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Maternal High Fiber Diet during Pregnancy and Lactation Influences Regulatory T Cell Differentiation in Offspring in Mice

Akihito Nakajima, Naoko Kaga,† Yumiko Nakanishi,‡ Hiroshi Ohno,‡ Junki Miyamoto,§ Ikio Kimura,§ Shohei Hori,*,† Takashi Sasaki, Keiichi Hiramatsu, Ko Okumura,** Sachiko Miyake,†† Sonoko Habu,** and Sumio Watanabe*

Short-chain fatty acids (SCFAs), the end products of dietary fiber, influence the immune system. Moreover, during pregnancy the maternal microbiome has a great impact on the development of the offspring’s immune system. However, the exact mechanisms by which maternal SCFAs during pregnancy and lactation influence the immune system of offspring are not fully understood. We investigated the molecular mechanisms underlying regulatory T cell (Treg) differentiation in offspring regulated by a maternal high fiber diet (HFD). Plasma levels of SCFAs in offspring from HFD-fed mice were higher than in those from no fiber diet–fed mice. Consequently, the offspring from HFD-fed mice had higher frequencies of thymic Treg (Tregthym) and peripheral Treg. We found that the offspring of HFD-fed mice exhibited higher autoimmune regulator (Aire) expression, a transcription factor expressed in the thymic microenvironment, suggesting SCFAs promote Treg differentiation through increased Aire expression. Notably, the offspring from HFD-fed mice had higher frequencies of thymic Treg (Tregthym) and peripheral Treg. We found that the offspring of HFD-fed mice exhibited higher autoimmune regulator (Aire) expression, a transcription factor expressed in the thymic microenvironment, suggesting SCFAs promote Treg differentiation through increased Aire expression. Notably, the receptor for butyrate, G protein–coupled receptor 41 (GPR41), is highly expressed in the thymic microenvironment and Aire expression is not increased by stimulation with butyrate in GPR41-deficient mice. Our studies highlight the significance of SCFAs produced by a maternal HFD for Treg differentiation in the thymus of offspring. Given that Aire expression is associated with the induction of Treg, the maternal microbiome influences Treg differentiation in the thymus of offspring through GPR41-mediated Aire expression. The Journal of Immunology, 2017, 199: 3516–3524.

Several hundred bacterial species inhabit the gastrointestinal tract of mammals and benefit the host immune system (1–3). Notably, gut microbiome–host cross-talk is closely linked to health and disease. In mice, maternal metabolism during pregnancy is altered to both maintain pregnancy and protect fetuses from infection. Thus, the composition of the gut microbiome in pregnant mice is different from that in normal control mice (4). Therefore, it is conceivable that alterations in the maternal gut microbiome influence the offspring’s immune system. In fact, the relative importance of maternal diet and the microbiome on the immune system and behavior of offspring has been previously demonstrated (5–7).

The thymus is a lymphoid organ crucial for T cell development; however, how the maternal gut microbiome impacts the fetal thymus and T cell development in offspring is poorly understood. Although T cell differentiation in the thymus is mostly dependent on genetic factors, we have previously reported that the expression of autoimmune regulator (Aire), a transcription factor expressed in the thymic microenvironment, is influenced by factors produced by commensal bacteria (8). In addition, recent studies suggest that alterations in the maternal gut microbiome also influence the immune system of offspring (9). Because the expression of Aire is associated with T cell differentiation and self-tolerance (10), these results suggest that the maternal gut

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A.N. designed the study and performed most experiments; N.K. performed measurements of the concentration of SCFAs in feces; Y.N. and H.O. performed measurements of the concentration of SCFAs in plasma; T.S. and K.H. performed 16S rDNA sequencing of feces; S. Hori provided Foxp3GFP reporter mice; J.M. and I.K. provided GPR41-deficient mice and interpreted the data; K.O., S.M., S. Habu, and S.W. supervised the study and interpreted the data; and A.N. and S. Habu wrote the manuscript.

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Abbreviations used in this article: Aire, autoimmune regulator; E, embryonic day; FTOC, fetal thymus organ culture; GC-MS, gas chromatography–mass spectrometry; GPR41, G protein–coupled receptor 41; HCD2, human CD2; HDAc3, histone deacetylase inhibitor; HFD, high fiber diet; Hprt, hypoxanthine-guanine phosphoribosyltransferase; MBTSTFA, N-(tert-butylimidethylsilyl)-N-trifluoroacetamide; mTEC, medullary TEC; ND, normal diet; NFD, no fiber diet; RANKL, receptor activator of NF-κB ligand; rDNA, rRNA-encoding DNA; SA, sodium acetate; SB, sodium butyrate; SCFA, short-chain fatty acid; SP, sodium propionate; TEC, thymic epithelial cell; Treg, regulatory T cell; TSA, trichostatin A; Tregthym, thymic Treg; Ulex europaeus agglutinin-1.

