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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

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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 (5×10^5) 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

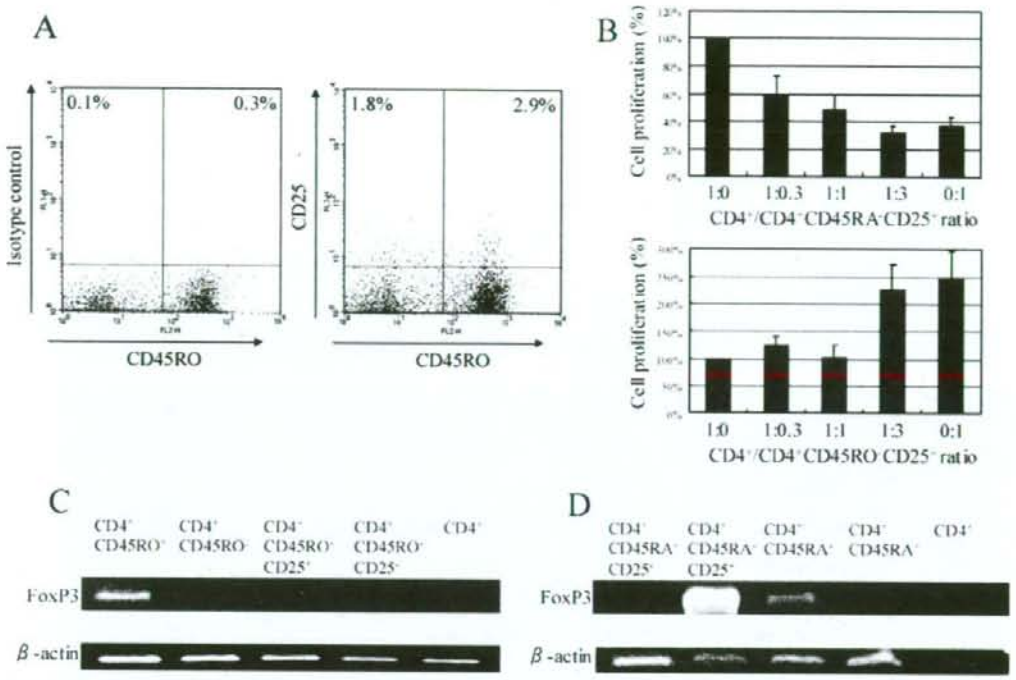

Fig 1. $CD4^+CD45RO^+CD25^+$ Treg cells in human PB. (A) Representative results of CD25 and CD45RO expression on $CD4^+$ T cells in the PB from normal healthy controls. Leukocytes were stained with FITC-conjugated anti-CD4, PE-conjugated anti-CD45RO, and Cy-Chrome-conjugated anti-CD25 and then analyzed 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, $60.2 \pm 12.2\%$; 1:1, $48.4 \pm 11.4\%$; 1:3, $31.8 \pm 5.5\%$). 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

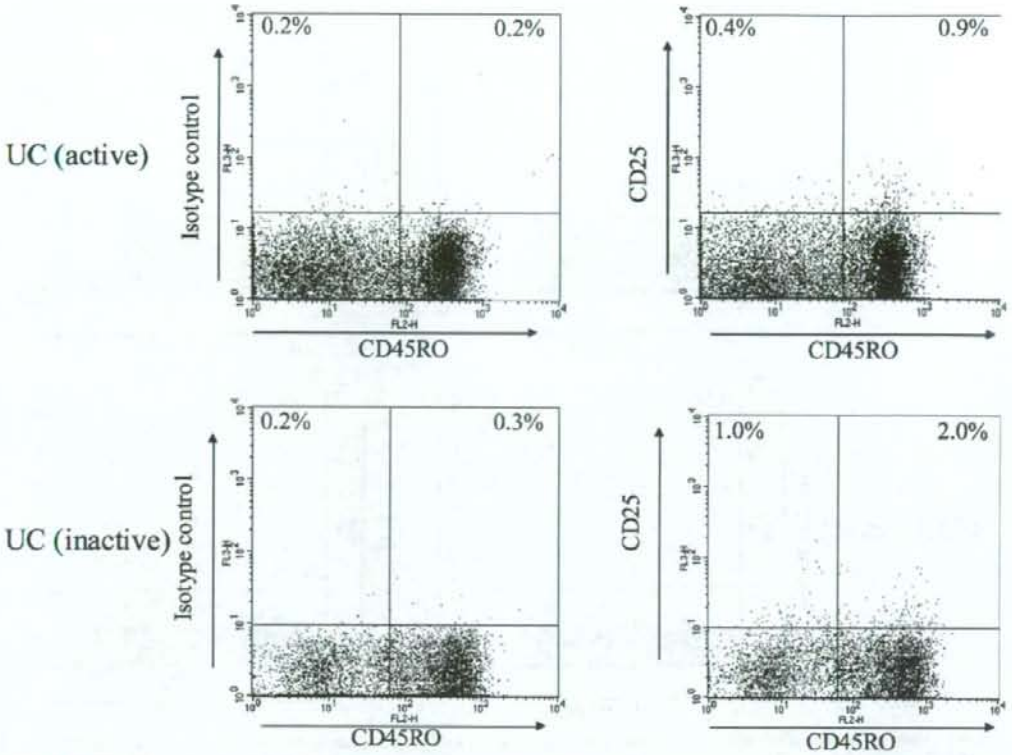

Fig 2. Flow cytometric analysis of $CD4^+CD45RO^+CD25^+$ Treg cells in the PB of UC patients. Representative results of CD25 and CD45RO expression on the peripheral $CD4^+$ T cells for active and inactive UC. The frequency of $CD45RO^+CD25^+$ (Treg) and $CD45RO^-CD25^+$ (non-Treg) fraction is expressed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

