Adalimumab

Adalimumab is a fully humanized anti-TNF monoclonal IgG1 antibody. This antibody is as efficacious as infliximab for the treatment of rheumatoid arthritis. *In vitro* studies revealed that this antibody is capable of inducing apoptosis in monocytes as infliximab[33]. As adalimumab does not contain a mouse peptide sequence, it is expected to be less immunogenic and more tolerable than infliximab. Two uncontrolled pilot studies of adalimumab with CD patients who had lost responsiveness or developed intolerance to infliximab[34,35] showed that subcutaneous adalimumab was well tolerated, thus suggesting a clinical benefit of adalimumab. In a phase 3, multicenter trial for active CD, clinical remission rates of patients who received adalimumab 160 mg at wk 0 and 80 mg at wk 2 was significantly higher than that of placebo at wk 4 (36% *vs* 12%)[36]. From these results, adalimumab is likely to be efficacious for the treatment of CD and it could thus be an alternative therapy for the patients who either lost responsiveness or developed intolerance to infliximab.

INHIBITION OF OTHER INFLAMMATORY CYTOKINES

Anti-IL-12 p40 antibody

IL-12, a heterodimeric molecule composed of IL-12 p40 and IL-12 p35 subunits, plays a central role in Th1 development. IL-12 is abundantly produced in the gut of CD patients[2]. In several animal models of Th1-mediated colitis, anti-IL-12 treatment effectively ameliorates intestinal inflammation[37,38]. IL-12 p40 subunit is also a component of another Th1 cytokine, IL-23, in which p40 forms a heterodimer with p19 subunit. IL-12 p40 is, therefore, a potential target for the treatment of CD in which intestinal inflammation is Th1-mediated. A double-blind, placebo-controlled randomized study of a humanized IgG1 monoclonal antibody against IL-12 p40 (ABT-874) was performed in 79 patients with active CD[39]. The patients were randomly assigned to receive seven weekly injections of 1 mg/kg anti-IL-12, 3 mg/kg anti-IL-12 or placebo subcutaneously either with or without 4 wk intervals between the first two injections. The patients who received 3 mg/kg anti-IL-12 for 7 wk showed a significantly greater clinical response rate than the patients treated with a placebo (75% *vs* 25%). The rates of remission were also higher in the 3 mg/kg anti-IL-12 group (38%) than in the placebo group (0%) but the difference did not reach statistical significance. The production of IL-12 and other Th1 and proinflammatory cytokines from patients' colonic lamina propria mononuclear cells dramatically decreased after the anti-IL-12 therapy. The most frequent adverse event was a local reaction at the injection site, which was observed with a greater rate in the anti-IL-12-treated group than in the placebo-treated group. Anti-drug antibody was formed in some patients who received anti-IL-12 antibody. No serious side effects requiring the discontinuation of the treatment, due to the anti-IL-12 therapy, were observed. Anti-IL-12 therapy is therefore considered to be a safe and effective treatment for active CD.

MRA (Anti-IL-6 receptor antibody)

IL-6 is one of the major inflammatory cytokines. IL-6 can transduce signals into cells without IL-6 receptor expression when IL-6 binds to soluble IL-6 receptor. The expression of IL-6 and soluble IL-6 receptor increases in patients with active CD[40,41]. A pilot randomized double-blind placebo-controlled trial of a humanized anti-IL-6 receptor monoclonal antibody, MRA, with active CD was performed[42]. Thirty-six patients were randomized biweekly to receive either a placebo, 8 mg/kg MRA or MRA/placebo alternately for 12 wk. The clinical remission rate with biweekly MRA was significantly higher than that with placebo (80% *vs* 31%). The acute phase responses such as ESR and CRP levels were rapidly suppressed 2 wk after the MRA injection. The incidence of adverse events was similar in all groups, and thus MRA treatment was generally well tolerated. It is, therefore, likely that anti-IL-6 receptor therapy is beneficial for active CD.

Fontolizumab (Anti-interferon-γ antibody)

Interferon-γ is a key cytokine that enhances the development of a Th1 immune response. Fontolizumab is a humanized monoclonal antibody directed against interferon-γ. A phase 2 study of fontolizumab at intravenous doses of 4 mg/kg or 10 mg/kg in 133 patients with moderate to severe active CD did not demonstrate efficacy at d 28. However, exploratory analyses based on 91 patients who received a second dose of fontolizumab at d 28 did demonstrate efficacy. This effect was most prominent in patients with elevated baseline concentrations of CRP[43]. An additional phase 2 study of fontolizumab at lower subcutaneous doses of 1.0 mg/kg or 4.0 mg/kg in 196 patients with active CD did not demonstrate efficacy at d 28[44]. These results indicate that a single dose may not be sufficient to achieve a significant improvement. Further clinical studies of fontolizumab for the induction and maintenance of remission in patients with CD are anticipated.

Anti-IL-2 receptor (CD25) antibodies

Daclizumab: IL-2 is a major T cell growth factor, which is secreted by activated T cells and acts via the high-affinity IL-2 receptor on T cells themselves to promote cell survival and proliferation. The IL-2 receptor α-chain (CD25) is a component of high-affinity IL-2 receptor and it is expressed on activated T cells. Daclizumab is a humanized monoclonal antibody to CD25, which blocks the binding of IL-2 to the IL-2 receptor. An open label pilot study of daclizumab suggested that it was beneficial for patients with active UC[45]. However, a recent placebo-controlled phase 2 trial of daclizumab at intravenous doses of 1 mg/kg twice with a 4-wk interval or 2 mg/kg every 2 wk for a total of four doses in 159 patients with active UC failed to show any efficacy[46].

Basiliximab: Basiliximab is a chimeric monoclonal antibody against CD25, which blocks the binding of IL-2 to the IL-2 receptor. Two uncontrolled pilot studies suggested that basiliximab in combination with steroids may be effective for steroid resistant UC[47,48]. A large random-

ized controlled trial is required to confirm the therapeutic benefit of this compound.

INHIBITION OF ADHESION MOLECULES

Many adhesion molecules play an important role in trafficking leukocytes into the inflamed gut wall and they are up-regulated in both CD and UC[49,50].

α4-integrins, predominantly expressed on lymphocytes, usually exist in combination with a β subunit and interact with adressins expressed on endothelium. α4β1 integrin binds to vascular cellular adhesion molecule 1 (VCAM-1) and α4β7-integrin binds to mucosal addressing cell adhesion molecule 1 (MAdCAM-1). The interaction between α4β7-integrin and MAdCAM-1 is important in mediating lymphocytes homing to the gut mucosa[51].

Leukocyte function-associated antigen 1 (LFA-1) expressed on leukocytes interacts with intercellular adhesion molecule 1 (ICAM-1), which is constitutively expressed at low levels on vascular endothelial cells and a subset of leukocytes, and they are up-regulated on many cell types in response to proinflammatory mediators[52].

Natalizumab

Natalizumab, a humanized IgG4 anti α4 integrin monoclonal antibody, inhibits both α4β7-integrin/MAdCAM 1 interaction and α4β1/VCAM-1 binding.

