



Cyclosporine protects from intestinal epithelial injury by modulating butyrate uptake via upregulation of membrane monocarboxylate transporter 1 levels

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ABSTRACT

Background and aims: A relationship between treatment outcomes and intestinal microbiota in patients with inflammatory bowel diseases has been demonstrated. Cyclosporine treatment leads to rapid improvement in severe ulcerative colitis. We hypothesized that the potent effects of cyclosporine would be exerted through relationships between intestinal epithelial cells (IECs) and the host microbiota. The present study was designed to elucidate the effects of cyclosporine on monocarboxylate transporter 1 (MCT1) regulation and butyrate uptake by IECs.

Methods: Colitis was induced in C57BL6 mice via the administration of 4% dextran sulfate sodium in drinking water, following which body weights, colon lengths, and histological scores were evaluated. To examine the role of butyrate in the protective effects of cyclosporine, MCT1 inhibitor and an antibiotic cocktail was administered and tributyrin (TB; a prodrug of butyrate) was supplemented; MCT1 protein expression and acetylated histone 3 (AcH3) signals in IECs, as well as the MCT1-membrane fraction of Caco-2 cells, were evaluated. To explore butyrate uptake, as s butyrate derivatives, 3-bromopyruvic acid (3-BrPA) and 1-pyrenebutyric acid were used.

Results: Treatment with cyclosporine inhibited body weight loss and colon length shortening. However, treatment with MCT1 inhibitor and the antibiotic cocktail negated the efficacy of cyclosporine, whereas TB supplementation restored its protective effect. Furthermore, cyclosporine upregulated MCT1 expression in the membrane and the AcH3 signal in IECs, while also inducing higher anti-inflammatory cytokine production compared to that in the vehicle-treated mice. The transcription level of *MCT1* mRNA in IECs and Caco-2 cells did not increase with cyclosporine treatment; however, cyclosporine treatment increased membrane MCT1 expression in these cells and uptake of butyrate derivative.

Conclusion: Cyclosporine treatment modulates butyrate uptake via the post-transcriptional upregulation of membrane MCT1 levels in IECs.

1. Introduction

The etiology and pathogenesis of inflammatory bowel disease (IBD) have not been fully elucidated; however, recent studies have revealed

that environmental factors are associated with disease onset. Ulcerative colitis (UC), a major form of IBD, is a chronic relapsing immune-mediated disorder. Intestinal dysbiosis has been considered one of the mechanisms of pathogenesis related to the environmental factors

Abbreviations: intestinal epithelial cells, IECs; tributyrin, TB; acetylated histone 3, AcH3; ulcerative colitis, UC; inflammatory bowel disease, IBD; dextran sulfate sodium, DSS; short-chain fatty acids, SCFAs; regulatory T cells, Tregs; antibiotic cocktail, abx.

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underlying IBD onset [1]. The gut microbiota also influences the outcome of treatment and can predict relapse [2]. Close relationships have also been demonstrated between treatment outcomes and intestinal dysbiosis [3].

Cyclosporine, a calcineurin inhibitor, inhibits T cell activation and is widely used as an immunosuppressant. The efficacy of intravenous cyclosporine treatment in patients with severe UC has been confirmed by several clinical trials [4,5]. We previously reported that the protective effect of cyclosporine is mediated by the transforming growth factor- β (TGF- β) signaling pathway in intestinal epithelial cells (IECs) [6]. However, the precise mechanisms underlying this protective effect remain to be clarified.

One candidate molecular player in this effect is thought to be butyrate, a short-chain fatty acid (SCFA) that is a major product of intestinal microbial fermentation. Butyrate is taken up by IECs and acts as an inhibitor of histone deacetylase, an energy source for IECs, as well as a Gpr109a agonist, exhibiting anti-inflammatory effects [7]. Furthermore, butyrate induces regulatory T cell (Treg) differentiation by enhancing the production of TGF- β in the colon via acetylation of the *Foxp3* promoter in naive T cells [8,9]. Tregs play an important role in colonic homeostasis by expressing interleukin (IL)-10 [10]. In the colon, butyrate is primarily transported into IECs by monocarboxylate transporter 1 (MCT1) under physiological conditions. The proportion of butyrate-producing bacteria and the expression of MCT1 in the colon is reportedly decreased in patients with IBD and in experimental colitis models [11–13]. Therefore, low butyrate availability and MCT1 expression in IECs are believed to be involved in the pathophysiology of UC. In the present study, we aimed to elucidate the effects of cyclosporine on the regulation of MCT1 and the uptake of butyrate by IECs, as well as the role of butyrate in cyclosporine treatment outcomes.

2. Materials and methods

2.1. Animals

Female C57BL/6J mice (aged 8–10 weeks, weighing 18–21 g) were purchased from CLEA Japan (Tokyo, Japan). All mice were maintained in a specific pathogen-free environment. All animal experiments were conducted in accordance with the Guidelines for Animal Experimentation, Hirosaki University (Permit number: M14009).

2.2. Induction of colitis

Colitis was induced in mice via the administration of 4% dextran sulfate sodium (DSS, molecular weight-5000; Wako Pure Chemical, Osaka, Japan) dissolved in distilled drinking water.