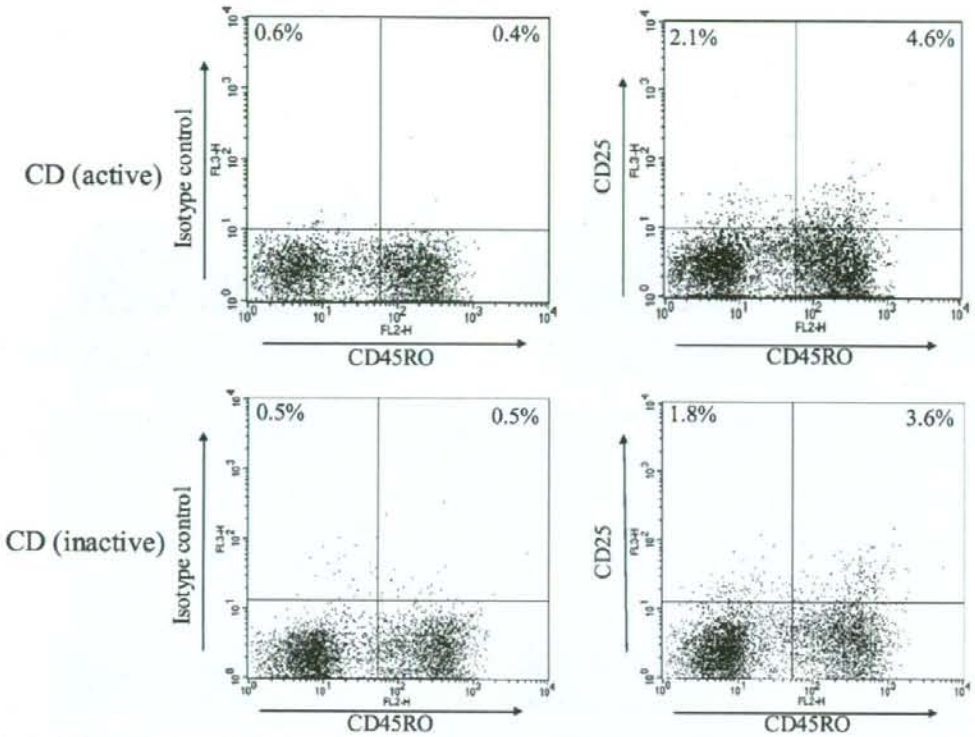

Fig 3. A flow cytometric analysis of $CD4^+CD45RO^+CD25^+$ Treg cells in the PB of CD patients. Representative results of CD25 and CD45RO expression on the peripheral $CD4^+$ T cells for active and inactive CD patients. The frequency of $CD45RO^+CD25^+$ (Treg) and $CD45RO^-CD25^+$ (non-Treg) fraction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

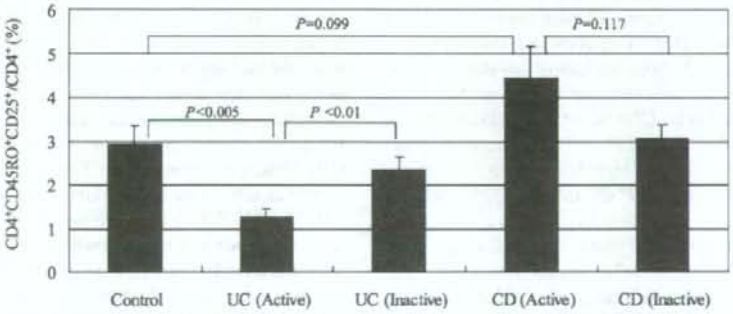

Fig 4. Percentage of $CD4^+CD45RO^+CD25^+$ Treg cells in the PB of IBD patients. PB samples from active UC ($n = 17$), inactive UC ($n = 10$), active CD ($n = 8$), inactive CD ($n = 19$), and healthy persons ($n = 10$) were evaluated to determine the frequencies of $CD4^+CD45RO^+CD25^+$ Treg cells. The 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).

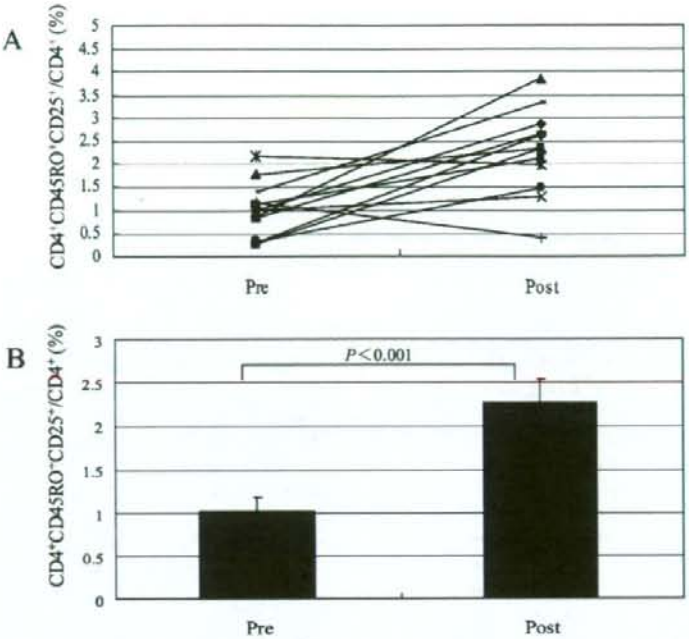

Fig 5. Comparison of the $CD4^+CD45RO^+CD25^+$ Treg cell proportions in UC patients before and after treatment. (A) The proportion of the $CD45RO^+CD25^+$ fraction in $CD4^+$ T cells increased after treatment in 10 subjects, while the value remained unchanged or tended to decrease in the remaining 2 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

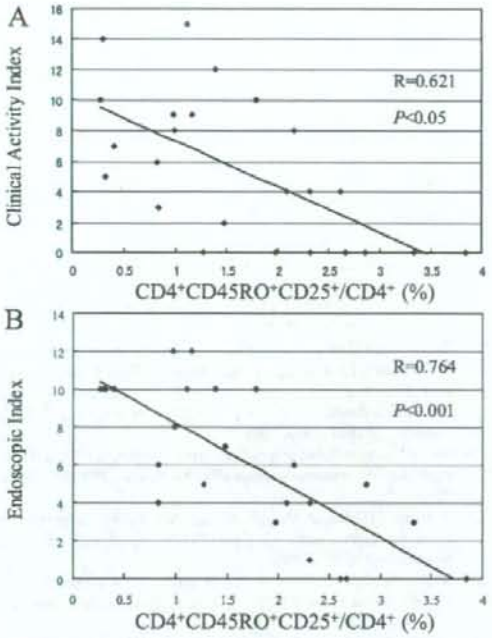

Fig 6. Correlation between the frequencies of $CD4^+CD45RO^+CD25^+$ Treg cells and disease activity of UC. Clinical Activity Index (Rachmilewitz), and Endoscopic Index (Rachmilewitz) were recorded in active UC patients ($n = 12$) before and after treatment. The relationship between the percentage of $CD45RO^+CD25^+$ 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.

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Expert Opinion

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Cell- & Tissue-based Therapy

The logics of leukocytapheresis as a natural biological therapy for inflammatory bowel disease

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Ulcerative colitis (UC) and Crohn's disease (CD) are debilitating idiopathic inflammatory bowel diseases (IBDs) with symptoms that impair ability to function and quality of life. The aetiology of IBD is inadequately understood and, therefore, drug therapy has been empirical instead of based on sound understanding of the disease mechanisms. This has been a major factor for poor drug efficacy and treatment-related side effects that often add to disease complications. The development of biologicals, notably infliximab, to block TNF-α reflects some progress, but there is major concern about their side effects and lack of long-term safety and efficacy profiles. However, IBD by its very nature is exacerbated and perpetuated by inflammatory cytokines, including TNF-α, IL-6 and IL-12, for which activated peripheral blood lymphocytes, monocytes/macrophages and granulocytes are major sources. Hence, activated leukocytes should be appropriate targets of therapy. At present, three strategies are available for removing excess and activated leukocytes by leukocytapheresis: centrifugation, Adacolumn® and Cellsorba™. Centrifugation can deplete lymphocytes or total leukocytes, whereas Adacolumn selectively adsorbs granulocytes and monocytes together with a smaller fraction of lymphocytes (FcγR- and complement receptor-bearing leukocytes), and Cellsorba non-selectively removes all three major leukocyte populations. Efficacy has ranged from 'none' to an impressive 93% together with excellent safety profiles and downmodulation of inflammation factors. Furthermore, leukocytapheresis has shown strong drug-sparing effects and reduced the number of patients requiring colectomy or exposure to unsafe immunosuppressants, such as cyclosporin A. Leukocytapheresis removes from the body cells that contribute to IBD and, therefore, unlike drugs, it is not expected to induce dependency or refractoriness.

