# 厚生科学研究費補助金難治性疾患克服研究事業
# 「炎症性腸疾患の画期的治療法に関する臨床研究」
# 平成15年度第1回総会プログラム

（敬称略）

開会（13：00）

Ⅰ．主任研究者挨拶・研究の進め方　　班長：渡辺　守

Ⅱ．研究報告

（1）上皮細胞の再生のための分子療法、細胞移植療法の確立（13：10～14：00）

・TNBS腸炎ラットに対するHGFの効果の検討　（分担研究者：坪内博仁）

坪内博仁[1)2)]、○沼田政嗣[1)2)]、井戸章雄[1)2)]、中西千尋[1)]、山本章二朗[1)]、宮田義史[1)]、宇都浩文[1)]　（[1)]宮崎大学医学部第2内科、[2)]京都大学医学部附属病院探索医療センター）

・難治性炎症性腸疾患に対するHGF遺伝子治療の検討　（分担研究者：鈴木健司）

○鈴木健司[1)]、河内裕介[1)]、朝倉　均[1)]、青柳　豊[1)]、丸山弘樹[2)]、宮崎純一[3)]

（[1)]新潟大学消化器内科、[2)]新潟大学腎・膠原病分野、[3)]大阪大学分子治療学幹細胞制御分野）

・骨髄由来細胞を利用した腸管上皮治療の可能性　（分担研究者：渡辺　守）

○岡本隆一[1)]、松本智子[1)]、山崎元美[1)]、中村哲也[1)]、金井隆典[1)]、渡辺　守[1)]、矢島知治[2)]、日比紀文[2)]　（[1)]東京医科歯科大学消化器内科、[2)]慶應義塾大学内科）

（2）腸管特異的免疫調節機構を標的とした治療法の開発（14：00～14：50）

・パネート細胞の自然免疫機能に基づく炎症性腸疾患の新規治療開発の可能性

（分担研究者：高後　裕）

○綾部時芳[1)]、河野　透[2)]、蘆田知史[1)]、高後　裕[1)]

（[1)]旭川医科大学第3内科、[2)]旭川医科大学第2外科）

・MIFの制御による炎症性腸疾患の新しい治療法の開発　（分担研究者：浅香正博）

○武田宏司、大川原辰也、浅香正博　（北海道大学大学院分子病態制御学）

・腸管上皮細胞の恒常性維持に関与するTLRの追究　（分担研究者：石川博通）

○石川博通（慶應義塾大学微生物学・免疫学）

（3）選択的細胞除去療法の開発（14：50～15：25）

・炎症性腸疾患に対する白血球除去療法の新展開　（分担研究者：日比紀文）

○桜庭　篤、芳沢茂雄、諸星雄一、泉谷幹子、緒方晴彦、岩男　泰、日比紀文

（慶應義塾大学内科）

・選択的白血球除去療法を目指して：ヒト末梢血における制御性T細胞分画に関する検討

（分担研究者：中村和彦）

○中村和彦、原田直彦、北村陽介、高橋　誠、本田邦臣、松井謙明、吉永繁高、名和田新

（九州大学大学院・医学研究院・病態制御内科）

（4）分子・細胞デリバリーシステムを用いた治療法確立（15：25～15：40）

・ラットにおけるデキサメサゾン含有ポリ乳酸マイクロカプセルの長期投与の安全性

（分担研究者：岡崎和一）

○岡崎和一[1)]、西尾彰功[2)]、仲瀬裕志[2)]、千葉　勉[2)]、田畑泰彦[3)]

（[1)]関西医科大学第3内科、[2)]京都大学消化器内科、[3)]京都大学再生医科学研究所）

事務局連絡

閉会の挨拶

# 平成15年度第1回総会出席者名簿

平成16年1月30日（金）
参加者38名（敬称略）

| | |
|---|---|
| 班　　長 | 渡辺　守（東京医科歯科大学消化器内科） |
| 分担研究者 | 日比紀文（慶應義塾大学内科） |
| | 坪内博仁（宮崎大学医学部第2内科） |
| | 高後　裕（旭川医科大学第3内科） |
| | 岡崎和一（関西医科大学第3内科） |
| | 石川博通（慶應義塾大学微生物学・免疫学） |
| | 中村和彦（九州大学大学院病態制御内科） |
| | 鈴木健司（新潟大学消化器内科） |
| 参加協力者 | 井上　詠、高石官均、桜庭　篤（慶應義塾大学内科） |
| | 武田宏司（北海道大学医学部第3内科） |
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| | 柴田　博、角　隆行（旭メディカル） |
| | 辻井勝哉（田辺製薬） |
| | 吉田義一、岸　雅人（中外製薬） |
| | 藤井克典、丸田展久、菅原慎一郎（日清キョーリン製薬） |
| | 国馬誠一郎、細井英治（日本抗体研究所） |
| | 金井隆典、中村哲也、岡本隆一、岡田英理子、戸塚輝治、山崎元美（東京医科歯科大学消化器内科） |
| 事　務　局 | 児玉　瞳、伊藤裕子（東京医科歯科大学消化器内科） |

# IX. 研究成果の刊行物・別刷

GASTROENTEROLOGY 2003;124:410–421

# Ameliorating Effect of Anti-inducible Costimulator Monoclonal Antibody in a Murine Model of Chronic Colitis

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*Background & Aims:* Inducible costimulator (ICOS)/B7RP-1 represents a newly described receptor/ligand pair involved in costimulation of T cells by antigen-presenting cells. We investigated the involvement of the ICOS/B7RP-1 interaction in the pathogenesis of colitis and the therapeutic potential of anti-ICOS monoclonal antibody (mAb) in experimental colitis. *Methods:* We administered anti-ICOS or anti–B7RP-1 mAb to mice with experimental colitis induced by transfer of $CD4^+CD45RB^{high}$ T cells from normal mice into SCID mice. The ability of $CD4^+CD45RB^{high}$ cells derived from ICOS−/− mice to induce colitis was assessed. Th2 cytokine production and apoptosis in infiltrating T cells was examined after administration of anti-ICOS mAb. *Results:* ICOS was strongly induced on $CD4^+$ T cells, and B7RP-1 was expressed by macrophages in the inflamed mucosa of colitic mice. Anti-ICOS mAb, but not anti–B7RP-1, ameliorated chronic colitis when administered in prevention or therapeutic protocols. Transfer of $CD4^+CD45RB^{high}$ T cells from ICOS−/− mice induced colitis. Treatment with anti-ICOS mAb did not enhance the production of Th2 cytokines, but a single dose of anti-ICOS mAb induced massive apoptosis of infiltrating ICOS-expressing T cells. *Conclusions:* ICOS/B7RP-1 interactions are not required for the development of colitis. However, treatment with anti-ICOS mAb can prevent and reverse intestinal inflammation by inducing apoptosis of ICOS-expressing T lymphocytes.

Crohn's disease (CD) and ulcerative colitis are 2 major forms of human inflammatory bowel disease. The cause of these diseases is unknown, but increasing evidence indicates that immune mechanisms play an important role.[1,2] These diseases are characterized by leukocytic infiltrates in inflamed intestinal mucosa, especially by activated T cells and macrophages, and the production of proinflammatory cytokines by lamina propria (LP) T cells and macrophages/dendritic cells. In general, CD is characterized by Th1 cytokines (interleukin [IL]-12, interferon [IFN]-γ, tumor necrosis factor α), whereas ulcerative colitis is characterized by Th2 cytokines (IL-4, IL-5).[3,4] Recently, various animal models of chronic intestinal inflammation have been established, which are useful to obtain new insights into the pathogenesis of inflammatory bowel disease. These include rats carrying transgenes of HLA-B27 and $\beta_2$-microglobulin,[5] mice carrying IL-7 transgenes,[6] and mice in which the genes for IL-2,[7] IL-10,[8] or the α or β chain of the T-cell receptor[9] have been inactivated by homologous recombination. In addition, adoptive transfer of $CD4^+CD45RB^{high}$ (naive) T cells from BALB/c mice to syngeneic SCID mice leads to the development of an inflammatory bowel disease–like syndrome that is characterized by diarrhea, weight loss, transmural inflammation in the proximal colon, and a Th1 immune response by LP $CD4^+$ T cells.[10] The clinical, histopathologic, and immunologic features of this model resemble those observed in human CD.

It is well known that the activation of T cells requires 2 distinct signals: one derived from the interaction between the T-cell receptor and peptide/major histocompatibility complex, and another designated as the costimulatory signal derived from the interaction between the costimulatory molecules of the CD28 family on T cells and their ligands of the B7 family on antigen-presenting cells.[11–13] CD28 is expressed by most resting T cells and interacts with the ligands B7-1 and B7-2 on antigen-presenting cells. CD28-mediated costimulation plays a critical role in pathogenic T-cell activation, as

*Abbreviations used in this paper:* ICOS, inducible costimulator; IFN, interferon; IL, interleukin; LP, lamina propria; LPMC, lamina propria mononuclear cell; mAb, monoclonal antibody; PE, phycoerythrin; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.


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shown by studies in which the severity of disease in animal models, such as experimental allergic encephalomyelitis, collagen-induced arthritis, and asthma, was markedly reduced when the CD28-B7 pathway was blocked.[14,15] However, although CD28-mediated costimulation seems to be essential for initial T-cell priming, some secondary or memory responses are CD28 independent, suggesting the presence of alternative costimulation pathways.

Inducible costimulator (ICOS)/activation-inducible lymphocyte immunomodulatory molecule is the third member of the CD28 family whose role in the immune responses remains largely unknown.[16,17] In contrast to CD28, ICOS is expressed after T-cell activation. The inducible expression of ICOS is interesting because ICOS may be particularly important in costimulation of activated T cells. A novel B7 family member (called B7h or B7RP-1)[18,19] was recently identified as the ligand for ICOS. Discovery of the ICOS/B7RP-1 pathway raises a number of interests about the physiologic and pathologic functions of this pathway. In the original studies, engagement of ICOS was particularly effective in costimulating production of IL-10 and IL-4 but not IL-2.[12] A series of studies has supported the concept that ICOS functions as a critical costimulatory pathway for Th2 responses.[16,18–21] However, recent studies show that ICOS also plays a substantial role in murine Th1-mediated disease models, such as acute allograft rejection and experimental allergic encephalomyelitis.[22,23] The contribution of ICOS to the pathogenesis of inflammatory bowel disease remains unknown.

In this study, we used the murine chronic colitis model induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells to SCID mice[10] to characterize the expression of ICOS/B7RP-1 in chronic intestinal inflammation and investigated the therapeutic potential of anti-ICOS monoclonal antibody (mAb).

## Materials and Methods

### Animals

Specific pathogen–free 6- to 8-week-old female BALB/c and C57BL/6 scid/scid (SCID) mice and female BALB/c mice were purchased from Japan Clea (Tokyo, Japan) and maintained in the animal facility at Tokyo Medical and Dental University. ICOS-deficient mice were generated by gene targeting (K.T., details will be described elsewhere), and ICOS−/− and ICOS+/− littermates (F3 interbred from 129/Ola × C57BL/6) were used as the donors of $CD4^+CD45RB^{high}$ T cells for C57BL/6 SCID mice.

