Upregulation of GRAIL is associated with remission of ulcerative colitis

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Egawa S, Iijima H, Shinzaki S, Nakajima S, Wang J, Kondo J, Ishii S, Yoshio T, Irie T, Nishida T, Kakiuchi Y, Yasumaru M, Yoshihara H, Kanto T, Tsujii M, Tsuji S, Hayashi N. Upregulation of GRAIL is associated with remission of ulcerative colitis. Am J Physiol Gastrointest Liver Physiol 295: G163–G169, 2008. First published May 8, 2008; doi:10.1152/ajpgi.90242.2008.—Abrogating tolerance against unidentified antigens is a critical step in the pathogenesis of ulcerative colitis (UC). T cell anergy, one of the main mechanisms of tolerance, has been shown to be induced by E3 ubiquitin ligases, such as gene related to anergy in lymphocytes (GRAIL), Itch, and c-Cbl in mice. However, it is not well known whether these E3 ligases play roles in human diseases. The pathophysiological role of the E3 ligases in patients with UC was investigated. At first, the expression of GRAIL, Itch, and c-Cbl in human anergic T cells was analyzed by quantitative RT-PCR and Western immunoblotting. Next, the mRNA expression of the E3 ligases was analyzed in peripheral CD4+ T cells of patients with UC and 10 healthy volunteers (HV). mRNA expression was analyzed in patients with active UC before and after treatment with prednisolone and leukocytapheresis. Anergic human CD4+ T cells expressed significantly higher levels of GRAIL, Itch, and c-Cbl than nonanergic cells. GRAIL expression was significantly higher in patients with UC in remission than in patients with active disease and in HV (P < 0.01). The level of GRAIL expression was also significantly increased in patients with active disease whose clinical activity index scores improved after treatment (P < 0.05). There were no significant differences in Itch and c-Cbl expression among patients with active UC, patients with UC in remission, and HV. These data suggest that GRAIL plays an important role in maintaining remission in patients with UC.

