for patients with hematologic malignancies undergoing allogeneic progenitor cell transplantation. Blood. 2001;97:631–7. doi:10.1182/blood.V97.3.631.

2. Slavin S, Nagler A, Naparstek E, et al. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. Blood. 1998;91:756–63.
3. McSweeney PA, Niederwieser D, Shizuru JA, et al. Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. Blood. 2001;97:3390–400. doi:10.1182/blood.V97.11.3390.
4. Izutsu K, Kanda Y, Ohno H, et al. Unrelated bone marrow transplantation for non-Hodgkin lymphoma: a study from the Japan Marrow Donor Program. Blood. 2004;103:1955–60. doi:10.1182/blood-2003-03-0937.
5. Bornhäuser M, Thiede C, Platzbecker U, et al. Dose-reduced conditioning and allogeneic hematopoietic stem cell transplantation from unrelated donors in 42 patients. Clin Cancer Res. 2001;7:2254–62.
6. Nagler A, Aker M, Or R, et al. Low-intensity conditioning is sufficient to ensure engraftment in matched unrelated bone marrow transplantation. Exp Hematol. 2001;29:362–70. doi:10.1016/S0301-472X(00)00655-X.
7. Chakraverty R, Peggs K, Chopra R, et al. Limiting transplantation-related mortality following unrelated donor stem cell transplantation by using a nonmyeloablative conditioning regimen. Blood. 2002;99:1071–8. doi:10.1182/blood.V99.3.1071.
8. Niederwieser D, Maris M, Shizuru JA, et al. Low-dose total body irradiation (TBI) and fludarabine followed by hematopoietic cell transplantation (HCT) from HLA-matched or mismatched unrelated donors and postgrafting immunosuppression with cyclosporine and mycophenolate mofetil (MMF) can induce durable complete chimerism and sustained remissions in patients with hematological diseases. Blood. 2003;101:1620–9. doi:10.1182/blood-2002-05-1340.
9. Maris MB, Niederwieser D, Sandmaier BM, et al. HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative conditioning for patients with hematologic malignancies. Blood. 2003;102:2021–30. doi:10.1182/blood-2003-02-0482.
10. Wong R, Giralt SA, Martin T, et al. Reduced-intensity conditioning for unrelated donor hematopoietic stem cell transplantation as treatment for myeloid malignancies in patients older than 55 years. Blood. 2003;102:3052–9. doi:10.1182/blood-2003-03-0855.
11. Kusumi E, Kami M, Yuji K, et al. Feasibility of reduced intensity hematopoietic stem cell transplantation from an HLA-matched unrelated donor. Bone Marrow Transplant. 2004;33:697–702. doi:10.1038/sj.bmt.1704425.
12. Rodriguez R, Parker P, Nademanee A, et al. Cyclosporine and mycophenolate mofetil prophylaxis with fludarabine and melphalan conditioning for unrelated donor transplantation: a prospective study of 22 patients with hematologic malignancies. Bone Marrow Transplant. 2004;33:1123–9. doi:10.1038/sj.bmt.1704493.
13. Inamoto Y, Oba T, Miyamura K, et al. Stable engraftment after a conditioning regimen with fludarabine and melphalan for bone marrow transplantation from an unrelated donor. Int J Hematol. 2006;83:356–62. doi:10.1532/IJH97.05168.
14. Baron F, Maris MB, Storer BE, et al. Unrelated donor status and high donor age independently affect immunologic recovery after nonmyeloablative conditioning. Biol Blood Marrow Transplant. 2006;12:1176–87. doi:10.1016/j.bbmt.2006.07.004.
15. Kim SW, Tanimoto TE, Hirabayashi N, et al. Myeloablative allogeneic hematopoietic stem cell transplantation for non-Hodgkin lymphoma: a nationwide survey in Japan. Blood. 2006;108:382–9. doi:10.1182/blood-2005-02-0596.
16. Sasazuki T, Juji T, Morishima Y, et al. Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. N Engl J Med. 1998;339:1177–85. doi:10.1056/NEJM199810223391701.
17. Petersdorf EW, Hansen JA, Martin PJ, et al. Major-histocompatibility-complex class I alleles and antigens in hematopoietic-cell transplantation. N Engl J Med. 2001;345:1794–800. doi:10.1056/NEJMoa011826.
18. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus conference on acute GVHD grading. Bone Marrow Transplant. 1995;15:825–8.
19. Sullivan KM, Agura E, Anasetti C, et al. Chronic graft-versus-host disease and other late complications of bone marrow transplantation. Semin Hematol. 1991;28:250–9.
20. Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. Stat Med. 1999;18:695–706. doi:10.1002/(SICI)1097-0258(19990330)18:6<695::AID-SIM60>3.0.CO;2-O.
21. Teshima T, Matsuo K, Matsue K, et al. Impact of human leucocyte antigen mismatch on graft-versus-host disease and graft failure after reduced intensity conditioning allogeneic haematopoietic stem cell transplantation from related donors. Br J Haematol. 2005;130:575–87. doi:10.1111/j.1365-2141.2005.05632.x.
22. Giralt S, Logran B, Rizzo D, et al. Reduced-intensity conditioning for unrelated donor progenitor cell transplantation: long-term follow-up of the first 285 reported to the national marrow donor program. Biol Blood Marrow Transplant. 2007;13:844–52. doi:10.1016/j.bbmt.2007.03.011.
23. Deeg HJ, Amylon ID, Harris RE, et al. Marrow transplants from unrelated donors for patients with aplastic anemia: minimum effective dose of total body irradiation. Biol Blood Marrow Transplant. 2001;7:208–15. doi:10.1053/bbmt.2001.v7.pm11349807.
24. Kröger N, Sayer HG, Schwerdtfeger R, et al. Unrelated stem cell transplantation in multiple myeloma after a reduced-intensity conditioning with pretransplantation antithymocyte globulin is highly effective with low transplantation-related mortality. Blood. 2002;100:3919–24. doi:10.1182/blood-2002-04-1150.
25. Ho AY, Pagliuca A, Kenyon M, et al. Reduced-intensity allogeneic hematopoietic stem cell transplantation for myelodysplastic syndrome and acute myeloid leukemia with multilineage dysplasia using fludarabine, busulphan, and alemtuzumab (FBC) conditioning. Blood. 2004;104:1616–23. doi:10.1182/blood-2003-12-4207.
26. van Besien K, Artz A, Smith S, et al. Fludarabine, melphalan, and alemtuzumab conditioning in adults with standard-risk advanced acute myeloid leukemia and myelodysplastic syndrome. J Clin Oncol. 2005;23:5728–38. doi:10.1200/JCO.2005.15.602.
27. Lim ZY, Ho AY, Ingram W, et al. Outcomes of alemtuzumab-based reduced intensity conditioning stem cell transplantation using unrelated donors for myelodysplastic syndromes. Br J Haematol. 2006;135:201–9. doi:10.1111/j.1365-2141.2006.06272.x.
28. Maruyama D, Fukuda T, Kato R, et al. Comparable anti-leukemia/lymphoma effects in non-remission patients undergoing allogeneic hematopoietic cell transplantation with a conventional cytoreductive or reduced-intensity regimen. Biol Blood Marrow Transplant. 2007;13:932–41. doi:10.1016/j.bbmt.2007.04.004.

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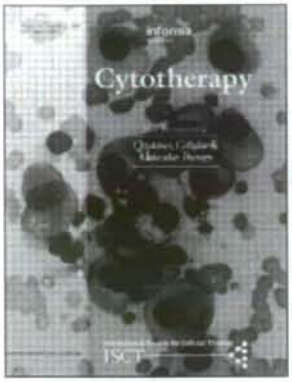

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Preparation of functionally preserved CD4+ CD25high regulatory T cells from leukapheresis products from ulcerative colitis patients, applicable to regulatory T-cell transfer therapy

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Preparation of functionally preserved $CD4^+$ $CD25^{high}$ regulatory T cells from leukapheresis products from ulcerative colitis patients, applicable to regulatory T-cell transfer therapy

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Background

Ulcerative colitis (UC) is an intractable disease; therefore new therapies need to be developed. $CD4^+$ $CD25^{high}$ regulatory T cells (Treg) significantly ameliorate colitis in animal models. In active UC patients, although Treg are functionally preserved, their proportion in peripheral blood decreases. Thus Treg transfer therapy is expected to be efficacious for UC. During leukapheresis for UC, Treg are depleted, as well as colitogenic effector leukocytes. We therefore designed a leukapheresis/Treg transfer therapy in which Treg are isolated from leukapheresis products and transfused to patients, and studied large-scale germ-free methods of Treg preparation.

Methods

Using the CliniMACS cell selection system, we conducted Treg isolation experiments from leukapheresis products in which B and $CD8^+$ T cells were depleted, followed by positive selection of $CD25^+$ cells. In some experiments, isolated Treg or non-Treg were expanded with interleukin-2 (IL-2) ± transforming growth factor (TGF)-β1. Expression of a Treg-specific marker, FOXP3, and gut-homing receptors, and suppressor activity of isolated or cultured cells, were analyzed.

Results

$CD4^+$ $CD25^{high}$ T cells were collected and efficiently enriched with a good recovery rate. Isolated cells preferentially expressed FOXP3 and significantly suppressed T-cell proliferation in vitro. *In addition, isolated Treg could be efficiently expanded, and Treg could be induced from non-Treg with TGF-β1* in vitro. *TGF-β1 significantly up-regulated αEβ7 and α4β7 integrins.*

Discussion

We have established a method of Treg isolation from leukapheresis products that can be used clinically; therefore, Treg transfer therapy is feasible in combination with leukapheresis for UC. Expansion or induction of Treg in vitro *may be another approach to Treg-based immunotherapy.*

Keywords

adoptive transfer, leukapheresis, regulatory T cell, ulcerative colitis.

Introduction

Ulcerative colitis (UC), a form of inflammatory bowel disease (IBD), is a chronic inflammatory disorder that causes persistent colonic inflammation. It mainly affects adolescents and young adults and deeply impairs their quality of life for a long period of time. Its etiology remains unknown; therefore, immunomodulators, including corticosteroids, which suppress immune responses in a non-specific manner, have been the mainstay of treatment for this disease. However, the development of innovative therapy is desperately needed because, despite conventional therapy, the majority of patients experience repeated recurrence of the disease following remission, and a considerable number of patients with severe UC ultimately undergo colectomy.