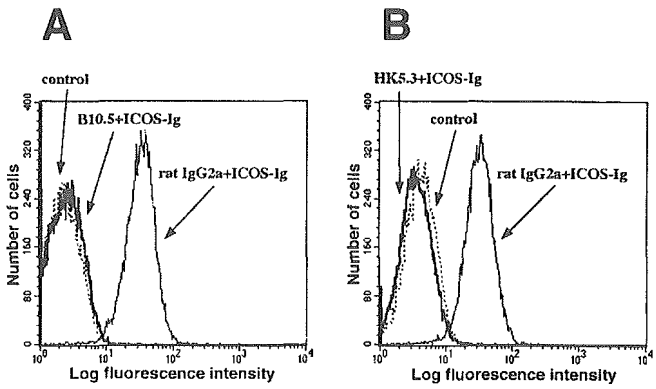


**Figure 1.** Characterization of B10.5 and HK5.3 mAbs. (*A*) B10.5 inhibits ICOS Ig binding to B7RP-1 transfectants. B7RP-1/L5178Y cells were stained with ICOS Ig, which was pretreated with B10.5 or rat IgG2a followed by PE-labeled goat anti-human IgG Ab. The control histogram represents background staining with human IgG1. (*B*) HK5.3 inhibits ICOS Ig binding to B7RP-1 transfectants. B7RP-1/L5178Y cells were pretreated with HK5.3 or rat IgG2a and then stained with ICOS Ig followed by PE-labeled goat anti-human IgG Ab. The control histogram represents background staining with human IgG1.

### Anti-ICOS and Anti–B7RP-1 mAbs

An anti-mouse ICOS mAb (B10.5 [JMAB51], rat immunoglobulin [Ig] G2a) was generated by immunizing Wistar rats with ICOS-transfected CHO-K1 cells.[24] The mAb was purified from culture supernatant using protein A columns and shown to contain <1 endotoxin unit/mg protein. This mAb blocked binding of mouse ICOS Ig to mouse B7RP-1 transfectants (Figure 1*A*). An anti-mouse B7RP-1 mAb (HK5.3, rat IgG1) was generated by immunizing SD rats with mouse B7RP-1–transfected L cells and screened for binding to mouse B7RP-1–transfected NRK cells. The mAb was purified from ascites in nude mice using protein A columns and shown to contain <1 endotoxin unit/mg protein. This mAb also blocked binding of mouse ICOS Ig to mouse B7RP-1 transfectants (Figure 1*B*).

### B7RP-1 Ig

B7RP-1 Ig fusion protein was produced from transfected CHO lines and purified over protein A/agarose columns. This construct consists of the extracellular domain of human B7RP-1 and the hinge, CH2, and CH3 domains of human IgG1. Control human IgG1 was purchased from Sigma (St. Louis, MO).

### Induction of Colitis and Treatments

Induction of colitis by adoptive transfer of $CD4^+CD45RB^{high}$ T cells into SCID mice was performed essentially as described previously.[25] $CD4^+$ T cells were isolated from spleen cells from BALB/c mice using the anti-CD4 (L3T4) magnetic-activated cell sorting system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Enriched $CD4^+$ T cells (96%–97% pure, as estimated by fluorescence-activated cell sorter) were then labeled with phycoerythrin (PE)-conjugated anti-mouse CD4 (RM4-5;

PharMingen, San Diego, CA) and fluorescein isothiocyanate–conjugated anti-CD45RB (16A; PharMingen) and sorted into $CD45RB^{high}$ (brightest staining, 30%) and $CD45RB^{low}$ (dullest staining, 30%) fractions on an FACS Vantage (Becton Dickinson, Sunnyvale, CA). Each BALB/c SCID mouse was injected intraperitoneally (IP) with 200 μL phosphate-buffered saline (PBS) containing $5 \times 10^5$ $CD4^+CD45RB^{high}$ T cells or $5 \times 10^5$ $CD4^+CD45RB^{low}$ T cells. These mice were administered 250 μg anti-ICOS mAb (B10.5) or anti–B7RP-1 mAb (HK5.3) IP in 250 μL PBS 3 times per week from the day of T-cell transfer over a 7-week period. An equivalent amount of control rat IgG (Sigma) was administered to control mice under the same conditions. To assess the role of ICOS signaling in the development of chronic colitis, mice were administered 100 μg B7RP-1 Ig fusion protein IP in 250 μL PBS 3 times per week from the day of T-cell transfer over a 7-week period. An equivalent amount of control human IgG (Sigma) was administered to control mice under the same conditions.

Because the loss of body weight in colitic SCID recipients started 3–5 weeks after T-cell transfer in preliminary experiments, we treated another group of SCID mice by IP injection with 250 μg anti-ICOS mAb 3 times per week from 3 weeks after T-cell transfer to determine the effect of delayed treatment with anti-ICOS mAb. Mice were killed 7 weeks after T-cell reconstitution and analyzed for bowel inflammation.

In some experiments, C57BL/6 SCID mice were injected IP with 200 μL PBS containing $5 \times 10^5$ $CD4^+CD45RB^{high}$ T cells from ICOS+/− or ICOS−/− littermate mice.

In another set of experiments, we treated 4 groups of mice with (1) 500 μg control IgG, (2) 250 μg anti-ICOS mAb + 250 μg control IgG, (3) 250 μg anti–IL-10 mAb (JES5-2A5) + 250 μg control IgG, or (4) 250 μg anti-ICOS mAb + 250 μg anti–IL-10 mAb 3 times per week from the day of T-cell transfer over a 7-week period to assess the contribution of IL-10 to the effect of anti-ICOS mAb.

## Adoptive Transfer of LP $CD4^+$ T Cells From Colitic Mice

LP $CD4^+$ T cells were isolated from BALB/c SCID colitic mice 6 weeks after transfer of $CD4^+CD45RB^{high}$ T cells by using magnetic-activated cell sorting magnetic beads as previously described (>95% $CD4^+$ as estimated by fluorescence-activated cell sorter). BALB/c SCID mice were injected IP with 200 μL PBS containing $1 \times 10^6$ LP $CD4^+$ T cells and were treated IP with 250 μg anti-ICOS mAb (B10.5) or control rat IgG in 250 μL PBS 3 times per week. Mice were killed 4 weeks after the pathogenic LP $CD4^+$ T-cell retransfer. The colon was removed and evaluated histologically.

## Histologic Examination and Immunohistochemical Staining

Tissue samples were fixed in PBS containing 6% neutral-buffered formalin. Paraffin-embedded sections (5 μm) were stained with H & E. The sections were analyzed without prior knowledge of the type of treatment. The degree of inflammation in the colon was graded according to the previously described scoring system.[26] Colonic samples for immunohistochemistry were embedded in OCT compound, snap frozen in liquid nitrogen, and stored at −80°C. Staining of the sections was performed using the avidin-biotin complex method. Six-micrometer sections were incubated with biotinylated anti-mouse ICOS mAb (B10.5), biotinylated anti-mouse B7RP-1 mAb (HK5.3), biotinylated anti-mouse CD4 mAb (RM4-5; rat IgG1, PharMingen), or biotinylated anti-mouse F4/80 mAb (rat IgG2b, PharMingen). Biotinylated isotype-matched control antibodies (PharMingen) were also used. Biotinylated antibodies were detected by streptavidin-biotinylated horseradish peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA), and visualized by diaminobenzidine. The sections were then counterstained with hematoxylin.

## Preparation of Lamina Propria Mononuclear Cells

Lamina propria mononuclear cells (LPMCs) were isolated from the colon as described previously.[27] Briefly, the entire intestine was opened longitudinally, washed with PBS, and cut into small pieces. The pieces were incubated 2 times with $Ca^{2+}/Mg^{2+}$-free Hank's balanced salt solution containing 1 mmol/L dithiothreitol (Sigma) for 30 minutes to remove mucus. The supernatants from these incubations were collected, pooled, and treated with 1 mg/mL collagenase (Worthington Biomedical Co., Freehold, NJ) and 0.01% deoxyribonuclease (Worthington Biomedical Co.) in medium for 2 hours. The cells were pelleted 2 times through a 40% isotonic Percoll solution and then further purified by Ficoll-Hypaque density gradient centrifugation (40%/75%) at the interface.

## Flow Cytometry

The isolated splenocytes or LPMCs were preincubated with an FcγR-blocking mAb (CD16/32; 2.4G2) for 20 minutes followed by incubation with fluorescein isothiocyanate–, PE-, or biotin-labeled mAbs for 30 minutes on ice. Biotinylated antibodies were detected with PE-streptavidin. Two-color flow cytometric analysis was performed on a FACScan (Becton Dickinson) using CellQuest software. Background fluorescence was assessed by staining with isotype-matched control mAbs.

## Cytokine Enzyme-Linked Immunosorbent Assay

LP $CD4^+$ cells were purified from LPMCs by using the anti-CD4 (L3T4) magnetic-activated cell sorting system. LP $CD4^+$ cells ($1 \times 10^5$) were cultured in 200 μL of 10% fetal calf serum/RPMI 1640 medium supplemented with 1 μg/mL soluble anti-CD28 mAb (37.51; PharMingen) in 96-well plates (Costar, Cambridge, MA) that were precoated with 10 μg/mL anti-CD3ε antibody (145-2C11, PharMingen) in PBS overnight at 4°C. Culture supernatants were collected after 48

hours and assayed for cytokine contents by specific enzyme-linked immunosorbent assay according to the manufacturer's instructions (R & D Systems, Minneapolis, MN).

### Detection of Apoptotic Cells

Detection of apoptotic cells in frozen sections was performed by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method using ApoTag kit (Intergen, Purchase, NY) according to the manufacturer's instructions. To quantify TUNEL-positive cells, the number of TUNEL-positive cells per 500 infiltrating LPMCs within 5 areas per section was counted under a microscope. Apoptosis index was expressed as the percentage of TUNEL-positive cells per 500 LPMCs counted. To identify the cell type of apoptotic cells, double staining was performed with PE-conjugated anti-CD4 mAb together with the TUNEL method by using an apoptosis detection kit (MBL, Nagoya, Japan).

### Statistical Analysis

The results are expressed as mean ± SD. Groups of data were compared by Mann–Whitney $U$ test. $P$ values $<0.05$ were considered statistically significant.

## Results

### Up-regulation of ICOS and B7RP-1 Expression in Colitic Mucosa

To investigate the role of ICOS and B7RP-1 in chronic colitis, we used a murine colitis model induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells to SCID mice. BALB/c SCID mice reconstituted with $CD4^+CD45RB^{high}$ T cells from normal BALB/c mice manifested progressive weight loss after 3–5 weeks. These mice had diarrhea with increased mucus in the stool, anorectal prolapse, and hunched posture by 6–8 weeks. The colons from these mice were enlarged and had a greatly thickened wall due to severe inflammation as described previously.[10] To assess whether ICOS was expressed or up-regulated in the inflamed colonic mucosa, LPMCs from colitic mice were analyzed by flow cytometry. As shown in Figure 1*A*, initially isolated $CD4^+CD45RB^{high}$ splenocytes before the transfer did not express ICOS. In contrast, almost all LP $CD4^+$ T cells from colitic mice at 7 weeks after transfer highly expressed ICOS, whereas only a part of LP $CD4^+$ T cells from normal BALB/c mice expressed ICOS at low to moderate levels (Figure 2*A*). In both normal and colitic LPMCs, some $CD4^-ICOS^+$ cells were DX5 positive, suggesting ICOS was also expressed on NK cells (data not shown). Immunohistochemical staining of colonic mucosa confirmed these results. The number of cells expressing ICOS was markedly increased in the colitic mice (Figure 2*B*). Most $ICOS^+$ cells were located in the LP and submucosa, but some were also observed in the tunica muscularis and subserosa. B7RP-$1^+$ cells were also increased in the inflamed colonic mucosa and colocalized with macrophages detected by F4/80 (Figure 2*B*). Flow cytometric analysis of LPMCs from colitic mice showed that B7RP-1 was expressed by a part of $F4/80^+$ macrophages (data not shown). This up-regulation of ICOS expression in infiltrated $CD4^+$ T cells and the existence of B7RP-1–expressing macrophages in colitic mucosa suggest the participation of ICOS and B7RP-1 in the pathogenesis of chronic colitis.

