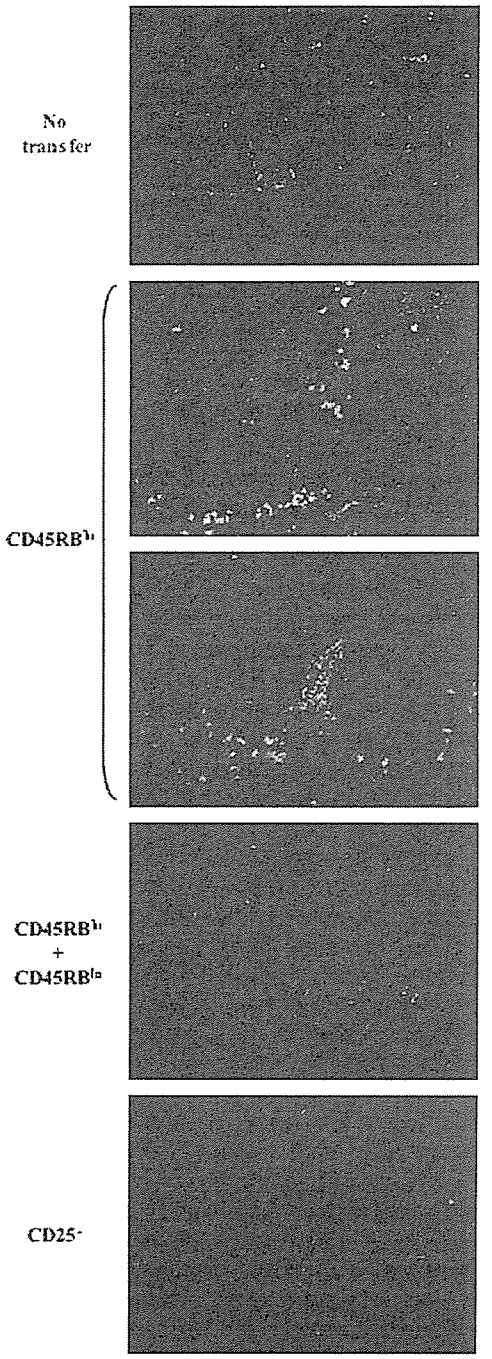

FIGURE 5. Colonic immunoglobulin-producing cells in mice reconstituted with $CD4^+CD45RB^{high}$ T cells 7 wk after transfer. The colonic sections from mice with no transfer, or reconstituted with $CD4^+CD45RB^{high}$ cells, $CD4^+CD45RB^{high}$ cells + $CD4^+CD45RB^{low}$ cells, or $CD4^+CD25^-$ cells 7 wk after transfer were stained IgG (red), IgM (green), and IgA (blue) by fluorescent immunohistochemistry. Original magnification ×200. These data were representative in 3 experiments.

answer lies in the fact that we isolated only the brightest 20% of $CD4^+CD45RB^{high}$ T cells from MACS-isolated $CD4^+$ T cells to avoid some contamination of $CD4^+CD45RB^{intermidiate}$ T cells, whereas Laroux and associates isolated the brightest 40% of $CD4^+CD45RB^{high}$ T cells. Although SCID mice that transfer with the brightest 40% of $CD4^+CD45RB^{high}$ T cells develop wasting disease and colitis,[23] it is possible that the brightest 20% to 40% of $CD4^+CD45RB^{intermidiate}$ T cells may suppress the development of colitis induced by the brightest 20% of $CD4^+CD45RB^{high}$ T cells.

It should also be noted that differences may be attributable to the use of immunodeficient recipients obtained from different animal facilities or to the environmental factors that drive the inflammatory response, because the nature of the endogenous microbiota in immunodeficient recipients may be an important and variable factor when comparing results between different laboratories. Indeed, *Helicobacter hepaticus* infection in an animal facility should be a critical point,[36] especially in the case of establishing a new animal colitis model. Although we performed the specific polymerase chain reaction for *Helicobacter* sp (including *H. hepaticus*) using stool samples from recipient mice in our facility, all of the data was negative for all *Helicobacter* species including *H. hepaticus* (data not shown). In light of this issue, we finally confirmed that nude mice transferred $CD4^+CD45RB^{high}$ T cells and bred in another independent animal facility at Tokyo Medical and Dental University also developed severe colitis (data not shown).

A second and related question that requires explanation is why lamina propria $CD4^+$ T cells from colitic nude mice induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells produced a large amount of T_H2 cytokines, IL-4 and IL-5, as well as T_H1 cytokines, although the classic colitis model induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells into SCID/Rag-1,2 mice contains a feature of T_H1- but not T_H2-type immune responses. One possibility relates to the markedly infiltrating lamina propria B cells in colitic nude mice. Because we previously demonstrated that infiltrating lamina propria $CD4^+$ T cells from SCID mice transferred with $CD4^+CD45RB^{high}$ T cells expressed ICOS, which preferentially induces T_H2 responses,[28] it may be possible that inducible co-stimulator ligand (B7-H1, B7h)-expressing B cells,[37,38] as with antigen-presenting cells, stimulate infiltrating $CD4^+$ T cells to produce T_H2 cytokines in the inflamed lamina propria in this nude colitis model but not in the SCID colitis model because of the lack of B cells. 2,4,6-Trinitrobenzene sulfonic acid (TNBS) colitis in mice, which is believed to be a model of Crohn's disease, was initially characterized by a T_H1-type inflammation that was mediated by IFN-γ driven by IL-12-producing antigen-presenting cells using SJL/J mice,[39] but studies of TNBS colitis in BALB/c mice or IFN-$\gamma^{-/-}$ mice showed that T_H2-type $CD4^+$ T cells also played a major role in IBD, accompanied by hypertrophy of colonic patches with distinct B and T cell zones

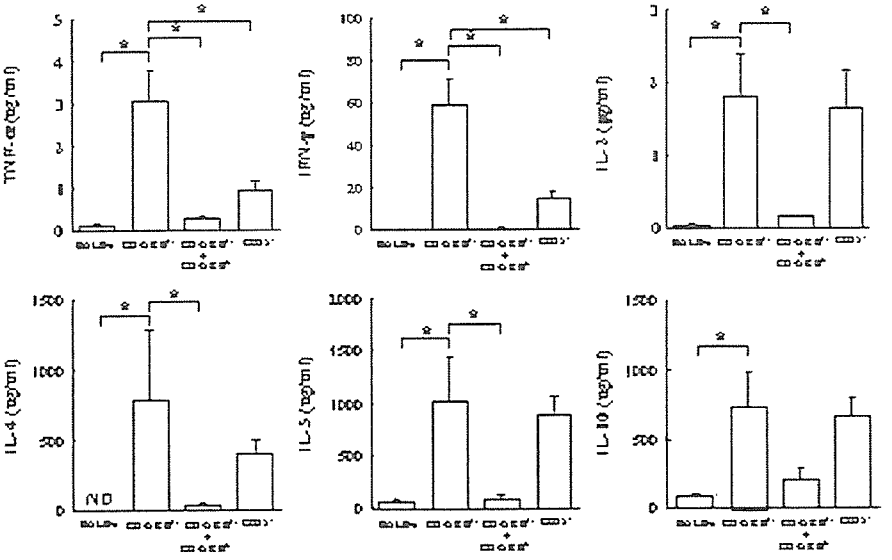

FIGURE 6. Cytokine production by lamina propria $CD4^+$ T cells. Lamina propria $CD4^+$ T cells were isolated from mice with no transfer, or reconstituted with $CD4^+CD45RB^{high}$ cells, $CD4^+CD45RB^{high}$ cells + $CD4^+CD45RB^{low}$ cells, or $CD4^+CD25^-$ cells 7 wk after transfer, and stimulated with anti-CD3 and anti-CD28 mAbs for 48 hours. IFN-γ, IL-2, IL-4, and IL-5 concentrations in culture supernatants were measured by CBA. IL-10 concentration was measured by a specific ELISA. Data are represented as the mean ± SEM of mice in each group. $^*P < .05$.

resembling Peyer's patches.[40] Furthermore, this dichotomy between T_H1- and T_H2-type mucosal inflammation is seen in human IBD. The histopathological features of Crohn's disease resemble those of experimental T_H1 cell-mediated colitis, whereas those of ulcerative colitis most resemble experimental T_H2-type mediated colitis[41]; however, it does not seem too simple to understand IBD by separating T_H1- and T_H2-type diseases because it is also well known the cytokine patterns for IBD patients are more complicated, and both T_H1- and T_H2-type immune responses should appear in individuals with IBD. To underscore true IBD pathogenesis, both T_H1- and T_H2-type immune responses in the present model may be valuable for future studies of intestinal inflammation.

