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The 15-lipoxygenase-1 expression may enhance the sensitivity to non-steroidal anti-inflammatory drug-induced apoptosis in colorectal cancers from patients who are treated with the compounds

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15-lipoxygenase-1, apoptosis, chemoprevention, colorectal cancer, NSAIDs.

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Abstract

Background and Aim: Non-steroidal anti-inflammatory drugs (NSAIDs) can prevent colorectal cancer (CRC), but their effect is limited. Recent studies have shown the involvement of 15-lipoxygenase-1 (15-LOX-1) in NSAID-induced apoptosis in colorectal carcinoma cells. We evaluate whether 15-LOX-1 expression influences the sensitivity of NSAID-induced apoptosis in CRCs.

Methods: In 22 CRC surgical samples from NSAID users who had been constant for more than 5 years and 28 CRC surgical samples from NSAID non-users, the expressions of 15-LOX-1, cyclooxygenase-2 (COX-2), beta-catenin, and p53 were analyzed using immunohistochemistry. TUNEL assay was also performed for samples. The effects of the transient transfection of 15-LOX-1 cDNA on indomethacin-induced apoptosis were certified in HCT-116 cells. The effects of adding 13-S-hydroxyoctadecadinoic acid (13-S-HODE) on indomethacin-induced apoptosis were also examined in HCT-116 cells. The levels of apoptosis were determined by the analysis of the floating-cells ratio and DNA gel electrophoresis.

Results: The expression of 15-LOX-1 on CRCs from NSAID users was significantly decreased compared with those from NSAID non-users; however, the expressions of other molecules were not significantly different between two groups. The levels of TUNEL scoring in samples from NSAID users were similar to those from NSAID non-users. Indomethacin (100 μM) induced less apoptosis in mocked cells, whereas the same concentrations of indomethacin enhanced the level of apoptosis in 15-LOX-1-transfected cells. 13-S-HODE also increased the level of indomethacin-induced apoptosis in cells.

Conclusion: Results suggest that 15-LOX-1 expression may be one of the mechanisms which enhance the sensitivity to NSAID-induced apoptosis in CRCs from patients who are treated with the compounds.

Introduction

Colorectal cancer (CRC) is a major health concern in many countries. CRC can be preventable by well-established screening and endoscopic or surgical techniques. However, the mortality of CRC remains high, calling for better compliance with screening guidelines.[1] Cancer chemoprevention, a promising strategy, strives to block, reverse, or delay carcinogenesis before the development of invasive disease by targeting key molecular derangements using pharmacological agents.[2] Because CRC is believed to develop over 10–20 years, providing the chance for interruption long before it poses clinical symptoms,[3] CRC can be a good target for cancer chemoprevention.

It has been established that non-steroidal anti-inflammatory drugs (NSAIDs) can show the chemopreventive effect on colorectal neoplasia. The chemopreventive mechanisms of NSAIDs are dependent on[4] or independent[5,6] of cyclooxygenase (COX) enzymes. More than 30 epidemiological studies confirm 40–50% reductions in colorectal adenomas, CRCs, and cancer-associated mortality among users of aspirin or other NSAIDs as compared with non-users. However, the clinical results of NSAIDs are variable.[7] Previous studies show that although sulindac reduces a number of adenomas in many patients with familial adenomatous polyposis, an increased number of adenomas are noted 6 months after the start of sulindac therapy in some patients.[8] To achieve the prompt effect of NSAID-induced chemoprevention on colorectal

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cancer, the mechanism of resistance should be evaluated. Several reports have proposed biomarkers, which predict resistance to chemopreventive treatment including K-ras,[9] bcl-2,[10] or BAX[11] *in vitro* or animal examination models.

Previous studies have shown that the enhancement of 15-lipoxygenase-1 (15-LOX-1), the main enzyme for metabolizing linoleic acid to 13-S-hydroxyoctadecadinoic acid (13-S-HODE), is critical for NSAID-induced apoptosis in colon[12,13] or esophageal[14] carcinoma cells. These studies suggest that the effectiveness of NSAID-induced chemoprevention may depend on the expression of 15-LOX-1 in patients with CRCs. Therefore, we sought to identify the impact of 15-LOX-1 expression in CRCs from patients who were treated with NSAIDs.

Materials and methods

Tissue samples

We analyzed 50 samples of primary human colorectal carcinomas and matched normal adjacent tissues that were obtained from patients who had undergone surgical or endoscopic resection in 2004 at the National Hospital Organization Beppu Medical Center. Eighty-five NSAID non-users with CRC had undergone surgical or endoscopic resection in 2004 at the hospital. Fifty-four of 85 patients gave their informed consent for participation in this study. We selected 28 age- and gender-matched patients from a group of 54 non-NSAID users who were then compared to NSAID users. The study group was composed of 22 NSAID users and 28 NSAID non-users. Twenty-two of the 50 samples were obtained from 22 patients who had been consistently treated with NSAIDs for more than 5 years (average 9.8 years) and had no colorectal lesions when starting at the therapy. They visited the clinic regularly to check their general condition and compliance. The other 28 samples were obtained from 28 age- and gender-matched patients who had been non-users of NSAIDs. Consecutive 4–6 μm tissue sections were cut from paraffin blocks of samples and placed on polylysine-coated slides for immunohistochemistry (IHC) analysis. The ethical committee of the National Hospital Organization Beppu Medical Center approved this protocol. All patients gave their informed consent for participation in this study. Their clinical characteristics are summarized in Table 1.

Table 1 Characteristics of patients with colorectal cancers

	NSAIDs	
	Users ($n = 22$)	Non-users ($n = 28$)
Age (average, year)	71.3	68.5
Gender (M/F)	14/8	18/10
Clinical stage[†]		
I	8	6
II	8	8
III	5	14
IV	1	0
Original disease		
Arthritis	4	0
Heart diseases	12	0
TIA	2	0
Unknown	4	0
NSAIDs		
Aspirin (100 mg/day)	14	0
Others[‡]	8	0

[†]TNM classification of colorectal cancer; [‡]Indomethacin, ibuprofen, and loxoprofen sodium were included.

Cell culture

Human colorectal carcinoma cells HCT-116 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HCT-116 cells were cultured as described previously.[15] Indomethacin (Sigma, St. Louis, MO, USA) was solubilized in less than 0.5% DMSO. 13-S-HODE (Cayman Chemical, Ann Arbor, MI, USA) was kept at −20°C and diluted with the appropriate media without FBS, just before the start of the experiment.