### Administration of Anti-ICOS mAb Prevents Colitis

To explore whether ICOS/B7RP-1 interaction is involved in the development of chronic colitis, we administered the anti-ICOS mAb to this colitis model. Anti-ICOS mAb or control rat IgG at a dose of 250 μg/mouse was injected IP 3 times per week, starting at the time of cell transfer and continuing over a 7-week period. The control IgG-treated mice developed severe colitis 4–7 weeks after the transfer, characterized by significant weight loss (Figure 3*A*), diarrhea, and thickening of the colonic wall with inflammation (Figure 3*B*). Average histologic scores, which were characterized by transmural inflammation with high numbers of lymphocytes in the LP and submucosa, as well as prominent epithelial hyperplasia with loss of goblet cells, were 5.9 ± 1.2 in those mice (Figure 3*C*). In contrast, mice treated with anti-ICOS mAb seemed healthy and did not exhibit any signs of colitis, with a gradual increase in body weight (Figure 3*A*) and no apparent thickening of the colonic wall (Figure 3*B*). No evident pathologic changes were observed in the bowel wall, with an average histologic score of 0.8 ± 0.7 (Figure 3*D*). The average LP $CD4^+$ T-cell recovery in the inflamed colon from colitic mice was $44 \pm 9 \times 10^5$ cells/colon, whereas that from mice treated with anti-ICOS mAb was $21 \pm 3 \times 10^5$ cells/colon ($P < 0.01$) (Figure 3*D*). This result shows that the anti-ICOS mAb prevented the development of chronic colitis.

### Administration of Anti–B7RP-1 mAb Did Not Prevent Colitis

We then examined the mechanism by which the anti-ICOS mAb prevented the development of colitis. If ICOS/B7RP-1 interaction plays an important role, blockade of B7RP-1 should also abrogate the disease. We treated the colitic mice with a blocking mAb against B7RP-1. Unexpectedly, the mice anti–B7RP-1 mAb–treated mice developed a severe colitis similar to that

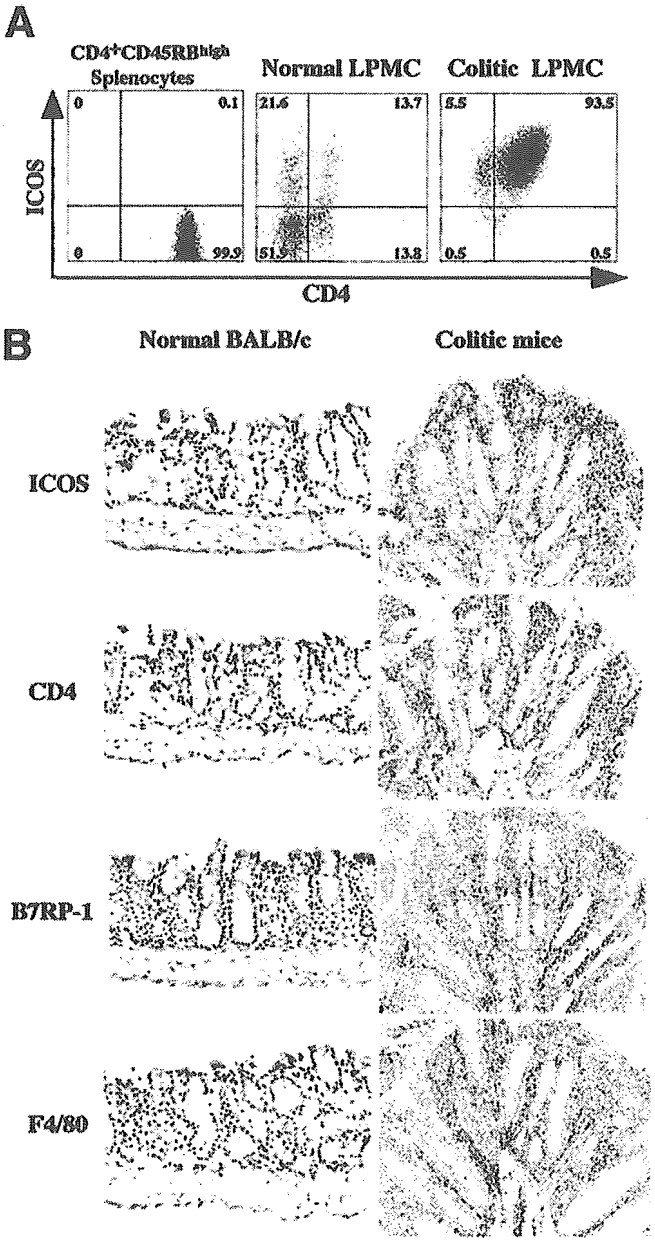


**Figure 2.** ICOS and B7RP-1 expression in inflamed colon from BALB/c SCID mice reconstituted with $CD4^+CD45RB^{high}$ T cells. (*A*) Flow cytometric analysis for ICOS expression. Initially isolated $CD4^+CD45RB^{high}$ splenocytes, LPMCs from normal BALB/c mice, and LPMCs from colitic mice at 6 weeks after transfer were stained with fluorescein isothiocyanate–labeled anti-CD4 mAb and biotin-labeled anti-ICOS mAb followed by PE-labeled streptavidin. (*B*) Cryostat sections of colonic samples from normal BALB/c mice and colitic SCID mice were stained with mAbs directed against ICOS, CD4, B7RP-1, and F4/80. (Original magnification 100×.)

seen in the control rat IgG–treated mice (Figure 3*A–C*). The number of LP $CD4^+$ T cells from the anti–B7RP-1—treated mice was also comparable to that from the control Ig-treated mice (Figure 3*D*). This suggested that the interaction of ICOS with B7RP-1 might not play a critical role in the development of colitis. To further investigate the blocking effect of the ICOS/B7RP-1 interaction, we treated the colitic mice with the human B7RP-1 Ig protein. Consistent with our results using blocking mAb against B7RP-1, hB7RP-1 Ig–treated mice developed a severe colitis similar to that seen in the control rat IgG–treated mice (Figure 4*A* and *B*). The number of LP $CD4^+$ T cells from the hB7RP-1 Ig–treated mice was also comparable to that from the control Ig-treated mice (Figure 4*C*).

### $CD4^+CD45RB^{high}$ T Cells From ICOS−/− Mice Induced Colitis

To further examine the requirement of ICOS for the development of colitis, we tested the ability of $CD4^+CD45RB^{high}$ cells derived from ICOS−/− mice to develop chronic colitis. During the 7-week period of observation, SCID mice reconstituted with either ICOS+/− or ICOS−/− $CD4^+CD45RB^{high}$ cells showed progressive weight loss from 3 to 5 weeks of reconstitution (data not shown). These mice had diarrhea with

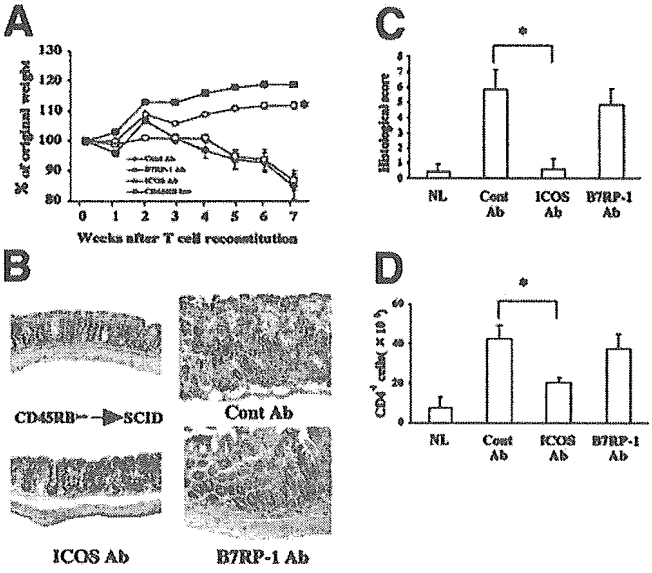


**Figure 3.** Effect of anti-ICOS and anti–B7RP-1 mAbs on the development of colitis. (*A*) BALB/c SCID mice were reconstituted with $CD4^+CD45RB^{high}$ T cells and treated with control rat IgG (●; n = 20), anti-ICOS mAb (○; n = 20), or anti–B7RP-1 mAb (□; n = 7) for a 7-week period. As a control, BALB/c SCID mice were reconstituted with $CD4^+CD45RB^{low}$ T cells and treated with control rat IgG (■; n = 7). Data are represented as the mean ± SD of 7–20 mice in each group. $^*P < 0.005$ compared with control IgG. (*B*) Histologic examination of the colon in mice reconstituted with $CD4^+CD45RB^{low}$ or $CD4^+CD45RB^{high}$ T cells and treated with control rat IgG, anti-ICOS mAb, or anti–B7RP-1 mAb at 7 weeks. (Original magnification 100×.) (*C*) Severity of colitis. Colons were removed from SCID mice 7 weeks after T-cell transfer and stained with H & E. Pathology was graded on a scale of 0–7. Data are represented as the mean ± SD of 7 mice in each group. $^*P < 0.0005$. NL, normal BALB/c mice. (*D*) Number of LP $CD4^+$ T cells. Data are represented as the mean ± SD of 7 mice in each group. $^*P < 0.01$.

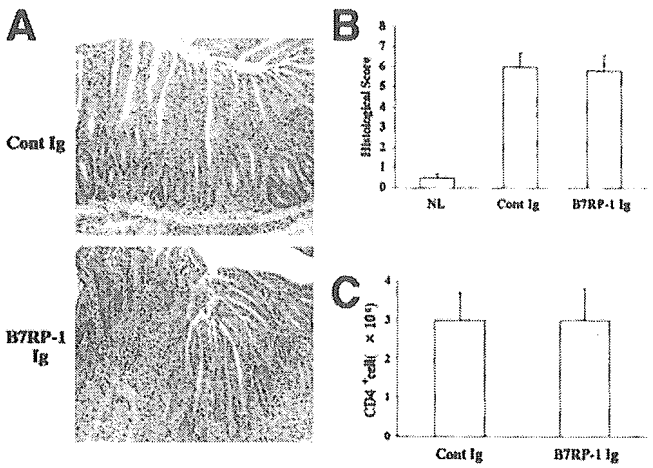


**Figure 4.** Blocking of ICOS/B7RP-1 interaction by B7RP-1 Ig does not affect the development of colitis. (*A*) BALB/c SCID mice were reconstituted with $CD4^+CD45RB^{high}$ T cells and treated with B7RP-1 Ig (n = 7) or control human IgG1 (n = 7) for up to 7 weeks. Histologic examination of the colon in mice reconstituted with $CD4^+CD45RB^{high}$ T cells and treated with B7RP-1 Ig or control human IgG1. (Original magnification 100×.) (*B*) Severity of colitis after the transfer. Colons were removed from SCID mice 7 weeks after T-cell transfer and stained with H & E. Pathology was graded on a scale of 0–7. Data represent the mean ± SD of mice in each group. NL, normal BALB/c mice. (*C*) Number of LP $CD4^+$ T cells isolated from the colons of individual mice. Data are represented as mean ± SD of 7 mice in each group.

increased mucus in the stool and hunched posture by 7 weeks. Histologic analysis of colonic sections showed transmural inflammation with large numbers of leukocyte infiltrates in the colon after the transfer of either ICOS+/− or ICOS−/− $CD4^+CD45RB^{high}$ cells (Figure 5*A* and *B*). This indicated that expression of ICOS on the pathogenic T cells was not required for the induction of colitis.