Finally, there is an implication for the evidence that the co-transfer of $CD4^+CD45RB^{low}$ T cells with $CD4^+CD45RB^{high}$ T cells ameliorated the infiltration of B cells as well as $CD4^+$ T cells in intestinal lamina propria in recipient mice, which is commonly observed in recipient mice transferred with only $CD4^+CD45RB^{high}$ T cells. The present report is the first to show that $CD4^+CD45RB^{low}$ T_R cells, which contain $CD4^+CD25^+$ T_R cells and other $CD4^+CD25^-$ T_R cells, mediate the suppression of B cell expansion in vivo. Indeed, it seems likely that the expansion of lamina propria B cells in colitic mice is induced by their interaction with activated lamina propria $CD4^+$ T cells because of, for example, the interaction between CD40-expressing B cells and CD40L-expressing T cells. Therefore, $CD4^+CD45RB^{low}$ T_R cells directly suppress the expansion of activated $CD4^+$ T cells; thereafter, the expansion of B cells is indirectly suppressed followed by the suppression of these $CD4^+$ T cells. Additional studies are needed to address this issue.

In conclusion, the present report establishes a new model of murine chronic colitis. The advantage of the present colitis model induced by the adoptive transfer of $CD4^+CD45RB^{high}$ T cells into nude mice is that one can investigate the roles of T_H2-type cells and B cells in a T_R cell–depleted condition. The disadvantage of a classic SCID-transfer model is the absence of normal B lymphocytes, which themselves should have pathological activity.

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REFERENCES

1. Powrie F, Leach MW. Control of intestinal inflammation by regulatory T cells. *Immunol Res.* 2001;182:190–200.
2. Blumberg RS, Saubermann LJ, Strober W. Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. *Curr Opin Immunol.* 1999;11:648–656.
3. Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. *Annu Rev Immunol.* 2002;20:495–549.
4. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. *Nature.* 1996;383:787–793.
5. O'Garra A. Cytokines induce the development of functionally heterogenous T helper cell subsets. *Immunity.* 1998;8:275–283.
6. Gershon RK. A disquisition on suppressor T cells. *Transplant Rev.* 1975;26:170–185.
7. Sakaguchi S, Sakaguchi N, Shimizu J, et al. Immunologic tolerance maintained by $CD25^+CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev.* 2001;182:18–32.
8. Curotto de Lafaille MA, Lafaille JJ. $CD4^+$ regulatory T cells in autoimmunity and allergy. *Curr Opin Immunol.* 2002;14:771–778.
9. Shevach EM. $CD4^+CD25^+$ suppressor T cells: more questions than answers. *Nat Rev Immunol.* 2002;2:389–400.
10. Sakaguchi S, Sakaguchi N, Asano M, et al. Immunological self-tolerance maintained by acute T cells expressing IL-2 receptor alpha chains (CD25).

Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol.* 1995;155:1151–1164.
11. Fehervari Z, Sakaguchi S. $CD4^+$ Tregs and immune control. *J Clin Invest.* 2004;114:1209–1217.
12. Fehervari Z, Sakaguchi S. Development and function of $CD25^+CD4^+$ regulatory T cells. *Curr Opin Immunol.* 2004;16:203–208.
13. Battaglia M, Blazar BR, Roncarolo MG. The puzzling world.of murine T regulatory cells. *Microbes Infect.* 2002;4:559–566.
14. Sakaguchi S. Regulatory T cells: key controllers of immunological self-tolerance. *Cell.* 2000;101:455–458.
15. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of $CD4^+CD25^+$ regulatory cells that control intestinal inflammation. *J Exp Med.* 2000;193:295–302.
16. Takahashi T, Tagami T, Yamazaki S, et al. Immunologic self-tolerance maintained by $CD4^+CD25^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med.* 2000;193:303–310.
17. Graca L, Thompson S, Lin CY, et al. Both $CD4^+CD25^+$ and $CD4^+CD25^-$ regulatory cells mediate dominant transplantation tolerance. *J Immunol.* 2002;168:5558–5565.
18. Van de Keere F, Tonegawa S. $CD4^+$ T cells prevent spontaneous experimental autoimmune encephalomyelitis in anti-myelin basic protein T cell receptor transgenic mice. *J Exp Med.* 1998;188:1875–1882.
19. Apostolou I, Sarukhan A, Klein L, et al. Origin of regulatory T cells with known specificity for antigen. *Nat Immunol.* 2002;3:756–763.
20. Lehmann J, Huehn J, de la Rosa M, et al. Expression of the integrin $\alpha_E\beta_7$ identifies unique subsets of $CD25^+$ as well as $CD25^-$ regulatory T cells. *Proc Natl Acad Sci U S A.* 2002;99:13031–13036.
21. Stephens LA, Mason D. CD25 is a marker for $CD4^+$ thymocytes that prevent autoimmune diseases in rats, but peripheral T cells with this function are found in both $CD25^-$ and $CD25^-$ subpopulations. *J Immunol.* 2000;165:3105–3110.
22. Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, et al. $CD25^+CD4^+$ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J Immunol.* 2001;166:3008–3018.
23. Powrie F, Leach MW, Mauze S, et al. Phenotypically distinct subsets of $CD4^+$ T cells induce or protect from chronic intestinal inflammation in C.B-17 scid mice. *Int Immunol.* 1993;5:1461–1471.
24. Powrie F, Leach MW, Mauze S, et al. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with $CD45RB^{hi}$ $CD4^+$ T cells. *Immunity.* 1994;1:553–562.
25. Claesson MH, Bregenholt S, Bonhagen K, et al. Colitis-inducing potency of $CD4^+$ T cells in immunodeficient, adoptive hosts depends on their state of activation, IL-12 responsiveness, and CD45RB surface phenotype. *J Immunol.* 1999;162:3702–3710.
26. Liu Z, Geboes K, Heremans H, et al. Role of interleukin-12 in the induction of mucosal inflammation and abrogation of regulatory T cell function in chronic experimental colitis. *Eur J Immunol.* 2001;31:1550–1560.
27. Sakaguchi S, Sakaguchi N, Asano M, et al. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol.* 1995;155:1151–1164.
28. Totsuka T, Kanai T, Iiyama R, et al. Ameliorating effect of anti-inducible costimulator monoclonal antibody in a murine model of chronic colitis. *Gastroenterology.* 2003;124:410–421.
29. Liu Z, Geboes K, Colpaert S, et al. Prevention of experimental colitis in SCID mice reconstituted with $CD45RB^{high}CD4^+$ T cells by blocking the CD40-CD154 interactions. *J Immunol.* 2000;164:6005–6014.
30. Kanai T, Watanabe M, Okazawa A, et al. Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease. *Gastroenterology.* 2001;121:875–888.
31. Kawamura T, Kanai T, Dohi T, et al. Ectopic CD40 ligand expression on B cells triggers intestinal inflammation. *J Immunol.* 2004;172:6388–6397.
32. Uraushihara K, Kanai T, Ko K, et al. Regulation of murine inflammatory bowel disease by $CD25^+$ and $CD25^-$ $CD4^+$ glucocorticoid-induced TNF receptor family-related gene$^+$ regulatory T cells. *J Immunol.* 2003;171:708–716.
33. Fagarasan S, Honjo T. T-independent immune response: new aspects of B cell biology. *Science.* 2000;6:89–92.
34. Oida T, Zhang X, Goto M, et al. $CD4^+CD25^-$ T cells that express latency-associated peptide on the surface suppress $CD4^+CD45RB^{high}$-induced colitis by a TGF-beta-dependent mechanism. *J Immunol.* 2003;170:2516–2522.
35. Laroux FS, Norris HH, Houghton J, et al. Regulation of chronic colitis in athymic nu/nu (nude) mice. *Int Immunol.* 2004;16:77–89.
36. Kullberg MC, Ward JM, Gorelick PL, et al. Helicobacter hepaticus triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12 and gamma interferon-dependent mechanism. *Infect Immun.* 1998;66:5157–5166.
37. Hutloff A, Dittrich AM, Beier KC, et al. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature.* 1999;397:263–266.
38. Coyle AJ, Gutierrez-Romos JC. The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. *Nat Immunol.* 2001;2:203–209.
39. Neurath MF, Fuss I, Kelsall BL, et al. Antibodies to interleukin 12 abrogate established experimental colitis in mice. *J Exp Med.* 1995;182:281–290.
40. Dohi T, Fujihashi K, Rennert PD, et al. Hapten-induced colitis is associated with colonic patch hypertrophy and T helper cell 2-type responses. *J Exp Med.* 1999;189:1169–1180.
41. Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol.* 2003;3:521–533.

