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Analysis of Peripheral $CD4^+CD25^+$ T Cells of Patients with Inflammatory Bowel Disease

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Background and Aim: $CD4^+CD25^{high}$ T cells act as regulatory T cells. CTLA-4 and GITR are associated with the suppressor function of regulatory T cells. The aim of this study was to assess any differences in the expression patterns of these molecules on regulatory T cells of patients with inflammatory bowel disease (IBD).

Methods: The study subjects were 15 patients with IBD (ulcerative colitis=7, Crohn's disease=8) and 7 healthy individuals. Peripheral blood mononuclear cells were obtained by centrifugation of fresh blood over Ficoll-paque and stained with anti-CD4, CD25, and GITR antibodies. For CTLA-4 staining, cells were fixed and permeabilized by saponin prior to incubation with anti-CTLA-4 antibody. Cell samples were analyzed on a FACScaliber. Each patient was investigated both in the active and inactive state.

Results: The percentage of $CD4^+CD25^+$ cells was significantly higher in active than in inactive ulcerative colitis. In contrast, the percentage of $CD4^+CD25^{high}$ cell was significantly higher in active Crohn's disease than in healthy individuals. CTLA-4^+ cell counts in $CD4^+CD25^{high}$ T cells were significantly lower in active Crohn's disease than in control and inactive Crohn's disease patients, although no difference was observed between active and inactive ulcerative colitis. In contrast, GITR expression was significantly higher in active than inactive Crohn's disease.

Conclusion: Our results suggest that $CD4^+CD25^{high}$ T cells are probably involved in the pathogenesis of Crohn's disease.

Key words: regulatory T cell, Crohn's disease, ulcerative colitis, CTLA-4, GITR

Introduction

Crohn's disease (CD) and ulcerative colitis (UC), collectively referred to as inflammatory bowel disease (IBD), are relatively common inflammatory diseases of the gastrointestinal tract. However, these two conditions have distinct histopathological and anatomical characteristics, with CD characterized by transmural inflammation throughout the GI tract, and UC by a more superficial inflammation confined to the colon and rectum. In human IBD tissue, $CD4^+$ T cells represent the vast majority of activated mononuclear cells infiltrating the gut. A large proportion of T cells bear the phenotypic characteristics of circulating naive lymphocytes and are recruited from the blood into the intestinal mucosa, probably as a result of enhanced expression of adhesion molecules and chemokines in the inflamed gut of IBD patients[1]. Evidence indicates that $CD4^+$ T cells play a key role in the pathogenesis of tissue damage in IBD, especially in CD[1)~3)]. The role of T cells in the pathogenesis of IBD is supported by clinical and pathophysiological observations, showing that the stable remission is observed in CD patients who develop symptomatic HIV infection or undergo bone marrow transplantation[2)4)].

Recent studies have demonstrated that $CD4^+$ regulatory T cells constitute an important component of the normal, healthy immune response[5]. These cells are engaged in the maintenance of immunologic self tolerance by actively suppressing

Table The baseline characteristics of all patients who participated in this study

		CD		UC	Control
Sex (M/F)		4/4		4/3	2/5
Mean age (y)		30.1 ± 9.5		49.4 ± 13.7	32.5 ± 10.0
Median duration of disease (y)		6.8 ± 11.1		5.2 ± 6.9	
Site of disease	Small bowel	1	Total colon	5	
	Colon	2	Leftsided	1	
	Small bowel & Colon	5	Proctitis	1	