## Cytokine Production by LP $CD4^+$ T Cells

All of these results suggest that the ameliorating effect of anti-ICOS mAb on colitis was not induced simply by the blocking of ICOS/B7RP-1 interaction. It has been reported that costimulation of anti–CD3-activated T cells with anti-ICOS mAb preferentially induced production of IL-4 and IL-10 but not IL-2 in vitro.[6] To determine the possibility that Th2 cytokine induction by treatment with anti-ICOS mAb in vivo led to the ameliorating effect of colitis, we measured cytokine production by LP $CD4^+$ T cells. As shown in Figure 6, treatment with anti-ICOS mAb did not enhance production of Th2 cytokines, including IL-4, IL-10, and IL-13. In contrast, production of IFN-γ was significantly decreased in mice treated with anti-ICOS mAb. Therefore, we concluded that treatment with anti-ICOS mAb did not induce Th2 deviation in vivo.

## Anti–IL-10 mAb Does Not Abolish the Effect of Treatment With Anti-ICOS mAb

Although treatment with anti-ICOS mAb did not induce increased IL-10 production by LP $CD4^+$ T cells in vitro, to further address the possible contribution of IL-10 to the effect of treatment with anti-ICOS mAb in vivo, we administered a neutralizing anti–IL-10 mAb to the mice treated with anti-ICOS mAb. As shown in Figure 7, coadministration of anti–IL-10 mAb did not significantly affect the ame-

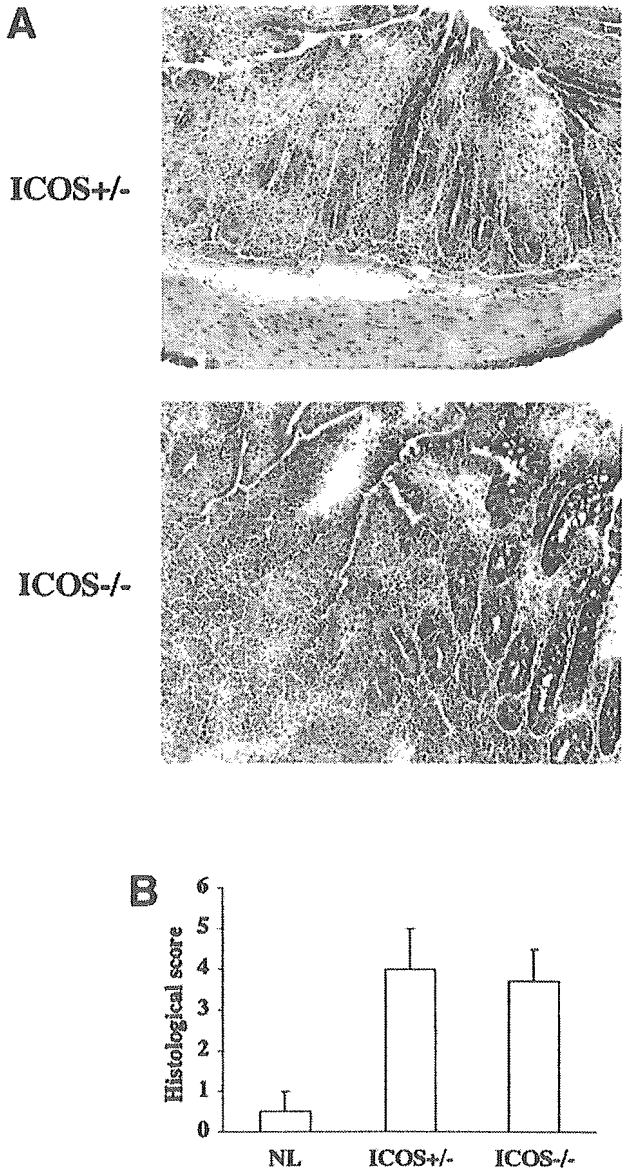


**Figure 5.** Development of colitis in ICOS-deficient T cells. (*A*) H & E staining of colon sections 6 weeks after transfer of $CD4^+CD45RB^{high}$ T cells from ICOS+/− or ICOS−/− mice. Representatives of 7 mice in each group. (Original magnification 100×.) (*B*) Severity of colitis. Colons were removed 6 weeks after T-cell transfer and stained with H & E. Pathology was graded on a scale of 0–7. Data are represented as the mean ± SD of 7 mice in each group.

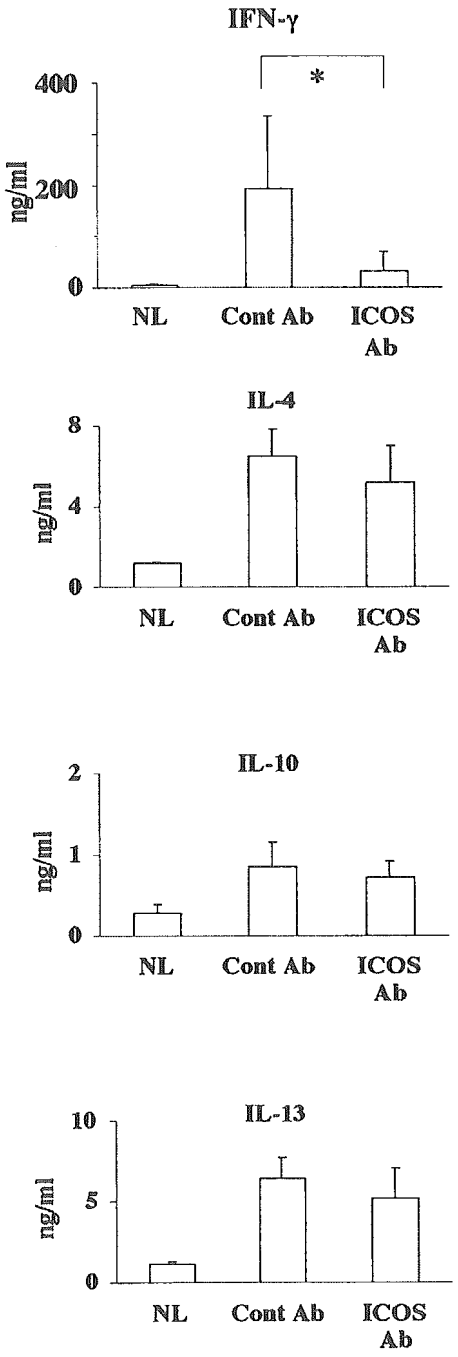


**Figure 6.** Cytokine production by LP $CD4^+$ T cells from SCID mice reconstituted with $CD4^+CD45RB^{high}$ T cells and treated with control rat IgG or anti-ICOS mAb. LP $CD4^+$ T cells were prepared from colons 7 weeks after T-cell transfer and stimulated with anti-CD3 and anti-CD28 mAbs for 48 hours. The indicated cytokines in these supernatants were measured by enzyme-linked immunosorbent assay. Data are represented as the mean ± SD of 7 mice in each group. NL, normal BALB/c mice; Cont Ab, control IgG-treated colitic mice; ICOS Ab, anti-ICOS mAb–treated colitic mice.

liorating effect of the anti-ICOS mAb on the development of colitis. This indicates that the effect of anti-ICOS mAb was not caused by the induction of IL-10–producing regulatory T cells in vivo.

## Induction of Apoptosis in Infiltrating Mononuclear Cells by Anti-ICOS mAb

We therefore examined whether the administration of anti-ICOS mAb could induce apoptosis in ICOS-expressing pathogenic T cells in vivo. We treated the mice with established colitis at 6 weeks after the transfer of $CD4^+CD45RB^{high}$ cells with a single dose of anti-ICOS mAb or control IgG and determined the number of apoptotic cells in tissue sections by the TUNEL method 24 hours later. As shown in Figure 8*A*, a marked increase in apoptotic cells, which were predominantly infiltrating mononuclear cells, was observed in the colonic mucosa of mice treated with anti-ICOS mAb compared with control IgG-treated mice. The quantitative apoptotic index in the mice treated with anti-ICOS mAb was significantly greater than that in control IgG-treated mice (Figure 8*B*). To identify whether LP $CD4^+$ T cells induced apoptosis by treatment with anti-ICOS mAb, double staining was performed using the TUNEL method and anti-CD4 mAb staining. As shown in Figure 8*C*, increased $CD4^+$ TUNEL-positive LP cells were observed in the inflamed mucosa of mice treated with anti-ICOS mAb compared with control IgG-treated mice. This suggests that the ameliorating effect of treatment with anti-ICOS mAb might be attributable to elimination of ICOS-expressing pathogenic T cells.

## Amelioration of Ongoing Colitis by Administration of Anti-ICOS mAb

We further evaluated whether delayed treatment with anti-ICOS mAb could improve ongoing disease. Because the wasting disease started 3–5 weeks after T-cell transfer and the infiltration of lymphocytes was detectable at 2 weeks in our model, we started treatment with anti-ICOS mAb 3 weeks after T-cell transfer. Colitic SCID mice were randomly assigned to be injected IP with either anti-ICOS mAb or control rat IgG, respectively, at a dosage of 250 μg/body wt 3 times weekly from week 3 after $CD4^+CD45RB^{high}$ T-cell transfer up to 8 weeks. As shown in Figure 9*A*, the mice treated with anti-ICOS mAb showed significantly improved weight loss and the absence of diarrhea. Histologic examination of colonic sections from the mice treated with anti-ICOS mAb showed significantly diminished granulomatous inflammation, leukocyte infiltration, and epithelial hyperplasia compared with the control IgG-treated mice. The inflammatory changes appeared to be focal, with only mild leukocyte infiltration in the LP and occasionally in the submucosa but not in muscular layers

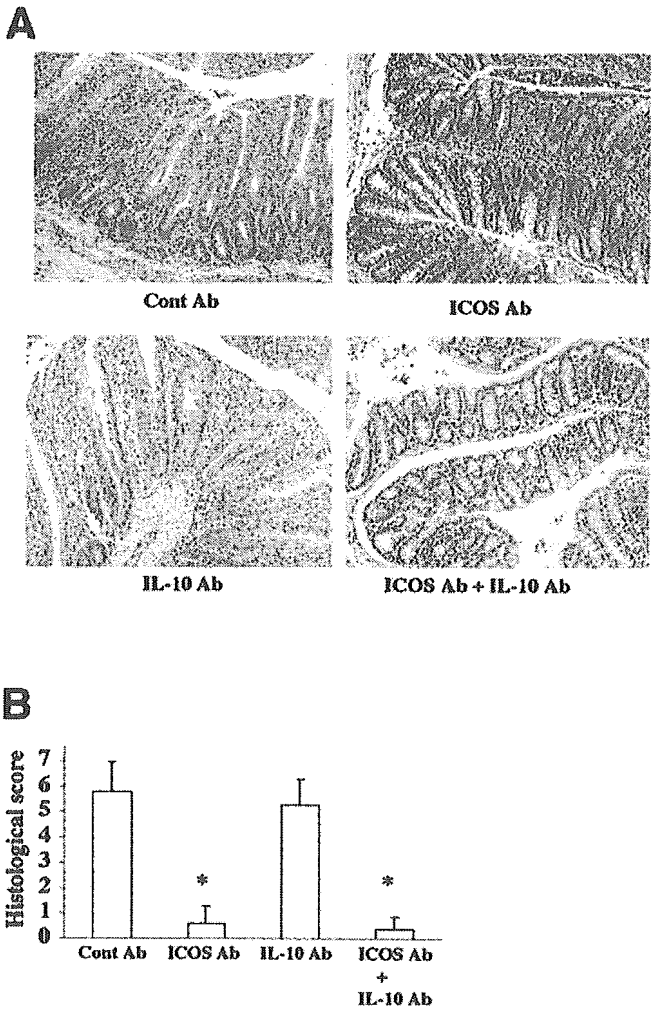


**Figure 7.** Neutralization of IL-10 does not affect the ameliorating effect of anti-ICOS mAb on colitis. (*A*) Histologic examination of the colon in SCID mice reconstituted with $CD4^+CD45RB^{high}$ T cells and treated with anti–IL-10 mAb (n = 7), anti-ICOS mAb (n = 7), anti–IL-10 + anti-ICOS mAbs (n = 7), or control rat IgG1 (n = 7) for 7 weeks. Representatives of 7 mice in each group. (Original magnification 100×.) (*B*) Severity of colitis. Colons were removed 7 weeks after T-cell transfer and stained with H & E. Pathology was graded on a scale of 0–7. Data are represented as the mean ± SD of 7 mice in each group. $*P < 0.05$ compared with control IgG.