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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

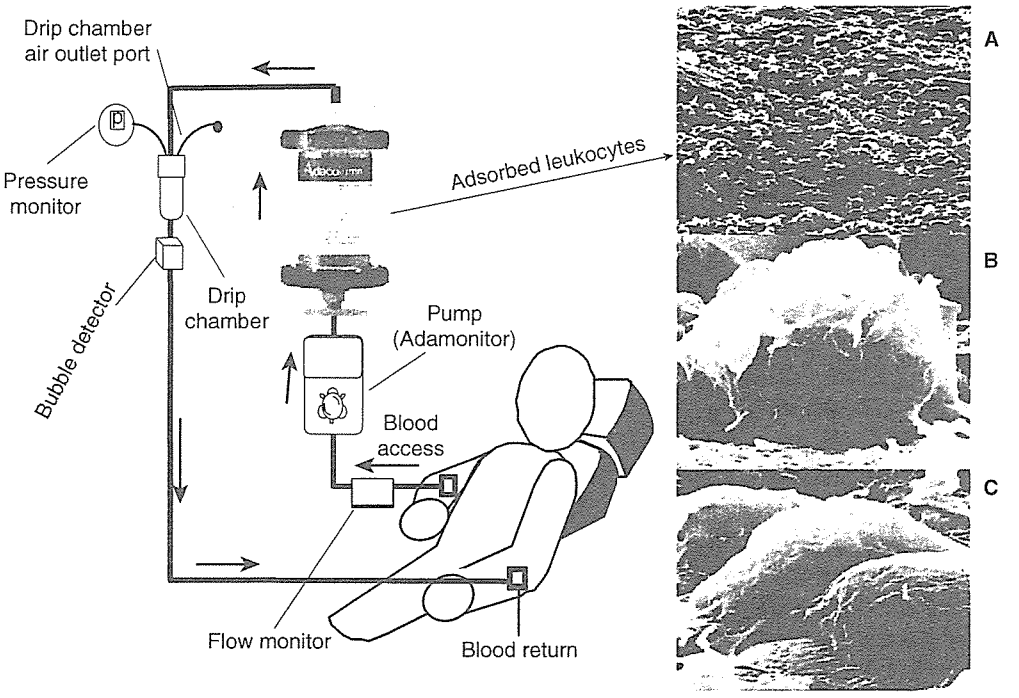

Figure 1. The operation outline for the Adacolumn selective leukocytapheresis system. The Adacolumn is filled with cellulose acetate beads as leukocytapheresis carriers that selectively adsorb granulocytes, monocytes/macrophages together with a small fraction of lymphocytes. These are the leukocytes 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

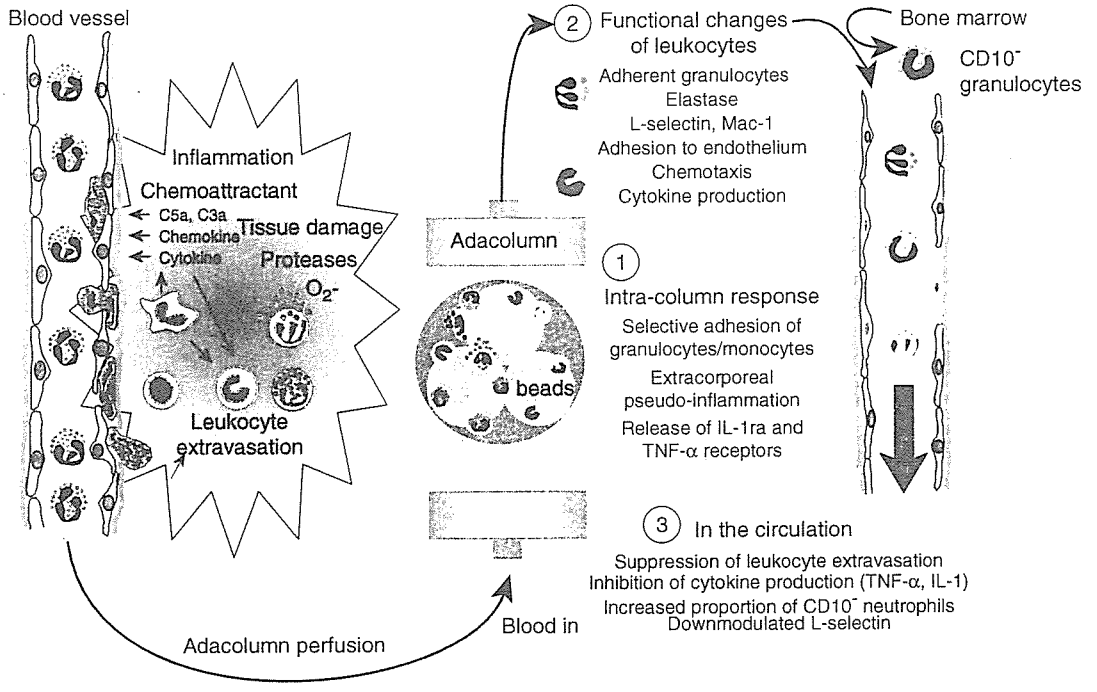

Figure 2. Summary of the anti-inflammatory/immunomodulatory actions of the Adacolumn selective leukocytapheresis. The scheme tentatively shows that in patients with IBD, leukocytes, in particular granulocytes, are elevated with activation behaviour, adhere to the vascular wall and extravasate to the 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

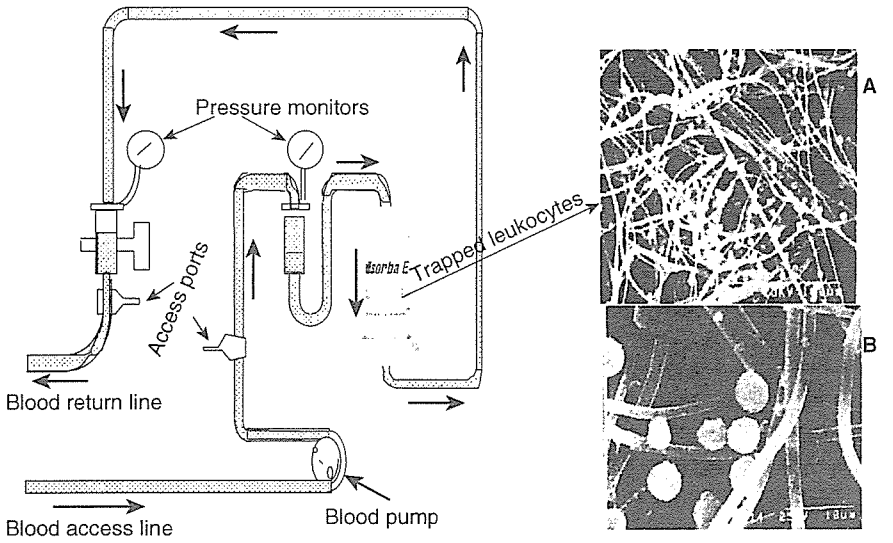

Figure 3. The operation outline for the Cellsorba filter column. The direction of blood flow indicated by the arrows shows that whereas blood inlet for the Adacolumn is from the lower port, for the Cellsorba it is from the top port. On the right hand side, scanning photomicrographs show leukocytes trapped 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

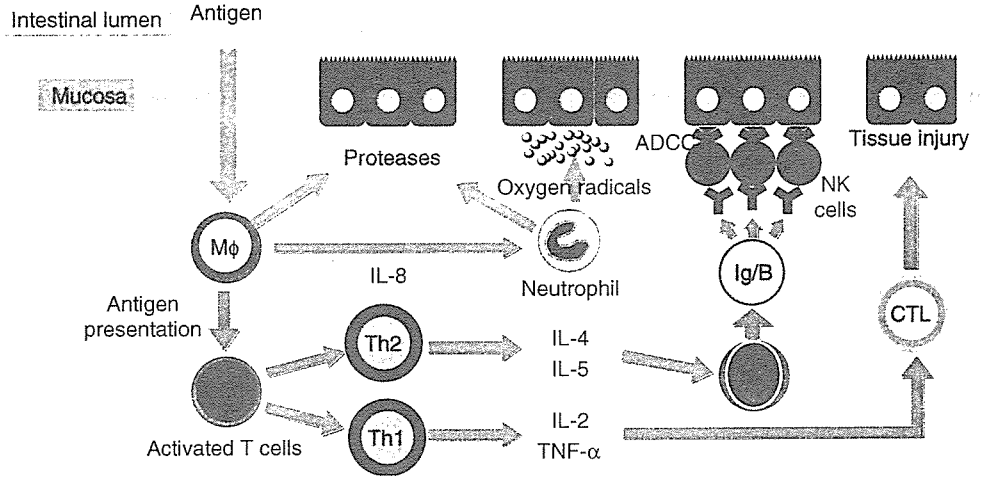

Figure 4. Summary of the anti-inflammatory/immunomodulatory actions of leukocytapheresis with the Cellsorba filter column. The scheme tentatively shows that monocytes/Mφs can be activated by dietary antigens (more likely in CD than in UC), bearing in mind that this is not the only pathway for leukocyte 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].