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Through much research, a gradual understanding of the pathophysiologic process of this disease has been gained, and the evidence suggests that an imbalance between colitogenic effector and the regulatory function of the intestinal immune system is responsible for colonic inflammation.

T cells with immunosuppressive capability play an important role in the maintenance of homeostasis by actively regulating immune reactions. One such subset of T cells, naturally occurring $CD4^+$ $CD25^+$ regulatory T cells (Treg), is characterized by their constitutive expression of CD25, cytotoxic T-lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor (TNF) receptor, and the transcription factor forkhead box P3 (FOXP3) [1]. Treg exhibit a hyporesponsiveness to stimuli through T-cell receptors, and possess the ability to suppress $CD4^+$ $CD25^-$ T-cell and $CD8^+$ T-cell proliferation *in vitro*. Treg are essential for the prevention of intestinal inflammation and thus for the maintenance of gut homeostasis. The transfer of naive $CD4^+$ $CD45RB^{high}$ T cells into immunodeficient recipient mice leads to the development of chronic persistent colitis, which resembles human IBD. This phenomenon is the result of the absence of Treg in the transferred cell population, and the co-transfer of Treg into such recipient mice clearly prevents and even cures colonic inflammation [2,3]. The existence of Treg was first demonstrated in mice [4–7] and they have also been identified in human peripheral blood [8,9]. In mice, Treg constitute a typically distinct population of $CD25^+$ cells that comprise 5–10% of thymic, lymph node and splenic $CD4^+$ T cells. In humans, however, up to 40% of peripheral blood $CD4^+$ T cells express CD25 to some extent. It has been shown that only a minority of human $CD4^+$ T cells that express the highest levels of CD25 ($CD25^{high}$) have suppressor activity [8]. The function of Treg seems to be qualitatively preserved in UC patients, as isolated Treg from peripheral blood or intestinal lamina propria in IBD patients reveal suppressor activity *in vitro*, similar to those in healthy subjects [10–12]. However, Treg seem to be quantitatively reduced, as the percentage of Treg is significantly reduced in peripheral blood of active UC patients, in inverse correlation with disease activity [13]. Thus Treg-based cell therapy is expected to be efficacious for the treatment of UC.

Apheresis that depletes large numbers of leukocytes from circulating peripheral blood has been developed for the treatment of UC [14–17]. It is now considered a standard therapy for patients with refractory UC who are steroid-resistant or -dependent in Japan, and its use is spreading to other countries. There are three methods of extracorporeal circulation for leukapheresis: (1) leukapheresis using a centrifugal cell separator; (2) leukapheresis by an adsorptive membrane filter (Cellsorba™); and (3) granulocyte and monocyte/macrophage apheresis by a column filled with cellulose diacetate beads (Adacolumn™). Each method removes different cell populations and thus seems to work through different mechanisms. The former two methods deplete a large number of lymphocytes. As the depletion of lymphocytes by these methods is non-selective, numerous Treg are removed during leukapheresis, together with colitogenic effector lymphocytes. We have considered a possible new therapy in which Treg are isolated from the lymphocytes removed during leukapheresis, and retransfused into UC patients, and named it leukapheresis/Treg transfer therapy. By such therapy, selective depletion of colitogenic effector T cells from UC patients and an increase in the Treg/effector ratio are theoretically possible. To conduct such adoptive transfer therapy with Treg, a large-scale Treg isolation method, which is clinically applicable, needs to be established. We chose the CliniMACS cell selection system from Miltenyi Biotec for this purpose. CliniMACS is a magnetic bead separation method developed for large-scale sterile isolation of cells for clinical application, and has already been applied clinically for the enrichment of $CD34^+$ cells for peripheral blood stem cell transplantation [18,19]. The aim of this study was to establish an appropriate method for *in vitro* Treg-enrichment from apheresis products from UC patients, which is applicable to Treg transfer therapy.

Methods

Patients

Four patients with moderate to severe UC, admitted to Kyushu University Hospital, Fukuoka, Japan, between 2006 and 2007, were enrolled in this study. The protocol of this study was approved by the institutional ethical committee at the Graduate School of Medical Sciences, Kyushu University. Written informed consent was obtained from all patients. Patients underwent centrifugal leukapheresis therapy weekly for 5–11 weeks. Clinical characteristics of the patients were summarized in Table 1. Patient 1 was given two separate courses of leukapheresis because she relapsed after remission induced by the first treatment. An isolation experiment of Treg was conducted

Table 1. Patient characteristics

CliniMACS run number	Patient number	Age (years)	Sex	Type of disease	Clinical activity index‡ at the initiation of the treatment	Concomitant treatment
1	1*	70	Female	Left-sided colitis	10	Prednisolone, 5-aminosalicylic acid
2	2	59	Female	Pancolitis	12	Total parenteral nutrition, prednisolone
3	1†	70	Female	Left-sided colitis	6	Prednisolone, 5-aminosalicylic acid
4	3	44	Male	Left-sided colitis	12	Total parenteral nutrition, prednisolone, 5-aminosalicylic acid
5	4	18	Male	Pancolitis	16	Total parenteral nutrition, prednisolone, azathioprine, cyclosporine A

**First treatment for patient 1.*
†Second treatment for patient 1.
‡Rachmilewitz criteria.

in each course of the leukapheresis for each patient. Concomitant pharmacotherapy included 5-aminosalicylates and peroral and intravenous prednisone and azathioprine. Patient 4 underwent leukapheresis after intravenous cyclosporine A.

Isolation of Treg from leukapheresis products using CliniMACS

Five large-scale cell-separation experiments with clinical-grade CliniMACS reagents were performed. The CliniMACS system (Miltenyi Biotec, Bergisch-Gladbach, Germany) includes an instrument, circuit tubes, clinical-grade magnetic beads reagents and computer programs. All of the procedures were done in sterile closed circuits. The leukapheresis product (LP) cells were washed, adjusted to 88 mL with phosphate-buffered saline (PBS)/ethylenediaminetetra-acetic acid (EDTA) buffer (Miltenyi Biotec), supplemented with 2% human serum albumin (HSA; Mitsubishi Pharma, Osaka, Japan) and one vial each of clinical-grade magnetic beads bearing anti-CD8 and anti-CD19 monoclonal antibodies (MAb) (CliniMACS CD8 MicroBeads and CliniMACS CD19 MicroBeads; Miltenyi Biotec), and incubated for 30 min at room temperature with frequent manual agitation in a cell-preparation bag. Then cells were centrifuged at 300 *g*, washed again, and resuspended in 60 mL PBS/EDTA/HSA. B cells and $CD8^+$ T cells were depleted with the CliniMACS^{plus} instrument (Miltenyi Biotec) by using the LS tubing set (Miltenyi Biotec) and the depletion program 2.1. B cell/$CD8^+$ T-cell depleted cells were suspended in 190 mL PBS/EDTA/HSA, labeled with 7.5 mL magnetic beads bearing anti-CD25 MAb (CliniMACS CD25 MicroBeads; Miltenyi Biotec) for 30 min at room temperature, washed, and resuspended in 100 mL PBS/EDTA/HSA. $CD25^+$ cells were isolated by three automatic cycles of positive selection using the enrichment program 3.1 of the CliniMACS device. Upon completion of the enrichment program, CD25-enriched (target cell fraction; TCF) and CD25-depleted cell fractions (non-target cell fraction; NTCF) were collected in separate collection bags. Aliquots before and after each step were taken for flow cytometric analysis.

Flow cytometry

Cells were resuspended in fluorescence-activated cell sorter (FACS) buffer, which consisted of PBS with 0.5% bovine serum albumin (BSA) at 10^7 cells/mL. For analysis of CD25 expression after magnetic cell separation, 1×10^6 cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (Miltenyi Biotec) and biotin-conjugated anti-human CD25 (4E3; Miltenyi Biotec) at 4°C in the dark for 15 min. Then the cells were washed and incubated with phycoerythrin (PE)-labeled anti-biotin antibody (Ab)

(Bio3-18E7; Miltenyi Biotec). Next, erythrocytolysis was performed using Pharm Lyse (BD Biosciences, San Jose, CA, USA). Cells were washed twice, briefly stained with 7-amino-actinomycin-D for non-viable cell exclusion, and subsequently analyzed by a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest software (Becton Dickinson). Expression of FOXP3 was detected using the FITC–anti-human FOXP3 staining kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. FITC-conjugated rat IgG2a (eBioscience) was used as an isotype control. The analysis was performed with a FACScan (Becton Dickinson) using CellQuest software. For detection of gut-homing receptors, expanded cells suspended in FACS buffer were incubated at 4°C with FITC-conjugated anti-integrin β7, PE-conjugated anti-αE integrin (CD103) or anti-α4 integrin (CD49d), PE–Cy5-conjugated anti-CD4 (L3T4) (eBioscience) in the dark for 20 min. FITC-conjugated rat IgG2a and PE-mouse IgG1 (eBioscience) were used as an isotype control. After washing, the cells were analyzed in the same way as other stained cells.

Suppressor function assays

Mononuclear cells were prepared from NTCF using Ficoll density-gradient centrifugation (Lymphocyte Separation Medium; ICN Biochemicals, Aurora, OH, USA). Mononuclear cells were incubated with magnetic beads bearing anti-CD4 MAb (CD4 MicroBeads; Miltenyi Biotec) and separated into $CD4^+$ $CD25^-$ and $CD4^-$ $CD25^-$ cells. 5×10^4 purified $CD4^+$ $CD25^-$ responder T cells were mixed with TCF cells or cultured cells at different ratios in a U-bottomed 96-well plate (Corning, Corning, NY, USA), stimulated with 1 μg/mL soluble anti-CD3 (BD Pharmingen, San Diego, CA, USA) and 1.5×10^5 mitomycin C-treated autologous $CD4^-$ $CD25^-$ cells, and cultured for 4 days. [^{3}H]thymidine (1 μCi) was pulsed to each well during the final 18 h of culture. Incorporation of [^{3}H]thymidine was measured by scintillation counting. Results are expressed in c.p.m. as the mean of triplicate cultures. Percentage proliferation was then calculated as (thymidine incorporation of each cell culture/thymidine incorporation of responder T Cells × 100.