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microbiome also influences the thymic development of offspring.

The composition of the gut microbiome is largely related to dietary fiber intake. Several studies have indicated that dietary fiber is fermented by certain bacteria, which results in the production of short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate (11). SCFAs are known to modulate immune responses in the gut and also in other organs. Interestingly, recent studies have demonstrated that SCFAs have a potential role in inducing regulatory T cells ($T_{reg}$) in the gut (12–14).

Accumulating evidence suggests that $T_{reg}$ prevents inflammation and maintains immune tolerance (15, 16). Depletion of $T_{reg}$ during day 0–10 in mice resulted in severe weight loss and a multiorgan autoimmune phenotype (17). $T_{reg}$ are divided into two distinct subtypes: thymic $T_{reg}$ ($T_{reg}^{th}$) and peripherally derived $T_{reg}$ ($T_{reg}^{ph}$, 18, 19). In the perinatal period, $T_{reg}^{th}$ are differentiated in the thymus and play a crucial role in preventing autoimmune diseases (20).

In the current study, we addressed whether SCFAs produced by the maternal microbiome during pregnancy influence $T_{reg}$ development in the thymus of offspring. We demonstrated that maternal dietary fiber intake during pregnancy and lactation influence $T_{reg}$ differentiation in offspring through G protein–coupled receptor 41 (GPR41)–mediated Aire expression in medullary thymic epithelial cells (mTECs).

**Materials and Methods**

**Mice and diets**

C57BL/6j mice and pregnant C57BL/6j mice were purchased from Japan SLC (Shizuoka, Japan). Foxp3<sup>CGD</sup> reporter mice for fetal thymus organ culture (FTOC) were obtained from RIKEN (Yokohama, Japan) (21). GPR41-deficient mice were as described previously (22). Mice were bred under specific pathogen-free conditions at the animal facility of Juntendo University (Tokyo, Japan) and Tokyo University of Agriculture and Technology (Fuchu, Japan). C57BL/6j mice were fed either a no fiber diet (NFD) (0% fiber; Research Diets, New Brunswick, NJ), a high fiber diet (HFD) (10% fiber; Research Diets), or a control diet (CD) (0% fiber; Research Diets) for 10 wk. C57BL/6j mice were housed individually. All animal experiments were approved by the Animal Experimentation Committee of Juntendo University.

**Reagents and Abs**

Mouse mAbs V421-CD8 (5H10-1), FITC-CD4 (RM4-5), allophycocyanin–CD45 (SB1), Pacific Blue–I-A/E-MS (114.15.2), PE-Foxp3 (FJK-16s), eFluor488-Aire (SH1-10), and biotinylated Ulex europaeus agglutinin-1 (UEA-1) were purchased from BioLegend (San Diego, CA). Miltenyi Biotec (Bergisch Gladbach, Germany), BD Biosciences (San Jose, CA), Tonbo Biosciences (San Diego, CA), eBioscience (San Diego, CA), and Vector Labs (Burlingame, CA). Recombinant receptor activator of NF-kB ligand (RANKL) was purchased from BioLegend. Sodium acetate (SA), sodium propionate (SP), sodium butyrate (SB), and CellSensor (WBK) were purchased from Wako (Osaka, Japan). Trichostatin A (TSA) was purchased from Sigma (St. Louis, MO).

**Flow cytometric analysis**

Total thymic cells were prepared by enzymatic digestion with Liberase TM (Roche, Basel, Switzerland). Cells were incubated for 30 min at 4°C, protected from light with a combination of appropriate fluorescently labeled mouse-specific Abs. After washing with 1% FBS/PBS, the cells were analyzed with a FACSVerse flow cytometer (BD Biosciences). For intracellular Foxp3, the Foxp3 Staining Buffer Set (eBioscience) was used in accordance with the manufacturer’s protocol. For Aire expression analysis in vivo, after CD4<sup>+</sup> cells were roughly enriched by depleting anti-CD45 microbeads by AutoMACS (Miltenyi Biotec), they were counted and stained with anti-I-A/E–Pacific Blue, anti–CD45-allophycocyanin, and UEA1-biotin and then streptavidin-PE. Then, for intracellular Aire staining, the cells were fixed and stained with anti–Aire–FITC or isotype control Ab. Data were analyzed with FlowJo (Tree Star, Ashland, OR).