In an initial small trial in 30 patients with active CD, a single 3 mg/kg intravenous infusion of natalizumab showed a short term effect in inducing remission at wk 2 and elevated circulating lymphocyte levels after the natalizumab infusion. Therefore, it is suggested that the natalizumab interrupted lymphocyte trafficking into the intestine[53]. In a large placebo-controlled randomized trial including 248 patients with moderate to severe CD, patients were treated twice at 4 wk intervals with 3 or 6 mg/kg of natalizumab or placebo. A significantly higher number of patients achieved remission at wk 6 only in the 3 + 3 mg/kg natalizumab group compared with the two infusions of placebo group (44% *vs* 27%). The clinical response rates at wk 6 in all treatment groups were significantly higher than that in the placebo-treated group (3 mg/kg natalizumab + placebo: 59%, 3 + 3 mg/kg natalizumab: 71%, 6 + 6 mg/kg natalizumab: 57% and the two infusions of placebo group, 38%)[54]. A larger phase 3 trial of ENACT-1 in 905 patients with moderate to severe CD failed to show a benefit for three intravenous infusions of 300 mg natalizumab every 4 wk. In a subgroup analysis, however, natalizumab-treated patients with concurrent immunosuppressive therapies, prior anti-TNF-α therapy or elevated CRP levels showed a significant response rate compared with placebo-treated patients[55]. Three hundred and thirty-nine patients with CD who responded to natalizumab in ENACT-1 were re randomized to maintenance therapy with natalizumab (300 mg) or a placebo for up to 12 additional monthly infusions. In this maintenance study (ENACT-2), natalizumab demonstrated a significant superiority over the placebo in its ability to sustain both the response and remission at all consecutive time points over a 6-mo period and enabled patients to be successfully withdrawn from steroids[56]. In an uncontrolled short term pilot study in 10 patients with active UC, a single 3 mg/kg intravenous infusion of natalizumab showed a short term benefit[57].

Natalizumab is efficacious in multiple sclerosis (MS) as well[58]. In MS, α4β1 integrin/VCAM-1 binding appears to be a crucial step because antiα4β1 integrin antibody prevented the development of experimental autoimmune encephalomyelitis, a model of human MS[59]. Against these effects of natalizumab in IBD and MS, 3 patients receiving repeated treatment with natalizumab developed JC virus related progressive multifocal leukoencephalopathy (PML)[60-62]. PML, which almost invariably occurs in patients with AIDS or leukemia or in organ-transplant recipients, is a fatal opportunistic infection of the central nervous system caused by the reactivation of a clinically latent JC polyomavirus infection. Two patients with MS had been receiving the concomitant administration of interferon β-1a[60,61] and 1 patient with CD had been treated with natalizumab monotherapy[62]. These observations force us to reconsider both the efficacy and the potential risks associated with an inhibition of lymphocytes trafficking by anti-α4 integrin therapy.

MLN-02

MLN-02, a humanized anti-α4β7-integrin blocks specifically the α4β7-integrin/MAdCAM 1 interaction.

A randomized placebo-controlled trial in 185 patients with mild to moderately active CD treated with placebo, 0.5 mg/kg MLN-02 or 2.0 mg/kg MLN-02 intravenously on d 1 and 29 demonstrated that on d 57, 2.0 mg/kg MLN-02 showed significantly greater remission rates over the placebo (36.9% *vs* 20.7%). There was no significant difference between the actively treated and placebo-treated groups regarding the clinical response rates. No obvious differences in adverse events were noted among the three groups[63].

A randomized placebo-controlled trial in 181 patients with moderately active UC treated with placebo, 0.5 mg/kg MLN-02, or 2.0 mg/kg MLN-02 intravenously on d 1 and 29 demonstrated that on d 43 the remission rates were significantly higher in the actively treated groups (0.5 mg/kg: 33%, 2.0 mg/kg: 34%) than in the placebo-treated group (15%). An infusion reaction occurred in one MLN-02-treated patient who developed mild angioedema[64].

MLN-02 appears to be a generally well tolerated and effective therapy especially for active UC, but further trials are necessary to confirm the efficacy of MLN-02 therapy for IBD.

Alicaforsen (ISIS 2302)

ISIS 2302 is a 20 base phosphorothioate oligodeoxynucleotide designed to specifically hybridize to the 3-untranslated region of the human ICAM-1 mRNA. Treatment of ISIS 2302 *in vitro* resulted in a highly specific reduction in ICAM 1 mRNA and, consequently, a marked decrease in ICAM-1 protein expression[65].

A pilot trial in patients with moderate CD (including 15 patients treated with 13 intravenous infusions of 0.5, 1.0 or 2.0 mg/kg ISIS 2302 *vs* 5 patients with placebo over 26 d) demonstrated a higher remission rate in ISIS 2302-treated group compared with the placebo-treated group on d 33

(47% *vs* 20%)[66]. However, a placebo-controlled trial in 75 patients with steroid refractory CD failed to demonstrate efficacy was showed that the subcutaneous administration of ISIS 2302 induced only 3% of ISIS 2302-treated patients to clinical remission with complete steroid taper (0% in placebo-treated patients)[67]. Another larger randomized placebo-controlled trial also failed to show any benefit of ISIS 2302 for active CD[68]. Two hundred and ninety-nine patients with moderately active, steroid-dependent CD received placebo or ISIS 2302 (2 mg/kg intravenously three times a week) for 2 or 4 wk in mo 1 and 3. There were no differences in the steroid-free remission rates at wk 14 between the ISIS 2302-treated groups (2 wk: 20.2%, 4 wk: 21.2%) and the placebo-treated group (18.8%). However, a subgroup analysis using the area under the curve (AUC) of ISIS 2302 plasma concentration demonstrated an improvement of the clinical response for the highest AUC group (AUC > 65 μg × h/mL). This suggested that ISIS 2302 may be effective when given in adequate doses. As a result, a dose ranging pilot trial of high dose ISIS 2302 in 22 patients with active CD was conducted. The patients, who were infused with high dose ISIS 2302 (250 mg to 350 mg) intravenously three times a week for 4 wk, showed a 41% remission rate. Five patients, however, withdrew due to infusion-related symptoms[69].

A randomized placebo-controlled trial in 40 patients with mild to moderately active distal UC treated with 60 mL alicaforsen enema (0.1, 0.5, 2, or 4 mg/mL or placebo) once daily for 28 consecutive days showed a beneficial effect at the highest dose. Four mg/mL alicaforsen enema group showed a significant improvement in the disease activity index on d 29 and mo 3 in comparison to the placebo enema group[70]. An open-label, uncontrolled study in 12 patients with chronic unremitting pouchitis treated with 240 mg alicaforsen enema nightly for 6 wk demonstrated a significant improvement in the pouchitis disease activity index and an endoscopic mucosal appearance at wk 6[71].

The most consistently reported side effects of ISIS 2302 in all clinical trials were infusion reactions and a moderate increase in activated partial thromboplastin time.

CONCLUSION

Infliximab has changed the medical treatment of CD dramatically. This agent has been proven to be clearly effective for the induction and maintenance of remission for active CD meanwhile it is also generally well-tolerated. However, there are some patients who fail to respond to infliximab. In addition, some responders either lose responsiveness to infliximab during long-term therapy or develop intolerance to infliximab. The next issue will therefore be how to treat such patients who cannot be treated with infliximab. Adalimumab may be an alternative to infliximab. Anti-IL-12 and anti-IL-6 receptor therapies seem to be promising. The selective blocking of α4 integrin and α4β7 integrin also demonstrated promising results, however, the side effects still need to be fully elucidated. We await the results of further clinical trials to include such novel compounds in the algorithm for the treatment of CD. Infliximab is likely to also be beneficial for UC. In addition, some other agents seem to be effective for the treatment of UC and further clinical development is also underway. As a result, the management of UC may also dramatically improve in the near future owing to the use of such novel agents. The systematization of novel biological therapies for UC is therefore an issue that needs to be addressed in the future.

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S- Editor Wang J **L- Editor** Zhao JB **E- Editor** Bai SH

Digestive Diseases and Sciences, Vol. 51, No. 4 (April 2006), pp. 677–686 (© 2006)
DOI: 10.1007/s10620-006-3191-2

An Inverse Correlation of Human Peripheral Blood Regulatory T Cell Frequency with the Disease Activity of Ulcerative Colitis

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Evidence suggests that $CD4^+CD25^+$ regulatory T cells play a crucial role in the suppression of intestinal inflammation. However, their role in the suppression of inflammatory bowel disease has not yet been addressed. We examined the proportion of regulatory T cells in inflammatory bowel disease. First, we isolated $CD4^+CD45RO^+CD25^+$ T cells from the peripheral blood of healthy persons and showed that these cells suppressed T cell proliferation profoundly and expressed FoxP3 abundantly, revealing that they are regulatory cells. Then the proportion of $CD45RO^+CD25^+$ in peripheral blood $CD4^+$ T cells was analyzed in patients and healthy controls by flow cytometry. $CD4^+CD45RO^+CD25^+$ T cell frequency was significantly lower in active ulcerative colitis than in the control and inactive ulcerative colitis. $CD4^+CD45RO^+CD25^+$ T cell frequency was inversely correlated with the clinical and endoscopic severity of ulcerative colitis. These results suggest that a deficiency of regulatory T cells is associated with the progression of ulcerative colitis.

