We found that the UC patients' levels of independence in daily life were influenced by age but not by FH, whereas those of the CD patients were affected by FH. Moreover, young and middle-aged CD patients experienced more difficulty in daily life than similarly aged UC patients. This difference suggests that disease severity is greater in CD patients with FH, particularly in young patients.

Interestingly, we found that 30–40% of both UC and CD patients in the highest disease severity categories were able to live normal lives. The reason for this counter-intuitive finding is unclear, but one possible reason is that there may have been a time lag between the time when disease severity was judged and the time when individual patients' independence in daily life was reported: in some cases, information on patients' daily lives may have been reported after their disease had been treated.

Our study has 2 limitations. The first is that we had no information on the patients' smoking habits. Smoking is thought to be a risk factor for CD and a determining factor in IBD severity [34, 35], although how it may be associated with FH is not known. The second limitation concerns the generalizability of our results. The information collected by prefectural governments is submitted only by those IBD patients applying for subsidization of their treatment costs and thereby agreeing to the use of their data for medical research. Patients who are in deprived circumstances and who are provided with public financial assistance do not need to be subsidized because the government covers all their medical expenses. In contrast, people who are wealthy enough and would not like to offer their personal information to the government may not apply for the subsidization. If wealthy patients were not included in the analysis, it is possible that the peak of the present age was estimated to be younger than the true value, because older people tend to have higher incomes. Also, if deprived patients were excluded from the analysis, the peak of the present age might also be estimated to be younger than the true value, because the percentage of people who receive public financial assistance is higher in the elderly population. In addition, these populations might include patients who live either in extremely clean or in unsanitary environments or have different lifestyle characteristics such as smoking habits and dietary habits. Thus, we should analyze the data carefully if the same population is again used to do a study to clarify IBD etiology, as there may be some bias regarding the socioeconomic status and condition of the patients whose data were analyzed. However, even if high- or low- income patients or those with mild disease conditions were missed by our study because they had no need of subsidization, the data at our disposal covered nearly 60% of IBD patients in Japan and probably constituted the most representative data on such patients available at the present time. Although the overall conversion rate from paper to electronic registration forms is rather low, we do not think this led to any bias, because data conversion to electronic forms depends only on the workforce allocated by local governments to perform the task and not on any factors relating to individual patients. The proportion of UC and CD patients with FHs was the same when we analyzed FH using data from local governments whose rates of conversion to electronic forms were 80% or more. Therefore, we think that the subjects on whom we obtained data are sufficiently representative.

In conclusion, we found that the presence of FH was associated with IBD severity, the onset age of UC, and the levels of independence in daily life of CD patients. On the other hand, in UC patients, we found no connection between FH and levels of independence in daily life or between FH and clinical course. The existence of FH may play an important role in the onset and severity not only of UC but also of CD. Furthermore, because the presence or absence of FH has a bearing on patients' activities of daily living, paying attention to FH should allow physicians to better predict an individual patient's disease course and prognosis.

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References

1. Pinsk V, Lemberg DA, Grewal K, Barker CC, Schreiber RA, Jacobson K. Inflammatory bowel disease in the South Asian pediatric population of British Columbia. Am J Gastroenterol. 2007;102:1077–83.
2. Loftus EV Jr. Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. Gastroenterology. 2004;126:1504–17.
3. Baumgart DC, Carding SR. Inflammatory bowel disease: cause and immunobiology. Lancet. 2007;369:1627–40.
4. Goh K, Xiao SD. Inflammatory bowel disease: a survey of the epidemiology in Asia. J Dig Dis. 2009;10:1–6.
5. Asakura K, Nishiwaki Y, Inoue N, Hibi T, Watanabe M, Takebayashi T. Prevalence of ulcerative colitis and Crohn's disease in Japan. J Gastroenterol. 2009;44:659–65.
6. Kitahora T, Utsunomiya T, Yokota A. Epidemiological study of ulcerative colitis in Japan: Incidence and familial occurrence. The Epidemiology Group of the Research Committee of Inflammatory Bowel Disease in Japan. J Gastroenterol. 1995;30(Suppl 8):5–8.
7. Ishige T, Tomomasa T, Takebayashi T, Asakura K, Watanabe M, Suzuki T, et al. Inflammatory bowel disease in children: epidemiological analysis of the nationwide IBD registry in Japan. J Gastroenterol. 2010;45:911–7.
8. Ben-Horin S, Avidan B, Yanai H, Lang A, Chowers Y, Bar-Meir S. Familial clustering of Crohn's disease in Israel: prevalence and association with disease severity. Inflamm Bowel Dis. 2009; 15:171–5.

9. Carbonnel F, Macaigne G, Beaugerie L, Gendre JP, Cosnes J. Crohn's disease severity in familial and sporadic cases. Gut. 1999;44:91–5.
10. Truelove SC, Witts LJ. Cortisone in ulcerative colitis. Final report on a therapeutic trial. Br Med J. 1955;2:1041–8.
11. de Dombal F, Softley A. IOIBD report no 1: Observer variation in calculating indices of severity and activity in Crohn's disease. International Organisation for the Study of Inflammatory Bowel Disease. Gut. 1987;28(4):474–81.
12. Jiang XL, Cui HF. An analysis of 10218 ulcerative colitis cases in China. World J Gastroenterol. 2002;8:158–61.
13. Park JB, Yang SK, Byeon JS, Park ER, Moon G, Myung SJ, et al. Familial occurrence of inflammatory bowel disease in Korea. Inflamm Bowel Dis. 2006;12:1146–51.
14. Monsén U, Broström O, Nordenvall B, Sörstad J, Hellers G. Prevalence of inflammatory bowel disease among relatives of patients with ulcerative colitis. Scand J Gastroenterol. 1987; 22:214–8.
15. Monsén U, Bernell O, Johansson C, Hellers G. Prevalence of inflammatory bowel disease among relatives of patients with Crohn's disease. Scand J Gastroenterol. 1991;26:302–6.
16. Yang H, McElree C, Roth MP, Shanahan F, Targan SR, Rotter JI. Familial empirical risks for inflammatory bowel disease: differences between Jews and non-Jews. Gut. 1993;34:517–24.
17. Roth MP, Petersen GM, McElree C, Vadheim CM, Panish JF, Rotter JI. Familial empiric risk estimates of inflammatory bowel disease in Ashkenazi Jews. Gastroenterology. 1989;96:1016–20.
18. Farmer RG, Michener WM, Mortimer EA. Studies of family history among patients with inflammatory bowel disease. Clin Gastroenterol. 1980;9:271–7.
19. Kirsner JB, Spencer JA. Family occurrences of ulcerative colitis, regional enteritis, and ileocolitis. Ann Intern Med. 1963;59: 133–44.
20. Lewkonia RM, McConnell RB. Progress report: familial inflammatory bowel disease: heredity or environment? Gut. 1976;17: 235–41.
21. Tysk C, Lindberg E, Järnerot G, Flodérus-Myrhed B. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins: a study of heritability and the influence of smoking. Gut. 1988;29:990–6.
22. Futami S, Aoyama N, Honsako Y, Tamura T, Morimoto S, Nakashima T, et al. HLA-DRB1*1502 allele, subtype of DR15, is associated with susceptibility to ulcerative colitis and its progression. Dig Dis Sci. 1995;40:814–8.
23. Yoshitake S, Kimura A, Okada M, Yao T, Sasazuki T. HLA class II alleles in Japanese patients with inflammatory bowel disease. Tissue Antigens. 1999;53:350–8.
24. Myung SJ, Yang SK, Jung HY, Chang HS, Park B, Hong WS, et al. HLA-DRB1*1502 confers susceptibility to ulcerative colitis, but is negatively associated with its intractability: a Korean study. Int J Colorectal Dis. 2002;17:233–7.
25. De La Concha EG, Fernandez-Arquero M, Santa-Cruz S, Lopez-Nava G, Figueredo MA, Diaz-Rubio M, et al. Positive and negative associations of distinct HLA-DR2 subtypes with ulcerative colitis (UC). Clin Exp Immunol. 1997;108:392–5.
26. Satsangi J, Welsh KI, Bunce M, Julier C, Farrant JM, Bell JI, et al. Contribution of genes of the major histocompatibility complex to susceptibility and disease phenotype in inflammatory bowel disease. Lancet. 1996;347:1212–7.
27. Cottone M, Bunce M, Taylor CJ, Ting A, Jewell DP. Ulcerative colitis and HLA phenotype. Gut. 1985;26:952–4.
28. Inoue N, Tamura K, Kinouchi Y, Fukuda Y, Takahashi S, Ogura Y, et al. Lack of common NOD2 variants in Japanese patients with Crohn's disease. Gastroenterology. 2002;123:86–91.
29. Leong RW, Armuzzi A, Ahmad T, Wong ML, Tse P, Jewell DP, et al. NOD2/CARD15 gene polymorphisms and Crohn's disease in the Chinese population. Aliment Pharmacol Ther. 2003;17: 1465–70.
30. Croucher PJ, Mascheretti S, Hampe J, Huse K, Frenzel H, Stoll M, et al. Haplotype structure and association to Crohn's disease of CARD15 mutations in two ethnically divergent populations. Eur J Hum Genet. 2003;11:6–16.
31. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature. 2001;411:603–6.
32. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cézard JP, Belaiche J, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature. 2001;411:599–603.
33. Annual report of the Research Committee of "intractable inflammatory bowel disease", 1992. The Ministry of Health and Welfare of Japan, Tokyo; 1993 (in Japanese).
34. Aldhous MC, Drummond HE, Anderson N, Baneshi MR, Smith LA, Arnott ID, et al. Smoking habit and load influence age at diagnosis and disease extent in ulcerative colitis. Am J Gastroenterol. 2007;102:589–97.
35. Smith BR, Arnott ID, Drummond HE, Nimmo ER, Satsangi J. Disease location, anti-*Saccharomyces cerevisiae* antibody, and NOD2/CARD15 genotype influence the progression of disease behavior in Crohn's disease. Inflamm Bowel Dis. 2004;10:521–8.

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Multicenter analysis of fecal microbiota profiles in Japanese patients with Crohn's disease

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Abstract
Background We analyzed the fecal microbiota profiles of patients with Crohn's disease (CD) at 4 inflammatory bowel disease (IBD) centers located in different districts in Japan.
Methods Terminal restriction fragment length polymorphism (T-RFLP) analysis was performed in 161 fecal samples from CD patients and 121 samples from healthy individuals. The bacterial diversity was evaluated by the Shannon diversity index (SDI).
Results There were no regional differences in the fecal microbiota profiles of the healthy individuals in Japan. A setting of similarity generated three major clusters of T-RFs: one included almost all the healthy individuals (118/121), and the other two clusters were mainly formed by CD patients at different stages of disease activity. The changes in simulated bacterial composition indicated that the class *Clostridia*, including the genus *Faecalibacterium*, was significantly decreased in CD patients with active disease and those in remission as compared with findings in the healthy individuals. In contrast, the genus *Bacteroides* was significantly increased in CD patients during the active phase as compared with findings in the healthy individuals. The genus *Bifidobacterium* was significantly decreased during the active phase of CD and increased to healthy levels during the remission phase. The bacterial diversity measured by the SDI was significantly reduced in CD patients during the active and remission phases as compared with findings in the healthy individuals. From the clinical data and T-RFLP analysis, we developed a logistic model to predict disease activity based on the fecal microbiota composition.
Conclusion Dysbiosis in CD patients was shown by a multi-IBD center study. The feasibility of using the fecal microbiota profile as a predictive marker for disease activity is proposed.

Keywords Inflammatory bowel disease · *Faecalibacterium* · *Firmicutes* · Shannon diversity index · T-RFLP

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Introduction

Inflammatory bowel diseases (IBDs) such as ulcerative colitis (UC) and Crohn's disease (CD) are chronic

intestinal disorders of unknown etiology [1–5]. The current hypothesis on the pathogenesis of IBD is that the mucosal immune system shows an aberrant response towards luminal antigens such as dietary factors and/or commensal microbiota in genetically susceptible individuals [4, 6–8]. In particular, the commensal microbiota are regarded as the major environmental factor relevant to IBD [6], and evidence for their role is provided through a variety of clinical and experimental studies [6, 8–12]. For example, IBD is mainly localized to areas of the intestines where most of the bacteria are found, such as the distal small intestine and colon. The presence of commensal microbiota was shown to be essential for the development of experimental colitis in several IBD animal models [7, 9]. In addition, the role of the microbiota is supported by recent genetic evidence that there are polymorphisms within the genes associated with innate immune responses to bacteria, such as NOD2 and autophagy-associated genes (e.g., *ATG16L1* and *IRGM*) in CD [13–15].

The global composition of the gut microbiota, rather than the presence of specific pathogens, is thought to be relevant to the etiology and pathogenesis of IBD (dysbiosis hypothesis) [6, 16]. Culture-based approaches have been used to analyze gut microbiota in IBD patients [17–19]. However, the cultivable bacteria represent only 20–30 % of the total fecal bacterial species [20, 21], and most gut microbiota cannot be cultured owing to their requirement for a complex anaerobic environment [20, 21]. Molecular approaches targeting 16S ribosomal(r) RNA, which enable the evaluation of uncultivable bacteria, have been employed for analyzing the gut microbiota of IBD patients [22], and significant differences in the diversity and composition of the gut microbiota have been reported [23].