**Purification of thymic epithelial cells and CD4<sup>+</sup> cells**

Thymic epithelial cells (TECs) were prepared by enzymatic digestion with Liberase TM (Roche) and DNase I (Roche) as described previously (23). For TEC isolation, CD4<sup>+</sup> cells from thymi were enriched by depleting CD4<sup>+</sup> cells using a magnetic cell sorter (Miltenyi Biotec). Then, for complete depletion of CD4<sup>+</sup> cells and obtaining MHC class II<sup>+</sup> cells, these CD4<sup>+</sup> cells were stained with allophycocyanin-conjugated Ab specific for CD45 (SB1) and Pacific Blue–conjugated Ab specific for I-A/E. CD4<sup>+</sup> I-A/E<sup>+</sup> TECs were isolated by sorting on a FACSaria (BD Biosciences).

**RNA isolation and quantitative mRNA analysis**

Total RNA was isolated with RNeasy Micro and Mini kits (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. cDNA was reverse-transcribed with RiiVaaTr (Toyobo, Osaka, Japan). Real-time PCR was performed with SYBR Green (Thermo Fisher Scientific, Waltham, MA) in accordance with the manufacturer’s protocol with 40 cycles of amplification. Results are derived from the relative quantification of target genes and the mean ± SD determined. The housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (Hprt) was used for normalization. The primers used were as follows: mouse Aire, 5'-ACCCAAACAGTTCGAAGACC-3', and 5'-GACAGCGCTTCACACAGATGA-3'; Gpr41, 5'-CTCTGGTGCTGGAGCACACTC-3' and 5'-TCCGGCTCCTCCC-GAAGTG-3'; Foxp3, 5'-GTGACATTGGGACACACTT-3' and 5'-CTCGGCGTGGTAGTGTATT-3'; mouse Hprt, 5'-GGAGACACCGCCC-AAATAAGG-3' and 5'-AAACAGATGTGGCCTGATCCAA-3'.

**FTOC**

FTOC was performed as previously described (8). Briefly, fetal thymic lobes from C57BL/6j, Foxp3<sup>CGD</sup> reporter mice and GPR41-deficient mice were removed from embryos (E17) and placed on Nucleopore filters (Whatman GE Healthcare Life Sciences, Buckinghamshire, U.K.) floating on RPMI 1640 medium (Sigma) supplemented with 10% FCS (Sigma), 500 U/ml penicillin, 500 μg/ml streptomycin, 10 mM HEPES buffer, and 1 mM 2-ME in the presence or absence of SB, SA, and SP (Wako).

**Measurement of the concentration of SCFAs in feces**

C57BL/6j mice at 5 wk of age were fed an NFD or HFD for 3 wk. Fresh feces (100 mg) from NFD-fed and HFD-fed mice was homogenized in 400 μl H<sub>2</sub>O containing hexanoic acid (methyl-d<sub>3</sub>) as an internal standard. Then, 80 μl of 25% meta-phosphoric acid was added to the homogenate and kept on ice for 30 min. Thereafter, samples were centrifuged at 17,500 × g for 15 min at 4°C. The supernatants were filtered using a Millipore Ultrafree MC PLHCC centrifugal filter (Merck Millipore, Billerica, MA) and analyzed by gas chromatography–mass spectrometry (GC-MS). One microliter of the sample was injected with a split mode (1:100) into a TRACE GC Ultra gas chromatograph equipped with a TQ5970 Quantum GC mass spectrometer (Thermo Fisher Scientific). A Nukol fused silica capillary column (0.25 mm internal diameter × 30 m, 0.25 μm film thickness; Supelco, Bellefonte, PA) was used for separation. The column temperature was programmed for 150°C for 2 min, increased to 200°C at a rate of 8°C/min, and then held at 200°C for 13 min. Helium was used as a carrier gas at a flow rate of 0.7 ml/min. The data were acquired in electron impact ionization mode at 70 eV.