Cell expansion

TCF cells purified by CliniMACS or $CD4^+$ $CD25^-$ T cells (1×10^6/well) were stimulated with Treg expander beads (Dynal Biotech ASA, Oslo, Norway) at a ratio of four beads per cell, and cultured for 10 days in complete X-vivo 15 medium (BioWhittaker, Walkersville, MD, USA), supplemented with 2 mM L-glutamine (Gibco BRL, Grand Island, NY, USA), 25 mM HEPES (Gibco BRL), 100 U/mL penicillin (Gibco BRL), 100 μg/mL streptomycin (Gibco BRL), 10% heat-inactivated human AB serum (Gemini Bioproducts, Woodland, CA, USA) and with 500 U/mL recombinant interleukin-2 (rIL-2; R&D Systems, Minneapolis, MN, USA) in the presence or absence of 2 ng/mL transforming growth factor (TGF)-β1 (R&D Systems). Cultures were split in half after 3 days. When the cell density was $> 2 \times 10^6$ cells/mL, the cultures were spilt to a density of $0.5–1.0 \times 10^6$ cells/mL in complete X-vivo 15 medium containing 500 U/mL rIL-2. Cells were separated from Dynabeads by a magnet and rested in complete X-vivo 15 medium containing 100 U/mL rIL-2 for 2 weeks for functional assays and flow cytometric analysis.

Statistical analysis

Data were analyzed by paired *t*-test. A *P*-value was considered to be significant when < 0.05.

Results

Enrichment of $CD4^+$ $CD25^{high}$ $FOXP3^+$ Treg by CliniMACS cell separation

To actualize Treg transfer therapy for UC patients in combination with leukapheresis, it is necessary to establish a method for large-scale isolation of Treg from LP that are clean enough to be used clinically. As mentioned above, we utilized the CliniMACS cell selection system. $CD25^+$ cells in human peripheral blood leukocytes comprise $CD4^+$ $CD25^+$ T cells that include Treg, $CD8^+$ $CD25^+$ T cells and $CD19^+$ $CD25^+$ B cells. Our strategy to isolate Treg from LP was to deplete $CD19^+$ B and $CD8^+$ cytotoxic T cells from LP as a first step, followed by positive selection of $CD25^+$ cells as a second step. After the first step depletion, all of the $CD25^+$ cells were theoretically $CD4^+$ $CD25^+$ T cells. Thus the second step selection of $CD25^+$ cells was considered to have enriched Treg. To test whether this isolation strategy was suited for potential clinical application, we conducted five experiments of Treg isolation with CliniMACS from LP from UC patients; the results are summarized in Table 2. The initial LP contained $1.89 \times 10^9–8.57 \times 10^9$ leukocytes (average 4.99×10^9). On average, 11.0% (range 3.3–21.2%) of the cells co-expressed CD4 and CD25, and 1.96% were $CD4^+$ $CD25^{high}$ T cells (range 1.0–3.3%).

Table 2. Results of isolation of $CD4^+$ $CD25^{high}$ Treg from LP from UC patients using the CliniMACS system

	LP								TCF					Collection rate (%)
	Percentage of total cells					Cell number		Percentage of total cells				Cell number		
CliniMACS run number	$CD4^+$	$CD4^+$ $CD25^+$	$CD4^+$ $CD25^{high}$	$CD8^+$	$CD20^+$	Total cells	$CD4^+$ $CD25^{high}$	$CD4^+$	$CD4^+$ $CD25^+$	$CD4^+$ $CD25^{high}$	$CD8^+$	$CD20^+$	Total cells	$CD4^+$ $CD25^{high}$	$CD4^+$ $CD25^{high}$
1	17.0	7.4	3.1	7.1	7.1	1.89×10^9	5.86×10^7	90.0	88.3	52.6	0	0	5.50×10^7	2.89×10^7	49.3
2	36.2	21.2	1.0	12.1	14.5	5.00×10^9	5.00×10^7	96.8	96.6	39.2	0.0017	0.0068	9.80×10^7	3.84×10^7	76.8
3	16.9	11.7	1.1	10.4	11.7	4.51×10^9	4.94×10^7	93.4	93.3	51.9	0.0028	0.053	7.30×10^7	3.78×10^7	76.5
4	17.9	11.5	3.3	12.3	9.8	8.57×10^9	2.81×10^8	82.5	81.6	65.5	0.0021	0.052	2.10×10^8	1.37×10^8	48.8
5	8.6	3.3	1.33	7.7	2.9	4.96×10^9	6.61×10^7	92.8	92.2	74.1	0.0081	0	7.80×10^7	5.78×10^7	87.4
Average	19.3	11.0	1.96	9.9	9.2	4.99×10^9	1.01×10^8	91.1	90.4	56.6	0.0029	0.022	1.03×10^8	6.00×10^7	67.8

After magnetic cell separation, 5.5×10^7–2.1×10^8 cells (average 1.03×10^8) were collected in the TCF (2.1% of the total LP cells on average; range 1.57–2.91%), which contained $< 0.01\%$ $CD8^+$ T cells (range 0–0.0081%), $< 0.06\%$ B cells (range 0–0.053%) and 91.1% $CD4^+$ T cells (range 82.5–96.8%). On average, 6.0×10^7 $CD4^+$ $CD25^{high}$ cells were collected in the TCF (range 2.89×10^7–1.37×10^8). Only 0.70% of the isolated cells were $CD4^+$ $CD25^-$ (range 0.11–1.66%), whereas 90.4% (range 81.6–96.6%) of the cells expressed both CD4 and CD25. The TCF was highly and preferentially enriched for $CD4^+$ $CD25^{high}$ T cells, which constituted 56.6% on average (range 39.2–74.1%) of the isolated cells. The recovery rate of $CD4^+$ $CD25^{high}$ T cells, as calculated from their frequencies in the initial LP and TCF, was 67.8% (range 49.3–87.4%). Representative FACS data illustrating the preferential retention of $CD4^+$ $CD25^{high}$ T cells after the magnetic enrichment for CD25-expressing cells are depicted in Figure 1A. In the LP (Figure 1A, left panel), $CD4^+$ $CD25^{high}$ T cells are identified not only by their high CD25 expression but also by their slightly lower levels of CD4 expression compared with those of $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^-$ T cells. After three repetitive enrichment cycles for CD25, an efficient retention of $CD4^+$ $CD25^{high}$ T cells in the TCF was achieved (Figure 1A, middle panel), whereas residual $CD4^+$ $CD25^{high}$ T cells were almost undetectable in the CD25-depleted NCTF (Figure 1A, right panel). In addition, the proportion of $CD4^-$ $CD25^+$ cells in the TCF was reduced because of the depletion of activated $CD25^+$ B cells and $CD8^+$ $CD25^+$ T cells (Figure 1A, middle panel).

To verify the enrichment of Treg in the TCF, LP, TCF and NTCF were analyzed at the single-cell level for expression of the Treg-specific markers FOXP3. As shown in Figure 1B, FOXP3 was preferentially expressed in $CD4^+$ $CD25^{high}$ T cells, whereas $CD4^+$ $CD25^{low}$ T cells contained less FOXP3, and the $CD4^+$ $CD25^-$ subpopulation did not contain FOXP3. As expected, the $CD4^+$ T cells in the TCF were highly enriched with $FOXP3^+$ cells (Figure 1B, middle panel) whereas the NTCF contained only very few $FOXP3^+$ cells (Figure 1B, right panel). The percentages of $CD4^+$ T cells expressing FOXP3 among the total $CD4^+$ T cells before and after isolation in each experiment are shown in Table 3. In LP, only 5.5% (range 0.42–10.4%) of $CD4^+$ T cells were $FOXP3^+$. In TCF, 56.4% (range 51.3–66.6%) of $CD4^+$ T cells expressed

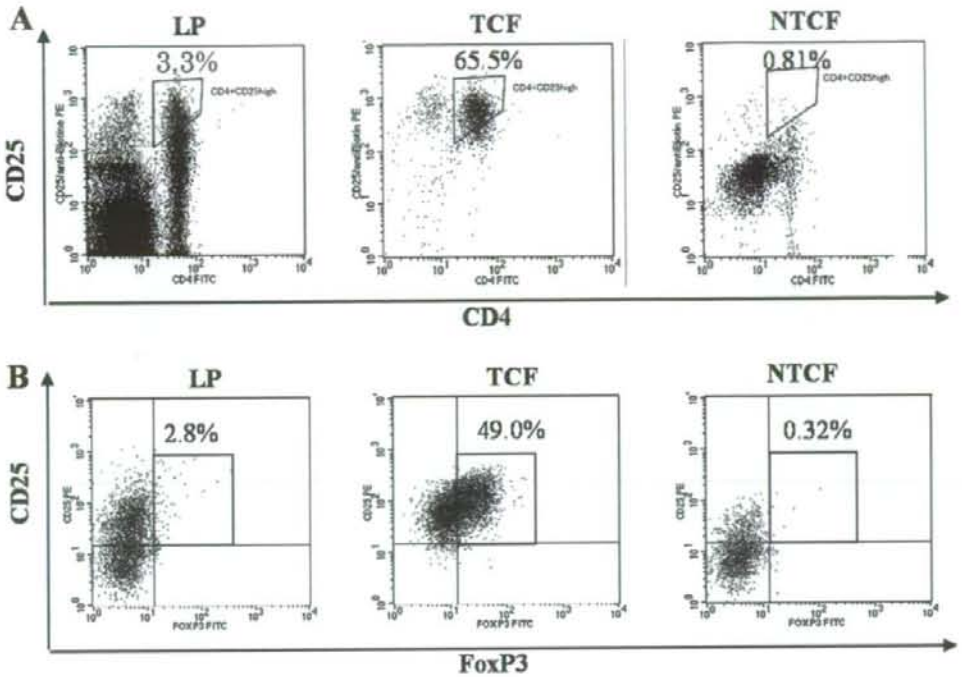

Figure 1. Flow cytometry of isolated cells by CliniMACS from LP from UC patients. LP were depleted of $CD8^+$ T and B cells and separated into $CD25^+$ TCF and NTCF. Before and after isolation, aliquots were taken and analyzed by flow cytometry. Representative data of five independent experiments are shown. (A) Cells were stained with FITC-conjugated anti-CD4 and biotin-conjugated anti-CD25 plus PE-conjugated anti-biotin Ab. Data in the quadrant indicate the percentage of $CD4^+$ $CD25^{high}$ T cells among the total number of cells. (B) Cells from each fraction were stained with Cy-Chrome-conjugated anti-CD4 and biotin-conjugated anti-CD25/PE-conjugated anti-biotin Ab, washed, fixed, permeabilized and stained with FITC-conjugated anti-FOXP3. The $CD4^+$ gate is shown. The percentages of $CD25^+$ $FOXP3^+$ among $CD4^+$ T cells are shown.