Keywords: biological therapy, granulocytes, inflammatory bowel disease, leukocytapheresis, lymphocytes, monocytes, ulcerative colitis

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1. Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) together represent the idiopathic inflammatory bowel diseases (IBDs) of the gut. IBD is characterised by inflammation in the intestinal mucosa followed by ulceration and extensive loss of the mucosal tissue if untreated. Both UC and CD are debilitating chronic disorders that afflict millions of individuals throughout the world and produce symptoms that impair ability to function and quality of life. Whereas UC is usually confined to the colon and the rectum, CD can affect any part of the gut, from the mouth to the perianal region [1-4]. The loss of the mucosal tissue as a consequence of inflammation and ulceration is associated with a multitude of clinical manifestations representing

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the expressions of IBD, including diarrhoea, rectal bleeding, abdominal discomfort, fever, anaemia and weight loss [1-3]. Both UC and CD tend to run a remitting–relapsing course affected by diverse environmental and genetic factors [1,3-5].

Despite the recognition of a strong genetic background together with environmental factors that at present are thought to translate into an inappropriate inflammatory response in patients with IBD [3,4,6], factors that cause IBD are not completely understood. Accordingly, until now, drug therapy of IBD has been empirical rather than based on sound understanding of disease aetiology. Thus, although the success of treatment is evident in most patients, it comes at the cost of significant side effects [7,8]. Hence, first-line medications for exacerbation of IBD include 5-aminosalicylic acid (5-ASA) or sulfasalazine in combination with a corticosteroid, with consideration of azathioprine and nutritional support for some patients [1,9-14]. Treatment failure in patients with severe disease has often been an indication for colectomy in up to 40% of steroid-refractory patients [10,15], although in recent years, cyclosporin A (CyA) has been introduced for corticosteroid-refractory UC [15,16]. Despite being moderately effective in this clinical setting in reducing colectomy rates, there remain concerns over long-term efficacy and toxicity of CyA [17].

2. Novel biologicals for the treatment of inflammatory bowel disease

IBD by its very nature is exacerbated and perpetuated by inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-12, and so on [18,19]. For unknown reasons, patients with IBD cannot maintain normal gut homeostasis. An understanding of the cytokine network and its role in promoting IBD pathogenesis is a crucial step towards finding a cure for this devastating disease [2,3]. Based on this understanding, in recent years, anticytokine antibodies, notably the TNF antibodies (infliximab, adalimumab), have been developed for the treatment of IBD [13]. The apparent success of infliximab in CD [20,21] has also been seen in patients with UC [22]. Following the introduction of infliximab, a plethora of other biologicals are being introduced as candidates for the treatment of IBD [13,23]. These include CDP571 [24], an immunoglobulin G4 monoclonal antibody that has been investigated in a pilot study of 15 patients with mild-to-moderate UC. However, the efficacy in UC was unsustainable. Another candidate is RDP58 [25], a novel p38/JNK inhibitor known to block TNF production and also inhibit the production of IL-2 and IL-12. RDP58 has been evaluated in a Phase II study of 127 patients with mild-to-moderate active UC [26]. Remission efficacy has been up to 72%. Antileukocyte adhesion agents also represent novel approaches to block infiltration of inflammatory leukocytes to the intestinal mucosal. Two recently developed candidates for UC are natalizumab, a monoclonal antibody to the α_4 integrin [27], and MLN-02, an $\alpha_4\beta_7$ integrin [28]. A large-scale study on natalizumab in patients with CD produced insignificant efficacy and had fatal adverse events (progressive multifocal leukoencephalopathy); the preparation has been halted [29].

The efficacy of CyA in severe UC is thought to be via inhibition of IL-2 production by T cells [13]. This has led to the development of anti-IL-2 receptor alpha monoclonal antibodies. Daclizumab and basiliximab are two such candidates [13,30]. One pilot study of daclizumab in 10 patients with refractory UC showed significant decreases in clinical activity scores after week 2 with a parallel decrease in C-reactive protein (CRP) and significantly reduced CD25+ cells in mucosal biopsy samples [30]. Likewise, certain interferons (IFNs) are expected to show efficacy in IBD. Based on this background, a pegylated IFN-α-2b was recently evaluated in 60 patients with UC [31]. Remission efficacy in one study was 58% compared with 40% for the placebo [31]. This study had a significant number of drop-outs (8 of 21 patients) due to side effects [13].

Growth factors such as epidermal growth factor (EGF) and keratinocyte growth factor (KGF) are known to regulate the integrity of the mucosa and maintain its barrier function. The potential use of these growth factors to heal and restore mucosal integrity has stimulated studies with EGF and KGF for the treatment of UC [13,32,33]. Despite this background, a placebo-controlled trial that enrolled 88 patients with active UC showed no significant benefit from KGF [33]. In contrast, when EGF was given to 12 patients as enemas (5 µg EGF in 100 ml of an inert vehicle), 10 experienced remission [13,33]. Clearly, additional studies together with long-term follow-up are necessary to fully assess the position of growth factors in the treatment of IBD. Further, potential benefits need to be balanced against the potential for upregulation of proto-oncogene expression and the risk of malignant transformation with EGF therapy in UC or adenomatous polyps [13].

The enthusiasm towards biologicals is dampened at present by concerns about their long-term efficacy and safety profiles [34-39]. Taking infliximab as one example that has been through extensive clinical evaluations, following the initial and subsequent administrations, antibodies to infliximab emerge that potentially can reduce its efficacy [39]. Concerning their side effects, the literature on biological therapy in general carries headlines such as *'Tumour necrosis factor antagonist therapy and lymphoma development'* [38]; *'Serious bacterial infections in patients with rheumatoid arthritis under anti-TNF-α therapy'* [37]; *'Treatment of rheumatoid arthritis with tumour necrosis factor inhibitors may predispose to significant increase in tuberculosis risk'* [36]; *'Adverse skin reactions to anti-TNF-α'* [34]; and so on. There is no shortage of many more warning statements.

