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Dietary Folic Acid Promotes Survival of Foxp3+ Regulatory T Cells in the Colon

Makoto Kinoshita,*†‡ Hisako Kayama,*†§ Takashi Kusu,* Tomoyuki Yamaguchi,¶ Jun Kunisawa,‖ Hiroshi Kiyono,‡¶ Shimon Sakaguchi,¶ and Kiyoshi Takeda*†‡

Dietary compounds as well as commensal microbiota contribute to the generation of a unique gut environment. In this study, we report that dietary folic acid (FA) is required for the maintenance of Foxp3+ regulatory T cells (Tregs) in the colon. Deficiency of FA in the diet resulted in marked reduction of Foxp3+ Tregs selectively in the colon. Blockade of folate receptor 4 and treatment with methotrexate, which inhibits folate metabolic pathways, decreased colonic Foxp3+ Tregs. Compared with splenic Tregs, colonic Tregs were more activated to proliferate vigorously and were highly sensitive to apoptosis. In colonic Tregs derived from mice fed with a FA-deficient diet, expression of anti-apoptotic molecules Bcl-2 and Bcl-xL was severely decreased. A general reduction of Tregs were more activated to proliferate vigorously and were highly sensitive to apoptosis. In colonic Tregs derived from mice fed with a FA-deficient diet, expression of anti-apoptotic molecules Bcl-2 and Bcl-xL was severely decreased. A general reduction of Tregs were more activated to proliferate vigorously and were highly sensitive to apoptosis.

A quiescent gut environment is achieved through proper colonization of gut commensal microbes and adequate composition of the diet (1). Imbalance in either one of these two is known to cause excess activation of proinflammatory immunity or insufficient development of regulatory T cells (Tregs), resulting in intestinal inflammation represented by inflammatory bowel diseases (2–4). Recent accumulation of data suggests that certain components in the diet have critical roles in the physiological development of the gut immune system (2, 5–7). Malnutrition is known to result in a higher susceptibility to intestinal infections or autoimmune-mediated gut inflammation (2, 8–10).

Among the numerous components of the human diet, dietary vitamins have attracted extra attention for their exclusiveness as a supply source to maintain various cellular metabolisms under physiological conditions (2, 11). More precisely, vitamin D, the best characterized vitamin regarding its immune-modulatory functions, has a wide spectrum of immune reactions. Vitamin D is known to inhibit lymphocyte proliferation (12), promote development of Tregs (13), and drive antimicrobial peptide expression through the vitamin D receptor expressed in immune cells (14). The active form of vitamin A has also been shown to exert a pivotal role in the homeostasis of the small intestine through imprinting of a gut-homing property on T cells and the development of de novo Tregs (15–18). Thus, the critical role of vitamins in the development of a proper gut immune system has been elucidated.

Vitamin B9, also known as folate, comprises a subtype of vitamins of type B. In accordance with other vitamins, the synthetic pathways for its de novo production are absent in mammals (19). Therefore, vitamin B9 is exclusively supplied by commensal microbes in the gut or by dietary supplementation (19, 20). Because a restricted diet devoid of folic acid (FA), a folate derivative, is known to cause manifestations of vitamin B9 deficiency characterized by anaemia, cancer, or cardiovascular diseases, intake of FA from the diet is considered to be essential to maintain our physiological homeostasis (19). However, it remains unknown whether dietary FA mediates the normal development of the gut immune system.

Because we previously reported preferential expression of folate receptor 4 (FR4) on Foxp3+ Tregs (21), we examined whether Foxp3+ Tregs are predominantly affected by a deficiency in dietary FA. Deficiency of FA in the diet resulted in a marked reduction of Foxp3+ Tregs selectively in the colon. Mice fed with a diet that contained no FA were more susceptible to intestinal inflammation. The preferential requirement of FA in the maintenance of colonic Tregs was confirmed by blockade of folate metabolism with the notable anti-folate agent methotrexate (MTX) and blockade of FR4 (22). FA was demonstrated to promote survival of Tregs, which are highly activated in the colon, by enhancing anti-apoptotic molecules Bcl-2 and Bcl-xL. These findings demonstrate the previously unappreciated role of FA in the maintenance of Foxp3+ Treg homeostasis in the colon.
Materials and Methods

Mice and diets

BALB/c mice were purchased from CLEA Japan. Foxp3 bicistronic reporter knock-in mice expressing EGFP were purchased from The Jackson Laboratory (Bar Harbor, ME). The AIN93G diet and the FA-deficient diet were manufactured by Oriental Yeast. Mice were fed with a standard Oriental MF diet to examine the effects of MTX and anti-FR4 Abs. For the FA-supplementation experiment, drinking water with FA (4 μg/ml; Sigma) was freshly prepared every other day. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Osaka University.