Disease activity	active	inactive	active	inactive
CDAI/UCDAI score	277.6	99.5	8.3	0.4
Medications				
No medication	4	0	2	7
Only Masalasine	0	2	1	0
corticosteroid	3	5	3	7
azathioprine	1	2	0	1
anti TNF-α Ab	1	0	—	—
cyclosporine A	—	—	1	1

the activation and expansion of self-reactive lymphocytes that may cause autoimmune disease[6)7)]. The majority of these regulatory T cells constitutively express CD25 (the IL-2 receptor alpha chain)[5)]. The removal or functional alteration of this population from normal rodents leads to the spontaneous development of various autoimmune diseases[6)7)]. Furthermore, it has reported that $CD4^+CD25^+$ regulatory T cells can reverse established intestinal inflammation in the $CD4^+CD45RB^{high}$ T cell transferred colitis model[8)]. In contrast to rodents, it has been reported that only $CD4^+CD25^{high}$ T cells exhibit strong regulatory function in humans[9)]. These $CD4^+CD25^{high}$ T cells inhibit the proliferation and cytokine secretion induced by TCR crosslinking of $CD4^+CD25^-$ responder T cells in a contact-dependent manner[9)].

$CD4^+CD25^+$ regulatory T cells constitutively express gene products of glucocorticoid-induced TNF receptor family-related receptors (GITR) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4)[10)~14)]. GITR, a member of the TNFR superfamily, is particularly abundant on $CD4^+CD25^+$ T cells[10)]. The high-level, basal expression by $CD4^+CD25^+$ regulatory T cells suggested an important role for GITR on this subset. CTLA-4 is a critical inhibitor of T cell activation as demonstrated by the lethal lymphoproliferation seen in CTLA-4 knockout mice[15)16)]. The involvement of CTLA-4 in the regulation mediated by $CD4^+CD25^+$ T cells has been described in mouse models[12)14)].

In the present study, we demonstrated the presence of low $CTLA\text{-}4^+CD4^+CD25^{high}$ T cell ratio and increased ratio of $GITR^+CD4^+CD25^{high}$ T cells in the peripheral blood of patients with active CD, but not in that with UC, however, there was no difference in the percentage of $CD4^+CD25^{high}/CD4^+$ between the two conditions. This finding suggests regulatory T cells might be involved in the pathogenesis of CD.

Material and Methods

Patients

A total of 15 patients with IBD (7 patients with UC and 8 patients with CD) and 7 healthy individuals participated in this study. Informed consent was obtained from patients before the study. The baseline characteristics of all patients are summarised in Table. Active CD was defined as a Crohn's disease activity index (CDAI) >150, while inactive disease as a CDAI <150. The activity of UC was determined with the UC disease activity index (UCDAI) that measures the frequency of bowel movements, blood in stool, endoscopic severity, and overall well-being. Active UC was defined as a UCDAI score >8 point, while inactive disease as a UCDAI score <3 point (UCDAI score: maximum 12 point).

Reagents

The following monoclonal antibodies (mAbs)