Leukocytes have the potential to initiate and amplify inflammation by releasing a cascade of pro-inflammatory cytokines, proteases and oxygen derivatives, leading to

extensive tissue injury [2]. In the face of the overwhelming evidence for the involvement of various cytokines in the immunopathogenesis of IBD and the fact that peripheral blood leukocytes are major sources of these cytokines, the leukocytes seem logical targets in the treatment of IBD. Indeed, histological examination of the mucosal tissue in biopsy specimens from patients with active IBD reveals a spectrum of pathological manifestations, among which presence of an abundance of neutrophils, lymphocytes and macrophages relates specifically to clinical disease activity and severity of the disease [1-3,40-42]. The circulating activated granulocytes and monocytes, which are major sources of inflammatory cytokines [43,44], are elevated with increased survival time in active IBD [45-52]. Paradoxically, corticosteroids that are given to most patients with active IBD increase neutrophil survival time [53]. In addition to their inflammatory cytokines that can exacerbate and perpetuate the inflammation in the mucosa, neutrophils and monocytes/macrophages can cause mucosal tissue injury via their proteases and reactive oxygen products [54].

This article reviews the therapeutic application of leukocytapheresis in IBD with a major focus on UC. The underlying rationale is that the removal of these cells that are otherwise destined for migration to the intestine reduces the inflammatory intensity, which in turn allows healing to take place. Also presented are arguments for why leukocytapheresis should be a safer and more effective biological therapy in IBD. Until now, three different leukocytapheresis systems have been applied to the treatment of IBD: centrifugation, an adsorptive carrier-based leukocytapheresis system (the Adacolumn®) and the Cellsorba™ filter column.

3. Leukocytapheresis in the treatment of inflammatory bowel disease

3.1 Earlier observations

Leukocytapheresis was first introduced to treat patients with chronic myelocytic leukaemia [55,56] and chronic lymphocytic leukaemia [57]. In 1975, thoracic duct drainage was associated with clinical improvement in 12 patients with rheumatoid arthritis [58]. In 1979, Tenenbaum and colleagues [59] successfully performed leukocytapheresis for rheumatoid arthritis using an IBM blood cell separator.

The logics of leukocytapheresis for autoimmune diseases was based on the expectation that removal of lymphocytes that are producing autoantibodies or stimulating antibody production should reduce the cause of an autoimmune disease such as rheumatoid arthritis. Recent evidence suggests that the efficacy of the therapy might not simply be attributed to cell removal *per se*, as contact activation of cells with the treatment surface or a change in the proportions of regulatory (suppressor) T cells and pathogenic macrophages might produce immunomodulatory effects. This notion has been further discussed in the following sections.

3.2 Centrifugal leukocytapheresis

In 1985, Bick and colleagues [60] reported the first clinical trial of centrifugal leukocytapheresis in IBD for patients with active CD. This uncontrolled trial together with their follow-up studies [61,62] suggested that leukocytapheresis had efficacy in patients with CD, but their preliminary observations were to be confirmed by subsequent studies in large cohorts of patients. In line with this assertion, in 1994, Lerebours and colleagues [63] assessed the efficacy of centrifugal lymphapheresis to suppress early relapse in patients with CD in clinical remission after steroid treatment for an acute attack. Twenty-eight patients were included in this randomised, multi-centre, prospective study. Before starting steroid tapering, patients were randomly assigned either to lymphapheresis (9 sessions within 4 – 5 weeks) or to a control group (no lymphapheresis). The primary judgment criterion was the cumulated recurrence rate after steroid discontinuation. All of the treated patients (12 of 12) were successfully withdrawn from corticosteroids together with 10 of 15 in the control group. At the end of an 18-month follow-up, the cumulated relapse rate was 83% in the lymphapheresis group and 62% in the control group. This study is so far the best controlled trial targeting peripheral blood lymphocytes in IBD, and showed that lymphapheresis alone is not an effective treatment for patients with CD. The authors' conclusion was '*although there was a trend towards a diminished incidence of corticosteroid dependence, centrifugal lymphapheresis did not prevent the occurrence of early relapses*'.

In 1997, Ayabe and colleagues [64] reported an open pilot study of centrifugal leukocytapheresis in patients with corticosteroid-refractory active UC, with focus on efficacy and safety. Fourteen patients with severe UC were treated by centrifugal leukocytapheresis. Patients received one leukocytapheresis session per week for three consecutive weeks. In each session, leukocyte-rich fractions of the buffy coat layers were removed from 2000 – 2400 ml of peripheral blood taken via an antecubital vein. Approximately 180 ml of ACD-A (acid citrate dextrose; 3%w/v citrate) was used as an anticoagulant. Thirteen patients (92.9%) achieved clinical remission within 4 weeks after leukocytapheresis and remained in remission for 8 months on average without any additional corticosteroid therapy. Both colonoscopic and histological examinations confirmed the efficacy of the treatment in terms of reduction of severe inflammation of the affected colon. No significant side effects were observed throughout the therapy. In addition, the expression of L-selectin and very late antigen-4α, which are target molecules on leukocytes for interactions with endothelial cells, were downmodulated. The same group conducted a second pilot study in which 23 patients with severe corticosteroid-refractory UC received centrifugal leukocytapheresis [65]. Of 23 patients, 18 (78.3%) achieved clinical remission. The third study by this group [66] was a multi-centre, open-label trial involving 50 patients with active corticosteroid-refractory UC conducted in 14 medical institutions. Using the Haemonectics' Component Collection

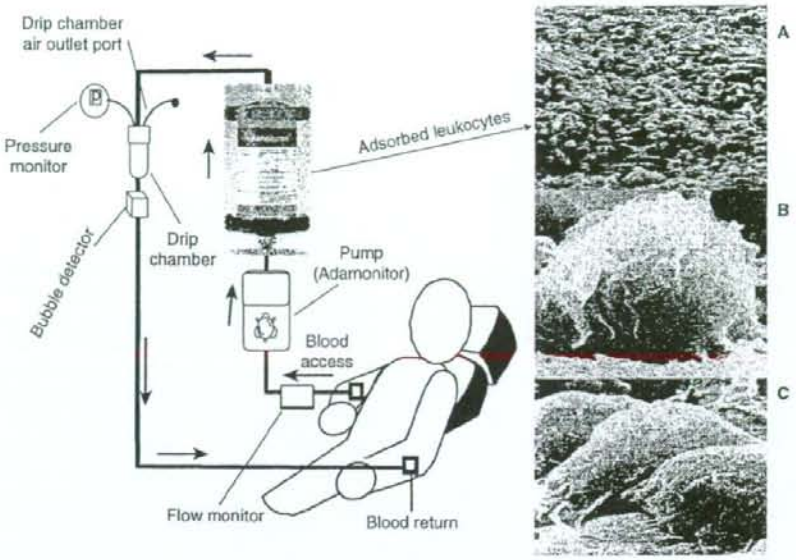

Figure 1. The operation outline for the Adacolumn selective leukocytapheresis system. The Adacolumn is filled with cellulose acetate beads as leukocytapheresis carriers that selectively adsorb granulocytes, monocytes/macrophages together with a small fraction of lymphocytes. These are the leukocytes that bear the FcγR and complement receptors. Arrows indicate the direction of blood flow during leukocytapheresis. On the right-hand side, scanning electron photomicrographs show adhesion of leukocytes to a carrier (**A**); the adsorbed leukocytes are primarily monocytes (**B**) and granulocytes (**C**). See text for comments on therapeutic effects and the mechanisms of actions.