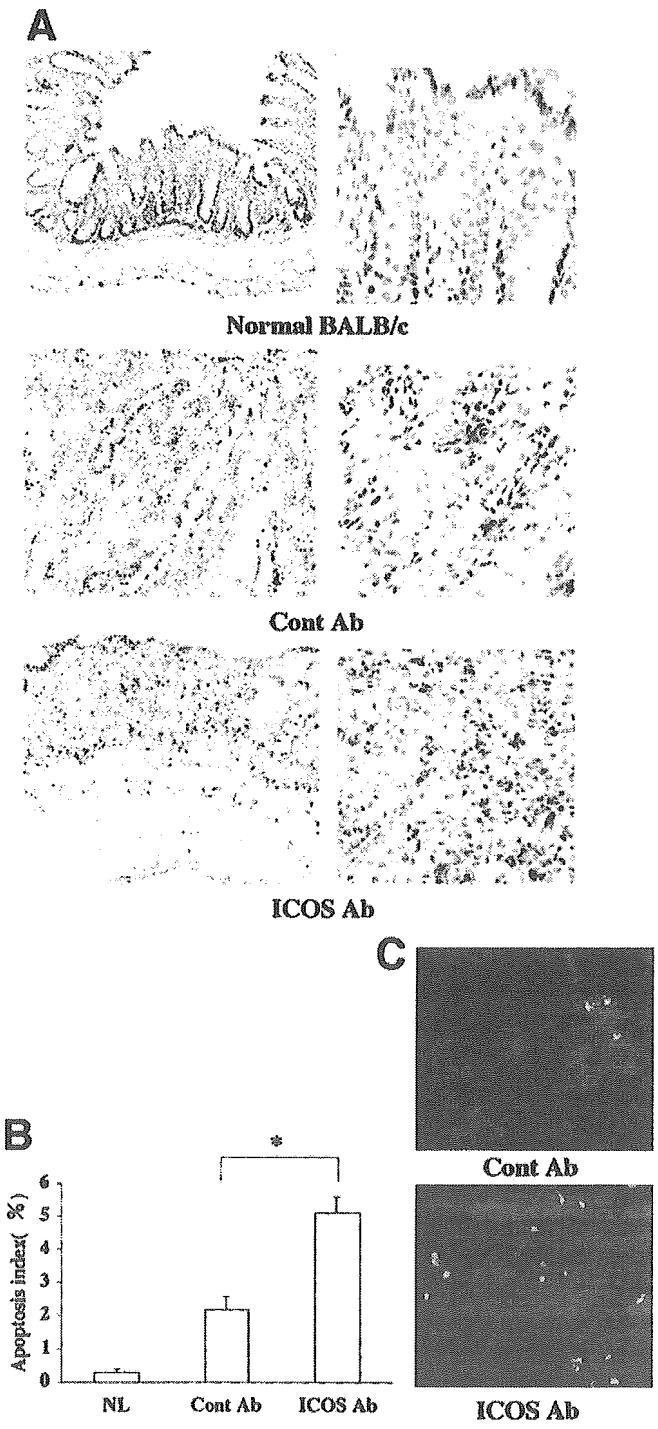


**Figure 8.** Induction of apoptosis in infiltrating mononuclear cells by anti-ICOS mAb. (*A*) Apoptotic cells in the colon from normal BALB/c mice or SCID mice reconstituted with $CD4^+CD45RB^{high}$ T cells and treated with control rat IgG or anti-ICOS mAb at 6 weeks were stained in brown by the TUNEL method. Representatives of 5 mice in each group. (Original magnification 200×.) (*B*) Apoptosis index was determined as percentage of TUNEL-positive cells per 500 mononuclear cells counted in several fields. Data are represented as the mean ± SD of 5 mice in each group. $*P < 0.05$. (*C*) Double staining of apoptotic cells with anti-CD4 mAb. LP $CD4^+$ cells were stained with PE-conjugated anti-CD4 mAb and subjected to the TUNEL method. (Original magnification 400×.)

(Figure 9*B*). Histologic score was significantly decreased in the mice treated with anti-ICOS mAb (1.62 ± 0.81) compared with that in the control IgG-treated mice (5.93 ± 1.23) ($P < 0.05$) (Figure 9*C*). The infiltration of $CD4^+$ T cells was also significantly decreased in the mice treated with anti-ICOS mAb (5.50 ± 0.7 × $10^6$ cells) compared with the control IgG-treated mice (31.3 ± 7.7 × $10^6$ cells) ($P < 0.05$) (Figure 9*D*). Furthermore, when LP $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs in vitro, production of IFN-γ was abolished in the mice treated with anti-ICOS mAb, whereas production of IL-4, IL-10, or IL-13 was not significantly affected (Figure 9*E*).

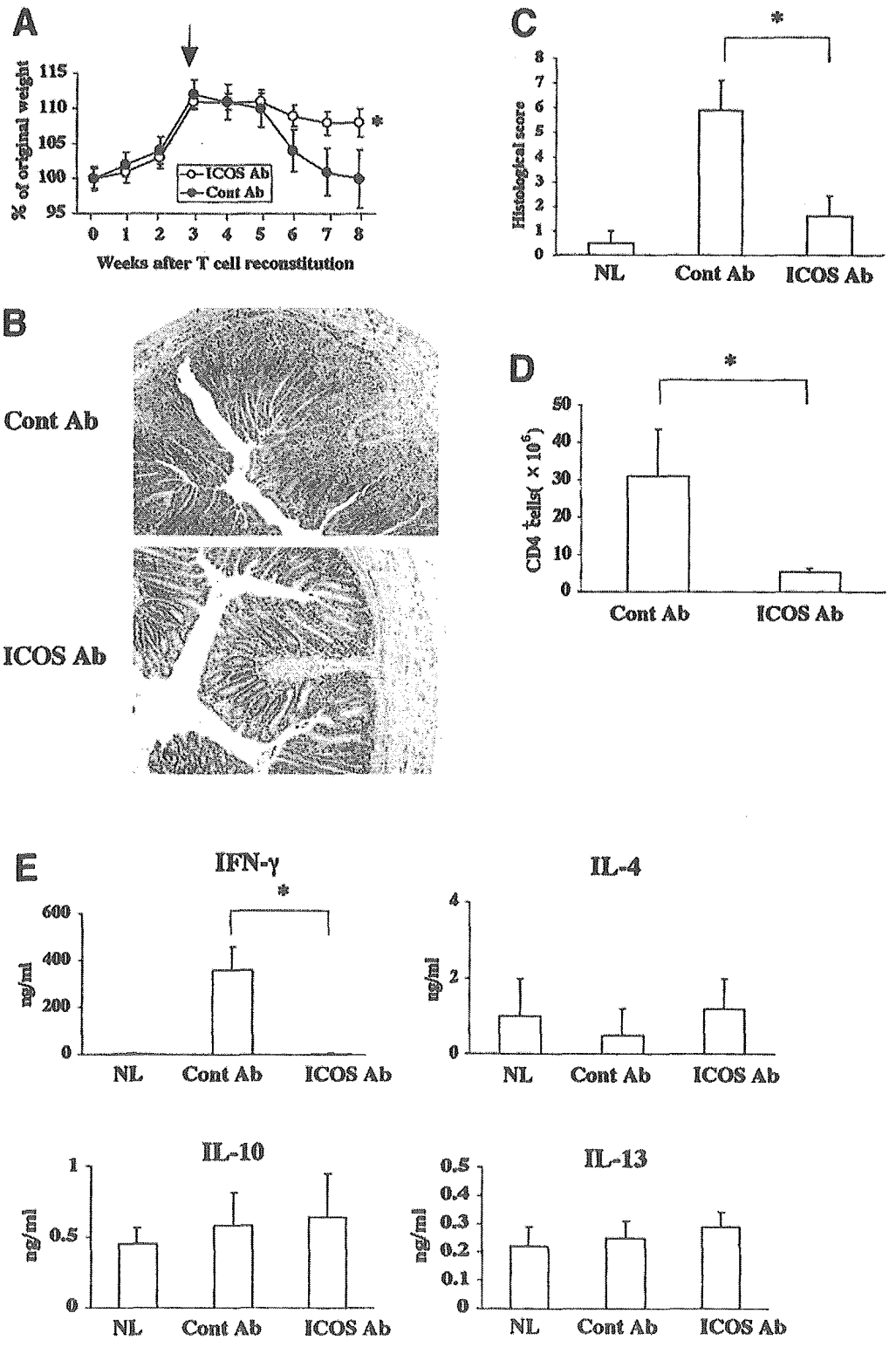


**Figure 9.** Improvement of colitis by delayed administration of anti-ICOS mAb. (*A*) Colitic SCID mice were treated with either anti-ICOS mAb or control rat IgG from 3 to 7 weeks after T-cell transfer. The change in weight over time is expressed as percent of the original weight. The *arrow* indicates the start of treatment. Data are represented as the mean ± SD of mice in each group. $*P <$ 0.005 compared with control IgG. (*B*) Histologic examination of the colons 7 weeks after T-cell transfer and treatment with control rat IgG or anti-ICOS mAb. (Original magnification 100×.) (*C*) Severity of colitis. Colons were removed 7 weeks after T-cell transfer and stained with H & E. Pathology was graded on a scale of 0–7. Data are represented as the mean ± SD of 5 mice in each group. $*P < 0.05$. NL, normal BALB/c mice. (*D*) Number of LP $CD4^+$ T cells. LP $CD4^+$ T cells were isolated from colons 7 weeks after T-cell transfer. Data are represented as the mean ± SD of 5 mice in each group. $*P < 0.05$. (*E*) Cytokine production by LP $CD4^+$ T cells. LP $CD4^+$ T cells were prepared from colons 7 weeks after T-cell transfer and stimulated with anti-CD3 and anti-CD28 mAbs for 48 hours. The indicated cytokines in the supernatants were determined by enzyme-linked immunosorbent assay. Data are represented as mean ± SD of 5 mice in each group. $*P < 0.01$ compared with control IgG.

### Anti-ICOS mAb Ameliorates Colitis Induced by Adoptive Transfer of LP $CD4^+$ T Cells From Colitic Mice

To further assess the effect of anti-ICOS mAb on pathogenic T cells in this model of colitis, we treated the recipient mice after adoptive transfer of LP $CD4^+$ T cells from colitic mice. The weight loss (Figure 10*A*) and histologic changes (Figure 10*B* and *C*) were significantly improved and the infiltration of $CD4^+$ T cells (Figure 10*D*) was greatly reduced in the mice treated with anti-ICOS mAb compared with the control IgG-treated mice.

