C57BL/6J mice 2 days after the 2 % DSS administration. The body weight, and the length and histology of the colon were evaluated on day 9.

Luciferase assay

Synthesized oligonucleotides of GRAIL-3′UTR construct, which contained putative miR-290-5p binding sites, were inserted downstream of Luciferase in a pMIR-REPORT Luciferase vector (Ambion). PGL4 *Renilla* Luciferase vector (Promega, Madison, WI, USA) was used to compensate for the transfection efficacy. These constructs and pre-miR 290 precursors (40 nM) or pre-miR^{TM} miRNA negative controls #1 (Ambion) were co-transfected into NIH-3T3 (murine fibroblast cell line) using Lipofectamine 2000 (Invitrogen). Firefly luciferase activity was measured 48 h after transfection using a Dual-Glo^{TM} Luciferase Assay System (Promega). The firefly luciferase activity was evaluated after the compensation by *Renilla* luciferase activity.

Histological analysis

Histological scores for DSS colitis were determined, as previously described, as the sum of the following scores [24]: changes in the epithelium (0, normal; 1, focal loss of goblet cells; 2, diffuse loss of goblet cells; 3, focal loss of crypts; 4, diffuse loss of crypts) and for cell infiltration (0, no increase; 1, around bases of the crypts; 2, diffuse infiltration along the layer of muscularis mucosae; 3, complete infiltration of the mucosal layer; 4, infiltration of both mucosal and submucosal layers).

Cytokine ELISA

DO11.10 cells were transfected with pAcGFP-GRAIL or an empty vector as control, and AcGFP^{+} T cells were sorted using flow cytometry 48 h after the transfection. Culture supernatants of the cells were collected after activation with plate-coated, anti-CD3 antibody (5 μg/ml) and soluble, anti-CD28 antibody (5 μg/ml), and were analyzed by IL-10, TGF-β, and IL-2 ELISA kit according to manufacturer's instructions (eBioscience, San Diego, CA, USA).

Statistical methods

Student's *t* test, the Wilcoxon signed-rank test and the Tukey HSD test were used for statistical analysis. The body weight of mice injected with DO11.10 was evaluated by a two-factor, repeated ANOVA. $P < 0.05$ was considered statically significant and data were presented as the mean + SEM.

Ethical considerations

The Institutional Review Board of Osaka University Hospital approved the human study protocol, and written informed consent was obtained from each participant. The Institutional Animal Committee on Animal Research approved the mouse protocol.

Results

Expression of GRAIL mRNA and protein in CD4^{+} T cells in the peripheral blood of the patients with CD

We examined the expression of GRAIL in the peripheral blood of 37 patients with CD and 22 healthy volunteers (HV). The characteristics of the subjects are listed in Table 1. The GRAIL mRNA levels in the CD4^{+} T cells were significantly lower in the peripheral blood of the patients with active CD than in the HV (Fig. 1a). In addition, the GRAIL mRNA levels were significantly lower even in the CD patients in remission than in the HV (Fig. 1a). Consistent with the lower GRAIL mRNA expression in CD, the protein levels of GRAIL were lower in the peripheral blood CD4^{+} T cells of the CD patients than in those of the HV (Fig. 1b). These results were different from our previous results of UC patients, where GRAIL mRNA in the remissive UC patients was higher than in healthy volunteers and the expressions of active UC patients and healthy volunteers were not statistically different [15]. We also investigated the expression of GRAIL in the peripheral blood of *Il-10*$^{-/-}$ mice. *Il-10*$^{-/-}$ mice develop spontaneous chronic intestinal inflammation at approximately 4 weeks of age, while *Il-10*$^{+/-}$ mice lack this inflammation. The levels of GRAIL mRNA in the peripheral blood CD4^{+} T cells were lower in *Il-10*$^{-/-}$ mice

Table 1 Clinical characteristics of the patients

	CD (active)	CD (remission)	HV
Gender (M/F)	13/4	14/6	16/6
Disease type (ileal/colonic/ileo-colonic)	9/2/6	8/7/5	–
Age[a]	38.2 (±11.7)	40.0 (±13.9)	35.6 (±7.7)
CDAI[a]	251.1 (±69.3)**	85.2 (±30.9)	–
CRP (mg/l)[a]	13.6 (±20.3)***	4.1 (±6.6)	–

CD Crohn's disease, *HV* healthy volunteers, *CDAI* Crohn's disease activity index, *CRP* C-reactive protein

[a] Mean (±SEM)