KEY WORDS: inflammatory bowel disease; colitis, ulcerative; CD4-positive T lymphocyte; interleukin-2 receptor α.

The etiology of inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), is still not fully understood. Studies of several animal models of IBD, however, have shed some light on this field and accumulating evidence now suggests that a dysregulation of immunity in the intestinal mucosa plays a pivotal role in the pathogenesis of IBD (1, 2). As the intestinal mucosa is a site where the immune system encounters abundant antigens, adequate control of immunity is required for homeostasis of the gut. In addition to increased effector cell function, a deficiency in the regulatory activity of the gut immune system may also be a cause of IBD (2, 3).

The immune system has developed several mechanisms to suppress or regulate immunity in order to protect the body from a sustained harmful immune response. One such mechanism is active suppression in the periphery by regulatory T (Treg) cells. $CD4^+CD25^+$ T cells have been demonstrated to be a cellular subset of Treg cells which is crucial for the regulation of a broad spectrum of immune reactions (4–8). Those cells seem to play an essential role in the regulation of mucosal immunity. Immunodeficient recipient mice injected with $CD4^+$ T cells which are devoid of a $CD25^+$ population have been shown to develop chronic persistent colitis. In addition, the transfer of $CD4^+CD25^+$ T cells into such recipient mice prevents and

Manuscript received June 22, 2005; accepted July 21, 2005.

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This work was supported in part by Health and Labour Science Research Grants from the Japanese Ministry of Health, Labour and Welfare and Research on Measures for Intractable Disease.

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even cures colonic inflammation (9, 10). $CD4^+CD25^+$ Treg cells are, therefore, considered to be one of the important cellular subsets of regulatory cells to control the immune response and maintain homeostasis in the gut.

The existence of $CD4^+CD25^+$ Treg cells was first demonstrated in mice (4–6, 11), and they have also recently been identified in human peripheral blood (PB) (12–15). As mouse $CD4^+CD25^+$ Treg cells have been shown to possess a strong and dominant capacity to regulate several experimental disease models (4, 7, 9, 10, 16, 17), and in vitro studies have also revealed numerous similarities between human $CD4^+CD25^+$ Treg cells and their murine counterparts (5, 6, 12–15), it is of great interest to elucidate whether these cells really play an important role in the prevention of human inflammatory diseases and can thus be potentially utilized in the treatment of such diseases. Human PB $CD4^+CD25^+$ Treg cells were found to decrease in systemic lupus erythematosus and juvenile idiopathic arthritis patients in comparison to healthy controls (18, 19). In rheumatoid arthritis patients, the frequency of $CD4^+CD25^+$ Treg cells in the PB was similar to that in healthy subjects, but they increased in number significantly in the inflamed joints (20). The regulatory function of $CD4^+CD25^+$ Treg cells was reported to be impaired in multiple sclerosis patients (21). Taken together, $CD4^+CD25^+$ Treg cells are thus strongly suggested to play a crucial role in limiting inflammation in some human diseases. Therefore, we consider clarifying the profile of $CD4^+CD25^+$ Treg cells in human IBD to be of great interest. To date, $CD4^+CD25^+$ Treg cells in IBD have not been reported. In this study, we analyzed the frequencies of Treg cells in the PB of IBD and demonstrate that Treg cells decreased in the active stage of UC but not in CD. Moreover, we show that the percentages of Treg cells negatively correlated with the disease activity of UC.

METHODS

Patients. All patients in this study were admitted to either Kyushu University Hospital, Saiseikai Fukuoka General Hospital, National Fukuoka-Higashi Medical Center, or Harasanshin Hospital between 2003 and 2004. The protocol was approved by the institutional ethical committee at all institutions. Written informed consent was obtained from all patients. We obtained PB samples from 17 active UC patients, 10 inactive UC patients, 8 active CD patients, and 19 inactive CD patients. In addition, 10 hospital employees were used as control subjects. Characteristics of the study groups are summarized in Table 1. For the diagnosis of UC, all patients underwent colonoscopy and a pathological examination of a colonic biopsy sample, and CD, ischemic colitis, and infectious colitis were thus ruled out. All active UC patients had either left-sided or pancolitis-type disease with more than moderate disease severity. Active UC patients had a first episode of the disease or a recurrence of colitis during maintenance therapy with oral mesalazine, salazosulfapyridine, or prednisolone at a dosage of less than 10 mg/day. Blood samples were obtained before remission induction therapy was initiated. Twelve patients were evaluated prior to and after treatment with 30 to 60 mg of prednisolone (oral or injection) and/or leukocytapheresis therapy (22, 23). Posttreatment samples were taken 4 to 6 weeks after prednisolone therapy was started or when one course (once a week, five times) of leukocytapheresis therapy was completed. In such patients, the disease activity was evaluated according to the Rachmilewitz Clinical Activity Index and the Endoscopic Index (24) before and after treatment. For a diagnosis of CD, the patients underwent a colonoscopy and/or an x-ray test of the small intestine at some time and a diagnosis was made based on the existence of the typical longitudinal ulcers and/or a cobblestone appearance and, in some cases, the detection of granuloma by histological examinations. UC, ischemic colitis, Bechet disease, and infectious enterocolitis were all ruled out. In active CD patients, the existence of an active open ulcer was confirmed by recent colonoscopy findings and/or an x-ray test of the small intestine.

Reagents. Fluorescein isothiocyanate (FITC)-conjugated anti-human CD4, phycoerythrin (PE)-conjugated anti-human CD45RO, Cy-Chrome-conjugated anti-human CD25, PE-conjugated mouse IgG2a, Cy-Chrome-conjugated mouse IgG1, anti-human CD3 mAb (UCHT1), and anti-human CD28 mAb (CD28.2) were purchased from BD PharMingen (San Diego, CA). Human $CD4^+$ T cell Isolation Kit, CD45RA Microbeads, CD45RO Microbeads, and CD25 Microbeads were purchased from Miltenyi Biotec (Auburn, CA).

Flow Cytometry. PB samples of UC patients, CD patients, and controls were obtained to analyze the proportion of the $CD45RO^+CD25^+$ fraction in $CD4^+$ T cells. Red blood cell lysis was performed using PharM Lyse (BD Biosciences, San Diego, CA) to obtain leukocytes. Next, 5×10^5 leukocytes were incubated with FITC-conjugated anti-CD4, PE-conjugated anti-CD45RO, and Cy-Chrome-conjugated anti-CD25 on ice in the dark for 20 min. Cells incubated with PE-conjugated mouse IgG2a and Cy-Chrome-conjugated mouse IgG1 were used as a control. The analysis was performed on a FACScan (Becton Dickinson) flow cytometer using the CellQuest software.

Cell Purification. Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized PB buffy coat from healthy volunteers using Ficoll density-gradient centrifugation. T cell subsets were isolated from PBMCs using the MACS separation system. Human $CD4^+$ T cells were purified using a $CD4^+$ T cell Isolation Kit II. $CD4^+$ T cells were incubated with CD45RA or CD45RO Microbeads and separated into $CD4^+CD45RA^+$ and $CD4^+CD45RA^-$, or $CD4^+CD45RO^+$ and $CD4^+CD45RO^-$, cells on negative selection columns. The isolated $CD4^+CD45RA^-$ and $CD4^+CD45RO^-$ T cell subsets were then incubated with CD25 Microbeads to obtain four subsets: $CD4^+CD45RA^-CD25^+$and $CD4^+CD45RA^-CD25^-$, or $CD4^+CD45RO^-CD25^+$ and $CD4^+CD45RO^-CD25^-$.

