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

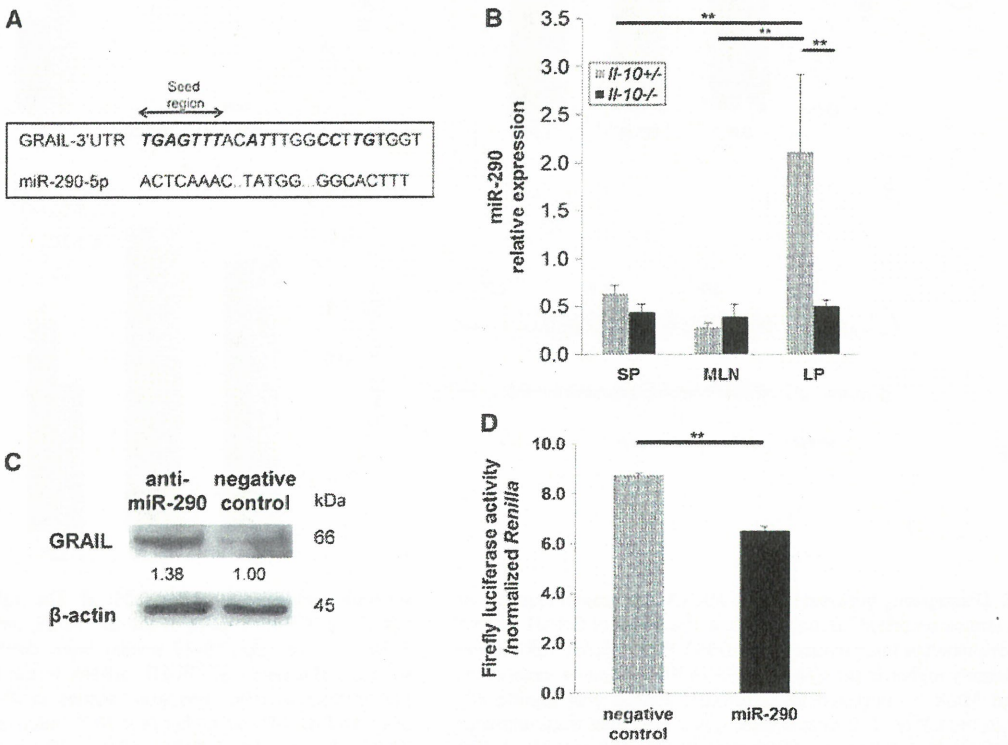

Fig. 3 miR-290-5p regulates GRAIL expression in vitro. **a** The sequences of miR-290-5p and 3′UTR of GRAIL. **b** Expression of miR-290-5p was determined by qRT-PCR. Expression of miR-290-5p was significantly higher in the LP of *Il-10*$^{+/-}$ mice than in the other lymphoid tissues and was significantly 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 ($n = 6$, $^{**}P < 0.01$)

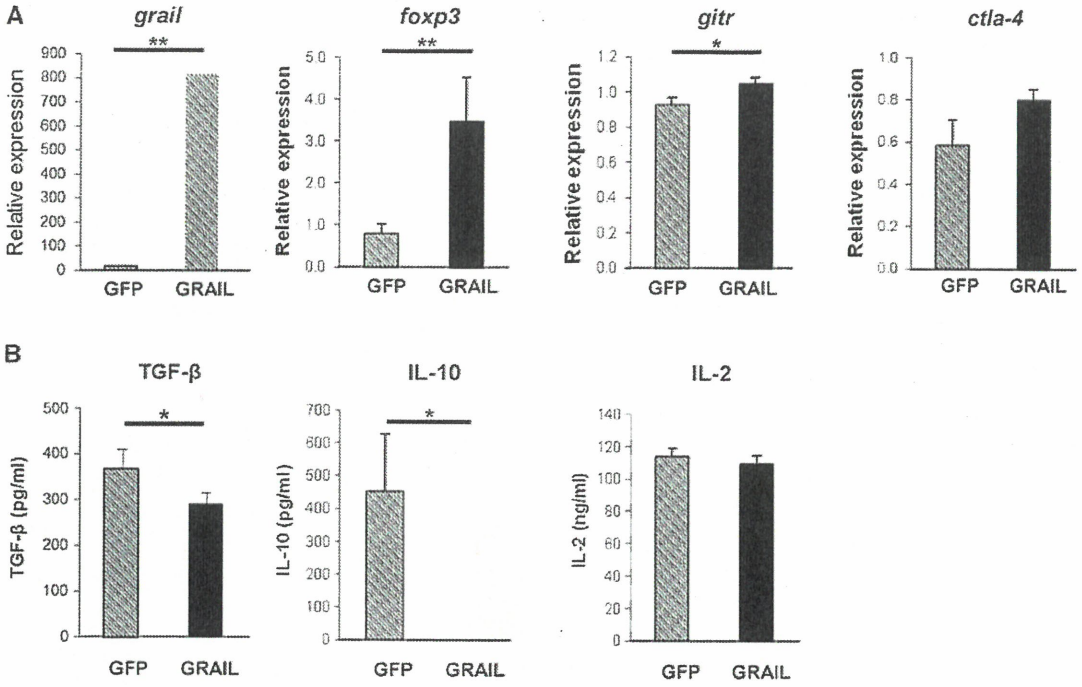

Fig. 4 Forced expression of GRAIL converts DO11.10 cells to a regulatory phenotype. **a** Either pAcGFP-GRAIL vector or empty pAcGFP vector was transfected to DO11.10 cells, and *grail*, *foxp3*, *gitr*, and *ctla-4* mRNA expressions in AcGFP positive cells were determined by qRT-PCR. Expressions of *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-over-expressing 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).