T cell anergy; E3 ubiquitin ligase; tolerance; inflammatory bowel disease

IMMUNOLOGICAL STATUS AGAINST LUMINAL BACTERIA AND FOOD ANTIGENS PLAYS A CRUCIAL ROLE IN THE DEVELOPMENT AND HEALING OF UC (22). IN HEALTHY SUBJECTS, MUCOSAL TOLERANCE IN THE GUT LUMEN PREVENTS BACTERIA AND FOOD ANTIGENS FROM EVOKING T CELL ACTIVATION BY ELIMINATING ACTIVATED ANTEN- SPECIFIC T CELLS THROUGH CLONAL DELETION, CLONAL ANERGY, AND ACTIVE SUPPRESSION (8). IN CONTRAST, PATIENTS WITH ACTIVE UC REACT TO THEIR OWN BACTERIAL FLORA AND VARIOUS DIETARY ANTIGENS DUE TO ABROGATION OF TOLERANCE (6, 11, 20). RECENTLY, DISRUPTIONS IN ORAL TOLERANCE IN PATIENTS WITH ACTIVE INFLAMMATORY BOWEL DISEASE (IBD) WERE FURTHER CONFIRMED WITH THE USE OF KEYHOLE LIMPET HEMOCYANIN AS A MODEL ANTIGEN (13). ACCORDING TO REPORTS OF STUDIES USING ANIMAL MODELS, GENETIC PREDISPOSITION ALSO CONtributes TO ABROGATION OF IMMUNE TOLERANCE AGAINST NORMAL ENTERIC FLORA (4, 7, 27). CLONAL ANERGY IS ONE OF THE IMPORTANT MECHANISMS GOVERNING IMMUNE TOLERANCE. DURING ANERGY, LYMPHOCYTES ARE INTRINSICALLY AND FUNCTIONALLY INACTIVATED FOLLOWING AN ANTIGEN ENCOUNTER AND REMAIN IN A HYPORESPONSIVE STATE FOR AN EXTENDED PERIOD (23). ANERGIC CELLS DO NOT PROLIFERATE OR EXPRESS INTERLEUKIN-2 FOLLOWING T CELL RECEPTOR-SPECIFIC STIMULATION BY THEIR COGNATE ANTIGENS, EVEN IN THE PRESENCE OF ADEQUATE COSTIMULATION. UNRESPONSIVENESS IS REPORTED TO BE CORRELATED WITH AN INCREASE IN INTRACELLULAR CALCIUM; HOWEVER, THE MECHANISM UNDERLYING INDUCTION OF T CELL ANERGY IS POORLY UNDERSTOOD (10). RECENTLY, SEVERAL E3 UBQUITIN LIGASES HAVE BEEN DEMONSTRATED TO BE ESSENTIAL FOR INDUCTION OF T CELL ANERGY (19). UBQUITIN IS A HIGHLY CONSERVED 76-AMINO ACID GLOBULAR PROTEIN THAT ATTACHES TO SUBSTRATE PROTEINS, THEREBY AFFECTING MULTIPLE CELLULAR PROCESSES, INCLUDING CELLULAR TRAFFICKING, TRANSCRIPTIONAL ACTIVATION, AND PROTEASEOMAL- AND LYSOSONAL-MEDIATED DEGRADATION. UBQUITINATION IS ACCOMPLISHED THROUGH A SERIES OF ENZYMATIC STEPS CATALYZED BY A UBQUITIN-ACTIVATING ENZYME (CALLED E1), A UBQUITIN-CONGUJATING ENZYME (E2), AND A UBQUITIN LIGASE (E3), WHICH MEDIATES THE TRANSFER OF UBQUITIN FROM THE E2 PROTEIN TO A LYSINE RESIDUE ON THE TARGET PROTEIN (29). ONLY ONE E1 HAS BEEN IDENTIFIED IN MAMMALS COMPARED WITH OVER 30 E2 ENZYMES AND MANY MORE E3 LIGASES. E3 UBQUITIN LIGASES PROVIDE SUBSTRATE SPECIFICITY IN UBQUITINATION REACTIONS AND CONtribute TO THE MANY CELLULAR PROCESSES CONTROLLED BY UBQUITIN MODIFICATION. THE GENE RELATED TO ANERGY IN LYMPHOCYTES (GRAIL), ONE OF THE WELL-CHARACTERIZED E3 UBQUITIN LIGASES, IS A TYPE I TRANSMEMBRANE PROTEIN THAT LOCALIZES TO THE ENDOCYTIC PATHWAY AND CONTAINS A REALLY INTERESTING NEW GENE (RING) FINGER MOTIF (1, 26). CONSTITUTIVE RETROVIRAL EXPRESSION OF GRAIL HAS BEEN SHOWN TO RENDER NAIVE CD4+ T CELLS ANERGIC TO ANTIGENIC CHALLENGE (24), AND THE EXPRESSION OF GRAIL IN RETROVIRALLY TRANSduced T CELL HYBRIDOMA CELLS SIGNIFICANTLY INHIBITS ACTIVATION-INDUCED IL-2 AND IL-4 CYTOKINE PRODUCTION (1). REMARKABLY, OVEREXPRESSION OF AN ENZYMATICALLY INACTIVE FORM OF GRAIL THAT INHIBITS ENDogenous GRAIL FUNCTION SUCCESSFULLY BLOCKS THE DEVELOPMENT OF ANERGY (24). ITCH IS ALSO REPORTED TO BE AN E3 UBQUITIN LIGASE RELATED TO T CELL ANERGY IN MICE (9). ITCH-DEFICIENT MICE DEVELOP A PROGRESSIVE AUTOIMMUNE-LIKE DISEASE CHARACTERIZED BY LYMPHOPROLIFERATION IN THE LYMPHOID ORGANS, SUCH AS SPLEEN, LYMPH NODES, AND A MEDULLA OF THE THYMUS. C-CBL WAS IDENTIFIED FROM THE GENOME OF A TRANSFORMING RETROVIRUS IN MOUSE PRE-B LYMPHOMA CELLS (15)

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and was the first E3 ubiquitin ligase linked to the development of the clonal anergy state (5).