Naive T cells preferentially recirculate between blood and secondary lymphoid tissues, entering lymph nodes from the blood by crossing high endothelial venules. After encountering the activated dendritic cells undergoing antigen presentation in the mesenteric lymph nodes, the naive T cells become activated, proliferate and differentiate into activated effector T cells. These effector T cells then acquire the gut-homing receptors, integrin $\alpha_4\beta_7$. Thus, colitogenic effector T cells, unlike naive T cells, can migrate efficiently to sites of inflammation [104], subsequently entering afferent lymphatic vessels and travelling to local lymph nodes [104-108]. In parabiotic mouse models, endogenous memory T cells in most peripheral tissues react in equilibrium with migrating blood-borne donor T cells within a week [109], suggesting that there is rapid recirculation of T cells in peripheral tissues. These recent understandings suggest that selective removal of these colitogenic activated effector T cells by leukocytapheresis should reduce the cellular components of IBD.

Factors believed to contribute to granulocyte activation and its increased survival time in IBD include inflammatory cytokines [110] and, paradoxically, corticosteroids [53], which are given to most patients with active IBD. Indeed, corticosteroids are known to reactivate quiescent UC and may precipitate the first UC attack [1]. These again in part explain why IBD shows poor response to drug therapy, and strengthen the assumption that activated leukocytes are involved in the initiation, exacerbation and perpetuation of IBD. Activated leukocytes and their cytokines, together with corticosteroids, might constitute a vicious cycle whereby leukocytes produce cytokines that then support the former in addition to promoting inflammation, and both are enhanced by corticosteroids. Hence, peripheral blood leukocytes should be the most appropriate target of therapy in IBD. Based on this thinking, leukocytapheresis should be equivalent to removing inflammatory cytokines at a point upstream of inflammatory drive.

To continue the above arguments, it could be said that the effectiveness of certain cytokine antagonists, such as infliximab, might be viewed as a solid evidence for the involvement of TNF-α (in this case) in the immunopathogenesis of IBD. Hence, given that major sources of TNF-α (and other inflammatory cytokines) include activated leukocytes, depleting these cells from patients' body should represent biological therapy, a natural medication that is safe and, as it removes the effector cells from the body rather than adding them, is not likely to cause refractoriness. Alternatively, leukocytapheresis as an adjunct to conventional medication should spare most patients with active IBD from additional drug therapy and reduce the number of patients who require colectomy.

The word apheresis means to take away, to purify. In fact, today's selective removal of activated peripheral blood leukocytes to achieve a therapeutic effect by apheresis is reminiscent of the rather crude practice of bloodletting (phlebotomy) and its therapeutic application at the time of Hippocrates (460 – 377 BC) in Ancient Greece. The perception then was that disease reflected the presence of disease-causing agents in the blood, and bloodletting was to expel the disease. Bloodletting was routinely and extensively performed for diseases such as inflammation, infection and fever. This practice was subsequently popularised by another Greek physician, Claudius Galen (129 – 203 AD), in Rome, who became a very well-respected authority on medicine for > 1500 years. In the age of modern medicine, bloodletting may be viewed as just a folly of the past, but it is difficult to imagine that this procedure would have been so widely practiced for such a long time if it had not been associated with efficacy.

5. Expert opinion

UC and CD together represent the chronic idiopathic IBDs, and produce 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 towards an ideal goal of having a medication with adequate efficacy margin, yet there is 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, IL-12, for which activated peripheral blood granulocytes and monocytes/macrophages are major sources. Furthermore, in patients with active IBD, peripheral leukocytes are elevated with activation behaviour, increased survival time and are believed to be major factors in the immunopathogenesis of IBD. Hence, depleting activated leukocytes should be considered as a safe and effective natural biological therapy, equivalent to reducing inflammatory cytokine release at an upstream point. Published data show leukocytapheresis producing impressive efficacy, strong drug-sparing effects, with potential to reduce the number of patients with severe disease who must undergo colectomy or be exposed to potent immunosupressors such as CyA. Based on data available at present, the safety of leukocytapheresis is no longer a concern; however, there are many other aspects of the treatment that require further investigation, not least the importance of large controlled studies to fully evaluate its therapeutic efficacy and its precise place in the treatment of IBD.

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Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. ALLISON MC, DHILLON AP, LEWIS WG, POUNDER RE: *Inflammatory Bowel Disease.* Allison MC, Dhillon AP, Lewis WG, Pounder RE (Eds), Mosby, London, UK (1998):9-95.

• **This book is rich in endoscopic and biopsy illustrations.**

2. FIOCCHI C: Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* (1998) 115:182-205.
3. PODOLSKY DK: Inflammatory bowel disease. *N. Engl. J. Med.* (2002) 347:417-429
4. HARRIS ML, BAYLESS TM: Dietary antigens as aggravating factors in Crohn's disease. *Dig. Dis. Sci.* (1989) 34:1613-1614.
5. SHANAHAN F: Crohn's disease. *Lancet* (2002) 359:62-69.
6. SARTOR RB: Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. *Am. J. Gastroenterol.* (1997) 92(Suppl.):5S-11S.
7. TAFFET SL, DAS KM: Sulphasalazine-adverse effects and desensitization. *Dig. Dis. Sci.* (1983) 28:833-842.
8. PRESENT DH: How to do without steroids in inflammatory bowel disease. *Inflamm. Bowel Dis.* (2000) 6:48-57.

• **This paper is a critical review on the limitations of corticosteroid therapy in IBD.**

9. RACHMILEWITZ D: Coated mesalazine (5-aminosalicylic acid) versus sulphasalazine in the treatment of active ulcerative colitis. (On behalf of an international study group). *Br. Med. J.* (1989) 298:82-86.
10. KORNBLUTH A, MARION JF, SALOMON P, JANOWITZ HD: How effective is current medical therapy for severe ulcerative colitis? *J. Clin. Gastroenterol.* (1995) 20:280-284.
11. TRUELOVE SC, JEWELL DP: Intensive intravenous regimens for severe attacks of ulcerative colitis. *Lancet* (1974) 1:1067-1070.

• **This is a first report of intensive corticosteroid therapy for UC.**

12. JARNEROT G, ROLNY P, SANDBERG-GERTZEN H: Intensive intravenous treatment of ulcerative colitis. *Gastroenterology* (1985) 89:1005-1013.
13. HANAUER SB: Medical therapy of ulcerative colitis. *Gastroenterology* (2004) 126:1582-1592.
14. O'KEEFE SJ: Nutrition and gastrointestinal disease. *Scand. J. Gastroenterol.* (1996) 220(Suppl.):52-59.
15. HYDE GM, THILLAINAYAGAM AV, JEWELL DP: Intravenous cyclosporin as rescue therapy in severe ulcerative colitis: time for a reappraisal? *Eur. J. Gastroenterol. Hepatol.* (1998) 10:411-413.
16. HANAUER SB: Can cyclosporine go it alone in severe ulcerative colitis. *Curr. Gastroenterol. Rep.* (2001) 3:455-456.
17. SERKOVA NJ, CHRISTIANS U, BENET LZ: Biochemical mechanisms of cyclosporine neurotoxicity. *Mol. Interv.* (2004) 4:97-107.
18. SCHREAIBER S, NIKOLAUS S, HAMPE J *et al.*: Tumour necrosis factor alpha and interleukin 1beta in relapse of Crohn's disease. *Lancet* (1999) 353:459-461.

• **One of the first reports directly implicating cytokines in the relapse of CD.**

19. PAPADAKIS KA, TARGAN SR: Role of cytokines in the pathogenesis of inflammatory bowel disease. *Annu. Rev. Med.* (2000) 51:289-298.
20. TARGAN SR, HANAUER SB, VAN DEVENTER SJ: A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. *N. Engl. J. Med.* (1997) 337:1029-1035.
21. HANAUER SB, FEAGAN BG, LICHTENSTEIN GR: Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* (2002) 359:1541-1549.
22. RUTGEERTS P, SANDBORN WJ, FEAGAN BG *et al.*: Infliximab for induction and maintenance therapy for ulcerative colitis.*N. Engl. J. Med.*. (2005) 353:2462-2476.
23. KORZENIK JR, PODOLSKY DK: Evolving knowledge and therapy of inflammatory bowel disease. *Nat. Rev. Drug Discov.* (2006) 5:197-209.
24. EVANS RC, CLARKE L, HEATH P, STEPHENS S, MORRIS AI, RHODES JM: Treatment of ulcerative colitis with an engineered human anti-TNF-alpha antibody CDP571. *Aliment. Pharmacol. Ther.* (1997) 11:1031-1035.
25. IYER S, KONTOYIANNIS D, CHEVRIER D *et al.*: Inhibition of tumor necrosis factor mRNA translation by a rationally designed immunomodulatory peptide. *J. Biol. Chem.* (2000) 275:17051-17057.
26. TRAVIS S, YAP L, HAWKEY CJ: Novel and effective therapy for ulcerative colitis: results of parallel, prospective, placebo-controlled trials (abstr). *Am. J. Gastroenterol.* (2003) 98:S239.
27. GORDON FH, HAMILTON MI, DONOGHUE S *et al.*: A pilot study of treatment of active ulcerative colitis with natalizumab, a humanized monoclonal antibody to alpha-4 integrin. *Aliment. Pharmacol. Ther.* (2002) 16:699-705.
28. FEAGAN B, GREENBERG GR, WILD G: A randomized controlled trial of a humanized alpha4beta7 antibody in ulcerative colitis (abstr). *Am. J. Gastroenterol.* (2003) 98:S248-S249.
29. SANDBORN WJ, COLOMBEL JF, ENNS R, FEAGAN BG, HANAUER SB: Natalizumab induction and maintenance therapy for Crohn's disease. *N. Engl. J. Med.* (2005) 353:1912-1925.