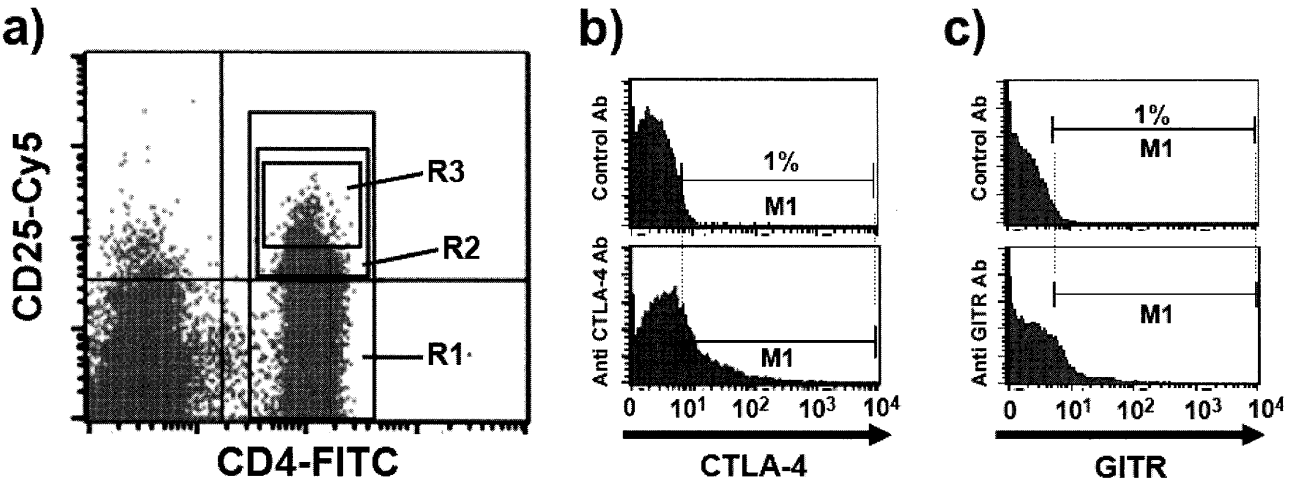

Fig. 1 Flow cytometric analysis of human peripheral blood mononuclear cells
a: Gating of CD4 T cells and determination of their CD25 expression in human peripheral blood lymphocytes. CD4 T cells were gated by their expression of CD4 (R1), CD25 (R2) and high expression of CD25 (R3).
b: Representative histogram of CTLA-4 positive cells in R3 ($CD4^+CD25^{high}$). Cells were stained with control antibody (upper panel) or anti-CTLA-4 / CD152 antibody (lower panel).
c: Representative histogram of GITR positive cells in R3 ($CD4^+CD25^{high}$). Cells were stained with control antibody (upper panel) or anti-GITR antibody (lower panel).
The threshold (M1) for counting CTLA-4 or GITR positive cells was put on the line which showed 1% positive in control antibody.

were used to analyze intracellular and surface antigens. Cy-Chrome-conjugated anti-human CD4, Cy5-conjugated anti-human CD25, PE-conjugated anti-human CTLA-4/CD152 were purchased from BD PharMingen (San Diego, CA, USA), PE-conjugated anti-human mouse IgG2a was purchased from Miltenyi Biotech (Auburn, CA, USA), anti-human mouse IgG2a negative control was from DAKO (Glostrup, Denmark), PE-conjugated anti-human GITR was purchased from R&D (Minneapolis, MN, USA). To stain and analyze intracellular antigens, peripheral blood mononuclear cells (PBMCs)were fixed and permealized with Cytofix/Cytoperm, and washed with Perm/wash buffer (BD Pharmingen).

FACS analysis of surface antigens and intracellular staining

PBMCs were obtained from healthy individuals and IBD patients by centrifugation of fresh blood over Ficoll-paque. PBMCs (5×10^5) were stained with Cy-Chrome-conjugated anti-human CD4, Cy5-conjugated anti-human CD25, and PE-conjugated anti-human GITR for 45 min at 4℃. After two washes, cell samples were run on a FACScaliber (BD Pharmingen) and analyzed using Cell Quest software. The isolated PBMCs (1×10^6) were stained with 2 colour, Cy-Chrome-conjugated anti-human CD4 and Cy5-conjugated anti-human CD25, for 30 min at 4℃. After cell surface labelling, cells were fixed and saponin permeabilized (fix/perm solution) for 20 minutes at 4℃. After washing twice and incubating with blocking antibody (anti-human mouse IgG2a negative control), cells were stained with PE-conjugated anti-human CTLA-4 / CD152 or PE-conjugated anti-human mouse IgG2a as an isotype control for 15 minutes at room temperature. After washing twice, cells samples were analyzed on a FACScaliber. Flow cytometry data were analyzed by Cell Quest software.

Lymphocytes were gated on cell optic characteristics (FSC, SSC). $CD4^+$ (R1), $CD25^+$ (R2), and $CD25^{high}$ (R3) cells were gated as shown in Fig. 1a, and the thresholds (M1) for counting CTLA-4 or GITR positive cells were put on the line which showed 1% positive in control antibody (Figs. 1b & c).

Statistical analysis

The results were expressed as mean ± SD.

Groups of data were analyzed by using the paired or unpaired t-test. P values <0.05 were considered significant.