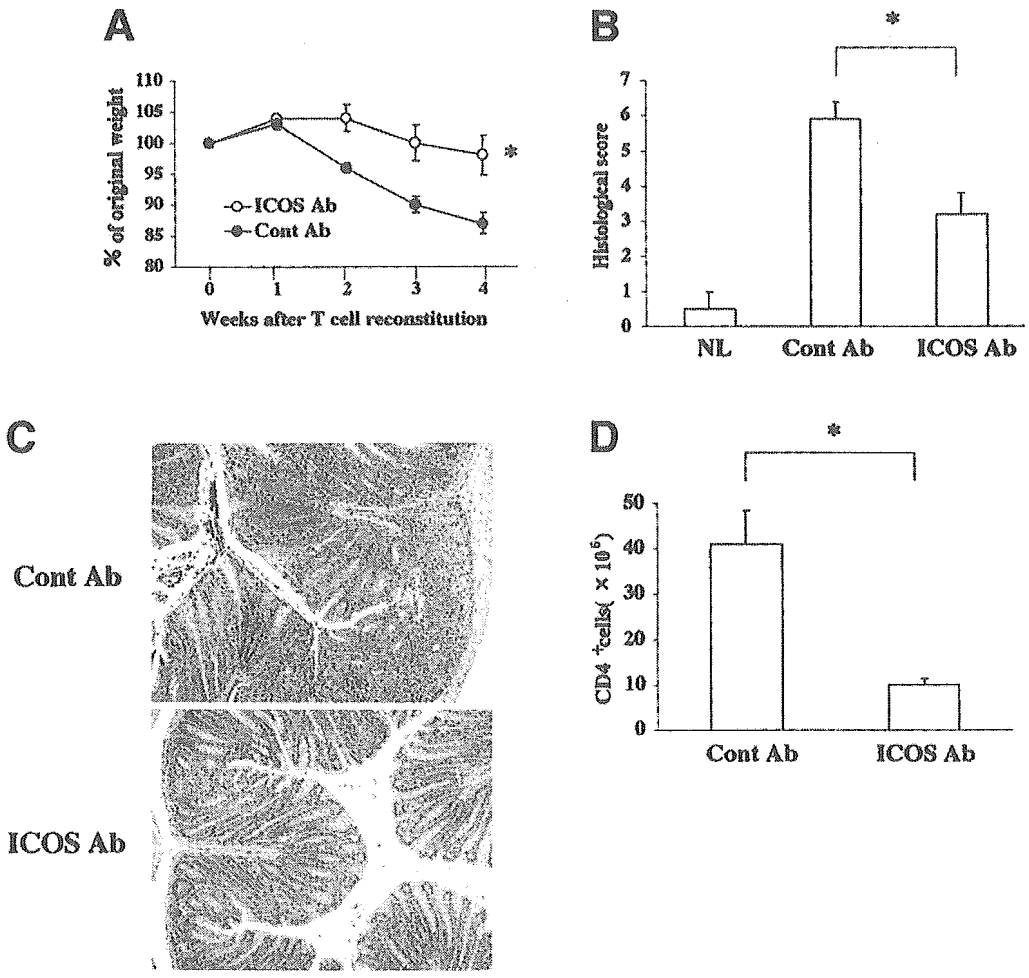


**Figure 10.** Effect of anti-ICOS mAb on colitis induced by adoptive transfer of LP $CD4^+$ T cells from colitic mice to SCID mice. LP $CD4^+$ T cells ($1 \times 10^6$) from colitic SCID mice 7 weeks after $CD4^+CD45RB^{high}$ T-cell transfer were injected IP into BALB/c SCID mice. Control rat IgG or anti-ICOS mAb were administered IP for 4 weeks. (*A*) Weight change. Data are represented as the mean ± SD of 7 mice in each group. $*P < 0.05$ compared with control IgG. (*B*) Histologic examination of the colon at 4 weeks. Representatives of 7 mice in each group. (Original magnification 100×.) (*C*) Severity of colitis. Colons were removed at 4 weeks and stained with H & E. Pathology was graded on a scale of 0–7. Data are represented as the mean ± SD of 7 mice in each group. NL, normal BALB/c mice. $*P < 0.05$. (*D*) Number of LP $CD4^+$ T cells. LP $CD4^+$ T cells were isolated from colons 4 weeks after transfer. Data are represented as the mean ± SD of 7 mice in each group. NL, normal BALB/c mice. $*P < 0.05$.

## Discussion

The most important findings in the present study were the up-regulation of ICOS expression on infiltrating T cells in colitic mucosa and the ameliorating effect of anti-ICOS mAb in the development of colitis. We used a Th1-mediated murine colitis model induced by adoptive transfer of $CD4^+CD45RB^{high}$ T cells into SCID mice. Because this model shares histopathologic and immunologic characteristics with human CD, a beneficial effect may be expected by targeting ICOS in the treatment of CD.

Because B7RP-1 was also detected in the colitic lesion, we first investigated whether the effect of treatment with anti-ICOS mAb was induced by blocking the ICOS/B7RP-1 interaction. However, the administration of neutralizing anti–B7RP-1 mAb did not prevent the colitis. Furthermore, the adoptive transfer of $CD4^+CD45RB^{high}$ T cells from ICOS−/− mice was as competent as those from ICOS+/− mice to induce the colitis, indicating that ICOS might not be essential for the priming, migration, and expansion of pathogenic T cells to induce the colitis. These results suggest that the effect of anti-ICOS mAb was not critically caused by the blocking of the ICOS/B7RP-1 interaction. However, an alternative explanation is that ICOS might have ligands other than B7RP-1 that are expressed in colitic mice, although anti–B7RP-1 mAb (HK5.3) blocked binding of mouse ICOS Ig to mouse B7RP-1 transfectants (Figure 1).

We next examined whether treatment with anti-ICOS mAb would induce a Th2 deviation of pathogenic T cells, because ICOS has been implicated in Th2 responses.[13,16,28] However, no significant change in production of IL-4, IL-10, or IL-13 was observed by in vitro stimulation of LP $CD4^+$ T cells from the mice treated with anti-ICOS mAb compared with the control IgG-treated mice. Interestingly, the Th2 cytokines in this model were higher compared with normal, even though production of IFN-γ was 100-fold higher than Th2 cytokines. Indeed, Powrie et al. showed that IL-4 is not required for the development of colitis in this model.[29] Elevated Th2 cytokines might be secondary to primary Th1-mediated immune responses in this model. The role

of Th2 cytokines in this model should be clarified by further studies.

Although treatment with anti-ICOS mAb did not increase production of IL-10 by LP $CD4^+$ T cells in vitro, it has been shown that IL-10 plays an important suppressive role in the pathogenesis of colitis.[8,30,31] To further address the possible contribution of IL-10 to the ameliorating effect of anti-ICOS mAb on colitis, we coadministered neutralizing anti–IL-10 mAb. However, this did not abrogate the effect of anti-ICOS mAb, excluding the contribution of IL-10. It is also possible that the anti–IL-10 mAb (JES5-2A5) in our experiment failed to sufficiently neutralize IL-10 activity in vivo. Recently, Asseman et al. showed that a blocking mAb reactive with the murine IL-10R (1B1.2) but not JES5-2A5 abrogated protection from colitis induced by the $CD4^+CD45RB^{low}$ population,[30] suggesting the relative ineffectiveness of JES5-2A5. Further studies using 1B1.2 are needed to address this point.

Enhanced T-cell resistance against apoptosis may contribute to perpetuation of CD.[32,33] Because most immunosuppressive drugs seem to mediate their clinical effects at least in part by the induction of T-cell apoptosis, such resistance against apoptosis may underlie insufficient short-term effects of immunosuppressive therapy in CD.[34] In the present study, we showed an unexpected function of anti-ICOS mAb to induce apoptosis in colon-infiltrating pathogenic T cells in vivo. It is possible that the ICOS-expressing cells in inflamed mucosa are killed by complement fixation. To address the possible contribution of complements, we tested whether anti-ICOS mAb (B10.5) fixes complements in vitro. However, B10.5 did not kill the ICOS transfectant in the presence of mouse complements (data not shown). Further studies are needed to assess the anti-ICOS mAb–inducing apoptosis mechanism.

In addition, the delayed administration of anti-ICOS mAb significantly inhibited production of IFN-γ by LP $CD4^+$ T cells. This suggests that the ameliorating effect of anti-ICOS mAb on colitis might be at least partly mediated by its apoptosis-inducing effect on pathogenic Th1 cells producing IFN-γ.

Of particular clinical importance, delayed treatment with anti-ICOS mAb ameliorated ongoing disease. In contrast to CD28, ICOS is specifically expressed on activated T cells and is thus an ideal target molecule for the treatment of CD. Infliximab (anti-human tumor necrosis factor α mAb) has been approved in the United States and Europe for the treatment of patients with CD refractory to conventional therapy. Interestingly, this mAb induces apoptosis of activated T lymphocytes and macrophages that express membrane-bound TNF.[35,36] Similarly, engagement of ICOS by this mAb might induce apoptosis of pathogenic T cells via ICOS-mediated signal transduction. Alternatively, anti-ICOS mAb may activate complement-mediated lysis or antibody-dependent cell-mediated cytotoxicity. Further studies are needed to address these possibilities.

In conclusion, our present study in a well-validated Th1-mediated mouse colitis model indicates that expression of ICOS and B7RP-1 is increased in activated T lymphocytes and macrophages, respectively, during experimental colonic inflammation. Furthermore, anti-ICOS mAb could both prevent and reverse established inflammation, suggesting a potential therapeutic application in human Th1-mediated intestinal inflammatory conditions such as CD. However, blockade of B7RP-1 did not have a protective effect, indicating that the interaction between these costimulatory molecules was not critical to the development of colitis. Anti-ICOS mAb induced apoptosis of activated T cells. Thus, selective induction of apoptosis of ICOS-expressing pathogenic T cells, rather than blockade of ICOS costimulatory activity, seems to be the therapeutic mechanism of this promising treatment strategy.

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ELSEVIER

# Prospects for regeneration of gastrointestinal epithelia using bone-marrow cells

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**One of the recent challenges in regenerative medicine is to reconstitute damaged human tissues using the pluripotency of adult-tissue-derived cells. We propose the new concept that bone-marrow-derived cells could promote the regeneration of damaged epithelia in the human gastrointestinal (GI) tract. In bone-marrow transplant (BMT) recipients, epithelial cells of donor origin were distributed throughout the GI tract. Furthermore, these cells substantially repopulated the GI tract of BMT recipients during epithelial regeneration after graft-versus-host disease or ulcer formation. These findings might lead to a novel therapy for the repair of damaged intestinal epithelia using the pluripotency of bone-marrow cells, and could provide an alternative therapy for refractory inflammatory bowel diseases.**

The epithelium of the gastrointestinal (GI) tract is a tissue in which rapid renewal continues throughout life. For example, epithelial cells of the small intestine are completely renewed in approximately 3–4 days [1,2]. This rapid regeneration is maintained by intestinal epithelial stem cells, which can self-renew and also give rise to the four main cell lineages in the intestinal epithelia: enterocytes, goblet cells, enteroendocrine cells and Paneth cells [2]. An estimated 4–5 intestinal epithelial stem cells are found at the bottom of each intestinal crypt, and these provide rapid-proliferating progenitor cells that reside in the mid-crypt region (Fig. 1) [3–5]. Proliferated immature epithelial cells are thought to become mature as they migrate along the crypt–villus axis, and drop off into the gut lumen when they reach the tip of the villus. However, the origin of intestinal epithelial cells, and the precise molecular mechanism of differentiation of intestinal epithelial cells, has yet to be fully characterized.

Despite rapid progress in regenerative medicine and stem-cell biology, the intestinal epithelia has received little attention to date, probably because they normally regenerate continuously without any manipulation. However, in clinical practice, we face some diseases in which regeneration of the damaged epithelia is disturbed. One example of such a condition is inflammatory bowel disease. In western countries and in Japan, the number of patients suffering from this disease is increasing [6–8], and some patient populations fail to regenerate the damaged intestinal epithelia. Complete remission cannot be achieved in these patients without proper regeneration of the epithelia and, therefore, we have sought to establish a novel therapy aimed at promoting this.