** $P < 0.05$

*** $P < 0.01$, compared with CD in remission

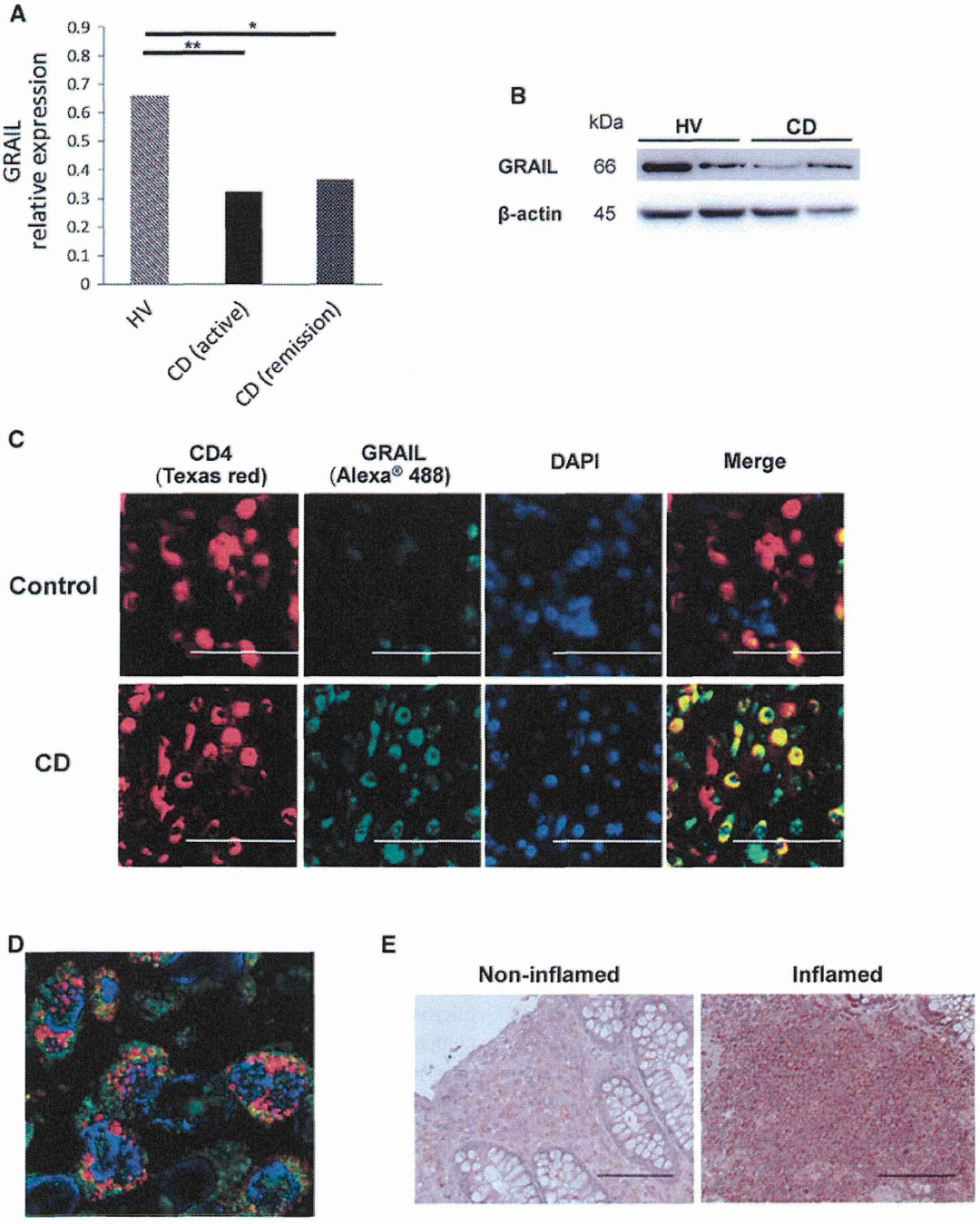

Fig. 1 Expression of GRAIL is lower in the peripheral $CD4^+$ T cells, but higher in the LP of Crohn's disease. **a** Levels of GRAIL mRNA in the peripheral blood $CD4^+$ T cells isolated from 22 healthy volunteers (HV), 17 active patients with CD, and 20 patients with CD in remission were analyzed by qRT-PCR. GRAIL mRNA was significantly lower in the $CD4^+$ T cells of active and remissive CD patients than in HV. Data are shown as the mean + SEM. $*P < 0.05$; $**P < 0.01$. **b** GRAIL protein expressions of the peripheral blood $CD4^+$ T cells isolated from HV and the patients of CD were analyzed by western blot. Representative blots from HV and active CD patients are shown. **c** Immunohistochemical staining of non-inflamed colonic mucosa of colon cancer patients (control) and inflamed mucosa of CD patients. Colonic tissues were stained with Texas red-labeled anti-CD4 antibody (*red*), Alexa®488-labeled anti-GRAIL antibody (*green*) and DAPI (*blue*). *Scale bar* 50 μm. **d** The LP mononuclear cells of patients with CD were stained with CD4-Texas red (*red*), GRAIL-Alexa®488 (*green*) and DAPI (*blue*), and subjected to three-dimensional, structured illumination microscopy. **e** Specimens from non-inflamed and inflamed colonic mucosa were stained by anti-GRAIL antibody and color was developed by 3, 3′-diaminobenzidine. GRAIL-stained cells (*brown*) were massively observed in the LP of inflamed lesion. *Scale bar* 100 μm (color figure online)

than in *Il-10*$^{+/-}$ mice, consistent with the reduced protein expression of GRAIL in *Il-10*$^{-/-}$ mice (Supplementary Fig. 1A, B).

Expressions of GRAIL mRNA and protein in CD4$^+$ T cells in the intestinal lamina propria of the patients with CD

Next, we examined GRAIL protein expression in the intestinal LP of CD patients and control subjects by immunohistochemistry. The GRAIL-positive cells were highly observed in the intestinal CD4$^+$ T cells of the patients with CD in contrast to the infrequent GRAIL-positive cells in control subjects (Fig. 1c, Supplementary Fig. 2A). When we investigated the GRAIL protein expression by three-dimensional, structured illumination microscopy, GRAIL was ubiquitously expressed in the cytoplasm, suggestive of endosomes, of CD4$^+$ T cells in the intestinal LP of the patients with CD (Fig. 1d). In addition, the expression of GRAIL was massively observed in severely inflamed lesions with erosions and ulcers, rather than in non-inflamed lesions (Fig. 1e). In contrast, GRAIL mRNA expressions were not significantly different between the colonic tissue samples of CD and the control subjects (Supplementary Fig. 2B). Thus, we observed discrepant patterns of GRAIL expression between the peripheral blood and the intestinal LP in patients with CD.

Discrepant GRAIL mRNA and protein expressions in the colonic CD4$^+$ T cells of dextran sodium sulfate-induced colitis

We next investigated the expression of GRAIL in the lymphoid organs of DSS-induced, acute murine colitis. The level of GRAIL mRNA was significantly lower in the LP CD4$^+$ T cells of mice with DSS colitis than in those of mice untreated with DSS (Fig. 2a). In contrast, the GRAIL protein level in the LP CD4$^+$ T cells of colitic mice was significantly higher than that of untreated mice (Fig. 2b, c). The level of GRAIL mRNA in CD4$^+$ T cells was significantly higher in the LP than in the SP or MLN of untreated mice (Fig. 2a). In contrast, the GRAIL protein level was lower in the CD4$^+$ T cells in the LP than in those in the SP and MLN of untreated mice (Fig. 2b, c).

Discrepant expressions between the GRAIL mRNA and protein in the colonic CD4$^+$ T cells of *Il-10*$^{-/-}$ mice

When the GRAIL mRNA was compared between *Il-10*$^{-/-}$ and *Il-10*$^{+/-}$ mice, the level of GRAIL mRNA was significantly lower in the LP CD4$^+$ T cells of colitic *Il-10*$^{-/-}$ mice than those of non-colitic *Il-10*$^{+/-}$ mice (Fig. 2d). In contrast, the level of GRAIL protein in the LP was significantly higher in *Il-10*$^{-/-}$ mice than in *Il-10*$^{+/-}$ mice (Fig. 2e, f). Thus, the GRAIL mRNA and protein expression patterns of *Il-10*$^{-/-}$ mice were similar to those of mice with DSS colitis in terms of the discrepancy of GRAIL mRNA and protein levels in the LP between colitic and non-colitic mice. In addition, there was a discrepancy of GRAIL mRNA and protein between the SP and LP in the non-colitic mice, showing high mRNA levels but low protein levels of GRAIL in the LP compared to the SP. It has been shown that the specific deubiquitinating enzyme (DUB), Otub-1, induces degradation of GRAIL [10, 25]. We speculated that the suppression of Otub-1 might upregulate GRAIL expression via the abrogation of deubiquitination of GRAIL in the LP of colitic mice. The levels of Otub-1 mRNA, however, were not significantly different among CD4$^+$ T cells in the SP, MLN, and LP of *Il-10*$^{+/-}$ and *Il-10*$^{-/-}$ mice (Supplementary Fig. 3A). In addition, the levels of ubiquitination of the GRAIL protein in the CD4$^+$ T cells of SP and LP were not different in both *Il-10*$^{+/-}$ and *Il-10*$^{-/-}$ mice (Supplementary Fig. 3B). These results indicated that Otub-1 does not play a major role for the regulation of GRAIL in murine colitis.