2.3. Treatment with cyclosporine, antibiotic cocktail, and inhibitor

Cyclosporine (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in pharmaceutical grade olive oil (vehicle) via sonication before use [14], and mice were intraperitoneally injected daily with 0.1 mL cyclosporine (20 mg/kg) or vehicle. This treatment began 1 day before DSS treatment. An antibiotic cocktail (abx, 200 μ L), consisting of ampicillin (1 g/L; Wako), vancomycin (500 mg/L; Wako), neomycin sulfate (1 g/L; Sigma-Aldrich), and metronidazole (1 g/L; Wako), was administered via oral gavage for 3 days. Following treatment with the abx cocktail, DSS administration was commenced. We then supplemented the abx-treated mice with 200 μ L of tributyrin (TB; Santa Cruz Biotechnology, Dallas, TX, USA) via oral gavage every day from day 0 (day of DSS administration), and the mice were euthanized and dissected 4 h after TB supplementation. In vivo experiment, α -cyano-4-hydroxycinnamic acid (CHC) (Sigma-Aldrich) was used as MCT1 inhibitor, which was injected (25 μ mol in 100 μ L) intraperitoneally twice a day [15]. The CHC treatment began 1 day before DSS treatment.

2.4. Evaluation of disease activity

The body weights of mice were measured every day. At day 6 and 7, the mice were sacrificed by cervical dislocation and dissected, at which point the colon was isolated. The length of the colon was measured and histological severity of colitis was graded on a scale of 0–3 (0, normal; 1, slight; 2, moderate; 3, severe) based on inflammatory cell infiltration into the lamina propria, the appearance of erosions, the decrease in the number of crypts and glands, and height of the epithelium, as previously described [16]. Histological evaluation was performed in a blinded fashion.

2.5. Isolation of IECs

IECs were purified as previously described [17]. Briefly, the mice were anesthetized through exposure to isoflurane and dissected to open the thoracic cavity. The left ventricle was perfused with 15 mL of 30 mmol/L EDTA in Hanks' balanced salt solution (HBSS). Following perfusion, the entire colon, excluding the cecum was removed, inverted, and placed in a cold tube containing 2 mL of cold 2 mmol/L EDTA in HBSS. The tube was shaken for 2 min using a mini-beadbeater (BioSpec Products, Bartlesville, OK, USA) and the tissue remnants were discarded. The suspension was centrifuged at 15,000 \times g (4 $^{\circ}$ C) for 20 min, and the pellet comprising IECs was suspended in RNA lysis solution (Thermo Fisher Scientific, Waltham, MA, USA) or used to extract protein.

2.6. Cell culture and treatment with cyclosporine

Caco-2 cells were obtained from the American Type Culture Collection (ATCC; Teddington, UK). These cells were seeded in a 148-cm² dish and cultured in Dulbecco's modified Eagle Medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), at 37 $^{\circ}$ C in 5% CO₂, with the medium being replaced every other day for 14–21 days. Caco-2 cells were treated with or without cyclosporine (1 μ g/mL) for 6 h.

2.7. Immunohistochemistry

For animal experiments, 24 h after intraperitoneally administering cyclosporine or the vehicle, paraffin-embedded sections of colon were obtained for immunohistochemical analysis. The sections were stained with an anti-SLC16A1 rabbit polyclonal antibody (TA 321555, OriGene, Rockville, MD, USA; 1:200) for 1 h, anti-acetyl-Histone H3 rabbit polyclonal antibody (06–599, Merck, Darmstadt, Germany; 1:500) for 1 h, anti- β 1 Na⁺/K⁺-ATPase antibody (ab134280, Abcam, Cambridge, UK; 1:100) for 1 h, Alexa Fluor 488 anti-rabbit IgG (A21206, Thermo Fisher Scientific; 1:500) for 30 min, and Alexa Fluor 555 anti-goat IgG (A21432, Thermo Fisher Scientific; 1:500) for 30 min. Nuclear staining and mounting were then performed using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; P36941, Thermo Fisher Scientific). Next, Caco-2 cells were stained with an anti-SLC16A1 mouse IgG1 monoclonal antibody (sc-365501, Santa Cruz Biotechnology; 1:200), anti-CD147 rabbit monoclonal antibody (abcam; 1:200), Alexa Fluor 488 anti-mouse IgG (A28175, Thermo Fisher Scientific; 1:200), Alexa Fluor 594 anti-rabbit IgG (A21207, Thermo Fisher Scientific; 1:200), and DAPI. The cells were stained at 14 days post-confluence, mounted on slides with coverslips, and observed using an IX73 (Olympus Co., Tokyo, Japan) and FV3000 (Olympus Co.).

2.8. Western blotting

IECs, colon sections and Caco-2 cells were lysed in lysis buffer containing 1 M tris-HCl, 500 mM EDTA, 0.5% Nonidet P-40 (Nakalai Tesque, Kyoto, Japan), 10% glycerol, 500 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), 100 mM sodium fluoride (Nakalai Tesque), 100 mM sodium vanadate (Sigma-Aldrich), 500 mM sodium

pyrophosphate (Sigma-Aldrich), and protease and phosphatase inhibitor cocktails (Roche Molecular Biochemicals, Mannheim, Switzerland), which was followed by incubation on ice for 30 min. Next, the cells were centrifuged at $15,000\times g$ (4°C) for 20 min and supernatants were collected as the protein fraction. The membrane fraction of Caco-2 cells was collected by ultracentrifugation at $27,000\times g$ (4°C) for 35 min, as described previously [18].