Measurement of FA

To prepare the luminal contents of the small intestine, 1 ml PBS was administered from the duodenum, and samples were collected from the ileum. Colonic luminal contents were collected by washing a longitudinally opened intestine in a 1.5-ml tube with 1 ml PBS. All the collected samples were subsequently centrifuged, and the upper solution was used for FA detection by RIDASCREEN (r-Biopharm).

Isolation of intestinal lymphocytes and myeloid cells

Lamina propria lymphocytes and myeloid cells were isolated using a previously described protocol (23). In brief, intestines were opened longitudinally, placed in HBSS with 5 mM EDTA, and incubated at 37˚C for 20 min in a shaking water bath. After washing in PBS, the tissues were cut into small pieces and incubated in RPMI 1640 containing 4% FBS, 1 mg/ml collagenase D (Roche), 0.5 mg/ml dispase (Invitrogen), and 40 μg/ml DNase I (Roche) for 1 h at 37˚C in a shaking water bath. The digested tissues were resuspended in 5 ml 40% Percoll (GE Healthcare) and then overlaid on 2.5 ml of 80% Percoll in a 15-ml tube. Percoll gradient separation was performed by centrifugation at 780 × g for 20 min at room temperature. The lamina propria lymphocytes were collected at the interface of the Percoll gradient and washed with PBS containing 2% FBS. For preparation of lamina propria myeloid cells, intestinal tissues digested with 1 mg/ml collagenase D, 0.5 mg/ml dispase, and 40 μg/ml DNase I for 40 min at 37˚C in a shaking water bath was filtered through a 40-μm cell strainer. For collection of intraepithelial lymphocytes, longitudinally opened intestines were shaken in HBSS containing 5 mM EDTA for 20 min at 37˚C. After filtration through nylon meshes, Percoll gradient sep-
aration was performed to obtain intraepithelial lymphocytes from the epithelial cell fraction.

**Flow cytometry and sorting**

The following Abs were used: PerCp/Cy5.5-conjugated anti-CD4, PE/Cy7-conjugated anti-IFR4, PE-conjugated anti-Helios, PE/Cy7-conjugated anti-GITR, FITC-conjugated anti-IFN-γ, Alexa Fluor 647-conjugated anti-CD62L, PE-conjugated anti-BrDU, Pacific blue-conjugated anti-CD11b, PE-conjugated anti-TCR γδ, and PE/Cy7-conjugated anti-TCR β-chain (purchased from BioLegend); Alexa Fluor 647-conjugated anti-Foxp3 (purchased from eBioscience); and PE-conjugated anti-CTLA-4, Alexa Fluor 647-conjugated anti-Foxp3 (purchased from BioLegend). For Foxp3 staining, the Foxp3 Staining Buffer Set (eBioscience) was used. Flow cytometric analysis, single-cell suspensions were stimulated for 4 h in the presence of calcium ionophore (5 μM; Sigma), PMA (50 ng/ml; Sigma), and Golgi Stop (BD Biosciences). The staining of intracellular cytokines was subsequently performed with Fixation and Permeabilization Buffers (eBioscience). For Foxp3 staining, the Foxp3 Staining Buffer Set (eBioscience) was used. Flow cytometric analysis was performed using a FACSCanto II flow cytometer (BD Biosciences). For intracellular cytokine analysis, single-cell suspensions were stimulated for 4 h in the presence of calcium ionophore (5 μM; Sigma), PMA (50 ng/ml; Sigma), and Golgi Stop (BD Biosciences). The staining of intracellular cytokines was subsequently performed with Fixation and Permeabilization Buffers (eBioscience). For Foxp3 staining, the Foxp3 Staining Buffer Set (eBioscience) was used. Flow cytometric analysis was performed using a FACSCanto II flow cytometer (BD Biosciences). CD4+ FR4+ T cells, CD4+ Foxp3-EGFP+ T cells, and CD11c+ dendritic cells (DCs) were sorted using a FACSaria system (BD Biosciences).