Immunohistochemistry (IHC) for 15-LOX-1

After deparaffinizing, slides were treated with 0.05% protease (type XXIV, Sigma, St. Louis, MO, USA) for 10 min at room temperature. Endogeneous peroxidase activity was blocked by incubation in 3% Hydrogen Peroxide for 5 min. Non-specific stainings were blocked by incubation with 10% porcine serum (GEMINI Bio-Products, Woodland, CA, USA) in PBS for 10 min at room temperature. Slides were incubated with 1:800 diluted anti15-LOX-1 polyclonal antibody (Cayman Chemical Company, Ann Arbor, MI, USA) overnight at 4°C. As a positive control for 15-LOX-1, sections of human bronchus were immunostained.[16] As a negative control, 10% porcine serum in PBS was applied. After slides were rinsed in phosphate-buffered saline (PBS), they were incubated with the biotinylated secondary IgG antibody for 30 min at room temperature. After being rinsed in PBS, diluted peroxidase-conjugated streptavidin (DAKO Cytometion, Glostrup, Denmark) was applied for 30 min at room temperature and developed with 20% 3,3′-diaminobenzidine,tetrahydrochloride and 6% hydrogen peroxide (DAB) for 5 min at room temperature.

IHC for COX-2, beta-catenin, and p53

For antigen retrieval, slides were microwaved four times in a 1 mM EDTA (pH 8.0) for 5 min in IHC for COX-2 and beta-catenin. In IHC for p53, slides were heated in an auto-clave with 1 mM EDTA (pH 8.0) at 121°C for 15 min. Staining was performed with each diluted primary antibody for 30 min on an automated immunostainer (Ventana NX System, Ventana Medical System, Inc. Tucson, AZ, USA) by using an endogeneous biotin blocking kit (Ventana Medical System, Inc. Tucson, AZ, USA) and Ventana DAB universal kit (Ventana Medical Systems, Inc., Tucson, AZ, USA). The immunostainer was constrained to perform all incubations at 37°C. Mouse monoclonal anti-COX-2 (the dilution of 1:100, DAKO Cytomation, Glostrup, Denmark), mouse monoclonal antibeta-catenin (1:100, DAKO Cytomation, Glostrup, Denmark), and mouse monoclonal antip53 (1:100, Novocastra, Newcastle, UK) were used for the study.

TUNEL staining

The activity of endogenous peroxidase was blocked with 0.3% Hydrogen Peroxide for 30 min. After treatment with 20 μg/mL

proteinase K for 10 min at room temperature, slides were incubated with reaction reagent 0.15 U/El of Terminal Transferase (Roche diagnostics, Indianapolis, IN, USA), 0.4 nM of Biotin-16-dUTP (Roche diagnostics, Indianapolis, IN, USA), and TUNEL Dilution Buffer (Roche diagnostics, Indianapolis, IN, USA) for 60 min at 37°C. They were covered with peroxidase-conjugated streptavidin (1:500, DAKO Cytomction, Glostrup, Denmark) and incubated with DAB for 5 min at room temperature. Finally, counterstaining was done with methyl green.

IHC and TUNEL scoring

Two observers who were unaware of the clinical characteristics scored the immunostained slides. 15-LOX-1 staining in the tumor cells was assessed, comparing it with normal mucosa to determine whether the staining was positive or negative. The extent of COX-2 staining was recorded based on the percentage of stained tumor epithelial cells: 0 = 1–20%; 1 = 21–70%; 2 = 70%–). Beta-catenin, membranous, cytoplasmic, and nuclear staining were evaluated individually. Membranous and cytoplasmic staining were compared with normal mucosa and assessed for decreased expression or over-expression respectively. Nuclear staining (absent in normal tissue) was scored semiquantitatively using a scale from 0 to 3 (0, no expression; 1, <5% positive nuclei; 2, <25% positive nuclei; and 3, >25% positive nuclei). p53 staining was considered positive when >10% of nuclei stained positive. For TUNEL scoring, 4 microscope fields (× 400) were randomly chosen; the number of TUNEL-positive cancer cells was counted and divided by the total number of cancer cells in each field. The level of apoptosis was expressed as the average percentage TUNEL positive cells of 4 fields.

15-LOX-1 transient transfection

HCT-116 cells were transfected with plasmid pcDNA 3/15-LOX-1-sense or vector alone as described previously.[15] After incubation, cells were lyzed and evaluated for 15-LOX-1, expression by immunoblot analysis. In some experiments, the culture media was measured for 13-S-HODE by ELISA.

13-S-HODE measurement by ELISA

HCT-116 cells (10^5/well) were transfected with 15-LOX-1 or vector for the indicated period of time in 6-well plates. These cells underwent serum starvation for 1 h and were then washed with PBS and exposed to 25 μM linoleic acid (LA, Cayman Chemical, Ann Arbor, MI, USA) in serum free media for 1 h at 37°C. 13-S-HODE was extracted and levels of 13-S-HODE were measured as described previously.[15]

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and immunoblot analysis

Cells were washed twice with ice-cold PBS and lyzed in a protein lysis buffer. Total protein (20–50 μg) from cell lysates was fractionated by SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes. Blots were probed with 15-LOX-1 primary antibody and processed by an enhanced chemiluminescence method, as described previously.[15]

Levels of floating-cell ratio measurement

Parental or 15-LOX-1-transfected HCT-116 cells were divided into 6-well plates (1×10^5/well). HCT-116 cells, transfected with pcDNA3-15-LOX-1 or pcDNA 3.1, were incubated for 48 h after seeding, then treated with 0 or 100 μM indomethacin for 1 day. For 13-S-HODE treatments, cells were grown to 60% confluence and then underwent serum starvation for 24 h. The bioactive lipid was then added to the cells in serum-free media for 1 day. Attached cells and floating cells were harvested separately and counted using a hemocytometer. The floating-cell ratio was determined as the ratio of the number of floating cells divided by the number of attached cells. Each experiment was done in duplicate and 3–5 separate samples were examined.