Protein content was determined using the BCA protein assay kit (Thermo Fisher Scientific). For western blotting, the following antibodies were used: anti-SLC16A1 rabbit polyclonal antibody (TA321555, origene; 1:2,000), anti-SLC16A1 mouse monoclonal antibody (sc-365501, Santa Cruz Biotechnology; 1:1,000), anti-TGF- β 1 rabbit monoclonal antibody (sc-146, Santa Cruz Biotechnology; 1:1,000), anti- $\text{Na}^{+}/\text{K}^{+}$ -ATPase rabbit polyclonal antibody (#3010, Cell Signaling Technology Inc., Danvers, MA, USA; 1:1,000), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) rabbit polyclonal antibody (#2118, Cell Signaling Technology Inc.; 1:1,000), HRP-conjugated goat anti-rabbit IgG (HAF008, R&D Systems, San Jose, CA, USA), and HRP-conjugated goat anti-mouse IgG (HAF007, R&D Systems). The density of each band was calculated using Image lab 3.0 (Bio-Rad, Hercules, CA, USA).

2.9. ELISA

Proteins were extracted from mice colonic tissue 24 h after the administration of cyclosporine or vehicle. Protein content was determined using the BCA protein assay kit (Thermo Fisher Scientific). The IL-10 level was measured in colons using an ELISA kit (Proteintech, Chicago, IL, USA) according to the manufacturer's instructions.

2.10. Real-time PCR

Total RNA from IECs and Caco-2 cells was isolated using the RNeasy mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The total RNA (500 ng) was reverse-transcribed to cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). Pre-designed primers and probes for human and mouse *MCT1* (Assay ID: Hs01560299, Mm01306379) and *GAPDH* (Assay ID: Hs02786624, Mm99999915) were purchased from Thermo Fisher Scientific. The reaction was carried out using a CFX connect (Bio-Rad) under the following conditions: 10 min at 95°C for initial polymerase activity, 40 cycles at 95°C for 15 s and 60°C for 1 min. Results were analyzed using the comparative Ct method and expression levels were normalized to those of *GAPDH*, as an internal standard.

2.11. Luminal SCFA concentration measurements

SCFAs in the cecum were extracted as previously described [19]. The concentration of SCFAs was measured via liquid chromatography-tandem mass spectrometry, which was performed using an ACQUITY UPLC I-Class (Waters Co., Milford, MA, USA) equipped with an ACQUITY UPLC HSS C18 Column (100 \AA , $1.8\text{ }\mu\text{m}$, $2.1\text{ mm}\times 100\text{ mm}$).

2.12. MCT1-mediated transport assesment

To determine the transport by MCT1 in Caco-2 cell transport, 3-bromopyruvate(3-BrPA)(Sigma-Aldrich), is a selective MCT1 substrate, induce to cell death by inhibiting GAPDH after transported into cell [20, 21]. Caco-2 cells were seeding to 24 well plate, and cultured in DMEM supplemented with 10% FBS, and with the medium being replaced every other day for 14–21 days. After treated with or without cyclosporine ($1\text{ }\mu\text{g}/\text{mL}$) for 6 h, cells were washed and preincubated with or without 1 mM CHC in DMEM supplemented with 2% FBS for 15 min, followed by added with $50\text{ }\mu\text{M}$ 3-BrPA or saline in same buffer for 24 h or 48 h at 37°C in 5% CO_2 . The supernatant was used to Cytotox 96 (Promega Co.

WI, USA) according to the manufacturer's instructions. To visualize MCT1 mediated transport, as a fluorescent derivative of butyrate, 1-pyrenebutyric acid was used. After 14–21 days after seeding at chamber slides, cells were treated with or without $1\text{ }\mu\text{g}/\text{mL}$ cyclosporine for 6 h, and preincubated with or without 1 mM CHC for 15 min in DMEM supplemented with 2% FBS, followed by added with 2 mM 1-pyrenebutyric acid or vehicle. After 1 min or 30 min, cells were washed twice by PBS, further mounted with Prolong Gold Antifade Reagent (Thermo Fisher Scientific). Intracellular 1-pyrenebutyric acid was observed by confocal laser scanning microscopy, FV3000 (Olympus Co.).

2.13. Statistical analyses

All data are presented as the mean \pm SEM. The data were analyzed with a Mann-Whitney *U* test using Graph Pad Prism v6.0 (Graph Pad, La Jolla, CA, USA). *P* values < 0.05 were considered statistically significant.

3. Results

3.1. Cyclosporine ameliorates DSS-induced colitis, which was counteracted by reduced intestinal butyrate

In the groups that were not treated with abx, body weight loss, colon shortening, and histological changes became worse following the administration of 4% DSS; however, these effects were significantly improved by cyclosporine treatment (Fig. 1A–D). The administration of abx significantly reduced the levels of cecal butyrate (Fig. 1E). However, as there was no difference in luminal butyrate levels between vehicle- and cyclosporine-treated mice, cyclosporine did not directly affect luminal butyrate levels. Hematoxylin and eosin staining of colon sections did not reveal inflamed mucosa on day 7 in mice treated with abx and without 4% DSS (data not shown). Alternatively, the administration of 4% DSS after treatment with abx equally exacerbated body weight loss from day 6 onward in both vehicle- and cyclosporine-treated mice (Fig. 1B). The colon lengths shortened and histological scores also became worse in the cyclosporine-treated group, compared to those in the vehicle-treated group (Fig. 1C and B). The efficacy of cyclosporine treatment for colitis was attenuated by abx pretreatment. The concentration of butyrate in the cecum increased sufficiently upon TB administration (Fig. 1E), and the protective effect of cyclosporine was restored (Fig. 1B–D). These results suggest that butyrate would play an important role in improving the efficacy of cyclosporine treatment for colitis.