Using terminal restriction fragment polymorphism (T-RFLP) analysis, we have previously demonstrated that the fecal microbiota profile of CD patients is different from those of healthy individuals [24, 25]. This difference was observed even in CD patients in whom the disease was inactive [24]. However, these studies have some limitations. The first is the relatively small number of patients recruited (approximately 15 CD patients each for the active and inactive phases). The second is that these studies were performed at a single medical center. De Filippo et al. [26] have recently demonstrated that the regional environment is important for populating the fecal microbiota, and this finding leads to the concern that our observations were limited to within the region where our center is located.

In the present study, for the further investigation of the fecal microbiota in CD patients, we designed a prospective study to analyze the fecal samples of CD patients recruited from 4 medical centers located in different districts of Japan. We tried to collect fecal samples from each patient at three clinically relevant time points (the first was prior to induction therapy, the 2nd was just after the achievement of remission, and the 3rd was at least 6 weeks after the achievement of remission, while remaining in remission). Further, using the T-RF pattern, we used multivariate logistic regression analysis to generate a model for predicting the disease activity of CD patients.

Patients, materials, and methods

Patients and setting

Sixty-seven patients with active CD [CDAI (Crohn's disease activity index reported by Best et al. [27]) >150] were recruited from 4 IBD centers in Japan [8 patients from the Hospital of Shiga University of Medical Science (Shiga prefecture), 16 patients from the Hospital of the Hyogo College of Medicine (Hyogo prefecture), 13 patients from the Fukuoka University Chikushi Hospital (Fukuoka prefecture), and 30 patients from the Toho University Sakura Medical Center (Chiba prefecture)]. The diagnosis of CD was based on clinical, endoscopic, and pathological criteria. One hundred and twenty-one healthy individuals living around each center were also enrolled (16 individuals from Shiga, 35 individuals from Hyogo and Osaka, 30 patients from Fukuoka and 40 individuals from Chiba). The location of each prefecture in Japan is shown in Supplementary Fig. 1.

Fecal samples were collected from each patient at 3 different clinical phases: (a) active phase at entry (active phase), (b) remission phase just after the achievement of remission (CDAI <150) (remission-achieved phase), and (c) remission phase maintained for at least 6 weeks (remission-maintained phase). The average period of remission between (b) and (c) was 15.7 ± 10.8 weeks (mean ± SD). Samples from patients with ileal stoma were excluded. Samples from patients who received surgical treatment or patients who failed to achieve remission during the study course were also excluded.

The Institutional Review Boards at all study sites approved the study, and written informed consent was obtained from each participant prior to enrolment.

DNA extraction

The fecal samples were suspended in buffer containing 4 M guanidinium thiocyanate, 100 mM Tris–HCl (pH 9.0), and 40 mM ethylenediamine tetraacetic acid (EDTA) and shaken in the presence of zirconia beads, using a FastPrep FP100A Instrument (MP Biomedicals, Irvine, CA, USA). Then the DNA was extracted from the beads-treated suspension using a Magtration System 12GC and GC series Magtration–MagaZorb DNA Common Kit 200N (Precision

System Science, Chiba, Japan). The final concentration of the DNA sample was adjusted to 10 ng/μl.

Polymerase chain reaction (PCR) amplification and T-RFLP analysis

The 16S rRNA gene was amplified from human fecal DNA using the 27 forward primer (5′-AGAGTTTGATCC TGGCTCAG-3′) and the 1492 reverse primer (5′-GGT TACCTTGTTACGACTT-3′) [28, 29]. The 5′-ends of the forward primers were labeled with 6′-carboxyfluorescein (6-FAM), which was synthesized by Applied Biosystems Japan (Tokyo, Japan). The PCR amplifications of the DNA samples (10 ng of each DNA) were performed according to a protocol previously described [28, 29] The amplified 16S rDNA genes were purified using MultiScreen PCR micro96 Plate (Merck Millipore, Tokyo, Japan) and dissolved in 40 μL of distilled water.

The restriction enzymes were selected according to Matsumoto et al. [28]. The purified PCR products (2 μl) were digested with 10 U of *Hha*I and *Msp*I at 37 °C for 3 h. The length of the T-RF fragments was determined with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan) in GeneScan mode. Standard size markers, such as GS 2500 ROX (Applied Biosystems) were used. The fragment sizes were estimated using the local Southern method in the GENESCAN 3.1 software (Applied Biosystems). Because the apparent size of identical T-RFs can vary over a range of 1–3 bp, major T-RFs similar in size to 1–3 bp were summarized to operational taxonomic units (OTUs). The major T-RFs were identified by computer simulation, which was performed using a T-RFLP analysis program [30], a phylogenetic assignment database for the T-RFLP analysis of human colonic microbiota (PAD-HCM) [28], and a Microbiota Profiler (InfoCom T-RFLP Database & Analysis Software, Infocom, Tokyo, Japan). T-RFs with a peak height of less than 50 fluorescence units were excluded from the analysis. Cluster analyses were performed using BioNumerics software (Applied Maths, Kortijk, Belgium) based on the *Hha*I or *Msp*I T-RFLP patterns. The distances were calculated to determine any similarity among the samples, and were represented graphically by constructing a dendrogram. Pearson's similarity coefficient analysis, and the unweighted pair-group methods with arithmetic means (UPGMA) were used to establish the type of dendrogram. Shannon–Wiener indexes [31] were used to compare the diversity among the different samples.

Statistical analysis

Differences between different samples were checked for statistical significance ($P < 0.05$) using non-parametric tests (the Kruskal–Wallis and Steel–Dwass tests). The data were analyzed using JMP 8.0 and Statview 5.0 (SAS Institute, Cary, NC, USA) software. Multivariate logistic regression analysis was performed with a stepwise method.

Results

The baseline characteristics of the 67 patients with active CD and the 121 healthy individuals are shown in Table 1. Fecal samples were collected from all subjects at entry. However, for several reasons (e.g., patients receiving surgical treatment during the study course or failure to achieve or keep remission, or inadequate sampling, such as missing samples or too small a volume for analysis), the numbers of available samples were reduced at the remission-achieved and remission-maintained phases. Consequently, 51 samples were available at the remission-achieved phase and 43 samples were available at the remission-maintained phase.

Initially, we assessed the possibility of the presence of regional differences in fecal microbiota profiles in Japan. The fecal microbiota profiles of 121 healthy individuals from different districts of Japan were analyzed. Based on the results of the *Hha*I/*Msp*I-digested T-RF patterns, the fecal microbial profiles were compared and illustrated by a dendrogram constructed by using a minimal variance algorithm. As shown in Fig. 1, there was no major cluster characteristic for each district, and we concluded that the regional differences in the fecal microbiota profiles in our study were negligible.

Next, we performed T-RFLP analyses of the 161 fecal samples from the CD patients (67 in active phase, 51 in

Table 1 Baseline characteristics of study population at entry

	Healthy individuals (n = 121)	CD patients (n = 67)
Female/male	52/69	26/41
Age (years) (mean ± SD)	32.1 ± 9.4	30.1 ± 11.6
CDAI (mean ± SD)	–	260.1 ± 59.3
Disease location		
Ileal	–	16
Ileocolonic	–	40
Colonic	–	11
Remission induction therapy		
5-ASA	–	42
Corticosteroid	–	11
Thiopurine	–	9
Infliximab	–	37
Enteral nutrition	–	26

CD Crohn's disease, *CDAI* Crohn's disease activity index, *5-ASA* 5-aminosalicylic acid

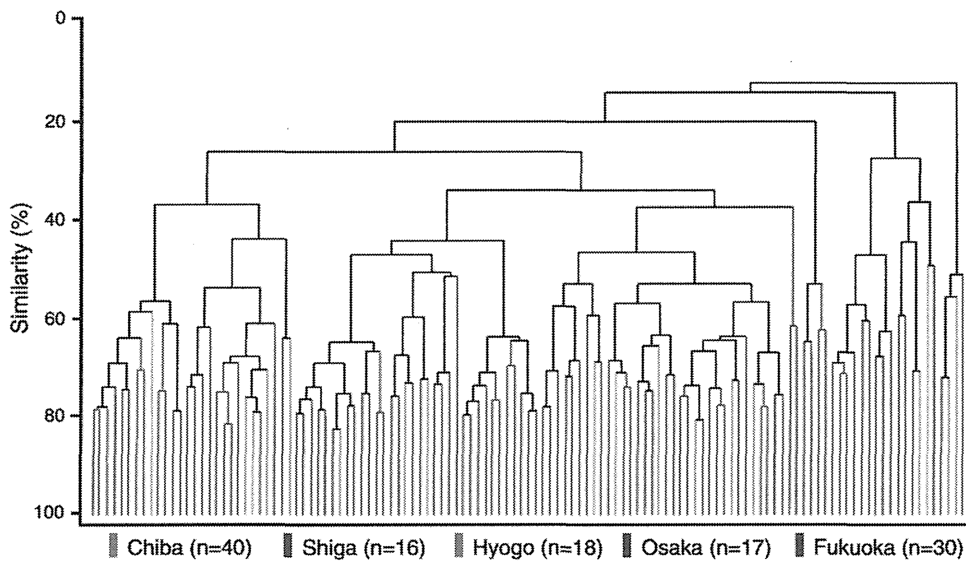

Fig. 1 Dendrogram of the fecal microbiota profiles of 121 healthy individuals from different districts in Japan (16 individuals from Shiga prefecture, 45 individuals from Hyogo and Osaka, 30 individuals from Fukuoka, and 40 individuals from Chiba). The location of each prefecture is illustrated in supplementary Fig. 1. The terminal restriction fragment length polymorphism (T-RFLP) patterns generated by *Hha*I and *Msp*I digestion were analyzed by Pearson's correlation and the unweighted pair-group methods with arithmetic means (UPGMA) algorithm

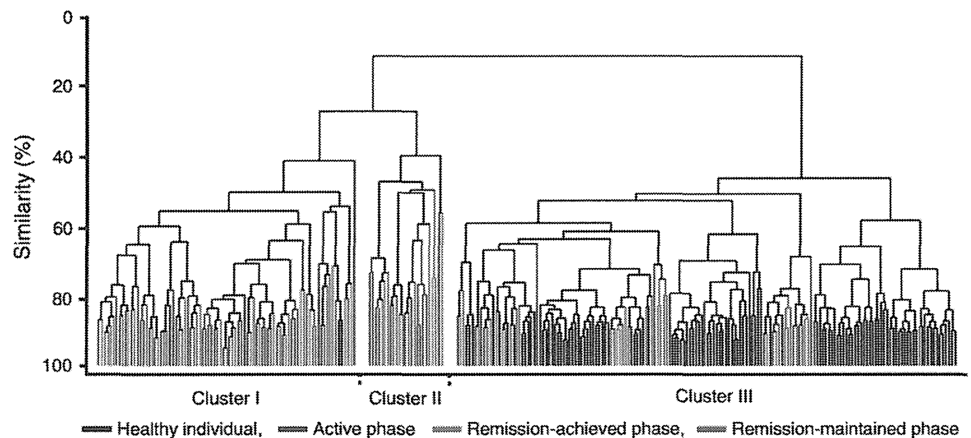

Fig. 2 Dendrogram of the fecal microbiota profiles of 161 fecal samples from Crohn's disease (CD) patients (67 active phase, 51 remission-achieved phase, and 43 remission-maintained phase) and 121 samples from healthy individuals. The T-RFLP patterns generated by *Hha*I and *Msp*I digestion were analyzed by Pearson's correlation and the UPGMA algorithm

remission-achieved phase, and 43 in remission-maintained phase) and the 121 samples from the healthy individuals (Fig. 2). A setting of similarity generated three major clusters. Almost all the healthy individuals (118/121) were included in cluster III, and the remaining three healthy individuals were classified in cluster I, while no healthy individuals were in cluster II (Table 2). On the other hand, the majority of CD patients during the active phase were classified in cluster I or II (38 + 12/67; 74.6 %). Similarly, the majority of patients in the remission-achieved (64.7 %) and maintained (60.5 %) phases were classified in cluster I or II. There were no specific clusters for disease activity. Thus, the fecal bacterial communities differed only between the healthy individuals and the CD patients.

The changes in representative OTUs after *Hha*I and *Msp*I digestion are shown in Tables 3 and 4. Each OTU was expressed as the percent AUC (percentage values of the individual OTU area per total OTU area), which is associated with the predominance of the bacterial composition of each peak. Some *Hha*I-associated OTUs predicting the class *Clostridia* [561-bp (*Clostridium* subcluster XIVa), 1064-bp (*Clostridium* cluster IV), and 1079-bp (*Clostridium* subcluster XIVa)] were significantly decreased in active-phase CD patients as compared with findings in the healthy individuals (Table 3), and these changes were maintained during the entire study course. Similar results were also observed in the changes of the *Msp*I-associated OTUs predicting the class *Clostridia* (206, 221, 225-bp OTUs)

(Table 4). These changes were also observed in other members of the class *Clostridia*. The genus *Faecalibacterium* (*Hha*I 32-bp OUT and *Msp*I 285-bp OTU), a member of the *Clostridium* cluster IV, was significantly decreased during both the active and remission phases of CD as compared with findings in healthy individuals. The genus *Ruminococcus* (89-bp *Hha*I OTU and 202-bp *Msp*I OTU) revealed a significant decrease during the active phase of CD as compared with findings in the healthy individuals, but the levels then increased in proportion to improvements in disease activity, reaching the same levels as those in the healthy individuals during the remission-maintained phase.