DNA gel electrophoresis

Harvested HCT 116 cells were suspended in a 0.5 mL lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% SDS, 400 mM NaCl). Cell lysates were incubated at 50°C for 2 h with 0.2 mg/mL proteinase K. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and re-extracted with choloroform/isoamyl alcohol. It was then precipitated with isopropanol at −70°C overnight. Precipitated DNA was centrifuged at 14 000 *g* for 30 min, washed with ice-cold 70% ethanol, resuspended in Tris-EDTA buffer (pH 8.0) and treated with 20 μg/mL DNAase-free Rnase (Boehringer, Mannheim, IN, USA). DNA samples were applied to 1.6% agarose gels and visualized using ethidium bromide staining.

Statistics

The data were expressed as the mean ± SEM. Mann–Whitney tests were used for assessment of differences for TUNEL scoring, COX-2, and nuclear beta-cantenin staining. Students' *t*-tests were used for evaluation of differences for levels of 13-HODE and the floating-cells ratio. Comparisons between the two groups were made by Fishers' exact test for 15-LOX-1 staining, membranous or cytoplasmic beta-catenin staining, and p53 staining. $P < 0.05$ was regarded as a significant difference.

Results

Patients characteristics

We performed a clinical study to confirm the role of 15-LOX-1 in NSAID-induced chemoprevention of CRCs. There were no significant differences in all categories of clinical characteristics between the two patients' groups (Table 1). The original diseases of NSAID users were controlled well. Of the NSAID users, two with CRC at stage I underwent the baseline colonoscopy before 5–7 years; others with CRC at stages II, III, or IV underwent the baseline colonoscopy before more than 10 years.

TUNEL scoring in CRCs from NSAID users and non-users

To evaluate the levels of apoptosis in CRC tissue, we determined the TUNEL scoring in CRCs from NSAID users and non-users. There were no statistical differences of TUNEL scores in CRCs

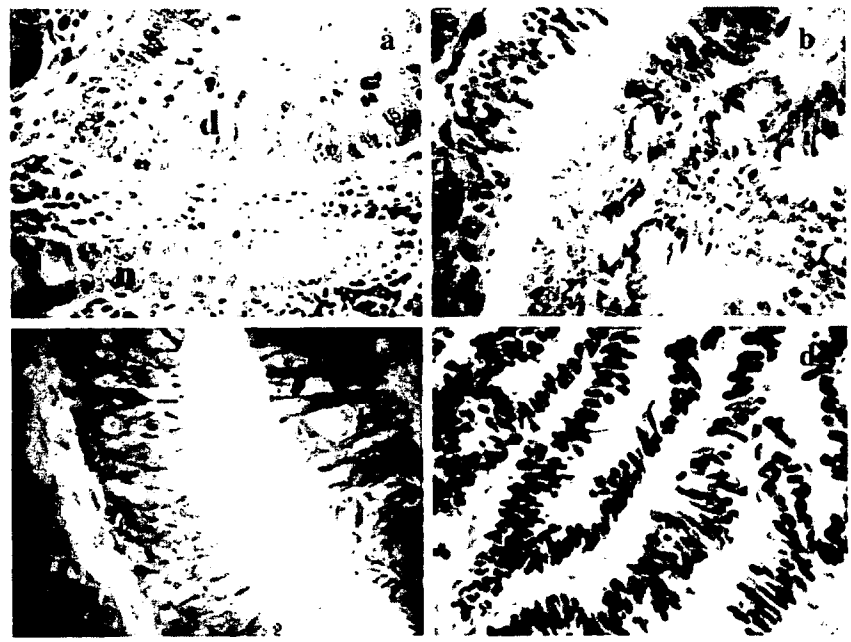

Figure 1 Immunohistochemical staining of 15-LOX-1, COX-2, beta-catenin, and p53 in colorectal cancers. (a) Cytoplastic staining of 15-LOX-1 was found in dysplastic epithelium (d) which was stronger than adjacent normal epithelium (n). (b) Cytoplastic staining of COX-2 was found in dysplasic epithelium. (c) Beta-catenin was found as a strong cytopastic staining and decreasing membranous staining in dysplastic epithelium. (c) Expression of p53 was detected in dysplastic epithelium.

between these two groups (NSAID users, 1.25 ± 0.56% *versus* non-users, 1.38 ± 0.46%).

Evaluation of the molecule expression in CRCs between NSAID users and non-users

We examined the immunohistochemistry for 15-LOX-1, COX-2, beta-catenin and p53 in CRCs from NSAID users and non-users. In all 50 samples, 15-LOX-1 was expressed in the epithelium of normal colonic tissue adjacent to the cancer. In 14 of the total 50 CRC tissues, 15-LOX-1 was expressed through the dysplasic epithelium as similar to or stronger than normal epithelium (Fig. 1a). In others, the expression of 15-LOX-1 was nearly lost compared with normal epithelium. The positive epithelial expression of 15-LOX-1 in CRCs from NSAID users was significantly decreased compared with those from non-users (9.1% *versus* 42.9%; $P < 0.05$ on Table 2). Moreover, the relationship between the 15-LOX-1 expression of CRCs and clinicopatological factors in the NSAID non-user group alone was evaluated. In the present study, there were no significant relationships between these two factors in NSAID non-users alone (data not shown). The expression of COX-2 was detected in tumor cells of CRC (Fig. 1b). In contrast, no COX-2 staining was found in the normal tissues adjacent to the cancers. The normal epithelium adjacent to the cancers showed strong membranous beta-catenin staining and weak cytoplasmic staining. CRCs displayed reduced membranous beta-catenin staining compared with the normal mucosa and an accumulation of cytoplasmic beta-catenin (Fig. 1c). However, no increased accumulation of nuclear beta-catenin staining in CRCs was found compared with normal tissue. The expression of p53 was detected in the nucleus of CRC cells (Fig. 1d). The normal epithelial cells were negative for p53. There were no differences in the expression of COX-2, beta-catenin, or p53 in CRCs between NSAID users and NSAID non-users (Table 2).

Table 2 Expression of 15-LOX-1, COX-2, beta-catenin, and p53 in CRCs between NSAIDs users and non-users

	NSAIDs	
	Users ($n = 22$) Median (range)	Nonusers ($n = 28$) Median (range)
COX-2	0.50 (0–2)	0.86 (0–2)
	n/n-tested (%)	n/n-tested (%)
15-LOX-1	2/22[†] (9.1)	12/28 (42.9)
Beta-catenin		
Membrane decreased	7/11 (63.6)	11/14 (78.6)
Cytoplasm overexpression	9/11 (81.8)	13/14 (92.9)
p53		
Positive	5/11 (45.5)	11/14 (78.6)

[†]$P < 0.05$; NSAID users *versus* non-users.