Results

Percentage of $CD4^+CD25^+$ T cells and $CD4^+CD25^{high}$ T cells in $CD4^+$ T cell population

We assessed the percentage of $CD4^+CD25^+/CD4^+$ and $CD4^+CD25^{high}/CD4^+$ cells in peripheral blood from the same IBD patients in both active and inactive phases. Although a comparison of the ratio of $CD4^+CD25^+/CD4^+$ between healthy individuals (7.46 ± 2.53%) and IBD patients showed no significant differences, $CD4^+CD25^+/CD4^+$ in the active phase was significantly higher than in the inactive phase in UC patients (UC; active: 8.96 ± 3.10% vs. inactive: 6.23 ± 2.26%, $p<0.05$). Although it was not significant, six of seven CD patients showed higher $CD4^+CD25^+/CD4^+$ in active phase (active: 10.35 ± 2.38%, inactive: 8.62 ± 2.67%, $p=0.11$) (Fig. 2a). We also examined the frequency of the peripheral $CD4^+CD25^{high}$ T cell population, which is thought to have function similar to that of mouse $CD4^+CD25^+$ regulatory T cells. $CD4^+CD25^{high}/CD4^+$ from CD patients in the active phase were significantly higher than in normal controls (Control: 1.46 ± 0.32% vs. CD; active: 2.62 ± 0.75%, $p<0.01$). However, in both UC and CD patients, no significant differences were found between the two phases, active and inactive (UC; active: 1.55% ± 0.58%, inactive: 1.07 ± 0.90%, CD; inactive: 2.07 ± 1.08%) (Fig. 2b).

CTLA-4 positive cells in regulatory T cells

The percentage of the $CD4^+CD25^{high}$ population with regulatory function in humans showed no correlation with disease activity in IBD. Next, we investigated the frequency of CTLA-4 positive cells that were reported to play an important role in the suppressive function of regulatory T cells[12]. Although there was no significant difference in the percentage of $CTLA\text{-}4^+CD4^+CD25^+/CD4^+CD25^+$ in both CD and UC (CD; active: 59.93 ± 12.28% vs. inactive: 72.46 ± 9.03%, UC; active: 58.67 ± 18.65% vs. inactive: 60.89 ± 15.89%, Fig. 3a), we found that $CTLA\text{-}4^+CD4^+CD25^{high}/CD4^+CD25^{high}$ from CD patients in the active phase was significantly lower than that from normal controls (Control; 94.66 ± 1.87%, CD; active: 87.46 ± 7.89%, $p<0.05$). Moreover, compared with the active phase and inactive phase in the same CD patient, the percentage of $CTLA\text{-}4^+CD4^+CD25^{high}/CD4^+CD25^{high}$ was also lower in the active phase. (CD; active: 87.46 ± 7.89%, inactive: 95.13 ± 2.36%, $p<0.01$, Fig. 3b). In contrast, there was no correlation with the frequency of CTLA-4 in UC patients.

Percentage of GITR positive cells in regulatory T cells from active CD was higher in inactive CD

Finally, we examined that the frequency of GITR positive cells. A recent report demonstrated that the engagement of GITR by anti-GITR Ab, both in vitro and in vivo, abrogated the suppressor activity of regulatory T cells[10]. $GITR^+CD4^+CD25^+/CD4^+CD25^+$ from IBD patients showed no correlation with disease activity (Fig. 4a). However, in the same CD patients, there was a significant difference in $CD4^+CD25^{high}GITR^+/CD4^+CD25^{high}$ between the active phase and inactive phase (CD; active: 36.07 ± 13.15%, inactive: 30.46 ± 12.36%, $p<0.05$, Fig. 4b). In contrast, no obvious tendency was observed in UC patients (UC; active: 34.14 ± 8.57%, inactive: 39.18 ± 9.45%).