3.2. Cyclosporine treatment upregulates levels of surface MCT1 in IECs and anti-inflammatory cytokines

To examine the effect of cyclosporine on MCT1 expression and butyrate uptake in IECs, C57BL/6 mice were administered vehicle or cyclosporine intraperitoneally, isolation of IECs and immunohistochemical analysis of colon sections were performed after 24 h. MCT1 protein level in IECs from cyclosporine-treated mice was significantly increased compared with that in IECs from vehicle-treated mice (Fig. 2A). In the cyclosporine-treated mice, the signals of MCT1 and ACh3 in IECs were also enhanced (Fig. 2B). To determine the localization of MCT1, double staining of $\text{Na}^{+}/\text{K}^{+}$ -ATPase, which is a membrane marker, and MCT1 was performed. In the cyclosporine-treated mice, the signals for MCT1 in IECs were significantly increased compared with those in vehicle-treated mice, and the increase in MCT1 signals coincided with surface staining of IECs (Fig. 2C). These results suggest that cyclosporine treatment facilitates butyrate uptake via the upregulation of surface MCT1 in IECs. To explore the mechanism underlying the upregulation of surface MCT1 by cyclosporine treatment, we performed RT-qPCR to examine the transcription levels of MCT1 mRNA in IECs 24 h after cyclosporine or vehicle treatment. However, the mRNA expression of MCT1 in cyclosporine-treated mice was not upregulated when

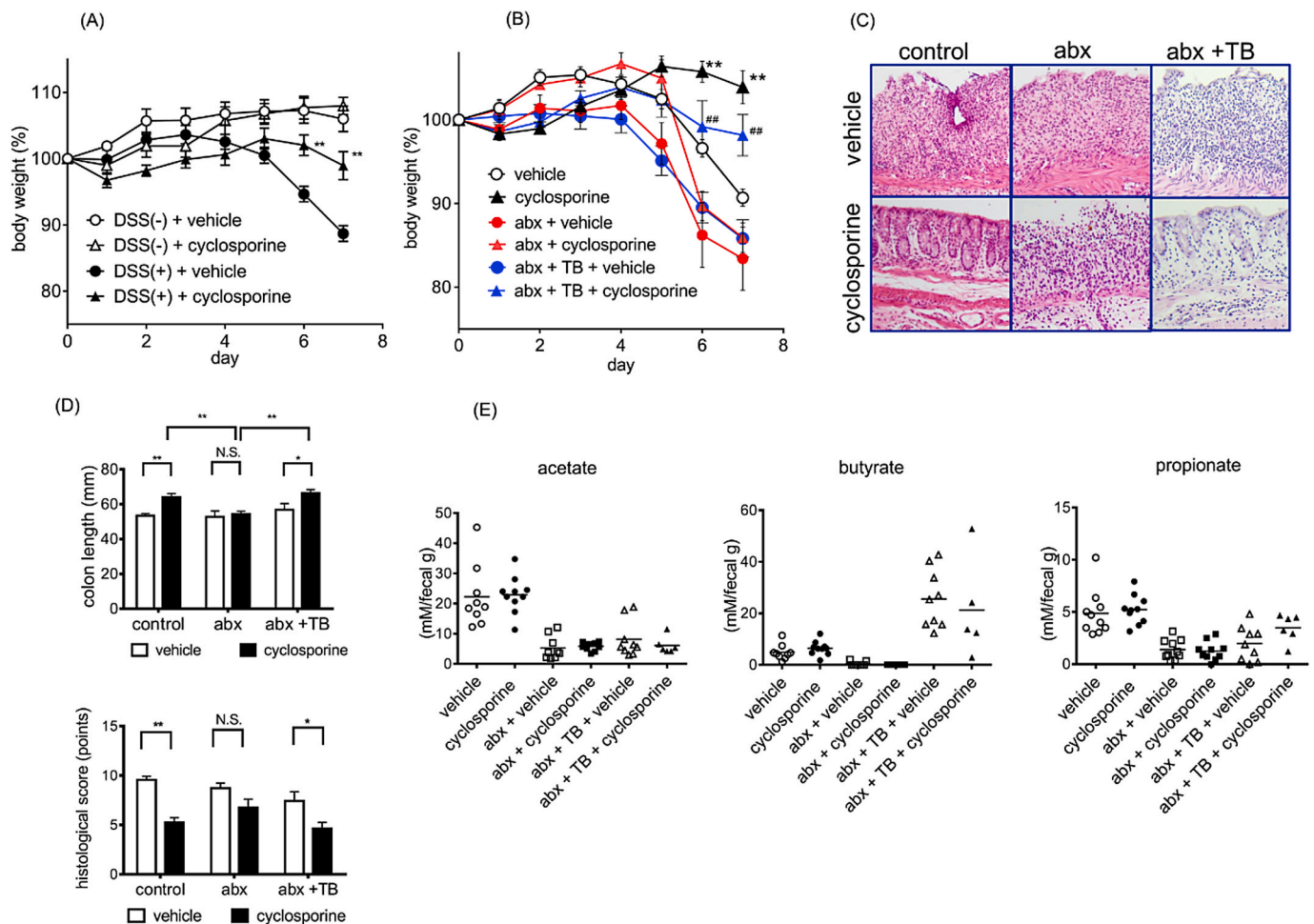


Fig. 1. Cyclosporine ameliorates dextran sulfate sodium (DSS)-induced mucosal damage and its protective effect requires intestinal butyrate. The administration of tributyrin (TB) to antibiotic cocktail (abx)-treated mice restored the efficacy of cyclosporine treatment. (A) (B) Cyclosporine inhibited body weight loss, and treatment with abx negated the inhibitory effect of cyclosporine. In contrast, the administration of TB restored the efficacy of cyclosporine. A graphical representation of the average percent weight loss in cyclosporine-treated and vehicle-treated mice compared to weights at the start of DSS treatment is provided. The data are presented as the mean \pm SEM of 8–12 mice per group. $^{**}P < 0.01$ vs. vehicle-treated mice. $^{##}P < 0.01$ vs. abx + TB + vehicle-treated mice. (C) Colonic sections of vehicle-treated mice and mice treated with cyclosporine plus only the abx or both the abx and TB were stained with hematoxylin and eosin on day 7 and observed under $200\times$ magnification. (D) Histological scores and colon lengths at day 7 in DSS-induced mice were calculated. Data are presented as the mean \pm SEM of 6–9 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle-treated mice. (E) Concentrations of acetate, butyrate, and propionate in the cecum were measured by liquid chromatography-tandem mass spectrometry. Data are presented as the mean \pm SEM of 5–10 mice per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle-treated mice.