Table 2 Distribution of fecal microbiota profiles in CD patients and healthy individuals, in three major clusters

	Cluster I	Cluster II	Cluster III
Healthy individuals ($n = 121$)	3 (2.4 %)	0 (0 %)	118 (97.6 %)
Crohn's disease			
Active phase ($n = 67$)	38 (56.7 %)	12 (17.9 %)	17 (25.3 %)
Remission-achieved phase ($n = 51$)	23 (45.0 %)	10 (19.6 %)	18 (35.3 %)
Remission-maintained phase ($n = 43$)	23 (53.5 %)	3 (6.8 %)	17 (39.5 %)

Based on the results of terminal restriction fragment length polymorphism (T-RFLP) analysis of fecal samples of CD patients and healthy individuals, a setting of similarity generated three major clusters, shown on the dendrogram in Fig. 2), in which the composition of each cluster is presented

In contrast to the decrease in the class *Clostridia*, the genus *Bacteroides*, predicted by the *Hha*I-associated 101-bp OTU and the *Msp*I-associated 94-bp OTU, was significantly increased during the active phase as compared with levels in the healthy individuals. The levels of this genus tended to decrease according to improvements in disease activity, although a significant difference from the levels in the healthy individuals remained even in the remission-maintained phase. The genus *Bifidobacterium*, predicted by the *Msp*I-associated 133-bp OTU, was significantly decreased during the active phase, but increased to healthy levels in the remission-maintained phase.

Using the Shannon diversity index (SDI) [31] to measure the bacterial diversity within the 161 fecal samples from the CD patients (67 active phase, 51 remission-achieved phase, and 43 remission-maintained phase) and the 121 samples from the healthy individuals (Fig. 3), we found that the healthy individuals generated the highest SDI figures, and that these values were significantly higher than those from the CD patients in both the active and remission phases. There was a trend towards a slight increase in the SDI according to improvements in disease activity, but these changes were not statistically significant.

Next, we attempted to predict the disease activity using the results from the T-RFLP analysis. From the clinical data and OTUs obtained from the *Hha*I and *Msp*I digestion, we selected 10 OTUs to form a model to predict the disease activity, using a stepwise model selection method (Table 5). A logistic model is generally given by the following formula [32]:

Table 3 OTUs with significant changes (*Hha*I digestion)

OTU	Representative bacteria predicted by T-RF length	Healthy individuals	Active phase	Remission - achieved phase	Remission - maintained phase	*P* value
32	*Faecalibacterium, Fusobacterium, Eubacterium, Clostridium* cluster IV	8.1 ± 4.3^{a}	4.0 ± 9.2^{b}	3.5 ± 6.1^{b}	4.4 ± 7.5^{b}	<0.0001
62	*Clostridium* cluster XI	0.6 ± 1.7^{b}	1.8 ± 5.4^{a}	2.0 ± 3.1^{a}	2.0 ± 2.0^{a}	<0.0001
64	*Clostridium* subcluster XIVa	2.5 ± 0.8^{a}	0.6 ± 1.0^{b}	0.3 ± 0.6^{b}	0.4 ± 1.1^{b}	<0.0001
101	*Bacteroides, Prevotella*	16.6 ± 5.2^{b}	21.6 ± 15.4^{a}	20.6 ± 12.3^{a}	19.9 ± 10.0^{ab}	0.0003
189	*Ruminococcus, Eubacterium, Clostridium* subcluster XIVa	3.8 ± 3.5^{a}	1.5 ± 3.1^{b}	1.4 ± 2.9^{b}	3.6 ± 6.0^{b}	<0.0001
373	*Bifidobacterium, Enterobacter, Escherichia, Proteus*	1.4 ± 2.0^{a}	0.7 ± 2.1^{b}	1.1 ± 3.6^{b}	0.5 ± 2.2^{b}	<0.0001
376	*Proteus*	3.0 ± 3.0^{b}	8.3 ± 13.2^{a}	7.7 ± 11.0^{ab}	9.0 ± 9.5^{a}	0.0002
561	*Clostridium* subcluster XIVa	2.1 ± 1.9^{a}	1.1 ± 1.4^{b}	1.2 ± 1.6^{b}	1.1 ± 1.5^{b}	0.0006
579	*Streptococcus*	1.1 ± 2.4^{a}	2.2 ± 6.2^{ab}	0.4 ± 1.6^{b}	2.4 ± 6.7^{a}	0.0235
1050	*Clostridium* cluster XI	0.5 ± 1.0^{b}	0.8 ± 1.4^{ab}	1.4 ± 3.3^{ab}	1.0 ± 1.2^{a}	0.0212
1064	*Clostridium* cluster XI	2.6 ± 3.0^{a}	0.5 ± 0.6^{b}	0.4 ± 1.0^{b}	0.3 ± 1.0^{b}	<0.0001
1079	*Ruminococcus, Clostridium* cluster IV	17.6 ± 11.5^{a}	9.3 ± 8.6^{b}	9.0 ± 9.3^{b}	9.9 ± 11.7^{b}	<0.0001
Others		37.6 ± 12.0^{b}	44.8 ± 16.8^{a}	47.8 ± 14.4^{a}	43.1 ± 12.8^{ab}	<0.0001

Each value indicates the percentage of the individual operational taxonomic unit (OTU) area per total OTU area after *Hha*I digestion. Values are expressed as means ± SD. Values not sharing a superscript letter are significantly different

Table 4 OTUs with significant changes (*Msp*I digestion)

OTU	Representative bacteria predicted by T-RF length	Healthy individuals	Active phase	Remission - achieved phase	Remission - maintained phase	*P* value
61	*Clostridium* subcluster XIVa	0.1 ± 0.3^{b}	0.8 ± 3.9^{a}	0.4 ± 0.7^{a}	0.7 ± 0.9^{a}	<0.0001
81	*Bacteroides*	0.1 ± 0.2^{b}	0.3 ± 0.8^{a}	0.5 ± 1.7^{a}	0.4 ± 1.4^{a}	0.0004
83	*Bacteroides, Ruminococcus*	0.1 ± 0.3^{b}	0.2 ± 0.6^{a}	0.5 ± 1.3^{a}	0.2 ± 0.6^{ab}	0.0001
94	*Bacteroides*	3.1 ± 2.2^{b}	9.6 ± 8.6^{a}	8.6 ± 7.3^{a}	8.0 ± 7.7^{a}	<0.0001
96	*Prevotella*	6.6 ± 4.0^{ab}	3.4 ± 4.7^{c}	4.0 ± 4.3^{bc}	6.5 ± 6.0^{a}	<0.0001
98	*Bacteroides, Prevotella*	6.4 ± 6.6^{a}	1.1 ± 4.9^{c}	1.8 ± 5.3^{bc}	2.7 ± 5.1^{b}	<0.0001
127	*Mesorhizobium* (α proteobacteria)	0.8 ± 1.0^{a}	0.5 ± 1.3^{b}	0.9 ± 3.2^{b}	0.3 ± 0.7^{b}	<0.0001
129	*Mesorhizobium* (α proteobacteria)	1.5 ± 1.7^{a}	0.3 ± 0.5^{b}	0.3 ± 1.0^{b}	0.2 ± 0.7^{b}	<0.0001
133	*Bifidobacterium*	2.6 ± 3.3^{a}	1.4 ± 3.2^{b}	2.1 ± 3.7^{ab}	2.6 ± 4.1^{ab}	0.0024
142	*Eubacterium*	2.0 ± 1.7^{a}	0.8 ± 1.4^{b}	1.0 ± 1.8^{b}	0.6 ± 1.3^{b}	<0.0001
144	*Clostridium* subcluster XIVa	0.9 ± 1.0^{a}	0.6 ± 3.2^{b}	0.1 ± 0.3^{b}	0.4 ± 1.7^{b}	<0.0001
147	*Clostridium* subcluster XIVa	0.5 ± 1.6^{b}	0.9 ± 2.6^{a}	0.6 ± 1.4^{ab}	2.5 ± 5.1^{a}	0.0002
190	*Lactobacillus*	1.3 ± 1.6^{b}	3.3 ± 7.3^{ab}	5.0 ± 8.5^{a}	3.9 ± 6.5^{ab}	0.0012
206	*Ruminococcus, Clostridium* subcluster XIVa	1.1 ± 1.5^{a}	0.1 ± 0.2^{b}	0.1 ± 0.3^{b}	0.3 ± 1.0^{b}	<0.0001
213	*Clostridium* subcluster XIVa	0.7 ± 0.8^{b}	1.9 ± 2.1^{a}	2.1 ± 3.6^{a}	2.1 ± 4.4^{ab}	0.0013
221	*Ruminococcus, Clostridium* subcluster XIVa	21.9 ± 6.5^{a}	15.4 ± 11.3^{b}	18.3 ± 13.4^{ab}	18.2 ± 11.1^{ab}	0.0013
225	*Clostridium* subcluster XIVa	3.9 ± 4.0^{a}	0.2 ± 1.3^{b}	0.2 ± 1.3^{b}	1.1 ± 3.8^{b}	<0.0001
285	*Faecalibacterium, Clostridium* cluster IV	8.5 ± 6.2^{a}	2.0 ± 3.7^{b}	1.5 ± 2.9^{b}	1.1 ± 2.8^{b}	<0.0001
302	*Veillonella, Clostridium* cluster IX, *Clostridium* cluster IV	4.7 ± 4.1^{a}	1.1 ± 1.9^{b}	1.8 ± 3.2^{b}	1.0 ± 1.7^{b}	<0.0001
305	*Clostridium* cluster IV	2.3 ± 3.0^{a}	1.8 ± 4.8^{b}	1.1 ± 2.0^{b}	1.5 ± 2.3^{ab}	0.0021
489	*Clostridium* subcluster XIVb	1.7 ± 1.1^{b}	3.4 ± 3.8^{a}	2.7 ± 2.4^{a}	2.2 ± 2.0^{ab}	0.0055
556	*Streptococcus, Leuconostoc*	3.3 ± 3.6^{b}	7.4 ± 7.7^{a}	5.9 ± 6.4^{a}	7.2 ± 8.3^{a}	<0.0001
563	*Streptococcus*	0.2 ± 0.6^{b}	4.7 ± 12.1^{a}	4.2 ± 8.2^{a}	2.9 ± 6.6^{a}	<0.0001
others		25.2 ± 9.7^{b}	36.3 ± 16.6^{a}	33.8 ± 18.6^{a}	31.4 ± 15.4^{ab}	<0.0001

Each value indicates the percentage of the individual OTU area per total OTU area after *Msp*I digestion. Values are expressed as means ± SD. Values not sharing a superscript letter are significantly different

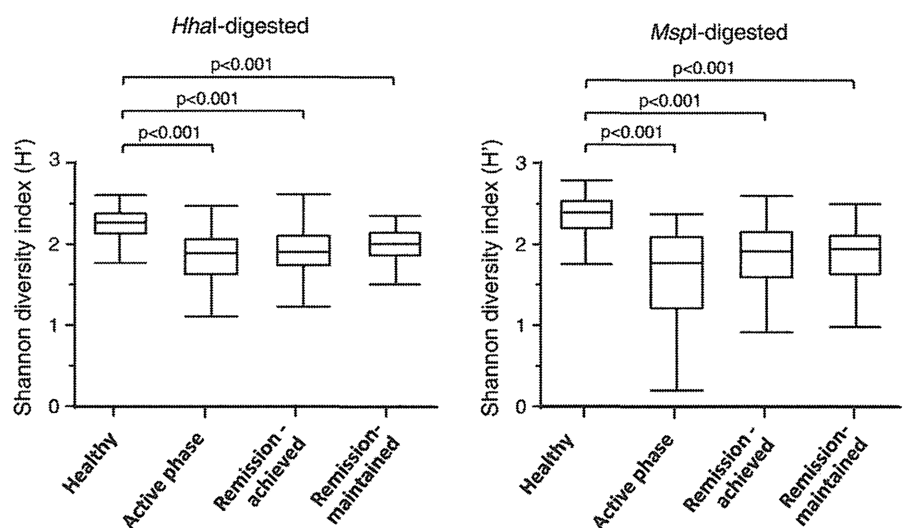

Fig. 3 Comparison of fecal bacterial diversity between CD patients and healthy individuals. The Shannon diversity index (SDI) [31] was calculated from the *Hha*I- and *Msp*I-digested T-RF patterns. A significantly reduced SDI as compared with healthy individuals is indicated by the *P* values

Table 5 Maximum likelihood estimates of logistic regression function

Variable	Estimated regression coefficient	Estimated SE	Wald χ^2	P	Estimated odds ratio	95 % confidence interval (CI) for odds ratio
Intercept	−3.939	0.903	19.034	<0.0001	–	–
*Hha*I 101	0.070	0.023	9.738	0.0018	1.073	1.026–1.121
*Hha*I 214	0.066	0.030	4.960	0.0259	1.069	1.008–1.133
*Hha*I 392	0.468	0.185	6.394	0.0114	1.596	1.111–2.294
*Hha*I 548	0.465	0.192	5.868	0.0154	1.592	1.093–2.318
*Hha*I 810	−0.514	0.145	12.620	0.0004	0.598	0.451–0.794
*Msp*I 64	0.274	0.092	8.832	0.0030	1.315	1.098–1.576
*Msp*I 456	−0.396	0.125	10.031	0.0015	0.673	0.527–0.860
*Msp*I 489	0.338	0.100	11.429	0.0007	1.401	1.152–1.704
*Msp*I 499	0.057	0.021	7.505	0.0062	1.058	1.016–1.102
*Msp*I 503	1.422	0.381	13.918	0.0002	4.145	1.964–8.748

Variables indicate *Hha*I- or *Msp*I-digested OTUs

$$\log(P/1-P) = \beta_0 + \beta_1\chi_1 + \beta_2\chi_2 + \cdots + \beta_n\chi_n = \beta X, P = e^{\beta X}/\left(1 + e^{\beta X}\right)$$

where P is the probability of the event, β_0 the intercept, β_n the parameter, and χ_n the variable. Our model to predict the disease activity of a CD patients is:

$$\log(P/1-P) = -3.939 + 0.070 \times \text{H101 (percent of } \textit{Hha}\text{I OTU101)} + 0.066 \times \text{H214} + 0.468 \times \text{H392} + 0.465 \times \text{H548} - 0.514 \times \text{H810} + 0.274 \times \text{M64 (percent of } \textit{Msp}\text{I OTU64)} - 0.396 \times \text{M456} + 0.337 \times \text{M489} + 0.057 \times \text{M499} + 1.422 \times \text{M503}.$$

When the cut-off score (P) was 0.5 [$P \geq 0.5$ for active disease (CDAI >150) and $P < 150$ for inactive disease (CDAI <150)], the accuracy of this model to predict disease activity was 0.826. Validations were performed using split data sets, in which the model was trained on a randomly selected subset of half of the data, and tested on the remaining data. The validation tests were repeated for different selections of the training and test data. The models produced were similar to the original, and performed nearly as well on the test data as on the training data.