miR-290-5p as a novel miRNA related to the regulation of GRAIL

miRNAs, a novel class of non-coding small RNAs, repress gene expression by binding to the 3′UTR of target messenger RNAs, thereby suppressing protein expression [26]. We screened miRNAs that were highly expressed in the LP relative to the SP by miRNA microarray, using samples of SP and LP CD4$^+$ T cells isolated from *Il-10*$^{+/-}$ mice, because there was a discrepancy between the mRNA and protein levels in these two lymphoid organs. The miRNA microarray revealed upregulation of 58 miRNAs in the LP, compared with SP (GEO accession number; GSE40891). Among these upregulated miRNAs, computational predictions revealed that miR-26b, miR-295, miR-330, miR-290-5p, and miR-290-3p had a potential to bind to the 3′UTR of GRAIL and miR-290-5p was expressed more than two-fold higher in the LP than in the SP (Table 2). The seed lesion of miR-290-5p completely coincided with the putative GRAIL-3′UTR (Fig. 3A). We next analyzed the expression of miR-290-5p in the CD4$^+$ T cells of the systemic and mucosal lymphoid tissues of *Il-10*$^{+/-}$ and *Il-10*$^{-/-}$ mice by qRT-PCR. miR-290-5p was confirmed to be expressed significantly higher in the LP than in the SP of *Il-10*$^{+/-}$ mice (Fig. 3b). In addition, the expression of miR-290-5p in the CD4$^+$ T cells of the LP was significantly higher in *Il-10*$^{+/-}$ mice than in *Il-10*$^{-/-}$ mice. The miR-290-5p levels in the SP and MLN were not significantly different between *Il-10*$^{+/-}$ and *Il-10*$^{-/-}$ mice (Fig. 3b).