compared to that in the vehicle-treated mice (Fig. 2D). Furthermore, the expression of anti-inflammatory cytokines such as TGF- β and IL-10 was also upregulated in cyclosporine-treated mice compared with that in vehicle-treated mice (Fig. 2E and F). To determine the involvement between MCT1 mediated butyrate uptake and DSS colitis, mice were injected CHC, which was pan MCTs inhibitor, before cyclosporine injection every day. By CHC administration, both vehicle- and cyclosporine-treated groups were deteriorated in body weight change, histological score (Fig. 2G, H, and I).

3.3. Cyclosporine increases membrane MCT1 levels in Caco-2 cells

To explore the mechanism underlying the upregulation of MCT1 expression in IECs, we assessed levels of MCT1 in the membrane fraction of Caco-2 cells. Caco-2 cells were treated with cyclosporine or vehicle for 6 h and the membrane fraction was collected. Results of western blot analysis indicated that MCT1 in the membrane fraction was significantly upregulated in the cyclosporine-treated group compared to expression in the vehicle-treated group (Fig. 3A). To confirm the levels of membrane MCT1, immunofluorescence double staining for MCT1 and CD147 was performed on Caco-2 cells, and observed by confocal laser scanning

microscopy. Immunofluorescence staining showed that the signals of surface MCT1 were significantly enhanced in the cyclosporine-treated group compared to that in the vehicle-treated group (Fig. 3B). In Caco-2 cells, we performed RT-qPCR for *MCT1* mRNA, however no difference was observed between the two groups (Fig. 3C).

3.4. Cyclosporine increases butyrate derivative in Caco-2 cells

To explore butyrate uptake, LDH releasing assay was performed by 3-BrPA, which is MCT1 selective substrate and lead to cell death [20,21]. Cyclosporine treatment increased LDH release in supernatant, however, it was canceled by CHC administration (Fig. 4A). Namely, the aggravation of cell death by cyclosporine treatment was due to 3-BrPA increased MCT1 mediated uptake. Furthermore, to visualize butyrate uptake, 1-pyrenebutyric acid, as a fluorescence tracer, was administered after cyclosporine treatment, and intracellular signals were observed by confocal laser scanning microscopy. Cyclosporine treatment increased intracellular 1-pyrenebutyric acid, and these uptake was canceled by CHC administration (Fig. 4D).