FOXP3, which showed that Treg were truly concentrated. In summary, these data revealed a preferential enrichment of $CD4^+$ $CD25^{high}$ $FOXP3^+$ T cells from LP by the two-step isolation strategy using the CliniMACS system.

Suppressor activity of enriched Treg by CliniMACS

To determine whether selective separation of functional Treg was done successfully by the CliniMACS cell selection, we assessed the suppressor activity of the isolated TCF cells. $CD4^+$ $CD25^-$ T cells and/or TCF were stimulated with anti-CD3 MAb and antigen-presenting cells (APC). As shown in Figure 2, cultures containing $CD4^+$ $CD25^-$ T cells alone proliferated vigorously, whereas cultures containing TCF alone were hypoproliferative, a characteristic of Treg. When $CD4^+$ $CD25^-$ T cells were co-cultured with equal numbers of TCF cells, T-cell proliferation was suppressed. This suppressive effect was enhanced by increasing the number of TCF cells (data not shown). These data demonstrated that successful enrichment of functional Treg in the TCF was achieved by CliniMACS cell separation. The isolated cells were, therefore, considered to be suitable for Treg transfer therapy.

In vitro expansion of Treg using anti-CD3/anti-CD28-coated beads

The intestine is not only the digestive organ but also the biggest lymphoid tissue in the body, containing numerous lymphocytes. Thus the question arises of how many Treg need to be transferred to regulate colonic inflammation. Although fairly large numbers of Treg could be recovered by one session of CliniMACS cell separation from LP, it may be necessary to transfer more Treg. Therefore, we conducted further experiments to investigate whether isolated Treg can be expanded *in vitro* by retaining their

Table 3. Percentages of FOXP3-expressing cells among $CD4^+$ T cells before and after CliniMACS selection

CliniMACS run number		1	2	3	4	5	Average
LP	$CD4^+$ $FOXP3^+$/$CD4^+$ T cell (%)	0.4	6.9	10.4	4.2	5.7	5.5
TCF	$CD4^+$ $FOXP3^+$/$CD4^+$ T cell (%)	54.3	66.6	51.3	52.4	57.3	56.4

immunoregulatory function. Poor proliferative potential upon stimulation through T-cell receptors is a well-known characteristic of Treg, and might be an obstacle for their use in sufficient numbers as an immunotherapeutic modality. Nevertheless, the anergic phenotype of Treg is not absolute and can be broken by synergistic stimulation through T-cell receptors with appropriate co-stimulatory signals or growth factors, such as CD28 signaling and IL-2 [5,6,20]. Therefore, we decided to use magnetic beads coupled with anti-CD3 and anti-CD28 Ab as artificial APC [21–23] in the presence of IL-2. As TGF-β has been reported to be an inducer of Treg development [24,25], we also analyzed the stimulation conditions in the presence of TGF-β1. We stimulated TCF cells and $CD4^+$ $CD25^-$ T cells as a control; the results are summarized in Table 4. TCF proliferated with anti-CD3/anti-CD28-coated beads and 500 U/mL IL-2 to a level comparable with $CD4^+$ $CD25^-$ T cells (Figure 3 and Table 4). Similarly,

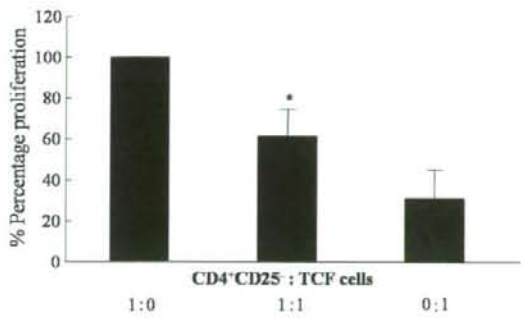

Figure 2. *Suppressor function of Treg isolated by CliniMACS from LP from UC patients. The TCF isolated by CliniMACS were hypoproliferative and showed suppressor activity. Autologous $CD4^+$ $CD25^-$ T cells and/or the TCF cells were co-cultured and stimulated with anti-CD3, in the presence of autologous APC for 4 days. Cells were pulsed with [^{3}H]thymidine (1 μCi) and the level of the proliferation is shown as the percentage proliferation, compared with the count with culture of $CD4^+$ $CD25^-$ T cells alone. Data represent the means ± SE of four independent experiments.* *$P < 0.05$ *for co-culture of $CD4^+$ $CD25^-$ and TCF cells compared with $CD4^+$ $CD25^-$ alone.*

in the presence of TGF-β1, TCF and $CD4^+$ $CD25^-$ T cells proliferated to a comparable level (Figure 3 and Table 4).

FACS analysis revealed that TCF cells expanded *in vitro* maintained higher levels of FOXP3 expression than cultured $CD4^+$ $CD25^-$ T cells (Figure 4, left and right center). When stimulated in the presence of TGF-β1, FOXP3 expression was up-regulated in both TCF and $CD4^+$ $CD25^-$ populations (Figure 4, left center and right). These results suggest that Treg can be expanded and maintained in cell culture in the presence of IL-2 and CD28 signaling, and that TGF-β1 induces the development of Treg from non-Treg.

We analyzed the function of expanded cells. Expanded TCF cells did not proliferate in response to stimulation in the same manner as freshly isolated Treg. In contrast, $CD4^+$ $CD25^-$ T cells proliferated well under these conditions, and their proliferation was suppressed in co-culture with *in vitro*-expanded TCF cells (Figure 5, left panel). The suppressor activity of bead-expanded TCF cells was stronger than that of freshly isolated TCF cells from the same patient (data not shown). In contrast, *in vitro*-expanded $CD4^+$ $CD25^-$ T cells proliferated vigorously and did not suppress the proliferation of $CD4^+$ $CD25^-$ T cells upon co-culture (Figure 5, right center). When cultured in the presence of TGF-β1, not only TCF cells but also $CD4^+$ $CD25^-$ cells revealed a regulatory capability. Cultured $CD4^+$ $CD25^-$ T cells became hyporesponsive to stimulation and suppressed the proliferation of $CD4^+$ $CD25^-$ T cells upon co-culture to a similar extent as TCF cells (Figure 5, left center and right). *In vitro* culture with TGF-β1 did not further enhance the regulatory activity of TCF (Figure 5, left and left center). Similar results were obtained with allogeneic stimulation (data not shown). Hence, Treg in the TCF expanded *in vitro* retained their characteristic phenotype of Treg, as well as their suppressive function. In addition, it is suggested that TGF-β1 induces the development of Treg *in vitro* from non-Treg.

Table 4. Results of expansion of $CD4^+$ $CD25^{high}$ Treg from LP from UC patients using the CliniMACS system

CliniMACS run number	TCF			TCF + TGFβ1			$CD4^+$ $CD25^-$			$CD4^+$ $CD25^-$ + TGFβ1		
	Fold-expansion	FOXP3$^+$ CD25$^+$ / $CD4^+$ T cells, (%)		Fold-expansion	FOXP3$^+$ CD25$^+$ / $CD4^+$ T cells, (%)		Fold-expansion	FOXP3$^+$ CD25$^+$ / $CD4^+$ T cells, (%)		Fold-expansion	FOXP3$^+$ CD25$^+$ / $CD4^+$ T cells, (%)	
2	15.8	53.3		10.8	73.3		5.5	11.9		7.6	59.0	
3	21.0	23.9		19.5	47.0		16.3	15.1		15.0	16.3	
4	20.8	49.8		32.0	59.1		16.0	4.8		24.4	66.1	
5	17.1	67.6		18.1	91.7		10.9	9.8		18.3	68.1	
Average	18.7	48.7		20.1	67.8		12.2	10.4		16.3	52.4	

For adoptive cell therapy for UC, it is hoped that expanded cells strongly express gut-homing receptors, such as α4(CD49d) β7 and αE(CD103)β7 integrins. As it has been previously reported that the expression of αE and β7 integrins is regulated by TGF-β1 [26,27], we investigated whether 10-day cultures in the presence or absence of TGF-β1 could up-regulate these integrins on the cell surface by flow cytometry. α4β7 and αEβ7 integrins were expressed only in the small percentage of expanded TCF cells and $CD4^+$ $CD25^-$ T cells without TGF-β1 (Figure 6, left and right center). The expression of αE and β7 integrins was up-regulated by TGF-β1 on both cell populations. The expression of α4 integrin was not altered significantly. In consequence, the expression of αEβ7 integrin and also α4β7 integrin on the surface of TCF cells and $CD4^+$ $CD25^-$ T cells was significantly increased by addition of exogenous TGF-β1 (Figure 6, left center and right). These results suggest that TGF-β1 enhances the expression of not only αEβ7 but also α4β7 integrin on the cell surface, and that treatment with TGF-β1 may be useful to enhance the Treg immunoregulatory capability to suppress intestinal inflammation by increasing the expression of gut-homing receptors and thus migration of injected Treg to the mucosal sites of inflammation.

Discussion

UC is an intractable disease that develops into chronic persistent colitis. As there are many patients who are refractory to conventional therapy, the generation of new, more effective therapies is desperately needed. Treg comprise a subset of $CD4^+$ T cells that possess regulatory activity of a broad range of immune reactions. As a result of their potent immunosuppressive capability, Treg are considered to be an attractive therapeutic tool for inflammatory human disorders. Extensive studies of animal models of IBD have revealed a potent and essential role of Treg for the prevention of colonic inflammation [2,3]. On account of their suppressive effect on experimental models of colitis, not only for prevention but also for therapeutic use, Treg are expected to be an attractive tool for the treatment of IBD. In humans, we and others have demonstrated that the proportion of Treg in the peripheral blood is significantly reduced in active UC [11,13]. As Treg are functionally preserved [10–12] and only quantitatively decreased in UC patients, Treg transfer

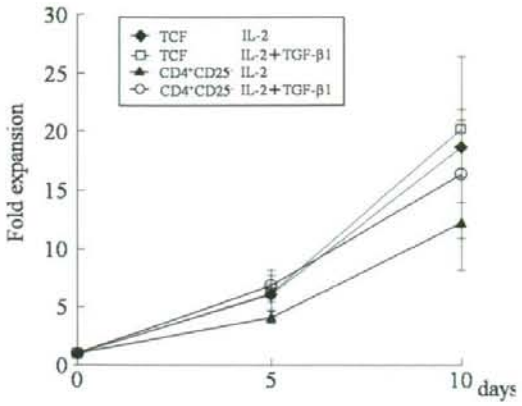

Figure 3. In vitro *expansion of Treg isolated by CliniMACS. Cells in the TCF of the CliniMACS selection and $CD4^+$ $CD25^-$ cells were activated with anti-CD3/anti-CD28-coated beads at a ratio of four beads per cell and 500 U/mL IL-2 in the presence or absence of 2 ng/mL TGF-β1. On days 5 and 10, cells were counted in aliquots of the cultures. The expansion of cells was demonstrated as a fold-increase compared with the original cell number at day 0 (1×10^6). Values represent means ± SE of four independent experiments.*

immunotherapy that enhances regulatory activity is thus expected to be efficacious for the treatment of UC.

