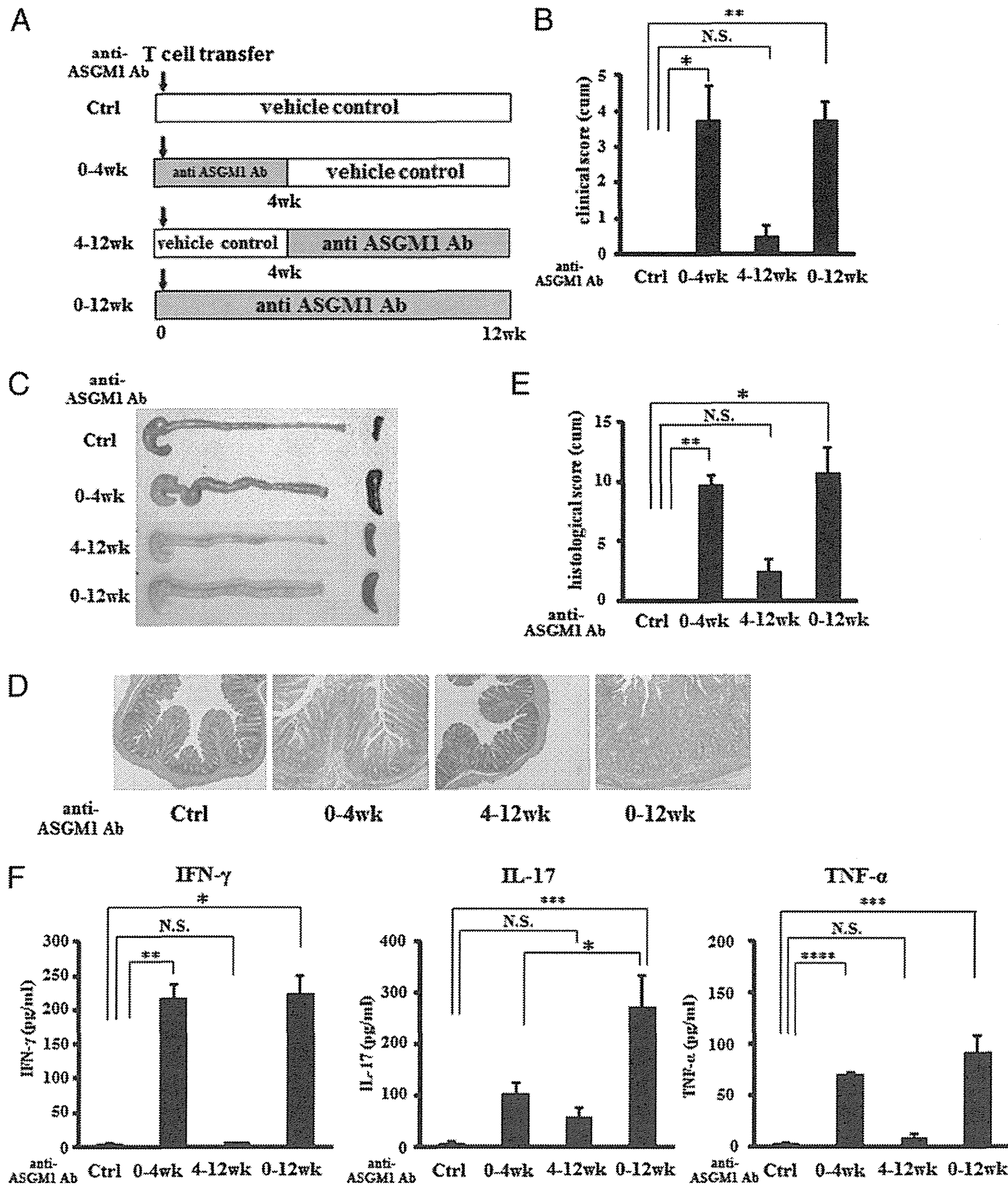

FIGURE 9. NK cell depletion at the early stage, but not at a late stage, in naive T cell-receiving IL-$7^{-/-}$RAG$^{-/-}$ mice results in the elicitation of massive colitis. (**A**) Protocol for NK cell depletion in a setting of chronic colitis. IL-$7^{-/-}$RAG$^{-/-}$ mice were injected with either vehicle control (Ctrl) or anti-ASGM1 Ab (0–12 wk) for 12 wk, anti-ASGM1 Ab for 4 wk followed by vehicle control for 8 wk (0–4 wk), or vehicle control for 4 wk followed by anti-ASGM1 Ab for 8 wk (4–12 wk). (**B**) Clinical scores of each group are shown. Data are expressed as means ± SEM from four mice. *$p < 0.05$, **$p < 0.005$. (**C**) Gross appearance of colons (*left*) and SP (*right*) from naive T cell-transferred IL-$7^{-/-}$RAG$^{-/-}$ recipients injected with either vehicle control for 12 wk (Ctrl), anti-ASGM1 for 4 wk and then vehicle control for 8 wk (0–4 wk), vehicle control for 4 wk and then anti-ASGM1 Ab for 8 wk (4–12 wk), or anti-ASGM1 Ab for 12 wk (0–12 wk). Representative features from four experiments are shown. (**D**) Histological feature of colons from naive T cell-transferred IL-$7^{-/-}$ RAG$^{-/-}$ recipients injected with either control for 12 wk (Ctrl), anti-ASGM1 for 4 wk and then control for 8 wk (0–4 wk), vehicle control for 4 wk and then anti-ASGM1 Ab for 8 wk (4–12 wk), or anti-ASGM1 for 12 wk (0–12 wk). Representative features of each group are shown. (**E**) Histological scores of each group are shown. Data are expressed as means ± SEM from four mice. *$p < 0.05$, **$p < 0.01$. (**F**) Cytokine production by LP T cells from each group is shown. Concentrations of IFN-γ (*left*), TNF (*middle*), and IL-17 (*right*) in the culture supernatant were measured by ELISA. Data are indicated as means ± SEM from four samples. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$.

infiltration in colonic tissues ~4 wk after the adoptive transfer into RAG$^{-/-}$ recipients (10). We therefore compared the effect of NK cell depletion by treatment with an anti-ASGM1 Ab at early (0–4 wk) or late stages (4–12 wk) after naive T cell transfer to treatment over the entire 12-wk period (0–12 wk) after transfer. Ab treatment at the early stage and over the entire 12 wk resulted in a similar degree of colitis exacerbation whereas Ab treatment at the late stage did not exacerbate colitis (Figs. 1, 9). Such exacerbation of colitis occurred relatively latent in the presence of IL-7 in the RAG$^{-/-}$ compared with the IL-$7^{-/-}$RAG$^{-/-}$ recipients when sacrificed at 12 wk after T cell transfer (Figs. 6, 7). However, the difference of colitis severity in the RAG$^{-/-}$ recipients with or without Ab treatment was interestingly remarkable when sacrificed at 6 wk after T cell receiving (Fig. 2). These results imply that NK cell function is critical for colitogenic T cell suppression at the early stage of colitis development.

Because the $CD4^+CD44^+CD62L^-$ colitogenic T_{EM} in the recipients were suggested to be suppressed at the early stage by NK cells (Figs. 1, 2, 9), we further analyzed the effect of NK cells on the development of $CD4^+$ T cells within a week after recon-

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stitution into the $RAG^{-/-}$ recipients (Fig. 3). The number of $CD4^+$ T cells in SPL and MLN was significantly increased 5–7 d after the transfer when NK cells were depleted compared with the control (Fig. 3A, 3B). Additionally, the significant increase of the $CD44^+CD62L^-$ T_{EM} subset was observed at this point when NK cells were depleted. $CD4^+CD44^+CD62L^-$ colitogenic T_{EM} are suggested to be susceptible to cell death when they are activated. We therefore analyzed the expression of several markers characteristic of NK cell targets on the $CD44^+CD62L^-$ T_{EM} subset, such as Fas, DR5, and Qa-1, which are the specific receptors or ligand for Fas ligand, TRAIL, and NKG2A, respectively (Fig. 4). As expected, this T cell subset expresses high levels of Fas and DR5, thereby making them susceptible to apoptosis (20). Additionally, these T cells also express some but not a significant level of Qa-1, which induces inhibitory signaling in NK cells via NKG2A. These data indicate that NK cells may suppress $CD4^+CD44^+CD62L^-$ colitogenic T_{EM} via apoptosis, and consistent with our previous observation of downregulated Bcl-2 and upregulated annexin V in $CD4^+$ T cells by the lack of IL-7 in vivo (10).