Discussion

To our knowledge, this is one of the largest T-RFLP studies investigating the microbiota profiles of healthy individuals and CD patients in the Japanese population. In contrast to earlier studies using T-RFLP analyses [24, 25, 33–35], which each examined a relatively small number of samples from a single institute, we sought instead to add to the current knowledge by obtaining a greater number of samples from multiple centers located in different geographic districts in Japan. T-RFLP analysis does not have the high resolution of the 16S rRNA clone library [16, 36], which inevitably handles a smaller number of samples owing to the depth of the molecular analysis required for each sample, but T-RFLP analysis is potentially useful for assessing a large number of samples and it enables rapid comparisons of the community structure between samples [29, 37]. In the present study, we provided data from a total of 282 fecal samples from both healthy individuals and CD patients.

De Filippo et al. [26] recently demonstrated a difference in gut microbiota between European and African children, suggesting that environmental factors such as diet, ethnicity, sanitation, and hygiene are important for shaping the gut microbiota [38]. This finding leads to the possibility of the presence of regional differences in the gut microbiota of the Japanese population, because there are different lifestyles in different regions in this country. In addition, prior to a multicenter study investigating the gut microbiota of CD patients, such a possibility should be assessed as a potential factor influencing the results from these patients. However, regional differences of the gut microbiota have not been previously investigated in Japan. Therefore, in the present study, we performed T-RFLP analyses of fecal samples from 121 healthy individuals living in different districts of Japan (Shiga, Hyogo, Osaka, Fukuoka, and Chiba prefectures); we observed that the gut microbiota profile characteristics for each of these districts were similar. Thus, in this multicenter study, regional differences in the gut microbiota were considered to be negligible.

Previous studies have reported that individual species such as *Mycobacterium avium* species or *Escherichia coli*

have been implicated in the disease etiology of CD [39, 40], but the emerging dysbiosis hypothesis instead emphasizes the importance of an overall imbalance of the global composition of the gut microbiota [6]. Numerous studies have attempted to characterize the microbial communities in IBD, and to compare these communities with those in healthy individuals. As demonstrated in the recent review by Nagalingam and Lynch [23], in individuals with IBD, bacterial diversity, temporal stability, and clusters are reduced when compared with findings in healthy controls [8, 16, 23, 24, 34, 36]. Compositional comparisons have generated inconsistent results, but have generally identified reductions in species from the phylum *Firmicutes*, mainly class *Clostridia*, and often, but not always, with concurrent increases in the phylum *Bacteroidetes* and the family *Enterobacteriaceae*, including *E. coli* [8, 16, 23, 24, 36]. Our observations from the present study, such as the reduced diversity of the microbiota community and reduced abundance of the class *Clostridia* (including the genus *Ruminococcus*), agree with the results from previous reports. We observed an increase in the relative abundance of the phylum *Bacteroidetes* in the CD patients, and this is compatible with a recent study of the clone library analysis for the mucosa-associated microbiota of IBD patients [36].

Among the above changes in the microbiota profiles, a decrease in the relative abundance of the class *Clostridia* is particularly significant for the etiology of CD. Recently, Atarashi et al. [41] have demonstrated that the genus *Clostridium* is a strong inducer of regulatory T cells (Tregs) in the colon. Tregs, which express the Foxp3 transcription factor, play a critical role in the maintenance of immune homeostasis [42]. Therefore, it has been suggested that the *Clostridium*-dependent induction of Tregs may be required for maintaining immune homeostasis in the gut, and that a decrease in the relative abundance of the genus *Clostridium* may lead to a disruption in mucosal homeostasis. Another etiological factor related to the reduction in the class *Clostridia* can be explained by the role of *Faecalibacterium prausnitzii*, a major member of the *Clostridium* cluster IV. Sokol et al. recently reported a reduction in *F. prausnitzii* in the gut microbiota of CD patients [43, 44]. *F. prausnitzii* possesses strong anti-inflammatory activities both in vitro and in vivo [43], and a reduced abundance of this bacterium was closely associated with relapses in CD activity. In the present study, the T-RFLP analysis showed that OTUs simulating the genus *Faecalibacterium* were significantly decreased in CD patients with active and inactive disease. As *F. prausnitzii* is the sole species reported as a member of the genus *Faecalibacterium* [45], this strongly suggests that our T-RFLP analysis demonstrated a decrease in *F. prausnitzii* in the patients' feces. Thus, a decrease in bacteria of the class *Clostridia*, including the genus *Faecalibacterium*, in the gut microbiota, was observed in our study. This decrease may translate into a reduction of bacteria-mediated anti-inflammatory activity in the mucosa– activity which is relevant to the pathophysiology of CD.

Recent studies have shown that the gut microbiota play a crucial role in energy harvesting, and that there is a remarkable difference in fecal microbiota composition between obese and lean individuals. Ley et al. [46] have reported that the proportion of the phylum *Bacteroidetes* relative to that of the phylum *Firmicutes* is decreased in obese people in comparison with lean people, suggesting that a decrease of the phylum *Bacteroidetes* relative to the phylum *Firmicutes* (*Clostridium*) is related to an increased capacity to harvest energy from the diet. In accordance with that report, our present observations of the decrease in the phylum *Firmicutes* relative to the phylum *Bacteroidetes* suggest reduced energy harvesting in CD patients. Future studies should evaluate the relationships among luminal metabolic alterations, quantitative changes in the gut microbiota composition, and the nutritional status of patients.

In the present study, we attempted to predict the clinical disease activity of CD patients from their fecal microbiota profiles during the active and remission phases. A predictive equation was developed using multivariate logistic regression analysis and the data from *Hha*I and *Msp*I-associated OTUs. We selected ten OTUs to form a model to predict the clinical disease activity, using a stepwise model selection method, and the accuracy of this model to predict clinical disease activity was 0.826. There are a number of limitations of the available diagnostic tests for CD: some do not always correlate with intestinal inflammation; an accurate diagnosis is often limited to active disease only; some tests are invasive requiring blood or biopsy collection; and none of the tests stands alone as a "gold standard", thus they are used as a supplement to endoscopy [47]. Therefore, it was encouraging that our predictive model based on fecal microbiota composition successfully determined the clinical disease activity of CD. This prediction model may make it possible to customize a therapeutic strategy according to the fecal microbiota composition of an individual CD patient.

Recently, we have reported that fecal calprotectin is a useful marker for the prediction of endoscopic disease activity in IBD patients [48]. This finding led us to evaluate the relationship between fecal calprotectin levels and fecal microbiota profiles. However, a preliminary study showed that there were only few OTUs exhibiting a weak correlation with fecal calprotectin levels (data not shown), indicating that the prediction of mucosal inflammatory status by fecal microbiota profiles may not be feasible at present. One factor contributing to this difficulty may be inter-individual differences in fecal microbiota profiles.

Thus, the fecal microbiota profile may be useful for the prediction of general disease activity such as the active or inactive phases determined by the CDAI, but the use of this profile is still insufficient for the precise prediction of activity that can be determined endoscopically. In the future, evaluation of fecal calprotectin and the microbiota profile together may enhance the accuracy of the prediction of disease activity.

In conclusion, we initially showed that there were no specific fecal microbiota profiles characterizing healthy individuals living in different districts of Japan. Based on this background, we performed T-RFLP analysis, and showed that CD patients from multiple IBD centers had different microbiota compositions and reduced bacterial diversity when compared with healthy controls. These changes in the CD patients were characterized by a reduced abundance of the class *Clostridia*, including the genus *Faecalibacterium*. In addition, we proposed a prediction model for the disease activity of CD patients based on their fecal microbiota profile. Further investigations into gut microbiota profiles will lead to novel therapeutic developments and improved diagnostics for IBD in the future.

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Conflict of interest The authors have no conflict of interests to declare in this study.

References

1. Sands BE. Inflammatory bowel disease: past, present, and future. J Gastroenterol. 2007;42:16–25.
2. Mayer L. Evolving paradigms in the pathogenesis of IBD. J Gastroenterol. 2010;45:9–16.
3. Hibi T, Ogata H. Novel pathophysiological concepts of inflammatory bowel disease. J Gastroenterol. 2006;41:10–6.
4. Podolsky DK. Inflammatory bowel disease. N Engl J Med. 2002;347:417–29.
5. Hamilton MJ, Snapper SB, Blumberg RS. Update on biologic pathways in inflammatory bowel disease and their therapeutic relevance. J Gastroenterol. 2012;47:1–8.
6. Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology. 2008;134:577–94.
7. Mizoguchi A, Mizoguchi E. Inflammatory bowel disease, past, present and future: lessons from animal models. J Gastroenterol. 2008;43:1–17.
8. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol. 2006;3:390–407.
9. Wirtz S, Neurath MF. Mouse models of inflammatory bowel disease. Adv Drug Deliv Rev. 2007;59:1073–83.
10. Braun J, Wei B. Body traffic: ecology, genetics, and immunity in inflammatory bowel disease. Annu Rev Pathol. 2007;2:401–29.
11. Seksik P, Sokol H, Lepage P, Vasquez N, Manichanh C, Mangin I, et al. Review article: the role of bacteria in onset and perpetuation of inflammatory bowel disease. Aliment Pharmacol Ther. 2006;24(Suppl 3):11–8.
12. Elson CO, Cong Y, McCracken VJ, Dimmitt RA, Lorenz RG, Weaver CT. Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. Immunol Rev. 2005;206:260–76.
13. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature. 2001;411:603–6.
14. Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature. 2008;456:259–63.
15. Kabi A, Nickerson KP, Homer CR, McDonald C. Digesting the genetics of inflammatory bowel disease: Insights from studies of autophagy risk genes. Inflamm Bowel Dis. 2012;18:782–92.
16. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci USA. 2007;104:13780–5.
17. Matsuda H, Fujiyama Y, Andoh A, Ushijima T, Kajinami T, Bamba T. Characterization of antibody responses against rectal mucosa-associated bacterial flora in patients with ulcerative colitis. J Gastroenterol Hepatol. 2000;15:61–8.
18. Lucke K, Miehlke S, Jacobs E, Schuppler M. Prevalence of *Bacteroides* and *Prevotella* spp. in ulcerative colitis. J Med Microbiol. 2006;55:617–24.
19. Swidsinski A, Ladhoff A, Pernthaler A, Swidsinski S, Loening-Baucke V, Ortner M, et al. Mucosal flora in inflammatory bowel disease. Gastroenterology. 2002;122:44–54.
20. Hayashi H, Sakamoto M, Benno Y. Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. Microbiol Immunol. 2002;46:535–48.
21. Langendijk PS, Schut F, Jansen GJ, Raangs GC, Kamphuis GR, Wilkinson MH, et al. Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl Environ Microbiol. 1995;61:3069–75.
22. Andoh A, Benno Y, Kanauchi O, Fujiyama Y. Recent advances in molecular approaches to gut microbiota in inflammatory bowel disease. Curr Pharm Des. 2009;15:2066–73.
23. Nagalingam NA, Lynch SV. Role of the microbiota in inflammatory bowel diseases. Inflamm Bowel Dis. 2012;18:968–84.
24. Andoh A, Imaeda H, Aomatsu T, Inatomi O, Bamba S, Sasaki M, et al. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. J Gastroenterol. 2011;46:479–86.
25. Andoh A, Tsujikawa T, Sasaki M, Mitsuyama K, Suzuki Y, Matsui T, et al. Fecal microbiota profile of Crohn's disease determined by terminal restriction fragment length polymorphism (t-rflp) analysis. Aliment Pharmacol Ther. 2009;29:75–82.
26. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc Natl Acad Sci USA. 2010;107:14691–6.
27. Best WR, Becktel JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index National Cooperative Crohn's Disease Study. Gastroenterology. 1976;70:439–44.
28. Matsumoto M, Sakamoto M, Hayashi H, Benno Y. Novel phylogenetic assignment database for terminal-restriction fragment length polymorphism analysis of human colonic microbiota. J Microbiol Methods. 2005;61:305–19.
29. Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y. Application of terminal RFLP analysis to characterize oral