System (Braintree, MA, USA), leukocytapheresis was performed once a week for five consecutive weeks, processing 2000 – 2400 ml of patients' blood per session as in their first study [64]. At the end of the study, stool frequency was decreased to < 4 times a day in 68.4% (26 of 38) of patients, and CRP level was normalised in 56.7% (17 of 30) of the patients. Colonoscopic remission was achieved in 57.7% (26 of 45) patients and histological improvement was noted in 54.1% (20 of 37) of patients tested. Following 5 – 6 leukocytapheresis sessions, improved disease activity was seen in 74% (37 of 50) of patients by general assessment criteria, but only 11 patients (22.0%) achieved clinical remission. It is not clear why this multi-centre study revealed lower rate of clinical remission as compared with the two earlier studies.

3.3 The Adacolumn selective leukocytapheresis system

The Adacolumn (Figure 1), which is featured in this section, is an example of a medical device that can selectively remove activated granulocytes and monocytes/macrophages together with small populations of lymphocytes [48,49,67,68]. The leukocytapheresis procedure is simple. Two large canulae are placed in the antecubital veins of the two arms (or other suitable sites) for direct blood access to the column, and return back to the patient. The blood flows into the column usually at 30 ml/minute and returns to the patient from the column outflow. The blood flow can be increased or decreased if necessary. Each session takes on average 60 min (can be prolonged or decreased if necessary). The column itself (Adacolumn) is filled with specially designed cellulose acetate beads of 2 mm in diameter as leukocytapheresis carriers. Pre- and postcolumn blood cell counts revealed that the carriers adsorb from the blood that passes through the column ~ 65% of granulocytes and 55% of monocytes/macrophages together with a smaller fraction of lymphocytes [49,68]. These are the leukocytes that bear the so-called FcγR and complement receptors [67,68]. These numerical data have been verified by scanning electron microscopy on the beads taken from the column following a leukocytapheresis session (Figure 1). The science and the therapeutic rationale behind the development of the Adacolumn have been broadly presented in two recent publications [49,68].

The first clinical trial of Adacolumn in patients with active UC was an open, multi-centre, controlled study conducted at 14 hospitals throughout Japan [69]. Of 105 eligible patients, 53 were in group I for Adacolumn and 52 in group II for conventional drug therapy. According to the study design, in group II, prednisolone (PSL) was increased to

63.1 ± 13.82 mg/day per patient at week 1 to promote remission compared with 23.5 ± 3.42 mg/day per patient in group I. In both groups, the PSL dose could be reduced if remission or improvements were observed. At week 7 (efficacy assessment time point), the average dose of PSL in group I was 14.2 ± 2.25 mg/day per patient versus 22.9 ± 2.07 mg/day in group II. Overall, 31 of 53 group I patients (58.5%) responded to Adacolumn leukocytapheresis therapy, 11 achieved remission, 20 had their symptoms improved and 22 did not respond. In group II, 23 of 52 patients (44.2%) responded to conventional drug therapy, 7 had remission, 16 had their symptoms improved and 29 did not respond. Likewise, in group I, a total of 8 adverse effects (flushing, light-headedness and so on) in 5 patients were reported, but no patient discontinued the apheresis treatment due to adverse reactions. In contrast, in group II, 40 adverse events in 24 patients were observed; 21 of 24 patients received medical treatment and 3 patients discontinued the treatment.

Subsequently, Hanai and colleagues [47,48] reported treating 41 patients with severe UC by using the Adacolumn to deplete their peripheral blood granulocytes and monocytes/macrophages. No additional drug therapy was initiated while their ongoing medications were tapered as symptoms improved. Pretreatment circulating neutrophil counts were very high, 9.3 ± 0.5 × 10^9/l, ~ 3 times the level seen in controls [48], and significant reductions were seen at week 12 of treatment, 4.9 ± 0.4 × 10^9/l. Haemoglobin at week 12 relative to baseline had increased by 25%, which might relate to the cessation of rectal bleeding following remission or improvements of clinical symptoms. Along with a fall in the patients' clinical activity index (CAI), disease activity index (DAI) and peripheral blood neutrophil counts, there was a comparable fall in CRP [48].

In one of the aforementioned studies by Hanai and colleagues [48], a total of 146 patients with active UC were given salicylates as the first-line medication. Ninety-two did not improve and were put on intensive corticosteroid (PSL) therapy. Among these 92 cases, 31 patients did not improve (steroid-refractory) and underwent Adacolumn leukocytapheresis therapy. These patients had a CAI of > 12, a DAI of > 10 and were treated twice-weekly for 2 – 3 consecutive weeks and then at one session per week. At the conclusion of five treatment sessions, ~ 50% of these steroid-refractory patients achieved remission or were significantly improved. At the conclusion of ten treatment sessions, the remission rate was 80%. The corticosteroid-refractory patients in this study represented a subgroup of patients with severe UC that are at significant risk of serious complications. Indeed, treatment failure after 5 – 10 days of intensive corticosteroids is often considered to be an indication for colectomy or exposure to CyA [15,16]. However, only 4 of the 31 (13%) patients underwent colectomy. At 12 months, 79% of patients had maintained their remission, which compares with a relapse rate of 60 – 80% for CyA [13], but, unlike CyA [17], Adacolumn was without major side effects. These initial response rates achieved by Hanai *et al.* have subsequently been reproduced both in Japan and in Europe [71-75]. One of these studies by Kanke *et al.* [71] reported that 90 min per Adacolumn session was significantly better than 60 min per session.

3.3.1 Adacolumn leukocytapheresis as first-line medication for steroid-naive patients

Of the 41 patients treated by Hanai *et al.* [48], 8 were steroid-naive at entry. All 8 (100%) went into a clinical remission with the Adacolumn treatment and remained steroid-naive during the treatment and follow-up time. Subsequently, Suzuki *et al.* [76,77] reported treating 20 steroid-naive patients with active UC by Adacolumn leukocytapheresis. These patients had moderate-to-severe UC; mean CAI was 8.8, range 5 – 17. At entry, all patients were on 5-ASA (1.5 – 2.25 g/day). Each patient was to receive up to a maximum of 10 Adacolumn sessions, at a frequency of 2 sessions per week. Efficacy was assessed 1 week after the last session. CAI fell to clinical remission levels (CAI < 4) in the majority of patients after 6 sessions; only 2 of the 20 patients required all 10 sessions. At post-treatment, the mean CAI was 3 (range 0 – 12; $p = 0.0001$), and 17 of 20 patients (85%) were in clinical remission. There were significant changes in total peripheral white blood cell counts (white blood cells × 10^9/l) (9.8 ± 1.0 [range 5.9 – 22.5] versus 7.0 ± 0.6 [range 3.5 – 15.3] for pre- and post-treatment, respectively; $p = 0.003$) together with decreases in CRP ($p = 0.0003$). During the Adacolumn leukocytapheresis therapy, two incidences of transient mild headache were reported. In both cases, the headache receded within 3 h without medication.