Cell Culture. The cells were cultured in RPMI-1640 medium supplemented with 5 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (GIBCO BRL, Auckland, NZ), 0.5 mM sodium pyruvate, 0.05 mM nonessential amino acids (GIBCO BRL), and 5% human AB serum (Omega Scientific Inc.).

$CD4^+CD45RA^-CD25^+$ or $CD4^+CD45RO^-CD25^+$ T cells were cocultured with $CD4^+$ T cells (5×10^3) at different ratios ($CD4^+$:$CD4^+CD45RA^-CD25^+$ or :$CD4^+CD45RO^-CD25^+$

TABLE 1. CLINICAL CHARACTERISTICS

	Control (n = *10*)	*UC (active)* (n = *17*)	*UC (inactive)* (n = *10*)	*CD (active)* (n = *8*)	*CD (inactive)* (n = *19*)
Male/female	5/5	11/6	4/6	5/3	13/6
Age, yr					
(Mean)	33.0	35.7	42.1	33.8	35.6
(Range)	(29–47)	(18–53)	(26–68)	(25–50)	(20–62)
Disease activity					
CAI*					
Mean		9.3	0		
(Range)		(5–12)			
CDAI†					
Mean				242.7	45.3
(Range)				(170.2–415.7)	(26.2–78.2)
Disease site					
(UC patients)					
Pancolitis		15	6		
Left-sided colitis		2	4		
CD patients					
Ileal				2	10
Ileal-colonic				4	5
Colon only				2	4
Medications‡					
Prednisolone		14	2	1	4
Mesalazine		15	9	7	16
Sulfasalazine		1	1		2
Azathioprine					5
Leukocyteapheresis		10			

*Clinical Activity Index.
†Crohn's Disease Activity Index.
‡Several patients were on a combination of two or more medications.

ratios: 1:0, 1:0.3, 1:1, 0:1) in a U-bottom 96-well plate (Corning Incorporated, Corning, NY) for in vitro suppression assays using [^{3}H] thymidine incorporation. The cells were stimulated with 5 μg/ml soluble anti-CD3 and 5 μg/ml soluble anti-CD28. All wells received 1×10^4 mitomycin C-treated $CD4^-CD8^-$ cells as antigen presenting cells. [^{3}H] thymidine (1 μCi) was added to each well during the final 16 hr of a 6- to 7-day assay, and proliferation was measured by scintillation counting.

RT-PCR Analysis. RNA was extracted from isolated cells using an RNeasy Mini Kit (Qiagen, Valencia, CA). In addition, the total RNA was reverse transcribed using a First-strand cDNA Synthesis Kit (Amersham Biosciences, Little Chalfont Buckinghamshire, UK). RT-PCR was performed using the following oligonucleotides: human FoxP3 forward, TTCATGCACCAGCTCTCAACGG (25); human FoxP3 reverse, TCGTCCATCCTCCTTTCCTTGATC (25); human β-actin forward, TCGTGCGTGACATTAAGGAG; and human β-actin reverse, GATGTCCACGTCACACTTCA.

Statistical Analysis. All data are expressed as mean ± SE. Statistical significance was determined by Student's t test for unpaired samples. $P < 0.05$ was considered to be statistically significant.

RESULTS

Human $CD4^+CD45RO^+CD25^+$ Cells in the PB Are Treg Cells. The human $CD4^+CD25^+$ Treg cell population is relatively indiscrete in comparison to the murine counterpart, probably because the human $CD4^+CD25^+$ fraction is a mixture of Treg and recently activated T cells. In human PB, only CD25-high (and not -low) positive $CD4^+$ T cells have been reported to possess immunoregulatory activity (15). We also confirmed that the regulatory capacity preferentially resides in the $CD4^+CD25^{high}$ fraction (26). However, the border line between $CD25^{high}$ and $CD25^{low}$ was obscure, so we had difficulty determining the cellular subsets of $CD25^{high}$ Treg cells when analyzing the Treg cell frequencies in the PB in IBD in our pilot study. Concerning the cell surface markers of human Treg cells, several reports have demonstrated that Treg cells also express a memory T cell marker, CD45RO (12–15). Indeed, the regulatory activity was demonstrated to be present only in the $CD45RO^+$ fraction, and not in the $CD45RO^-$ fraction, of $CD4^+CD25^+$ cells (12, 27). In this study, we therefore utilized triple staining of CD4, CD45RO, and CD25 to identify Treg cells. Figure 1A shows representative staining findings for CD45RO and CD25 of $CD4^+$ T cells in PB obtained from normal healthy controls, showing that 2.9% of $CD4^+$ T cells were $CD45RO^+CD25^+$ and 1.8% were $CD45RO^-CD25^+$. We next analyzed the immunoregulatory function of both fractions. For this purpose, we measured the activity to suppress T cell proliferation in vitro, which was

