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Supplemental Information

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

So-ichiro Hirata, Kento Sawane, Jun Adachi, Junko Isoyama, Yuki Sugiura, Ayu Matsunaga, Koji Hosomi, Takeshi Tomonaga, Makoto Suematsu, Takahiro Nagatake, and Jun Kunisawa

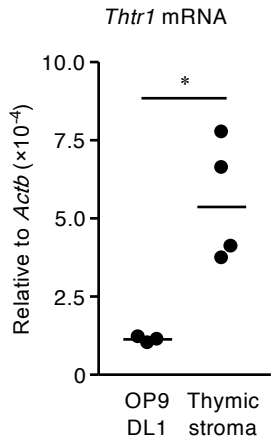

Figure S1. The requirement for vitamin B1 is higher in thymic stromal cells than in OP9-DL1 cells, *Related to Figure 2.*

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).

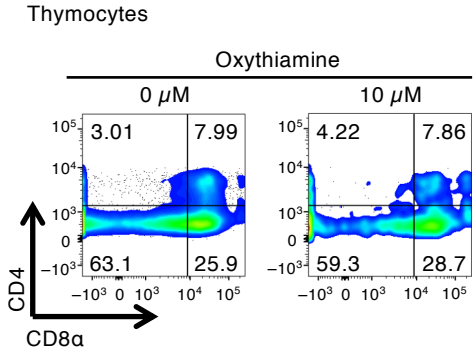

Figure S2. Vitamin B1 inhibition did not reduce DP thymocytes in fetal thymic organ cultures, *Related to Figure 2.*

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.