•• **This paper reports death from progressive multifocal leukoencephalopathy, associated with the JC virus in the natalizumab group.**

30. VAN ASSCHE G, DALLE I, NOMAN M *et al.*: A pilot study on the use of the humanized anti-interleukin-2 receptor antibody daclizumab in active ulcerative colitis. *Am. J. Gastroenterol.* (2003) 98:369-376.
31. TILG H, VOGELSANG H, LUDWICZEK O: A randomized placebo-controlled trial of pegylated interferon alpha in active ulcerative colitis (abstr). *Gastroenterology* (2003) 124:A62.
32. BECK PL, PODOLSKY DK: Growth factors in inflammatory bowel disease. *Inflamm. Bowel Dis.* (1999) 5:44-60.
33. SANDBORN WJ, SANDS BE, WOLF DC *et al.*: Repifermin (keratinocyte growth factor-2) for the treatment of active ulcerative colitis: a randomized, double-blind, placebo-controlled, dose-escalation trial. *Aliment. Pharmacol. Ther.* (2003) 17:1355-1364.
34. DEVOS SA, VAN DEN BOSSCHE N, DE VOS M, NAEYAERT JM: Adverse skin reactions to anti-TNF-α. *Dermatology* (2003) 206:388-390.

35. SEEGERS D, BOUMA G, PENA AS: Review article: a critical approach to new forms of treatment of Crohn's disease and ulcerative colitis. *Aliment. Pharmacol. Ther.* (2002) **16**(Suppl. 4):53-58.

36. GOMEZ-REINO JJ, CARMONA L, VALVERDE VR, MOLA EM, MONTERO MD: Treatment of rheumatoid arthritis with tumor necrosis factor inhibitors may predispose to significant increase in tuberculosis risk: a multicenter active-surveillance report. BIOBADASER Group. *Arthritis Rheum.* (2003) **48**:2122-2127.

37. KROESEN S, WIDMER AF, TYNDALL A, HASLER P: Serious bacterial infections in patients with rheumatoid arthritis under anti-TNF-alpha therapy. *Rheumatology* (2003) **42**:617-621.

38. BROWN SL, GREENE MH, GERSHON SK, EDWARDS ET, BRAUN MM: Tumor necrosis factor antagonist therapy and lymphoma development: twenty-six cases reported to the Food and Drug Administration. *Arthritis Rheum.* (2002) **46**:3151-3158.

39. ATZENI A, ARDIZZONE S, SARZI-PUTTINI P *et al.*: Autoantibody profile during short-term infliximab treatment for Crohn's disease: a prospective cohort study. *Aliment. Pharmacol. Ther.* (2005) **22**:453-461.

40. TIBBLE JA, SIGTHORSSON G, BRIDGER D, FAGERHOL MK, BJARNASON I: Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. *Gastroenterology* (2000) **119**:15-22.

•• **This is the first major report on the use of faecal neutrophil protein (calprotectin) as a biomarker of relapse in both UC and CD.**

41. LIMBURG P, DAVID M, AHLQUIST A, SANDBORN WJ: Faecal calprotectin levels predict colorectal inflammation among patients with chronic diarrhoea referred for colonoscopy. *Am. J. Gastroenterol.* (2000) **95**:2831-2837.

42. ROSETH AG, SCHMIDT PN, FAGERHOL MK: Correlation between faecal excretion of Indium-111-labelled granulocytes and calprotectin, a granulocyte marker. *Scand. J. Gastroenterol.* (1999) **34**:50-54.

43. CASSATELLA MA: The production of cytokines by polymorphonuclear neutrophils. *Immunol. Today* (1995) **16**:21-26.

44. NIKOLAUS S, BAUDITZ J, GIONCHETTI P: Increased secretion of pro-inflammatory cytokines by circulating polymorphonuclear neutrophils and regulation by interleukin-10 during intestinal inflammation. *Gut* (1998) **42**:470-476.

45. MCCARTHY DA, RAMPTON DS, LIU YC: Peripheral blood neutrophils in inflammatory bowel disease: morphological evidence of *in vivo* activation in active disease. *Clin. Exp. Immunol.* (1991) **86**:489-493.

46. MAHIDA YR: The key role of macrophages in the immunopathogenesis of inflammatory bowel disease. *Inflamm. Bowel Dis.* (2000) **6**:21-33.

47. HANAI H, WATANABE F, SANIABADI A, MATSUSHITA I, TAKEUCHI K, IIDA T: Therapeutic efficacy of granulocyte and monocyte adsorption apheresis in severe active ulcerative colitis. *Dig. Dis. Sci.* (2002) **47**:2349-2353.

48. HANAI H, WATANABE F, TAKEUCHI K, SANIABADI A, BJARNASON I: Leukcocyte adsorptive apheresis for the treatment of active ulcerative colitis: a prospective uncontrolled pilot study. *Clin. Gastroenterol. Hepatol.* (2003) **1**:28-35.

•• **This is the first major study on the efficacy of selective leukocytapheresis in patients with severe steroid-refractory UC.**

49. SANIABADI AR, HANAI H, BJARNASON I, LOFBERG R: Adacolumn, an adsorptive carrier based granulocyte and monocyte apheresis device for the treatment of inflammatory and refractory diseases associated with leukocytes. *Ther. Apher. Dial.* (2003) **7**:48-59.

50. RUGTVEIT J, BRANDTZAEG P, HALSTENSEN TS, FAUSA O, SCOTT H: Increased macrophage subsets in inflammatory bowel disease: apparent recruitment from peripheral blood monocytes. *Gut* (1994) **35**:669-674.

51. MEURET G, BITZI A, HAMMER B: Macrophage turnover in Crohn's disease and ulcerative colitis. *Gastroenterology* (1978) **74**:501-503.

52. BRANNIGAN AE, O'CONNELL PR, HURLEY H: Neutrophil apoptosis is delayed in patients with inflammatory bowel disease. *Shock* (2000) **13**:361-366.

53. MEAGHER LC, COUSIN JM, SECKL JR, HASLETT C: Opposing effects of glucocorticoids on the rate of appoptosis in neutrophilic and eosinophilic granulocytes. *J. Immunol.* (1996) **156**:4422-4428.

54. GRISHAM MB, YAMADA T: Neutrophils, nitrogen oxides and inflammatory bowel disease, neuro-immuno-physiology of the gastrointertinal mucosa. *Ann. NY Acad. Sci.* (1992) **664**:103-115.

•• **This paper is an excellent source of review and analysis of the mechanisms by which neutrophils contribute to mucosal tissue injury in IBD.**

55. MORSE EE, CARBONE PP, FREIREICH EJ, BRONSON W, KLIMAN A: Repeated leukapheresis of patients with chronic myelocytic leukemia. *Transfusion* (1966) **6**:175-182.

56. BUCKNER D, GRAW RG JR, EISEL RJ, HENDERSON ES, PERRY S: Leukapheresis by continous flow centrifugation (CFC) in patients with chronic myelocytic leukemia (CML). *Blood* (1969) **33**:353-369.

57. CURTIS JE, HERSH EM, FREIREICH EJ: Leukoapheresis therapy of chronic lymphocytic leukemia. *Blood* (1972) **39**:163-175.

58. PEARSON CM, PAULUS HE, MACHLEDER HI: The role of the lymphocyte and its products in the propagation of joint disease. *Ann. NY Acad. Sci.* (1975) **256**:150-168.

59. TENENBAUM J, UROWITZ MB, KEYSTONE EC, DWOSH IL, CURTIS JE: Leukoapheresis in severe rheumatoid arthritis. *Ann. Rheum. Dis.* (1979) **38**:40-44.

60. BICKS RO, GROSHART KW, CHANDLER RW: The treatment of severe chronically active Crohn's disease by T8 (suppressor cell) lymphapheresis (Abstr). *Gastroenterology* (1985) **88**:A1325.