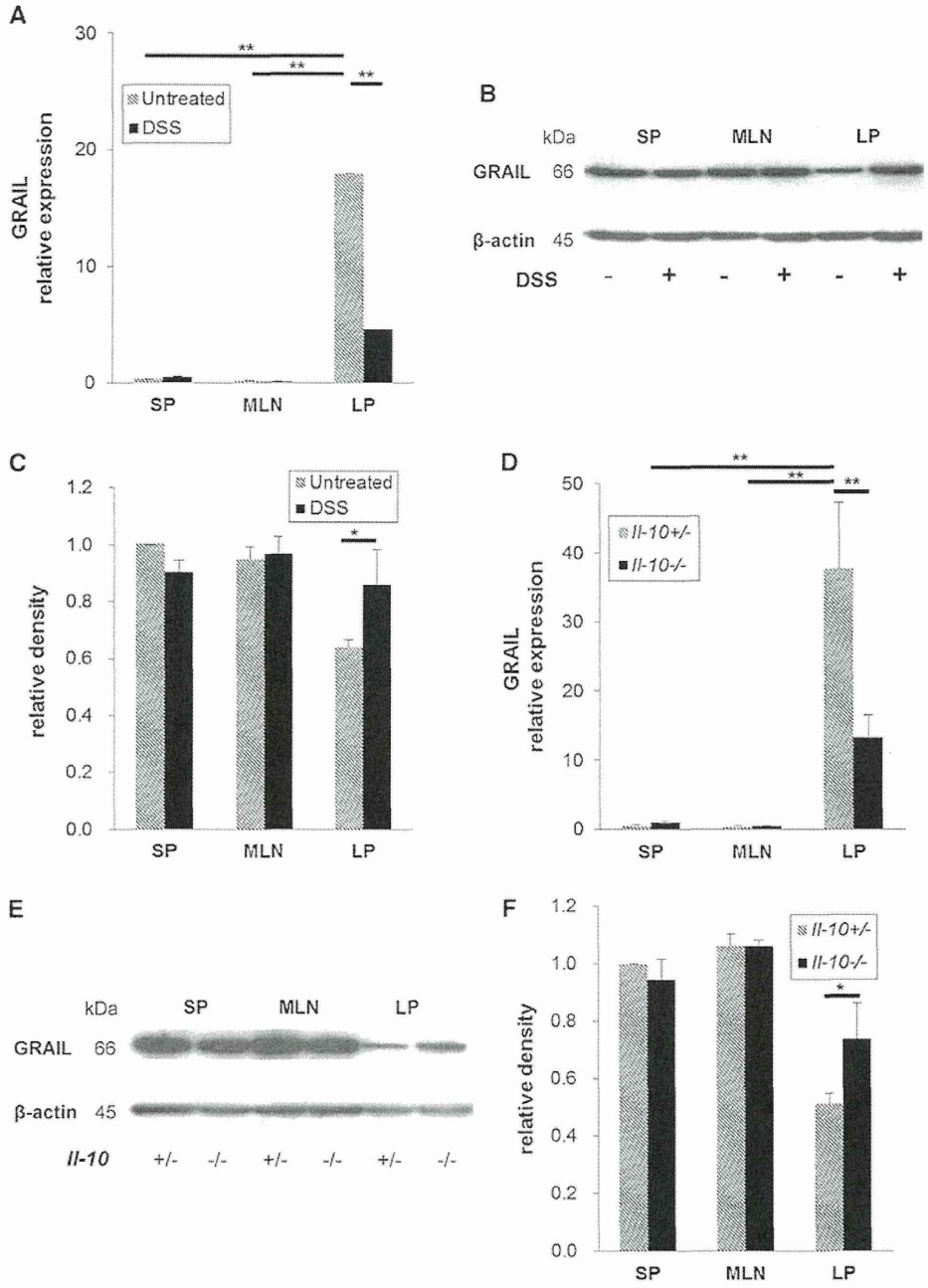

Fig. 2 Discrepancy between GRAIL mRNA and protein expressions in the lamina propria of murine colitis. **a** The levels of GRAIL mRNA were evaluated in mice induced with DSS colitis. GRAIL mRNA was significantly higher in the $CD4^+$ T cells of the LP than in those of the SP and MLN in untreated mice. GRAIL mRNA was significantly lower in the LP of DSS-treated mice (*solid bar*) than mice untreated with DSS (*shaded bar*; $n = 8$ in each group; $^{**}P < 0.01$). **b** The GRAIL protein levels in the $CD4^+$ T cells isolated from lymphoid tissues were analyzed by western blot in mice treated with DSS (+) or without DSS (−). Representative pictures of three independent experiments are shown. **c** Band intensities were quantified by densitometry. The data were expressed as mean + SEM from three separate experiments. $^*P < 0.05$. **d** The mRNA expressions of GRAIL in $CD4^+$ T cells of the SP, MLN, and LP of *Il-10*$^{+/-}$ and *Il-10*$^{-/-}$ mice (age 10–12 weeks) were determined by qRT-PCR ($n = 8$). The levels of GRAIL mRNA in the LP were significantly higher than in other lymphoid tissues in *Il-10*$^{+/-}$ mice. GRAIL mRNA of the LP was higher in *Il-10*$^{+/-}$ mice (*shaded bar*) than in *Il-10*$^{-/-}$ mice (*solid bar*; $^{**}P < 0.01$). E. The levels of GRAIL protein in $CD4^+$ T cells of the SP, MLN, and LP of *Il-10*$^{+/-}$ and *Il-10*$^{-/-}$ mice were analyzed by western blot. Representative pictures from three independent experiments are shown. **f** Band intensities were quantified by densitometry. The data were expressed as mean + SEM from three separate experiments. $^*P < 0.05$

Table 2 miRNAs upregulated in the lamina propria and predicted to bind to GRAIL

miRNA	Ratio (LP/SP)
mmu-miR-290-5p	2.15
mmu-miR-295	1.26
mmu-miR-330	1.18
mmu-miR-290-3p	1.16
mmu-miR-26b	1.15

We introduced DO11.10 cells with an anti-miR-290 inhibitor to confirm that miR-290-5p inhibits GRAIL protein expression. The expression of GRAIL protein was increased after the introduction of an anti-miR-290 inhibitor (Fig. 3c). Next, to confirm that miR-290-5p can inhibit the translation of GRAIL mRNA, we generated a plasmid vector, pMIR-GRAIL-3′UTR, in which the putative miR-290-5p binding site of GRAIL was inserted downstream of the firefly luciferase open-reading frame, and conducted a luciferase reporter assay using a pMIR-GRAIL-3′UTR vector co-transfected with the miR-290 precursor. Firefly luciferase activity was significantly decreased by transfection of the miR-290 precursor compared to the negative control (Fig. 3d). These results confirmed that miR-290-5p can inhibit expression of GRAIL protein via binding to the GRAIL-3′UTR.

T cells overexpressing GRAIL express regulatory markers

The plasmid vector designed to express pAcGFP1 and GRAIL was transfected in DO11.10 cells and we evaluated the expression of markers for regulatory T cells, e.g., FoxP3, GITR, and CTLA-4 [27]. The *grail* mRNA was confirmed to be expressed in the cells transfected with pAcGFP1-GRAIL (Fig. 4a). The *foxp3* and *gitr* mRNA levels were significantly higher in the cells transfected with pAcGFP1-GRAIL than in those transfected with the control vector (Fig. 4a). The protein levels of IL-10 and TGF-β were significantly lower in the cells with transfection of pAcGFP-GRAIL than in the cells transfected with the control GFP vector (Fig. 4b). These results are consistent

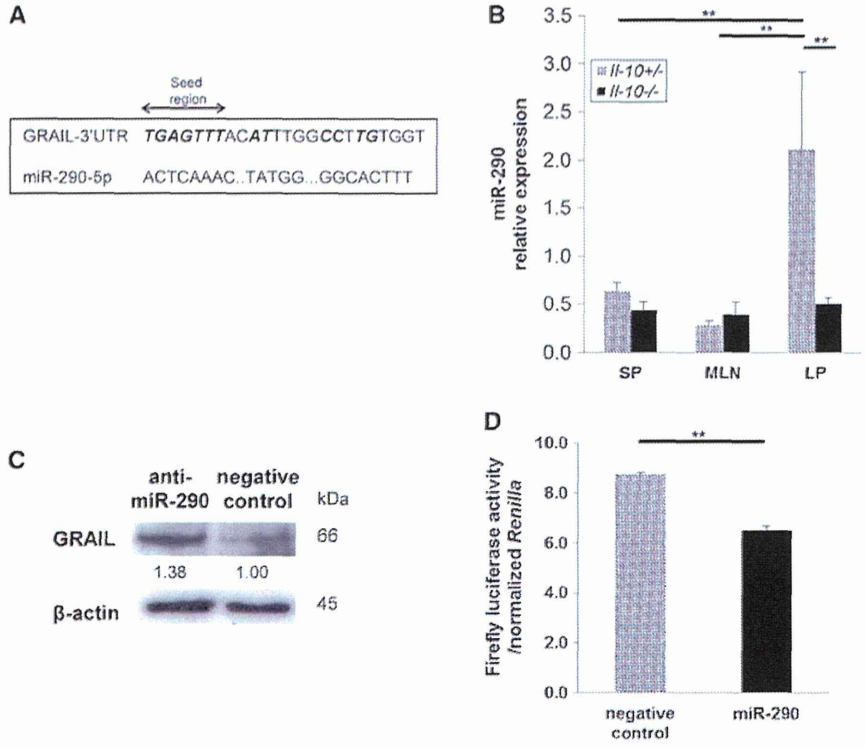

Fig. 3 miR-290-5p regulates GRAIL expression in vitro. **a** The sequences of miR-290-5p and 3′UTR of GRAIL. **b** Expression of miR-290-5p was determined by qRT-PCR. Expression of miR-290-5p was significantly higher in the LP of *Il-10*$^{+/-}$ mice than in the other lymphoid tissues and was significantly lower in the LP of *Il-10*$^{-/-}$mice than of *Il-10*$^{+/-}$ mice. The levels of miR-290-5p normalized by snoRNA135 are shown ($n = 8$, $^{**}P < 0.01$). **c** Expression of GRAIL protein was determined by western blotting after the transfection of DO11.10 cells with an anti-miR-290-5p inhibitor. Relative protein levels were determined by densitometry. **d** Luciferase activity was determined by the transfection of NIH-3T3 with GRAIL-3′UTR reporter together with miR-290 precursor. Luciferase activity was significantly decreased by miR-290 precursor compared with the pre-miR^{TM} miRNA negative control ($n = 6$, $^{**}P < 0.01$)