Furthermore, we also observed an increased unique T cell subset, $CD44^-CD62L^-$, when NK cells were depleted (Fig. 3C–E). We were able to observe these cells in the SPL and MLN within 2 wk after T cell transfer into RAG mice, and subsequently they were not detectable afterward (Fig. 7B, 7C). The fact that the $CD44^-$ $CD62L^-$ T cell subset was only observed at the beginning of colitogenic T cell development would suggest that this interesting population may be associated with the importance of early stage at the pathogenic T cell development in this chronic colitis model. This T cell subset, which is distinct from $CD44^+CD62L^-$ T_{EM}, is likely to be a second target of NK cells. However, the expressions of Fas and DR5 are lower on these cells compared with those of the $CD44^+CD62L^-$ T_{EM} (Fig. 4). The expression of Qa-1 in $CD44^-CD62L^-$ is not greatly different from that of the $CD44^+$ $CD62L^-$ subset. This phenotype of the $CD44^-CD62L^-$ subset does not suggest that it is a target of NK cells. However, a recent report showed that CD44 expression on Th1 cells is required to prevent apoptosis via Fas signaling (35). Thus, the $CD44^-$ $CD62L^-$ subset may be susceptible to apoptosis, since these cells still express some level of Fas on their surface. This may be one of the reasons why early stage of T cell development in this colitis model is targeted by NK cells. Additionally, this possibility may be a potential reason why Th1 cells fail to survive when transferred into $IL\text{-}7^{-/-}RAG^{-/-}$ mice. It is also possible that NK cells may regulate $CD44^+CD62L^-$ and $CD44^-CD62L^-$ cells by different mechanisms. Analysis of IL-7R expression levels of the $CD44^-CD62L^-$ subset revealed two distinct populations: $IL\text{-}7R^{hi}$ and $IL\text{-}7R^{lo}$ (indicated with an arrow in Fig. 4). The $IL\text{-}7R^{lo}$ population in this subset could potentially arise due to transient downregulation of IL-7R expression during differentiation. Unfortunately, the scarcity of these cells prohibited their further analysis and characterization. However, these cells still need to be further studied.

Our recent studies suggested that $IL\text{-}7^{-/-}RAG^{-/-}$ mice were able to induce colitis when parabiosed with colitic $RAG^{-/-}$ recipient mice that had received naive T cells 6 wk previously (15). Moreover, deparabiosed $IL\text{-}7^{-/-}RAG^{-/-}$ mice, which were surgically separated from T cell-receiving $RAG^{-/-}$–$IL\text{-}7^{-/-}RAG^{-/-}$ parabionts 6 wk after the initial surgery, still maintained chronic colitis for at least another 12 wk (16). The latter finding is similar to our present observation that $IL\text{-}7^{-/-}RAG^{-/-}$ recipient mice, which had been depleted of NK cells at an early stage during induction, showed elicited colitis, even after completion of the anti-ASGM1 Ab treatment (Fig. 9). However, the mechanism by which the colitogenic T cells are maintained in the $IL\text{-}7^{-/-}RAG^{-/-}$ mice after the establishment of massive colitis is still unclear. One potential interpretation is that the pathogenic T cells can continue to proliferate, resulting in induction of colitis when the T cell number exceeds the capacity of the NK cells to suppress the T cells. A second possibility is based on the recent report that IL-17 inhibits NK cell-suppressive ability (36). It has been suggested that the increased IL-17 production from T cells that occurs when the severity of the colitis increases may affect NK cell function. The latter possibility is supported by one of our observations that NK cell depletion starting at the late stage of colitis development failed to exacerbate colitis (Supplemental Fig. 1).

We observed that the characteristics of NK cells are not modified by the lack of IL-7 in $RAG^{-/-}$ mice (Fig. 5F). This observation is consistent with a previous report by Vosshenrich et al. (37) showing that the lack of IL-7 does not affect the growth, phenotype, or effector functions of NK cells in vivo, although IL-7 had been reported to influence NK cell differentiation. Consistent with this, we also observed that the differentiation of NK cells, which is characterized by the expression of CD11b and CD27 (31), is not altered in the same mice (Fig. 5G). Additionally, there is no significant difference between NK cells derived from $RAG^{-/-}$ and $IL\text{-}7^{-/-}RAG^{-/-}$ mice in terms of their cytotoxic activities against the target cells such as T cells and YAC-1 cells (Fig. 5D, 5E, 5H) as well as the production of IFN-γ (Fig. 5I). These data indicate that the dramatic difference in the severity of colitis between $IL\text{-}7^{-/-}RAG^{-/-}$ and $RAG^{-/-}$ recipients following NK depletion is not caused by a difference in NK function between NK cells derived from $RAG^{-/-}$ and $IL\text{-}7^{-/-}RAG^{-/-}$ mice.

The $IL\text{-}7^{-/-}RAG^{-/-}$ recipient mice that received naive T cells failed to induce colitis even though the cytotoxicity of NK cells was not altered. One potential explanation of this result is that the susceptibility of T cells to apoptosis is increased in these mice. It has been reported by others that the expression of Bcl-2, an antiapoptotic molecule, in T cells is downregulated in $IL\text{-}7^{-/-}$ mice (38, 39). We have also reported that Bcl-2 expression is downregulated in T cells injected into $IL\text{-}7^{-/-}RAG^{-/-}$ recipient mice (10). A second explanation is based on our previous report that IL-7 contributes to the expansion of colitogenic T cells (39). Thus, these data suggest that colitogenic T cells are not able to survive in the mice due to their reduced expansion and increased susceptibility to apoptosis at the early stage of colitis development.

In this study, we demonstrate NK cell-mediated regulation of T cell development, which is associated with the pathogenesis of chronic colitis. Although the detailed mechanism still remains to be elucidated, an insight into such a mechanism is significant for understanding the regulation of mucosal immune responses.

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Disclosures

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

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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 peripheral Tregs was induced by a neutralizing Ab against IL-2, but a further decrease by additional FA deficiency was observed exclusively in the colon. Mice fed with an FA-deficient diet exhibited higher susceptibility to intestinal inflammation. These findings reveal the previously unappreciated role of dietary FA in promotion of survival of Foxp3⁺ Tregs that are in a highly activated state in the colon. *The Journal of Immunology*, **2012, 189: 2869–2878.**

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 anemia, 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.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CMTMR, 5-(and-6)-{[(4-chloromethyl)benzoyl] amino}tetramethylrhodamine; DC, dendritic cell; FA, folic acid; FF-diet, diet free of folic acid; FR4, folate receptor 4; MLN, mesenteric lymph node; MTX, methotrexate; NAb, neutralizing Ab; TNBS, 2,4,6-trinitrobenzene sulfonic acid; Treg, regulatory T cell.

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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-

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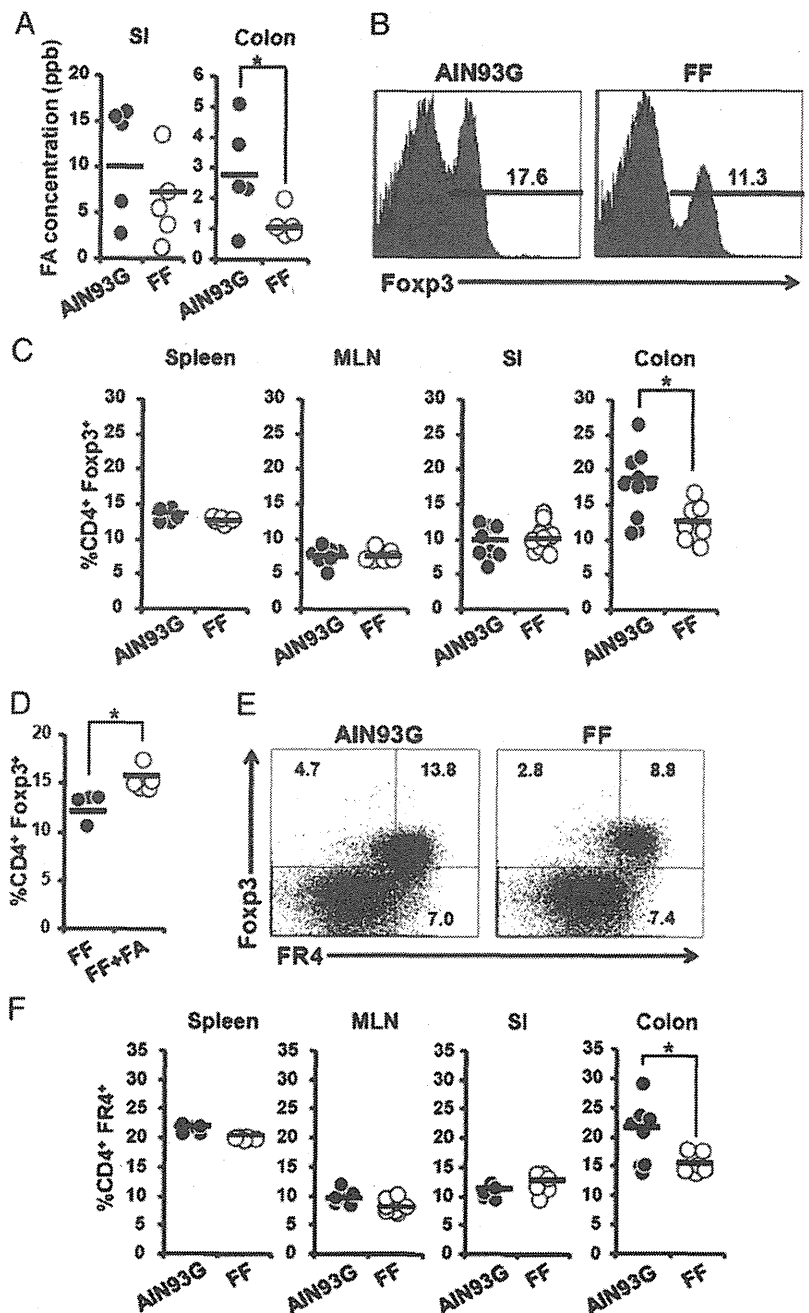

FIGURE 1. Dietary FA deficiency decreases Foxp3$^+$ Tregs in the colon. (**A–F**) Mice were given either the AIN93G diet or FF-diet from day 14 during the gestation period, and their litters were analyzed 4 or 6 wk after birth. (A) FA concentrations in the small intestine and the colon of the indicated mice. (B) Representative FACS histograms showing colonic Foxp3$^+$ CD4$^+$ T cells in the indicated mice. (C) Percentages of Foxp3$^+$ CD4$^+$ T cells in various organs are shown. (D) FF-diet mice were given FA-supplemented drinking water (FF + FA) for 4 wk, and mice were subsequently analyzed by FACS. Percentages of colonic Foxp3$^+$ CD4$^+$ T cells are shown. (E) Representative FACS dot plots showing expression of Foxp3 and FR4 in colonic CD4$^+$ T cells of the indicated mice. (F) Percentages of FR4$^+$ CD4$^+$ T cells in various organs are shown. Data are of three independent experiments. *$p < 0.05$. AIN93G, AIN93G diet; FF, FF-diet; SI, small intestine.