**Measurement of the concentration of SCFAs in plasma**

Extraction and measurement of SCFAs were as previously described by Osman et al. (24) with slight modifications. Approximately 50 μl of plasma was added to 5 μl of a solution containing internal standards (2.2 mM [1,2,3–<sup>13</sup>C<sub>3</sub>] acetate, 2.2 mM [1,2,3–<sup>13</sup>C<sub>3</sub>] butyrate, and 2.2 mM crotonate). Then, 27.5 μl hydrochloric acid and 110 μl diethyl ether were added and mixed well. After centrifugation at 3000 × g for 10 min, 50 μl of the organic layer was transferred to a glass vial and 1 μl N-tert-butyl(dimethyl)silyl-N-trifluoroaceticamide (MTBSTFA) (Sigma) was added to derivatize the samples. The vials were incubated at 80°C for 20 min and allowed to stand for 48 h before injection. Analysis was performed using gas chromatography–tandem mass spectrometry platforms on a GCMS-QTOF8030 Triple Quadrupole Mass Spectrometer (Shimadzu, Kyoto, Japan) with a capillary column (DB-5; Agilent Technologies, Santa Clara, CA). The gas chromatography oven was programmed as follows: 80°C held for 3 min, increased to 180°C (8°C/min), and then 300°C (20°C/min) and held for 3 min. The detector and injector temperatures were 250 and 260°C, respectively, and the carrier gas was helium (1.18 ml/min). Injection volume was set at 1 μl.

**Microbiome analysis based on 16S rRNA amplicon sequencing**

The gut content samples were diluted 10-fold in TE buffer (10 mM Tris, 1 mM EDTA [pH 8]) and frozen at −80°C until use. Five hundred microliters of each diluted sample was used for DNA extraction. After pretreatment in TE buffer with 50 U of achromopoditase (Wako) at 50°C for 30 min, phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) was used for DNA purification.
We performed microbiome analysis at the phylum level based on 16S rDNA amplicon sequencing, as previously reported (25). We used 16S universal primers without adapter sequences, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3'), which amplified almost the entire length of 16S rRNA-encoding DNA (rDNA) (26). The PCR reaction mixture consisted of 2 μL of DNA extract in a total volume of 25 μL containing 1× KAPA HotStart ReadyMix (Kapa BioSystems, Boston, MA) and 10 pmol of each primer. Reaction mixtures were thermally cycled once at 95˚C for 2 min; then 25–30 times at 95˚C for 30 s, 65˚C for 30 s, and 72˚C for 90 s; and then once at 72˚C for 2 min. DNA libraries were prepared by transposon-based fragmentation of the 1.5-kbp 16S rDNA using a Nextera XT DNA Sample Prep Kit (Illumina, San Diego, CA). Sequencing was performed using a Miseq Reagent Kit v3 (600 Cycle) and a paired-end 2×300 bp cycle run on an Illumina MiSeq sequencing system.

After sequencing, the obtained reads were filtered and trimmed by removing bases with quality value scores of 20 or less and reads shorter than 100 bases. The reads were converted from FASTQ to FASTA format using FastX-Toolkit version 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Only read 1 reads were used for taxonomic assignments of 16S rDNA. Analyses of the trimmed sequencing reads were performed using blastn by blast+ 2.5.0, with an e-10 e-value cutoff (27). Taxonomic classification was performed using MEGAN version 5 (28).

**Statistical analysis**

Statistical analysis was performed using a Student t test (GraphPad Prism 4; GraphPad Software, La Jolla, CA). A p value <0.05 was considered statistically significant.

**Results**

The offspring from HFD-fed mice display increased plasma levels of SCFAs

To determine the levels of SCFAs in the maternal gut after dietary fiber intake, C57BL/6 pregnant mice were fed either an NFD or HFD containing 10% purified inulin. Then, fresh fecal samples were collected from each group of mice and the levels of SCFAs determined by GC-MS. The results clearly showed that the levels of fecal SCFAs, including acetate, propionate, and butyrate, were higher in HFD-fed mice when compared with NFD-fed mice (Fig. 1A). Given that the composition of the maternal gut microbiome exhibits dramatic changes during pregnancy (4), we next examined the changes in circulating SCFAs in maternal plasma in detail. Wild-type pregnant mice were divided into three stages: the early (embryonic day [E] 0–E6), middle (E7–E14), and late stages (E15–E19) of pregnancy. Then, the levels of SCFAs, including acetate, propionate, and butyrate, in the plasma of each mouse were measured by GC-MS. Although there were no changes in plasma acetate levels, we found elevated levels of propionate and butyrate. Notably, the levels of butyrate were significantly higher in the late stage of pregnancy (Fig. 1B). Based on increased plasma butyrate levels, we hypothesized that maternal SCFAs were transferred to fetuses throughout pregnancy and to offspring during lactation. To explore this possibility, we measured plasma levels of SCFAs in offspring from NFD- and HFD-fed, and ND-fed mice after birth (from day 1 to day 30) by GC-MS. The offspring from NFD-fed mice showed lower plasma levels of acetate, propionate, and butyrate, when compared with HFD-fed or ND-fed mice at day 1 after birth (Fig. 1C). Because little gut microbiota is observed at day 1 after birth, the circulating SCFAs were due to maternal transfer during pregnancy or breast milk after birth. Plasma levels of acetate and propionate were increased in offspring from HFD-fed mice, whereas those in offspring from NFD-fed mice were decreased. The difference in SCFAs levels between NFD and HFD continued until day 22 after birth.