**In vitro cell survival assay**

Cells were cultured in FA-sufficient or FA-null RPMI 1640 media supplemented with 10% dialyzed FBS. All the products were purchased from Invitrogen. The FA concentrations after FBS supplementation were 34.9 ppb and 0.2 ppb, respectively. For the cell survival assay, 5 × 10^5 sorted cells were cultured in 96-well plates. After the indicated days of incubation with plate-bound anti-CD3 Ab (2 μg/ml; BioLegend), soluble anti-CD28 Ab (2 μg/ml; BioLegend), and recombinant IL-2 (500 U/ml; PeproTech), cells were stained using an annexin V–FLUOS staining Kit (Roche).

**Cell proliferation experiments**

Spleenic CD4+ Foxp3+ T cells isolated from Foxp3-EGFP mice were labeled with 5-(and-6)-[1,4-chloromethyl]benzoylamino]tetramethylrhodamine (CMTMR) (Invitrogen). CMTMR-labeled T cells (1 × 10^7) were cultured with 5 × 10^4 CD11c+ DCs isolated from the spleen and the mesenteric lymph node (MLN) in the presence of soluble anti-CD3 Ab (2 μg/ml), anti-CD28 Ab (2 μg/ml), and recombinant IL-2 (250 U/ml) for 24 h. Proliferation of CD4+ Foxp3+ cells was examined by dilution of CMTMR intensity. For in vivo analysis of CD4+ Foxp3+ T cell proliferation, Foxp3-EGFP mice were given BrdU (0.8 mg/ml; Nakalai Tesque) in their drinking water for 5 or 8 d. Incorporation of BrdU by colonic Foxp3+ T cells was analyzed by flow cytometry using a BrdU Flow Kit (BD Biosciences).

**Neutralizing Abs experiments**

Anti-IL-2 and anti-FR4 Abs were prepared as previously described (21, 24). Fab fragments of anti-FR4 Abs were prepared with the Fab Preparation Kit (Pierce) according to the manufacturer’s instructions. Undigested Ab was subsequently removed with HiTrap Protein G columns (GE Healthcare). Anti-IL-2 Abs (0.48 mg) or anti-FR4 Fab fragment (37 μg) were intravenously injected into each recipient.

**MTX experiment**

For the MTX experiment, MTX (0.16 mg; Sigma) was given to the recipient mice every day using oral catheters.

**RT-PCR**

Samples were pooled from each of four mice receiving the AIN93G diet or the diet free of folic acid (FF-diet), respectively. Total RNA was isolated using Isogen (Nippon Gene), and the collected RNA was reverse-transcribed using a SuperScript II cDNA synthesis kit (Invitrogen). Real-time RT-PCR was performed on an ABI 7300 system (Applied Biosystems) using the GoTaq qPCR Master Mix. All values were normalized to the expression of Gapdh encoding glyceraldehyde-3-phosphate dehydrogenase, and the fold difference in expression relative to that for Gapdh is shown. Amplification conditions were 50°C (2 min), 95°C (10 min), and 40 cycles of 95°C (15 s) and 60°C (60 s). Primer sets for Gapdh used were as previously described (23). Sequences for Bcl-2 and Bcl-xL are as follows: Bcl-2, 5'-CACATCCAAATAAGAGC-3' and 5'-ACCCCATCTGAAAGAGTT-3'; Bcl-xL, 5'-AGCCAGCGATGTTGAACAC-3' and 5'-GAACCACCAAGCCCAAGTCA-3'.