3.3.2 Adacolumn leukocytapheresis suppressed relapse in asymptomatic patients

Bjarnason and colleagues in London have evaluated the efficacy of Adacolumn leukocytapheresis to suppress IBD relapse in asymptomatic patients at high risk of experiencing a clinical relapse. A preliminary analysis was presented at the United European Gastroenterology Week, UEGW2005 [78]. This approach reflects a fundamental change in the philosophy of treating IBD. Instead of treating active disease, asymptomatic patients are identified solely on the basis of a very high faecal calprotectin concentration, a neutrophil selective protein that provides a quantitative measure of intestinal inflammatory activity [40-42]. The high calprotectin levels (> 250 µg/g) place them in a very high-risk group for relapse of their disease [40]. This multi-centre, prospective, randomised, controlled study randomly assigned patients to Adacolumn leukocytapheresis, undergoing five once-weekly out-patient sessions, or to unchanged treatment. Follow-up was monthly for 6 months for clinical relapse. Thirty patients who met the inclusion criteria were recruited from 244 potential subjects who underwent screening. In the Adacolumn group, 62% maintained their remission compared with 24% in the control group ($p < 0.04$). Life

table analysis demonstrated that mean survival in the Adacolumn group was 181 days, whereas in the control group it was 104 days ($p = 0.01$). It seems likely that the five weekly sessions of Adacolumn in such patients will have a significant effect and potentially avoid the morbidity associated with severe clinical relapses and the subsequent drug therapy.

3.3.3 *Adacolumn leukocytapheresis in the treatment of Crohn's disease*

The vast majority of studies with Adacolumn have been in patients with UC. However, there is evidence to suggest that Adacolumn leukocytapheresis is effective in patients with CD as well. The first study in CD was reported by Matsui and colleagues [79]. In that study, 7 patients with CD refractory to conventional medication, including nutritional therapy, each received five Adacolumn sessions. Five of seven patients achieved remission. In the follow-up study by Fukuda *et al.* [80], 21 patients with severe drug and nutritional therapy-refractory CD received five Adacolumn sessions each. Efficacy rate was 52.4% in these severe patients. More recently, Domenech *et al.* [75] reported treating 12 steroid-dependent patients with CD. The remission rate in this study was 70%, which is higher than in the study reported by Fukuda *et al.* [80]. Finally, Lofberg and colleagues [81] have reported treating 7 patients with CD who were refractory or had relapsed despite medication. Six had received infliximab, but without success. Adacolumn leukocytapheresis was performed at one session per week for 5 weeks. Efficacy was assessed at week 7 and 12 months. The median value of Crohn's disease activity index (CDAI) scores decreased from 290 at week 1 to 184 at week 7 ($p = 0.031$). At the 12-month follow-up, CDAI had decreased further to 129 ($p = 0.0016$).

3.3.4 *Immunomodulation associated with Adacolumn leukocytapheresis*

Although the aim of treatment with Adacolumn has been to remove excess and activated granulocytes and monocytes from the circulation, it has been difficult to explain why some patients continue to improve long after the treatment is concluded. In addition, the low relapse rate reported by Hanai *et al.* [48] cannot be fully explained by our current understanding of neutrophil function *per se*. Alternative mechanisms of actions have therefore been sought. Adacolumn is filled with cellulose acetate beads to which leukocytes that bear the FcγR and complement receptors adhere [67,68]. The adsorbed leukocytes release an array of active substances both toxic and non-toxic, but some anti-inflammatory as well. Most of these substances are of short half-life and may not reach the patients' circulation in significant amounts. Several investigators have carried out analyses on blood samples taken from the Adacolumn inflow and outflow (blood return line to patients) during leukocytapheresis. Both Hanai *et al.* [82] and Suzuki *et al.* [76] found a significant increase in blood levels of soluble TNF-α receptors I and II. Soluble TNF receptors are reported to neutralise TNF without invoking TNF-like actions [83]. Similarly, several studies report a marked decrease in the capacity of peripheral blood leukocytes to release inflammatory cytokines, including TNF-α, IL-1β, IL-6 and IL-8, following Adacolumn leukocytapheresis [47,49,84,85]. The procedure appears to produce a similar effect on leukocyte trafficking receptors. Thus, the expressions of both L-selectin [47,49,84,85] and the chemokine receptor CXCR3 [67,85] were dramatically reduced and were sustained well beyond the last leukocytapheresis session, whereas the expression of the leukocyte integrin Mac-1 (CD11b/CD18) was upregulated [49,68]. These actions should suppress leukocyte extravasation. Furthermore, *in vitro* studies by Takeda *et al.* [87] show that incubation of human blood with the Adacolumn carriers for 60 min results in the release of significant amounts of IL-1 receptor antagonist (IL-1ra) and hepatocyte growth factor (HGF) in the incubation medium. In contrast, the authors did not detect significant amounts of TNF-α or IL-1β in the same test samples. IL-1ra has an essential role in the control of inflammation in the intestinal mucosa, while HGF is known to promote mucosal epithelial cell regeneration, which is an essential step in ulcer healing [87,88]. Finally, a study by Kashiwagi *et al.* [85] shows that the proportion of naive or immature neutrophils ($CD10^-$ neutrophils) in the circulation significantly increases during Adacolumn leukocytapheresis. Figure 2 summarises the immunomodulatory actions of Adacolumn leukocytapheresis. At present, an investigation into the effects of Adacolumn leukocytapheresis on Toll-like receptors and $CD14^+CD16^+HLA\text{-}DR^{++}$ monocytes (pro-inflammatory) is in progress.

3.4 The Cellsorba leukocyte removal system

The Cellsorba leukocyte removal filter column (Figure 3) was developed by Asahi Kasei Medical in Japan and has been comprehensively described by Sawada *et al.* [89]. This system is also a direct blood perfusion device. Blood access is from the antecubital vein in one arm and returns via the antecubital vein in the contralateral arm. Alternative access sites may be used if necessary. Cellsorba uses a filter consisting of polyester non-woven fabric that non-selectively removes ~ 13.0×10^9 leukocytes and 5.2×10^{11} platelets from the circulating blood during one treatment session [90]. The column is capable of removing almost 100% of neutrophils and monocytes, including macrophages, and 30 – 60% of lymphocytes when measured between the inlet and the outlet of the column [89].