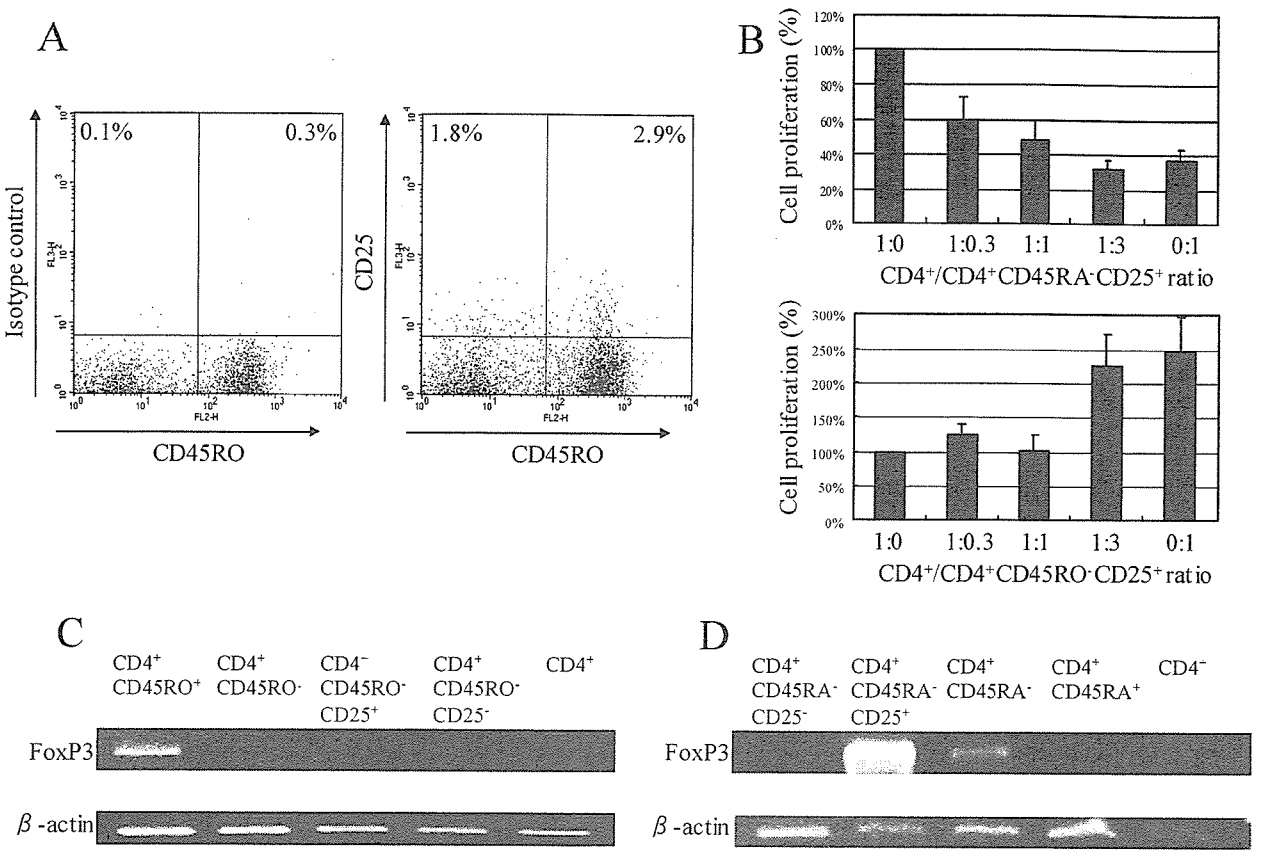

Fig 1. $CD4^+CD45RO^+CD25^+$ Treg cells in human PB. (A) Representative results of CD25 and CD45RO expression on $CD4^+$ T cells in the PB from normal healthy controls. Leukocytes were stained with FITC-conjugated anti-CD4, PE-conjugated anti-CD45RO, and Cy-Chrome-conjugated anti-CD25 and then analyzed by flow cytometry. The expression of CD45RO and CD25 in the $CD4^+$ gate is shown. The frequency of the $CD45RO^+CD25^+$ and $CD45RO^-CD25^+$ fractions is expressed as a percentage of whole $CD4^+$ T cells. Cy-Chrome-conjugated mouse IgG1 was used as an isotype-matched control for anti-CD25. (B) $CD4^+CD45RA^-$ ($CD45RO^+$) $CD25^+$ but not $CD4^+CD45RO^-CD25^+$ T cells suppress the proliferation of $CD4^+$ T cells in vitro. Different doses of $CD4^+CD45RA^-CD25^+$ T cells or $CD4^+CD45RO^-CD25^+$ T cells were added to wells containing 5×10^3 $CD4^+$ T cells and then were stimulated with soluble anti-CD3 and soluble anti-CD28 in the presence of 1×10^4 mitomycin C-treated antigen presenting cells. An increasing number of $CD4^+CD45RA^-CD25^+$ T cells resulted in a greater suppression of proliferation. In contrast, $CD4^+CD45RO^-CD25^+$ T cells did not suppress the proliferation of T cells. Representative results of three independent experiments are shown. (C, D) The exclusive expression of FoxP3 message in $CD45RA^-$($CD45RO^+$) $CD25^+$ population in $CD4^+$ T cells. In C, $CD45RO^+$, $CD45RO^-$, $CD45RO^-CD25^+$, $CD45RO^-CD25^-$, and the whole fraction of $CD4^+$ T cells were purified. In D, $CD45RA^-CD25^-$, $CD45RA^-CD25^+$, $CD45RA^-$, $CD45RA^+$ and the whole fraction of $CD4^+$ T cells were isolated. RNA was purified from each sample. The expression of FoxP3 and β-actin message was analyzed by RT-PCR. The representative results of six independent experiments are shown.

most widely used to assess the regulatory function in previous studies (12–15, 27). $CD4^+CD45RO^+CD25^+$ and $CD4^+CD45RO^-CD25^+$ cells were purified by magnetic beads from the buffy coat of healthy volunteers. To isolate $CD4^+CD45RO^+CD25^+$ cells, we purified $CD45RO^+$ cells by negative selection of $CD45RA^-$ cells instead of $CD45RO^+$ positive selection, as the consecutive positive selections of $CD45RO^+$ and $CD25^+$ were technically difficult to perform. CD45RA is a marker of naïve T cells and the expression of CD45RA and CD45RO is reciprocal. We confirmed almost all negatively selected $CD45RA^-$ cells (>98%) to be $CD45RO^+$ and the majority of positively selected $CD25^+$ cells to be $CD45RO^+CD25^+$ (>82%) of $CD4^+$ T cells by flow cytometric analysis in our pilot study (data not shown). As shown in Figure 1B, $CD4^+CD45RA^-CD25^+$ T cells were cocultured with $CD4^+$ T cells at different ratios ($CD4^+$/$CD4^+CD45RA^-CD25^+$: 1:0, 1:0.3, 1:1, 1:3, 0:1). $CD4^+CD45RA^-CD25^+$ T cells proliferated poorly and suppressed the proliferation of $CD4^+$ T cells in a dose-dependent fashion (percentage proliferation compared with that at culturing with $CD4^+$ alone: 1:0.3, $60.2\pm12.2\%$; 1:1, $48.4\pm11.4\%$; 1:3, $31.8\pm5.5\%$). In contrast, $CD4^+CD45RO^-CD25^+$ T cells proliferated vigorously and did not suppress $CD4^+$ T cell growth at all. To further confirm these results, we analyzed the expression of

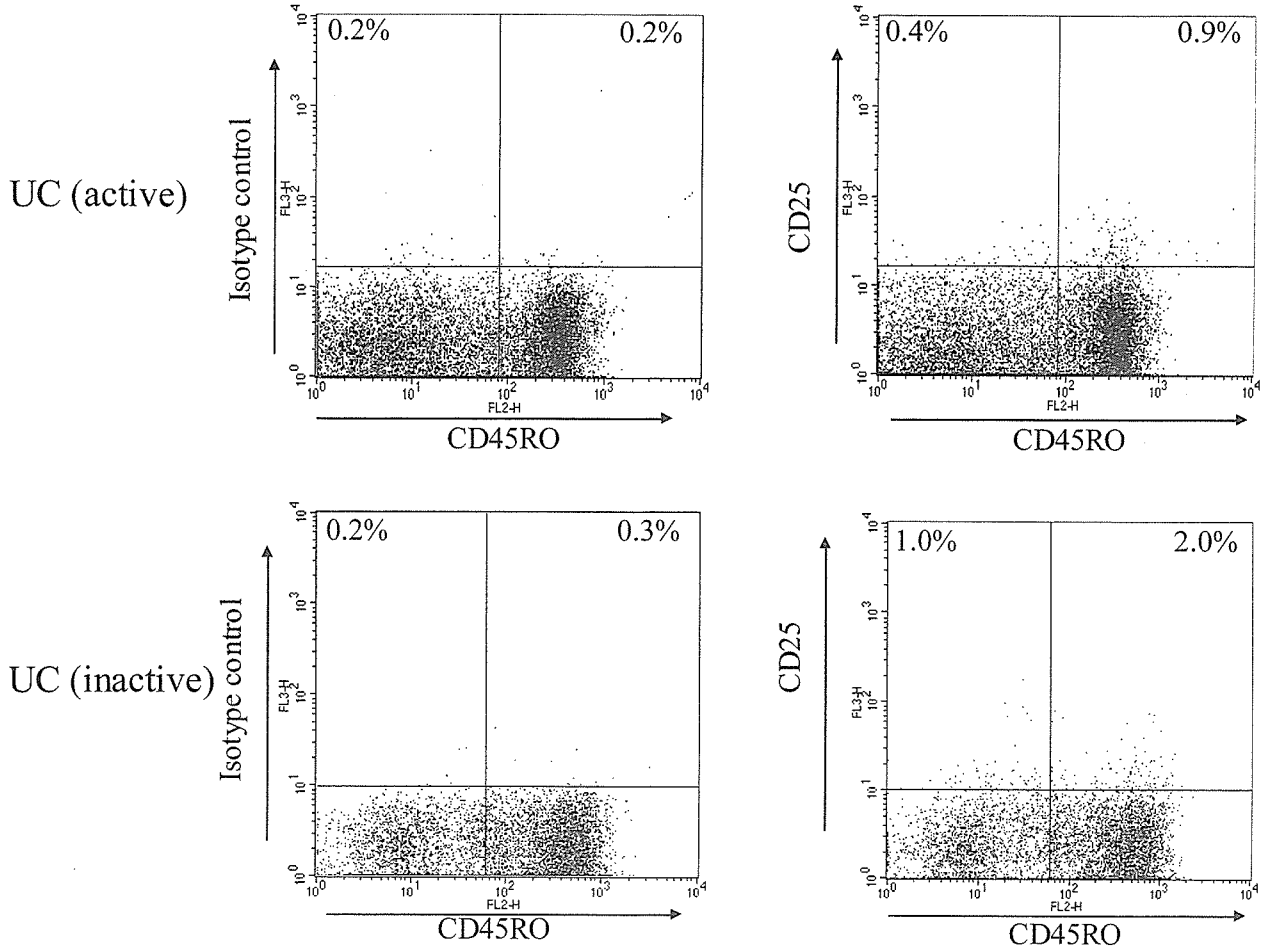

Fig 2. Flow cytometric analysis of $CD4^+CD45RO^+CD25^+$ Treg cells in the PB of UC patients. Representative results of CD25 and CD45RO expression on the peripheral $CD4^+$ T cells for active and inactive UC. The frequency of $CD45RO^+CD25^+$ (Treg) and $CD45RO^-CD25^+$ (non-Treg) fraction is expressed as a percentage of whole $CD4^+$ T cells.