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-FR4, 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 γ/δ, 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–IL-17, PE-conjugated anti–IL-10, FITC-conjugated anti-CD11c, PE-conjugated anti-Ki67, FITC-conjugated anti-CD25, FITC-conjugated anti-CD3, and PE-conjugated anti-B220 (purchased from 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, $CD4^+$ $FR4^-$ 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, 5×10^4 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

Splenic $CD4^+$ $Foxp3^+$ T cells isolated from Foxp3-EGFP mice were labeled with 5-(and-6)-{[(4-chloromethyl)benzoyl]amino}tetramethylrhodamine (CMTMR) (Invitrogen). CMTMR-labeled T cells (1×10^5) 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 (Promega). All values were normalized to the expression of *Gapdh* encoding glyceraldhyde-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′-CACATCCAATAAAAGAGC-3′ and 5′-ACCCCATCCTGAAGAGTT-3′; *Bcl-xL*, 5′-AGGCAGGCGATGAGTTTGAAC-3′ and 5′-GAACCACACCAGCCACAGTCA-3′.

2,4,6-Trinitrobenzene sulfonic acid-induced colitis and transfer experiment

Colitis was induced in the AIN93G and FF-diet mice at 8–9 wk of age as previously described (25). In brief, mice were anesthetized, and a 3.5-French catheter was inserted 4 cm into the colon. 2,4,6-Trinitrobenzene sulfonic acid (TNBS; WAKO) dissolved in 30% ethanol was administered

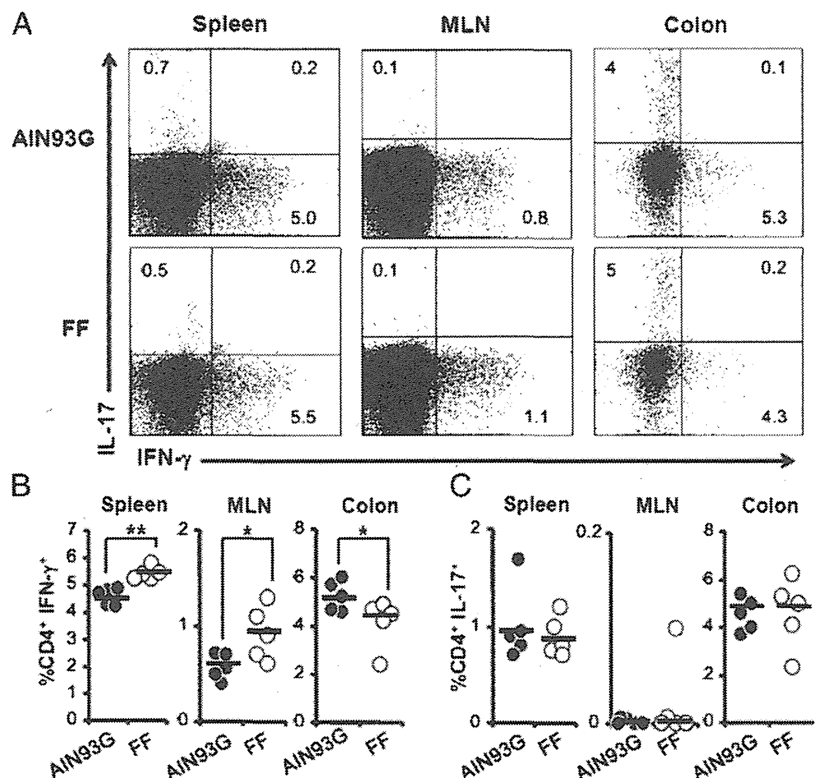

FIGURE 2. Deficiency of dietary FA has little effect on inflammatory T cell subsets. (**A–C**) IL-17– and IFN-γ–producing $CD4^+$ T cells in various organs of the AIN93G diet and FF-diet mice were analyzed 6 wk after birth. (A) Representative FACS dot plots showing expression of IL-17 and IFN-γ gated 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 of two independent experiments. $*p < 0.05$, $**p < 0.01$. AIN93G, AIN93G diet; FF, FF-diet.

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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 FR4^{int} 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). Colons 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 αβ$^+$ or γδ$^+$ intraepithelial lymphocytes, CD11c$^+$ CD11b$^-$ or CD11c$^+$ CD11b$^+$ DCs,

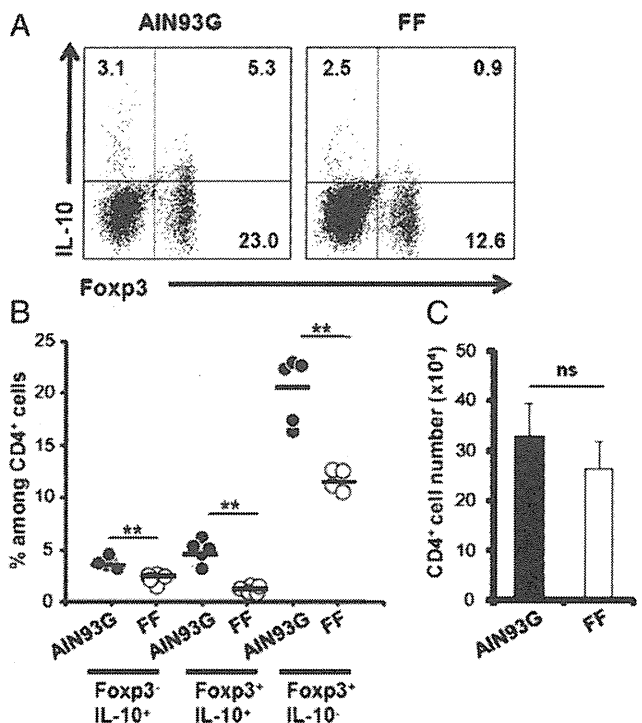

FIGURE 3. FA deficiency decreases Foxp3-expressing Tregs. **(A–C)** Intestinal CD4$^+$ T cells were analyzed by FACS for Foxp3 and IL-10 expression in the AIN93G diet or FF-diet Foxp3-GFP mice 8 wk after birth. (A) Representative FACS dot plots showing the expression of Foxp3 and IL-10 in CD4$^+$ T cells in the indicated mice. (B) Percentages of IL-10$^+$ Foxp3$^-$, IL-10$^+$ Foxp3$^+$, and IL-10$^-$ Foxp3$^+$ among CD4$^+$ T cells in the colon of the indicated mice are shown. (C) Total numbers of colonic CD4$^+$ T cells of the AIN93G diet and FF-diet mice are shown. Data are of two independent experiments. $^{**}p < 0.01$. AIN93G, AIN93G diet; FF, FF-diet.

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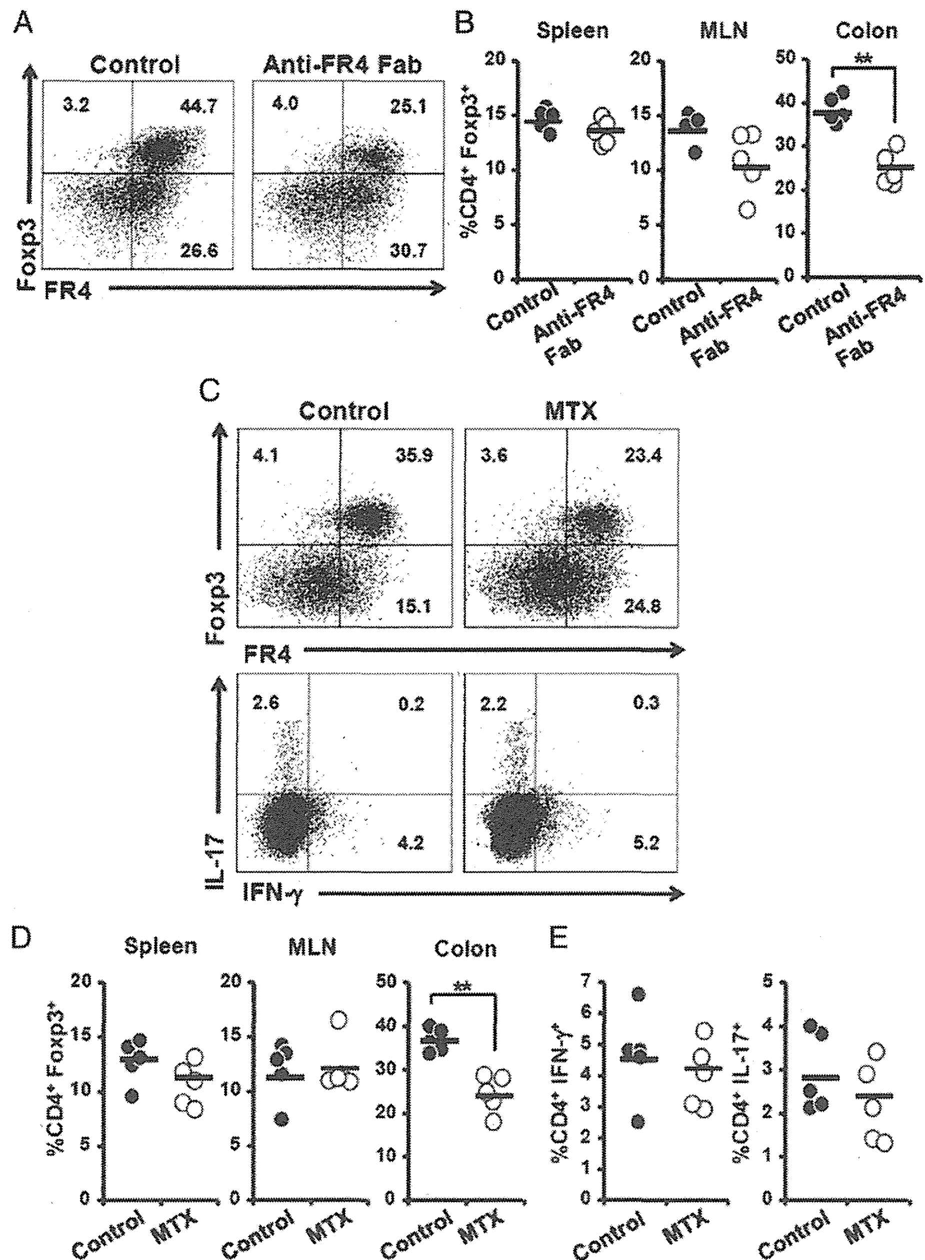