To exclude the possibility that the microbiota of offspring affects the production of SCFAs, we first measured the level of SCFAs in the gut contents of offspring at day 1 after birth by GC-MS. The concentration of acetate was much lower than that of mother mice and there was no difference between the offspring from NFD- and HFD-fed mothers. Furthermore, propionate and butyrate were not detected (data not shown). We then performed 16S rDNA–based microbiome analysis of feces. From DNA libraries of 16S rDNA PCR products, we obtained an average of 310,527 reads per sample. Nine unique phyla (Actinobacteria, Bacteroidetes/ Chlorobi group, Chlamydiae/Verrucomicrobia group, Cyanobacteria, Deferribacteres, Firmicutes, Proteobacteria, Spirochaetes, and Tenericutes) were assigned in the taxonomic classification of 16S rDNA (Supplemental Table I). As shown in Fig. 1D, at the phylum level the average relative abundance of Bacteroidetes in NFD-fed mice clearly decreased compared with that in HFD-fed mice. In contrast, the level of Firmicutes was lower in HFD-fed mice compared with NFD-fed mice. In addition, we also analyzed the microbiota of their offspring. The gut microbiota composition of offspring was completely different from adult mice. There were almost no Bacteroidetes present and the dominant phylum of the gut microbiota was Firmicutes in both NFD and HFD offspring. Interestingly, there is no difference in the composition of the phyla between the offspring from NFD-fed and HFD-fed mothers (Fig. 1D). Previous studies have suggested that Bacteroides are crucial for the production of SCFAs (29, 30). These results suggest that SCFAs derived from the maternal gut were transferred to fetuses throughout pregnancy and to offspring during lactation.

\[ \text{SCFAs induce } \text{Foxp3}^+ \text{hCD2}^+ \text{in FTOC} \]

Given that maternal dietary fiber intake during pregnancy and lactation affects the differentiation of \( \text{Foxp3}^+ \) cells in offspring, we examined whether SCFAs can induce \( \text{Foxp3}^+ \) cells in vivo using a FTOC system, which is commonly used to analyze T cell development and the thymic microenvironment (35). We used the fetal thymus of Foxp3 reporter mice, which express human CD2 (hCD2) on the \( \text{Foxp3}^+ \) surface corresponding to the thymus and peripheral organs of offspring (Fig. 2E).
to Foxp3 expression in the nucleus (21). E17 fetal thymi were treated with butyrate for 72 h and the expression of CD2 by CD4+ cells detected by flow cytometry. Interestingly, butyrate significantly increased the number of Foxp3+ tTregs in a dose-dependent manner (Fig. 3A, 3B). Moreover, the other SCFAs tested, acetate and propionate, also significantly increased Foxp3+ tTregs (Fig. 3C). These results suggest that SCFAs have a potential role in inducing tTregs in the thymus.

**Butyrate enhances Foxp3 expression through the GPR41 receptor**

We next investigated the mechanism by which butyrate enhances Foxp3 expression in the thymus. Previous studies have shown that there exist at least two possibilities to explain the properties of butyrate. The first possibility is that butyrate acts as a histone deacetylase inhibitor (HDACi) and activates histone H3 acetylation at the Foxp3 gene locus (12), resulting in increased Foxp3 expression.
expression in peripheral organs such as the gut. To address this
possibility, fetal thymi of Foxp3 reporter mice were treated with
TSA, a common HDACi, under FTOC conditions. Consequently,
our results demonstrated that the expression of Foxp3 is not up-
regulated by TSA (Fig. 4A), suggesting that butyrate does not function as an HDACi to activate Foxp3 expression in the thymus.