The impact of 15-LOX-1 on indomethacin-induced apoptosis in HCT-116 cells

We performed transient transfection of 15-LOX-1 cDNA in HCT-116 cells. The expression of 15-LOX-1 was certified in the protein levels and the activity of 15-LOX-1 was also confirmed by evaluating the levels of 13-S-HODE, a 15-LOX-1 major metabolite as described previously.[15] We compared the levels of 100 μM indomethacin (Indo)-induced apoptosis by measuring the floating-cell ratio. The floating-cell ratio was 2.21 ± 0.48% in mocked (15-LOX [–]) cells without Indo; 2.48 ± 0.97% in 15-LOX-1-transfected (15-LOX [+]) cells without Indo; 6.49 ± 2.11% in 15-LOX-1 (+) cells with Indo; 2.41 ± 1.11% in 15-LOX (–) cells with Indo. The ratio in 15-LOX (+) HCT-116 cells with Indo significantly exceeded that of 15-LOX (–) cells with Indo

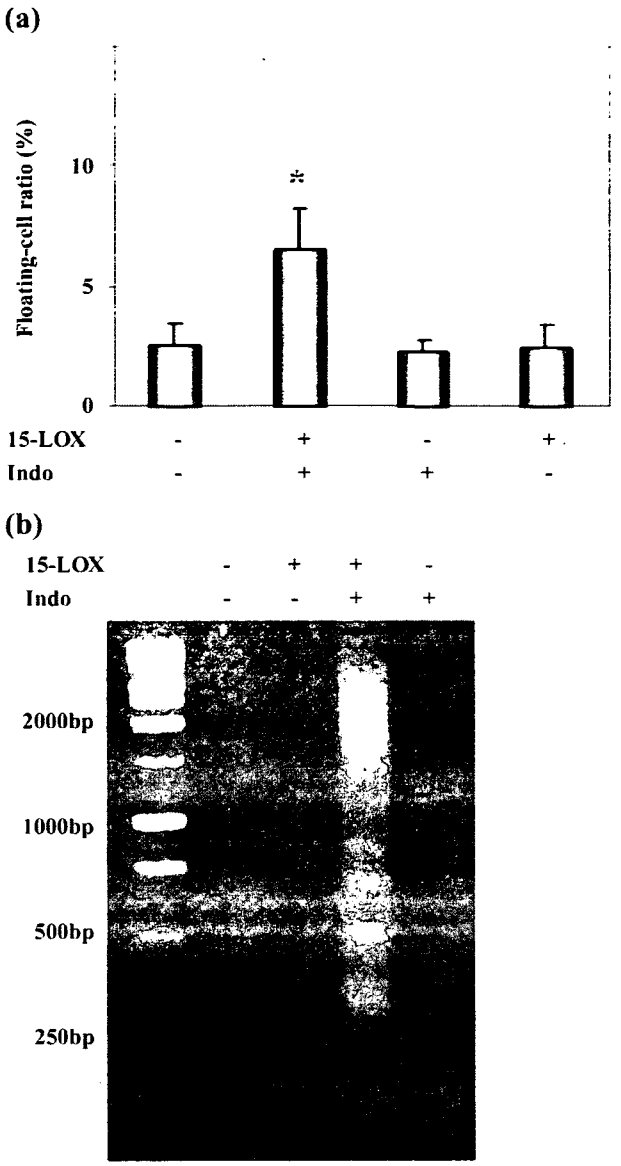

Figure 2 Impacts of 15-LOX-1 overexpression on indomethacin-induced apoptosis in HCT-116 cells. (a) Zero (Indo –) or 100 μM indomethacin (Indo +) was treated for 24 h in 15-LOX-1-transfected (15-LOX +) or mocked (15-LOX –) HCT 116 cells. *$P < 0.05$, 15-LOX (+)/Indo (–) *versus* 15-LOX (–)/Indo (+). (b) DNA gel electrophoresis for the assessment of apoptosis. Zero (Indo -) or 100 μM indomethacin (Indo +) was treated for 24 h in 15-LOX-1-transfected (15-LOX +) or mocked (15-LOX –) HCT 116 cells. First line was DNA marker.

($P < 0.05$ in Fig. 2a). Figure 2b indicates that 15-LOX-1-transfected HCT-116 cells with 100 μM indomethacin showed DNA fragmentation into a typical ladder pattern of 200 bp integer multiples. We evaluated the floating-cell ratio in HCT-116 cells, adding 100 μM indomethacin and/or 10 μM 13-S-HODE. Figure 3 shows that the levels of floating-cells ratio in HCT-116 cells with added indomethacin and 13-S-HODE were increased

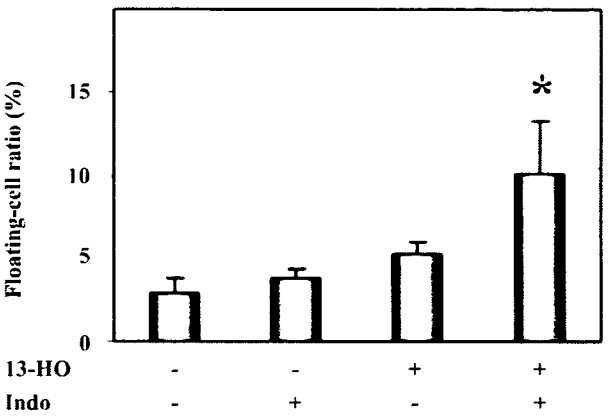

Figure 3 Effects of 13-S-HODE on indomethacin-induced apoptosis in HCT-116 cells. Zero, 100 μM indomethacin (Indo +) and/or 0, 10 μM 13-S-HODE (13-HO) were treated for 24 h in HCT 116 cells. *$P < 0.05$, 13-HO 10 μM/Indo (+) *versus* 13-HO 0 μM/Indo (+).

significantly compared with those in cells to which only indomethacin had been added (10.06 ± 3.27% *versus* 3.81 ± 0.58%, $P < 0.05$).

Indomethacin (100 μM) did not show any influences on 15LOX-1 expression in carcinoma cells (data not shown).