The first major application of Cellsorba for the treatment of UC was performed in 1995 by Sawada *et al.* [91]. Cellsorba leukocytapheresis was administered 5 times at 1-week intervals for 5 consecutive weeks during intensive therapy, and 5 times at ~ 1-month intervals for 5 months during maintenance therapy to 13 patients with IBD (8 UC and 5 CD patients). Improved clinical response during the intensive therapy was seen in 11 of 13 patients (84.6%), 6 of

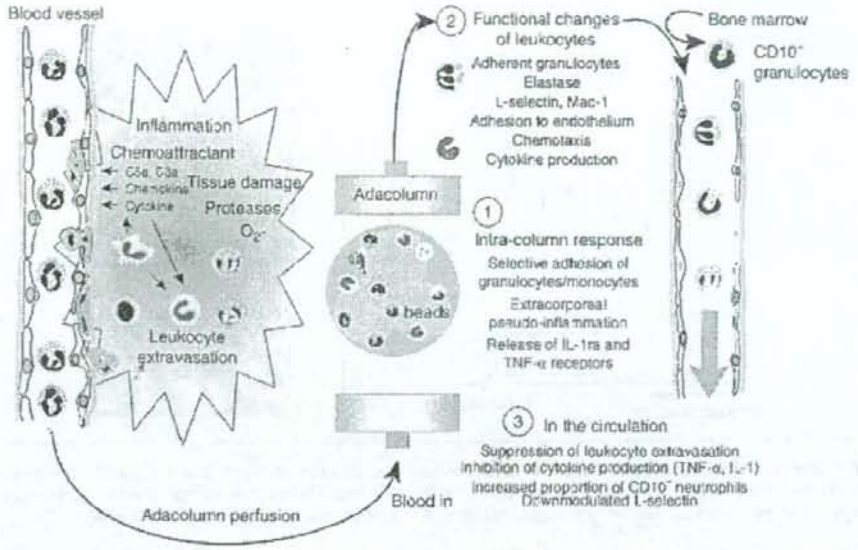

Figure 2. Summary of the anti-inflammatory/immunomodulatory actions of the Adacolumn selective leukocytapheresis. The scheme tentatively shows that in patients with IBD, leukocytes, in particular granulocytes, are elevated with activation behaviour, adhere to the vascular wall and extravasate to the mucosal tissue where they promote inflammation and tissue injury. During passage of blood through the Adacolumn, most of the activated leukocytes adhere to the carriers, and those that pass through are downmodulated. Additional immunomodulatory or anti-inflammatory actions are depicted in the scheme. Further comments and analyses are presented in the text.

IBD: Inflammatory bowel disease; IL-1ra: Interleukin-1 receptor antagonist.

8 UC patients (75.0%) and 5 of 5 CD patients (100%). The remission was maintained in 8 of 13 patients (61.5%) during the maintenance therapy.

A nationwide multi-centre trial was carried out in Japan to assess the efficacy and safety of Cellsorba versus corticosteroid therapy in patients with active UC refractory to conventional medication [89]. This was a controlled multi-centre study with randomised assignment of 76 patients with UC to two groups. The 39 patients in the Cellsorba group received weekly leukocytapheresis for 5 consecutive weeks as an intensive therapy, which was added to the ongoing drug therapy, while steroids were maintained, but not increased. Leukocytapheresis was gradually reduced to one session every 4 weeks as maintenance therapy. In the high-dose PSL group ($n = 37$), PSL was added or increased to 30 – 40 mg/day for moderately severe patients and to 60 – 80 mg/day for severe patients, and was then gradually tapered. The Cellsorba group showed a significantly higher efficacy compared with PSL (74 versus 38%; $p = 0.005$), and a lower incidence of side effects (24 versus 68%; $p < 0.001$).

Furthermore, Sawada and colleagues [93] recently investigated the efficacy of Cellsorba leukocytapheresis in a multi-centre trial using active and sham devices in a double-blind study with focus on assessing the placebo effect of extracorporeal circulation. Twenty-five patients with active UC of severe or moderately severe intensity were assigned to the active treatment or sham treatment. Six patients who did not meet the inclusion criteria were excluded at screening and 19 (10 in the active group and 9 in the sham group) were included. Cellsorba leukocytapheresis was performed once-weekly for 5 weeks, followed by two additional sessions during the following 4 weeks at 2-week intervals. Corticosteroids and other medications were continued at the same dosage for 4 weeks. CAI showed that the active group achieved a significantly greater improvement (80%, 8 of 10 patients) compared with the sham apheresis group (33%, 3 of 9 patients; $p < 0.05$). Although there was a significant advantage in favour of the active treatment, the total number of patients was rather small in this study. Likewise, patients had active UC refractory to conventional drug therapy, and most of them were receiving concomitant corticosteroids. A similar study with a large cohort of patients with strict control of their concomitant medications is warranted to confirm the results of this study.

Sawada *et al.* [93] further reported the efficacy and safety of Cellsorba in treating patients with severe or fulminant UC or toxic megacolon. Six patients were included and Cellsorba leukocytapheresis was performed 3 times per week for

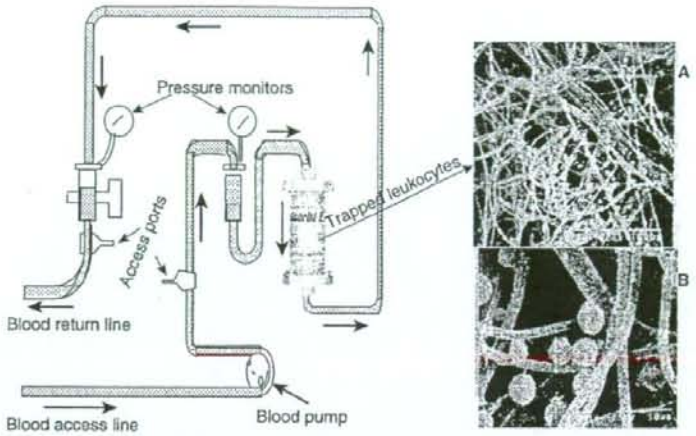

Figure 3. The operation outline for the Cellsorba filter column. The direction of blood flow indicated by the arrows shows that whereas blood inlet for the Adacolumn is from the lower port, for the Cellsorba it is from the top port. On the right hand side, scanning photomicrographs show leukocytes trapped in the Cellsorba filter (**B** is a higher magnification view of part of **A**).

2 weeks, followed by 4 further sessions in the following 4 weeks. Four of six patients improved and achieved remission; the remaining two patients had to undergo colectomy although their symptoms had been reduced by Cellsorba. Further larger studies are essential to fully assess the efficacy of Cellsorba in this clinical setting.

In earlier studies, Sawada *et al.* [94] and Yamaji *et al.* [95] reported fluctuations in the leukocyte count in the peripheral blood during Cellsorba leukocytapheresis. The count fell to 20 – 40% of the baseline level at 20 – 30 min after the start of each session. Cellsorba itself had a sustained removal performance in excess of 90% of the baseline value for the circulating blood leukocytes [96]. Therefore, it appears that leukocytes from the marginal pools including the bone marrow, spleen and vessel walls compensate for the lost leukocytes during a session. This finding led to the concept and investigation of Cellsorba as a therapy for UC. It is believed that activated peripheral blood leukocytes serve as 'primed reserve cells', which might include leukocytes that originally have been activated in the lymph nodes. During active IBD, this pool provides a sustainable supply of activated leukocytes for infiltration into the colonic mucosa. By depleting this pool, leukocytapheresis can in effect influence the source of activated leukocytes in the marginal pools as well. Indeed, infiltration of activated leukocytes into the intestinal mucosa has been considered as a major factor in the aetiology of IBD [1,40,45].

