxyguanosine for 1 day, and sorted DN1–3 thymocytes (2000 cells/well) from 8-week-old mice were reconstituted in FTOC by hanging-drop culture for 2 days. To treat FTOCs with oxythiamine, reconstituted FTOCs were incubated for 10 days on floating filters in the presence of oxythiamine, branched-chain α-keto acids, or K02288 (1 μM; Sigma–Aldrich) alone or in combination. To treat FTOCs with branched-chain α-keto acids, reconstituted FTOCs were incubated on floating filters for 24 h in the presence of branched-chain α-keto acids or K02288 (1 μM; Sigma–Aldrich) or both; afterward, fresh medium without branched-chain α-keto acids or K02288 was added, and cultures were incubated for 9 days. Stained cells underwent flow cytometry, and data were analyzed by using FlowJo software.

Immunohistochemistry

Frozen thymic tissues were evaluated histologically according to a modification of a previous protocol (Kunisawa et al., 2015). The tissues were embedded in Optimal Cutting Temperature compound (Sakura Finetechnical). For the detection of THTR1, cryostat sections (thickness, 7 μm) were fixed in cold acetone for 1 min without paraformaldehyde fixation. Fixed sections were preblocked by using an anti-CD16/CD32 Ab (Biolegend) for 15 min at room temperature and then stained by using a rabbit anti-SLC19A2 (THTR1) polyclonal Ab (Atlas Antibodies) followed by fluorescent-conjugated Ab specific for EpCAM (Biolegend, 118207), AF488-conjugated anti-rat IgG Ab (Jackson ImmunoResearch Laboratories), or Cy3-conjugated donkey anti-rabbit IgG Ab (Jackson ImmunoResearch Laboratories). Slides were counterstained by using DAPI (Sigma–Aldrich). The specimens were analyzed by using a fluorescence microscope (model BZ-9000, Keyence).

In vitro analysis of thymic stromal cells

Thymic stromal cells (0.5×10^6 cells/mL; 1 or 2 mL/well) were incubated in serum-free DMEM with or without oxythiamine or branched-chain α-keto acids at indicated durations and concentrations. The supernatants and cells were collected separately and underwent metabolite analysis by using ion chromatography–MS (described in the next section). mRNA levels were evaluated through reverse transcription–PCR analysis, as described earlier.

Ion chromatography (IC)–MS analysis

The protocol was established and modified according to the manufacturer's protocol (Thermo Scientific) and a previous report (Kunisawa et al., 2015). As an internal control, succinic acid-2,2,3,3-d4 (Santa Cruz) was added to the hydrophilic fraction, which then was extracted from the mixture of supernatant, methanol, and chloroform. Hydrophilic fractions were column-purified (UFC3LCCNB-HMT; Human Metabolome Technologies), and purified fractions were dried overnight in an evaporator. The solid residue was dissolved in water and subjected to IC-MS.

For metabolome analysis focused on glucose metabolic central pathways, namely glycolysis, TCA cycle, and pentose phosphate pathway, anionic metabolites were measured by using an orbitrap-type MS instrument (Orbitrap Elite and Thermo Tune Plus, Thermo Scientific) connected to high-performance IC system (ICS-5000+, Thermo Fisher Scientific), thus enabling us to perform highly selective and sensitive metabolite quantification owing to the features of IC separation and Fourier Transfer MS (Hu et al., 2015; Miyazawa et al., 2017).

The IC device was equipped with an anion electrolytic suppressor (Thermo Scientific Dionex AERS 500) to convert the potassium hydroxide gradient into pure water before the sample entered the MS instrument. The separation was performed by using a Dionex IonPac column (Thermo Scientific AS11-HC; particle size, 4 µm). IC flow rate was 0.20 mL/min supplemented post-column with a 0.20 mL/min makeup flow of acetonitrile. The potassium hydroxide gradient conditions for IC separation were to 1 mM (0–1 min), 1 mM to 100 mM (1–75 min), and 100 mM (75–80 min) at a column temperature of 30°C.

The Orbitrap MS was operated under an ESI negative mode for all detections. Full mass scan (*m/z* 50－900) was used at a resolution of 60,000. The automatic gain control (AGC) target was set at 1×10^6 ions, and maximum ion injection time (IT) was 10 ms. Source ionization parameters were optimized with the spray voltage at 3 kV, and other parameters were as follows: transfer temperature, 250°C; S-Lens level, 70%; heater temperature, 500°C; sheath gas, 50 arbitary units; and auxiliary gas at 15 arbitary units.

Statistics

Statistical significance was assessed by using the Mann–Whitney *U*-test, or an two-tailed unpaired Student's *t*-test was used for comparing two groups (Prism, GraphPad Software). *P* values less than 0.05 were considered to be significant.

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