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## Pluripotency of adult-tissue-derived cells

There are two main sources of cells for regenerating human tissues: embryonic stem (ES) cells and adult-tissue-derived pluripotent cells. ES cells are extremely immature cells derived from a developing blastocyst [9,10], and have the potential to differentiate into a wide variety of cell types, including epithelial cells [10,11]. However, they have to be taken from a developing embryo, and several *in vivo* studies have shown that these cells have the potential to develop teratomas [9]. Therapeutic use of ES cells will require several ethical and technical problems to be solved.

The adult-tissue-derived pluripotent cells include a wide variety of tissue-specific stem cells. Among these, bone-marrow cells have been well studied and are thought to have the pluripotency to differentiate into cells outside of the haematopoietic lineage in certain conditions [12,13]. Indeed, a recent report has suggested that certain bone-marrow-derived cells cultured *in vitro* have almost the same pluripotency as ES cells, but do not generate teratomas [14,15]. However, other reports present contradictory results on the pluripotency of bone-marrow cells [16–18] and, hence, this issue will require further studies before it can be confirmed and well understood [19]. One of the alternative explanations for the observed pluripotency of bone-marrow cells is cell fusion [16,18]. This possibility was proposed on the basis of *in vitro* studies, and the importance of cell fusion *in vivo* remains controversial [20–24]. However, our recent findings clearly suggest that bone-marrow-derived cells have therapeutic potential in regeneration of the GI epithelia in humans [25].

## Bone-marrow-derived cells repopulate the GI epithelia of bone-marrow transplant recipients

### *Donor-derived epithelial cells were distributed throughout the GI tract*

In our recent study, we sought to determine whether human bone-marrow cells have the potential to repopulate the GI epithelia and could be therefore considered as a

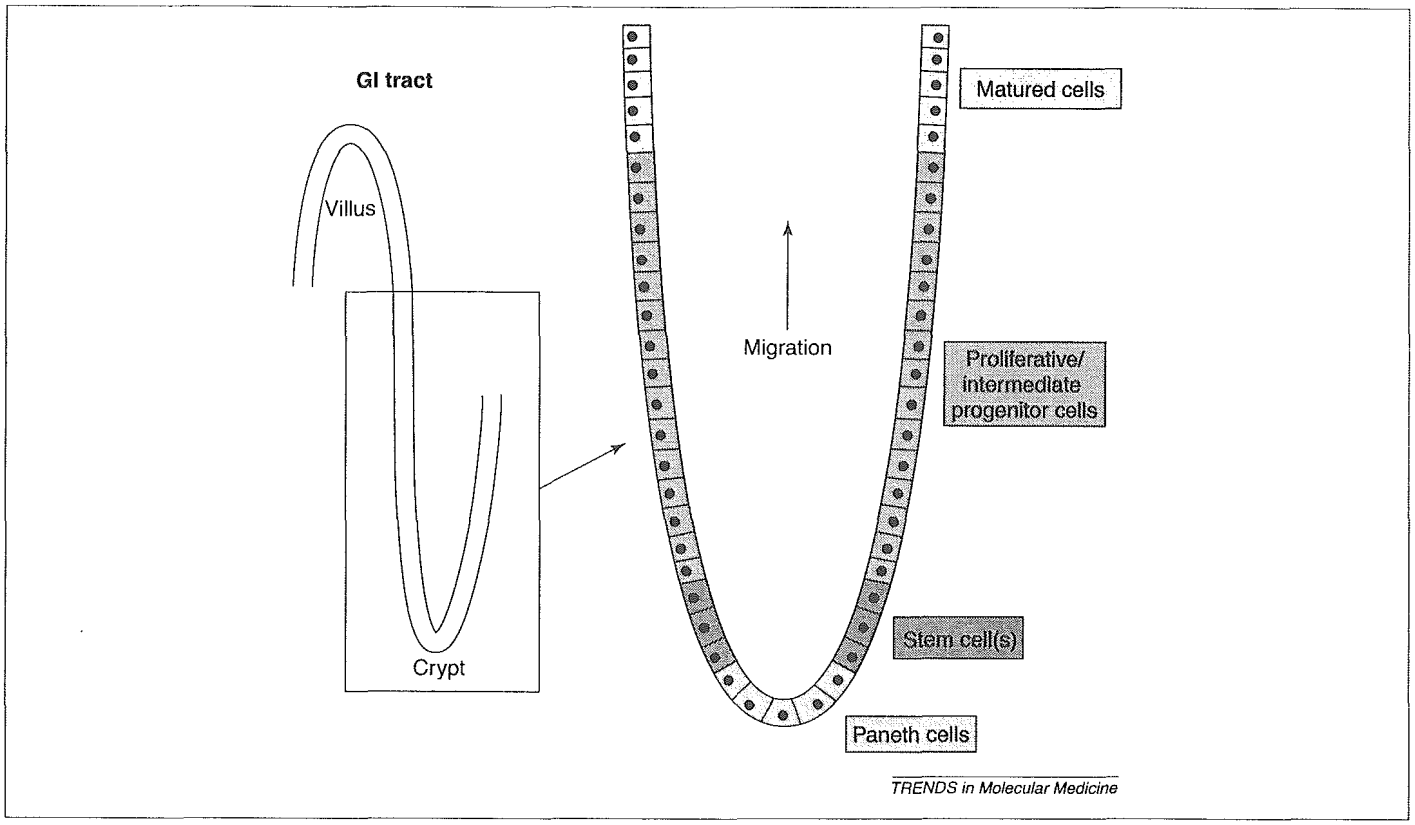


**Fig. 1.** Structure of a crypt in the gastrointestinal (GI) tract. A crypt functions as a single unit to maintain and regenerate GI epithelia. Intestinal stem cells reside in the lower part of the crypt and, as well as self-renewal, they provide proliferating progenitor cells that mature into the four main lineages of the GI epithelia: enterocytes, goblet cells, enteroendocrine cells and Paneth cells.

therapeutic source for intestinal diseases [25]. We examined biopsy specimens of the GI tract, taken during endoscopic examinations of females who had received a bone-marrow transplant (BMT) from male donors. Fluorescent *in situ* hybridization (FISH) was used to identify, in the GI tract of each of the subjects, epithelial cells that contained a Y chromosome and, hence, must have descended from transplanted bone-marrow cells. We examined 29 biopsy specimens taken during 12 endoscopic examinations of four females who had received a BMT from male donors, and detected donor-derived epithelial cells in every part of the GI tract (Fig. 2). The donor-derived epithelial cells were not clustered, and there were no linear clusters in the longitudinal sections of the intestinal crypt. Instead, these cells were distributed individually in diffuse patches around the epithelia. Furthermore, donor-derived epithelial cells were detected in tissue samples obtained more than eight years after BMT, suggesting that these cells are continuously generated from bone-marrow cells and are continuously renewed.

Our findings are supported by an another report, which showed that intestinal epithelial cells of suspected extra-intestinal origin exist within the graft of a small-intestinal transplantation recipient [26]. Although there is evidence of cell fusion *in vivo* [22,24], several results from our specimens suggest that cell fusion *in vivo* does not contribute substantially to our observations of GI epithelia [25].

*Donor-derived epithelial cells repopulated the GI tract of transplant recipients during epithelial regeneration*

By taking GI-tract biopsy specimens from each patient at a series of time points, we were able to correlate the changes in the population of donor-derived epithelial cells with regeneration of the damaged epithelia [25]. We obtained specimens at four time points, from the small intestine of a female BMT recipient who developed acute graft-versus-host disease (GVHD) (Fig. 3). No donor-derived cells were detected in the tissue obtained 25 days before BMT. The number of donor-derived epithelial cells was low (0.4 per 100 nucleated cells) 26 days after BMT, at the beginning of an episode of GVHD, but this had increased ninefold (to 3.6 per 100 nucleated cells) by day 77 as GVHD ran its course. By day 777 after BMT, the patient had recovered from GVHD, but donor-derived epithelial cells were still present, although they were less abundant (1.0 per 100 nucleated cells). We also examined another case, in which the patient developed a gastric ulcer by day 586 after BMT. At this time, there were 40–50 × as many donor-derived epithelial cells in the regenerating epithelia of the stomach as in histologically normal epithelia.

These findings suggest that when tissue repair is required to reconstitute human GI epithelia, bone-marrow-derived cells can assist in the healing process by actively repopulating the regenerating epithelia, thereby promoting the repair of mucosa damaged by GVHD or gastric ulcer.

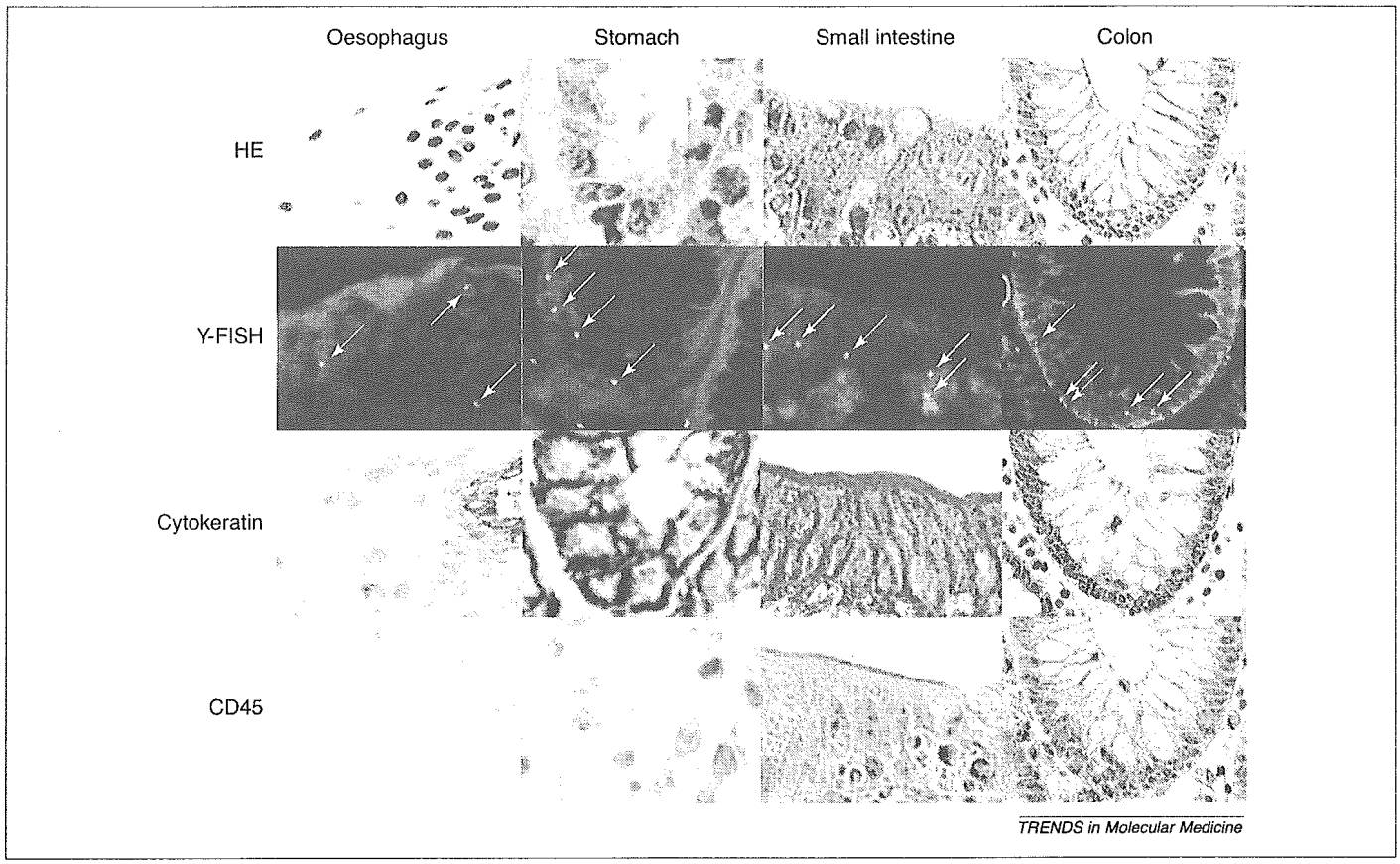


**Fig. 2.** Bone-marrow-derived cells are distributed in the epithelia of the entire gastrointestinal (GI) tract after bone-marrow transplantation (BMT). Serial section analysis was performed by Y-chromosome fluorescent *in situ* hybridization (Y-FISH) and immunoperoxidase staining using endoscopic biopsy specimens of GI tract tissues from female recipients 26–381 days after BMT from male donors. Y-chromosome-positive epithelial cells were confirmed by their location and expression of cytokeratin but not CD45. Y-chromosome-positive cells (white arrows) were predominantly found in cytokeratin$^{+}$CD45$^{-}$ epithelial cells, in every part of GI tract, including the oesophagus, stomach, small intestine and colon. A serial section stained by haematoxylin and eosin (HE) is also shown. Magnifications are 800 × for oesophagus and colon, 1600 × for stomach and 1200 × for small intestine. Adapted with permission from [25].