FoxP3 in each fraction. FoxP3, a forkhead/winged-helix transcription factor, is specifically expressed in Treg cells and is considered to be a master control protein for the generation of Treg cells (28–30). We performed RT-PCR of FoxP3 and β-actin. As shown in Figure 1C, FoxP3 mRNA was not detectable in whole $CD4^+$ T cells. However, in the $CD45RO^+$ fraction of $CD4^+$ T cells, we detected a definite band of FoxP3 mRNA. In contrast, the FoxP3 message was not detectable in the $CD45RO^-$ fraction. Furthermore, even after a further separation of the $CD25^+$ and $CD25^-$ populations of $CD45RO^-$, we could not detect a band of FoxP3 in either lane. In each lane, we detected a comparable level of β-actin bands. These results show that Treg cells expressing FoxP3 are present in the $CD45RO^+$ population. Finally, we attempted to confirm that FoxP3 expressing cells are present in the $CD25^+$ fraction of $CD45RO^+$ ($CD45RA^-$). As shown in Figure 1D, $CD45RA^-$ cells of the $CD4^+$ T cell population expressed FoxP3 mRNA, whereas $CD45RA^+$ cells did not, which is consistent with the results in Figure 1C. In addition, the $CD45RA^-CD25^+$ fraction expressed the FoxP3 message abundantly, while the $CD45RA^-CD25^-$ fraction did not contain detectable FoxP3 mRNA. The intensity of the FoxP3 message band in $CD45RA^-CD25^+$ was much higher than that in the whole fraction of $CD45RA^-$. A comparable amount of the β-actin message was detected in each lane. Taken together, Treg cells in human PB were thus found to preferentially reside in the $CD4^+CD45RO^+CD25^+$ fraction. This method is, therefore, useful to identify an enriched Treg cell population in the human $CD4^+CD25^+$ fraction.

The Percentage of $CD4^+CD45RO^+CD25^+$ Treg Cells in IBD Patients. We obtained samples of PB from IBD patients and healthy persons and determined the frequency of $CD4^+CD45RO^+CD25^+$ Treg cells. In Figures 2 and 3 representative three-color staining results

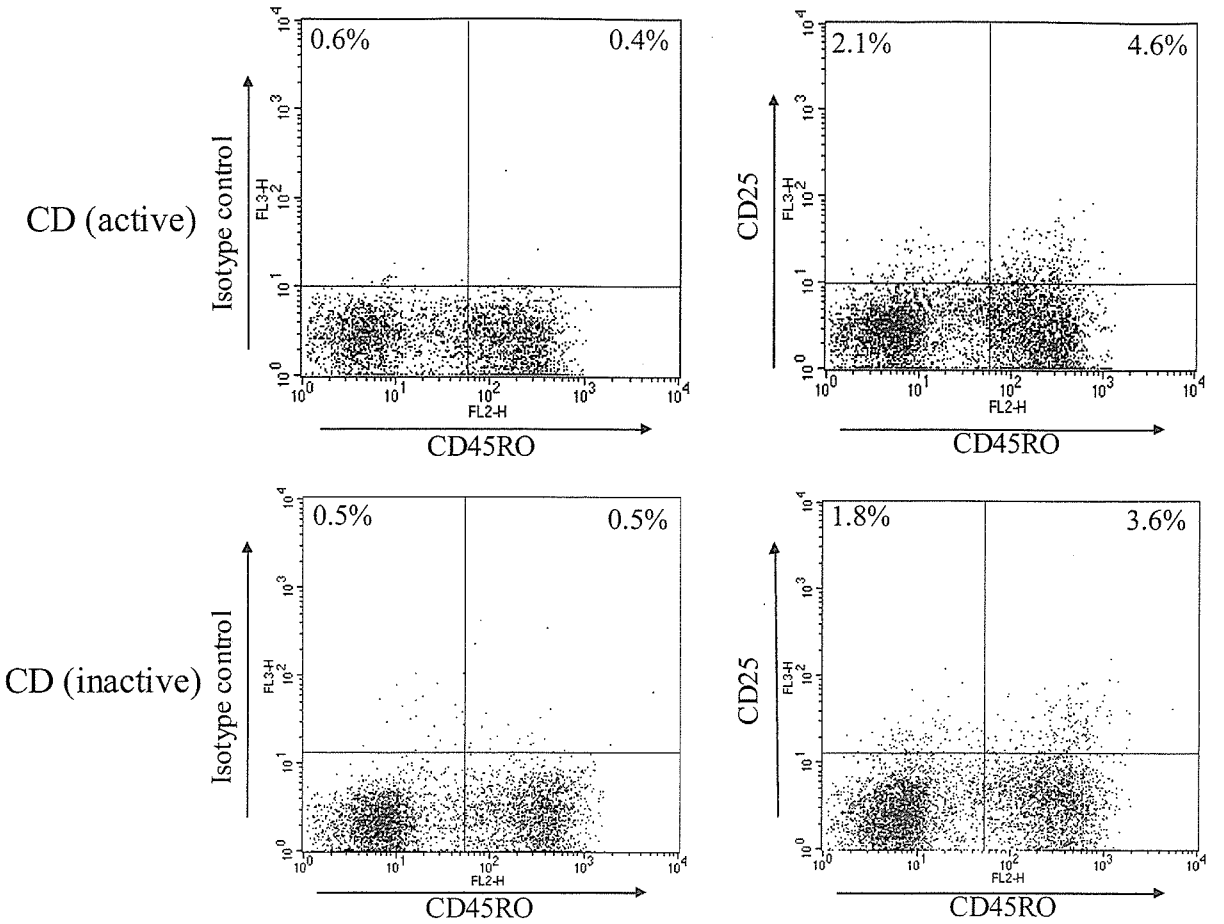

Fig 3. A flow cytometric analysis of $CD4^+CD45RO^+CD25^+$ Treg cells in the PB of CD patients. Representative results of CD25 and CD45RO expression on the peripheral $CD4^+$ T cells for active and inactive CD patients. The frequency of $CD45RO^+CD25^+$ (Treg) and $CD45RO^-CD25^+$ (non-Treg) fraction is expressed as a percentage of whole $CD4^+$ T cells.

of CD4/CD45RO/CD25 for active and inactive UC and active and intactive CD samples are shown. The percentages of each fraction for each group are summarized in Table 2. As shown in Table 2 and Figure 4, the percentage of $CD4^+CD45RO^+CD25^+$ Treg cells was significantly lower in active UC patients (mean ± SE, 1.3 ± 0.2%) than that in normal controls (2.9 ± 0.4%) or inactive UC patients (2.3 ± 0.3%) ($P < 0.005$ and $P < 0.01$, respectively). In contrast, the frequency of $CD4^+CD45RO^+CD25^+$ Treg cell was higher in active CD patients (4.4 ± 0.7%) than in normal controls or inactive CD patients (3.1 ± 0.3%), but the difference was not statistically significant ($P = 0.099$ and $P = 0.117$, respectively).