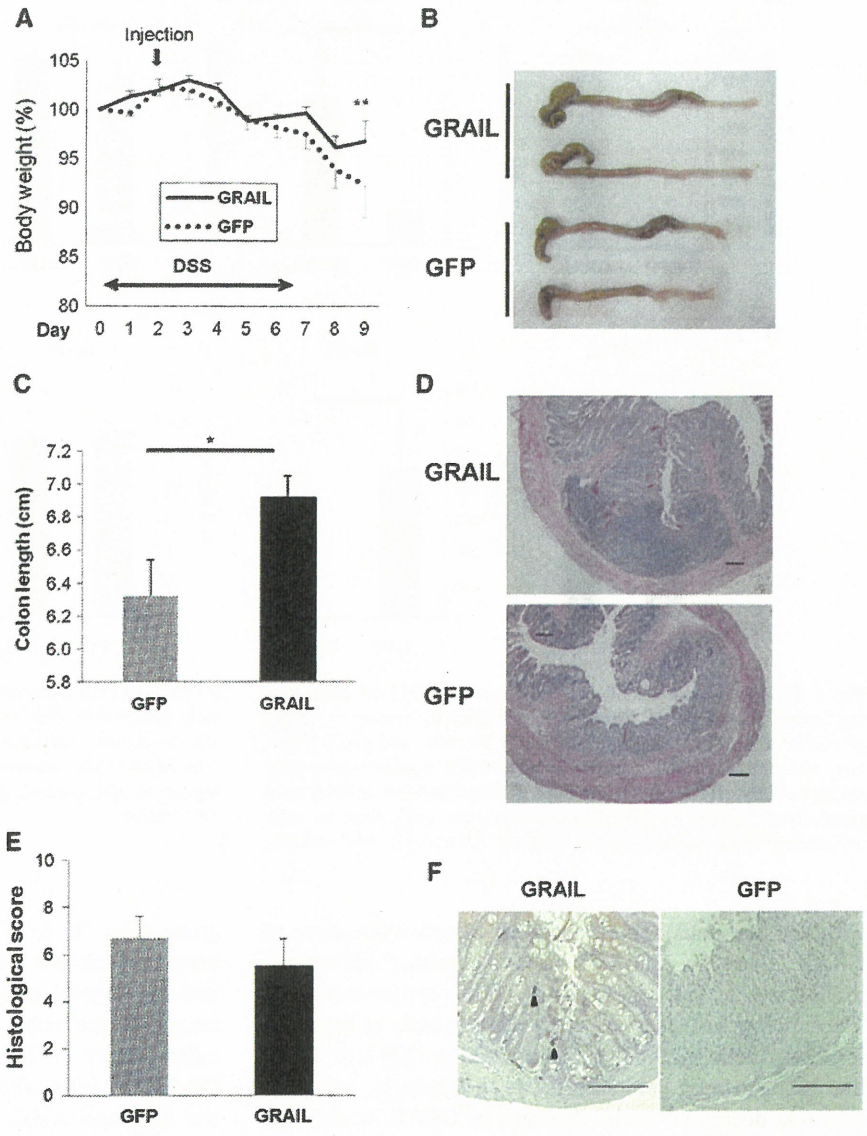

Fig. 5 Transfer of GRAIL^{high} T cells suppresses colonic inflammation induced by dextran sodium sulfate. **a** Mice induced with DSS colitis were injected with either GRAIL^{high} DO11.10 (GRAIL) or the cells transfected with the GFP-expressing empty vector (GFP) on day 2. Mice injected with GRAIL^{high} 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, ×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)

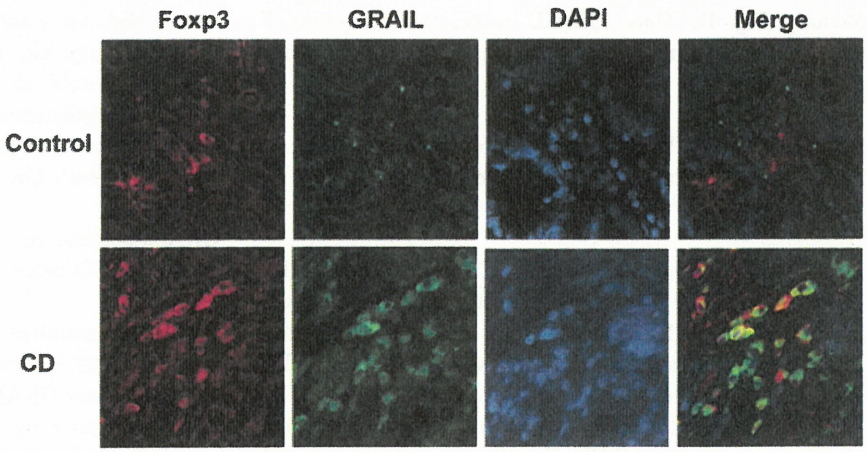

Fig. 6 Immunohistochemical staining of FoxP3 and GRAIL in the non-inflamed, colonic mucosa of colon cancer patients (control) and in the inflamed mucosa of CD patients. Colonic tissues were stained with anti-GRAIL antibody followed by Alexa®488-labeled anti-rabbit IgG (*green*), mouse anti-FoxP3 antibody 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.