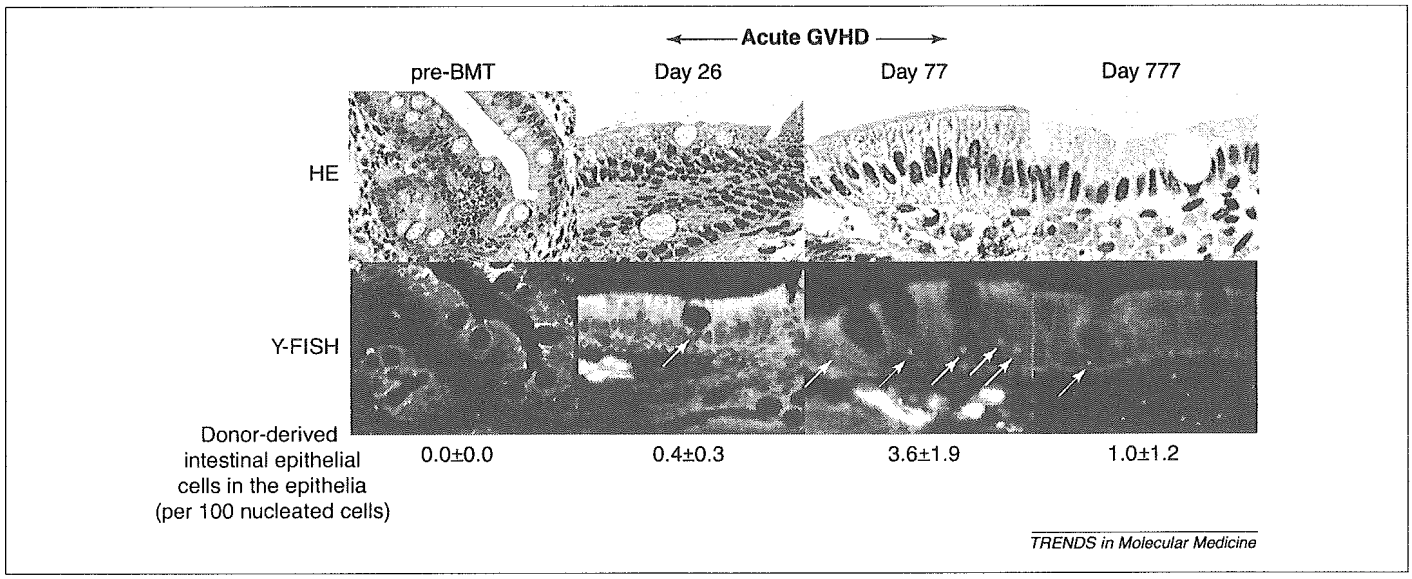


**Fig. 3.** The number of bone-marrow-derived epithelial cells increased during regeneration of the gastrointestinal epithelia. Serial section analysis was performed by Y-chromosome fluorescent *in situ* hybridization (Y-FISH) and immunoperoxidase staining in specimens of small-intestinal tissues taken at four different time points from a female patient before and after bone-marrow transplantation (BMT) from a male donor. No Y-chromosome-signal-positive cells were detected in a tissue specimen taken 25 days before BMT (pre-BMT). The frequency of Y-chromosome-signal-positive epithelial cells (white arrows) was quite low on day 26 after BMT, when sudden onset of acute graft-versus-host disease (GVHD) occurred, but it increased ninefold by day 77, as GVHD ran its course. On day 777 after BMT, when GVHD had gone, Y-chromosome-signal-positive epithelial cells were decreased but still present at low frequency. A serial section stained by haematoxylin and eosin (HE) is also shown. Magnifications are 400 × for pre-BMT, 800 × for day 26 and 1200 × for days 77 and 777. Adapted with permission from [25].

## How can intestinal epithelial cells be generated from bone-marrow cells?

The mechanism by which bone-marrow-derived epithelial cells are generated remains to be elucidated, but we can suggest two possible pathways (Fig. 4). Because these cells show a patchy distribution, the first potential pathway is somehow to be integrated into the epithelia as considerably differentiated epithelial cells but at extremely low frequency. The second possible pathway is to be integrated as cells that maintain some degree of stem-cell property, but with proliferation and differentiation strictly suppressed by the residing competitors. The increase in bone-marrow-derived epithelial cells during epithelial regeneration could result from an increase in the frequency of cells entering the intestinal epithelia, or from rapid proliferation within the epithelia as a result of weakened suppression. The frequency of bone-marrow-derived epithelial cells falls to almost the same level as in normal conditions after regeneration is completed, and this could be explained by restriction in entry or proliferation when normal structure is restored.

We hope to clarify whether either of these pathways exists as a means of generating human intestinal epithelia. Recently, a reliable molecular marker for intestinal epithelial stem cells and progenitor cells was reported [27,28]. Clarification of whether the donor-derived cells express these stem-cell markers will provide evidence as to which of the two pathways is more likely. In another study, human bone-marrow cells have also been shown to differentiate into pericryptal myofibroblasts [29], and these bone-marrow-derived myofibroblasts might be one of the cells that provides the 'niche' for bone-marrow cells to enter the intestinal epithelia [30]. However, we do not have any satisfactory results to confirm this idea.

## Future prospects

Several case reports have suggested that BMT (either allogeneic or autologous) has therapeutic effects for autoimmune diseases, including inflammatory bowel diseases [31–35]. A recent study of two cases of Crohn's disease demonstrated that autologous haematopoietic stem-cell transplantation, following high-dose immunosuppressive therapy, induced long-term remission in refractory patients [36]. So far, the therapeutic effect in these cases has been thought to derive from elimination of pathogenic immune cells. However, considering our results [25], bone-marrow cells might also have an important role in regenerating the damaged epithelia in these cases. All of our reported cases received a BMT that included heterogeneous cell types, but another report has demonstrated that patients receiving $CD34^+$ haematopoietic stem-cell transplantation show similar results [13]. This suggests that $CD34^+$ cells might be the origin of the bone-marrow-derived intestinal epithelial cells, at least in humans, and could be the therapeutic source for regenerating epithelia. However, this remains controversial [17].

Whether BMT itself is the best way to use bone-marrow-derived cells to regenerate intestinal epithelia is unknown. If there are humoral factors that lead to the introduction of bone-marrow cells into the intestinal epithelia then these might also be used. A possible candidate is granulocyte–macrophage colony-stimulating factor, which has recently been reported to have therapeutic effects with Crohn's disease patients [37]. A precise study of the molecular mechanisms by which bone-marrow-derived epithelial cells are generated might help us to uncover these factors. Finding a molecule that promotes the introduction of bone-marrow cells into intestinal epithelia will be one of the major subjects of future study.

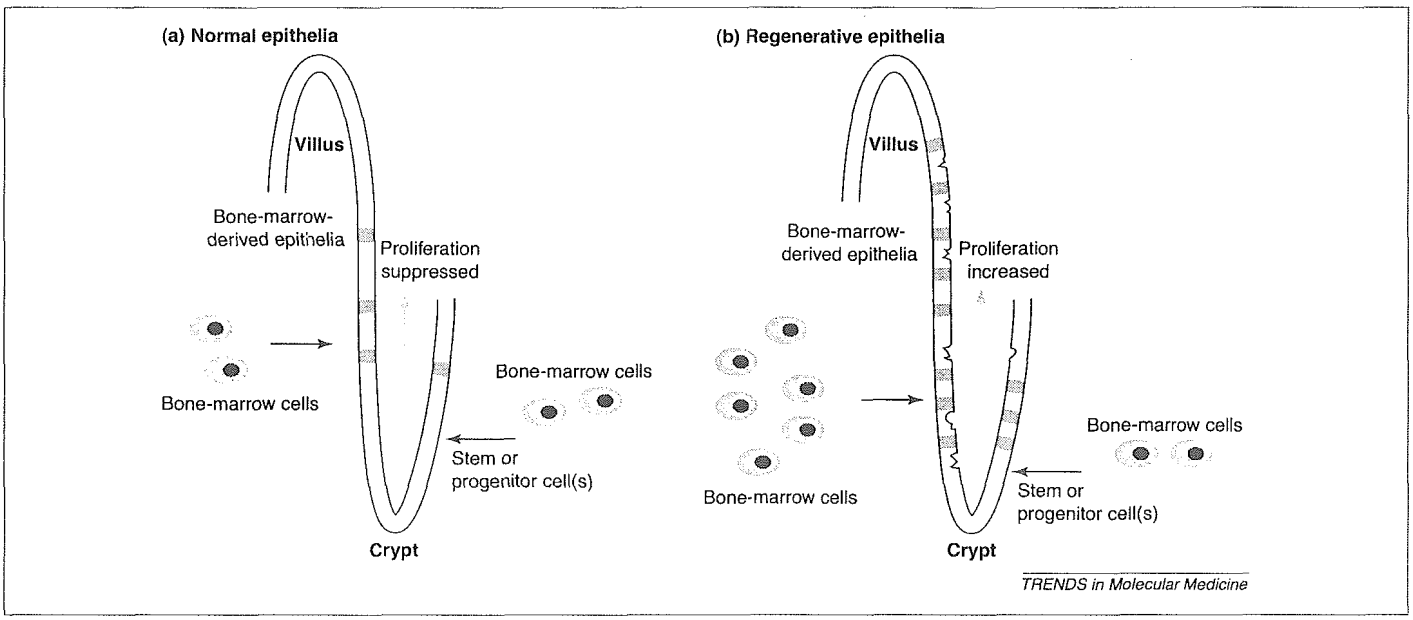


**Fig. 4.** Possible pathways by which bone-marrow cells could repopulate the gastrointestinal epithelia. (a) At steady state, when epithelial cells are continuously generated from the residing stem cells, bone-marrow cells (blue) enter the epithelia as relatively differentiated epithelial cells, at extremely low frequency. Alternatively, they enter as stem-cell-like progenitor cells, with proliferation strictly suppressed by residing competitive stem cells. (b) When regeneration is required, the frequency of bone-marrow cells entering the epithelia increases substantially, or suppression of the proliferation of bone-marrow-derived stem-cell-like progenitor cells is weakened. As a result, the relative frequency of bone-marrow-derived epithelial cells increases, thereby regenerating the damaged epithelia.

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