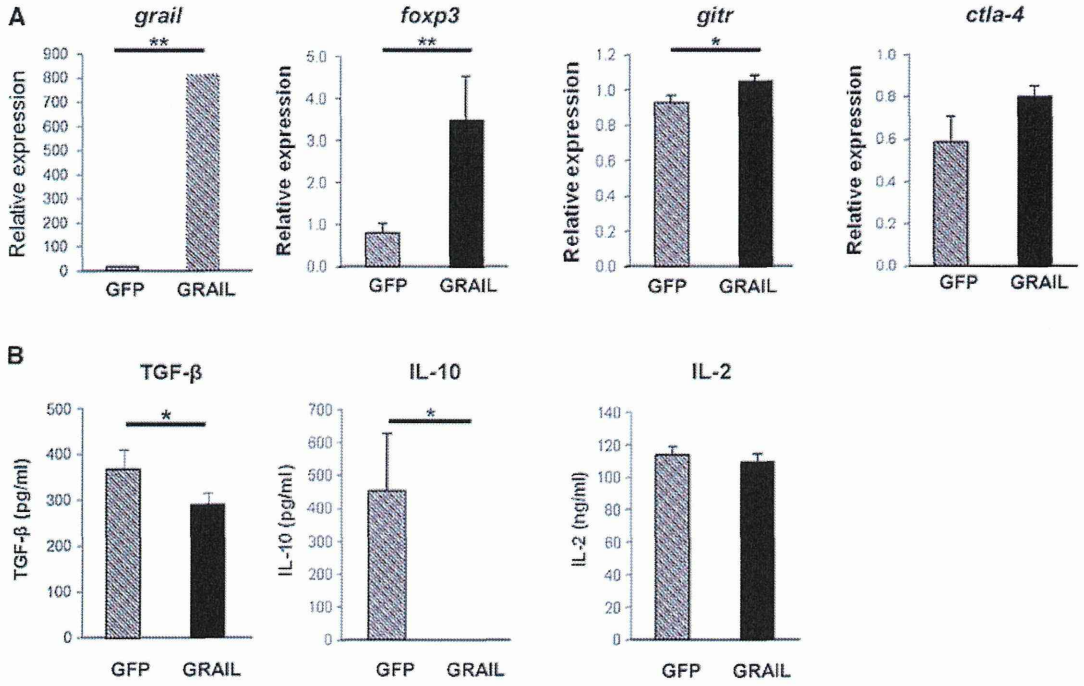

Fig. 4 Forced expression of GRAIL converts DO11.10 cells to a regulatory phenotype. **a** Either pAcGFP-GRAIL vector or empty pAcGFP vector was transfected to DO11.10 cells, and *grail*, *foxp3*, *gitr*, and *ctla-4* mRNA expressions in AcGFP positive cells were determined by qRT-PCR. Expressions of *foxp3* and *gitr* mRNA were significantly higher in GRAIL-overexpressing cells than in cells transfected with control vector ($n = 5$; $^{*}P < 0.05$; $^{**}P < 0.01$). **b** DO11.10 cells transfected with pAcGFP1-GRAIL or empty vector were cultured in vitro with anti-CD3 and anti-CD28 mAb for 24 h and the culture supernatants were analyzed for TGF-β, IL-10, and IL-2 by ELISA. The expressions of TGF-β and IL-10 were significantly higher in the control group than in the GRAIL group ($n = 5$; $^{*}P < 0.05$)

with the previous report showing forced expression of GRAIL converts DO11.10 to a suppressor phenotype, irrespective of the suppressive cytokine expressions [12]. The suppressive effect on proliferative activity of the naïve responder cells by the GRAIL-expressing DO11.10 T cell line has also been demonstrated [12]. Similarly, cell division was decreased in the presence of GRAIL-transfected cells when compared to the GFP control cells (Supplementary Fig. 4). Thus, GRAIL expression converted T cells to a regulatory phenotype independent of regulatory cytokines, such as IL-10 and TGF-β.

Effect of GRAIL^{high} T cell-transfer on intestinal inflammation in murine colitis induced by dextran sodium sulfate

To assess the function of GRAIL, C57BL/6J mice orally administered 2 % DSS were injected with GRAIL-overexpressing DO11.10 (GRAIL^{high}) T cells. The injection of GRAIL^{high} T cells significantly suppressed the body-weight loss of mice with DSS colitis (Fig. 5a). The length of the colon was significantly longer in mice transferred with GRAIL^{high} T cells than in the control group (Fig. 5b, c). The cellular profile was not different between recipient mice transferred with GRAIL^{high} cells and the control mice (Supplementary Fig. 5). The histology of the colon section showed less infiltration of inflammatory cells in mice transferred with the GRAIL^{high} cells when compared with the control group, and myeloperoxidase activity tended to be lower in the GRAIL^{high} group than the control group, but these results did not reach statistical significance (Fig. 5d, e, Supplementary Fig. 6A). The GRAIL^{high} T cells were detected mainly in the abdomen by in vivo imaging system (Supplementary Fig. 6B) and GFP-positive cells were confirmed to be located in the colonic LP of mice transferred with GRAIL^{high} T cells (Fig. 5f).

GRAIL-positive cells express FoxP3 in the lamina propria of CD patients

We further examined the expression of FoxP3 and GRAIL in the colonic lamina propria of CD patients and control subjects. Many GRAIL-positive cells were co-stained with anti-FoxP3 antibody, especially in the inflamed, colonic LP of the patients with CD (Fig. 6).