Discussion

The present study shows that CRCs from NSAID users showed the loss of 15-LOX-1 expression compared with those from NSAID non-users. In *in vitro* studies, over-expression of 15-LOX-1 enhances NSAID-induced apoptosis in colorectal carcinoma cells.[12–14,17,18] However, this conclusion is not proved in *in vivo* studies. To the best of our knowledge, the present study is the first study indicating that 15-LOX-1 is less expressive in CRCs from patients who are resistant to NSAID-induced chemoprevention.

In the present study, the levels of TUNEL scoring in CRCs from NSAID users were similar to those from non-users. The previous study showed that NSAID (sulindac sulfone) enhances the apoptotic (TUNEL) labeling index and reduces numbers of rectal polyps in familiar adenomatous polyposis.[19] NSAID users in our study visited the clinic regularly to be checked for compliance. Treatment with NSAIDs was effective in causing users' original disease to remain in a stable condition. These results indicate that CRCs from NSAID users are regarded as resistant to NSAID-induced apoptosis. Our study design may only indirectly evaluate the impact of 15-LOX-1 expression on NSAIDs-induced apoptosis *in vivo* for CRCs. For the direct evaluation, we should have divided our CRC patients into two groups (15-LOX-1 [+] or [–]), treated them with NSAIDs, and then evaluated the levels of apoptosis. However, this kind of design was refused, because patients were in danger of CRC progression if they had 'NSAID-resistant' CRCs. Therefore, we certified the impact of 15-LOX-1 induction on NSAID-induced apoptosis *in vitro*. In the present study, 100 μM indomethacin alone did not induce apoptosis in HCT-116 cells transfected with vector; however, the same concentration of

indomethacin induced apoptosis in HCT-116 cells transfected with 15-LOX-1. This result certifies that the expression of 15-LOX-1 decreases the resistance to indomethacin-induced apoptosis in colorectal carcinoma cells.

In the present study, more than half of NSAID users were treated with aspirin (100 mg per day). Recent studies have shown that optimal chemoprevention for CRCs requires long-term use of aspirin doses substantially higher than those recommended for the prevention of cardiovascular disease.[20,21] These studies are excellent RCTs, but have only been evaluated for US women. Further studies will be needed for men or those of other ethnicities. Moreover, previous studies have shown that low-doses of aspirin will prevent colorectal neoplasm to induce chemoprotective effects on CRCs.[22,23]

Furthermore, the dose-related risk of gastrointestinal bleeding must be also considered.[20,21] If we can improve the sensitivity to NSAID-induced apoptosis on CRC tissue, we will reduce the risk of gastrointestinal bleeding by decreasing the dosage and/or shortening the treatment duration of NSAID in the chemoprevention for CRCs. The present results suggest that the increase of 15-LOX-1 expression may enhance the sensitivity to NSAID-induced apoptosis in human CRCs. Therefore, the induction of 15-LOX-1 in colorectal tumor tissues may strongly contribute to improving the effectiveness of NSAID-induced chemoprevention of CRCs. Previous studies have shown that high dosages of NSAIDs can induce 15-LOX-1 expression in carcinoma cells,[12–14,17,18] but this type of effect of NSAIDs on CRCs was not defined in the present study (data not shown). We plan to evaluate which compounds can induce 15-LOX expression in colorectal neoplasia.

In conclusion, we have shown that 15-LOX-1 is less expressive in CRCs resistant to NSAID-induced chemoprevention. Our preliminary results suggest that 15-LOX-1 expression may enhance the sensitivity to NSAID-induced apoptosis in CRCs in patients treated with the compounds, although further examination is needed.

Acknowledgments

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Ethics approval

The ethical committee of the National Hospital Organization Beppu Medical Center approved this protocol. All patients gave their informed consent for participation in this study.

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BASIC–ALIMENTARY TRACT

A Critical Role of CD30 Ligand/CD30 in Controlling Inflammatory Bowel Diseases in Mice

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Background & Aims: **A CD30-ligand (CD30L) is a 40-kilodalton, type II membrane-associated glycoprotein belonging to the tumor necrosis factor family. Serum levels of soluble CD30 increased in inflammatory bowel diseases (IBD), suggesting that CD30L/CD30 signaling is involved in the pathogenesis of IBD. In this study, we investigated the role of CD30L in oxazolone (OXA)- and trinitrobenzene sulfonic acid (TNBS)-induced colitis in CD30L knockout (KO) mice.** ***Methods:*** **Colitis was induced by OXA or TNBS in CD30LKO mice with BALB/c or C57BL/6 background, respectively, and diverse clinical signs of the disease were evaluated. Cytokine production from lamina propria T cells of the colon was assessed by enzyme-linked immunosorbent assay. Anti-interleukin (IL)-4 monoclonal antibody (mAb) or agonistic anti-CD30 mAb was inoculated in mice with colitis induced by OXA or TNBS.** ***Results:*** **CD30LKO mice were susceptible to OXA-induced colitis but resistant to TNBS-induced acute colitis. The levels of T helper cell 2 type cytokines such as IL-4 and IL-13 in the LP T cells were significantly higher, but the levels of interferon γ were lower in OXA- or TNBS-treated CD30LKO mice than in wild-type mice. In vivo administration of agonistic anti-CD30 mAb ameliorated OXA-induced colitis but aggravated TNBS-induced colitis in CD30LKO mice.** ***Conclusions:*** **These results suggest that CD30L/CD30 signaling is involved in development of both OXA- and TNBS-induced colitis. Modulation of CD30L/CD30 signaling by mAb could be a novel biologic therapy for IBD.**

Human inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are characterized by inflammation in the large and/or small intestine associated with uncontrolled innate and adaptive immunity against normal constituents, including commensal bacteria and various microbial products.[1–4] The responding T cells exhibit a T helper cell (Th) 1 phenotype capable of producing interferon (IFN)-γ in CD, whereas Th2 cytokines are closely associated with UC.[5–7] Among various experimentally induced colitis models in mice, spontaneous colitis in interleukin (IL)-10-deficient mice,[8] colitis in recombination-activating gene (RAG)-deficient mice transferred with $CD4^+CD45RB^{high}$ T cells,[9] and hapten-induced colitis in mice caused by intrarectal administration of trinitrobenzene sulfonic acid (TNBS)[10–13] are thought of as a Th1-type colitis animal model resembling CD. On the other hand, spontaneous colitis in IL-2-deficient mice[14] or in T-cell receptor (TCR) α-deficient mice[15] resembles UC with Th2-like responses. Another hapten-induced colitis caused by intrarectal administration of oxazolone (OXA) is characterized by inflammation with increased Th2-type cytokine secretion and is thought to be associated with Th2-like responses mediated by $CD4^+$ T cells including natural killer (NK) T cells.[16,17]