bacterial flora in saliva of healthy subjects and patients with periodontitis. J Med Microbiol. 2003;52:79–89.
30. Marsh TL, Saxman P, Cole J, Tiedje J. Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. Appl Environ Microbiol. 2000;66:3616–20.
31. Hill TC, Walsh KA, Harris JA, Moffett BF. Using ecological diversity measures with bacterial communities. FEMS Microbiol Ecol. 2003;43:1–11.
32. Chiang JK, Cheng YH, Koo M, Kao YH, Chen CY. A computer-assisted model for predicting probability of dying within 7 days of hospice admission in patients with terminal cancer. Jpn J Clin Oncol. 2010;40:449–55.
33. Andoh A, Sakata S, Koizumi Y, Mitsuyama K, Fujiyama Y, Benno Y. Terminal restriction fragment length polymorphism analysis of the diversity of fecal microbiota in patients with ulcerative colitis. Inflamm Bowel Dis. 2007;13:955–62.
34. Nishikawa J, Kudo T, Sakata S, Benno Y, Sugiyama T. Diversity of mucosa-associated microbiota in active and inactive ulcerative colitis. Scand J Gastroenterol. 2009;44:180–6.
35. Nomura T, Ohkusa T, Okayasu I, Yoshida T, Sakamoto M, Hayashi H, et al. Mucosa-associated bacteria in ulcerative colitis before and after antibiotic combination therapy. Aliment Pharmacol Ther. 2005;21:1017–27.
36. Walker AW, Sanderson JD, Churcher C, Parkes GC, Hudspith BN, Rayment N, et al. High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease. BMC Microbiol. 2011;11:7.
37. Schutte UM, Abdo Z, Bent SJ, Shyu C, Williams CJ, Pierson JD, et al. Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. Appl Microbiol Biotechnol. 2008;80:365–80.
38. Kurokawa K, Itoh T, Kuwahara T, Oshima K, Toh H, Toyoda A, et al. Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. DNA Res. 2007;14:169–81.
39. Feller M, Huwiler K, Stephan R, Altpeter E, Shang A, Furrer H, et al. *Mycobacterium avium subspecies paratuberculosis* and Crohn's disease: a systematic review and meta-analysis. Lancet Infect Dis. 2007;7:607–13.
40. Barnich N, Darfeuille-Michaud A. Adherent-invasive *Escherichia coli* and Crohn's disease. Curr Opin Gastroenterol. 2007;23:16–20.
41. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, et al. Induction of colonic regulatory T cells by indigenous *Clostridium* species. Science. 2011;331:337–41.
42. Hall JA, Bouladoux N, Sun CM, Wohlfert EA, Blank RB, Zhu Q, et al. Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. Immunity. 2008;29:637–49.
43. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, et al. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci USA. 2008;105:16731–6.
44. Sokol H, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, et al. Low counts of *Faecalibacterium prausnitzii* in colitis microbiota. Inflamm Bowel Dis. 2009;15:1183–9.
45. Duncan SH, Hold GL, Harmsen HJ, Stewart CS, Flint HJ. Growth requirements and fermentation products of *Fusobacterium prausnitzii*, and a proposal to reclassify it as *Faecalibacterium prausnitzii* gen. nov., comb. nov. Int J Syst Evol Microbiol. 2002;52:2141–6.
46. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature. 2006;444:1022–3.
47. Aomatsu T, Imaeda H, Matsumoto K, Kimura E, Yoden A, Tamai H, et al. Faecal chitinase 3-like-1: a novel biomarker of disease activity in paediatric inflammatory bowel disease. Aliment Pharmacol Ther. 2011;34:941–8.
48. Aomatsu T, Yoden A, Matsumoto K, Kimura E, Inoue K, Andoh A, et al. Fecal calprotectin is a useful marker for disease activity in pediatric patients with inflammatory bowel disease. Dig Dis Sci. 2011;56:2372–7.

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HIF-1 in T cells ameliorated dextran sodium sulfate-induced murine colitis

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ABSTRACT

HIF-1 is active in hypoxia, such as inflamed mucosa, and HIF-1 in epithelium has been reported to control inflamed mucosa in IBD models. Although T cells play an important role for pathogenesis of IBD, the function of HIF-1 in T cells remains to be elucidated. We aimed to clarify the function of HIF-1 in T cells in IBD with focus on the balance between Treg and Teff. Double immunohistochemistry of colonic mucosa in IBD patients showed that HIF-1 was expressed in T cells infiltrating the inflamed mucosa, suggesting that HIF-1 in T cells is involved in the pathogenesis. DSS administration to T cell-specific HIF-1α KO mice showed more severe colonic inflammation than control mice with the up-regulation of Th1 and Th17. Hypoxic stimulation in vitro increased Treg activation in WT T cells but not in HIF-1-deleted T cells. In contrast, hypoxic stimulation increased Th17 activation, and the degree was higher in HIF-1-deleted cells than in control cells. These results show that hypoxia controls intestinal inflammation by regulating cytokine balance in an HIF-1-dependent manner, suggesting that strengthening HIF-1 induction in T cells at the sites of inflammation might be a therapeutic strategy for IBD regulation. *J. Leukoc. Biol.* 91: 000–000; 2012.

Introduction

The etiology of IBD, including UC and CD, is still unknown. Dysregulated immune responses are thought to be one of the important factors for development of IBD [1], and the involvement of Treg, Th1, and Th17 has been demonstrated [2–4]. Functions of T cells have been studied in animal models of colitis, such as a DSS-induced murine model of colitis, and the involvement of Th1 and Th17 in the pathogenesis of this model has been suggested [5, 6].

In inflamed mucosa, oxygen concentration decreases as a result of vascular change and increased consumption of oxygen by inflammatory cells [7, 8]. In this condition, hypoxia leads to the activation of alternative metabolic pathways that do not require molecular oxygen. Hypoxia-dependent regulation occurs at the transcriptional level and is mediated by HIF-1 [9], which consists of an oxygen-dependent α-subunit and a constitutively expressed β-subunit. Under normoxic conditions, HIF-1α is hydroxylated by HIF PHDs. Under hypoxic conditions, HIF-1α hydroxylation is abrogated, leading to its cellular accumulation to become HIF subunits heterodimerized, aimed to a wide range of target genes [9]. HIF-1 is expressed in many kinds of cells, and its specific function in each cell is distinct, playing a key role in control of the vascular system, maturation of RBCs, energy metabolism, cell proliferation, and viability under the condition of hypoxia [9]. Roles of HIF-1 in immunomodulation have also been reported. HIF-1 has been shown to be essential for the development of granulocytes and monocytes/macrophages [10], and another study has shown that T cell-targeted deletion of HIF-1 could rescue mice from LPS-induced septic death [11]. In a colitis model, HIF-1 in epithelial cells has been found to work as a barrier for protection against mucosal insult [12]. Furthermore, some PHD inhibitors that increase HIF-1 levels by inhibiting HIF-1 hydroxylation ameliorate intestinal inflammation in colitis models, and the mechanism underlying the amelioration of intestinal inflammation has been thought to be enhance-

Abbreviations: CD=Crohn's disease, DSS=dextran sulfate sodium, EAE=experimental autoimmune encephalitis, Foxp3=forkhead box p3, FSC=forward-scatter, HIF-1=hypoxia-inducible factor-1, HIF-1α$^{-/-}$=HIF-1α deletion in lck-Cre-positive mice, HIF-1α$^{+/+}$=HIF-1α-positive lck-Cre-negative mice, IBD=inflammatory bowel disease, KO=knockout, LPMNC=lamina propria mononuclear cell, NDMC=National Defense Medical College, PGK=phosphoglycerate kinase, PHD=prolyl hydroxylase, PSGL-1=P-selectin glycoprotein ligand-1, RBC=red blood cell, RORγt=retinoic acid receptor-related orphan nuclear receptor γt, SSC=side-scatter, Teff=effector T cell, Treg=regulatory T cell, UC=ulcerative colitis

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ment of the barrier function of colonic mucosa by PHD inhibitors [13, 14].

Recently, it has been reported that hypoxia induces an anti-inflammatory program by increasing the number of Tregs and by enhancing suppressive properties of Tregs through HIF-1 activation [15]. Thus, we hypothesized that HIF-1 in T cells, not only in the epithelium, might also play an important role in the regulation of IBD. The aim of this study was to clarify the role of T cell-specific HIF-1 in colitis with focus on the balance between Treg and Teff, including Th1 and Th17.

MATERIALS AND METHODS

Double immunohistochemistry in intestinal mucosa of IBD patients

Samples were prepared as described previously [16]. Primary antibodies include: rabbit polyclonal anti-HIF-1α antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-human T cell CD3 antibody (Dako, Denmark). Secondary antibodies include: biotilate goat anti-rabbit IgG antibody and PE-labeled anti-mouse IgG (BD PharMingen, San Diego, CA, USA). We used a fluorescence microscope, BZ-8000 (Keyence, Osaka, Japan). This study was approved by the Ethics Committee of the NDMC (Saitama, Japan; No. 821).

Animals

Mice with T cell-targeted deletion of the HIF-1α gene have been generated as described previously [17], allowing for a T cell lineage-specific HIF-1$\alpha^{-/-}$. The lck-Cre-negative littermate was used as WT control (HIF-1$\alpha^{+/+}$).

Induction of colitis

DSS (Lot No. 7399J; ICN Biochemicals, Cleveland, OH, USA) was dissolved in water (3.0%). The experimental protocol was approved by the Animal Research Committee of NDMC (No. 10022).

Isolation of LPMNCs

LPMNCs were isolated using a modified protocol, as described previously [18].

Hypoxic study

Cells were cultured for 16 h in a GasPak 100 system using BBL GasPak Plus anaerobic systems envelope with palladium catalyst (Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C (0.2% O_2 after 100 min activation).

T cell isolation

We isolated CD3-positive cells from splenocytes using Pan T Cell Isolation Kit by MACS (Miltenyi Biotec, Auburn, CA, USA) method.

Flow cytometry analysis

In an hypoxic study, we examined expressions of Foxp3 (Mouse Foxp3 Buffer Set, BD PharMingen) and IL-17 secretion (Mouse IL-17 Secretion Assay Detection Kit, Miltenyi Biotec) using the following antibodies: anti-mouse FITC-CD4 (Santa Cruz Biotechnology), anti-mouse PE-Foxp3 (BD PharMingen), and anti-mouse IL-17 mAb conjugated to biotin (Miltenyi Biotec).

FSC and SSC were adjusted to exclude debris and dead cells. Leukocytes were first stained with CD3. Single-parameter histogram of CD3-positive cells was determined compared with nonspecific binding of isotype control. Then, gated, CD3-positive cells were expressed in a FSC/SSC dot plot. The FSC/SSC dot plot gate of lymphocytes was defined as an area of CD3-positive cells.

Preparations for intravital observation

T cells (~1×10^7) were incubated with carboxyfluorescein diacetate succinimidyl ester (CFDSE; Molecular Probes, Eugene, Oregon, USA) as described previously [19]. An ileal segment was prepared, and migration was observed as described previously [19].

mRNA expression by RT-PCR

Total RNA was extracted as described previously [20]. Primer and probes were purchased from Applied Biosystems (Foster City. CA, USA): TNF-α (Mm00443258), IFN-γ (Mm00801778), Foxp3 (Mm00475156), RORγt (Mm01261022), TGF-β (Mm03024053), IL-1β (Mn00434226), IL-6 (Mm00446190), IL-10 (Mm00439616), IL-12a (Mm00434165), IL-17a (Mm00439619), IL-23a (Mm00518984), PGK-1 (Mm00435617), and VEGFA (Mm00437304). The results were standardized to mouse GAPDH in vivo study and mouse β-actin in vitro study.

Statistics

The differences between groups were examined for statistical significance ($P<0.05$) using Mann-Whitney U test or one-way factorial ANOVA, followed by Scheffe's F test.

RESULTS AND DISCUSSION

In this study, we investigated the role of HIF-1 in T cells in colonic inflammation and hypoxic condition from the viewpoint of Treg, Th1, and Th17, and we showed the protective role of HIF-1 in T cells.