Another possibility is that butyrate acts through GPR41. Bu-
tyrate is a potent ligand of GPR41 and its activation is known to be associated with energy consumption and metabolic homeostasis in the gut (22, 36). GPR41 is also expressed by sympathetic ganglia and is involved in the activation of the sympathetic nervous system. However, the function of GPR41 in the thymus is largely

![Figure 2](https://example.com/Figure2.png)

**FIGURE 2.** tTregs and splenic Tregs are increased in offspring from HFD-fed mothers. (A) Flow cytometric analysis of Foxp3^+ tTregs in the thymus. Total thymocytes of offspring from NFD- and HFD-fed mice at day 3 were stained with anti-CD4 and anti-CD8 Abs, then fixed and stained with anti-Foxp3 Ab. Foxp3^+ tTregs (gated on lower panel) were analyzed on gated CD4^+ CD8^+ cells (upper panel). The frequency of Foxp3^+ cells gated on CD4^+ CD8^+ cells was analyzed by FACS Verse. The figure shows data representative of at least three independent experiments. (B) The frequency and number of Foxp3^+ tTregs of offspring born to NFD- and HFD-fed mothers at day 3, 5, and 11 after birth were estimated from total thymic cell numbers. n = 8 per group. Data are presented as the mean ± SD. The figure shows data representative of at least three independent experiments. (C) The frequency and number of Foxp3^+ Tregs in the spleen of offspring born to NFD- and HFD-fed mothers at day 5 and 11 after birth were estimated from whole spleen cell numbers. n = 8 per group. Data are shown as the mean ± SD. (D) Whole spleen cells were stained with Abs against CD4, CD8, neuropillin-1, and Foxp3. Gated cells were CD4^+ CD8^- Panels show representative results of offspring born to NFD- and HFD-fed mothers at day 8 after birth. Results are representative of at least three independent experiments. (E) Summary of percentage of Foxp3^+ tTregs in the thymus (left) and the spleen (right) of offspring from NFD- and HFD-fed mothers after birth. Data are presented as the mean ± SD. *p < 0.05, **p < 0.01.
unknown. To clarify whether GPR41 is expressed in the thymus, TECs and CD4+ CD8− T cells were isolated from E17 fetal thymi from wild-type mice and GPR41-deficient mice. To address whether GPR41 is associated with the induction of iTreg differentiation by butyrate, E17 fetal thymi from GPR41-deficient mice were treated with butyrate for 72 h. Notably, iTregs were not induced in FTOC (Fig. 4B). This result suggests that iTreg differentiation by butyrate is GPR41 dependent, but HDACi independent. We then performed PCR analysis of the expression of the GPR41 gene and revealed that GPR41 is highly expressed by TECs, but not by CD4+ CD8− T cells (Fig. 4C). These results prompted us to investigate the possibility that butyrate activates TECs through GPR41 to promote iTreg differentiation. TECs play a critical role in T cell tolerance and are divided into two populations, cortical TECs and mTECs. In accordance with previous observations indicating the involvement of mTECs in iTreg differentiation (37), we next confirmed that butyrate promotes iTreg differentiation through GPR41 in mTECs.

**Butyrate regulates GPR41-mediated Aire induction in mTECs**

Recent evidence suggests that Aire is mainly expressed in mTECs, and its expression is involved in the negative selection of T cells and immunotolerance (10, 38). Aire is also associated with Foxp3+ Treg differentiation in the thymus (17, 37, 39). We therefore hypothesized that butyrate would enhance Aire expression through GPR41 in mTECs and promote iTreg differentiation in fetuses throughout pregnancy and in neonates during lactation. To investigate this hypothesis, the expression of Aire was analyzed by real-time PCR.

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**FIGURE 3.** SCFAs induce Foxp3+ iTregs in FTOC. (A) Fetal thymus lobes (E17) from Foxp3hCD2 reporter mice were cultured with or without butyrate (50 μM) for 3 d in FTOC. Thymocytes from FTOC were stained with Abs against CD4, CD8, and Foxp3. The figure shows data representative of at least three independent experiments. (B) The frequency (left) and the number (right) of Foxp3+ tTregs stimulated with or without SB. Graphs were compiled from three independent experiments. Data are presented as the mean ± SD. (C) The frequency of Foxp3+ tTregs stimulated with or without SA (left) and SP (right). Graphs were compiled from three independent experiments. Data are shown as the mean ± SD. *p < 0.05.