**FIGURE 2.** Deficiency of dietary FA has little effect on inflammatory T cell subsets. (A–C) IL-17– and IFN-γ–producing CD4+ T cells in various organs of the AIN93G diet and FF-diet mice were analyzed 6 wk after birth. (A) Representative FACS dot plots showing expression of IL-17 and IFN-γ gated on CD4+ T cells. (B and C) Percentages of IFN-γ– or IL-17–producing CD4+ T cells in the spleen, the MLN, and the colon are shown. Data are from two independent experiments. *p < 0.05, **p < 0.01. AIN93G, AIN93G diet; FF, FF-diet.
at 20 mg/kg via the catheter into the lumen using a 1-ml syringe. Animals were monitored daily for loss of body weight and survival. For the adoptive transfer experiment, CD4+ T cells were purified using CD4 MicroBeads (Miltenyi Biotec), and the FR4+ population was subsequently sorted by a FACSARia system eliminating FR4int CD25+ cells representing central memory T cells (21). Sorted cells (∼5 × 10^5) were intravenously injected into each recipient mouse.

**Histological analysis**

Colon samples embedded in Tissue-Tek OCT compound were sectioned and stained with H&E. Severity of colitis was evaluated by the standard scoring system as previously described (26). Colonos were graded semiquantitatively from 0 (no change) to 5 (most severe change). The grading represents an increasing incidence and degree of inflammation, goblet cell loss, ulceration and fibrosis in the lamina propria. The scoring was performed in a blinded manner. Images of H&E staining were taken using Biozero (Keyence).

**Statistical analysis**

The Student t test or one-way ANOVA and Bonferroni’s multiple comparison tests were used to determine the significance of experiments, and p values <0.05 were considered significant.

**Results**

**Dietary FA deficiency causes decrease of Foxp3+ Tregs in the colon**

Among the various lineages of CD4+ T cells, Foxp3+ Tregs preferentially express FR4, a specific folate receptor, on the surface (21). To analyze whether dietary FA affects the homeostasis of Tregs, mice were given a diet supplemented with FA (AIN93G) or a diet strictly free of FA (FF-diet). Maternal milk is known to contain substantial amounts of FA (27); therefore, the FF-diet was started from day 14 during the gestation period, and litters were analyzed 6 wk after birth. Mice given the FF-diet showed a profound decrease in FA luminal concentrations at 4 wk after birth in the colon but not in the small intestine (Fig. 1A). Foxp3+ Tregs were markedly decreased in the colonic lamina propria in the FF-diet mice (Fig. 1B, 1C, Supplemental Fig. 1A). The specific impairment of colonic Tregs was in contrast to a sustained Treg population in other tissues, such as the spleen, MLN, and even the small intestine (Fig. 1C). The indispensable role of dietary FA for colonic Tregs was also confirmed by an observation that supplementing FA in the drinking water of the FF-diet mice increased colonic Tregs (Fig. 1D). Because we previously reported specific expression of FR4 on Foxp3+ Tregs, we examined whether T cells expressing FR4 are predominantly affected by a deficiency in dietary FA. The selective decrease of FR4+ cells in the colon was observed in the FF-diet group (Fig. 1E, 1F). Thus, dietary FA was required for the maintenance of colonic Tregs.

We next examined whether FA deficiency also affects development of other T cell subsets. Although there was a slight increase in the IFN-γ-producing T cell population in the spleen and MLN or a decrease in the colon (Fig. 2A, 2B), Th17 cells were not influenced in any tissues examined (Fig. 2A, 2C). Together, these observations suggest that FA is exclusively required for the maintenance of Foxp3+ Tregs in the colon.

In the gut immune system, distinct Treg subsets mutually collaborate to prevent an excess inflammatory response against foreign Ags (28). Besides Foxp3+ Tregs, there is Foxp3− IL-10−producing Tr1 cells (29). To discriminate between the effects of dietary folate deficiency on each Treg subset, colonic CD4+ T cells of the FF-diet mice were analyzed for expression of Foxp3 and IL-10. In the FF-diet mice, Foxp3+ T cells were profoundly decreased irrespective of the IL-10 expression patterns (Fig. 3A, 3B). IL-10− Foxp3+ Tregs among the CD4+ T cells decreased to nearly 11% in the FF-diet mice, in contrast to ∼20% in the control mice. IL-10− Foxp3+ Tregs in the FF-diet mice were also reduced to nearly 1%, whereas those in the control group showed ∼5% (Fig. 3A, 3B). Only a marginal decrease in the population of IL-10+ Foxp3+ cells was observed (Fig. 3A, 3B). These findings indicate that Tregs expressing Foxp3 are susceptible to dietary FA deficiency.