Double immunohistochemistry in IBD patients

First, we examined whether HIF-1 was induced in T cells infiltrating inflamed mucosa of IBD patients, including patients with UC (n=6; average age was 44.8, 31~71) and CD (n=6; average age was 33.8, 24~56). Biopsy specimens were taken from colonic mucosa of active lesion. The control mucosa was taken from normal colonic mucosa of colonic polyp patients. **Figure 1** shows representative images of immunostaining for HIF-1α and CD3 in inflamed mucosa of IBD patients (Fig. 1A and B) and in normal mucosa of non-IBD patients (Fig. 1C). At the sites of inflamed intestine in Fig. 1A, HIF-1 immunoreactivity was observed mainly in the lamina propria (green, Fig. 1Aa), and there were also several CD3-positive cells (red, Fig. 1Ab). Some CD3-positive cells coexpressed HIF-1 (yellow, Fig. 1Ac). In the normal control mucosa, 6.0 ± 3.2% of $CD3^+$ cells were positive for HIF-1. In IBD patients, the ratio of HIF-1-expressing cells among $CD3^+$ cells was 35.2 ± 2.4% in CD patients and 22.9 ± 4.8% in UC patients, respectively, and those statistically significant increased compared with that of normal control mucosa. It has been reported that inflamed mucosa becomes hypoxic because of alteration in vascular anatomy and increased inflammatory cell activity [7, 8], and our results, showing that HIF-1 was expressed in inflamed mucosa, are consistent with the results reported previously [21]. Furthermore, our results showing that some T cells expressed HIF-1 suggest that HIF-1 is involved in impaired T cell function in IBD.

Examination of the characteristics of HIF-1-deleted T cells

To investigate the function of HIF-1 in T cells in inflamed colitis, we used T cell- specific conditional KO mice. We matched

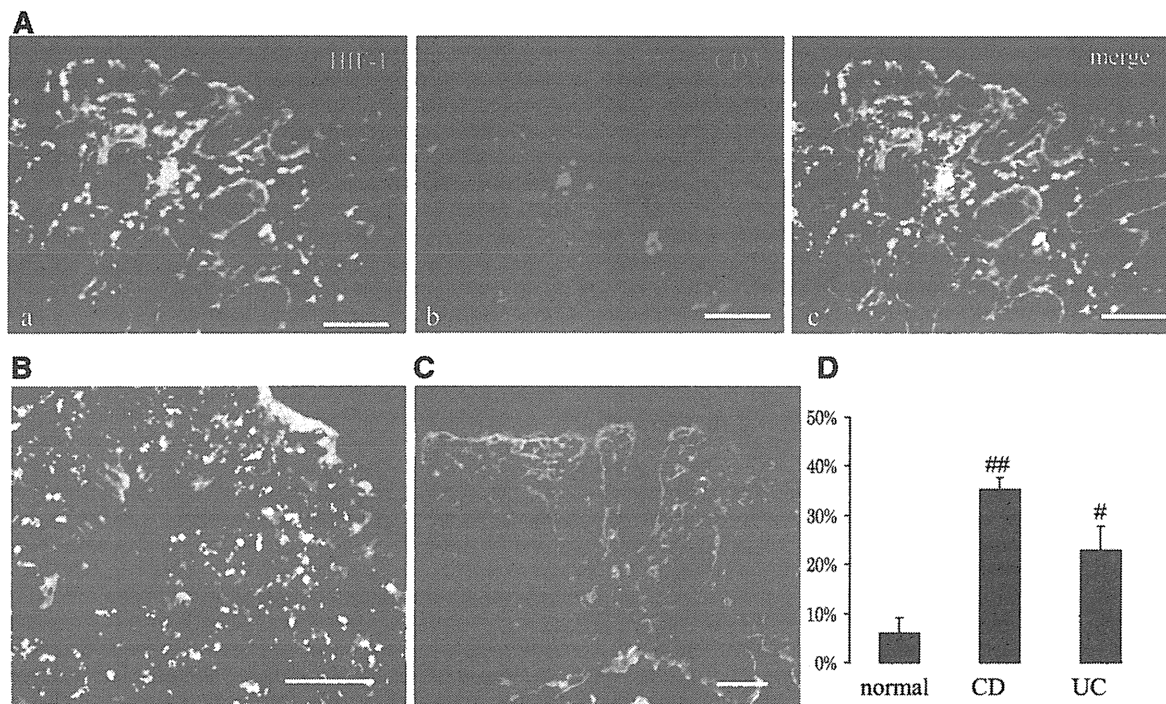

Figure 1. HIF-1 expression is increased in T cells of colonic mucosa of IBD patients. (A) Representative image of an active lesion of colonic mucosa in a CD patient. HIF-1-positive cells (green, Aa), CD3-positive cells (red, Ab), and double-positive cells (yellow, Ac). (B) Double staining of an active lesion of colonic mucosa in an UC patient. (C) Double staining of a colonic mucosa in normal mucosa. (D) We examined what percentage of $CD3^+$ cell was positive for HIF-1 immunohistochemically in all the subjects (n=6/group), and we analyzed two visual fields in each subject under a fluorescence microscope. Data are indicated as mean ± SEM (n=6/group). $^{\#}P < 0.05$ versus normal control; $^{\#\#}P < 0.01$ versus normal control (Mann-Whitney U test). Original bars, 100 μm.

number, sex, age (8–10 weeks), and weight in each group. As it has been reported that HIF-1 deletion in myeloid cells results in profound impairment of myeloid cell migration [10] and as it is generally accepted that T cell recruitment is a critical factor in the pathogenesis for IBD, we examined whether deletion of HIF-1 changes the recruitment ability of T cells. For this purpose, characteristic differences of adhesion molecules and a chemokine receptor in T cells were examined using antibodies, as follows, by flow cytometry analysis: FITC-anti mouse CD62 ligand (L-selectin, Invitrogen, Carlsbad, CA, USA), PE-anti mouse CD162 (PSGL-1, Invitrogen), Fib 504 (β7-integrin, BD PharMingen), and fluorescein-conjugated anti-mCCR9 (R&D Systems, Minneapolis, MN, USA). It was found that there were only slight differences in the intensities of expressions examined (**Fig. 2A**). We next investigated, by using intestinal microscopy, whether these minor expression changes cause functional differences in recruitment to intestinal microvessels and Peyer's patches in vivo. Isolated T cells from WT and HIF-1$\alpha^{-/-}$ mice were injected into nontreated recipient WT mice, and it was found that migratory behaviors were almost the same in the two groups; few T cells attached to intestinal venules, and many T cells attached to Peyer's patches (Fig. 2B), suggesting that HIF-1 in T cells has little relations with their migratory ability.

Effect of DSS administration

After we confirmed that there was no remarkable impairment in the migratory ability of HIF-1-deleted T cells, 3% DSS was administered to each group to investigate the role of HIF-1 in T cells at the site of inflammation. DSS administration induced diarrheal bloody stools in both groups and significantly reduced body weights compared with those in nontreated controls (**Fig. 3A**). The degree of body weight loss caused by DSS administration was significantly higher in HIF-1$\alpha^{-/-}$ mice than in WT mice on Days 4 and 5. Colonic length in the DSS-treated HIF-1$\alpha^{-/-}$ mice was shorter, and swelling was greater than those in the DSS-treated WT mice macroscopically on Day 5 (Fig. 3B). In microscopic examinations by H&E staining (Fig. 3C), shortening of crypts and focal thickening of the epithelium with moderate inflammation were observed frequently in the DSS-treated WT mice, and severe inflammation in the lamina propria and submucosa with loss of epithelium was observed frequently in the DSS-treated HIF-1$\alpha^{-/-}$ mice. Histological damage of distal colon was assessed with H&E staining, according to a previous report from Grade 1 to 4 (Cooper's score) [22]. Figure 3D showed that DSS administration induced inflammation in WT and HIF-1$\alpha^{-/-}$ mice, which was significantly higher in the HIF-1$\alpha^{-/-}$ mice than in the WT mice. Double immunohistochemistry was performed to con-

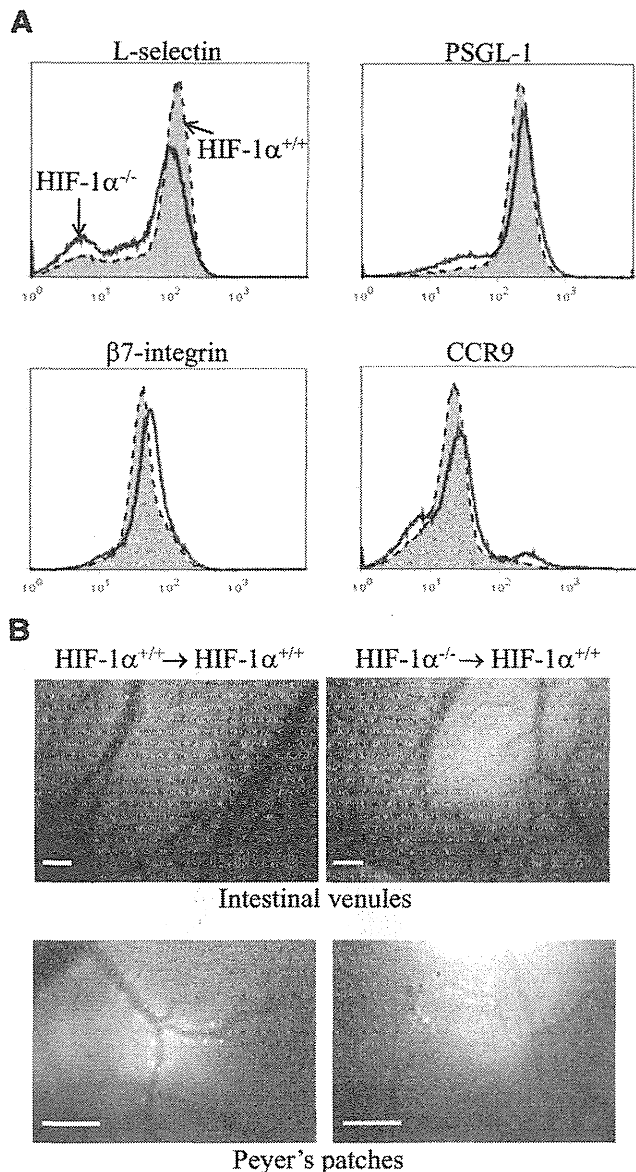

Figure 2. Migratory ability of HIF-1-deleted T cells does not change. (A) Characteristic differences of T cells (CD3-positive cells isolated from splenocytes) between the WT and the HIF-1$\alpha^{-/-}$ mice, examined by flow cytometry analysis. Representative data from three individual measurements. Dotted lines: HIF-1$\alpha^{+/+}$ mice; solid lines: HIF-1$\alpha^{-/-}$ mice. (B) The behavior of T cell in submucosal venules and Peyer's patches was observed from the serosal side using an intravital microscope. Representative pictures of migratory T cells (CD3-positive cells) isolated from WT mice and HIF-1$\alpha^{-/-}$ mice, ~2 h after i.v. injection of T cells, respectively. Original bars, 100 μm.

firm the involvement of HIF-1 in T cells in the pathogenesis of DSS colitis (data not shown). In the normal control mucosa, 2.7 ± 1.8% of CD3$^+$ cells were positive for HIF-1. In the inflamed mucosa of WT mice induced by DSS administration, the ratio of HIF-1-expressing cells among CD3$^+$ cells increased significantly up to 33.7 ± 2.7%. It suggested that DSS colitis in our model is valid for evaluation of the function of T cell-specific HIF-1.

Expression of cytokines in colonic mucosa of DSS colitis

To clarify the mechanism by which T cell-specific HIF-1 deletion exacerbated DSS colitis at the sites of inflammation, we examined cytokine expression on Days 0, 3, and 5 with focus on Treg-, Th1-, and Th17-related genes (**Fig. 4A**): Foxp3, TGF-β, IL-10, RORγt, IL-1β, IL-6, IL-17, IL-23, IFN-γ, IL-12a, and TNF-α [23–28]. The results showed that there were significantly increased expressions of Foxp3, IL-10, IL-1β, IL-6, IL-17, IL-23, IFN-γ, IL-12a, and TNF-α in HIF-1$\alpha^{-/-}$ mice compared with WT mice on Day 5. However, RORγt, known as a key regulator of Th17 differentiation, decreased as colitis progressed in HIF-1$\alpha^{-/-}$ mice. Considering that IL-17 and other Th17-related cytokines, such as IL-1β, IL-6, and IL-23, were increased significantly in HIF-1$\alpha^{-/-}$ mice, and the recent report that RORγt expression precedes Th17 differentiation and decreases when IL-17 is actually induced in vitro [25], our results suggest that Th17 was up-regulated significantly in HIF-1$\alpha^{-/-}$ mice and that RORγt expression is not always parallel to IL-17 expression in vivo. In addition, Th1-related cytokines, including IFN-γ and IL-12a, are elevated significantly in HIF-1$\alpha^{-/-}$ mice. However, expressions of Foxp3, a key regulator of Treg differentiation, and IL-10, a suppressive cytokine induced mainly by Treg, also increased in HIF-1$\alpha^{-/-}$ mice as colitis progressed. It is generally accepted that Th17 and Treg cells are complementary, and the increase and decrease of Foxp3 and IL-17 expression reflect the balance between Treg and Th17. Therefore, although we could not measure the net effect of pro- and anti-inflammatory cytokines, we assessed the balance of Th17 and Treg by calculating the ratio of mRNA IL-17 and mRNA Foxp3 (IL-17:Foxp3) in each mouse (Fig. 4B). In HIF-1$\alpha^{-/-}$ mice, the ratio was elevated higher than that in WT mice as early as Day 3 with significance (approximately four times), suggesting that the balance of Th17 and Treg leaned toward Th17 in the HIF-1α KO condition. In addition, we isolated LPMNCs from colonic mucosa to investigate more specifically which cells were producing Foxp3 and IL-17, as several kinds of cells other than T cells can produce them (**Fig. 5**; n=4 in each). After administration of DSS for 5 days, Foxp3 mRNA in T cells from WT mice was higher than that from HIF-1$\alpha^{-/-}$ mice, although it was not statistically significant (P=0.34). The expression level of mRNA IL-17 was higher in HIF-1$\alpha^{-/-}$ mice than WT on Day 5 (P=0.40). The ratio of IL-17:Foxp3 was ~10 times higher in HIF-1$\alpha^{-/-}$ mice (P=0.21). There was no significant difference of increased IFN-γ in both groups (P=0.52). All of these results suggest that HIF-1 deficiency might lead to the up-regulation of Teff, especially Th17, in colonic inflammation, implying some possibilities that HIF-1 might exert its beneficial effects on the degree of inflammation via controlling the levels of Treg or direct inhibition of differentiation of naïve T cells into Th17 or Th1. In addition, increased expression of IL-10 in HIF-1$\alpha^{-/-}$ mice can be explained by the study reported recently that Th17 can produce IL-10 to limit host tissue injury [29]. TGF-β,