**FIGURE 4.** Butyrate enhances Foxp3 expression through the GPR41 receptor. (A) Fetal thymus lobes (E17) from Foxp3hCD2 reporter mice were cultured with TSA or butyrate (50 μM) for 3 d in FTOC. The frequency of Foxp3+ tTregs is shown. Graphs were estimated from three independent experiments. Data are shown as the mean ± SD. *p < 0.05. (B) Fetal thymus lobes (E17) from GPR41-deficient mice were cultured with butyrate for 3 d in FTOC. The frequency of Foxp3+ tTregs is shown. Graphs were compiled from three independent experiments. (C) CD4+ CD8− T cells and TECs from wild-type and GPR41-deficient mice were isolated as described in the Materials and Methods. Gene expression of Gpr41 and Hprt by CD4+ CD8− T cells and TECs was analyzed by real-time PCR.
using the FTOC system in the presence or absence of butyrate. For this purpose, TEC populations defined as CD45⁻ MHC class II⁺ cells were prepared from thymi and the expression of Aire in TECs was determined by real-time PCR. Compared with RANKL stimulation as a positive control to induce Aire expression (40, 41), butyrate significantly enhanced Aire expression; whereas the expression of β5t, mainly expressed by cortical TECs, was not altered (Fig. 5A). Using flow cytometry, we revealed an increased frequency of Aire-positive cells in UEA-1-positive mTECs in a dose-dependent manner with SB in FTOC (Fig. 5B, Supplemental Fig. 2A). Notably, Aire expression was significantly increased in the presence of 50 μg of SB. We could thus confirm that butyrate stimulates Aire expression, at both the mRNA and protein levels.

To address the contribution of GPR41 to Aire induction in mTECs, we stimulated fetal thymi from GPR41-deficient pregnant mice with butyrate for 72 h under FTOC conditions and confirmed Aire expression by real-time PCR. The results clearly showed that Aire expression is not increased by butyrate stimulation, but was upregulated by RANKL as a positive control, in GPR41-deficient mice (Fig. 5C). These results further suggest that butyrate regulates GPR41-mediated Aire induction in mTECs.

Finally, we compared Aire expression between offspring from NFD- and HFD-fed mice and the contribution to tTreg elevation in vivo used in the same mice. Consistent with the result in Fig. 2B, the number of Foxp3⁺ tTregs was increased in offspring from HFD-fed mice compared with those from NFD-fed mice at day 8 (Supplemental Fig. 2B). Aire expression in mTECs was detected as shown in Supplemental Fig. 2C. Interestingly, both tTreg frequency and Aire expression were increased in the offspring of HFD-fed mice compared with in those of NFD-fed mice (Fig. 5D, Supplemental Fig. 2B). Therefore, we concluded that tTreg induction through GPR41 and Aire expression are correlated in vivo (Supplemental Fig. 2B). These findings collectively led us to conclude that SCFAs derived from maternal dietary fiber intake are transferred to the fetus during pregnancy and affect the number of Foxp3⁺ tTregs in offspring through Aire expression in mTECs.

**Discussion**

In the current study, we have demonstrated that maternal dietary fiber intake during pregnancy and lactation influences Foxp3⁺ tTreg differentiation in offspring. To confirm pregnancy-related changes in the maternal gut microbiome (4), we first determined the levels of SCFAs in the maternal gut after feeding either a HFD or NFD.
The resultant levels of SCFAs in HFD-fed mice were much higher than those in NFD-fed mice. In agreement with this finding, we revealed elevated plasma levels of SCFAs such as propionate and butyrate in pregnant mice. Interestingly, the highest concentration of butyrate was found in the late stage of pregnancy. These results led us to analyze plasma levels of SCFAs in offspring from HFD-fed mice. Consequently, we found that SCFAs, especially butyrate, were increased in the plasma of offspring during lactation. These observations support the idea that the alteration of the maternal gut microbiome is not only for maintaining pregnancy but also for generating the immune system of offspring. Despite the fact that neonates harbor little gut microbiota on day 1 and do not consume any dietary fibers during lactation, the presence of circulating SCFAs including acetate, propionate, and butyrate were detected.

According to previous studies, *Bacteroides* may be crucial for the production of SCFAs (9, 29, 30). However, there is little *Bacteroides* present in offspring just after birth, and there is no difference between offspring from NFD-fed and offspring from HFD-fed mice. Moreover, we could not detect propionate and butyrate in the gut contents of offspring. These results suggest that the circulating SCFAs in offspring are mostly due to maternal transfer during pregnancy and lactation.