Discussion

Despite accumulating evidence for the immunoregulatory properties of $CD4^+CD25^+$ regulatory T cells, the role of these cells in the pathogenesis of human disease has not yet to be thoroughly demonstrated. Several investigators reported that the frequency or function of $CD4^+CD25^+$ changed in autoimmune disease, however, the role of regulatory T cells in human disease remains controversial[17)~21)]. For example, Kukreja et al[17] revealed that the percentage of $CD4^+CD25^+$ T cell in peripheral blood of type 1 diabetes patients was lower than that of healthy control, although others reported that no difference in $CD4^+CD25^+$ T cell was observed and that $CD4^+CD25^{high}$ T cells were increased in type 1 diabetes patients[18]. Cao et al[19] investigated the ratio of $CD4^+CD25^{high}$ T cells in patients with rheumatoid arthritis (RA), and reported no observed differences between patients with RA and healthy controls. In contrast, Amelsfort et al[20]

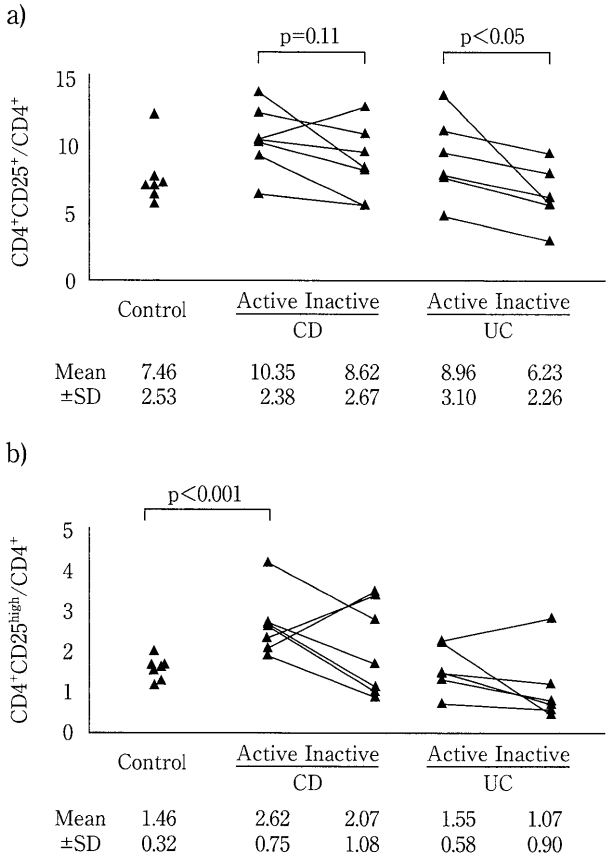

Fig. 2 Flow cytometric analysis of $CD4^+$ $CD25^+$ T cells and $CD4^+$ $CD25^{high}$ T cells of peripheral blood from healthy individuals (n = 7), CD patients (active and inactive, n = 7), and UC patients (active and inactive, n = 6).
In IBD patients, the same patient was analyzed in both active and inactive state.
a: Percentages of $CD4^+$ $CD25^+/CD4^+$ T cells were significantly increased in active UC than inactive UC (active: 8.96 ± 3.10% vs. inactive: 6.23 ± 2.26% , $p < 0.05$). In CD patients, there was an increasing but non-significant trend in the percentage of $CD4^+$ $CD25^+/CD4^+$ T cells in active state (active: 10.35 ± 2.38%, inactive: 8.62 ± 2.67%, $p = 0.11$).
b: Percentages of $CD4^+$ $CD25^{high}$ T cells were significantly increased in active CD patients than in healthy individuals (Control: 1.46 ± 0.32% vs. CD; active: 2.62 ± 0.75% , $p < 0.01$). No significant difference was observed between active and inactive state of both CD and UC patients.