**Folic metabolic pathway maintains Foxp3+ Tregs in the colon**

Blocking of FR4 by Fab fragment of neutralizing Abs (NAbs) previously was shown to decrease the number of peripheral CD25+ Tregs (21). The effect of FR4 blockade was most remarkable in the colon compared with the spleen and MLN (Fig. 4A, 4B, Supplemental Fig. 1B). Colonic Foxp3+ Tregs among the CD4+ T cells in the treated group decreased to nearly half of the control group. Thus, FR4 is functionally involved in the maintenance of Foxp3+ Tregs residing in the colon.

MTX, the most notable anti-folate, is a widely used chemotherapeutic drug for its potent capacity to induce cell death and inhibit cell proliferation (22). Once MTX is transported into the cytosol, it inhibits various folate enzymes, such as dihydrofolate reductase, thereby interfering with folic metabolic pathways (22). As FA was shown to have a critical role in the maintenance of colonic Foxp3+ Tregs, we examined whether folic metabolic pathways are specifically required for the maintenance of Tregs residing in the colon. Mice given oral administration of MTX for 5 d showed a remarkable decrease of Foxp3+ Tregs in the colon but not in the spleen or MLN (Fig. 4C, 4D, Supplemental Fig. 2A). MTX had little effect on IFN-γ− or IL-17−producing T cells (Fig. 4C, 4E). No alteration was observed in αβ2 or γδ1 intraepithelial lymphocytes, CD11c+ CD11b− or CD11c+ CD11b+ DCs,
CD11c+ CD11b+ macrophages (Supplemental Fig. 3). Colonic epithelial cells were also unaffected by the MTX treatment (Supplemental Fig. 2B). Thus, Foxp3+ Tregs in the colon possess a unique feature of requiring folic metabolic pathways.

FA promotes survival of colonic Tregs by inhibiting apoptosis

To examine the precise mechanism by which FA maintains Tregs in the colon, FR4+ and FR42 T cells were isolated from the spleen and the colon as Treg and non-Treg populations, respectively. These cells were then cultured in either FA-sufficient or FA-null conditions. When equal numbers of FR4+ T cells were cultured with TCR stimulation for 3 d, the viability of colonic FR4+ T cells in the FA-null condition dramatically decreased, whereas splenic FR4+ T cells showed only a marginal reduction (Fig. 5A). Moreover, when colonic FR4+ and FR42 T cells were cultured separately with TCR stimulation for 2 d, FR4+ T cells were more prone to die compared with FR42 T cells (Fig. 5B). In addition, a dose-dependent promotion of colonic FR4+ T cell survival by FA was observed (Fig. 5C). In accordance with these observations, colonic FR4+ T cells under the FA-null condition showed the highest frequency of annexin V staining, indicating that colonic FR4+ T cells are highly sensitive to apoptosis in the absence of FA (Fig. 5D, 5E).

To examine further how FA provides protective activity against apoptosis, expression of anti-apoptotic molecules was examined. When T cells isolated from the spleen and the colon of the FF-diet mice were compared, FR4+ T cells in the colon expressed a remarkably reduced level of Bcl-2 compared with those in the spleen (Fig. 5F). Furthermore, FR4+ T cells isolated from the colon of the FF-diet mice showed a severely lower expression level of Bcl-2 and Bcl-xL than AIN93G mice (Fig. 5G). Thus, colonic Tregs in the absence of FA are highly sensitive to apoptosis with a reduced expression of Bcl-2 and Bcl-xL.