The correlation between anergy and these E3 ubiquitin ligases has been well described in mouse models. Also, the correlation of GRAIL expression and T cell function has been recently reported in human peripheral T cells (12); however, the expression of the E3 ligases in human diseases has not been reported. In this study, we examined the expression of GRAIL, Itch, and c-Cbl in human CD4+ T cells isolated from patients with active and remitting UC and identified the role of these E3 ubiquitin ligases in patients with UC.

MATERIALS AND METHODS

Subjects. Twenty patients with UC who visited or were hospitalized in Osaka University Hospital and Osaka Rosai Hospital were enrolled in this study (Table 1). Ten age-matched healthy volunteers (HV) were recruited and served as the control group. The diagnosis of UC was based on conventional clinical, endoscopic, and histopathological criteria. The clinical activity index (CAI) of UC was determined according to the criteria of Rachmilewitz et al. (21), and patients with UC whose CAI scores were equal or greater than 6 were considered active (14). Eight patients who had been asymptomatic and whose CAI score had been zero for longer than 3 mo without using corticosteroids or immunomodulators (azathioprine or 6-mercaptopurine) were enrolled as patients in remission. All of the subjects provided written informed consent, and the study protocol was approved by the Ethics Committee of Osaka University Graduate School of Medicine and Osaka Rosai Hospital.

Reagents and antibodies. Unlabeled anti-human CD3 and anti-CD28 monoclonal antibodies (mAbs), phycoerythrin (PE)-conjugated anti-CD25 (M-A251), CD45RA (HI100), FITC-conjugated anti-CD4 (RPA-T4), CD45RO (UCHL1), PECy5-conjugated anti-CD4 (RPA-T4), and isotype control Abs were purchased from BD Biosciences (San Jose, CA).

Induction of anergy in human CD4+ T cells in vitro. Peripheral blood mononuclear cells (PBMC) were prepared from heparinized peripheral blood of HV by density-gradient centrifugation using Ficoll-paque PLUS (GE Healthcare Bio-Sciences, Piscataway, NJ). CD4+ T cells were purified by positive selection using CD4 microbeads and a magnetic cell sorting system (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany). The purified CD4+ T cells (2 × 10^6/ml) were treated with 1.5 µM ionomycin (Sigma-Aldrich, St. Louis, MO) at 37°C for 6 h (mRNA) or 18 h (protein). Cells were washed, and cell pellets were prepared for quantitative RT-PCR or Western blot analysis. To confirm the induction of anergy, 5 × 10^5 cells were examined for proliferation. After 18 h of anergic stimuli, the cells were cultured in vitro in 96-well plates precoated with anti-CD3 mAb (2 µg/ml) together with soluble anti-CD28 mAb (1 µg/ml) for 72 h. The cells were pulsed for the final 16 h with [3H]thymidine, and the radioactivity was counted in a β-counter.

Cell purification. Memory T cells and CD4+CD25+ T cells were isolated using the Memory CD4+ T cell isolation kit and CD4+CD25+ regulatory T cell isolation kit, respectively (Miltenyi Biotech), according to the manufacturer’s protocol. Naïve T cells were isolated by positive selection of PE-conjugated anti-human CD45RA with anti-PE microbeads from unlabelled CD4+ T cells. Before positive selection of naïve T cells, the unlabeled CD4+ T cells were purified by negative selection with the CD4+ T cell isolation kit II (Miltenyi Biotech). The purity of the cells was >97%, as analyzed by flow cytometry.

Real-time quantitative RT-PCR. Total RNA was extracted from 25 µl of peripheral blood or collected materials during centrifugal leukocyteapheresis (CFLA) using guanidine thiocyanate-phenol chloroform extraction (ISOGEN; Wako, Osaka, Japan) and subsequently reverse transcribed with the use of random hexamer primers and the SuperScript 3 First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time quantitative RT-PCR for GRAIL, Itch, c-Cbl, and β-actin was performed using ready-to-use assays (Applied Biosystems, Foster City, CA) in the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Probes and primer sets specific for the GRAIL, Itch, and c-Cbl cDNA were purchased from Applied Biosystems. All reactions were performed in duplicate. The thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The quantities of each E3 ligase were expressed as the E3 ligase/β-actin arbitrary units (AU).