FIGURE 4. Folic metabolic pathway maintains $Foxp3^+$ Tregs in the colon. (**A** and **B**) Six-week-old mice fed with a standard MF diet were given i.v. injections of either PBS (control) or Fab fragment of NAbs against FR4 (Anti-FR4 Fab). Mice were given NAbs once per week and analyzed 2 wk after the start of the experiments. (A) Representative FACS dot plots showing colonic $CD4^+$ T cells gated on Foxp3 and FR4. (B) Percentages of $Foxp3^+$ $CD4^+$ T cells in the spleen, the MLN, and the colon of the indicated mice. (**C–E**) Six-week-old mice fed with a standard MF diet were given oral administration of either PBS or MTX for 5 d. (C) Representative FACS dot plots of colonic $CD4^+$ T cells analyzed for the expression of Foxp3 and FR4 in the indicated mice (*upper panels*). Representative FACS dot plots of IL-17– and IFN-γ–producing $CD4^+$ T cells in the indicated mice (*lower panels*). (D) Percentages of $Foxp3^+$ $CD4^+$ T cells in the spleen, the MLN, and the colon are shown. (E) Percentages of IFN-γ– and IL-17–producing cells in the colonic $CD4^+$ T cells are shown. Data are of two independent experiments. $^{**}p < 0.01$.

and $CD11c^-$ $CD11b^+$ 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 $FR4^-$ 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 $FR4^-$ T cells were cultured separately with TCR stimulation for 2 d, $FR4^+$ T cells were more prone to die compared with $FR4^-$ 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

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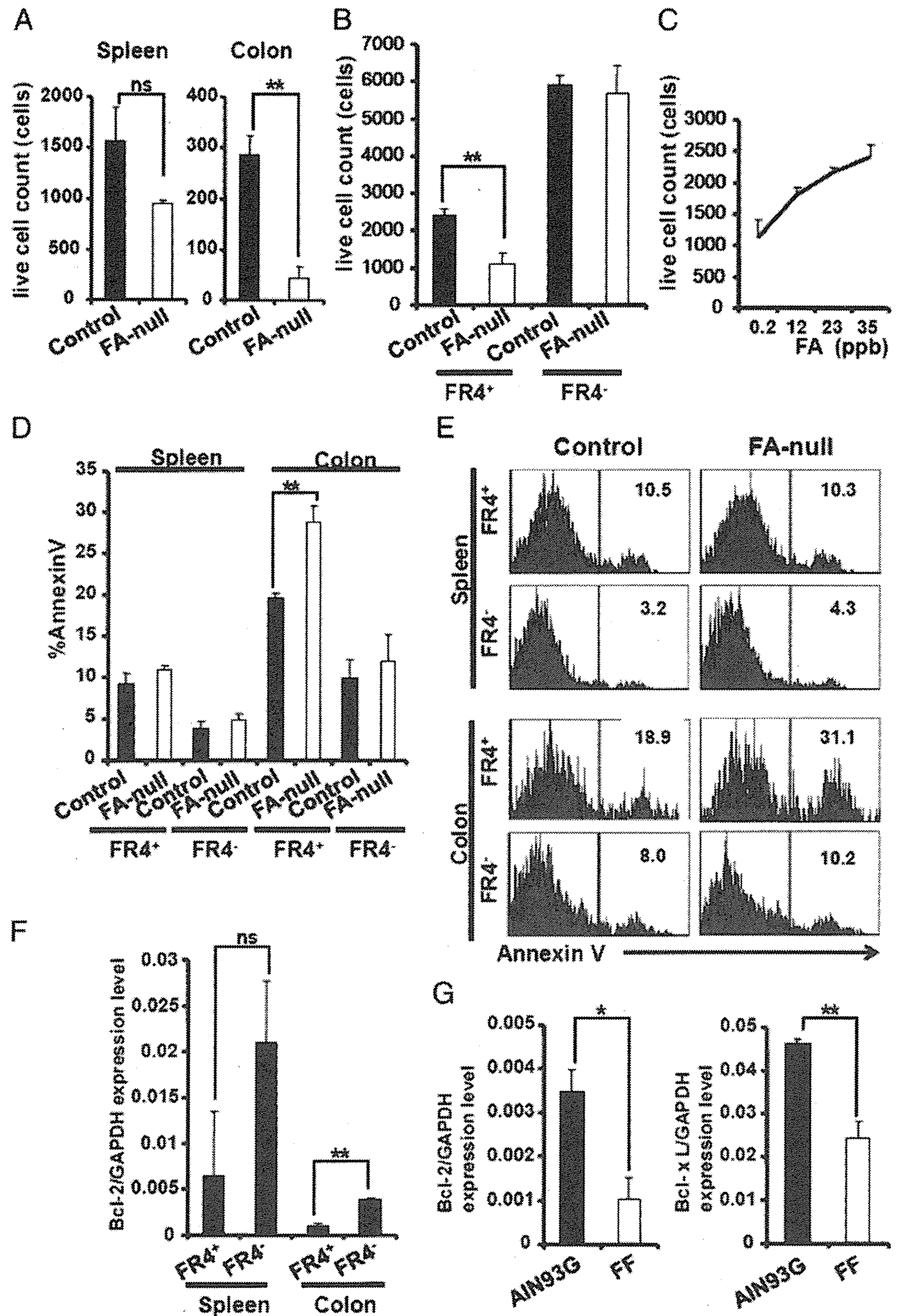

FIGURE 5. FA promotes survival of colonic FR4$^+$ T cells by inhibiting apoptosis. (**A**) Splenic or colonic FR4$^+$ CD4$^+$ T cells isolated from the normal diet mice were cultured under either FA-sufficient (control) or FA-null conditions. Isolated T cells were cultured for 3 d. The numbers of live 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-xL* expression levels normalized to that of *Gapdh*. Mean values ± SD of duplicate or triplicate determinations. All data are of two independent experiments. $*p < 0.05$, $**p < 0.01$.

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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 colonic T cells. At 5 or 8 d after exposure to BrdU, colonic Foxp3$^+$ Tregs of the control and FF-diet mice incorporated BrdU almost to the same level (Fig. 6A, 6B). To confirm further the dispensability of FA for Treg proliferation, Foxp3$^+$ T cells isolated from Foxp3-GFP mice were labeled with CMTMR and examined for the proliferative capacity under FA-sufficient and FA-null conditions. Foxp3$^+$ T cells stimulated with CD11c$^+$ DCs showed a comparable proliferative capacity irrespective of the FA conditions (Fig. 6C). Thus, growth activity of colonic Tregs was not altered by the FA deficiency.