A CD30 ligand (CD30L, CD153) is a 40-kilodalton, type II membrane-associated glycoprotein belonging to the tumor necrosis factor (TNF) family[18] and is expressed on both $CD4^+$ Th1 and Th2 cells, although there are several lines of evidence for expression on macrophages, dendritic cells (DC), and B cells.[19–22] CD30, a receptor for CD30L, is expressed preferentially by activated or memory Th2 cells but not by resting B or T cells.[23–26] There are several lines of evidence showing that the CD30L/CD30 signaling is involved in Th2 cell responses and Th2-associated diseases.[23–26] However, a number of recent studies suggested that CD30L/CD30 signaling is also linked to Th1 cell responses and Th1-associated diseases.[27–30] Serum levels of soluble CD30 (sCD30) increased in UC, suggesting that CD30L/CD30 signaling is involved in the pathogenesis of IBD.[31] However, little is known about the roles of CD30L/CD30 signaling in IBD.

In the present study, to verify the roles of CD30L in IBD, we examined susceptibility of CD30L knockout

Abbreviations used in this paper: Ag, antigen; APC, allophycocyanin; CD, Crohn's disease; CD30L, CD30 ligand; DC, dendritic cell; KO, knockout; LPL, lamina propria lympocyte; mAb, monoclonal antibody; OXA, oxazolone; TNBS, trinitrobenzene sulfonic acid; UC, ulcerative colitis.

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(KO) to OXA- or TNBS-induced colitis, 2 representative colitis models for IBD. CD30LKO mice were highly susceptibile to OXA-induced colitis but resistant to TNBS-induced acute colitis. In vivo administration of agonistic anti-CD30 monoclonal antibody (mAb) ameliorated OXA-induced colitis but aggravated TNBS-induced acute colitis in CD30LKO mice. These results suggest that CD30L is involved in development of both types of IBD. Implications of these findings for a novel biologic therapy in controlling IBD are discussed.

Materials and Methods

Mice

Age- and sex-matched BALB/c or C57BL/6 male mice were obtained from Charles River Laboratories (Atsugi, Japan). The generation of CD30LKO mice with BALB/c background were described previously.[32,33] CD30LKO mice with C57BL/6 background were backcrossed into C57BL/6 mice more than 8 times, and their littermates were used as control. This study was approved by the Committee of Ethics on Animal Experiment in Faculty of Medicine, Kyushu University.

Induction of Colitis

Colitis was induced by intrarectal administration of OXA or TNBS according to the methods described previously.[13,16] Briefly, mice were anesthetized with diethyl ether (Nacalai Tesque, Inc, Kyoto, Japan) and then administered TNBS (3 mg, Sigma-Aldrich Japan Co, Tokyo, Japan) or OXA (0.8% or 1%, 4-ethocymethylene-2-phenyl-2- oxazolin-5-one) (Sigma Chemical Co, St Louis, MO) dissolved in 45% ethanol intrarectally via a 3.5-French catheter equipped with a 1-mL syringe. The catheter was inserted so that the tip was 4 cm proximal to the anal verge, and the haptenating agent was injected with a total volume of 150 μL. To ensure distribution within the entire colon and cecum, mice were held in a vertical position for 30 seconds after the injection. Control mice were administered an ethanol solution without haptenating agent using the same technique.

Histology Assessment of Colitis

The middle parts of colons were removed and fixed with 10% neutral buffered formalin and then embedded in paraffin. After cutting in round slices, the thin tissue sections were stained with H&E. Histology was scored as follows: epithelium (E): 0, normal morphology; 1, loss of globlet cells; 2, loss of globlet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas; and infiltration (I): 0, no infiltrate; 1, infiltrate around the crypt basis; 2, infiltrate reaching the L muscularis mucosae; 3, extensive infiltration reaching the L muscularis mucosae and thickening of the mucosa with abundant edema; 4, infiltration of the L submucosa.[34] The total histologic score was given as E + I.

Antibodies and Reagents

FITC-conjugated anti-CD3ε, anti-CD4, anti-CD11c, anti-NK (DX5), anti-CD11b, anti-Foxp3, anti-CD25, and anti-IL-10 mAbs; PE-conjugated anti-Ly-6G, anti-CD30L, anti-CD30, anti-CD8α, anti-CD4, anti-CD25, anti-IFN-γ, anti-IL-10, and anti-IL-4 mAbs; allophycocyanin (APC)-conjugated anti-CD3ε and anti-CD44 mAbs; and biotin-conjugated anti-F4/80 (BM8) and anti-CD30L and APC-conjugated streptavidin (SA-APC) mAbs were purchased from e-Bioscience (San Diego, CA). PE-conjugated anti-TCRγδ (GL-3) mAb and PerCP-Cy5.5-labeled anti-CD4 were purchased from BD PharMingen (San Diego, CA). Armenian Hamster IgG1 was purchased from Wako Pure Chemicals (Osaka, Japan).

In Vivo Treatment of Mice With Abs

Agonistic anti-CD30 mAb (clone 30.1) and rat anti-mouse IL-4 (clone 11B11) mAb were obtained by growing hybridoma cells in CELLine CL-1000 (BD, Biosciences, San Diego, CA) with serum-free medium (medium 101; Nissui Pharmaceutical, Tokyo, Japan) and collecting these antibodies by HiTrap Protein G HP (Amersham Biosciences). The purity of the preparation was confirmed by SDS-PAGE, and the concentration of Ab was determined by the Lowry method. The mAbs, diluted to 1 mg/mL in phosphate-buffered saline (PBS), were stored at −70°C until use. For in vivo neutralization, various doses of rat anti-mouse IL-4 or isotype control (rat IgG; e-Bioscience) were intraperitoneally (IP) injected into mice at the time of disease induction with OXA. For in vivo activation, 100 μg agonistic anti-CD30 mAb or isotype control (hamster IgG1; BD Biosciences) was injected IP into mice before or after induction of colitis.