Immunoblot analysis. Cells were washed with cold PBS and lysed in RIPA buffer (1× Tris-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.004% sodium azide). Lysates were centrifuged at 12,000 revolution/min for 20 min, and protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Cleared lysates were resolved by SDS-PAGE and absorbed to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Proteins were detected with GRAIL (IMGENEX, San Diego, CA), Itch, and c-Cbl (BD Biosciences)-specific Abs. Blots were developed with horseradish peroxidase (HRP)-conjugated secondary Abs and enhanced chemiluminescence (GE Health Care).

Treatment and blood sampling by CFLA. CFLA was performed in eight patients with active UC with a Haemonetics CCS (Haemonetics, Braintree, MA) (Table 2). The access and return line was connected to the patients’ cubital vein, and apheresis was performed at a blood flow rate of 50 ml/min. In each session, the leukocyte-rich fractions were removed from 2,000 to 2,400 ml of patients’ peripheral blood. Mononuclear cells were isolated from the buffy coat using Ficoll-paque. Each patient was treated with CFLA once per week, and the treatment continued for 4–5 wk.

Flow cytometry. The mononuclear cells of patients with active UC obtained at CFLA were subjected to flow cytometry using specific surface antibodies. For flow cytometry, single-cell suspensions were stained for 30 min at 4°C with PE-, FITC-, and PECy5-conjugated mAbs. The stained cells were washed twice in fluorescence-activated cell sorting (FACS) buffer and the cells were resuspended in PBS containing 1% paraformaldehyde. The fixed cells were then analyzed on a FACScan flow cytometer (BD Biosciences). Data from 10^6 cells were analyzed with the Cell Quest software (BD Biosciences).

Statistical analysis. The Student’s t-test, Mann-Whitney U-test, the Kruskal-Wallis test followed by the Mann-Whitney U-test with Bonferroni correction, and the Wilcoxon signed-rank test were used for statistical analysis, using Stat View software Version 5.0 (SAS Institute, Cary, NC). A P value <0.05 was considered statistically significant.
GRLA UPREGULATION IN ULCERATIVE COLITIS IN REMISSION

Table 2. Characteristics of the subjects received CFLA

<table>
<thead>
<tr>
<th>No.</th>
<th>Gender</th>
<th>Age</th>
<th>Type</th>
<th>Corticosteroid</th>
<th>Immunosuppressant</th>
<th>CRP, mg/l</th>
<th>CAI</th>
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<tr>
<td>1</td>
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<td>37</td>
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<td>yes</td>
<td>29.0</td>
<td>15</td>
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<td>2</td>
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<td>40</td>
<td>left-sided</td>
<td>yes</td>
<td>no</td>
<td>5.0</td>
<td>9</td>
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<tr>
<td>3</td>
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<td>18</td>
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<td>7</td>
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<tr>
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<td>no</td>
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<td>7</td>
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</table>

*CAI score was increased after centrifugal leukocytapheresis (CFLA).

RESULTS

GRLA, Itch, and c-Cbl mRNA and protein expression are upregulated in human anergic CD4+ T cells. Human CD4+ T cells freshly isolated from HV were cultured in the anergic or activated condition, and mRNA expression of these E3 ubiquitin ligases was analyzed. The calcium ionophore ionomycin has previously been used to mimic early events in the induction of T cell anergy (10, 16). The mRNA levels of the E3 ubiquitin ligases (GRLA, Itch, and c-Cbl) were significantly higher in the ionomycin-treated CD4+ T cells than in the nontreated cells (Fig. 1A). In addition, protein expression of these E3 ubiquitin ligases in ionomycin-treated cells was upregulated compared with untreated cells, as determined by Western immunoblotting (Fig. 1B). The anergic state was confirmed by proliferation assay; the CD4+ T cells cultured in vitro with ionomycin showed significantly lower proliferation rates than the nontreated cells (Fig. 1C). Alternatively, T cell anergy can be induced by stimulation with anti-CD3 mAb in the absence of anti-CD28 mAb stimulation, whereas T cells are activated by the costimulation with anti-CD3 mAb and anti-CD28 mAb (25). Under anergic conditions (induced by anti-CD3 mAb treatment alone), GRLA mRNA expression in human T cells was significantly higher than that of cells costimulated with anti-CD3 mAb and anti-CD28 mAb (P < 0.05, data not shown).