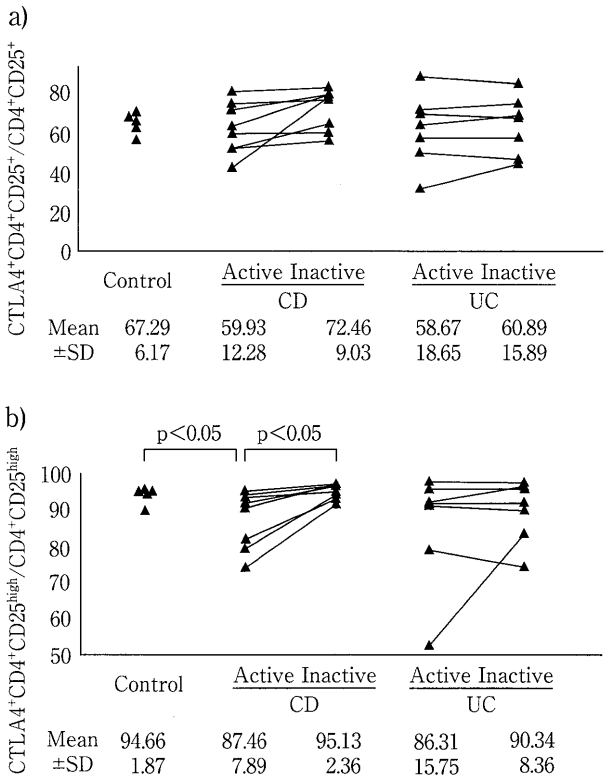

Fig. 3 Flow cytometric analysis of CTLA-4^+ $CD4^+CD25^+$ T cells and CTLA-$4^+CD4^+CD25^{high}$ T cells from healthy individuals (n = 5), CD patients (n = 8), and UC patients (n = 7).
In IBD patients, the same patient was analyzed in both active and inactive state.
a: Percentages of CTLA-$4^+CD4^+CD25^+/CD4^+CD25^+$ cells. No differences were seen between active and inactive phases in both UC and CD patients, or between control and IBD patients.
b: Percentages of CTLA-$4^+CD4^+CD25^{high}/CD4^+CD25^{high}$ cell. CTLA-$4^+CD4^+CD25^{high}$ T cells were significantly decreased in the active than inactive state only in CD patients (active: 87.9 ± 7.5%, inactive: 95.13 ± 2.36%, $p < 0.01$) or than healthy control (Control; 94.66 ± 1.87%, $p < 0.05$).

reported an increased number of $CD4^+CD25^{high}$ T cells in the synovial fluid of RA patients.

The percentage of $CD4^+CD25^+$ T cells and $CD4^+$ $CD25^{high}$ T cells in the total $CD4^+$ T cell population in human peripheral blood was reported to be approximately 5-10% and 1-2%, respectively[9]. Similar results were observed in our study, and the ratio of $CD4^+CD25^+/CD4^+$ was higher in the active phase than inactive phase of IBD patients. Although this population of T cells was reported as the regulatory T cells in mice, it has been revealed that $CD25^+$ is not the marker of regulatory T cells in humans. CD25 is upregulated upon activation of T cells, and the majority of CD25 positive cells are $CD25^{low\text{-}intermediate}$ cells in humans[9)22)]. These cells are most likely to be activated cells with CD25 expression resulting from an encounter with foreign antigens. Therefore, anti-