Leukapheresis therapy is one of the standard therapies for UC in Japan and is mainly applied to steroid-resistant patients. During leukapheresis, it is considered that Treg, as well as colitogenic effector cells, are removed from the peripheral blood. It is, therefore, reasonable to expect better efficacy when Treg are isolated from LP and transferred to the patient. Thus, we chose a strategy to isolate and transfuse Treg following leukapheresis. For the application of leukapheresis, there are adsorptive and centrifugal methods [14–17]. The former utilizes columns to which leukocytes adhere. We chose the latter because intact leukocytes can be collected by this method. Centrifugal leukapheresis for patients with UC manages *c.* 2000–2500 mL peripheral blood per session, which is *c.* 40–45% of the total blood volume. In the present study, one session of centrifugal leukapheresis removed on average 9.67×10^8 $CD4^+$ T cells from the body and 6.0×10^7 Treg were isolated from LP. When isolated Treg are returned to patients just after leukapheresis, it is possible to achieve an increase of 1–3% in the ratio of Treg/total $CD4^+$ cells in the peripheral blood, which could add a suppressive effect of Treg to the conventional leukapheresis effect.

For clinical application, it is essential to isolate Treg safely and hygienically after leukapheresis. Although magnetic enrichment of $CD4^+$ $CD25^+$ Treg using anti-CD25 Ab-coated beads has already been applied in many laboratories for experimental purposes [9,28], it is unclear

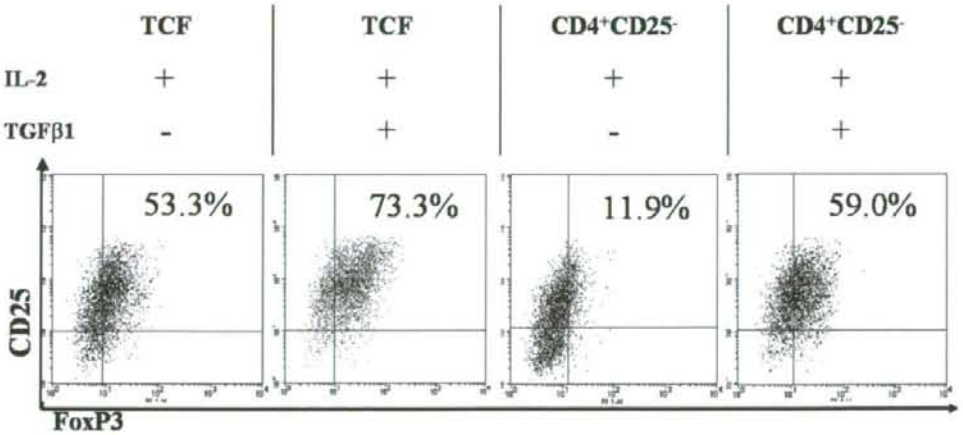

Figure 4. *FOXP3 expression in the* in vitro*-expanded cells. Cells in the TCF of the CliniMACS selection and $CD4^+$ $CD25^-$ cells were stimulated with anti-CD3/CD28 beads and IL-2 in the presence or absence of TGF-β1. Flow cytometry for FOXP3 expression was performed on day 24 (after resting for 2 weeks). Cells were stained with PE–anti-CD25 and PE-Cy5–anti-CD4, fixed, permeabilized, stained with FITC–anti-FOXP3 and analyzed by flow cytometry. Expression of CD25 and FOXP3 on the $CD4^+$ gate is shown. Data are representative of four independent experiments.*

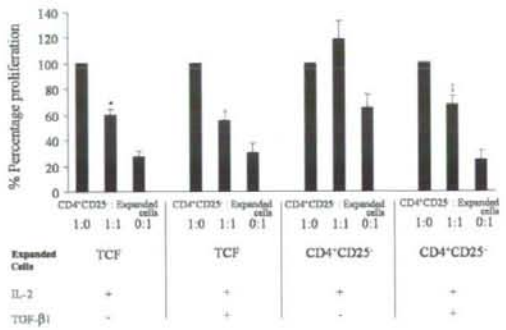

Figure 5. *Suppressor function of the* in vitro*-expanded cells. Cells in the TCF of the CliniMACS selection and $CD4^+$ $CD25^-$ cells were stimulated with anti-CD3/CD28 beads and IL-2 in the presence or absence of TGF-β1. After 10 days, cells were separated from beads by a magnet and rested for 2 weeks.* In vitro*-expanded TCF cells, TCF cells in the presence of TGF-β1, $CD4^+$ $CD25^-$ cells and $CD4^+$ $CD25^-$ cells in the presence of TGF-β1 were mixed with $CD4^+$ $CD25^-$ T cells and stimulated with anti-CD3 and mitomycin-C-treated APC. Proliferation was determined by [^{3}H]thymidine uptake, and the percentage proliferation was calculated by comparing with cultures of $CD4^+$ $CD25^-$ T cells alone. Data represent the means ± SE of four independent experiments.* *$P < 0.05$ *for co-culture of $CD4^+$ $CD25^-$ and each expanded cell compared with $CD4^+$ $CD25^-$ alone,* †$P < 0.05$ *for co-culture of $CD4^+$ $CD25^-$ and each expanded cell compared with $CD4^+$ $CD25^-$ alone,* ‡$P < 0.05$ *for co-culture of $CD4^+$ $CD25^-$ and each expanded cell compared with $CD4^+$ $CD25^-$ alone.*

what would be suited to the large-scale purification of Treg under sterile conditions for clinical applications. To this end, we sought to develop a strategy that ensures the efficient and reliable enrichment of human $CD4^+$ $CD25^{high}$ T cells from LP. We chose the CliniMACS cell selection system for this purpose. This system is a magnetic bead-based cell isolation system developed by Miltenyi Biotec for clinical purposes, and has been used clinically for such procedures as $CD34^+$ stem cell transplantation [18,19]. Our protocol comprised one cycle of $CD8^+$ T/B-cell depletion with anti-CD8 and anti-CD19 beads, followed by three repetitive enrichment cycles for $CD25^+$ cells. Pre-depletion was included to eliminate activated $CD25^+$ B cells and $CD8^+$ $CD25^+$ T cells. These cells would contaminate Treg-enriched cells to various degrees, depending on the blood composition of each patient. Therefore, after almost all $CD4^-$ $CD25^+$ cells were deleted, the TCF could include Treg at high purity. In fact, B cells and $CD8^+$ T cells were almost undetectable in the cell products after depletion, and a low percentage of $CD4^-$ $CD25^+$ cells was only present in the TCF. It has recently been shown that a single magnetic cell-separation step efficiently depletes human Treg from LP but is insufficient for their enrichment [29]. Hoffmann *et al.* [30] have reported a protocol for Treg enrichment that comprises B-cell depletion with anti-CD19 beads followed by enrichment for $CD25^+$ cells. However, the low percentage of $CD8^+$ T cells still contaminates the TCF by their method. We consider that it is safer to deplete $CD8^+$ $CD25^+$ T cells because such cells may act as colitogenic factors. In our protocol, the enrichment for CD25 ensured not only the preferential retention of cells with high CD25 expression but also an almost-complete elimination of $CD4^-$ $CD25^+$ cells. As expected from their CD25 expression profile, TCF contained a high percentage of FOXP3-expressing Treg, and potently suppressed non-Treg proliferation in functional assays. In consequence, Treg could be isolated in fairly good numbers and with good recovery rates and, more importantly, isolated Treg retained their immunoregulatory activity. Thus Treg can be isolated from LP in large numbers that are clean enough for clinical use, and Treg transfer therapy is feasible in combination with centrifugal leukapheresis for UC.

The mechanism of Treg-mediated suppression of intestinal inflammation is not fully understood. Most *in vitro* studies have failed to identify a soluble suppressor cytokine for $CD4^+$ $CD25^+$ Treg activity [5,6]. *In vivo*, however, an experimental model of colitis revealed that suppression of colonic inflammation depends on immunosuppressive cytokines, IL-10 and TGF-β, as administration of Ab-neutralizing TGF-β [2] and IL-10 [31] to recipient mice abrogated the protective effect of Treg. We have previously demonstrated that Treg express a high level of TGF-β1 [32]. We have shown that Treg-produced TGF-β1 is essential for the suppression of colitis, as Treg isolated from TGF-β1-deficient mice fail to ameliorate the disease [33]. In contrast, it is not always required for the inhibition of T-cell proliferation *in vitro* [33]. Recently, Li *et al.* [34] have reported that Treg from T-cell specific TGF-β1 knockout mice failed to suppress a $CD4^+$ $CD45RB^{high}$ T-cell transfer model of colitis, which demonstrates that TGF-β1 produced from Treg is essential for gut homeostasis. It is therefore of interest whether