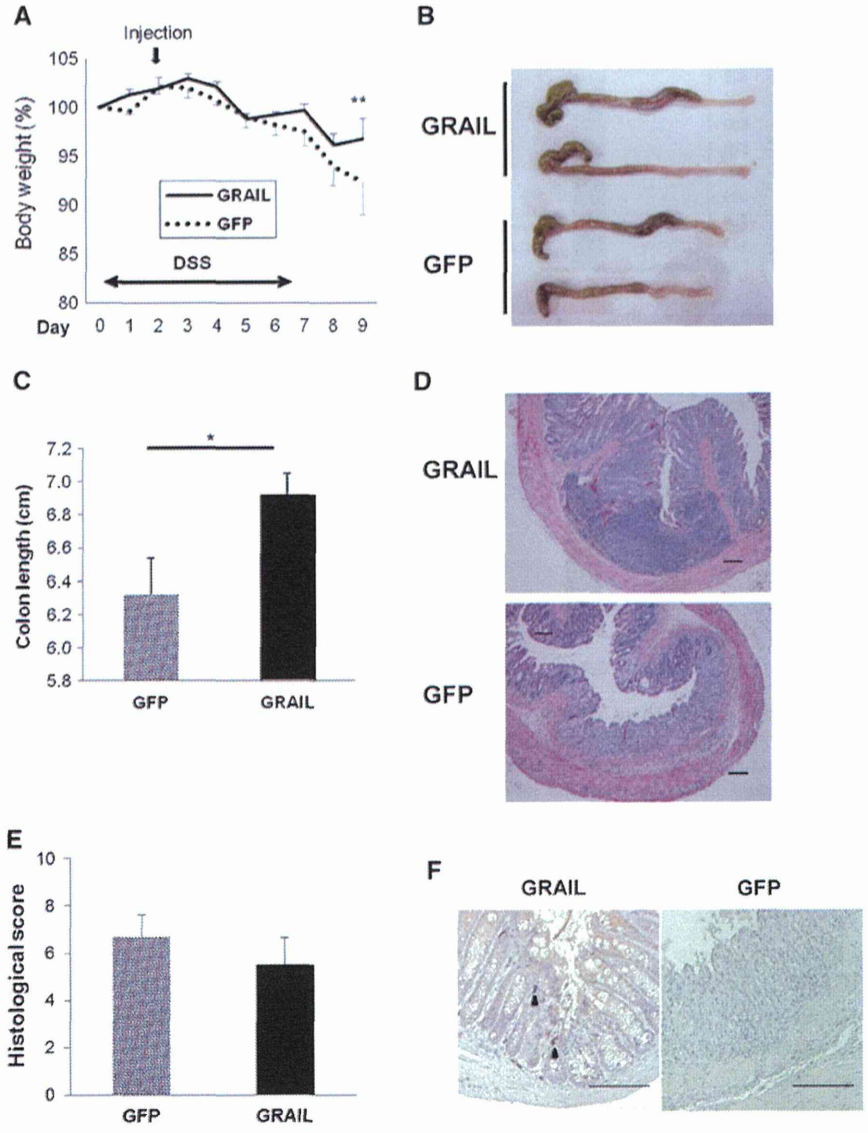

Fig. 5 Transfer of GRAIL^{high} T cells suppresses colonic inflammation induced by dextran sodium sulfate. **a** Mice induced with DSS colitis were injected with either GRAIL^{high} DO11.10 (GRAIL) or the cells transfected with the GFP-expressing empty vector (GFP) on day 2. Mice injected with GRAIL^{high} cells were significantly protected from body-weight loss compared to the control group ($n = 6$; $^{**}P < 0.01$). **b** Macroscopic pictures of the colon with or without GRAIL^{high} cell-transfer. **c** The colon lengths of mice with GRAIL^{high} cell-transfer were significantly longer than those of the control group ($n = 6$; $^{*}P < 0.05$). **d** Representative H&E sections of the distal colon of mice injected with either GRAIL^{high} or control cells (magnification, $\times 10$). *Bar* 100 μm. **e** Histological scores of GRAIL^{high} and control groups ($n = 6$, not significant). **f** Colonic sections of mice with DSS colitis, injected with GRAIL^{high} cells or control cells, were stained with an anti-GFP antibody. The GFP-positive cells expressing GRAIL (*arrow*) were observed in the colonic lamina propria (*scale bar* 100 μm)

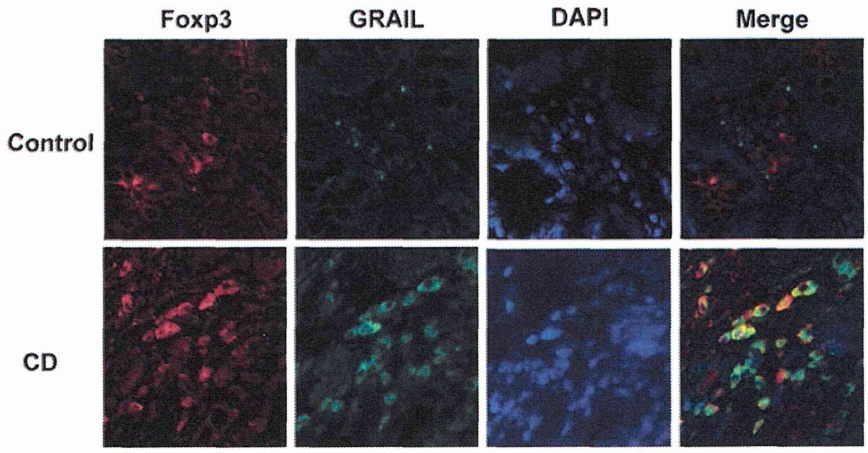

Fig. 6 Immunohistochemical staining of FoxP3 and GRAIL in the non-inflamed, colonic mucosa of colon cancer patients (control) and in the inflamed mucosa of CD patients. Colonic tissues were stained with anti-GRAIL antibody followed by Alexa®488-labeled anti-rabbit IgG (*green*), mouse anti-FoxP3 antibody followed by Texas red-labeled anti-mouse IgG (*red*), and DAPI (*blue*). The representative pictures are shown (color figure online)

Discussion

Our present study demonstrated for the first time the expression of GRAIL in the peripheral blood of CD patients and mice with experimental colitis. We also demonstrated the unique local regulation of GRAIL by miRNA. We have previously reported that GRAIL mRNA expression was increased in the peripheral blood $CD4^+$ T cells of patients with UC in the remissive state, while the levels of GRAIL were not different between HV and UC in the active state [15]. In the present study, we demonstrated that GRAIL mRNA and protein expressions were lower in the peripheral blood $CD4^+$ T cells of the patients with CD than in those of healthy subjects, irrespective of the activity of the patients with CD. Thus, the expression pattern of GRAIL in the peripheral blood is completely different between UC and CD. Our present results suggest that systemic T-cell anergy is abrogated in patients with CD, even in patients who have established clinical remission. Decrease of GRAIL expression in the peripheral blood of $CD4^+$ T cells may be used as a new biomarker of CD. Our present results showing difference in the expression of GRAIL between CD and UC are consistent with the fact that T-cell cycle abnormalities are present in CD, indicating a state of hyperreactivity compatible with a loss of tolerance in contrast to a hyporeactive state in UC [28]. The precise mechanisms of the difference of T cell states are unknown, and the differences in anergic immune status between CD and UC need to be further investigated.