FA is dispensable in the proliferation of colonic Tregs

Apart from promoting cell survival, FA also is known to be important for cell proliferation (19). To analyze whether FA also...
provides the proliferative capacity of colonic Foxp3+ Tregs, AIN93G or FF-diet Foxp3-GFP mice were given BrdU in their drinking water and subsequently examined for BrdU incorporation by flow cytometry. (B) FR4+ or FR4− colonic CD4+ T cells were cultured for 2 d under the indicated conditions, and live cells were counted. (C) Colonic FR4+ T cells were cultured under different FA concentrations as indicated for 2 d, and live cells were counted. (D and E) Splenic or colonic FR4+ CD4+ T cells isolated from the normal diet mice were cultured under either FA-sufficient (control) or FA-null conditions for 2 d. Percentages of annexin V+ cells among live cells gated on forward scatter and side scatter were analyzed. (F) Real-time quantitative PCR analysis of mRNA expression of Bcl-2 normalized to that of Gapdh in the FF-diet mice. (G) Colonic FR4+ T cells isolated either from the AIN93G diet (AIN93G) or FF-diet (FF) mice were analyzed for Bcl-2 and Bcl-3L expression levels normalized to that of Gapdh. Mean values ± SD of duplicate or triplicate determinations. All data are of two independent experiments. *p < 0.05, **p < 0.01.

Colonic Tregs exhibit constitutively activated phenotype

We previously reported that Ag stimulation increases the Bcl-2low population that highly express CTLA-4 among FR4+ Tregs (21). Because the gut environment is constitutively exposed to foreign Ags derived from the diet and commensal microbes, we next examined whether colonic Tregs are in a highly activated state compared with splenic Tregs. Splenic Foxp3+ Tregs expressed CD62L. However, CD62L expression was severely decreased in colonic Foxp3+ Tregs. Furthermore, colonic Foxp3+ Tregs showed increased expression of Treg activation markers such as CTLA-4.

FIGURE 5. FA promotes survival of colonic FR4+ T cells by inhibiting apoptosis. (A) Splenic or colonic FR4+ CD4+ T cells isolated from the normal diet mice were cultured under either FA-sufficient (control) or FA-null conditions. Isolated T cells were cultured for 3 d. The numbers of live cells gated on forward scatter and side scatter were analyzed by flow cytometry. (B) FR4+ or FR4− colonic CD4+ T cells were cultured for 2 d under the indicated conditions, and live cells were counted. (C) Colonic FR4+ T cells were cultured under different FA concentrations as indicated for 2 d, and live cells were counted. (D and E) Splenic or colonic FR4+ CD4+ T cells isolated from the normal diet mice were cultured under either FA-sufficient (control) or FA-null conditions for 2 d. Percentages of annexin V+ cells among live cells gated on forward scatter and side scatter were analyzed. (F) Real-time quantitative PCR analysis of mRNA expression of Bcl-2 normalized to that of Gapdh in the FF-diet mice. (G) Colonic FR4+ T cells isolated either from the AIN93G diet (AIN93G) or FF-diet (FF) mice were analyzed for Bcl-2 and Bcl-3L expression levels normalized to that of Gapdh. Mean values ± SD of duplicate or triplicate determinations. All data are of two independent experiments. *p < 0.05, **p < 0.01.
and GITR compared with splenic Tregs (Fig. 7A, 7C). In addition, a markedly higher percentage of Foxp3+ Tregs was positive for Ki67, a common marker of proliferation, in the colon (Fig. 7B, 7D). These results demonstrate the distinct feature of colonic Foxp3+ Tregs, which are constitutively in an activated state in comparison with splenic Tregs.

**FIGURE 6.** FA is dispensable for the proliferation of colonic Tregs. (A and B) AIN93G diet or FF-diet Foxp3-GFP mice were given BrdU in their drinking water for 5 or 8 d. BrdU incorporation by colonic CD4+ T cells was analyzed by FACS. (A) Representative FACS dot plots of the indicated mice. (B) Percentages of BrdU+ cells among Foxp3+ CD4+ T cells are shown. (C) CMTMR-labeled Foxp3+ T cells were cultured with CD11c+ DCs. Proliferation of CD4+ Foxp3+ T cells was examined by dilution of CMTMR intensity. Data are of three independent experiments. AIN93G, AIN93G diet; FF, FF-diet.