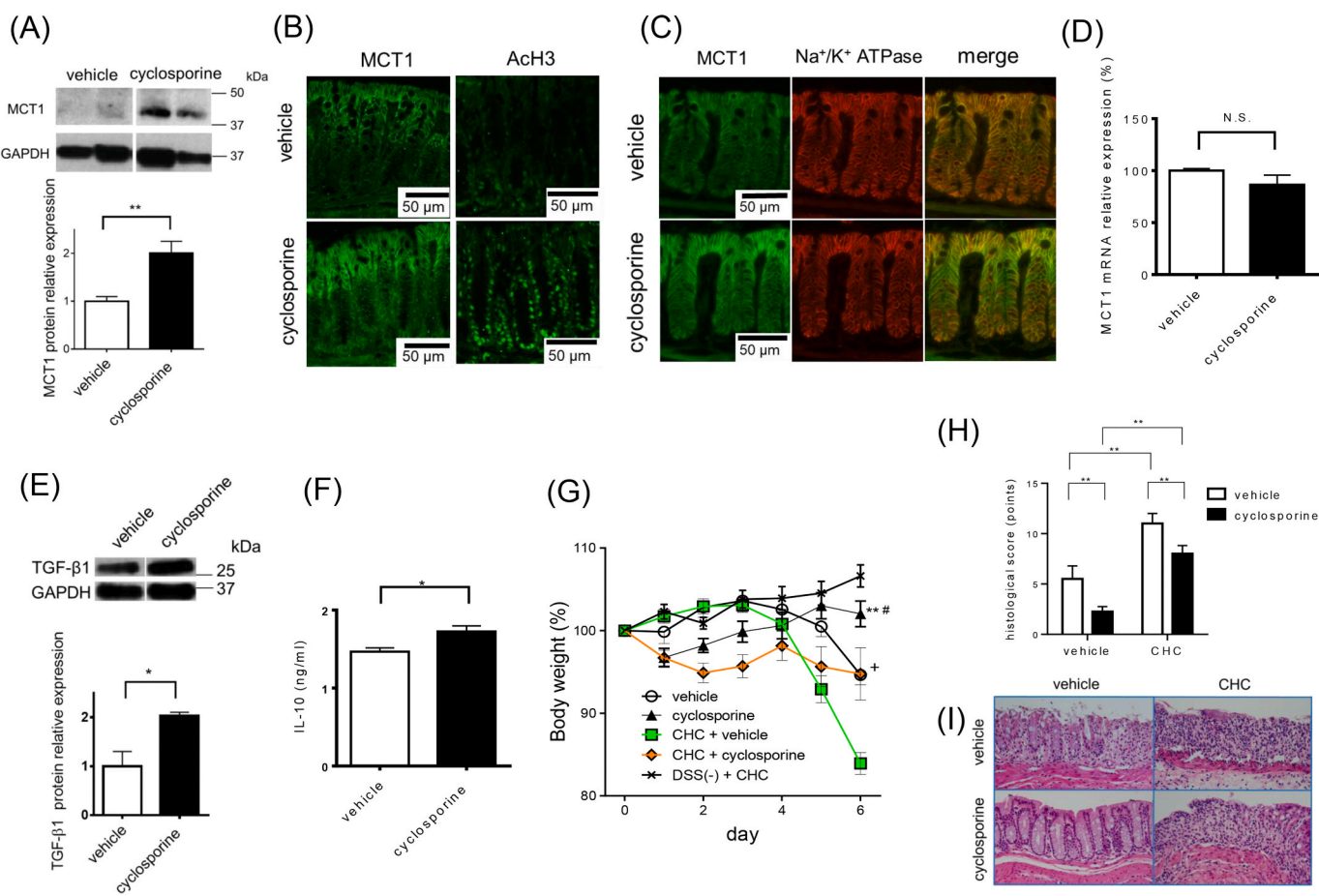


Fig. 2. Cyclosporine upregulates surface monocarboxylate transporter 1 (MCT1) expression and acetylated histone 3 (ACh3) in intestinal epithelial cells (IECs), and does not influence mRNA expression of *MCT1*. Cyclosporine upregulated the colonic expression of transforming growth factor- β (TGF- β)-1 and interleukin (IL)-10. Inhibition of MCT1, by α -cyano-4-hydroxycinnamic acid (CHC) treatment, both vehicle-treated and cyclosporine-treated groups became worse. (A) MCT1 protein level in IECs at 24 h after drug administration. Results are presented as mean \pm SEM of 4–6 mice in each group. $^{***}P < 0.01$ vs. vehicle-treated mice. (B, C) Immunohistochemical staining for MCT1, ACh3, and Na⁺/K⁺-ATPase in the colonic sections of mice treated with or without cyclosporine. (D) Effects of cyclosporine on MCT1 mRNA expression in IECs. The results are presented as the mean \pm SEM of 4–5 mice in each group (E, F). The expression of TGF- β 1 and IL-10 in the entire colon in samples obtained at 24 h after the administration of cyclosporine. The results are presented as the mean \pm SEM of 4–6 mice in each group. $^{***}P < 0.01$ vs. vehicle-treated mice. Vehicle-treated and cyclosporine-treated mice were injected CHC treatment (25 μ mol in 100 μ l) intraperitoneally twice a day. (G) Body weight change, (H) Histological score at day 6. The results are presented as the mean \pm SEM of 4–8 mice in each group. $^{***}P < 0.01$ vs. vehicle-treated mice. $^{\#}P < 0.05$ vs. cyclosporine plus CHC-treated mice. $^{\#}P < 0.05$ vs. vehicle plus CHC-treated mice. (I) Colonic sections of vehicle-treated mice and mice treated with cyclosporine plus CHC were stained with hematoxylin and eosin on day 6 and observed under 200 \times magnification.

4. Discussion

Herein, we describe a novel mechanism of action for cyclosporine; specifically, the efficacy of cyclosporine against DSS-induced colitis is associated with the upregulation of membrane MCT1, which facilitates the uptake of butyrate in IECs. Severe UC is a life-threatening disease and requires immediate aggressive treatment, sometime including total colectomy. The continuous intravenous infusion of cyclosporine exhibits considerable efficacy against severe UC, leading to rapid restitution of IECs. With respect to these rapid effects, we focused on the direct effect of cyclosporine on IECs. Moreover, we previously described that cyclosporine ameliorated mucosal damage via upregulation of TGF- β . We hypothesized that the upregulation of TGF- β 1 by cyclosporine may contribute to the active incorporation of butyrate into IECs. With cyclosporine treatment, the expression of MCT1 of apical membrane was upregulated in IECs of mice, and the transport of butyrate to the IECs was promoted. Therefore, the efficacy of cyclosporine treatment may be a result of the promoted uptake of butyrate, as well as the inhibition of T-cell activation. Studies have reported that germ-free and abx-treated mice are susceptible to epithelial damage and exacerbated hemorrhage in DSS-induced colitis [22]. In the present study, the protective

effect of cyclosporine was negated in mice treated with abx. Further, the treatment of abx-administered mice with butyrate restored the protective effect of cyclosporine. MCT1 expression was significantly down-regulated in abx-administered mice and MCT1 upregulation by cyclosporine cancelled. Restoration of butyrate recovered both MCT1 upregulation and resolution of mucosal damages by cyclosporine (data not shown). Therefore, intestinal butyrate is sure to play an important role in the favorable effects of cyclosporine treatment. Studies have shown that the membrane expression of MCT1 in other tissues is regulated by cyclosporine treatment, without inducing a change at the transcriptional level [23]. The present study is the first to demonstrate that cyclosporine upregulates the membrane expression of MCT1 in IECs without inducing changes at the transcriptional level. We postulate that the following underlying mechanisms are involved in this activity: (1) a recycling mechanism [24] and (2) protein trafficking systems [25–27] considering that other studies have suggested that calcineurin regulates cAMP degradation and affects cytoskeleton function [24]. Moreover, MCT1 forms a complex with various other proteins, including CD147 and cyclophilin A [27]. Cyclophilin A has a peptidyl-prolyl *cis*-*trans*-isomerase, which is associated with protein trafficking and functions as an adaptor protein for cyclosporine [28]. Hence, trafficking via