Flow Cytometry Analysis and Intracellular Cytokine Synthesis Analysis

Lamina propria (LP) cells in the large intestine were isolated by a modified method described previously.[34] LP lymphocytes (LPLs) were purified on a 45%/66.6% discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient at 600*g* for 20 minutes. For flow cytometry analysis, isolated cells were preincubated with an Fcγ receptor-blocking mAb (CD16/32; 2.4G2) for 15 minutes at 4°C then incubated with saturating amounts of FITC-, PE-, APC-, and biotin-conjugated mAbs for 30 minutes at 4°C. To detect biotin-conjugated mAb, cells were stained with APC-conjugated streptavidin, and the stained cells were analyzed by using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). For intracellular cytokine staining, 10^6 LP cells were simulated with phorbol myristate acetate (PMA) and ionomycin for 5 hours at 37°C. Brefeldin A (10 μg/mL; Sigma Chemical Co) was included during the final 4 hours of stimulation. These cells were harvested, washed, and incubated for 30 min at 4°C with mAbs for surface staining then cells were

subjected to intracellular cytokine staining using the Fast Immune Cytokine System according to the manufacturer's instructions (Becton Dickinson Co). The data were analyzed with CellQuest software (BD Biosciences).

Culture of LP Cells for Assay of Cytokine Production

To measure cytokine production by LP cells, 10^6 LP cells from mice were cultured without any stimulation for 24 hours at 37°C under 5% CO_2 in 96-well flat-bottomed plates in a volume of 0.2 mL RPMI containing 10% fetal bovine serum (FBS). For cytokine production by LPT cells, LP cells purified as described above were loaded into uncoated culture wells or wells coated with 10 μg/mL anti-CD3ε mAb and 1 μg/mL soluble anti-CD28 mAb and cultured for 48 hours. The culture supernatants were then harvested and assayed for cytokine concentration by enzyme-linked immunosorbent assay (ELISA) using an ELISA Development Kit (Genzyme Diagnostics, Cambridge, MA).

Statistical Analysis

The difference in survival rates was evaluated by the log-rank test (Mantel-Cox). Disease activity index and histologic scores were statistically analyzed using the Mann-Whitney *U* test. Differences in parametric data were evaluated by Student *t* test. Differences of $P < .05$ were considered statistically significant.

Results

CD30LKO Mice Are Susceptible to OXA-Induced Colitis

To examine the role of CD30L in development of OXA-induced colitis in mice, CD30LKO mice with BALB/c background were subjected to induction of colitis by intrarectal administration of 1.0% or 0.8% OXA. We first examined the expression levels of CD30L and CD30 on LPL in the colon from naive mice and mice with colitis. The CD30L was expressed mainly on a part of $CD4^+$ T cells from freshly isolated LPL in naive BALB/c mice and mice treated with 0.8% OXA 4 days previously (see Supplemental Figure 1*A* and 1*B* online at www.gastrojournal.org). Although the CD30 expression was not detected on freshly isolated LPL cells in the colon of naïve mice or OXA-treated mice (data not shown), appreciable numbers of $CD30^+$ cells were detected in the $CD4^+$ T-cell population from naïve and OXA-treated mice after 24-hour in vitro culture with or without anti-CD3 mAb stimulation (see Supplemental Figure 1*C* online at www.gastrojournal.org).

The survival rates were significantly decreased in CD30LKO mice compared with those in WT mice after 1% OXA administration (Figure 1*A*, $*P < .05$). CD30LKO mice showed exacerbated colitis as indicated by the significant weight loss from day 3 to day 10 after 0.8% OXA administration (Figure 1*B*, $*P < .05$). Macroscopic examinations on day 2 after 0.8% OXA administration revealed that the colon was shorter in CD30LKO mice than that in WT mice (Figure 1*C* and 1*D*, $*P < .05$). On histologic examination of involved colon of 0.8% OXA-treated wild-type (WT) mice, we observed a superficial inflammation characterized by the presence of epithelial cell loss and patchy ulceration, pronounced depletion of mucin-producing goblet cells, and reduction of the density of the tubular glands. In addition, in the LP, a mixed inflammatory cell infiltrate consisting of lymphocytes and granulocytes was associated with an exudation of cells into the bowel lumen. These histopathologic changes of OXA-induced colitis were more serious in the colon of CD30LKO mice than in WT mice, and the histologic score of the colon was significantly higher in CD30LKO mice than in WT mice on day 2 after OXA administration (Figure 1*E* and *F*, $*P < .05$). Thus, CD30LKO mice were highly susceptible to OXA-induced colitis compared with control WT mice.

Cell Accumulation in the Colon Mucosa of CD30LKO Mice With OXA-Induced Colitis

Populations of LP cells in the large intestines from CD30LKO mice before and on day 4 after 0.8% OXA administration were analyzed by flow cytometry. As shown in Supplemental Figure 2*A* (see Supplemental Figure 2*A* online at www.gastrojournal.org), the proportions of $CD11b^+Gr\text{-}1^+$ and $CD11b^+F4/80^+$ cells were significantly higher than OXA-treated WT mice ($*P < .05$), whereas the proportions of $\gamma\delta TCR^+CD3^+$ and $DX5^+CD3^-$ cells had slightly decreased in OXA-treated CD30LKO mice compared with OXA-treated WT mice (see Supplemental Figure 2*B* online at www.gastrojournal.org, $*P < .05$ or $**P < .01$). The relative numbers of $CD4^+CD25^+$ and $CD4^+CD44^+$ T cells were significantly lower in CD30LKO mice before or after OXA-administration (see Supplemental Figure 2*C* online at www.gastrojournal.org, $*P < .05$). Intracellular staining analysis for expression of Foxp3 revealed that $CD4^+CD25^+$ T cells were divided into 2 populations on the basis of Foxp3 expression.[35] $CD4^+CD25^+Foxp3^-$ T cells were selectively reduced in CD30LKO mice before and after OXA administration (see Supplemental Figure 2*D* online at www.gastrojournal.org, $*P < .05$). CD30L was expressed by $CD4^+CD25^+Foxp3^-$ T cells but not by $CD4^+CD25^+Foxp3^+$ T cells (see Supplemental Figure 2*D* online at www.gastrojournal.org).