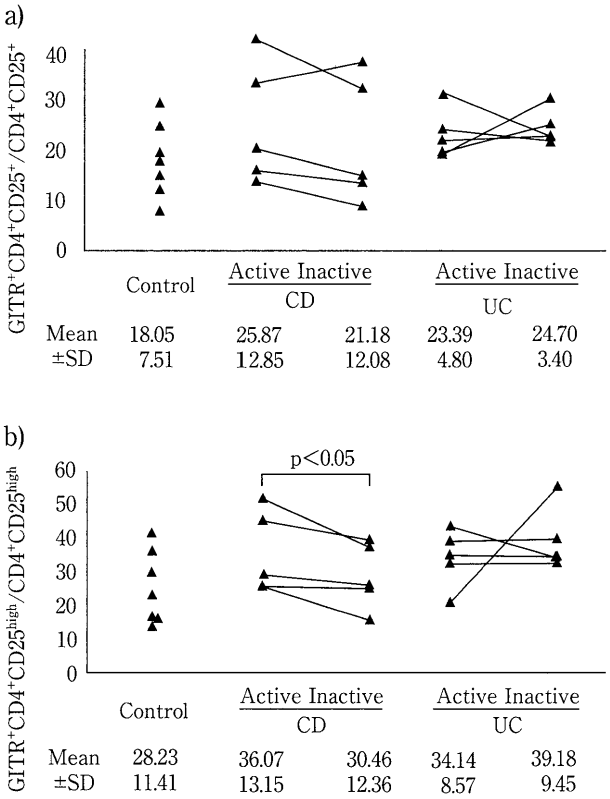

Fig. 4 Flow cytometric analysis of GITR$^+$ CD4$^+$ CD25$^+$ T cells and GITR$^+$ CD4$^+$ CD25^{high} T cells of from healthy individuals (n = 5), CD patients (n = 5), and UC patients (n = 5).
In IBD patients, the same patient was analyzed in both active and inactive state.
a: Percentages of GITR$^+$ CD4$^+$ CD25$^+$/CD4$^+$CD25$^+$ cells. No differences were seen between active and inactive phases in both UC and CD patients, or between control and IBD patients.
b: Percentages of GITR$^+$ CD4$^+$ CD25^{high}/CD4$^+$ CD25^{high} cell. GITR$^+$ CD4$^+$ CD25^{high} T cells were significantly increased in active CD than inactive CD (CD; active: 36.07 ± 13.15%, inactive: 30.46 ± 12.36%, $p < 0.05$). No differences were observed between active and inactive UC.

CD25 antibody (basiliximab) is widely used to control acute rejection after organ transplantation, and the efficacy of this therapy has been well proven[23)24)]. The high frequency of CD4$^+$CD25$^+$/CD4$^+$ in the active phase of IBD may reflect the existence of active inflammation in patients with active phase IBD.

In humans, CD4$^+$CD25^{high} T cells have regulatory functions[9)]. In present study, the ratio of CD4$^+$ CD25^{high}/CD4$^+$ was increased in active CD in comparison to the controls, but not between active and inactive phases in CD and UC. Maul et al[25)] reported that CD4$^+$CD25^{high} T cells in peripheral blood were significantly decreased in active CD and UC patients. This report was not in agreement with our results, in which no differences were observed. We cannot explain the reason of this difference, but one possibility might be the difference in sample background. Recently, three independent studies, one from the United States[26)], and two from Europe[27)28)], showed that three variants in the coding region of Nod2 gene are associated with CD. However, none of the common variants in the Nod2 gene were identified in Japanese CD patients[29)]. This suggests that the pathogenesis of CD is not completely explained by a single gene mutation, but may be different between races.

Makita et al[30)] isolated CD4$^+$CD25^{bright} T cells from lamina propria (LP), and showed that the ratio of LP CD4$^+$CD25^{bright} T cells was similar to that in peripheral blood, and the cells retained their regulatory function. The transfer of CD4$^+$CD45RB^{high} T cells into immunodeficient mouse results in colitis, and co-transfer of CD4$^+$CD25$^+$ T cell can prevent this colitis[12)]. Furthermore, CD4$^+$CD25$^+$ T cells can cure established colitis in mice[8)]. These findings suggest that CD4$^+$CD25^{high} cells are relevant to the establishment of colitis, and moreover peripheral blood regulatory T cells play a pivotal role in the prevention of colitis.