61. BICKS RO, GROSHART KW, CHANDLER RW: The treatment of severe chronically active Crohn's disease by T lymphocyte apheresis (Abstr). *Gastroenterology* (1986) **90**:A1346.

62. BICKS RO, GROSHART KW, LUTHER RW: Total parenteral nutrition (TPN) plus T-lymphocyte apheresis (TLA) in the treatment of severe chronic active Crohn's disease (Abstr). *Gastroenterology* (1987) **94**:A34.

63. LEREBOURS E, BUSSEL A, MODIGLIANI R *et al.*: Treatment of

Crohn's disease by lymphocyte apheresis: a randomized controlled trial. Groupe d'Etudes Therapeutiques des Affections Inflammatoires Digestives. *Gastroenterology* (1994) 107:357-361.

•• **This is the first controlled study showing lack of clinical benefit from selective lymphocytapheresis.**

64. AYABE T, ASHIDA T, TANIGUCHI M *et al.*: A pilot study of centrifugal leukocyte apheresis for corticosteroid-resistant active ulcerative colitis. *Intern. Med.* (1997) 36:322-326.

65. AYABE T, ASHIDA T, KOHGO Y: Centrifugal leukocyte apheresis for ulcerative colitis. *Ther. Apher.* (1998) 2:125-128.

66. KOHGO Y, HIBI T, CHIBA T *et al.*: Leukocyte apheresis using a centrifugal cell separator in refractory ulcerative colitis; a multicenter open label trial. Study group for Alternative Therapies in Ulcerative Colitis Patients. *Ther. Apher.* (2002) 6:255-260.

67. HIRAISHI K, TAKEDA Y, SANIABADI A *et al.*: Studies on the mechanisms of leukocyte adhesion to cellulose acetate beads: an *in vitro* model to assess the efficacy of cellulose acetate carrier-based granulocyte and monocyte adsorptive apheresis. *Ther. Apher. Dial.* (2003) 7:334-340.

68. SANIABADI AR, HANAI H, SUZUKI Y, BJARNASON I, LOFBERG R: Adacolumn for selective leukocytapheresis as a non-pharmacological treatment for patients with disorders of the immune system: an adjunct or an alternative to drug therapy?. *J. Clin. Apher.* (2005) 20:171-184.

• **This paper is a comprehensive review on the application of selective leukocytapheresis to treat inflammatory diseases.**

69. SHIMOYAMA T, SAWADA K, TANAKA T *et al.*: Granulocyte and monocyte apheresis with the G-1 column in the treatment of patients with active ulcerative colitis. *Jpn. J. Apher.* (1999) 18:117-131.

70. STACK WA, LONG RG, HAWKEY CJ: Short and long-term outcome of patients treated with cyclosporin for severe acute ulcerative colitis. *Aliment. Pharmacol. Ther.* (1998) 12:973-978.

71. KANKE K, NAKANO M, HIRAISHI H, TERANO A: Evaluation of granulocyte/monocyte apheresis therapy for active ulcerative colitis. *Dig. Liv. Dis.* (2004) 36:512-518.

72. NAGANUMA M, FUNAKOSHI S, SAKURABA A *et al.*: Granulocytapheresis is useful as an alternative therapy in patients with steroid-refractory or -dependent ulcerative colitis. *Inflamm. Bowel Dis.* (2004) 10:251-257.

73. YAMAMOTO T, UMEGAE S, KITAGAWA T, YASUDA Y, YAMADA Y, TAKAHASHI D: Granulocyte and monocyte adsorptive apheresis in the treatment of active distal ulcerative colitis: a prospective, pilot study. *Aliment. Pharmacol. Ther.* (2004) 20:783-792.

74. PREMCHAND P, TAKEUCHI K, BJARNASON I: Granulocyte, macrophage, monocyte apheresis for refractory ulcerative proctitis. *Eur. J. Gastroenterol. Hepatol.* (2004) 16:943-945.

75. DOMENECH E, HINOJOSA J, ESTEVE-COMAS M, GASSULL A: Spanish Group for the Study of Crohn's Disease and Ulcerative Colitis (GETECCU). Granulocyteaphaeresis in steroid-dependent inflammatory bowel disease: a prospective, open, pilot study. *Aliment. Pharmacol. Ther.* (2004) 20:1347-1352.

76. SUZUKI Y, YOSHIMURA N, SANIABADI AR, SAITO Y: Selective neutrophil and monocyte adsorptive apheresis as a first line treatment for steroid naïve patients with active ulcerative colitis: a prospective uncontrolled study. *Dig. Dis. Sci.* (2004) 49:565-571.

77. COHEN RD: Treating ulcerative colitis without medications – 'Look Mom, No Drugs!' *Gastroenterology* (2005) 128:235-236:

• **This is a good commentary on the use of selective leukocytapheresis instead of drug therapy.**

78. MAIDEN L, BAUR R, TAKEUCHI K *et al.*: Adacolumn apheresis reduces relapse rates in patients at significant risk of clinical relapse (Abstr). *Gut* (2005) 54:A57.

79. MATSUI T, NISHIMURA T, MATAKE H, OHTA T, SAKURAI T, YAO T: Granulocytapheresis for Crohn's disease: a report on seven refractory patients. *Am. J. Gastroenterol.* (2003) 98:511-512.

80. FUKUDA Y, MATSUI T, SUZUKI Y, KANKE K, HIBI T: granulocyte and monocyte apheresis for refractory Crohn's disease: an open multicenter prospective study. *J. Gastroenterol.* (2004) 39:1158-1164.

81. MURATOV V, LUNDAHL J, ULFGREN AK *et al.*: Downregulation of interferon- parallels clinical response to selective leukocyte apheresis in patients with inflammatory bowel disease. A 12-month follow-up study. *Int. J. Colorectal Dis.* (2006) (In Press).

82. HANAI H, WATANABE F, SANIABADI A *et al.*: Correlation of serum soluble TNF-alpha receptors I and II levels with disease activity in patients with ulcerative colitis. *Am. J. Gastroenterol.* (2004) 99:1532-1538.

83. MOHLER KM, TORRANCE DS, SMITH GA, WIDMER MB: Soluble tumour necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoximia and function simultaneously as both TNF carriers and TNF antagonists. *J. Immunol.* (1993) 151:1548-1561.

84. KASHIWAGI N, HIRATA I, KASUKAWA R: A role for granulocyte and monocyte apheresis in the treatment of rheumatoid arthritis. *Ther. Apher.* (1998) 2:134-141

85. KASHIWAGI N, SANIABADI A, SHIMOYAMA T *et al.*: Immunomodulatory effects of granulocyte and monocyte adsorption apheresis as a treatment for patients with ulcerative colitis. *Dig. Dig. Sci.* (2002) 47:1334-1341.

86. BISWAS P, MANTELLI B, HASSON H, SANIABADI A, LAZZARIN A, BERETTA A: *In vivo* modulation of leukocyte trafficking receptor following therapeutic purging of myeloid cells: implications for treatment of HIV infection and other immune disorders. *Clin. Immunol.* (2003) 109:355-358.

87. TAKEDA Y, SHIOBARA N, SANIABADI AR, ADACHI M, HIRAISHI K: Adhesion dependent release of hepatocyte growth factor and interleukin-1 receptor antagonist from human blood granulocytes and monocytes: evidence for the involvement of plasma IgG, complement C3 and β2 integrin. *Inflamm. Res.* (2004) 53:277-283.

88. TAHARA Y, IDO A, YAMAMOTO S, MIYATA Y, UTO H, HORI T: Hepatocyte growth factor facilitates colonic mucosal repair in experimental ulcerative colitis in rats. *J. Pharmacol. Exp. Ther.* (2003) 307:146-151.

89. SAWADA K, MUTO T, SHIMOYAMA T *et al.*: Multicenter randomized controlled trial for the treatment of ulcerative colitis

with a leukocytapheresis colum. *Curr. Pharm. Des.* (1997) 9:307-321.