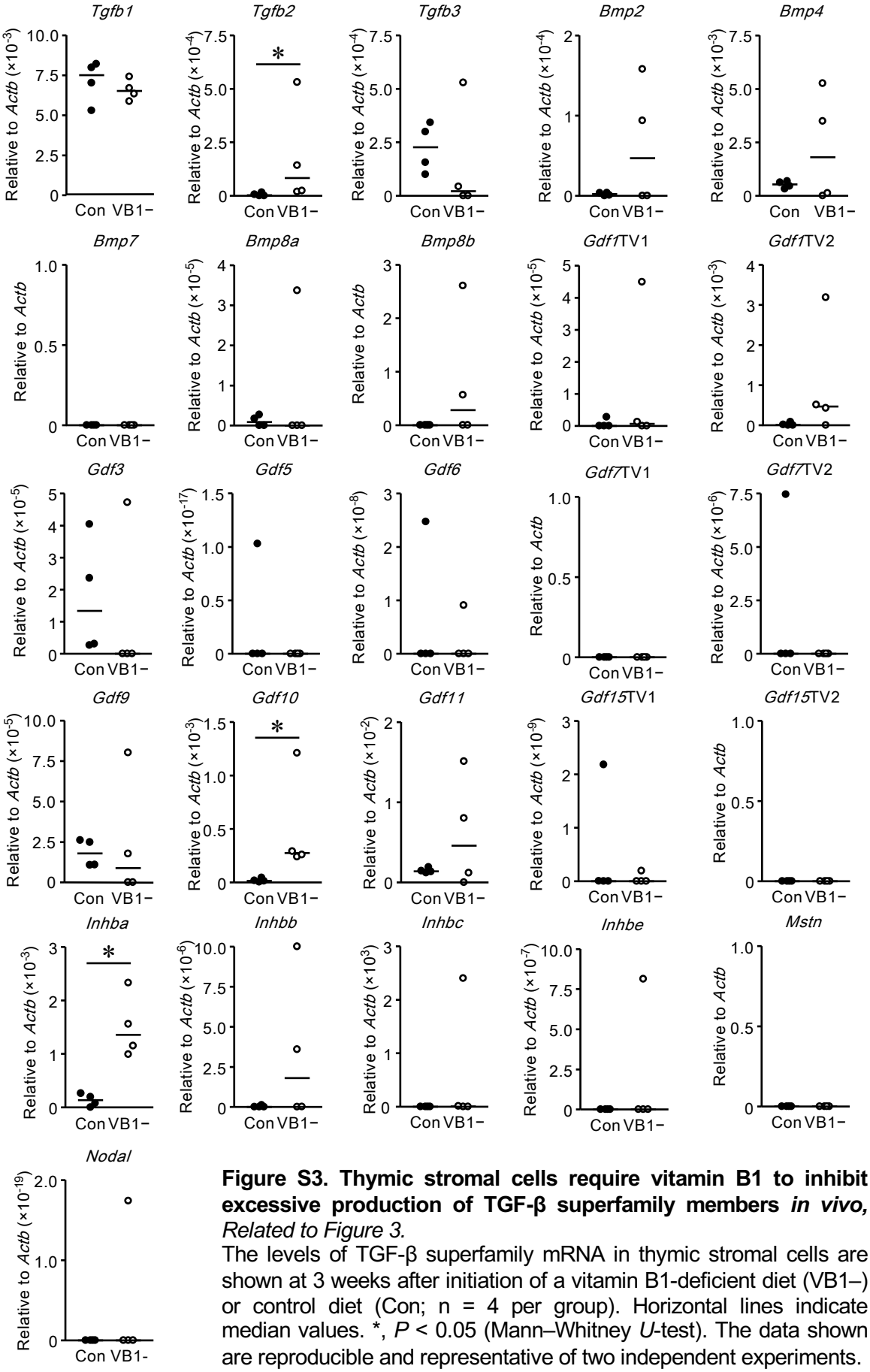

Figure S3. Thymic stromal cells require vitamin B1 to inhibit excessive production of TGF-β superfamily members *in vivo,* *Related to Figure 3.*

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.