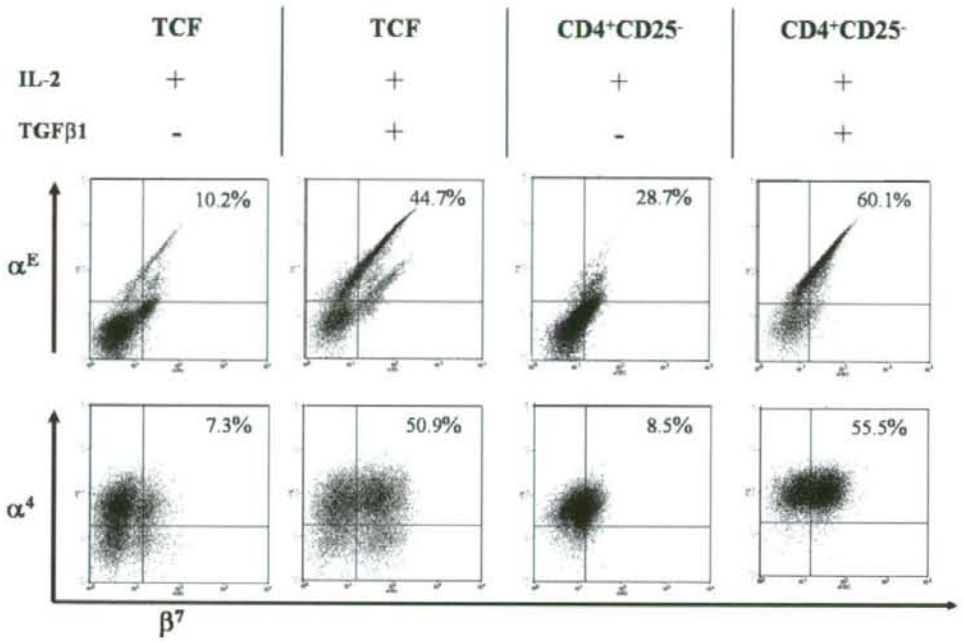

Figure 6. *αEβ7 and α4β7 expression on the* in vitro*-expanded cells. Cells in the TCF of the CliniMACS selection and $CD4^+$ $CD25^-$ T cells were stimulated with anti-CD3/CD28 beads and IL-2 in the presence or absence of TGF-β1. αEβ7 and α4β7 expression was analyzed by FACS after a 10-day culture. Cells were stained with PE–Cy5-anti-CD4, PE–anti-CD103 (αE) or PE–anti-CD49d (α4) and FITC–anti-β7 and analyzed by flow cytometry. Expression of αEβ7 and α4β7 on the $CD4^+$ gate is shown. Data are representative of two independent experiments.*

Treg mobilized to the intestine in IBD patients express high levels of TGF-β1 and IL-10.

In Treg transfer therapy for UC, the number of Treg that are considered sufficient for amelioration of colonic inflammation is unclear. Collection of 1×10^8 $CD4^+$ $CD25^{high}$ cells per session of CliniMACS separation from LP is fairly good. However, more Treg may be needed to suppress intestinal inflammation as the intestine is the organ that contains the most immune cells in the body. Thus we conducted *in vitro* expansion of Treg. Treg isolated using CliniMACS could be expanded *in vitro* up to 18-fold in 10 days. Treg retained their immunosuppressive activity during *in vitro* culture. These results suggest that more Treg can be transferred to patients after *in vitro* expansion, at least theoretically. However, we need to pay much more attention to the safety of the transfer of such cultured cells than that for simple transfusion of isolated cells.

Treg develop in the thymus and were originally considered to be of a different lineage from conventional $CD4^+$ T cells [1]. Recently, however, TGF-β1 has been found to induce development of Treg from non-Treg *in vitro* [24,25]. Thus we tested whether CliniMACS-isolated Treg can be further enriched during cultivation in the presence of TGF-β1, and whether Treg can be induced from non-Treg by TGF-β1. Culture with TGF-β1 increased the percentage of $CD4^+$ $FOXP3^+$ cells in the TCF, although TGF-β1-treated TCF cells did not show a significant increase in suppression. Cultivation of $CD4^+$ $CD25^-$ T cells in the presence of TGF-β1 *in vitro* significantly increased the proportion of $CD4^+$ $FOXP3^+$ cells, and such cells showed suppressor activity to reduce T-cell proliferation. Thus, as reported previously in healthy subjects [24,25], TGF-β1 can induce Treg differentiation *in vitro* from non-Treg obtained from UC patients. Induction of Treg *in vitro* may be an alternative approach to conduct Treg-based immunotherapy.

Lymphocyte trafficking is mainly regulated by adhesion molecules, L-selectin and integrins. The integrin α4β7 is a homing receptor for cellular migration of T lymphocytes to mucosal sites [35,36]. The α4β7 integrin binds to

vascular addressins, selectively expressed by venules in mucosal tissues. Expression of the other β7 integrin, αEβ7, on T cells is thought to be important for intestinal intraepithelial T-lymphocyte (IEL) entry into the epithelial compartment [37,38]. It has been postulated that lymphocytes entering the gastrointestinal tract from the peripheral blood do so via an interaction of α4β7 on their surface with the mucosa-associated cell adhesion molecule on endothelial cells. Subsequent to migration, the αE subunit is up-regulated by TGF-β1 in the microenvironment of the intestine. The αEβ7 integrin is suggested to interact with E-cadherin on the enterocyte surface, thereby mediating selective localization or retention of IEL in the epithelium. Therefore, we analyzed α4β7 and αEβ7 expression of expanded cells. In contrast to the TGF-β1 untreated cells, we could detect higher levels of expression of αEβ7 and α4β7 on expanded TCF and $CD4^+ CD25^-$ T cells in the presence of TGF-β1, and each of TGF-β1-treated cells (Treg and non-Treg) showed suppressive properties for conventional $CD4^+ CD25^-$ T cells *in vitro*. Taken together, the treatment with TGF-β1 may support clinical cell therapy for mucosal inflammation using *in vitro*-induced or -expanded human Treg by up-regulating the expression of gut-homing receptors and thus the trafficking of infused Treg to the gut.

In summary, we have established a protocol to isolate Treg from LP from UC patients in clinically relevant numbers under sterile conditions, and this enables clinical trials of Treg transfer therapy for UC patients, in combination with leukapheresis. Successful expansion or induction of Treg *in vitro* demonstrates the future possibility of transfer therapy of cultivated Treg.

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References

1 Sakaguchi S. Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004;22:531–62.

2 Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* 2000;192:295–302.

3 Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by $CD4^+$ $CD25^+$ regulatory T cells. *J Immunol* 2003;170:3939–43.

4 Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 1996;184:387–96.

5 Thornton AM, Shevach EM. $CD4^+$ $CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 1998;188:287–96.

6 Takahashi T, Kuniyasu Y, Toda M *et al.* Immunologic self-tolerance maintained by $CD25^+$ $CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 1998;10:1969–80.

7 Itoh M, Takahashi T, Sakaguchi N *et al.* Thymus and autoimmunity: production of $CD25^+$ $CD4^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 1999;162:5317–26.

8 Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. $CD4^+$ $CD25^{high}$ regulatory cells in human peripheral blood. *J Immunol* 2001;167:1245–53.

9 Jonuleit H, Schmitt E, Stassen M *et al.* Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 2001;193:1285–94.

10 Makita S, Kanai T, Oshima S *et al.* $CD4^+$ $CD25^{bright}$ T cells in human intestinal lamina propria as regulatory cells. *J Immunol* 2004;173:3119–30.

11 Maul J, Loddenkemper C, Mundt P *et al.* Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. *Gastroenterology* 2005;128:1868–78.

12 Holmen N, Lundgren A, Lundin S *et al.* Functional $CD4^+$ $CD25^{high}$ regulatory T cells are enriched in the colonic mucosa of patients with active ulcerative colitis and increase with disease activity. *Inflamm Bowel Dis* 2006;12:447–56.

13 Takahashi M, Nakamura K, Honda K *et al.* An inverse correlation of human peripheral blood regulatory T cell

frequency with the disease activity of ulcerative colitis. *Dig Dis Sci* 2006;51:677–86.

14 Shimoyama T, Sawada K, Hiwatashi N *et al.* Safety and efficacy of granulocyte and monocyte adsorption apheresis in patients with active ulcerative colitis: a multicenter study. *J Clin Apheresis* 2001;16:1–9.

15 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;100:1362–9.

16 Hanai H, Watanabe F, Takeuchi K *et al.* Leukocyte adsorptive apheresis for the treatment of active ulcerative colitis: a prospective, uncontrolled, pilot study. *Clin Gastroenterol Hepatol* 2003;1:28–35.

17 Kohgo Y, Hibi H, Chiba T *et al.* Leukocyte apheresis using a centrifugal cell separator in refractory ulcerative colitis: a multicenter open label trial. *Therapeutic Apheresis* 2002;6:255–60.

18 Handgretinger R, Lang P, Ihm K *et al.* Isolation and transplantation of highly purified autologous peripheral CD34(+) progenitor cells: purging efficacy, hematopoietic reconstitution and long-term outcome in children with high-risk neuroblastoma. *Bone Marrow Transplant* 2002;29:731–6.

19 Lang P, Klingebiel T, Bader P *et al.* Transplantation of highly purified peripheral-blood $CD34^+$ progenitor cells from related and unrelated donors in children with nonmalignant diseases. *Bone Marrow Transplant* 2004;33:25–32.

20 Levings MK, Sangregorio R, Roncarolo MG. Human CD25(+)CD4(+) T regulatory cells suppress naive and memory T cell proliferation and can be expanded *in vitro* without loss of function. *J Exp Med* 2001;193:1295–302.

21 Godfrey WR, Ge YG, Spoden DJ *et al. In vitro*-expanded human CD4(+)CD25(+) T-regulatory cells can markedly inhibit allogeneic dendritic cell-stimulated MLR cultures. *Blood* 2004; 104:453–61.

22 Hoffmann P, Eder R, Kunz-Schughart LA *et al.* Large-scale *in vitro* expansion of polyclonal human CD4(+)CD25high regulatory T cells. *Blood* 2004;104:895–903.

23 Karakhanova S, Munder M, Schneider M *et al.* Highly efficient expansion of human $CD4^+$ $CD25^+$ regulatory T cells for cellular immunotherapy in patients with graft-versus-host disease. *J Immunother* 2006;29:336–49.

24 Chen WJ, Jin WW, Hardegen N *et al.* Conversion of peripheral $CD4^+$ $CD25^-$ naive T cells to $CD4^+$ $CD25^+$ regulatory T cells by TGF-β induction of transcription factor Foxp3. *J Exp Med* 2003;198:1875–86.

25 Fantini MC, Becker C, Monteleone G *et al.* Cutting edge: TGF-beta induces a regulatory phenotype in $CD4^+$ $CD25^-$ T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 2004;172:5149–53.

26 Smith TJ, Ducharme LA, Shaw SK *et al.* Murine M290 integrin expression modulated by mast cell activation. *Immunity* 1994; 1:393–403.