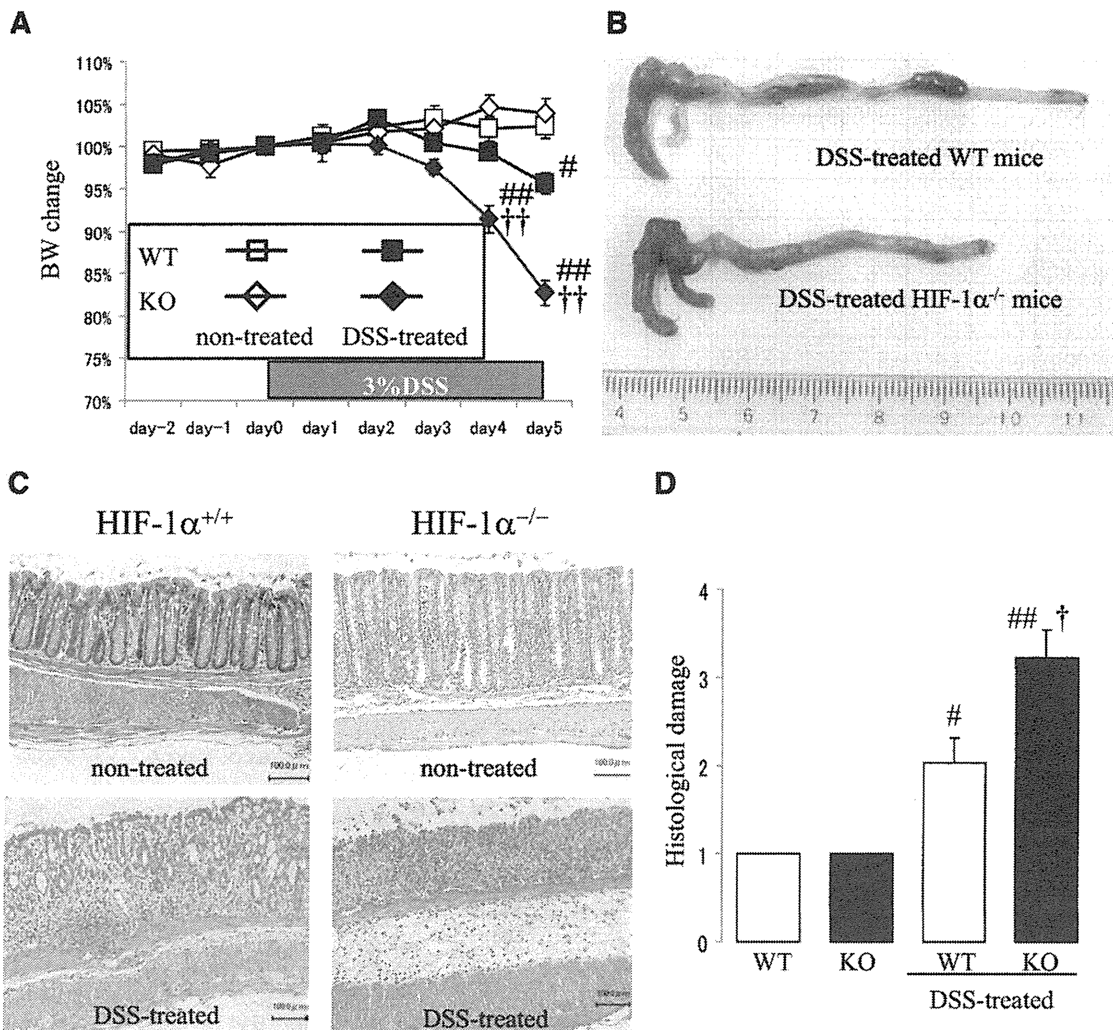

Figure 3. DSS administration induces severe inflammation in HIF-1$\alpha^{-/-}$ mice. (A)Time-course changes in body weights (BW) in DSS-treated WT mice and HIF-1$\alpha^{-/-}$ (KO) mice. Body weight change is expressed in percentage of body weight on Day 0 in each mouse. Data are indicated as mean ± SEM (n=8/group). $^{\#}P < 0.05$ versus nontreated group; $^{\#\#}P < 0.01$ versus nontreated group; $^{\dagger\dagger}P < 0.01$ versus DSS-treated WT mice (Scheffe's F test). (B)Macroscopic findings in DSS colitis. Representative macroscopic findings of removed, total colons in DSS-administered mice are shown. (C) Representative microscopic findings of the distal colon assessed by H&E staining in each group are shown. Original bars, 100 μm. (D) Assessment of histological damage in each group according to Cooper's score. Data are indicated as mean ± SEM (n=8/group). $^{\#}P < 0.05$ versus nontreated group; $^{\#\#}P < 0.01$ versus nontreated group; $^{\dagger}P < 0.05$ versus DSS-treated WT mice (Scheffe's F test).

known as a suppressive cytokine, is slightly higher in HIF-1$\alpha^{-/-}$ mice than WT mice, but we are not sure if this increase worked in an anti-inflammatory way, as TGF-β can also produce Th17 when combined with IL-6 [30].

Changes in the expression levels of Foxp3 and IL-17 induced by hypoxia

To confirm that a hypoxic condition is directly involved in changes in cytokine expression through HIF-1 induction in T cells, splenocytes obtained from WT and HIF-1$\alpha^{-/-}$ mice were cultured under a hypoxic condition for 16 h to examine the difference in the expression levels of Treg and Teff in vitro. Splenocytes were cultured after RBCs were lysed using RBC lysis buffer (BioLegend, San Diego, CA, USA) in RPMI-1640 medium with 5% FBS and MEM nonessential amino acids (Invitrogen) containing penicillin and streptomycin (100 μg/ml, Invitrogen) on a tissue-culture dish coated with 1 μg/ml anti-CD3 mAb (clone 145-2C11, Imgenex, San Diego, CA, USA) and anti-CD28 mAb (Beckman Coulter, Brea, CA, USA) for 16 h. Validity of the hypoxic study was confirmed in advance by Western blotting of HIF-1α and by RT-PCR of HIF-1 target genes, PGK-1, and VEGFA (data not shown). Then, we performed flow cytometry analysis to investigate the hypoxic effects on Treg (**Fig. 6A**) and Th17 (Fig. 6B) populations. In splenocytes from WT mice, hypoxic stimulation increased the intensity

Figure 4. Cytokine expressions are increased in colonic mucosa of DSS-treated HIF-1α$^{-/-}$ mice. (A) Expression levels of mRNAs at the inflamed distal colon in DSS-treated WT and HIF-1α$^{-/-}$ mice. Relative quantity of each mRNA standardized to GAPDH is expressed. (B) The ratio of IL-17:Foxp3 in each mouse was calculated to assess cytokine balance. Data are indicated as mean ± SEM (n=6 on Day 0; n=4 on Day 3; n=8 on Day 5 in each group). $^{\#}P < 0.05$ versus nontreated group; $^{\#\#}P < 0.01$ versus nontreated group; $^{\dagger}P < 0.05$ versus DSS-treated WT mice; $^{\dagger\dagger}P < 0.01$ versus DSS-treated WT mice (Scheffe's F test).

of Foxp3 expression in CD4-positive cells compared with that before the hypoxic stimulation. On the other hand, intensity of Foxp3 expression in CD4-positive, HIF-1-deleted cells was not changed by hypoxic stimulation compared with that before hypoxic stimulation. In the examination of IL-17 expression, the percentage of Th17 cells (IL-17- and CD4-positive cells) was almost the same before hypoxic stimulation in WT mice and HIF-1α$^{-/-}$ mice (0.3% among CD4-positive cells). After exposure to hypoxia, the percentage of Th17 cells increased in both groups, but the degree of increase was higher in the HIF-1α$^{-/-}$ mice (5.1% among CD4-positive cells) than in the WT mice (1.4% among CD4-positive cells). In addition, expression levels of mRNA IL-17 and IFN-γ were examined in each group (Fig. 6C). IL-17 was not detected before hypoxic exposure in either group. However, after exposure to hypoxia for 16 h, IL-17 was de-

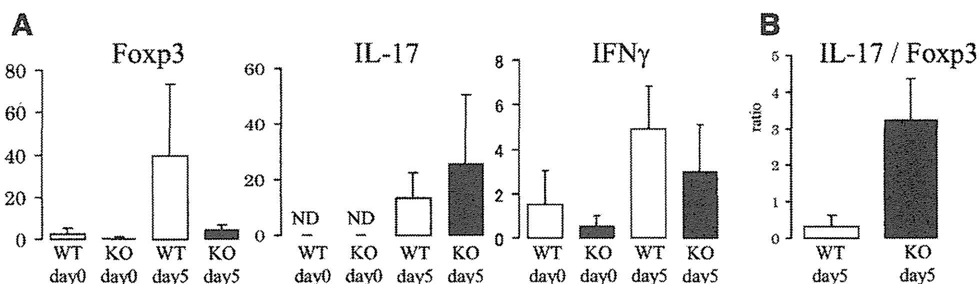

Figure 5. Balance of Treg and Th17 changes in DSS-treated HIF-1α$^{-/-}$ mice. (A) Expression levels of mRNAs of isolated LPMNCs from colonic mucosa in WT and HIF-1α$^{-/-}$ (KO) mice, before (Day 0) and after (Day 5) administration of 3% DSS for 5 days. Relative quantity of each mRNA standardized to GAPDH is expressed. Data are indicated as mean ± SEM (n=4/group). (B) The ratio of IL-17:Foxp3 in each mouse was calculated to assess cytokine balance.

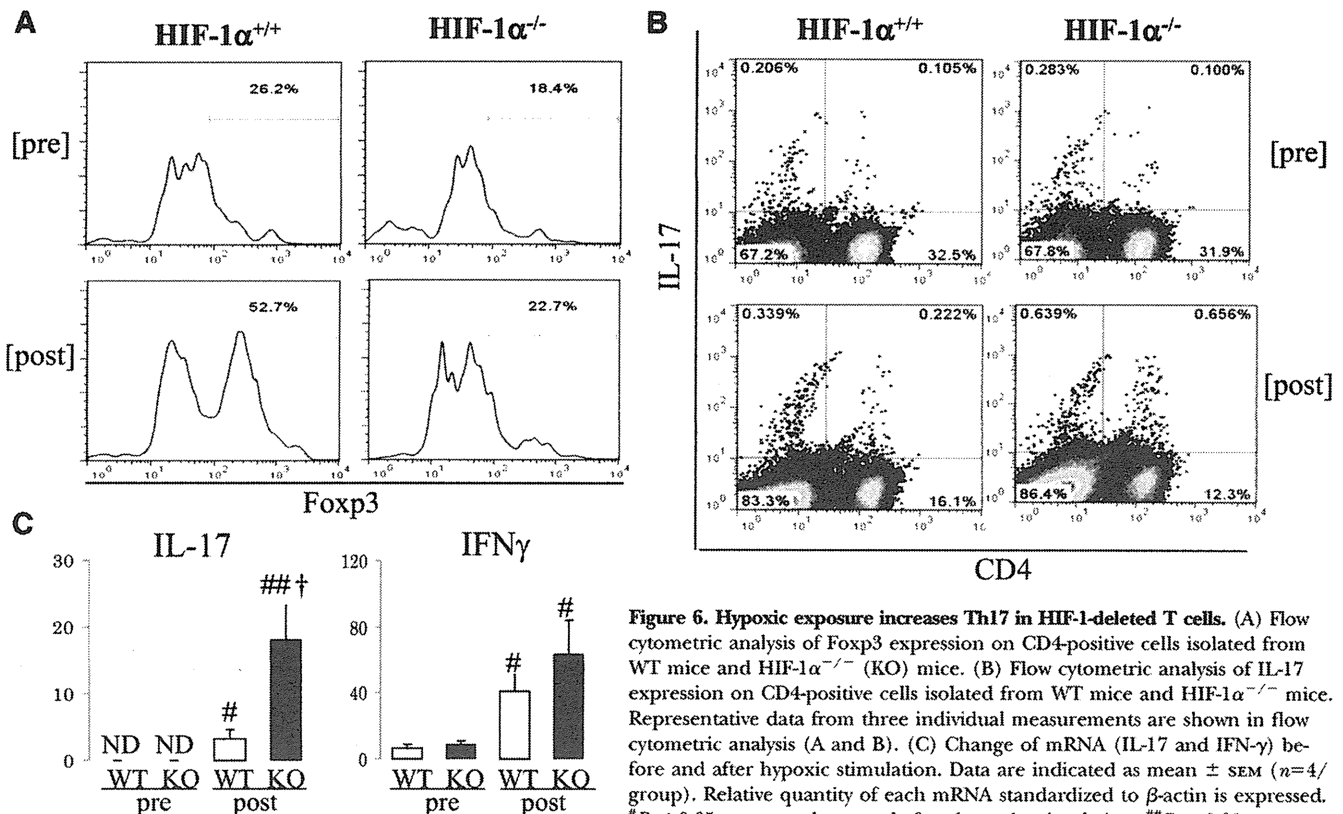

Figure 6. Hypoxic exposure increases Th17 in HIF-1-deleted T cells. (A) Flow cytometric analysis of Foxp3 expression on CD4-positive cells isolated from WT mice and HIF-1$\alpha^{-/-}$ (KO) mice. (B) Flow cytometric analysis of IL-17 expression on CD4-positive cells isolated from WT mice and HIF-1$\alpha^{-/-}$ mice. Representative data from three individual measurements are shown in flow cytometric analysis (A and B). (C) Change of mRNA (IL-17 and IFN-γ) before and after hypoxic stimulation. Data are indicated as mean ± SEM ($n=4$/group). Relative quantity of each mRNA standardized to β-actin is expressed. $^{\#}P < 0.05$ versus each group before hypoxic stimulation. $^{\#\#}P < 0.01$ versus each group before hypoxic stimulation; $^{\dagger}P < 0.05$ versus WT mice after hypoxic stimulation (Scheffe's F test). pre, Before hypoxic stimulation; post, after 16 h hypoxic stimulation.