Recent studies have revealed that several molecules expressed on CD4$^+$CD25^{high} cells, such as CTLA-4 and GITR, are relevant to the regulatory function of these cells. CTLA-4 negatively modulates T cell proliferation and cytokine production[31)], and GITR, which is preferentially expressed on the surface of regulatory T cells in mice and humans, potentially provides a signal that abrogates regulatory T cell suppression[10)11)]. Read et al reported that the preventative effect of CD4$^+$CD45RB^{low} T cell against colitis induced by CD4$^+$CD45RB^{high} T cell was abrogated by anti-CTLA-4 antibody[12)]. This indicates that CTLA-4 plays a key role to prevent the effect of regulatory T cells in this form of colitis, which is considered to be a mouse model of human CD. In this study, only peripheral blood from patients with active CD, and not from patients from

UC, decreased CTLA-4 expression and increased GITR expression were observed in the subset of $CD4^+CD25^{high}$ T cell population. We also assessed the peripheral blood of patients with infectious colitis and ischemic colitis, and found no difference in CTLA-4 and GITR expression between active and remission state (n=3, data not shown). It was interesting to note that the expressing molecules, which correlate with the function of regulatory T cells, were changed to downregulate its function only in active CD, in which $CD4^+$ T cell plays a key role in the pathogenesis.

In conclusion, we demonstrated that the percentage of $CTLA\text{-}4^+CD4^+CD25^{high}$ T cells was decreased and $GITR^+CD4^+CD25^{high}$ T cells increased in peripheral blood of active CD patients. These findings indicate the importance of $CD4^+CD25^{high}$ T cell function in CD pathogenesis.

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炎症性腸疾患における末梢血 CD4$^+$CD25$^+$ T 細胞の解析

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CD4$^+$CD25^{high} T 細胞は調節性 T 細胞（Treg）と称される．炎症性腸疾患（IBD）モデルマウスへの Treg 移入が腸炎発症を抑制することが報告され，Treg が IBD の病因に関与している可能性が示された．そこで我々は病期の異なる同一 IBD 患者末梢血 Treg（CD4$^+$CD25$^+$ T 細胞，CD4$^+$CD25^{high} T 細胞）の割合，Treg に恒常的に発現し抑制機能の鍵とされる GITR および細胞内 CTLA-4 陽性細胞の割合を調べ，ヒト Treg が IBD の病態に関与しているか否かを検討した．〔対象および方法〕対象は潰瘍性大腸炎（UC）7 人，クローン病（CD）8 人，健常人 7 人とした．末梢血単核球を採取し，CD4，CD25，に対するモノクローナル抗体を用いてフローサイトメトリーにより解析した．CD4$^+$ T 細胞中の CD25$^+$細胞，CD25^{high}細胞の割合（Treg/CD4$^+$）を CD，UC，健常人において測定した．患者群では同一患者における緩解，活動期にそれぞれ測定を行い，また細胞表面 GITR，細胞内 CTLA-4 も同時に測定し差異を認めるか否かを検討した．〔結果〕CD4$^+$CD25$^+$/CD4$^+$（%）は同一 UC 患者で，緩解期に比べ活動期で有意に高かった．また CD4$^+$CD25^{high}/CD4$^+$（%）は健常人と比較し，活動期 CD 患者で有意に高かった．一方，活動期 CD 患者の CD4$^+$CD25^{high}細胞中 CTLA-4 陽性細胞の割合（CD4$^+$CD25^{high}CTLA-4$^+$/CD4$^+$CD25^{high}（%））は健常人や CD 緩解期に比べて有意に低く，GITR 陽性細胞の割合（CD4$^+$CD25^{high}GITR$^+$/CD4$^+$CD25^{high}（%））は CD 緩解期に比べて CD 活動期で有意に高かった．〔結論〕強い細胞増殖抑制機能を持つ CTLA-4 は CD 活動期 CD4$^+$CD25^{high}で有意に低く，逆に Treg の抑制機能を無効とする GITR は有意に高かった．また同様の傾向は UC には認められなかった．同一 CD 患者の異なる病期で Treg の機能に関与する molecule の変化を認めた事実は CD の病因に Treg が関与している可能性を強く示唆させた．