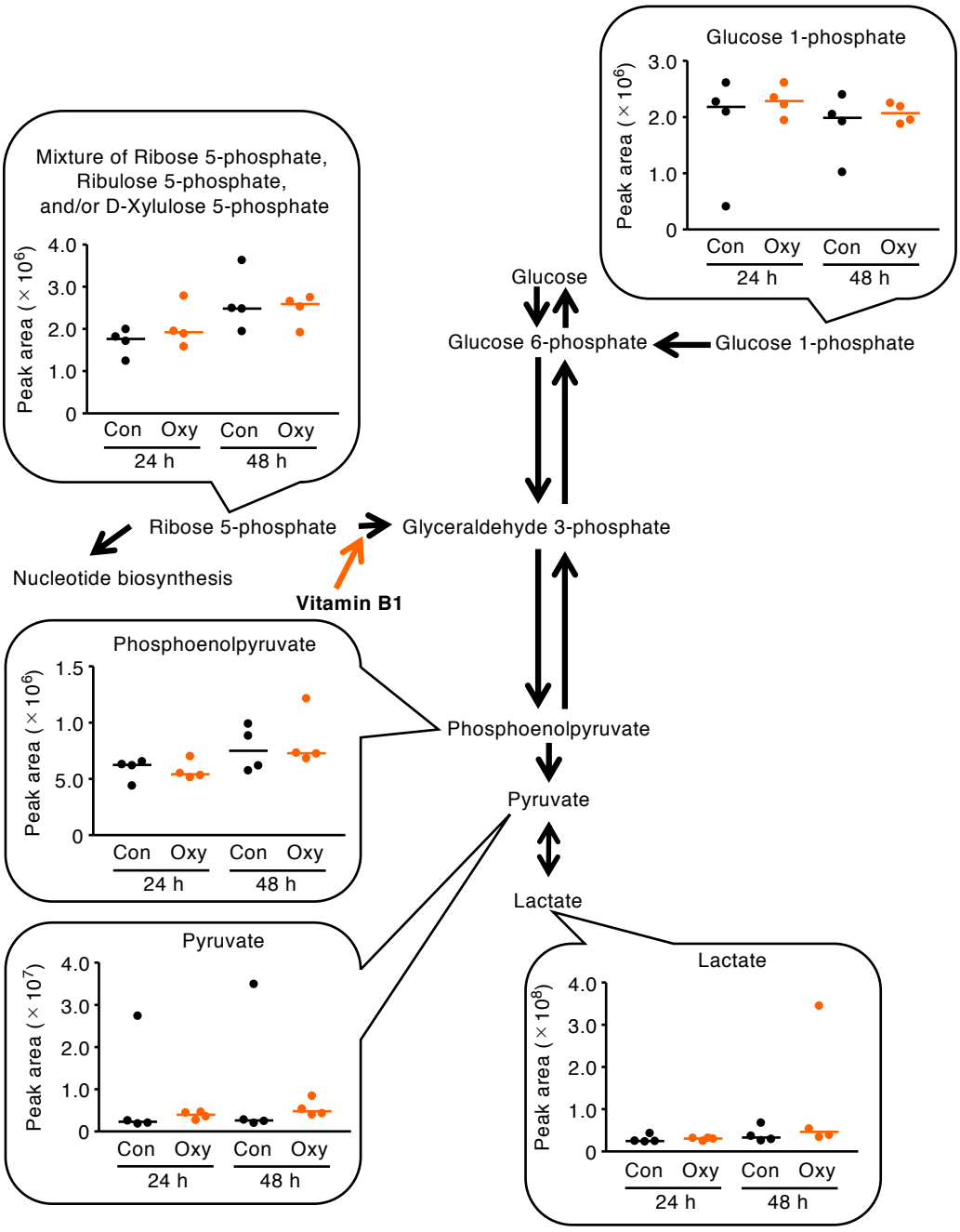

Figure S4. Glycolysis-related metabolic profiles in vitamin B1 inhibition of thymic stroma *in vitro,* *Related to Figure 4.*
We used IC-MS to investigate glycolysis-related metabolites in the supernatants from murine thymic stromal cells that had been incubated in the presence of oxythiamine (Oxy) or in its absence (control, Con) for 24 or 48 h. Horizontal lines indicate median values.