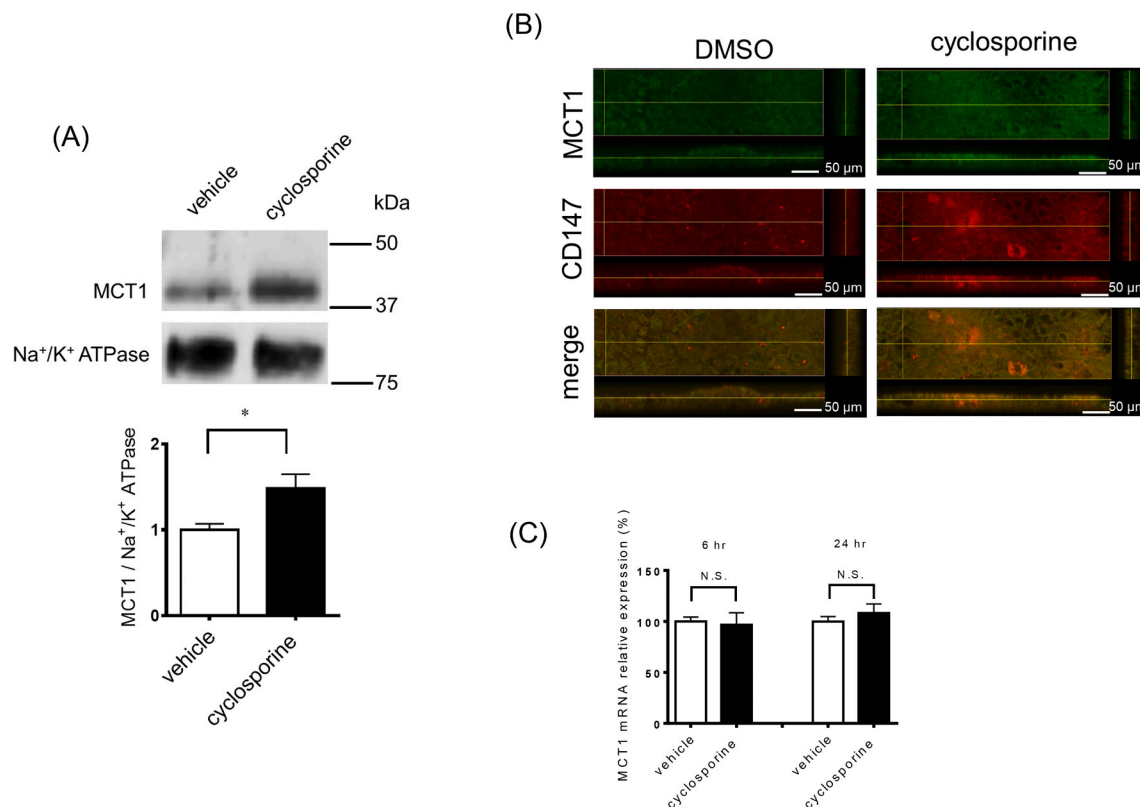


Fig. 3. Cyclosporine increases surface monocarboxylate transporter 1 (MCT1) levels in Caco-2 cells treated with cyclosporine (1 μg/mL) for 6 h, without mRNA expression change. (A) The membrane fraction of Caco-2 cells was collected by ultracentrifugation, and western blot analysis for MCT1 was performed. The results are presented as the mean ± SEM of five independent experiments. *P < 0.05. (B) Confocal microscopy images of Caco-2 cells with or without cyclosporine treatment. Cells were stained for MCT1 (green) and CD147 (red), x-y, y-z, and x-z images were shown. (C) Effects of cyclosporine on MCT1 mRNA expression in Caco-2 cells. The results are presented as the mean ± SEM of four independent experiments. *P < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cyclophilin A might serve as a mechanism underlying the upregulation of membrane MCT1 following cyclosporine treatment. However, the precise mechanism remains unclear, which comprises the primary limitation of this study, and thus, this requires further analysis.

Cyclosporine is known to be an inducer of TGF-β signaling pathway. Butyrate is also an inducer of TGF-β, which is absorbed by IECs and is metabolized as an energy source. Consequently, butyrate upregulates the expression of Foxp3 in T cells via histone acetylation and enhances the production of anti-inflammatory cytokines such as TGF-β and IL-10 [8]. Our data indicated that butyrate-uptake through MCT1 was closely related to TGF-β upregulation in IECs by cyclosporine. Moreover, butyrate plays a key role in IEC protection and inhibitory effect of cancer development [29]. One of key issues to be resolved in cyclosporine treatment is whether cyclosporine would increase cancer risk via TGF-β signaling pathway. Enhanced butyrate uptake may reduce risk of cancer development and further studies are needed to clarify the effect.

The precise localization of MCT1 in the colon is controversial, as its expression has been observed on both the apical and basolateral sides [30,31]. The results of the present study showed that MCT1 in IECs is expressed at the apical membrane. Furthermore, the upregulation of MCT1 expression in IECs after cyclosporine treatment was also observed on the apical side and exerted the protective effect against intestinal injury. Our data suggests the MCT1 expression in apical membrane may be important for butyrate-mediated resolution of mucosal damage.