27 Lim SP, Leung E, Krissansen GW. The beta7 integrin gene (Itgb-7) promoter is responsive to TGF-beta1: defining control regions. *Immunogenetics* 1998;48:184–95.

28 Dieckmann D, Plottner H, Berchtold S *et al. Ex vivo* isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med* 2001;193:1303–10.

29 Powell DJ, Parker LL, Rosenberg SA. Large-scale depletion of $CD25^+$ regulatory T cells from patient leukapheresis samples. *J Immunother* 2005;28:403–11.

30 Hoffmann P, Boeld TJ, Eder R *et al.* Isolation of $CD4^+$ $CD25^+$ regulatory T cells for clinical trials. *Biol Blood Marrow Transplant* 2006;12:267–74.

31 Asseman C, Mauze S, Leach MW *et al.* An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 1999;190:995–1004.

32 Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med* 2001;194:629–44.

33 Nakamura K, Kitani A, Fuss I *et al.* TGF-beta 1 plays an important role in the mechanism of $CD4^+$ $CD25^+$ regulatory T cell activity in both humans and mice. *J Immunol* 2004; 172:834–42.

34 Li MO, Wan YSY, Flavell RA. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity* 2007;26:579–91.

35 Berlin C, Bargatze RF, Campbell JJ *et al.* Alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell* 1995;80:413–22.

36 Berlin C, Berg EL, Briskin MJ *et al.* Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. *Cell* 1993;74:185–95.

37 Shibahara T, Si-Tahar M, Shaw SK, Madara JL. Adhesion molecules expressed on homing lymphocytes in model intestinal epithelia. *Gastroenterology* 2000;118:289–98.

38 Agace WW, Higgins JMG, Sadasivan B *et al.* T lymphocyte–epithelial cell interactions: integrin alpha(E)(CD103)beta(7), LEEP-CAM and chemokines. *Curr Opin Cell Biol* 2000;12:563–8.

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OTOLOGY

The engraftment of transplanted bone marrow-derived cells into the inner ear

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Abstract To investigate whether bone marrow-derived cells (BMC) would migrate and engraft into the sensory epithelium of the inner ear, BMC of green fluorescence protein (GFP) mice were transplanted into lethally irradiated recipient mice. Then the recipient mice were treated with streptomycin and immunohistochemical staining was performed to evaluate the migration and engraftment of donor BMC into the sensory epithelium of the inner ear. Immunohistochemical staining for GFP was found initially in the vascular epithelium and oral mucosa but not in the sensory epithelium of the inner ear. In the case of mouse, BMC may not migrate and be engrafted into the sensory epithelium of the inner ear.

Keywords Bone marrow · Transplantation · Inner ear · Engraftment · GFP mice

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Introduction

Many non-mammalian species continually produce new sensory hair cells of the inner ear and regenerate them in response to damage. The regenerated sensory hair cells arise from adjacent non-sensory (supporting) cells, by a proliferative response whereby supporting cells re-enter the mitotic cycle or direct transdifferentiation of supporting cells to the sensory cells without dividing [1, 2]. Unfortunately, in adult mammals, several authors have reported that in vivo differentiation of newly produced cells into the hair cells of the inner ear is rare or non-existent [3, 4]. Recent advances in techniques used to study non-mammalian sensory cells regeneration promise to broaden our understanding of molecular signaling to trigger supporting cells to generate new sensory cells [5]. Another new area of study in tissue-engineering also has recently been developed and this technique may provide a new way to regenerate the injured sensory cells of mammalian inner ear. Numerous studies have shown that bone marrow-derived cells (BMC) display tremendous transdifferentiation [6–11] and also we have demonstrated that BMC can be engrafted in the olfactory epithelium and then differentiate into olfactory neuron cells [12]. In case of the inner ear, intravenously transplanted mouse-BMC into irradiated adult mice was found in the spiral ligament and the locations normally occupied by fibrocytes and mesenchymal cells [13]. Naito et al. [14] isolated chinchillas' BMC, labeled with Dil (Molecular Probes, Eugene, OR), and injected it directly into the inner ear of the same animals, which had been treated with gentamicin. Matsuoka et al. [15] isolated BMC from mice and injected it directly into the inner ear of Mongolian gerbils. In those studies, the BMC directly transplanted into the cochlea were found in the scala tympani, scala vestibule, spiral ligament or modiolus [14, 15]. To our

knowledge, there have been few reports, which suggest the possibility of BMC migrating into the sensory epithelium of the inner ear [16]. We performed an experiment in which BMC of green fluorescence protein (GFP) mice were transplanted into lethally irradiated recipient mice followed by treatment with streptomycin. Since Lang et al. [13] have reported that adult mice receiving unmanipulated BMC showed inner ear engraftment levels about four times higher than those receiving clonal hematopoietic stem cells we used unmanipulated BMC including mesenchymal stem cells. The efficacy of adult stem cell engraftment increases in damaged organs [17]. Thus, we tried to add damage to the inner ear by streptomycin injection after radiation. Some studies have suggested that patients can recover vestibular function after severe vestibular function loss caused by gentamicin and streptomycin ototoxicity [18, 19]. The vestibular epithelium in adult rodents shows a small but significant increase in cell proliferation in response to hair cell damage in vivo [20]. These reports might suggest that the vestibular epithelium of mammals including humans appear to contain cells with stem cell properties. We are interested in whether BMC would supply the stem cells of the vestibular sensory cells and contribute to the recovery of injured vestibule. We focused on the sensory epithelium of the vestibule in order to evaluate the migration and engraftment of BMC into the sensory cells of the inner ear.

Materials and methods

Animals and animal care

Female GFP transgenic mice (C57BL/6 TgN (act-EGFP)OsbC14-Y01-FM131) were gifted by Dr. Okabe [21] and female C57BL/6 mice were purchased from Okayama University Animal Center. All animals used in the present study were housed, supervised, and handled according to the Okayama University Graduate School of Medicine and Dentistry guidelines for the care and use of laboratory animals. This research was approved by the Animal Experiment Control Committee of the Okayama University Graduate School of Medicine and Dentistry, under No. 05-006-099.

Bone marrow transplantation

Bone marrow transplantation was carried out according to a standard protocol described previously [22]. Briefly, immediately after 8-week-old female C57BL/6 recipient mice had undergone 10 Gy of lethal whole-body-irradiation split into two doses separated by 6.5 h to minimize gastrointestinal toxicity, they were transplanted with bone marrow cells harvested from the femurs of age- and sex-matched GFP donors. Donor bone marrow cells were resuspended in Hank's balanced salt solution and 5×10^6 bone marrow cells were injected into the tail vein of the recipients. The recipient mice were maintained in a specific pathogen-free environment and received normal chow and hyper-chlorinated drinking water for the first 3 weeks after bone marrow transplantation. To determine the engraftment of the donor bone marrow, the bone marrow was examined 4 weeks after transplantation by immunohistochemical staining for GFP [23].

Injection of streptomycin and tissue preparation

The recipient mice were treated with streptomycin (intraperitoneal injection, 10 mg/day × 10 days) at 1 month after the bone marrow transplantation. They were sacrificed under diethyl-ether anesthesia at 1 ($n = 4$) and 2 ($n = 4$) months after the streptomycin injection. The head of each mouse was cut into two halves along the sagittal plane and the brain tissues were removed. After overnight fixation in 4% paraformaldehyde and decalcification for 14 days in 10% ethylenediamine-tetraacetic acid (EDTA), the tissue samples were embedded in paraffin and 5 μm thick, consecutive cross-sections were prepared.

Immunohistochemistry for GFP

The sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked by incubation for 30 min in 0.3% H_2O_2 in methanol to block endogenous peroxidase. The sections were then incubated in 0.1% trypsin (Difco Laboratories, Detroit, Michigan) for 5 min at 37°C. After blocking of non-specific binding sites by incubation in 10% normal swine serum for 15 min, the sections were incubated overnight at 4°C in anti-GFP (Santa Cruz Biotechnology Inc, Santa Cruz, California) polyclonal rabbit antibody at a dilution of 1:100. The sections were then rinsed in TBS followed by treatment with swine anti-rabbit immunoglobulin (Dako, Copenhagen, Denmark) antibody for 30 min. After rinsing in TBS, the sections were incubated in streptavidin peroxidase (Dako, Copenhagen, Denmark) at room temperature for 30 min, followed by staining with DAB for 15 min. Staining was visualized using a light microscope (BX51-54; Olympus). Sections from the olfactory epithelium of the donor mice were used as positive controls, while negative control sections were processed in an identical way with the exception that the primary antibody was excluded.

Results

Four weeks after transplantation, the bone marrow of the recipient mice was occupied fully with GFP-positive cells derived from donor bone (Fig. 1), a finding that

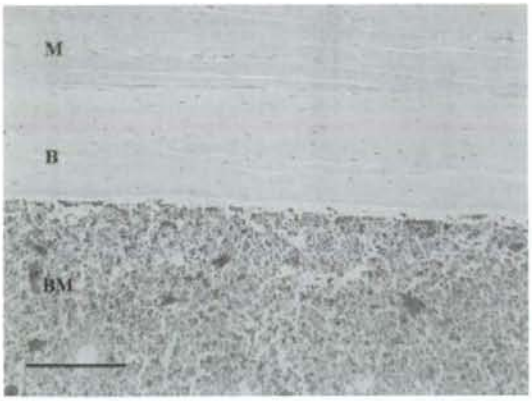

Fig. 1 Numerous GFP-positive cells were seen in the bone marrow of the recipient mice 1 month after transplantation. The recipient bone marrow was reconstructed by self-renewal of transplanted donor bone marrow-derived cells. *M* muscle layer, *B* bone layer, *BM* bone marrow, *scale bar* = 100 μm