Colonic Tregs exhibit constitutively activated phenotype

We previously reported that Ag stimulation increases the Bcl-2^{low} 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

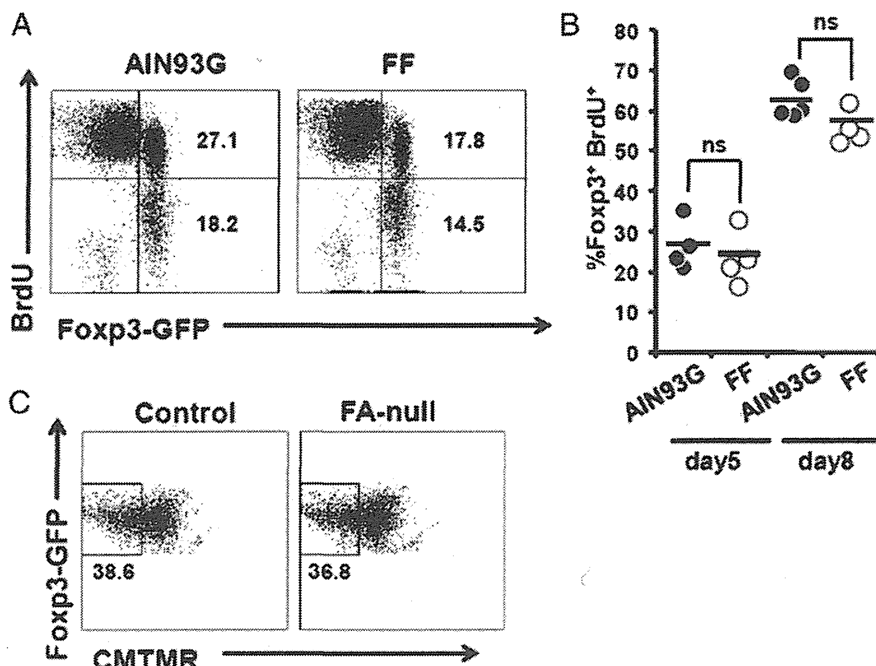

FIGURE 6. FA is dispensable for the proliferation of colonic Tregs. (**A** and **B**) AIN93G diet or FF-diet Foxp3-GFP mice were given BrdU in their drinking water for 5 or 8 d. BrdU incorporation by colonic CD4$^+$ T cells was analyzed by FACS. (A) Representative FACS dot plots of the indicated mice. (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.

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.

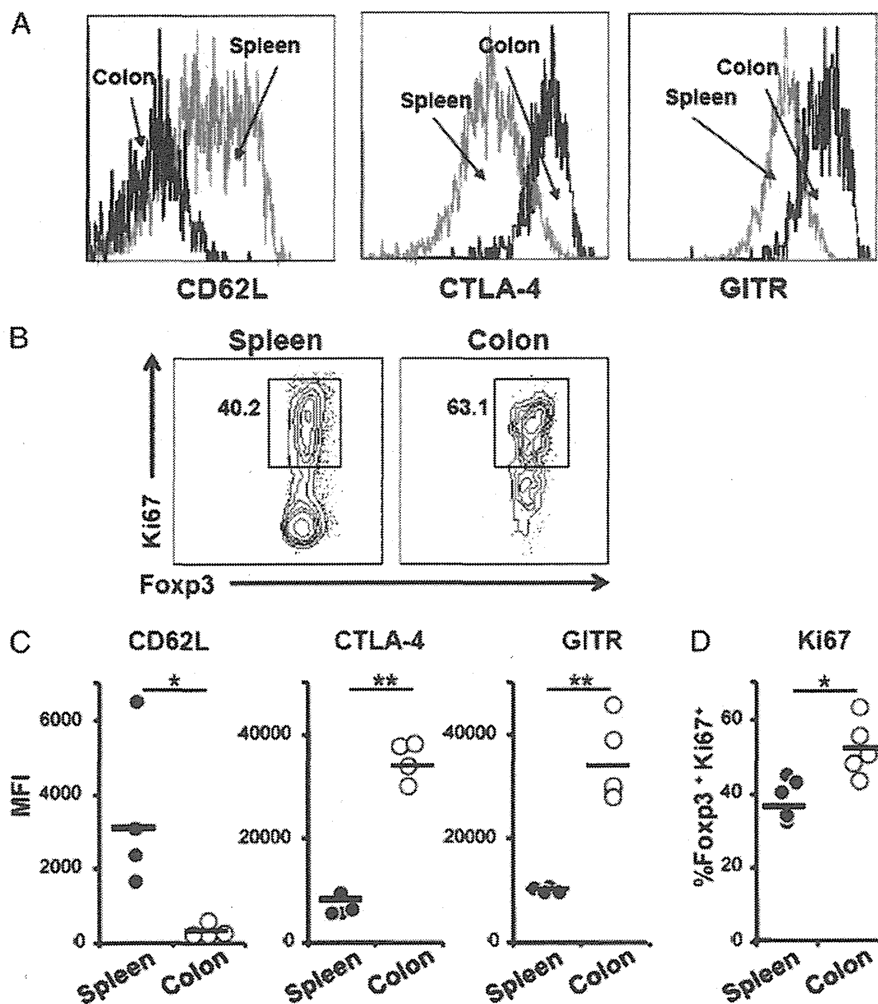

FIGURE 7. Colonic Tregs exhibit constitutively activated phenotype. (**A**) Representative histograms of the indicated surface markers on splenic or colonic Foxp3$^+$ Tregs isolated from mice fed with the normal diet are shown. (**B**) Representative dot plots of Ki67 staining in splenic or colonic Foxp3$^+$ 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. *$p < 0.05$, **$p < 0.01$.

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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, thus 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

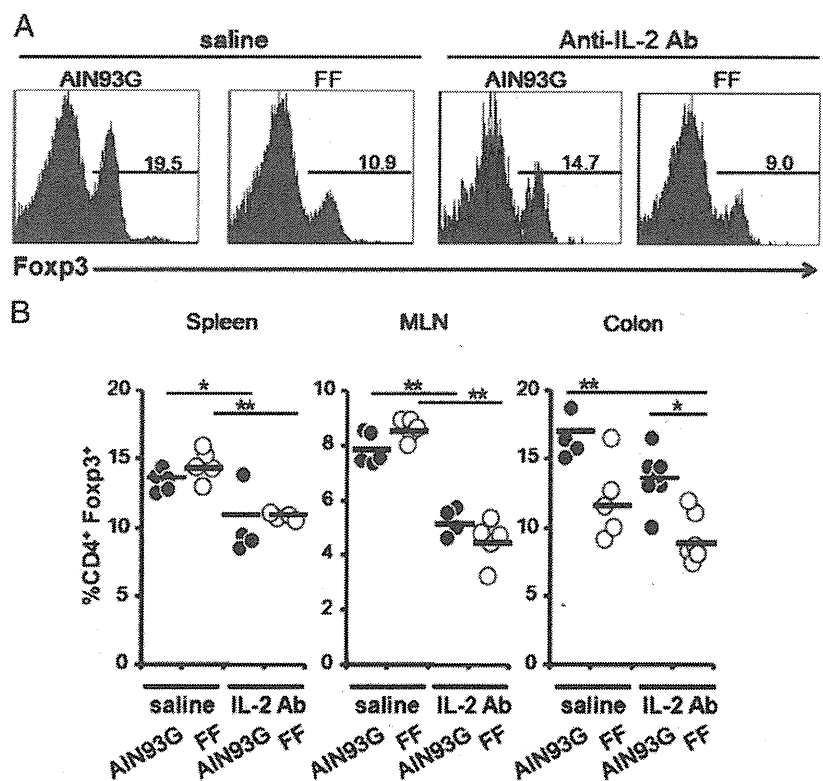

FIGURE 8. Cooperative regulation of Tregs by IL-2 and FA is observed in the colon. (**A** and **B**) Six-week-old AIN93G diet or FF-diet mice were treated with i.v. injections of saline or anti–IL-2 NAbs. One week after the treatment with anti–IL-2 NAbs, Foxp3⁺ CD4⁺ T cells in the spleen, MLN, and the 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.

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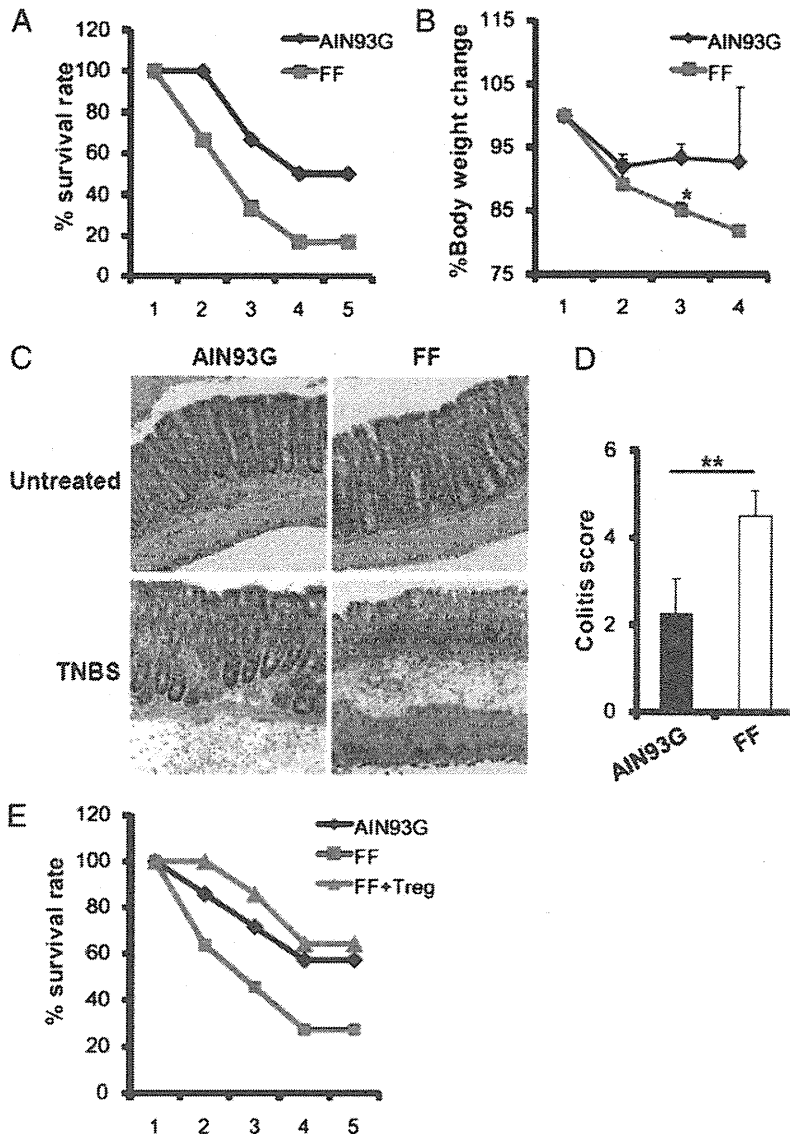

FIGURE 9. FA prevents excess inflammation in a TNBS-induced colitis model. (**A–E**) TNBS-colitis was induced in mice of AIN93G diet or FF-diet at 8–9 wk of age. (A) Survival rate of the indicated mice is shown. Data are of six mice in each group. (B) Body weight changes relative to the value prior to the colitis induction are shown. Data are of six mice in each group. (C) H&E staining of the indicated mice. Untreated mice represent mice without TNBS-colitis induction. Original magnification ×20. (D) Histological scores of TNBS-colitis in the indicated mice. Data are of four mice in each group. (E) Two days prior to TNBS-colitis induction, 5×10^5 $FR4^+$ $CD4^+$ T cells were adoptively transferred to the FF-diet mice. The graph shows the survival rate of the indicated mice. Data are of 11–14 mice in each group. Error bars denote mean values ± SD. Data are of two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$. AIN93G, AIN93G diet; FF, FF-diet.