**FIGURE 7.** Colonic Tregs exhibit constitutively activated phenotype. (A) Representative histograms of the indicated surface markers on splenic or colonic Foxp3+ Tregs isolated from mice fed with the normal diet are shown. (B) Representative dot plots of Ki67 staining in splenic or colonic Foxp3+ Tregs isolated from mice fed with the normal diet. (C) The mean fluorescent intensities (MFIs) of the indicated surface markers on splenic or colonic Foxp3+ Tregs are shown. (D) Ki67 positivity among splenic or colonic Foxp3+ Tregs is shown. Data are of two independent experiments. *p < 0.05, **p < 0.01.
Distinct and cooperative regulation of Tregs is mediated by IL-2 and FA

IL-2 is known to play critical roles in promoting Treg survival and proliferation (30–32). Indeed, NAbs against IL-2 were shown to decrease CD25+ Tregs in the spleen and peripheral lymph nodes (24). In accordance with the previous reports, anti–IL-2 NAbs decreased Foxp3+ Tregs in the spleen, MLN, and the colon of AIN93G mice (Fig. 8A, 8B, Supplemental Fig. 4). In contrast, further reduction of Foxp3+ Tregs by dietary FA deficiency was observed only in the colon (Fig. 8A, 8B, Supplemental Fig. 4). Together, these findings indicate distinct and cooperative regulation of Tregs by IL-2 and FA.

FA prevents TNBS-induced colitis by maintaining Tregs

Dysregulation of Tregs is known to accelerate various inflammatory responses, including intestinal inflammation (33). Thus, we examined whether FA deficiency in the diet would augment inflammatory responses in a TNBS-induced colitis model. When the AIN93G or FF-diet mice were exposed to TNBS, the FF-diet mice showed a substantially higher rate of mortality and a decrease in the body weight (Fig. 9A, 9B). Histological analysis confirmed the pronounced inflammation in the colon of the FF-diet mice (Fig. 9C, 9D). To examine further whether the exacerbation of colitis in the FF-diet mice was mediated by a reduced number of Tregs, FR4+ T cells were transferred into the FF-diet mice prior to the colitis induction. The transfer of FR4+ T cells improved the survival rate (Fig. 9E), suggesting that dietary FA is a critical nutrient to maintain colonic Tregs, thereby preventing excessive inflammation in the colon.

Discussion

In this study, we demonstrated that dietary FA is indispensable for physiological homeostasis of the gut immune system. Although nutritional status in the gut critically influences the profile of commensal microbes (6), it is unlikely that alteration of the gut flora by FA deficiency affected the Tregs in our model. This speculation is supported by our observations in two different settings: both MTX administration and the blocking of FR4 with Fab NAbs diminished colonic Tregs in mice that harbor unaltered gut flora. Although the decrease in the number of splenic CD4+ T cells in FF-diet mice indicates the reduction of total number of Tregs in the spleen, the systemic effect on Tregs, most of the FF-diet mice showed a slight decrease in the size of the spleen for some reason. Therefore, the reduction of the splenic Treg number would simply reflect the difference in the size of this organ rather than the specific effect of FA on systemic Tregs. Taking this fact into consideration, the increase in the frequency of the IFN-γ-producing T cell population in the spleen and MLN might actually indicate that FA deficiency causes the systemic activation of the inflammatory T cell subset. It would be interesting to elucidate how FA deficiency leads to the activation of IFN-γ-producing T cells exclusively in the spleen and MLN.

Colonic mucosal surfaces outnumber other immune compartments in the amount of commensal microbes. Thus, well-balanced immune regulation by Tregs is especially required to counteract numerous foreign Ags (28, 33). In accordance with previous reports (34, 35), colonic Tregs showed more highly activated features than splenic Tregs. Colonic Tregs showed low expression of CD62L and elevated expression patterns of CTLA-4 and GITR. Ki67 staining also revealed that colonic Tregs are more vigorously proliferating in contrast to splenic Tregs. Thus, colonic Tregs might particularly require FA in large amounts because of a highly activated metabolic state. The high requirement of folic metabolism by colonic Tregs was confirmed by using MTX in our study. Although the common usage of MTX as a therapeutic drug for patients with inflammatory bowel diseases might apparently contradict with our current observations (22), excess activation of inflammatory T cell subsets is the hallmark of these diseases. This is in great contrast to the quiescent intestinal state where only a small fraction of T cells presents inflammatory characteristics as