Expression of GRLA, Itch, and c-Cbl in CD4+ T cell subsets. To determine the cell types that express GRLA, Itch, and c-Cbl in human PBMCs of HV, we isolated CD4+ T cells from PBMCs and compared the expression of the E3 ligases in CD4+ T cells and CD4+ cells. GRLA was more highly expressed on CD4+ T cells than on CD4- cells (P < 0.05, Fig. 2A). However, the levels of Itch and c-Cbl mRNA were not higher in CD4+ T cells than in CD4- cells (Fig. 2A). Because GRLA was highly expressed in CD4+ T cells, we analyzed the expression of GRLA in memory/naive and regulatory/non-regulatory T cell subsets of CD4+ T cells. However, there were no significant differences in the levels of GRLA mRNA expression between the naive CD4+CD45RA+ T cell subset and the memory CD4+CD45RO+ T cell subset (Fig. 2B). In contrast, the expression of Itch and c-Cbl in naive CD4+CD45RA+ T cells was significantly higher than that in memory CD4+CD45RO+ T cells. Because murine regulatory CD4+CD25+ T cells are reported to be anergic (28), the level of GRLA mRNA in human peripheral CD4+CD25+ T cells was examined and compared with that in CD4+CD25+ T cells. However, there was no significant difference in the level of GRLA expression between these two subsets (Fig. 2C). The expression of Itch and c-Cbl also revealed no significant difference between the two subsets.

Patients with UC in remission express high levels of GRLA mRNA. We next analyzed the expression of GRLA, Itch, and c-Cbl mRNA in human CD4+ T cells isolated from patients with UC. The patients’ profile and disease characteristics are presented in Table 1. When we compared the levels of GRLA, Itch, and c-Cbl expression between all UC patients (both active and in remission) and HV, we found no significant differences in the levels of E3 ligases between the two groups (data not shown). However, the level of GRLA expression was significantly higher in UC patients in remission than in HV (median 1.25 AU vs. 0.23 AU, P = 0.0021, Fig. 3A). In addition,
GRAIL expression was significantly higher in patients with UC in remission than in patients with active UC (median 1.25 AU vs. 0.16 AU, \( P = 0.0015 \)). The levels of GRAIL expression in patients with active UC were similar to those of HV. Although there was a trend toward increased levels of Itch and c-Cbl expressions in patients in remission compared with other groups, there were no significant differences in the levels of Itch and c-Cbl mRNA among the three groups (Fig. 3A).

The correlation between the level of GRAIL expression and extension of the disease in patients with UC was analyzed further. GRAIL expression was relatively lower in patients with pancolitis than in patients with left-sided colitis, but the results were not statistically significant (Fig. 3B). Although we analyzed the correlation between the level of GRAIL expression and severity of UC, GRAIL expression did not correlate with CAI (data not shown).

CAI scores, GRAIL expression, and T cell subsets at the initial and final CFLA. We next analyzed GRAIL mRNA expression during treatment of UC. Eight patients with active UC were treated with prednisolone (initial dose was 40–80 mg/day) and CFLA (once per wk) for 4–5 wk. In these eight patients, CAI scores at the final CFLA were significantly decreased compared with the scores at the first CFLA (Fig. 4A, \( P < 0.05 \)). In these patients, the levels of GRAIL mRNA expression were not significantly different between the samples obtained at the first and the final CFLA (Fig. 4B). However, when a patient who did not respond to the treatment (○ in Fig. 4, A and B) was eliminated from the analysis, the levels of GRAIL mRNA expression were significantly higher in CD4^+ T cells obtained at the final CFLA (average 1.22 AU) than in those obtained at the first CFLA (average 0.98 AU) in patients who responded to the treatment (● in Fig. 4, A and B, \( P < 0.05 \)). In a patient whose CAI increased despite treatment, GRAIL expression was decreased after the treatment (○ in Fig. 4, A and B).