Recent reports have suggested that propionate and butyrate induce the proliferation of intestinal T_{regs} (12, 13). We therefore considered the possibility that the maternal microbiome in pregnancy influences the immune responses of offspring. Consequently, our flow cytometric analysis revealed that Foxp3^{+} T_{regs} are increased in offspring from HFD-fed mice. We then assessed the mechanism by which maternal SCFAs influence Foxp3^{+} T_{reg} differentiation in offspring. Recent studies have shown that the transcription factor Aire expressed by mTECs is involved in Foxp3^{+} T_{reg} differentiation in the thymus (17, 37, 39). Therefore, we analyzed the expression of Aire using an FTOC system in the presence or absence of butyrate and found that increased levels of butyrate can enhance Aire expression in mTECs. These results are consistent with studies indicating a crucial role for Aire in inducing Foxp3^{+} T_{reg} (37). Notably, the number of T_{regs} in the thymus in neonatal Aire-deficient mice is lower than that of neonatal wild-type mice (17). This suggests that Aire expression might be crucial for the induction of T_{reg} in neonates. Based on this finding, we hypothesize that higher Aire expression in the offspring from HFD-fed mothers was related to the increased induction of T_{reg}. Notably, there is a small but significant difference in Aire expression between NFD and HFD mice (Fig. 5D). Thus, increased Aire expression might be one possible mechanism for the induction of T_{reg} in the thymus. Further studies are required to elucidate the effect of Aire in T_{reg} induction. In recent years, much attention has been focused on the link between Aire expression and autoimmune diseases (10, 42). Prenatal Aire expression is considered essential for the induction of tolerance and prevention of autoimmune diseases (43). These findings support our observations that perinatal Aire expression is crucial for the development of the offspring’s Foxp3^{+} T_{regs}.

To further investigate the mechanism by which butyrate influences thymic Foxp3^{+} T_{reg} differentiation in offspring, we focused on GPR41. GPR41 is a butyrate receptor and essential for energy metabolism in the gut and the nervous system (22). Interestingly, we revealed that GPR41 is highly expressed by mTECs, but not by CD4^{+}CD8^{+} T cells. Furthermore, we found that butyrate does not stimulate Aire expression in GPR41-deficient mice in FTOC (Fig. 5C). To address the contribution of GPR41 to T_{reg} induction in vivo, we analyzed the frequency of Foxp3^{+} T_{regs} in 3-d-old offspring of GPR41-deficient mice fed either an NFD or an HFD. As shown in Supplemental Fig. 1A, Foxp3^{+} T_{reg} levels in the CD4^{+}CD8^{+} T cell population were detected by flow cytometry. Although the mice were deficient in GPR41, Foxp3^{+} T_{reg} levels in the offspring of HFD-fed mice were higher than those in the offspring of NFD-fed mice (Supplemental Fig. 1A, 1B). We hypothesized that other receptors of SCFAs such as GPR109a and GPR43 might be involved in inducing Foxp3^{+} T_{reg} to compensate for the lack of GPR41. According to previous studies, GPR109a and GPR43 function as receptors for butyrate, propionate, and acetate. It has been reported that GPR109a and GPR43 are related to the induction of T_{reg} and the immune response against allergy and inflammation (14, 44). When we explored this hypothesis, we found that Foxp3^{+} T_{reg} levels were increased not only by butyrate but also by propionate and acetate in FTOC (Fig. 3C). Notably, in this revised experiment with GPR41-deficient mice, the difference between the offspring of NFD- and HFD-fed mice (*p < 0.05) was statistically smaller than that observed in the experiments using wild-type mice (Fig. 2A) (**p < 0.01). We believe that the smaller difference between the offspring of NFD- and HFD-fed GPR41-deficient mice is a result of the contribution of GPR43 to Foxp3^{+} T_{reg} induction. GPR41 thus has a pivotal role in mTECs. These results suggest that butyrate regulates GPR41-mediated Aire induction in mTECs and support our previous evidence that the thymic microenvironment is partially affected by environmental factors such as the microbiome (8).

In conclusion, the present studies demonstrate that the maternal microbiome during pregnancy and lactation influences T_{reg} differentiation in offspring through GPR41-mediated Aire expression in mTECs. Therefore, we believe maternal microbiome–mediated T_{reg} differentiation is an intriguing topic to be explored in further studies on protecting against allergic and autoimmune diseases.

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Disclosures
The authors have no financial conflicts of interest.

References