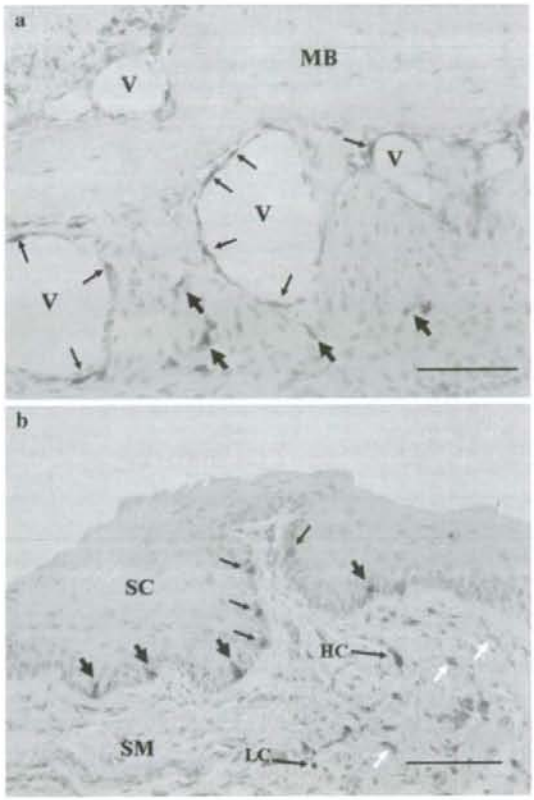

Fig. 2 Two months after bone marrow transplantation, GFP-positive cells were found in **a** the endothelial cells (*arrows*) of the vessels (*V*) near the mandibular bone (*MB*), fibroblasts (*wide arrows*) and **b** epithelial cells (*black arrows*) and dendritic cells which might be the Langerhans cells (*wide black arrows*) of the oral squamous cell (*SC*) layer, and some hemagioendothelial cells (*HC*), fibroblasts (*white arrows*) and a few lymphatic cells (*LC*) of the submucosal (*SM*) layer, *scale bars* = 50 μm

demonstrated the recipient bone marrow had been reconstructed by transplanted donor marrow cells. GFP-positive cells were found in the vascular endothelia (Fig. 2a) and oral mucosa (Fig. 2b) of recipient mice both 2 and 3 months after the bone marrow transplantation. The vestibular epithelium cells were partially vacuolated and the arrangement of them was disordered. However, we could not find any GFP-positive cells among them (Fig. 3). Immunostaining for GFP was found in all cells of the olfactory epithelium of the positive controls, whereas no positive staining was found in the sections of the negative control animals.

Discussion

If BMC migrate into the sensory epithelia of the inner ear, similar to what occurs in epithelia of other organs, this would represent one of the first steps of therapy for vestibular dysfunction or sensorineural hearing loss. However, in the present study, we could not find any BMC among the sensory epithelium of the vestibule. This observation suggested that BMC might not be engrafted into the inner ear, even if there were stem cells in the sensory epithelium of the inner ear in mature mammals. Krause et al. [8] reported that the engraftment rate of the transplanted cells depends on (1) the degree of tissue damage induced by the transplant, (2) the residual tissue-specific stem cell capacity within each organ, and/or (3) the normal rate of cell turnover in each organ. The sensitivity of each organ to radiation or severe injury like graft versus host disease (GVHD) may influence epithelial engraftment. For example, the number of bone marrow-derived epithelial cells in the gastrointestinal (GI) tract was increased remarkably during epithelial regeneration after radiation, GVHD or ulcer formation [24]. When compared with the GI or respiratory epithelia, the sensory cells of the inner ear may be more resistant to irradiation and GVHD. Although we added damage to the inner ear using streptomycin injection, it might have been not enough for efficacy of BMC-engraftment. Previously we have demonstrated that BMC can be engrafted in the olfactory epithelium and then differentiate into olfactory neuron cells [12]. Olfactory neurons have an unusual lifelong ability to continue neurogenesis and they differentiate from stem cells (globose basal cells). The transplanted donor BMC may have been engrafted as the stem cells of olfactory neuron cells. On the other hand, the rate of cell turnover among the sensory epithelium of the inner ear is very low, even in the avian vestibular epithelium

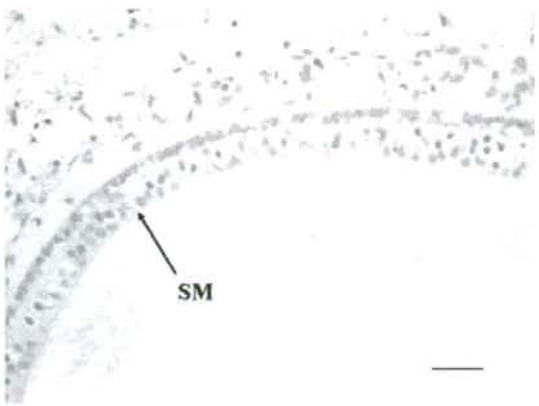

Fig. 3 Three months after marrow transplantation (two months after streptomycin injection), there were no GFP-positive cells in the saccular epithelium although damage induced by streptomycin was observed. *SM* saccular macula, *scale bar* = 50 μm

[25]. Although it has been reported that BMC might have ability to generate sensory cells of the inner ear [16] and BMC directly transplanted into the cochlea can survive after 3 weeks [14], we could not observe the engraftment of BMC into the sensory epithelium of the inner ear. However, even the spontaneous engraftment rate of donor BMC into the olfactory epithelium is about 0.15% [12]. Considering the difference in the number of sensory cells between the olfactory epithelium and the inner ear epithelium, there might be a possibility that the engraftment rate of BMC into the mouse-vestibular-epithelium had been too small to detect in the present study.

Conclusion

This study shows no evidence, which suggests the possibility of BMC, serving as a source of precursor cells for the sensory cells of the inner ear.

Conflict of interest We have no conflict of interest, potential conflicts or financial relationships.

References

1. Corwin JT, Cotanche DA (1988) Regeneration of sensory hair cells after acoustic trauma. Science 240:1772–1774. doi:10.1126/science.3381100
2. Roberson DW, Alosi JA, Cotanche DA (2004) Direct transdifferentiation gives rise to the earliest new hair cells in regenerating avian auditory epithelium. J Neurosci Res 78:461–471. doi:10.1002/jnr.20271
3. Oesterle EC, Cunningham DE, Westrum LE et al (2003) Ultrastructural analysis of (3H) thymidine-labeled cells in the rat utricular macula. J Comp Neurol 463:177–195. doi:10.1002/cne.10756
4. Ogata Y, Slepecky NB, Takahashi M (1999) Study of the gerbil utricular macula following treatment with gentamicin, by use of bromodeoxyuridine and calmodulin immunohistochemical labeling. Hear Res 133:53–60. doi:10.1016/S0378-5955(99)00057-X
5. Hawkins RD, Bashiardes S, Helms CA et al (2003) Gene expression differences in quiescent versus regenerating hair cells of avian sensory epithelia: implications for human hearing and balance disorders. Hum Mol Genet 12:1261–1272. doi:10.1093/hmg/ddg150
6. Brazelton TR, Rossi FMV, Keshet GI et al (2000) From marrow to brain: expression of neuronal phenotypes in adult mice. Science 290:1775–1779. doi:10.1126/science.290.5497.1775
7. Ferrari G, Angelis GCD, Coletta M et al (1998) Muscle regeneration by bone marrow-derived myogenic progenitors. Science 279:1528–1530. doi:10.1126/science.279.5356.1528
8. Krause DS, Theise ND, Collector MI et al (2001) Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 105:369–377. doi:10.1016/S0092-8674(01)00328-2
9. Mezey É, Chandross KJ, Harta G et al (2000) Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 290:1779–1782. doi:10.1126/science.290.5497.1779
10. Orlic D, Kajstura J, Chimenti S et al (2001) Transplanted adult bone marrow cells repair myocardial infarcts in mice. Ann New York Acad Sci 938:221–230
11. Petersen BE, Bowen WC, Patrene KD et al (1999) Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170. doi:10.1126/science.284.5417.1168
12. Tsujigiwa H, Nishizaki K, Teshima T et al (2005) The engraftment of transplanted bone marrow-derived cells into the olfactory epithelium. Brain Res 1052:10–15. doi:10.1016/j.brainres.2005.05.061
13. Lang H, Ebihara Y, Schmiedt R et al (2006) Contribution of bone marrow hematopoietic stem cells to adult mouse inner ear: mesenchymal cells and fibrocytes. J Comp Neurol 496:187–201. doi:10.1002/cne.20929
14. Naito Y, Nakamura T, Nakagawa T et al (2004) Transplantation of bone marrow stromal cells into the cochlea of chinchillas. Neuroreport 15:1–4. doi:10.1097/00001756-200401190-00001
15. Matsuoka AJ, Kondo T, Miyamoto RT et al (2006) In vivo and in vitro characterization of bone marrow-derived stem cells in the cochlea. Laryngoscope 116:1363–1367. doi:10.1097/01.mlg.0000225986.18790.75
16. Jeon SJ, Oshima K, Heller S et al (2007) Bone marrow mesenchymal stem cells are progenitors in vitro for inner ear hair cells. Mol Cell Neurosci 34:59–68. doi:10.1016/j.mcn.2006.10.003
17. Orlic D, Kajstura J, Chimenti S et al (2001) Bone marrow cells regenerate infracted myocardium. Nature 410:701–705. doi:10.1038/35070587
18. Winkel O, Hansen MM, Kaaber K et al (1978) A prospective study of gentamicin ototoxicity. Acta Otolaryngol 86:212–216. doi:10.3109/00016487809124738
19. Fee WE Jr (1980) Aminoglycoside ototoxicity in the human. Laryngoscope 90(10 Pt 2 Suppl 24):1–19. doi:10.1288/00005537-198010001-00001
20. Warchol ME, Lambert PR, Goldstein BJ et al (1993) Regenerative proliferation in inner ear sensory epithelia from adult guinea pigs and humans. Science 259:1619–1622. doi:10.1126/science.8456285
21. Okabe M, Ikawa M, Kominami K et al (1997) 'Green mice' as a source of ubiquitous green cells. FEBS Lett 407:313–319. doi:10.1016/S0014-5793(97)00313-X
22. Wall DA, Hamberg SD, Reynolds DS et al (1988) Immunodeficiency in graft-versus-host disease. I. Mechanism of immune suppression. J Immunol 140:2970–2976
23. Zijlmans JM, Visser JW, Laterveer L et al (1998) The early phase of engraftment after murine blood cell transplantation is mediated

by hematopoietic stem cells. Proc Natl Acad Sci USA 95:725–729. doi:10.1073/pnas.95.2.725

24. Okamoto R, Yajima T, Yamazaki M et al (2002) Damaged epithelia regenerated by bone marrow-derived cells in the human gastrointestinal tract. Nat Med 8:1011–1017. doi:10.1038/nm755

25. Stone JS, Choi YS, Woolley SM et al (1999) Progenitor cell cycling during hair cell regeneration in the vestibular and auditory epithelia of the chick. J Neurocytol 28:863–876. doi:10.1023/A:1007022205821