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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-2^{low} 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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Disclosures

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ORIGINAL ARTICLE

Poor Recall of Prior Exposure to Varicella Zoster, Rubella, Measles, or Mumps in Patients with IBD

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Background: Few studies have measured the levels of antibodies specific for measles, mumps, rubella, and varicella zoster/chickenpox viruses in inflammatory bowel disease (IBD) patients undergoing treatment with immunomodulators/biologics.

Methods: We prospectively recruited 139 IBD outpatients. Enzyme-linked immunosorbent assays were used as the serological tests for measles, mumps, rubella, and varicella zoster. We defined anti-rubella IgG <10 IU/mL, anti-measles IgG <16 IU/mL, and anti-mumps/varicella zoster IgG <4 IU/mL as seronegative for viruses. We also asked participants about past immunizations against or infections with measles, mumps, rubella, and varicella zoster viruses.

Results: The proportion of patients with seronegative levels of antibodies specific for varicella zoster, rubella, measles, and mumps viruses was 5%, 30%, 34%, and 37%, respectively. Approximately 40% of the IBD patients did not remember whether they had previously been infected with any of the viruses, and almost one-third of the patients could not remember whether they had previously been vaccinated. Almost 30% of the patients with a past history of rubella or measles did not have seropositive antibody levels. A total of 54% of the patients being treated with immunosuppressant displayed seronegative levels of antibodies specific for at least one of the viruses.

Conclusions: Many IBD patients were unaware of whether they had previously been vaccinated against or infected with the viruses causing varicella zoster, rubella, measles, or mumps. Therefore, measuring the current levels of antibodies specific for such viruses is useful for determining whether patients have seropositive antibody levels before immunomodulators/biologics are used for therapy.

(Inflamm Bowel Dis 2012;000:000–000)

Key Words: inflammatory bowel disease, serological test for virus, immunomodulators, biologics

Recent European Crohn's and Colitis Organization (ECCO) guidelines reported that ≈80% of inflammatory bowel disease (IBD) patients are treated with corticosteroids, 40% of IBD patients are treated with thiopurine, and 20% of IBD patients are treated with anti-tumor necrosis factor alpha (TNF-α) therapy.[1] TNF-α plays a pivotal role in inducing apoptosis of infected cells.[2,3] Thus, anti-TNF therapy increases the risk of infection. In patients being treated with immunomodulators and/or biologics, the possibility of disseminated disease resulting from the administration of a live vaccine should be considered.[4,5] In fact, 25% of liver- and intestine-transplant recipients receiving immunomodulators developed a vesicular rash after varicella zoster vaccinations; among these patients, 75% required oral acyclovir treatment.[6] A previous case report described a patient on long-term steroid therapy who suffered from fatal paralytic poliomyelitis after the patient's daughter received the live polio vaccine.[7] Another study indicated that the tacrolimus serum level trough should be less than 5 ng/mL, and the cyclosporine serum level should be less than 50 ng/mL when live vaccines are given[8]; however, these levels are below the levels considered therapeutic in previous studies.[9,10] Thus, it is not practical for patients on immunomodulators/biologics to be given live vaccines. Live vaccinations are contraindicated in IBD patients on immunomodulator therapy.[1]

There have been few studies to measure antibodies specific for measles, mumps, rubella, and varicella zoster viruses in IBD patients who are undergoing treatment with immunomodulators or biologics. The aim of this study was to investigate the levels of antibodies specific for these viruses in IBD patients.

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PATIENTS AND METHODS

Study Design and Patient Populations

A total of 141 patients were asked to participate in the study from January to April 2011. Of these patients, 139 agreed to be included in the study. This was a prospective study of IBD patients (ulcerative colitis [UC] = 77, Crohn's disease [CD] = 59, indeterminate colitis = 3) at the clinic of the Tokyo Medical and Dental University Hospital. None of the subjects were inpatients. Blood tests were performed to measure levels of antibodies specific for measles, mumps, rubella, and varicella zoster viruses. Because most of the patients were undergoing blood tests at each clinic visit to check for anemia, inflammatory markers (e.g., C-reactive protein) and liver/renal functions, the tests to measure the antibodies were performed during regular blood tests.

Evaluation of Antibodies Specific for Measles, Mumps, Rubella, and Varicella Zoster/Chickenpox Viruses

The antibody levels specific for measles, mumps, rubella, and varicella zoster viruses were measured using enzyme-linked immunosorbent assays (ELISAs) (SRL, Tokyo, Japan). The antibody levels for all of the patients were classified as either seropositive or seronegative. According to a previous report[11] and the guidelines of the Japanese Society of Environmental Infections, we defined anti-rubella IgG <10 IU/mL, anti-measles IgG <16 IU/mL, and anti-mumps/varicella zoster IgG <4 IU/mL as seronegative antibody levels.

Data Acquisition

Physicians collected patient information, including current age and age at diagnosis, type of disease, and medical treatments using medical charts. All patients were classified as UC or CD according to a previous report.[12] The use of infliximab was recorded if patients had been treated with this medication within 8 weeks of the survey. The use of adalimumab was recorded if it had been administered within 2 weeks of the survey. In this study, adalimumab was received every other week in all eight patients and infliximab was received every 6–8 weeks in most patients. All patients who were classified as being on adalimumab received it within 2 weeks. Five of 46 patients received infliximab a few days beyond 8 weeks prior to participation in this study, but they were classified as being on biologics. The use of 5-aminosalicylates, corticosteroids, and tacrolimus was recorded if these medications had been administered within 2 weeks of the survey. The use of other medications (e.g., azathioprine [AZA], 6-mercaptopurine [6-MP], or methotrexate) was recorded if these had been administered within 12 weeks of the survey. The dose of AZA ranged from 25–250 mg daily. The dose of 6-MP ranged from 10–100 mg daily. Only one patient used 10 mg of 6-MP and three patients used 25 mg of AZA. In these patients, doses were reduced due to adverse effects. In general, in our institution 30–70 mg of 6-MP and 50–150 mg of AZA was used and adjusted according to patients' body weight and number of white blood cells. The enzyme level of TPMT in Japanese patients is significantly lower than those in Caucasians.[13] Thus, relatively low dosages of thiopurine were used.

We also asked the patients about their histories of measles, mumps, rubella, and chickenpox/varicella zoster infection and their histories of the respective vaccinations (each patient answered yes, no, or unknown) when conducting blood tests to measure antibody levels.

Data Analysis and Statistical Methods

The proportions of patients with seropositive and seronegative antibody levels against varicella zoster, rubella, measles, and mumps were compared between males and females and between UC and CD diagnoses. The mean ages of patients with seropositive and seronegative antibody levels were also compared. The proportion of patients with seronegative antibody levels was assessed in patients who were treated with mesalamine, corticosteroids, thiopurine, biologics (e.g., infliximab, adalimumab), methotrexate, or tacrolimus.

The percentage of patients who had previously been infected with each virus (varicella zoster, rubella, measles, and mumps) or who had been vaccinated was assessed. A correlation between the presence of antibodies specific for each virus and a past history of infection/vaccination was also assessed.

The protocols in this study were approved by the ethics review boards of Tokyo Medical and Dental University (No. 864 in 2010). For statistical analyses, the differences in ages between two groups were tested using a *t*-test, and the differences in the incidence of other factors (e.g., sex, and type of disease) between two groups were tested using chi-squared tests and Fisher's exact test. The significance level in all tests was <5%. All data were analyzed using SPSS v. 18 (Tokyo, Japan).

RESULTS

The clinical features of the patients and the medications used for IBD are listed in Table 1. Mean age in this study was 35.4 years old (range 16–71). A total of 139 patients with IBD were included in this study.

The number of patients with seropositive levels of antibodies specific for varicella zoster/chickenpox, rubella, measles, and mumps viruses is shown in Table 2. The mean age of patients with seronegative levels of antibodies specific for mumps virus was comparable to the mean age of patients with seropositive antibody levels. In contrast, the mean age was significantly lower in patients with seronegative antibody levels specific for varicella zoster, rubella, and measles viruses than in patients with seropositive antibody levels (Table 3). The proportion of males with seronegative antibody levels specific for each virus was comparable to that of females.