tected in both groups and the degree was significantly higher in the HIF-1$\alpha^{-/-}$ mice than in the WT mice. As for IFN-γ, there were the same increasing tendencies between two groups, but more increase was observed in HIF-1-deleted cells. This hypoxic study showed that the induction of Foxp3 on HIF-1-deleted T cells was lower than WT T cells, and it was accompanied with elevated Teff activation. Our hypoxic study also showed that Treg induction was suppressed, and Th1 and Th17 were elevated under HIF-1-deleted conditions, indicating that hypoxia-induced Treg differentiation depends on T cell-specific HIF-1, in agreement with the results of a previous study [15].

In addition, to evaluate the change of migratory ability under hypoxia, we examined the mRNA expression changes of CCR9 in both groups (data not shown). The expression of CCR9 in WT and HIF-1$\alpha^{-/-}$ mice was nearly the same level before hypoxia treatment, which reduced the expression of CCR9 in WT and HIF-1$\alpha^{-/-}$ mice to the same degree, showing that there was no significant difference in the hypoxic effect on the chemokine receptors on T cells with or without HIF-1.

Taken together, our results suggested that HIF-1 in T cells might contribute to suppress inflammation in colitis by controlling the balance of Treg/Teff cells. But, a recent study showed a contradictory result about the role of HIF-1 in Treg and Th17 balance [31]. It showed that EAE did not get worse in HIF-1$\alpha^{-/-}$ mice and that HIF-1 in T cells plays an important role to up-regulate Th17 in Th17-skewing condition via the HIF-1/STAT3/RORγt/IL-17 pathway, suggesting that HIF-1 in T cells works in a proinflammatory way. We think there are mainly two reasons for the contradictory result. First, the different character of each in vivo model might be one reason. In encephalitis, Th17 works as a proinflammatory factor [32], and Th1 works as an anti-inflammatory factor and plays a suppressive role against Th17 [33–35]. But, in colitis, Th1 and Th17 are considered proinflammatory factors. It is also reported that there is plasticity between Th1 and Th17 in a colitis model [36]. These facts show that the roles and relationships of Th17 and Th1 are extremely complicated in colitis, leading to different regulation of Treg/Th17 balance from EAE. Second is the different expression of RORγt. According to the referenced study, expression of RORγt is sustained to induce IL-17 under the Th17-skewing condition in vitro. However, it is reported from another group that expression of RORγt in Th17 cells was temporary and decreased thereafter when the expression of IL-17 was induced in another condition in vitro, suggesting that sustained expression of RORγt was not always necessary for IL-17-maintained expression [25]. Our result showed that RORγt was decreased when IL-17 was induced, resembling the latter study, and the decrease was significantly more rapid in HIF-1$\alpha^{-/-}$ mice. Furthermore,

there is another report that Foxp3 deletion from T cells resulted in a marked increase in IL-17 without an increase in RORγt expression [37], suggesting that IL-17 expression does not always parallel RORγt expression. Therefore, it is possible that the expression of RORγt and other cytokines to maintain Th17 might be different between colitis and EAE models. These differences might be responsible for the altered role of HIF-1 on Th17 expressions.

Our results indicate that HIF-1 in T cells is induced in IBD patients; plays a protective role in intestinal inflammation by controlling cytokine balance; and induces Treg differentiation to suppress Th1 and Th17 activation under a condition of hypoxia. Dysregulated immune responses are thought to be one of the important factors for development of IBD [1], and as the potential role of Th17 cells in an IBD animal model, as well as in human IBD, has been proven recently, target therapy aimed at the Th17/IL-17 axis may have a therapeutic role in the treatment of intestinal mucosal inflammation [38]. From this viewpoint, strengthening HIF-1 induction in T cells at the sites of intestinal inflammation, which could lead to up-regulation of Treg and suppression of Th1 and Th17, might be another therapeutic strategy for IBD regulation. Previous studies have already shown that some PHD inhibitors work to support mucosal barrier dysfunction by activation of HIF-1 in the epithelium on the inflamed intestine [13, 14]. Considering the fact that PHD inhibitors are not selective for particular PHD isoforms, our results suggest that HIF-1 activated by PHD inhibitors in T cells might also have played an important role in control of inflammation in those previous studies.

Conclusion

In this study, we clarified one of the important roles of HIF-1 in T cells for the cytokine balance in the inflamed intestine. Active induction of HIF-1 in T cells might be a new therapeutic target for IBD patients.

AUTHORSHIP

M.H., R.H., M.S., N.G., and S.M. contributed to the conception; M.H., R.H., and S.M., the design; and M.H., R.H., H.H., C.K., T.U., C.W., K.T., M.N., S.K., Y.O., A.K., and S.N., the performance of the paper.

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DISCLOSURES

The authors declare no financial or commercial conflict of interest.

REFERENCES

1. Xavier, R. J., Podolsky, D. K. (2007) Unravelling the pathogenesis of inflammatory bowel disease. *Nature* **448,** 427–434.
2. Boden, E. K., Snapper, S. B. (2008) Regulatory T cells in inflammatory bowel disease. *Curr. Opin. Gastroenterol.* **24,** 733–741.
3. Ahern, P. P., Izcue, A., Maloy, K. J., Powrie, F. (2008) The interleukin-23 axis in intestinal inflammation. *Immunol. Rev.* **226,** 147–159.
4. Brand, S. (2009) Crohn's disease: Th1, Th17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease. *Gut* **58,** 1152–1167.
5. Jiang, H. R., Gilchrist, D. S., Popoff, J. F., Jamieson, S. E., Truscott, M., White, J. K., Blackwell, J. M. (2009) Influence of Slc11a1 (formerly Nramp1) on DSS-induced colitis in mice. *J. Leukoc. Biol.* **85,** 703–710.
6. Bai, A., Yong, M., Ma, A. G., Ma, Y., Weiss, C. R., Guan, Q., Bernstein, C. N., Peng, Z. (2010) Novel anti-inflammatory action of 5-aminoimidazole-4-carboxamide ribonucleoside with protective effect in dextran sulfate sodium-induced acute and chronic colitis. *J. Pharmacol. Exp. Ther.* **333,** 717–725.
7. Hatoum, O. A., Binion, D. G., Gutterman, D. D. (2005) Paradox of simultaneous intestinal ischaemia and hyperaemia in inflammatory bowel disease. *Eur. J. Clin. Invest.* **35,** 599–609.
8. Taylor, C. T., Colgan, S. P. (2007) Hypoxia and gastrointestinal disease. *J. Mol. Med.* **85,** 1295–1300.
9. Zagorska, A., Dulak, J. (2004) HIF-1: the knowns and unknowns of hypoxia sensing. *Acta Biochim. Pol.* **51,** 563–585.
10. Cramer, T., Yamanishi, Y., Clausen, B. E., Forster, I., Pawlinski, R., Mackman, N., Haase, V. H., Jaenisch, R., Corr, M., Nizet, V., Firestein, G. S., Gerber, H. P., Ferrara, N., Johnson, R. S. (2003) HIF-1α is essential for myeloid cell-mediated inflammation. *Cell* **112,** 645–657.
11. Thiel, M., Caldwell, C. C., Kreth, S., Kuboki, S., Chen, P., Smith, P., Ohta, A., Lentsch, A. B., Lukashev, D., Sitkovsky, M. V. (2007) Targeted deletion of HIF-1α gene in T cells prevents their inhibition in hypoxic inflamed tissues and improves septic mice survival. *PLoS One* **2,** e853.
12. Karhausen, J., Furuta, G. T., Tomaszewski, J. E., Johnson, R. S., Colgan, S. P., Haase, V. H. (2004) Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. *J. Clin. Invest.* **114,** 1098–1106.
13. Robinson, A., Keely, S., Karhausen, J., Gerich, M. E., Furuta, G. T., Colgan, S. P. (2008) Mucosal protection by hypoxia-inducible factor prolyl hydroxylase inhibition. *Gastroenterology* **134,** 145–155.
14. Cummins, E. P., Seeballuck, F., Keely, S. J., Mangan, N. E., Callanan, J. J., Fallon, P. G., Taylor, C. T., Cummins, E. P., Seeballuck, F., Keely, S. J., Mangan, N. E., Callanan, J. J., Fallon, P. G., Taylor, C. T. (2008) The hydroxylase inhibitor dimethyloxalylglycine is protective in a murine model of colitis. *Gastroenterology* **134,** 156–165.
15. Ben-Shoshan, J., Maysel-Auslender, S., Mor, A., Keren, G., George, J. (2008) Hypoxia controls CD4+CD25+ regulatory T-cell homeostasis via hypoxia-inducible factor-1α. *Eur. J. Immunol.* **38,** 2412–2418.
16. Okada, Y., Tsuzuki, Y., Miyazaki, J., Matsuzaki, K., Hokari, R., Komoto, S., Kato, S., Kawaguchi, A., Nagao, S., Itoh, K., Watanabe, T., Miura, S. (2006) *Propionibacterium freudenreichii* component 1.4-dihydroxy-2-naphthoic acid (DHNA) attenuates dextran sodium sulphate induced colitis by modulation of bacterial flora and lymphocyte homing. *Gut* **55,** 681–688.
17. Lukashev, D., Klebanov, B., Kojima, H., Grinberg, A., Ohta, A., Berenfeld, L., Wenger, R. H., Sitkovsky, M. (2006) Cutting edge: hypoxia-inducible factor 1α and its activation-inducible short isoform I.1 negatively regulate functions of CD4+ and CD8+ T lymphocytes. *J. Immunol.* **177,** 4962–4965.
18. Fujimori, H., Miura, S., Koseki, S., Hokari, R., Komoto, S., Hara, Y., Hachimura, S., Kaminogawa, S., Ishii, H. (2002) Intravital observation of adhesion of lamina propria lymphocytes to microvessels of small intestine in mice. *Gastroenterology* **122,** 734–744.
19. Higashiyama, M., Hokari, R., Matsunaga, H., Takebayashi, K., Watanabe, C., Komoto, S., Okada, Y., Kurihara, C., Kawaguchi, A., Nagao, S., Itoh, K., Miura, S. (2008) P-selectin-dependent monocyte recruitment through platelet interaction in intestinal microvessels of LPS-treated mice. *Microcirculation* **15,** 441–450.
20. Matsunaga, H., Hokari, R., Ueda, T., Kurihara, C., Hozumi, H., Higashiyama, M., Okada, Y., Watanabe, C., Komoto, S., Nakamura, M., Kawaguchi, A., Nagao, S., Sekiyama, A., Miura, S. (2011) Physiological stress exacerbates murine colitis by enhancing proinflammatory cytokine expression that is dependent on IL-18. *Am. J. Physiol. Gastrointest. Liver Physiol.* **301,** G555–G564.
21. Giatromanolaki, A., Sivridis, E., Maltezos, E., Papazoglou, D., Simopoulos, C., Gatter, K. C., Harris, A. L., Koukourakis, M. I. (2003) Hypoxia inducible factor 1α and 2α overexpression in inflammatory bowel disease. *J. Clin. Pathol.* **56,** 209–213.
22. Cooper, H. S., Murthy, S. N., Shah, R. S., Sedergran, D. J. (1993) Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab. Invest.* **69,** 238–249.
23. Hori, S., Nomura, T., Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299,** 1057–1061.
24. Zhou, L., Lopes, J. E., Chong, M. M., Ivanov, I. I., Min, R., Victora, G. D., Shen, Y., Du, J., Rubtsov, Y. P., Rudensky, A. Y., Ziegler, S. F., Litt-