Collectively, we have demonstrated that treatment with cyclosporine modulates the uptake of butyrate by post-transcriptionally upregulating the levels of membrane MCT1 in IECs. Our results indicate the pathway is one of mechanisms involved in the protective effects of cyclosporine against intestinal epithelial damage. Intestinal butyrate availability may

affect the efficacy of cyclosporine treatment in UC and could be used a predictive marker for the response to cyclosporine treatment. Our results also suggest that concurrent supplementation with butyrate or butyrate-producing bacteria would improve the prognosis of patients with severe UC who were refractory to cyclosporine treatment. Further analysis of the mechanism underlying the effects of cyclosporine on IECs may lead to the development of new therapies that function by altering host-microbiota interactions.

RediT authorship contribution statement

Shinji Ota: Writing - original draft, Funding acquisition, Drafting the manuscript and data acquisition, Formal analysis, Analysis of colitis model, Analysis of Cell lines, Formal analysis, Analysis of protein levels, Analysis of mRNA expressions, Immunohistochemistry. **Hirotake Sakuraba:** Conceptualization, Guarantor of this article and conceptualization, Formal analysis, Analysis of colitis model, Isolation of IECs, Analysis of Cell lines, Histological assessment, Immunohistochemistry, Analysis by confocal laser scanning microscopy. **Hiroto Hiraga:** Formal analysis, Analysis of colitis model, Isolation of IECs. **Shukuko Yoshida:** Formal analysis, Analysis of Cell lines, Analysis of protein levels, Analysis of mRNA expressions, Analysis by confocal laser scanning microscopy. **Miwa Satake:** Formal analysis, Analysis of colitis model, Isolation of IECs, Analysis of Cell lines. **Yui Akemoto:** Histological assessment. **Nahoko Tanaka:** Formal analysis, Analysis of colitis model, Immunohistochemistry. **Rina Watanabe:** Formal analysis, Analysis of colitis model, Immunohistochemistry. **Maeda Takato:** Formal analysis, Analysis of colitis model, Analysis of mRNA expressions. **Yasuhisa Murai:** Formal analysis, Analysis of colitis model. **Kayo Ueno:** Formal

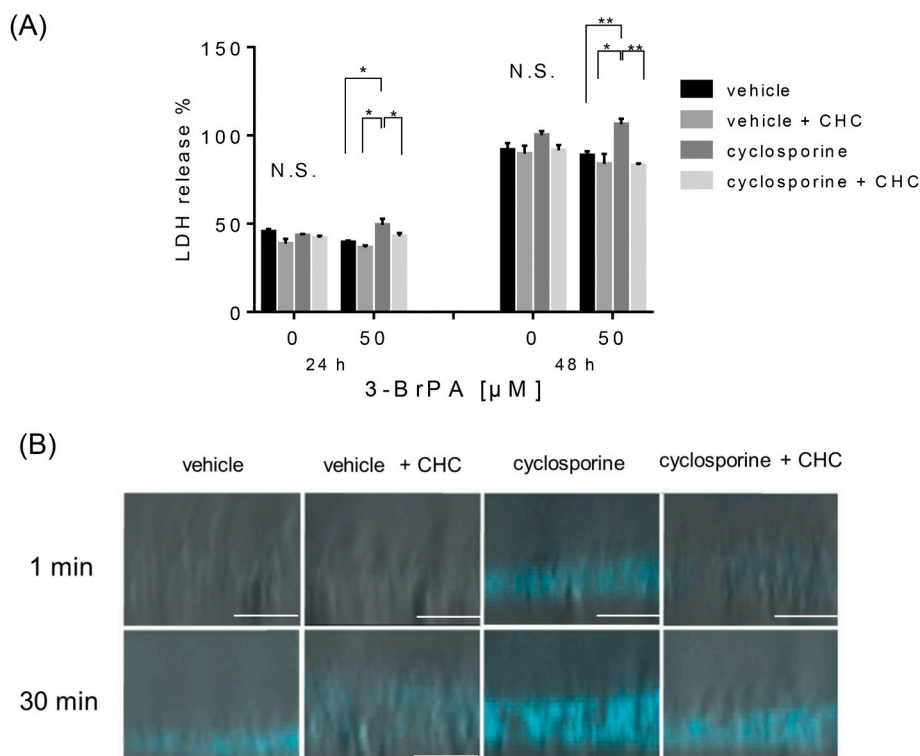


Fig. 4. Cyclosporine increases uptake of butyrate derivatives in Caco-2 cells. Cyclosporine treatment enhanced 3-Bromopyruvate (3-BrPA) mediated cell death, and uptake of 1-pyrenebutyric acid. (A) After Caco-2 cells treated with cyclosporine (1 μg/mL) for 6 h, preincubated with or without 1mM α-cyano-4-hydroxycinnamic acid (CHC) for 15 min and 3-BrPA (0, 50 μM) was added. After 24 h or 48 h, LDH of the supernatant were examined by cytotox kit. The results are presented as the mean ± SEM of four independent experiments. *P < 0.05, **P < 0.01. (B) After Caco-2 cells treated with cyclosporine (1 μg/mL) for 6 h, preincubated with or without 1mM CHC for 15 min, 2 mM 1-pyrenebutyric acid added. After 1 min or 30 min, cells were observed by confocal microscopy. Scale shown is 20 μm.

analysis, Analysis of short-chain fatty acid. **Takenori Niioka:** Formal analysis, Analysis of short-chain fatty acid. **Makoto Hayakari:** Formal analysis, Analysis of short-chain fatty acid. **Yoh Ishiguro:** Isolation of IECs. **Shinsaku Fukuda:** Supervision, Approval of final manuscript: all authors.

Declaration of competing interest

The authors declare no conflicts of interest associated with this manuscript.

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