TABLE 1. Clinical Features of the IBD Patients

Gender (male: female)	84:55	
Median age (yrs)	35.4+/−13.6	(16-71)
Median duration of disease (yrs)	7.3+/−6.9	(0-26)
IBD subtype		
Ulcerative colitis	77	(55%)
Crohn's disease	59	(42%)
Indeterminate colitis	3	(2%)
Previous surgery	19	(14%)
Medication		
Mesalamine/SASP	68	(49%)
Steroids	10	(26%)
Thiopurine	81	(58%)
Methotrexate	6	(4%)
Tacrolimus	11	(8%)
Infliximab	46	(33%)
Adalimumab	7	(5%)

Next, we assessed the relationship between antibody levels and past immunization against or infection with each virus. As Figure 1 shows, many IBD patients were unaware of whether they have previously been vaccinated against or infected with the viruses. With the exception of varicella zoster virus (97.1% vs. 92.8%), the proportions of patients with seropositive levels of antibodies were significantly higher in patients who had a history of vaccination than in those who had no history or an unknown history of vaccination (Fig. 2). Seropositive antibody levels were observed in only 70% of the patients who reported having a past rubella or measles virus infection (Fig. 3). The other 30% of patients had seronegative levels of antibodies specific for each virus, even though they reported having a previous infection. Among the patients who reported having a past history of mumps, 19% of the patients (11 patients) had seronegative antibodies for mumps virus (Fig. 3). These results suggest that antibody measurements are useful for confirming whether patient recall for history of infection is accurate and whether the patient has seropositive antiviral antibody levels.

Finally, we assessed the proportion of patients who had seronegative levels of antibodies specific for the four viruses among those who were being treated with steroids, thiopurine, biologics, or other immunomodulators. The proportion of those with seropositive antibody levels specific for varicella zoster viruses was comparable between

TABLE 2. Serum Antibodies Against Varicella, Rubella, Measles, and Mumps Viruses

	Seropositive Level	
Varicella zoster	132	95.0%
Rubella	98	70.5%
Measles	92	66.2%
Mumps	88	63.3%

We defined anti-rubella IgG <10 IU/mL, anti-measles IgG <16 IU/mL, and anti-mumps/varicella zoster IgG<4 IU/mL as seronegative antibody levels.

TABLE 3. Mean Ages of Patients With/Without Antibodies Against Varicella Zoster, Rubella, Measles, and Mump Viruses

	Patients with Seropositive Antibody	Patients with Seronegative Antibody	*P*-value
Varicella zoster	35.9+/−13.5 (16-71) yrs	24.6+/−8.2 (19-41) yrs	0.03
Rubella	37.3+/−13.6 (16-71) yrs	30.9+/−12.4 (16-64) yrs	0.01
Measles	38.0+/−13.9 (17-71) yrs	30.3+/−11.4 (16-64) yrs	0.001
Mumps	35.9+/−13.2 (19-71) yrs	34.5+/−14.3 (16-66) yrs	0.54

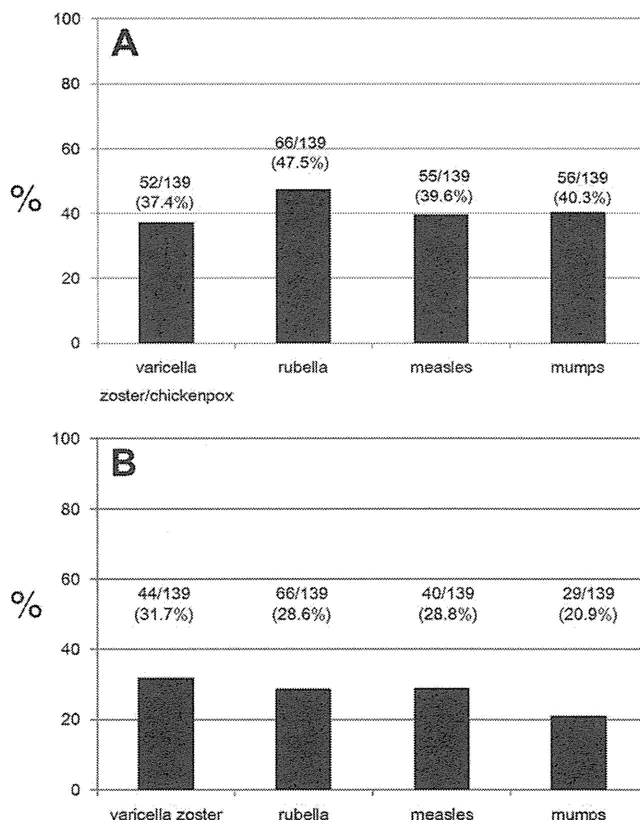

FIGURE 1. Percentages of patients who did not remember: (a) whether they had been infected with each virus; and (b) whether they had been vaccinated against each virus.

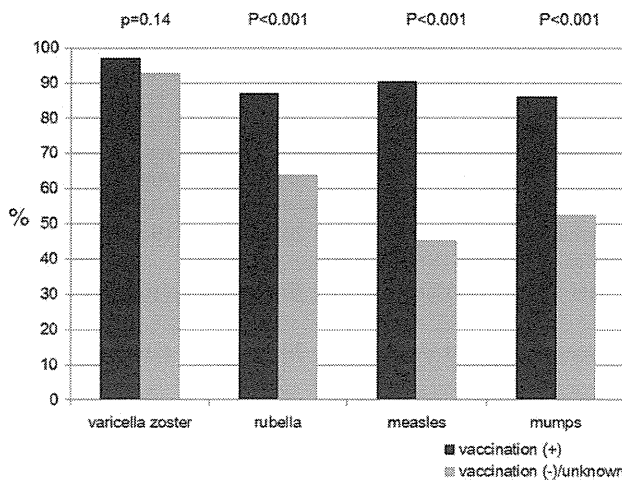

FIGURE 2. Percentages of patients with/without previous vaccination and with seropositive antibody levels against varicella zoster, rubella, measles, and mumps viruses.

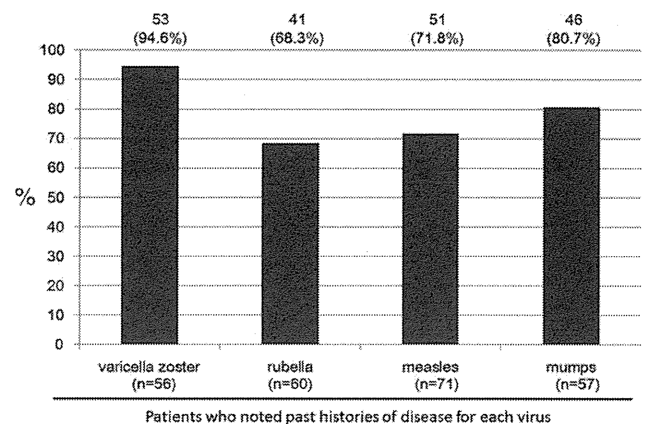

FIGURE 3. Percentages of patients who reported having a past history of infection and who had seropositive antibody levels against varicella zoster, rubella, measles, and mumps viruses. Almost 30% of the patients had seronegative antibody levels against rubella and measles viruses, even though they reported having previous infections with these diseases.

patients who were administered thiopurine and those on 5-ASA/no medication (Table 4). A similar result was obtained for all four viruses in patients who received biologics and those on 5-ASA/no medication. A total of 6 (5.4%), 33 (29.8%), 36 (32.4%), and 43 (38.8%) patients being treated with any immunosuppressants (e.g., steroids, thiopurine, biologics, tacrolimus or methotrexate) had seronegative antibody levels for varicella zoster, rubella, measles, and mumps virus, respectively (Table 4). More than half of patients receiving an immunosuppressant (54.0%) had seronegative antibody levels against at least one virus. For rubella, 17 female patients who were less than 35 years of age displayed seronegative levels of rubella-specific antibodies; among the female patients, 11 patients (65%) were receiving thiopurine and/or biologics.

DISCUSSION

Live vaccinations are generally contraindicated in immunocompromised patients, and this raises concerns because of recent guidelines recommending the use of immunomodulators or biologics in refractory IBD patients.[14,15] It is generally recommended that live vaccinations should be avoided for at least 3 months after treatment with immunomodulators have ceased.[16] Immunomodulators should also be withheld for at least 3 weeks after live vaccines have been given. Regarding the use of live vaccines for patients on biologics, such vaccines should be administered after at least 5 half-lives of TNF-α antagonists (e.g., up to 6 months for infliximab).[3] These results suggest that live vaccines are best given before immunomodulator treatment.[1]

Most IBD patients (especially CD patients) require steroids, immunomodulators, or biologics within the first 10 years of diagnosis of the disease. Because it is not easy for patients to receive live vaccinations if treatments with immunomodulators/biologics have already begun, IBD patients and their physicians should know the measles, mumps, varicella zoster, and rubella histories of the

TABLE 4. Seropositive Rates in Patients with Thiopurine, Biologics, or Any Immunosuppressant (Use of at Least 1 Immunomodulator, Including Steroids, Thiopurine, Tacrolimus, Methotrexate, and Biologics)

	No Medication /5-ASA Only (n=28)	Thiopurine (n=81)		Biologics (n=53)		Any Immunosuppressants (n=111)	
		(+)	P-value*	(+)	P-value*	(+)	P-value*
Varicella zoster	27 (96.4%)	76 (93.8%)	0.51	50 (94.3%)	0.57	105 (94.6%)	0.57
Rubella	20 (71.4%)	60 (74.1%)	0.48	38 (71.7%)	0.48	78 (70.2%)	0.55
Measles	17 (60.7%)	58 (71.6%)	0.28	37 (69.9%)	0.41	75 (67.6%)	0.49
Mumps	20 (71.4%)	56 (69.1%)	0.82	32 (60.4%)	0.32	68 (61.2%)	0.32

*Compared to proportion of patients who were not treated with any medication or treated with 5-aminosalitylates (5-ASA).

patients and whether vaccinations have been administered before treatment with immunomodulators/biologics is started. However, in our study almost 40% of the IBD patients did not remember whether they had previously been infected with varicella zoster, rubella, measles, and mumps viruses. Furthermore, almost one-third of the IBD patients did not remember whether they had been vaccinated against these diseases. These results suggest that IBD patients were unaware whether they had been previously infected with varicella zoster, rubella, measles, and mumps viruses. Our study also indicated that among the patients who reported having a past history of rubella and measles, almost 30% did not have seropositive antibody levels. This result may have been due to failure to raise a strong immune response to primary/secondary vaccination or failure to accurately remember past immunizations. Because patients cannot necessarily remember infections/vaccinations, antibody measurements are critical for determining whether patients have sufficient antibody levels.