90. SHIROKAZE J: Leukocytapheresis using a leukocyte removal filter. *Ther. Apher.* (2002) 6:261-266.
91. SAWADA K, OHNISHI K, FUKUI S: Leukocytapheresis therapy performed with leukocyte removal filter for inflammatory bowel disease. *J. Gastroenterol.* (1995) 30:322-329.
92. SAWADA K, KUSUGAMI K, SUZUKI Y *et al.*: Leukocytapheresis in ulcerative colitis: results of a multicenter double-blind prospective case-control study with sham apheresis as placebo treatment. *Am. J. Gastroenterol.* (2005) 100:1362-1369.
93. SAWADA K, EGASHIRA A, OHNISHI K, FUKUNAGA K, KASUKA T, SHIMOYAMA T: Leukocytapheresis (LCAP) for management of fulminant ulcerative colitis with toxic megacolon. *Dig. Dis. Sci.* (2005) 50:767-773.
94. SAWADA K, MUTO T, SHIMOYAMA T: Leukocytapheresis with leukocyte removal filter as new therapy for ulcerative colitis. *Ther. Apher.* (1997) 1:207-211.
95. YAMAJI K, YANG K, TSUDA H, HASHIMOTO H: Fluctuations in the peripheral blood leukocyte and platelet counts in leukocytapheresis in healthy volunteers. *Ther. Apher.* (2002) 6:402-412.
96. SHIBATA H, KURIYAMA T, YAMAWAKI N: Cellsorba. *Ther. Apher. Dial.* (2003) 7:44-47.
97. KING C, ILIC A, KOELSCH K, SARVETNICK N: Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* (2004) 117:265-277.

•• **Based on the findings of these authors, an intervention that induces lymphopenia potentially may trigger homeostatic T cell expansion-associated autoimmune disease.**

98. SLEASMAN JW: The association between immunodeficiency and the development of autoimmune disease. *Adv. Dent. Res.* (1996) 10:57-61.

•• See [97].

99. NOGUCHI M, HIWATASHI N, HAYAKAWA T, TOYOTA T: Leukocyte removal filter-passed lymphocytes produce large amounts of interleukin-4 in immunotherapy for inflammatory bowel disease: role of bystander suppression. *Ther. Apher.* (1998) 2:109-114.
100. ANDOH A, TSUJIKAWA T, INATOMI O *et al.*: Leukocytaoheresis therapy modulates circulating T cell subsets in patients with ulcerative colitis. *Ther. Apher. Dial.* (2005) 9:270-279.
101. BRESNIHAN B, CUNNANE G: Infection complications associated with the use of biologic agents. *Rheum. Dis. Clin. North Am.* (2003) 29:185-202.
102. EGAN LJ, SANDBORN WJ: Advances in the treatment of Crohn's disease. *Gastroenterology* (2004) 126:1574-1581.
103. SHAND A, FORBES A: Potential therapeutic role for cytokine or adhesion molecule manipulation in Crohn's disease: in the shadow of infliximab? *Int. J. Colorectal Dis.* (2003) 18:1-11.
104. CAMPBELL DJ, DEBES GF, JOHNSTON B, WILSON E, BUTCHER EC: Targeting T cell responses by selective chemokine receptor expression. *Semin. Immunol.* (2003) 15:277-286.
105. MACKAY CR, MARSTON WL, DUDLER L: Naïve and memory T cells show distinct pathways of lymphocyte recirculation. *J. Exp. Med.* (1990) 171:801-817.
106. OLSZEWSKI WL: The lymphatic system in body homeostasis: physiological conditions. *Lymphat. Res. Biol.* (2003) 1:11-21.
107. BROMLEY SK, THOMAS SY, LUSTER AD: Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat. Immunol.* (2005) 6:895-901.
108. DEBES GF, ARNOLD CN, YOUNG AJ *et al.*: Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat. Immunol.* (2005) 6:889-894.
109. KLONOWSKI KD: Dynamics of blood-borne CD8 memory T cell migration *in vivo*. *Immunity* (2004) 20:551-562.
110. LEE A, WHYTE M, HASLETT C: Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *J. Leukoc. Biol.* (1993) 54:283-288.

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FTY720 suppresses CD4+CD44highCD62L– effector memory T cell-mediated colitis

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Fujii, R., T. Kanai, Y. Nemoto, S. Makita, S. Oshima, R. Okamoto, K. Tsuchiya, T. Totsuka, and M. Watanabe. FTY720 suppresses $CD4^+CD44^{high}CD62L^-$ effector memory T cell-mediated colitis. *Am J Physiol Gastrointest Liver Physiol* 291: G267–G274, 2006. First published March 30, 2006; doi:10.1152/ajpgi.00496.2005.—FTY720, a sphingosine-derived immunomodulator, causes immunosuppression via enhancement of lymphocyte sequestration into secondary lymphoid organs, thereby preventing their antigen-activated T cell egress to sites of inflammation. FTY720 is highly effective in inhibiting autoimmunity in various animal models. However, there is little known about how FTY720 controls the migration property of memory T cells. Here, we demonstrated that FTY720 prevents the development of colitis induced by the adoptive transfer of lamina propria (LP) colitogenic effector memory $CD4^+$ T cells (T_{EM} cells; $CD45RB^{low}CD44^{high}CD62L^-$) into severe combined immunodeficiency (SCID) mice and suppresses interferon-γ, interleukin-2, and tumor necrosis factor-α production by LP $CD4^+$ T cells. The numbers of spleen, peripheral blood, mesenteric lymph node, and LP $CD4^+$ T cells in FTY720-treated mice were significantly reduced compared with those in control mice. Notably, LP $CD4^+$ T_{EM} cells as well as splenic $CD4^+CD45RB^{high}$ T cells expressed several spingosine-1-phosphate receptors that are targets for FTY720. Furthermore, FTY720 also prevented the development of colitis induced by the adoptive transfer of splenic $CD4^+CD45RB^{high}$ T cells into SCID mice. Collectively, the present data indicate that FTY720 treatment may offer the potential not only to prevent the onset of disease but also to treat memory T cell-mediated autoimmune diseases including inflammatory bowel diseases.

therapy; migration; I cell

2-AMINO-2-[2-(4-OCTYLPHENYL)ETHYL]-1,3-PROPANEDIOL HYDROCHLORIDE (FTY720) is a chemical derivative of myriocin (thermozymocidin), a substance found in the fungi *Myriococcum albomyces* and *Isaria sinclairii* (1, 2, 5, 6). FTY720 is a potent immunomodulator that has been shown to prevent graft rejection in various animal allotransplantation models (23, 24), autoimmune disease animal models (11, 16, 20), and viral infection animal models (17, 21). Good synergy with cyclosporine and sirolimus has been reported (3, 28, 30). FTY720 elicits a lymphopenia resulting from a reversible redistribution of naïve lymphocytes from the circulation to secondary lymph nodes (4) without evoking a generalized immunosuppression (31). Another study (21) has suggested that low blood lymphocyte counts may reflect reduction in the emigration of effector cells to the periphery.

After naïve lymphocytes enter a secondary lymphoid organ from the blood, they travel to separate subcompartments, where they survey for antigen. In the absence of an antigen encounter, cells leave the organ via the efferent lymphatics or, in the case of the spleen, via the red pulp. Timely egress ensures that the cells travel rapidly to further lymphoid organs to continue their antigen surveillance process. Recent studies (1, 4, 22) have demonstrated that FTY720 blocks the egress of lymphocyte from the lymph nodes, Peyer's patches, and thymus. In vivo, FTY720 is immediately phosphorylated, and in vitro analysis has established that FTY720-phosphate is an agonist for sphingosine-1-phosphate (S1P) receptors ($S1P_1$–$S1P_5$ except for $S1P_4$) (2, 5, 13). The $S1P_1$ ligand, S1P, is present at high concentrations (100–300 nM) within blood and body fluids (26). It has been suggested that the egress-blocking activity of FTY720 can be explained by its $S1P_1$ downmodulating activity in lymphocytes (8, 15).

In inflammatory bowel diseases (IBDs), it is believed that memory T cells are intermittently reactivated in secondary lymphoid organs and thereafter return to inflammatory tissues, such as, in this case, the gut (22). These memory T cells can survive for a long period, and they provide the basis for long-term immunological memory. However, it is little known how memory T cells are controlled by FTY720 in IBDs. In this study, we evaluated the effect of FTY720 using our recently established $CD4^+CD44^{high}CD62L^-$ effector memory T cell (T_{EM} cell)-mediated chronic colitis model.

MATERIALS AND METHODS

Animals. Female BALB/c and CB-17 severe combined immunodeficiency (SCID) mice were purchased from Japan Clear (Tokyo, Japan). Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. The Institutional Committee on Animal Research of Tokyo Medical and Dental University approved the experiments.

Antibodies. The following monoclonal antibodies were used for the purification of cell populations and flow cytometric analysis: RM4-5, CyChrome- or phycoerythrin (PE)-conjugated anti-mouse CD4 (BD PharMingen; San Diego, CA); 16A, FITC-conjugated anti-mouse CD45RB (BD PharMingen); IM7, PE-conjugated anti-mouse CD44; and MEL-14, PE- or FITC-conjugated anti-mouse CD62L.