Perhaps a word of caution is warranted in relation to any leukocytapheresis procedure that depletes lymphocytes. Thus, a recent study by King and colleagues [97] indicates that the state of lymphopenia may promote the development of autoimmunity. Likewise, it is known that human diseases of autoimmune aetiology often present with lymphopenia [98]. These findings led to the hypothesis that transient lymphopenia during Cellsorba leukocytapheresis potentially may trigger homeostatic T cell expansion-associated autoimmune disease. Accordingly, if a patient with UC develops autoimmune disease following exposure to Cellsorba, the transient lymphopenia can be suspected to have predisposed to the condition.

3.4.1 *Immunomodulation associated with Cellsorba leukocytapheresis*

In the first major study by Sawada *et al.* [91] in patients with IBD, flow cytometry revealed that patients who improved had a higher percentage of HLA-DR$^+$, HLA-DR$^+$CD3$^+$ and HLA-DR$^+$CD8$^+$ cells (pro-inflammatory) at entry. The levels of these cells, CRP and erythrocyte sedimentation rate (ESR) decreased to within the normal range by the end of therapy. In contrast, patients who showed poor response to leukocytapheresis, CRP and ESR did not change. Cellsorba leukocytapheresis also affected cytokine production [94,95]. The levels of pro-inflammatory cytokines TNF-α, IL-1β, IL-2, IL-8 and IFN-γ were high in responders at entry and were significantly reduced by leukocytapheresis [96]. These cytokines are mainly secreted by activated peripheral blood leukocytes [43,44]. In addition, the level of IL-4, an immunoregulatory cytokine, increased after leukocytapheresis [99]. These observations indicate that Cellsorba leukocytapheresis is associated with changes in cytokine profile in the disease state, returning to normality via inhibition of several pro-inflammatory cytokines and by stimulation of an immunoregulatory cytokine.

Andoh *et al.* [100] recently evaluated the alterations in circulating T cell subsets after Cellsorba leukocytapheresis

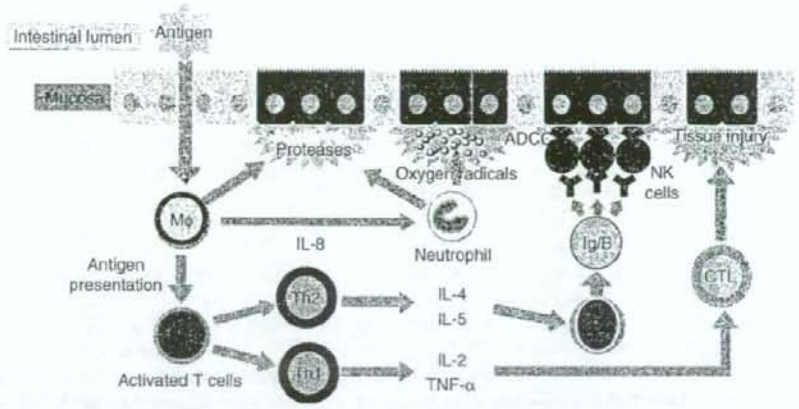

Figure 4. Summary of the anti-inflammatory/immunomodulatory actions of leukocytapheresis with the Cellsorba filter column. The scheme tentatively shows that monocytes/Mφs can be activated by dietary antigens (more likely in CD than in UC), bearing in mind that this is not the only pathway for leukocyte activation. After receiving the antigen, monocytes interact with T lymphocytes, mucosal tissue, as well as with neutrophils via IL-8. The net effect of this step includes appearance of Th1, Th2, neutrophil-derived proteases and active oxygen derivatives (mucosal tissue damage), as well as release of IL-4, IL-5, IL-2 and TNF-α. The subsequent step includes B lymphocyte activation, interaction with Ig and activation of NK cells via Ig-based antibodies. The damage to the mucosal tissue is depicted to be via ADCC. The impact of TNF and IL-2 on the mucosal tissue is shown to be via CTLs.
ADCC: Antibody-dependent cell-mediated cytotoxicity; CD: Crohn's disease; CTL: Cytotoxic T lymphocyte; Mφ: Macrophage; NK: Natural killer; UC: Ulcerative colitis.

therapy in 18 patients with UC. Peripheral blood was obtained within 5 min before and 5 min after leukocytapheresis therapy. The average number of lymphocytes, T and B cells were significantly decreased after Cellsorba ($p < 0.01$). The number of $CD4^+$ and $CD8^+$ T cells were also significantly decreased ($p < 0.01$), but the $CD4^+/CD8^+$ ratio did not change. In addition, the number of $CD45RO^+CD4^+$ memory T cells significantly decreased. Using an intracellular cytokine staining method, it was shown that IFN-γ-expressing (Th1) cells had significantly decreased after leukocytapheresis, whereas there was no significant change in the number of IL-4-expressing (Th2) cells. The Th1/Th2 ratio was significantly decreased after Cellsorba. Figure 4 summarises the immunomodulatory phenomenon associated with Cellsorba leukocytapheresis.

4. The science behind leukocytapheresis as a natural biological therapy

IBD may be viewed as the consequence of an overexuberant immune activity triggered and maintained by inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-12, and so on [18,19]. This might be a major factor for IBD showing poor response to conventional drug therapy [1,8,13,48]. Indeed, administrations of these agents, often at high doses over long periods of time, can produce additional complications [1,7,8,17,101]. Furthermore, it is true to say that for decades drug therapy of IBD has been empirical rather than based on sound understanding of the disease mechanisms (poorly understood aetiology). The current view is that treatment interventions targeted at inflammatory mediators (such as biologicals) should be more effective and produce minimal side effects. Accordingly, the present era of antibody-based therapy targeting specific cytokines, chemokines and adhesion molecules represents some progress, albeit only truly effective in the minority of treated patients [13,102,103]. Cytokines in particular represent the best validated therapeutic targets, and it is logical to view cytokines as major causes of persistent intestinal inflammation. However, major sources of inflammatory cytokines include lymphocytes, monocytes/macrophages and granulocytes [43,44], which in IBD are elevated [48,49] with activation behaviour [45], prolonged survival time [52], and are found in vast numbers within the inflamed intestinal mucosa [1,40]. Granulocyte infiltration into the mucosal tissue can indeed predict relapse of both UC and CD [40,78]. This indicates that during quiescent IBD, activated leukocytes infiltrate the intestinal mucosa and have a major role in mucosal inflammation, injury and IBD relapse [1,40,45,78]. Indeed, leukocyte activation and prolonged survival is a feature of persistent inflammation, and neutrophil-mediated mucosal damage has been shown to be associated with the development of IBD [40,45,50,51]. Accordingly, selective depletion of activated peripheral blood leukocytes by centrifugation, Adacolumn or Cellsorba has been associated with dramatic efficacy and a marked reduction of inflammatory cytokines produced by leukocytes [47,48,68,94,95].