There are limitations to this study. First, precise information regarding the disease and vaccination history for each patient was lacking. Some of the patients were unaware of whether they had previously been infected or had received vaccinations. Second, the sample size in our study was too small to detect the equivalence of seronegative rate in patients with or without immunomodulator treatment. Third, we did not perform a case–control study using healthy controls in this study. Finally, there have been no definitive cutoff levels to protect against each virus. Since there has been no clear definition with supporting evidence that low titers of antibody for viruses cause more infection, clinically relevant cutoff levels should be clear in future studies.

Our study indicated that the seronegative rates for rubella, measles, and mumps viruses were almost 30%. Thus, better awareness and education of patients and physicians would be needed regarding poor recall of prior exposure to the viruses. In our study, some of the younger patients did not have enough antibodies to prevent infection with the measles, mumps, or rubella viruses. Therefore, IBD patients, especially younger patients, might benefit from being tested for antibodies against viruses at the time of IBD diagnosis. Vaccinations against these viruses can be given to patients with seronegative antibody levels before immunomodulators or biologics are used if patients want to receive vaccinations.

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ORIGINAL ARTICLE

Interval of Less Than 5 Years Between the First and Second Operation Is a Risk Factor for a Third Operation for Crohn's Disease

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Background: Previous studies have shown various risk factors for the initial and/or the second operation for Crohn's disease (CD). However, limited data are available with regard to the risk factors for a third operation. We aimed to clarify the risk factors for a third operation for CD.

Methods: A total of 200 CD patients who underwent a second intestinal surgery at 13 institutions were examined. We performed univariate and multivariate analyses to examine the influence of independent variables on the cumulative rate of needing a third operation.

Results: A total of 95 patients underwent a third operation. The overall 5-year and 10-year cumulative rates for the third operation were 42.2% and 71.0%, respectively. In univariate analysis, the interval between the initial and the second operation ($P = 0.0069$), postoperative administration of infliximab ($P = 0.0030$), and the anatomical site of the disease ($P = 0.0132$) were significant risk factors for the third operation. In multivariate analysis, the interval between the initial and the second operation ($P = 0.0287$) and postoperative administration of infliximab ($P = 0.0297$) remained significant risk factors for the third operation. The cumulative 5-year third operation rate was significantly higher in patients with an interval of less than 5 years between the first and second operations than for those with an interval of 5 years or more (47.8% versus 35.2%, $P = 0.0232$).

Conclusions: An interval of less than 5 years between the first and the second operations is a significant risk factor for a third operation in patients with CD.

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Key Words: Crohn's disease, surgery, reoperation, second surgery, risk factor, time trend, time changes

Approximately 50%–80% of patients with Crohn's disease (CD) require surgery at some point during their lifetime.[1–3] Postoperative recurrence is common in CD, and after the initial operation some patients need a second and/or multiple operations. Reoperation rates for recurrence range from 48%–71% at 20 years after the initial surgery.[4] Furthermore, the risk of needing a third operation reaches 40% at 10 years after the second operation.[5,6] Therefore, prevention of recurrence remains one of the major goals in the treatment of CD patients.

In order to prevent recurrence in CD, identification of patients at high risk for future recurrence is important because intensive therapy may be given to such patients to decrease recurrence needing surgical intervention. To identify such high-risk patients, previous studies evaluated various factors that potentially influenced the recurrence rates in CD patients, including age, gender, smoking, steroid use, duration of preoperative history, perforating disease, perianal disease, ileocolic disease, etc.[1,2,7–13] However, these studies have focused on identifying risk factors for the initial or the second surgery. To date, few data have been generated with regard to the risk factors for the third operation except for one study with a comparatively small number of patients.[14] Therefore, in the present study we aimed to evaluate risk factors for a third intestinal operation in a larger number of CD patients. We

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examined a total of 200 CD patients and showed that a shorter interval between the initial and the second operation was a significant risk factor for needing a third operation. To our knowledge, this is the first study that has shown that the interval between the initial operation and the second operation was a significant risk factor for a third operation. To the best of our knowledge, this is also the largest study of patients who underwent a second operation that has focused on the risk of a third intestinal operation for CD.

PATIENTS AND METHODS

Patients and Criteria for Diagnosis

A total of 200 CD patients who underwent initial and second intestinal surgeries were examined. Their onset of disease was between 1963–2003, and the diagnosis of CD was made according to the criteria provided by the Investigation and Research Committee for Intractable Inflammatory Bowel Disease organized by the Japanese Ministry of Public Welfare as described previously.[15] The first and the second operation included intestinal surgery consisting of resection or strictureplasty. Surgeries for perianal disease or other minor surgical procedures without intestinal surgery were excluded from the initial and the second operations included in our study. This study was approved by the local Ethics Committee.

Data Management and Definitions

Case records were collected from 13 institutions which are participating in the Investigation and Research Committee for Intractable Inflammatory Bowel Disease organized by the Japanese Ministry of Public Welfare and scrutinized retrospectively. Data included the patient date of birth, date of onset of symptoms, date of diagnosis, disease localization at diagnosis, type of disease, type of surgery and date of initial/second surgery, and date of final follow-up, which were transferred to a data file (Microsoft Office Excel, Redmond, WA). The indications for surgery included acute abdominal pain, medical intractability, intestinal obstruction, palpable mass/abscess, internal fistulas, colonic dilatation, etc. The disease localization was established at the time of diagnosis and was classified into three groups: 1) small bowel disease (inflammation of the small bowel); 2) ileocolic disease (inflammation involving both the small bowel and the colon); 3) colorectal disease (inflammation confined to the colon or rectum or both). The type of disease was classified into perforating or nonperforating disease, as described previously.[11] Perforating disease included patients who underwent their first operation due to perforating disease, whereas nonperforating disease patients were those who underwent the initial operation due to another cause, such as intestinal obstruction, medical intractability, hemorrhage, etc. Perforating disease was classified as perforating, regardless of the concomitant presence of additional nonperforating disease. The primary outcome measure of this study was the rate of patients needing a third intestinal resection or strictureplasty.

Statistical Analysis

The statistical analysis was performed using the JMP software program (SAS Institute, Cary, NC). The cumulative third operation rate was calculated by the Kaplan–Meier method and compared by log-rank test. Univariate and multivariate analyses were performed by Cox proportional hazards regression models in order to examine the influence of independent variables on the cumulative probability of the third operation. Variables with $P < 0.1$ in univariate analysis were entered into each multivariate analysis. $P \leq 0.05$ was considered statistically significant in all analyses. Probability values and confidence intervals were calculated at the 95% level.

RESULTS

Patient Characteristics

Table 1 shows the characteristics of patients. In the 200 CD patients who underwent a first and second intestinal operation, 95 patients underwent a third intestinal surgery after a median of 3.5 years. The frequency of ileocolic disease or administration of infliximab was significantly higher in patients who underwent the third operation than for those who did not. The overall 5-year and 10-year cumulative rates of needing a third operation were 42.2% and 71.0%, respectively (Fig. 1).

Risk Factors for Reoperation and Cumulative Rate of Reoperation

The impact of possible risk factors that may have influenced the frequency of the third operation was evaluated by univariate and multivariate analyses (Table 2). In an analysis of duration of disease, we evaluated the following three different types of disease duration with respect to the risk of a third operation: first, the period between disease onset and the first operation; second, the period between disease onset and the second operation; and last, the interval between the first and the second operation. In a univariate analysis, significant risk factors for the third operation were the interval between the first and the second operation, the anatomical site of the disease, and postoperative administration of infliximab. The cumulative risk of the third operation was significantly higher in patients whose interval between the first and second operations was less than the median interval (4.7 years). We next examined whether the same trend could be observed when we divided patients according to the interval of either shorter or longer than 5 years between the surgeries. Patients whose interval between the initial and the second operation was 5 years or less also showed a higher risk of requiring a third operation (hazard ratio = 0.617 (95% confidence interval [CI], 0.401–0.935, $P = 0.0226$) compared to the patients whose interval was longer than 5 years. With regard to the anatomical site of the disease, patients with ileocolic disease showed significantly higher risk of