T cell reconstitution and FTY720 treatment. FTY720 (Novartis Pharma; Basel, Switzerland) was dissolved in sterile distilled water (DW). For in vivo treatment, FTY720 was administered via per os gavage. To exclude the possibility that in vivo FTY720 treatment might be cytotoxic for $CD4^+$ T cells, we treated normal BALB/c mice with a single dose of FTY720 (0, 0.3, and 3.0 mg/kg). Seventy-two hours after the treatment, dead and apoptotic cells were detected by annexin V-FITC/propidium iodide (PI) staining (MBL; Nagoya, Japan). As shown in Fig. 1, there were no significant differences in the

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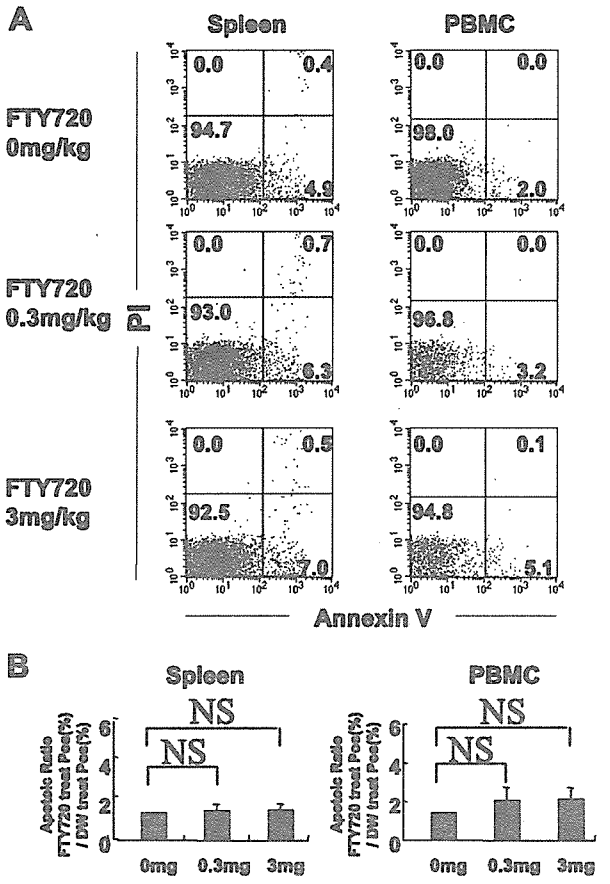

Fig. 1. FTY720 treatment does not induce apoptosis of peripheral blood (PB) monoclonal cells (PBMC) and splenic CD4+ cells. Normal BALB/c mice were administerred with FTY720 at a single dose of 0, 0.3, or 3.0 mg/kg of FTY720. *A*: 48 h after the administration, the numbers of dead or apoptotic cells were determined by annexin V-FITC/propidium iodide (PI). Data are from 5 mice/group. NS, not significantly different. *B*: data are means ± SE of the percentages of PI^+ and annexin V^+ (dead) cells and PI^- and annexin V^+ (apoptotic) cells from 5 mice/group.

numbers of dead (PI^+ and annexin V^+) or apoptotic (PI^- and annexin V^+) cells between mice treated with each dose. Thus we adopted the 0.3 mg/kg dose of FTY720 in a line of in vivo experiments.

Colitis was induced in CB-17 SCID mice by the adoptive transfer of syngeneic $CD4^+CD45RB^{high}$ T cells as described previously (10). Briefly, $CD4^+$ T cells were isolated from splenocytes from normal BALB/c mice using the anti-CD4 (L3T4) MACS magnetic separation system (Miltenyi Biotec; Auburn, CA). Enriched $CD4^+$ T cells were labeled with PE-conjugated anti-mouse CD4 monoclonal antibodies and FITC-conjugated anti-CD45RB monoclonal antibodies and the isolated $CD45RB^{high}$ (highest staining: 30%) fraction on a FACS Vantage (Becton-Dickinson; Sunnyvale, CA). Each SCID mouse was injected intraperitoneally with syngeneic 3×10^5 $CD4^+CD45RB^{high}$ T cells. Colitic mice were killed at 5–7 wk after the transfer to isolate the colitogenic lamina propria (LP) memory $CD4^+$ T cells (10). The entire length of the colon was opened longitudinally, washed with PBS, and cut into small pieces. The dissected mucosa was incubated with Ca^{2+}-and Mg^{2+}-free HBSS containing 1 mM DTT (Sigma) for 30 min to remove mucus and then treated with 1 mg/ml collagenase (Worthington Biomedical; Freehold, NJ) and 0.01% DNase (Worthington) for 2 h. Cells were pelleted two times through a 40% isotonic Percoll solution and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched $CD4^+$ LP T cells were obtained by positive selection using anti-CD4 (L3T4) MACS magnetic beads. The resultant cells, when analyzed by FACS Calibur, contained >96% $CD4^+$ cells. To investigate LP memory $CD4^+$ T cell migration properties and the preventive effect by FTY720, we next induced a colitogenic LP memory $CD4^+$ T cell-mediated colitis as previously described (25). In brief, seven SCID mice from each group were injected intraperitoneally with 200 μl PBS containing 3×10^5 colitic LP $CD4^+$ T cells and were treated with DW or FTY720 (0.3 mg/kg) daily starting 1 day before the transfer over a period of 4 wk. In another experiment, seven SCID mice from each group were injected intraperitoneally with 200 μl PBS containing 3×10^5 normal splenic $CD4^+CD4RB^{high}$ T cells and were treated with DW or FTY720 (0.3 mg/kg) daily starting 1 day before T cell transfer over a period of 5 wk. SCID mice after transfer were weighed initially and then three times per week thereafter. They were observed for the following clinical signs of illness: hunched over appearance, piloerection of the coat, diarrhea, and blood in the stool. The mice were then killed and assessed for a clinical score, that is, the sum of the following four parameters: hunching and wasting, *0* or *1*; colon thickening, *0–3* (*0*, no colon thickening; *1*, mild thickening; *2*, moderate thickening; and *3*, extensive thickening); and stool consistency, *0–3* (*0*, normal beaded stool; *1*, soft stool; and *2*, diarrhea; and an additional point was added if gross blood was noted) (29).

Histological examination and immunohistology. Tissue samples were fixed in PBS containing 6% neutral buffered formalin. Paraffin-embedded sections (5 μm) were stained with hematoxylin and eosin. Three tissue samples from the middle part of the colon were prepared. Sections were analyzed without prior knowledge of the type of T cell reconstitution or treatment. The area most affected was graded by the number and severity of lesions. The mean degree of inflammation in the colon was calculated using a modification of the previously described scoring system (29).

Flow cytometry. Flow cytometry three-color analysis was performed. Isolated peripheral blood, spleen, mesenteric lymph node (MLN), peripheral lymph node (PLN; inguinal, axillary, and lateral axillary), and LP cells obtained from FTY720- and DW-treated mice were preincubated with Fcγ receptor-blocking monoclonal antibodies for 20 min followed by an incubation with CyChrome-conjugated anti-mouse CD4, PE-conjugated anti-CD44, and FITC-conjugated anti-CD62L monoclonal antibodies for 30 min on ice. After the cells had been stained, flow cytometry and data analysis were performed using FACS Calibour and CELLQUEST software (BD Biosciencesl San Jose, CA).

Cytokine production assay. To measure cytokine production, 1×10^5 LP $CD4^+$ T cells were cultured in 200 μl culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar; Cambridge, MA) precoated with 5 μg/ml hamster anti-mouse CD3ε monoclonal antibodies (145-2C11, BD PharMingen) and hamster 2 μg/ml anti-mouse CD28 monoclonal antibodies (37.51, BD PharMingen) in PBS overnight at 4°C. Culture supernatants were removed after 48 h and assayed for cytokine production. Cytokine concentrations were determined by a specific ELISA [interleukin (IL)-10] (R&D; Minneapolis, MN) or a mouse T helper (Th)1/Th2 cytokine bead array kit [IL-2, IL-4, IL-5, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ] (BD Biosciences) per the manufacturer's recommendation.

RT-PCR. Total cellular RNA was extracted from 7×10^5 cells using the RNeasy Mini Kit (Qiagen; Valencia, CA). Five micrograms of total RNA were reverse transcribed using SuperScript reverse transcriptase (Invitrogen). S1P receptor ($S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, and $S1P_5$) levels were measured by a QuantiTect SYBER green PCR kit using an ABI7500 real-time PCR system and 7500 system SDS software (Applied Biosystems; Foster city, CA). The following primers were used: $S1P_1$, forward 5′-GTG TAG ACC CAG AGT CCT GCG-3′ and reverse 5′-AGC TTT TCC TTG GGA GAG-3′; $S1P_2$, forward 5′-GGC CTA GCC AGT GCT CAG C-3′ and reverse 5′-CCT TGG TGT AAT TGT AGT GTT CCA GA-3′; $S1P_3$, forward 5′-GGA GCC CCT AGA CGG GAG T-3′ and reverse 5′-CCG ACT GCG GGA AGA GTG T-3′; $S1P_4$, forward 5′-CCT GGA ACT CAC