It has been reported that \( \sim 10^9 \) cells can be eliminated from the blood circulation in each CFLA session (3), and there is a possibility that change of GRAIL expression is induced by alteration of cellular population by CFLA. Because CD4^+ T cells express higher levels of GRAIL compared with CD4^- cells (Fig. 2A), it is possible that increase of the CD4^+ T cell population in the peripheral blood corresponds with the elevation of GRAIL after CFLA. However, the proportion of CD4^+ T cells obtained in the buffy coat was not significantly different between the first and the final CFLA (Fig. 4C). During analysis of the surface markers of buffy coat cells, we found that the proportion of naïve T cells was significantly increased and the proportion of memory T cells was significantly decreased after the treatment (\( P < 0.05 \), Fig. 4, D and E). However, the levels of GRAIL mRNA expression were not different between memory and naïve CD4^+ T cells, as shown in Fig. 2A. It is also possible that regulatory T cells (Tregs) were increased and contribute to the improvement of CAI scores by CFLA. However, there was no significant difference in the proportion of regulatory CD4^+CD25^{high} T cells between the first and the final CFLA (Fig. 4F).

GRAIL expression and duration of maintaining remission in UC after the treatment by CFLA. One may suspect that GRAIL expression is not only driving but also maintaining remission in UC. We therefore examined the GRAIL expression and the
duration of remission in the patients with UC after the treatment by CFLA. GRAIL expression was relatively higher in those who maintained long-term remission than those who encountered early relapse, although not statistically significant (Fig. 5). The average of GRAIL expression levels was 0.18 AU in patients who maintained remission less than 6 mo and was 1.95 AU in patients who maintained remission more than 6 mo.

**DISCUSSION**

Induction of clonal anergy in T cells is associated with global defects in T cell receptor (TCR) signaling, including reduced phosphorylation of TCR-ζ and -ε chains, poor activation of p56Lck, 70-kDa zeta-associated protein (Zap70), Ras, c-JNK, ERK, and defective transactivation at the IL2 gene by NF-κB, activating protein 1 (AP-1), and NF-AT(19). E3 ubiquitin ligases, such as GRAIL, Itch, and c-Cbl, have been shown to be involved in the process of T cell anergy in animals (1, 19, 24). In this study, we found that GRAIL, Itch, and c-Cbl mRNA and protein were highly expressed on anergic human CD4+ T cells. These E3 ubiquitin ligases appear to reflect the status of T cell anergy not only in mice but also in human CD4+ T cells. We demonstrated that GRAIL expression in CD4+ T cells of patients with UC in remission was significantly higher than that in patients with active UC or in HV (Fig. 3A). Because the anergic CD4+ T cells express high levels of GRAIL (Fig. 1), it is possible that patients in remission have more profoundly anergic CD4+ T cells than do patients with active UC or HV. In addition, because the level of GRAIL expression was not significantly different regardless of the inflamed location (Fig. 3B) or activity of UC, low GRAIL expression is suggested to be involved even in the mild inflammation of UC. The expression of Itch and c-Cbl in UC patients in remission was relatively higher than in patients with active UC and in HV; this result further supports the involvement of anergy in the remission of UC.