Our study demonstrated the unique expression and regulation of GRAIL in the LP in physiological and inflammatory conditions. In non-colitic mice, GRAIL protein expression was lower in LP $CD4^+$ T cells, regardless of the high expression of GRAIL mRNA, in comparison to the SP $CD4^+$ T cells. In terms of the discrepancy between mRNA and protein levels of GRAIL in the LP, we initially speculated that GRAIL protein degradation by Otub-1 might mediate the discrepancy of GRAIL mRNA and protein expressions [25]. Otub-1 is a member of the deubiquitinating enzymes group (DUBs) with the capacity to cleave proteins at the ubiquitin-protein bond using its cysteine protease domain. GRAIL expression is reduced by Otub-1 expression via the abrogation of ubiquitin-specific protease (USP) 8-mediated deubiquitination and stabilization of GRAIL [14, 29]. Otub-1 expression, however, was not found to be associated with the local regulation of GRAIL in this study. Recent studies have shown that miRNAs are associated with the pathogenesis of CD using plasma and colonic tissues [30], but the functions of these miRNAs are not yet clarified. In the present study, we firstly identified a novel system to suppress GRAIL protein expression by miR-290 in the normal intestinal mucosa. Elevation of miR-290 was observed in the LP $CD4^+$ T cells of control mice, but not in those of inflamed colon or in other lymphoid organs (SP, MLN). In the non-inflammatory condition, translation of GRAIL was suggested to be suppressed by miR-290. In contrast, the GRAIL protein expression might not be decreased because the expression of miR-290 was not increased in the inflammatory condition. Thus, GRAIL protein expression turned out to be higher in the LP $CD4^+$ cells from colitic mice than in those from control mice. In addition to the regulation by miRNA, the $GRAIL^{high}$ T cells migrated to the inflamed intestine (Fig. 5f, Supplementary Fig. 6B). The alteration of cellular migration after the expression of GRAIL must be further investigated.

The regulatory role of $GRAIL^{high}$ DO11.10 cells has already been demonstrated in vitro [12]. We confirmed a regulatory function of $GRAIL^{high}$ T cells by investigating the proliferative activity of fluorescent-labeled T cells (Supplementary Figure 4). $GRAIL^{high}$ T cells did not exhibit either the upregulation of regulatory cytokines or the decrease of the effector cytokines (Fig. 4b). These results were consistent with the results showing that $GRAIL^{high}$ T cells exhibit a regulatory function without the induction of regulatory cytokines [12]. GRAIL-positive T cells were also shown to highly express FoxP3. In addition, we demonstrated that transfer of $GRAIL^{high}$ T cells partially reduced intestinal inflammation in murine colitis. These results indicated that GRAIL-expressing T cells have a potential to prevent acceleration of inflammation in the intestinal mucosa. Although cells co-expressing FoxP3 and GRAIL were highly detected in the inflamed mucosa of the colon of CD patients, intestinal inflammation was evident in the CD patients. An ameliorative effect of GRAIL may not be so powerful as to establish a complete reduction of inflammation. We may have to investigate whether the $GRAIL^{high}$ cells still maintain a regulatory function in the local inflammatory site. The pathophysiological roles of GRAIL-expressing T cells are yet to be further investigated in relation to luminal antigens and commensal bacteria that reside in the intestine.

It should be noted as a limitation of our study that no identical miRNA for murine miR-290 was found in the database for humans (miRBase). Because the GRAIL expression pattern in the LP $CD4^+$ T cells was similar in patients with CD and *Il-10*$^{-/-}$ mice, it was speculated that the GRAIL of CD patients is regulated by a similar mechanism to that of *Il-10*$^{-/-}$ mice. This regulatory mechanism of GRAIL by miRNA can be novel for controlling immune status via the alteration of the anergic status of $CD4^+$ T cells, and more investigation is required to search for the miRNAs that control GRAIL in humans.

In conclusion, our study revealed a reduction of GRAIL expression in the peripheral blood of patients with CD. We also showed the presence of GRAIL-expressing $CD4^+$

T cells in the inflamed intestinal mucosa and a novel regulatory mechanism of GRAIL by miRNA. Our understanding of miRNA biogenesis will provide insight into the cellular functions in the inflammatory processes of IBD. A more precise investigation of the regulatory mechanisms of GRAIL would help to explore powerful therapeutic strategies for controlling inflammation by inducing anergy.

Acknowledgments We thank Drs. Takatoshi Nawa, Satoshi Shimizu (Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine), and Hiroaki Yamanaka, Ph.D. (Pattern Formation Group, Osaka University Graduate School of Frontier Bioscience) for technical advice about generating plasmid vectors and AcGFP fusion protein. This work was supported by a Grant in Aid from the Japan Society for the Promotion of Science (Grant No. 23590941).

Conflict of interest The authors declare that they have no conflicts of interest.