An Inverse Correlation Between the $CD4^+CD45RO^+CD25^+$ Treg Cell Frequency and the Disease Activity of UC. We next compared the $CD4^+CD45RO^+CD25^+$ Treg cell proportions in UC patients before and after treatment. As shown in Figure 5A, in most of the cases, the percentage of Treg cells increased after treatment. The mean proportion of Treg cells was significantly higher posttreatment (2.3 ± 0.3%) than prior to treatment (1.0 ± 0.2%; $P < 0.001$) (Figure 5B).

We next analyzed the relationship between the $CD4^+CD45RO^+CD25^+$ Treg cell frequency and the disease activity. As shown in Figure 6A, there was an intermediate reverse correlation between the Clinical Activity Index and the percentage of $CD4^+CD45RO^+CD25^+$ Treg cells ($R = 0.621$). In addition, there was also an intermediate inverse correlation between the Endoscopic Index and the percentages of $CD4^+CD45RO^+CD25^+$ Treg cells ($R = 0.764$) (Figure 6B).

DISCUSSION

The role of $CD4^+CD25^+$ Treg cells in the suppression of intestinal inflammation in human IBD has not yet been

TABLE 2. PERCENTAGES OF EACH CD4/CD45RO/CD25 FRACTION FOR CONTROL, UC, AND CD GROUPS

	% $CD4^+CD45RO^-CD25^+/CD4^+$	% $CD4^+CD45RO^+CD25^+/CD4^+$	% $CD4^+CD45RO^-CD25^-/CD4^+$	% $CD4^+CD45RO^+CD25^-/CD4^+$
Control	2.5 ± 0.4	2.9 ± 0.4	53.3 ± 3.3	41.2 ± 3.7
UC				
Active	1.7 ± 0.4	1.3 ± 0.2*†	56.0 ± 3.3	40.0 ± 3.8
Inactive	1.9 ± 0.4	2.3 ± 0.3	48.7 ± 4.1	46.0 ± 4.5
CD				
Active	2.6 ± 0.4	4.4 ± 0.7‡§	38.5 ± 5.7	53.9 ± 5.6
Inactive	2.2 ± 0.3	3.1 ± 0.3	44.0 ± 4.0	50.1 ± 4.0

Note. Data are mean ± SE (%). $*P < 0.005$ vs. control. $†P < 0.01$ vs. UC (inactive). $‡P = 0.099$ vs. control. $§P = 0.117$ vs. CD (inactive).

addressed. In this study, we demonstrated for the first time a link between the frequency of Treg cells and the activity of human IBD. As a result, the percentages of Treg cells in the PB decreased in active UC patients. In addition, the Treg cell frequency increased after treatment and also was inversely correlated with the clinical and endoscopic severity of UC. Our results strongly indicate that a reduction in the number of Treg cells in the PB is associated with an enhancement of colonic inflammation in UC, which thus suggests that Treg cells actually suppress colonic inflammation in humans. In contrast to UC, in CD patients, the Treg cell frequency did not decrease and instead tended to increase, although the difference was not statistically significant. These results suggest that the mechanism of the development of intestinal inflammation and the relevance of the regulatory function of Treg cells are quite different in UC and CD.

We analyzed the frequency of $CD4^+CD45RO^+CD25^+$ cells as a Treg cell population instead of measuring $CD4^+CD25^{high}$ T cells. The reason we chose this method is that we considered it difficult to determine the border between $CD25^{high}$ Treg and $CD25^{low}$ non-Treg cells in our pilot study. As it was necessary to detect small changes in the Treg cell frequencies, such as 1% vs. 3%, we therefore needed a more obvious way to set a borderline. Previous reports have demonstrated that only $CD4^+CD45RO^+CD25^+$, and not $CD4^+CD45RO^-CD25^+$, T cells possess immunoregulatory activity (12, 27). The results of our T cell proliferation assay are consistent with those of previous reports. We provided further evidence regarding the Treg cell population by also analyzing FoxP3 expression. FoxP3, specifically expressed in $CD4^+CD25^+$ Treg cells, has been shown to be a master control gene for the development

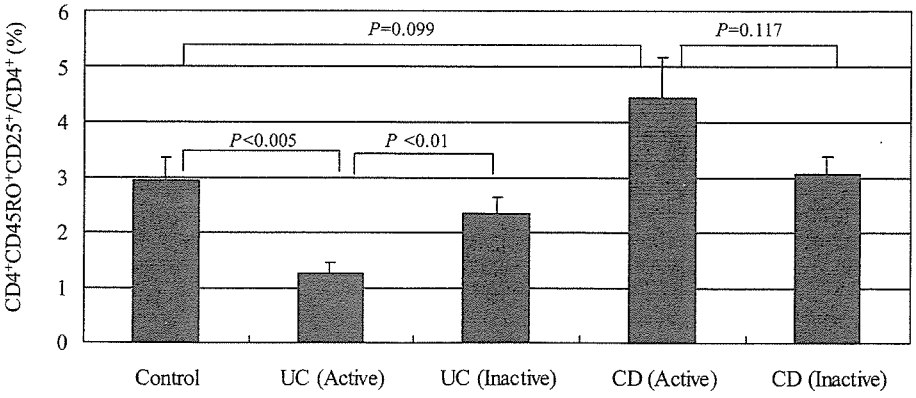

Fig 4. Percentage of $CD4^+CD45RO^+CD25^+$ Treg cells in the PB of IBD patients. PB samples from active UC ($n = 17$), inactive UC ($n = 10$), active CD ($n = 8$), inactive CD ($n = 19$), and healthy persons ($n = 10$) were evaluated to determine the frequencies of $CD4^+CD45RO^+CD25^+$ Treg cells. The mean percentage of $CD45RO^+CD25^+$ in $CD4^+$ T cells in each group is shown. The percentage of $CD4^+CD45RO^+CD25^+$ Treg cells was significantly lower in active UC (mean ± SE, 1.3 ± 0.2%) than in the normal controls (2.9 ± 0.4%) or in inactive UC (2.3 ± 0.3%) ($P < 0.005$ and $P < 0.01$, respectively). In contrast, the frequency of $CD4^+CD45RO^+CD25^+$ Treg cell tended to be higher in active CD (4.4 ± 0.7%) than in normal controls and inactive CD (3.1 ± 0.3%) ($P = 0.099$ and $P = 0.117$, respectively).

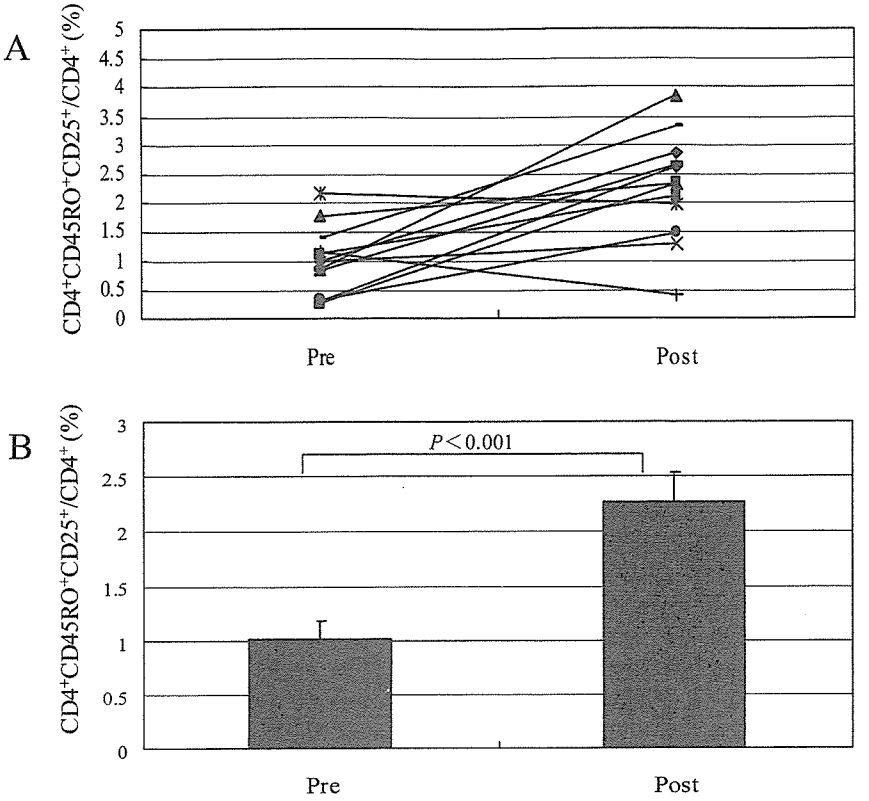

Fig 5. Comparison of the $CD4^+CD45RO^+CD25^+$ Treg cell proportions in UC patients before and after treatment. (A) The proportion of the $CD45RO^+CD25^+$ fraction in $CD4^+$ T cells increased after treatment in 10 subjects, while the value remained unchanged or tended to decrease in the remaining 2 subjects. (B) The mean proportion of the $CD45RO^+CD25^+$ fraction in $CD4^+$ T cells was $1.0 \pm 0.2\%$ prior to treatment and $2.3 \pm 0.3\%$ after treatment, and there was a significant difference between the two ($P < 0.001$).