**FIGURE 8.** Cooperative regulation of Tregs by IL-2 and FA is observed in the colon. (A and B) Six-week-old AIN93G diet or FF-diet mice were treated with i.v. injections of saline or anti–IL-2 NAbs. One week after the treatment with anti–IL-2 NAbs, Foxp3+ CD4+ T cells in the spleen, MLN, and the colon were analyzed by FACS. (A) Representative histograms of colonic Foxp3+ CD4+ T cells of the indicated mice are shown. (B) Percentages of Foxp3+ CD4+ T cells are shown. Data are of two independent experiments. *p < 0.05, **p < 0.01. AIN93G, AIN93G diet; FF, FF-diet.
observed in our experiments. Folate metabolism is most notable among cells under activation (36). Thus, in the quiescent gut environment, MTX might preferentially reduce colonic Tregs, which show a highly activated phenotype.

With regard to the precise mechanism by which FA maintains colonic Tregs, pivotal roles of FA for inhibiting Treg apoptosis were suggested. Even under FA-sufficient conditions, colonic Tregs more readily underwent apoptotic processes after TCR stimulation in contrast to splenic Tregs. This apoptosis-prone feature of colonic Tregs became even clearer under FA-null conditions. This might be partly explained by the low expression levels of Bcl-2 and Bcl-xL, the major anti-apoptotic factors, in colonic Tregs. We previously showed that Bcl-2low cells increase after Ag stimulation among FR4+ Tregs (21). Given that colonic Tregs are constitutively exposed to a large amount of luminal Ags in contrast to splenic Tregs, the apoptosis-prone feature of colonic Tregs might reflect their highly activated phenotype after continuous stimulation by various Ags. The essential role of FA in promoting colonic Treg survival, as shown in our study, complies with reports showing that Tregs in the inflammatory milieu require a high amount of survival signaling to maintain Bcl-2 expression (37, 38). It would be interesting to investigate in future studies how expression of anti-apoptotic molecules is exactly regulated by FA.

Blockade of FR4 with NAbs was shown previously to decrease peripheral Tregs (21). However, the physiological role of FR4 in the context of folic metabolism has not been elucidated. FR4 is a member of the folate receptor family that more efficiently incorporates FA than other folate transporters (39). The striking decrease of colonic Foxp3+ T cells, which also are positive for FR4, in mice treated with anti-FR4 NAbs suggests that FR4 might serve as functional machinery to provide a sufficient amount of FA to Tregs in a highly activated state.

IL-2 is a well-characterized factor to promote peripheral Treg survival (40), and mice deficient in IL-2 spontaneously develop colitis (41). Contrary to the multifunctional roles of IL-2, such as Treg proliferation, survival, and differentiation (30–32, 40), FA is particularly indispensable for inhibiting apoptosis of colonic Tregs. Accordingly, the cooperative effect of FA and IL-2 was observed exclusively in the colon where Tregs are highly susceptible to cell death. In contrast, IL-2 was shown to be generally essential for Treg maintenance in the periphery. Therefore, IL-2 might serve as a fundamental factor for Treg survival even in a quiescent state, and FA provides additional anti-apoptotic signals only in the presence of excess inflammation.

Finally, the indispensable role of FA in preventing excess inflammation was shown in the TNBS-colitis model. Dysregulation
of Tregs is known to augment various inflammatory responses, including intestinal inflammation (33, 42). Therefore, FA was speculated to prevent colitis by maintaining Tregs. It is possible that FA deficiency might have caused fragility of intestinal epithelial cells, thereby augmenting immune responses against luminal Ags. However, the improvement in the survival rate by transfer of FR4+ Tregs in the FF-diet mice highlights the pivotal role of FA on Treg maintenance under intestinal inflammation.

In summary, our study demonstrated the previously unappreciated role of dietary FA as a critical nutrient that maintains Tregs in the colon. Our observation will have particularly important implications in manipulation of intestinal inflammation, represented by inflammatory bowel disease. Improvement of malnutrition with dietary FA might provide a novel therapeutic strategy for those who suffer from an excess of inflammation in the gut.

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References