References

1. Leone V, Chang EB, Devkota S. Diet, microbes, and host genetics: the perfect storm in inflammatory bowel diseases. J Gastroenterol. 2013;48:315–21.
2. Hamilton MJ, Snapper SB, Blumberg RS. Update on biologic pathways in inflammatory bowel disease and their therapeutic relevance. J Gastroenterol. 2012;47:1–8.
3. Arimura Y, Isshiki H, Onodera K, Nagaishi K, Yamashita K, Sonoda T, et al. Characteristics of Japanese inflammatory bowel disease susceptibility loci. J Gastroenterol. 2013. doi:10.1007/s00535-013-0866-2.
4. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol. 2006;3:390–407.
5. Anandasabapathy N, Ford GS, Bloom D, Holness C, Paragas V, Seroogy C, et al. GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells. Immunity. 2003;18:535–47.
6. Iweala OI, Nagler CR. Immune privilege in the gut: the establishment and maintenance of non-responsiveness to dietary antigens and commensal flora. Immunol Rev. 2006;213:82–100.
7. Kraus TA, Toy L, Chan L, Childs J, Mayer L. Failure to induce oral tolerance to a soluble protein in patients with inflammatory bowel disease. Gastroenterology. 2004;126:1771–8.
8. Mueller DL. E3 ubiquitin ligases as T cell anergy factors. Nat Immunol. 2004;5:883–90.
9. Lineberry NB, Su LL, Lin JT, Coffey GP, Seroogy CM, Fathman CG. Cutting edge: the transmembrane E3 ligase GRAIL ubiquitinates the costimulatory molecule CD40 ligand during the induction of T cell anergy. J Immunol. 2008;181:1622–6.
10. Su LL, Iwai H, Lin JT, Fathman CG. The transmembrane E3 ligase GRAIL ubiquitinates and degrades CD83 on CD4 T cells. J Immunol. 2009;183:438–44.
11. Nurieva RI, Zheng S, Jin W, Chung Y, Zhang Y, Martinez GJ, et al. The E3 ubiquitin ligase GRAIL regulates T cell tolerance and regulatory T cell function by mediating T cell receptor-CD3 degradation. Immunity. 2010;32:670–80.
12. MacKenzie DA, Schartner J, Lin J, Timmel A, Jennens-Clough M, Fathman CG, et al. GRAIL is up-regulated in CD4+ CD25+ T regulatory cells and is sufficient for conversion of T cells to a regulatory phenotype. J Biol Chem. 2007;282:9696–702.
13. Schartner JM, Singh AM, Dahlberg PE, Nettenstrom L, Seroogy CM. Recurrent superantigen exposure in vivo leads to highly suppressive CD4+ CD25+ and CD4+ CD25$^-$ T cells with anergic and suppressive genetic signatures. Clin Exp Immunol. 2009;155:348–56.
14. Whiting CC, Su LL, Lin JT, Fathman CG. GRAIL: a unique mediator of CD4 T-lymphocyte unresponsiveness. FEBS J. 2011;278:47–58.
15. Egawa S, Iijima H, Shinzaki S, Nakajima S, Wang J, Kondo J, et al. Upregulation of GRAIL is associated with remission of ulcerative colitis. Am J Physiol Gastrointest Liver Physiol. 2008;295:G163–9.
16. MacDonald TT, Monteleone I, Fantini MC, Monteleone G. Regulation of homeostasis and inflammation in the intestine. Gastroenterology. 2011;140:1768–75.
17. Lennard-Jones JE. Classification of inflammatory bowel disease. Scand J Gastroenterol Suppl. 1989;170:2–6; discussion 16–9.
18. Podolsky DK. Inflammatory bowel disease (1). N Engl J Med. 1991;325:928–37.
19. 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.
20. Fujihashi K, Taguchi T, Aicher WK, McGhee JR, Bluestone JA, Eldridge JH, et al. Immunoregulatory functions for murine intraepithelial lymphocytes: gamma/delta T cell receptor-positive (TCR+) T cells abrogate oral tolerance, while alpha/beta TCR+ T cells provide B cell help. J Exp Med. 1992;175:695–707.
21. Takahashi I, Kiyono H, Hamada S. CD4+ T-cell population mediates development of inflammatory bowel disease in T-cell receptor alpha chain-deficient mice. Gastroenterology. 1997;112:1876–86.
22. Sudo H, Mizoguchi A, Kawauchi J, Akiyama H, Takizawa S. Use of non-amplified RNA samples for microarray analysis of gene expression. PLoS ONE. 2012;7:e31397.
23. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, et al. Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nat Genet. 2001;29:365–71.
24. Dohi T, Ejima C, Kato R, Kawamura YI, Kawashima R, Mizutani N, et al. Therapeutic potential of follistatin for colonic inflammation in mice. Gastroenterology. 2005;128:411–23.
25. Soares L, Seroogy C, Skrenta H, Anandasabapathy N, Lovelace P, Chung CD, et al. Two isoforms of otubain 1 regulate T cell anergy via GRAIL. Nat Immunol. 2004;5:45–54.
26. Sinkkonen L, Hugenschmidt T, Berninger P, Gaidatzis D, Mohn F, Artus-Revel CG, et al. MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. Nat Struct Mol Biol. 2008;15:259–67.
27. Corthay A. How do regulatory T cells work? Scand J Immunol. 2009;70:326–36.
28. Sturm A, Leite AZ, Danese S, Krivacic KA, West GA, Mohr S, et al. Divergent cell cycle kinetics underlie the distinct functional capacity of mucosal T cells in Crohn's disease and ulcerative colitis. Gut. 2004;53:1624–31.
29. Lin JT, Lineberry NB, Kattah MG, Su LL, Utz PJ, Fathman CG, et al. Naive CD4 t cell proliferation is controlled by mammalian target of rapamycin regulation of GRAIL expression. J Immunol. 2009;182:5919–28.
30. Dalal SR, Kwon JH. The role of MicroRNA in inflammatory bowel disease. Gastroenterol Hepatol (N Y). 2010;6:714–22.

腸内細菌異常と腸管感染症(偽膜性腸炎など)

Alterations of intestinal bacterial flora and intestinal infectious diseases

飯島英樹
Hideki IIJIMA
大阪大学大学院医学系研究科消化器内科学

◎ヒトには非常に多数の腸内細菌が共生しているが，抗菌薬などの使用による菌交代現象の結果，腸内細菌の構成は大きく変化し，腸管炎症を発症することがある．その代表的なものは *C. difficile* 感染症(CDI)や急性出血性大腸炎などである．欧米において従来の治療法で制御することが困難な難治性 CDI が激増し，大きな問題となっている．これに対して便移植という非常にユニークな治療法が行われ，従来の治療法を上まわる効果をあげている．世界中で腸内細菌の研究は精力的に行われており，腸内細菌のバランスの維持の重要性，およびそれが破綻した際の疾患の発症について急速に解明が進んでいる．

Key word：腸内細菌異常，菌交代現象，偽膜性腸炎，*Clostridium difficile*，糞便移植

ヒト腸内にはヒト自身の細胞数(60 兆個)を超える 100 兆個以上の常在菌が共生しており，吸収，代謝，栄養素の産生，免疫などを介して，ヒトの健康と疾患に直接にかかわっていることが明らかとなってきた[1]．腸内細菌叢の構成は食事やプレバイオティクス，プロバイオティクスの投与により緩徐に変化するが，抗菌薬が投与された場合には劇的な変化が生じる．

本稿ではおもに，抗菌薬の投与後に腸内細菌の変化により引き起こされる代表的な腸管感染症の診断および治療の現況について概説する．

*C. difficile*関連性腸炎の診断と治療

Clostridium difficile(以下，*C. difficile*)は偏性嫌気性グラム陽性桿菌で，健康な乳児の糞便からは 20～40%の割合で検出されるが，加齢とともに *C. difficile* の検出は減少する．抗菌薬の使用などにより健康成人ではあまり検出されない *C. difficile* の異常増殖により *C. difficile* 感染症(*C. difficile* infection：CDI)状態となり，大腸炎を発症する．CDI は入院患者，とくに長期臥床者，悪性腫瘍や腹部手術後，免疫抑制剤使用者などの compromised host に対して抗菌薬を使用した場合に多く発症する(図 1)．抗菌薬の開始数日から数週後に比較的急性に下痢(ときに血性)，発熱，腹痛を発症した場合は本症を念頭におくことが必要である．原因となる抗菌薬はニューキノロン系やラクタム系などが多いが，CDI の治療に使われるメトロニダゾールやバンコマイシンなども原因となりうる．CDI は院内感染として発症することがあるが，*C. difficile* により汚染された室内では芽胞が数カ月から数年間残存し，医療従事者を介した感染拡大の原因となるといわれている．

大腸内視鏡では典型的には特徴的な白苔(偽膜)がみられることから偽膜性腸炎といわれるが(図 2)，偽膜を形成しない場合もある．Tonna らは CDI を，①無症候性保菌者，②抗菌薬関連下痢症，③非偽膜性慢性下痢症，④偽膜性大腸炎，⑤劇症偽膜性大腸炎，の 5 型に分類している[2]．*C. difficile* が産生する毒素には，①エンテロトキシンとして作用するトキシン A と，②サイトトキシンとして作用するトキシン B，の 2 つが知られており，腸炎の発症には両者が共同的に働く．トキシン A，B とも glycosyltransferase 活性をもち，低