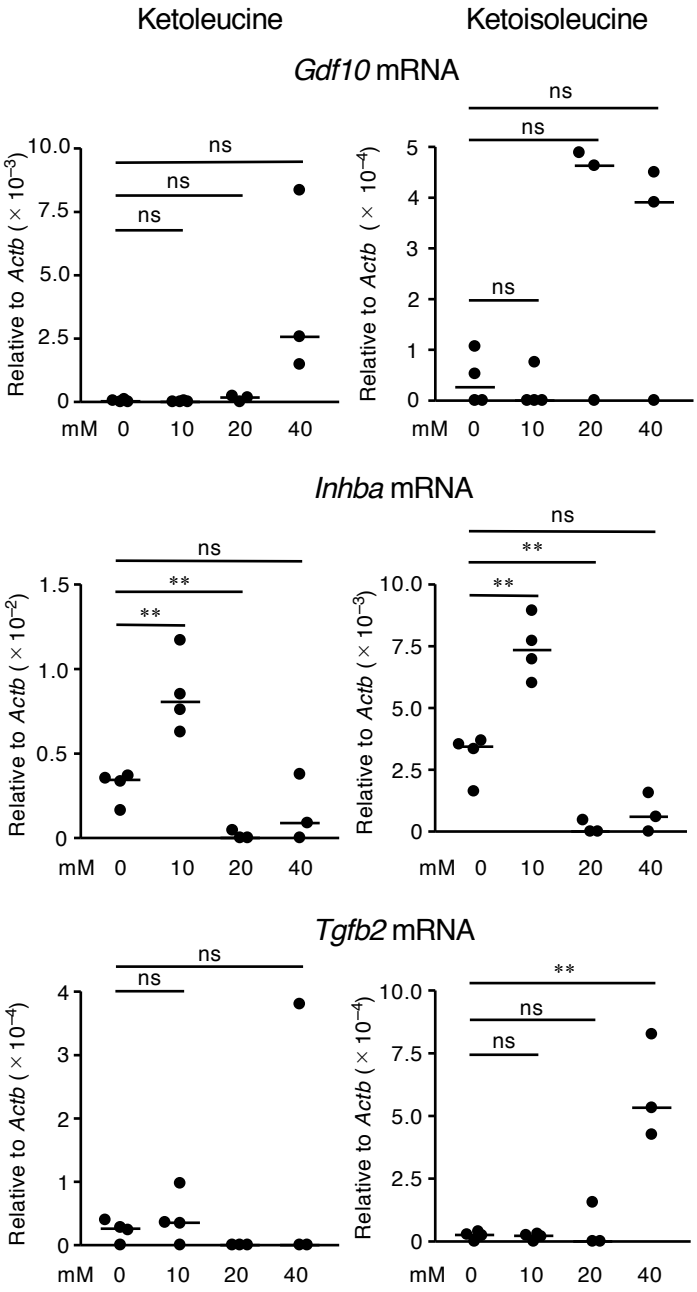

Figure S5. The effect of 12-h exposure to branched chain α-keto acids on production of the TGF-β superfamily in thymic stromal cells, *Related to Figure 5.*

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.

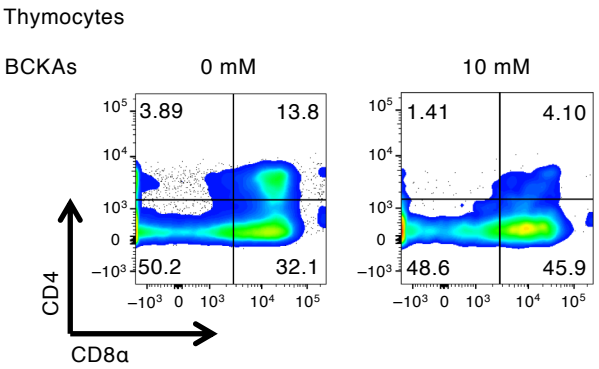

Figure S6. Treatment with branched chain α-keto acids (BCKAs) decreased DP thymocytes in fetal thymic organ culture, *Related to Figure 6.*

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.

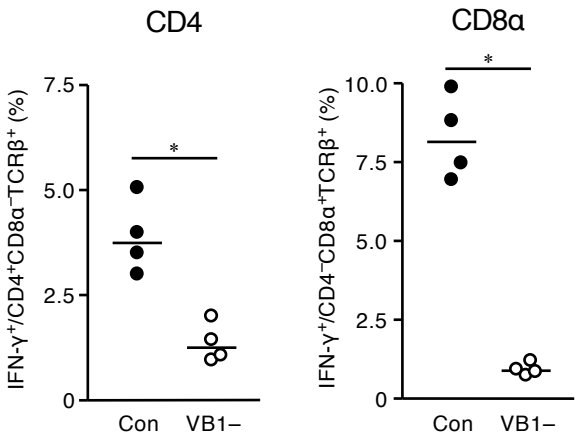

Figure S7. Vitamin B1 is necessary for IFN-γ production from peripheral T lymphocytes, *Related to Figure 7.*
The proportion of IFN-γ^+ gated on CD4 or CD8$\alpha\beta$ TCRβ^+ T lymphocytes in spleen at 3 weeks after mice began to receive vitamin B1-deficient (VB1–) or control (Con) chow. Horizontal 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 α4β7 (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 TCRγδ (Biolegend, 118120); FITC-conjugated anti-mouse TCRγδ (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′ -gaatcgagatcaacgccttt-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′ -tcaatataaaggggcgtaca-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.

Supplemental References

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