We demonstrated that GRAIL expression in CD4+ T cells of patients with UC in remission was significantly higher than that in patients with active UC or in HV (Fig. 3A). Because the anergic CD4+ T cells express high levels of GRAIL (Fig. 1), it is possible that patients in remission have more profoundly anergic CD4+ T cells than do patients with active UC or HV. In addition, because the level of GRAIL expression was not significantly different regardless of the inflamed location (Fig. 3B) or activity of UC, low GRAIL expression is suggested to be involved even in the mild inflammation of UC. The expression of Itch and c-Cbl in UC patients in remission was relatively higher than in patients with active UC and in HV; this result further supports the involvement of anergy in the remission of UC. It was difficult to evaluate the status of T cell anergy in patients with UC because the antigen(s) responsible for the induction of inflammation in UC is (are) unknown. However, our data, in addition to other previous information about E3 ligases (1, 19), suggest that analyzing the E3 ubiquitin ligases will provide information about T cell anergic status. In addition to the driving property of GRAIL to remission of UC, GRAIL might be important for maintaining remission of UC. Considering clinical relevance of maintaining remission in UC, we further examined GRAIL level and duration of the remission in the patients treated with CFLA. Although the number of the subjects was limited to confirm this hypothesis, the expression of GRAIL was relatively higher in UC patients who maintained long-term remission than those who encountered early relapse. These preliminary results suggest the association of GRAIL with remission of UC, which remained to be investigated in further study involving the larger number of patients with UC.
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It is also necessary to analyze the expression of the E3 ligases in the colonic mucosa to further clarify the roles of the E3 ligases in the mucosal T cell anergy. However, we could not detect the E3 ligase mRNA expressions either from intestinal biopsy samples or from peripheral blood samples from patients with Crohn’s disease by the conventional method. Evaluation of the E3 ligases in the mucosa and blood samples of Crohn’s disease by modified methods is ongoing.

It was expected that CD4+ T cells of HV show higher levels of anergic status than that of patients with active UC. However, in this study, GRAIL expression in CD4+ T cells of HV was not significantly higher than that of patients with active UC. One potential explanation is that HV do not encounter anergic or pathogenic stimulation from the environment. The exact mechanisms regulating the expression levels of the E3 ligases are unknown and need further investigation.

In addition to the observation of high GRAIL expression in patients with UC in remission, GRAIL expression was significantly increased after treatment in patients with active UC whose CAI scores improved after treatment with CFLA and prednisolone. Previous reports demonstrated that the number of memory CD4+CD45RO+ T cells of patients with UC decreases after leukocytapheresis with the use of a leukocyte removal filter (2). We obtained similar results showing a significant decrease in memory CD4+CD45RO+ T cells and significant increase in naive CD4+CD45RA+ T cells at the final CFLA compared with the initial CFLA (Fig. 4, D and E). However, we observed no significant difference in the expression of GRAIL between memory and naive CD4+ T cells. It is also reported, in patients with IBD, CD4+CD25high Tregs increase during remission but decrease during active disease (18). Therefore, it was speculated that Tregs were attributed for decrease in the CAI scores after the CFLA. However, we found that the percentages of CD4+CD25high T cells before and after CFLA were not significantly different. In addition, GRAIL expression was not significantly different between CD4+CD25+ T cells and CD4+CD25− T cells. These results suggest that GRAIL expression increased in patients with UC independently of the proportion of naive, memory, and Tregs.

We also found some differences in the expression of the E3 ligases between humans and mice. High GRAIL expression was reported for murine CD4+CD25+ T cells (17). However, our observations indicate that GRAIL expression in human CD4+CD25+ T cells was not higher than that in CD4+CD25− T cells (Fig. 2C). In human CD4+ T cells, there was no significant difference in the levels of GRAIL expression between naive and memory CD4+ T cell subsets (Fig. 2B). In contrast, GRAIL expression in murine memory CD4+ T cells was significantly higher than that in naive CD4+ T cells (S. Egawa, unpublished observations). The E3 ligase expression variations could be caused by species-specific differences or by differences in antigen exposure between humans and mice, since mice used for experimentation are exposed to a limited number of antigens in pathogen-free animal facilities.

Our present study demonstrated that GRAIL was upregulated in human CD4+ T cells following the induction of anergy in vitro, and GRAIL expression was enhanced in patients with remission stage of UC compared with patients with active disease. Previous basic research has shown that the anergic phenotype can be obtained by introducing GRAIL into murine T cells (27), indicating that GRAIL is not only a marker for T
cell anergy but also plays a key role in inducing anergy in CD4+ T cells. Taken together, GRAIL is suggested to be useful to reflect the status of human T cell anergy in UC and also to be a potential therapeutic target of UC.

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