of $CD4^+CD25^+$ Treg cells (28–30), as the inactivation of this gene in both humans and mice leads to a lack of conventional $CD4^+CD25^+$ Treg cells, thus leading to systemic inflammatory diseases including intestinal inflammation (31–33). We demonstrated FoxP3 mRNA to be strongly expressed in the $CD4^+CD45RO^+CD25^+$ but not in the $CD4^+CD45RO^-CD25^+$ cell fraction, thus showing that only the former includes a Treg cell population. An analysis of $CD4^+CD45RO^+CD25^+$ cells as Treg cells in PB is thus considered to be a useful and reasonable method to investigate Treg cell frequencies in humans. While we were preparing this article, another report supporting our strategy was published showing that only human $CD4^+CD45RO^+CD25^+$, and not $CD4^+CD45RO^-CD25^+$, T cells are regulatory and express FoxP3 mRNA and protein (34).

Treg cells injected into immunodeficient recipient mice passed through and were present in the colon (10). Treg cells are thus suggested to circulate throughout the body from the PB to the lamina propria (LP) of the intestine and then probably from the LP to the systemic circulation like other lymphocytes. In this regard, PB Treg cells may be the source of such cells in the intestine. We therefore analyzed the Treg cell frequency in the PB of IBD patients as the first step in studying the role of Treg cells in IBD. However, the changes in the number of Treg cells in the PB do not necessarily parallel those in the target organ (19). Hence, the frequency of Treg cells in the gut of IBD should also be analyzed. Makita *et al.* (25) reported that $CD4^+CD25^{high}$ Treg cells are present in the LP of the intestine and the proportion of those cells is up-regulated but not reduced in UC as well as in CD. It should therefore be clarified in further studies whether or not a reduction in the number of PB $CD4^+CD45RO^+CD25^+$ Treg cells in UC is accompanied by a reduction in the number of LP Treg cells or whether such a discrepancy is due to a different detection method of Treg cells, such as $CD4^+CD25^{high}$ vs. $CD4^+CD45RO^+CD25^+$. A reduction of Treg cells in the PB may not directly result in a profound reduction of regulatory activity in the intestine. However, our results

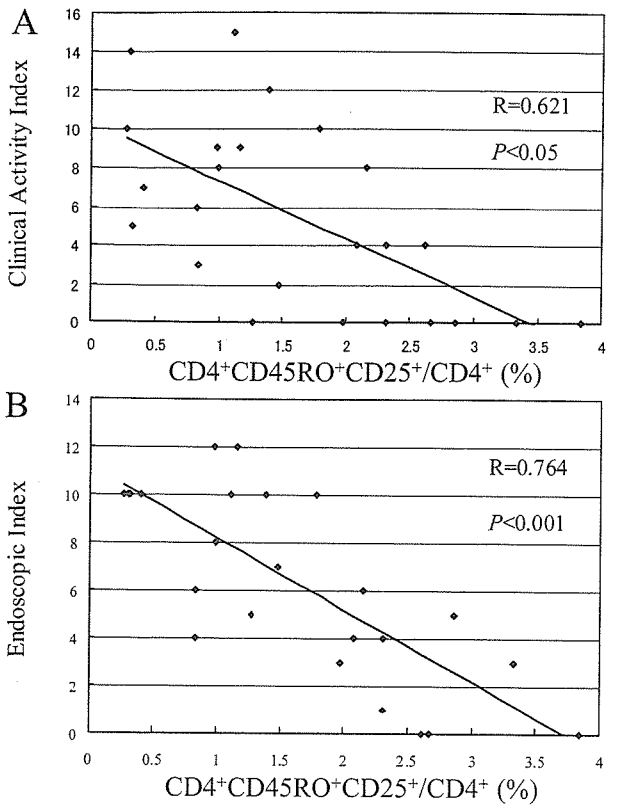

Fig 6. Correlation between the frequencies of $CD4^+CD45RO^+CD25^+$ Treg cells and disease activity of UC. Clinical Activity Index (Rachmilewitz), and Endoscopic Index (Rachmilewitz) were recorded in active UC patients ($n = 12$) before and after treatment. The relationship between the percentage of $CD45RO^+CD25^+$ Treg cells in PB $CD4^+$ T cells and the disease activity was analyzed. (A) There was an intermediate inverse correlation between the Clinical Activity Index and the frequency of $CD4^+CD45RO^+CD25^+$ Treg cells. (B) There was also an intermediate inverse correlation between the Endoscopic Index and the frequency of $CD4^+CD45RO^+CD25^+$ Treg cells.

revealed a decrease in Treg cells in the PB to be associated with an augmentation of the disease activity of UC, thus strongly indicating that a depletion of Treg cells in the PB leads to a failure of immunoregulation in the gut and an acceleration of colonic inflammation.

In further studies, it should be determined whether the function of Treg cells is altered in UC patients. Although the frequency of PB Treg cells did not decrease in inactive UC patients, it is possible that the function of these cells is impaired, which could be one of the causes of the onset of this disease. Alternatively, Treg cells may be functionally normal in UC and just quantitatively decrease during the development of the disease. In either case, the sum of the Treg cell capacity is considered to decrease during the active stage of UC as the proportion of Treg cells decreases. It is, therefore, of great interest whether Treg cells can be utilized for the treatment of UC. In Japan, leukocytapheresis therapy was developed as a treatment for active UC and it has been proven to be effective (22, 23). One possible way to utilize Treg cells is that, in combination with such leukocytapheresis, Treg cells can be purified from removed leukocytes and thereafter can be returned to the patients. The second possibility is that Treg cells could be isolated from patients, cultured, expanded in vitro, and then injected into the patients.

The mechanism of Treg cell suppression in colonic inflammation in IBD remains to be elucidated. In in vivo studies, the $CD45RB^{high}$ transfer model of colitis revealed suppression of colonic inflammation to be dependent on immunosuppressive cytokines, IL-10 and TGF-β (9, 35). We previously demonstrated that Treg cells express a high level of TGF-β1 (36) and the production of this cytokine is inevitable for the suppression of colitis, while it is not always required for the inhibition of T cell proliferation in vitro (26). Further analyses regarding the suppression mechanism of intestinal inflammation in human IBD will hopefully provide more useful information for the generation of Treg cell-based immunotherapy.

In this study, we demonstrated the Treg cell frequency to decrease in the active stage of UC and it correlated inversely with the disease activity. Our results strongly indicate a reduction of the Treg cell capability to be associated with the disease progression, and a new therapy which can enhance the regulatory activity of Treg cells may thus be effective for the treatment of UC.

ACKNOWLEDGMENTS

The authors thank Prof. Tsukuru Umemura and Dr. Koichiro Muta for their excellent advice. We also appreciate the valuable technical assistance of Ms. Akiyo Kondo.

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