Cytokine Production by LP Cells of CD30LKO Mice With OXA-Induced Colitis

The spontaneous release levels of IFN-γ, TNF-α, IL-12p40, and IL-10 were significantly lower in CD30LKO mice than in WT mice before and on day 4 after 0.8% OXA administration (Figure 2*A*, $*P < .05$ or $**P < .01$), but the levels of IL-1β and IL-6 were significantly higher in CD30L KO mice than in WT mice ($*P < .05$ and $**P < .01$). The secretion of IL-4 and IL-13 were significantly higher, but the levels of IL-10 and IFN-γ were significantly lower in naïve and OXA-treated

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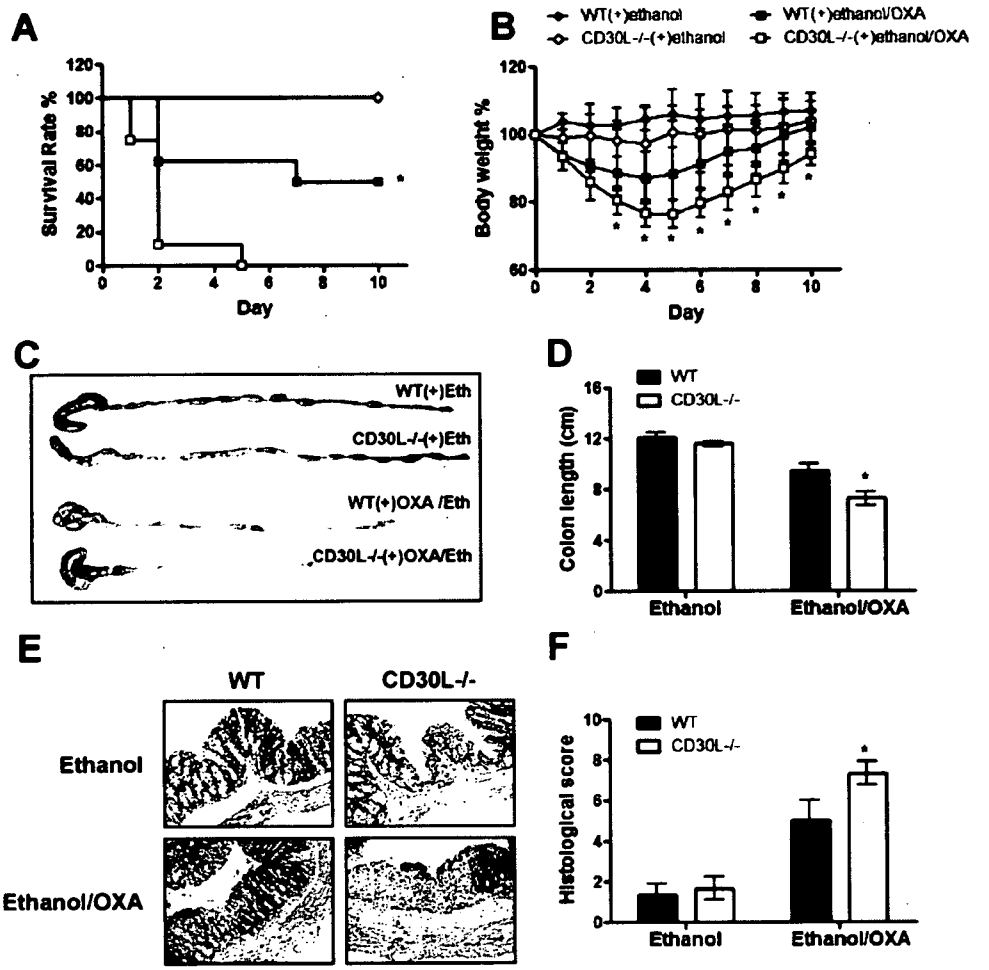

Figure 1. Susceptibility of CD30L$^{-/-}$ mice to OXA-induced colitis. Survival rate (*A*) and weight loss (*B*) of mice after intrarectal administration of 1% or 0.8% OXA, respectively. (*C*) Macroscopic changes of colons on day 2 after administration of 0.8% OXA. (*D*) Colon length measured on day 2 after 0.8% OXA administration. (*E*) Histologic analysis of the colons from mice on day 2 after 0.8% OXA administration (original magnification, ×200). (*F*) Histologic scores of the colons from ethanol- and OXA-treated WT and CD30L$^{-/-}$ mice. Data shown represent mean values ± SD of 24 mice of each group obtained from 3 independent experiments. Statistically significant differences from the value for OXA-treated WT mice are shown (*$P < .05$).

CD30LKO mice than WT mice upon stimulation with anti-CD3/anti-CD28 mAbs (Figure 2*B*, *$P < .05$ or **$P < .01$).

To identify T-cell populations producing IFN-γ, IL-4, or IL-10, we examined intracellular cytokine flow cytometry analysis on LP T cells in OXA-induced colitis. CD4$^+$ T cells were major producers of IL-4 and IL-10 (Figure 3*A* and 3*C*), whereas CD4$^-$ T cells produced an appreciable level of IFN-γ in addition to CD4$^+$ T cells (Figure 3*B*). The absolute numbers of IL-4$^+$CD4$^+$ T cells were significantly higher in naïve and OXA-treated CD30LKO mice than in WT mice (Figure 3*A*, *$P < .05$ or **$P < .01$). The absolute numbers of IFN-γ$^+$CD4$^+$ T cells and IL-10$^+$CD4$^+$ T cells were significantly lower in OXA-treated CD30LKO mice compared with OXA-treated WT mice (Figure 3*B* and *C*, **$P < .01$). We further characterized these CD4$^-$ T cells in the LP of the colon in mice with OXA-induced colitis. IFN-γ was produced mainly by the CD4$^+$CD25$^-$ T-cell population, whereas CD4$^+$CD25$^+$ T cells mainly produced IL-10. CD4$^+$CD30L$^-$ T cells preferentially produced IFN-γ and IL-10 (see Supplemental Figure 2*E* and *F* online at www.gastrojournal.org).

In Vivo Treatment With Anti-IL-4 mAb Ameliorates OXA-Induced Colitis in CD30LKO Mice

To determine the involvement of elevated IL-4 in the pathogenesis of OXA-induced colitis in CD30LKO mice, we examined the effect of in vivo administration of anti-IL-4 mAb on OXA-induced colitis in CD30L KO mice. In vivo injection of more than 0.3 mg anti-IL-4 mAb significantly ameliorated OXA-induced colitis in CD30LKO mice (Figure 4*A–C*, *$P < .05$ or **$P < .01$). The levels of IL-10 and IFN-γ were significantly higher in anti-IL-4 mAb- and OXA-treated CD30LKO mice than in control IgG- and OXA-treated

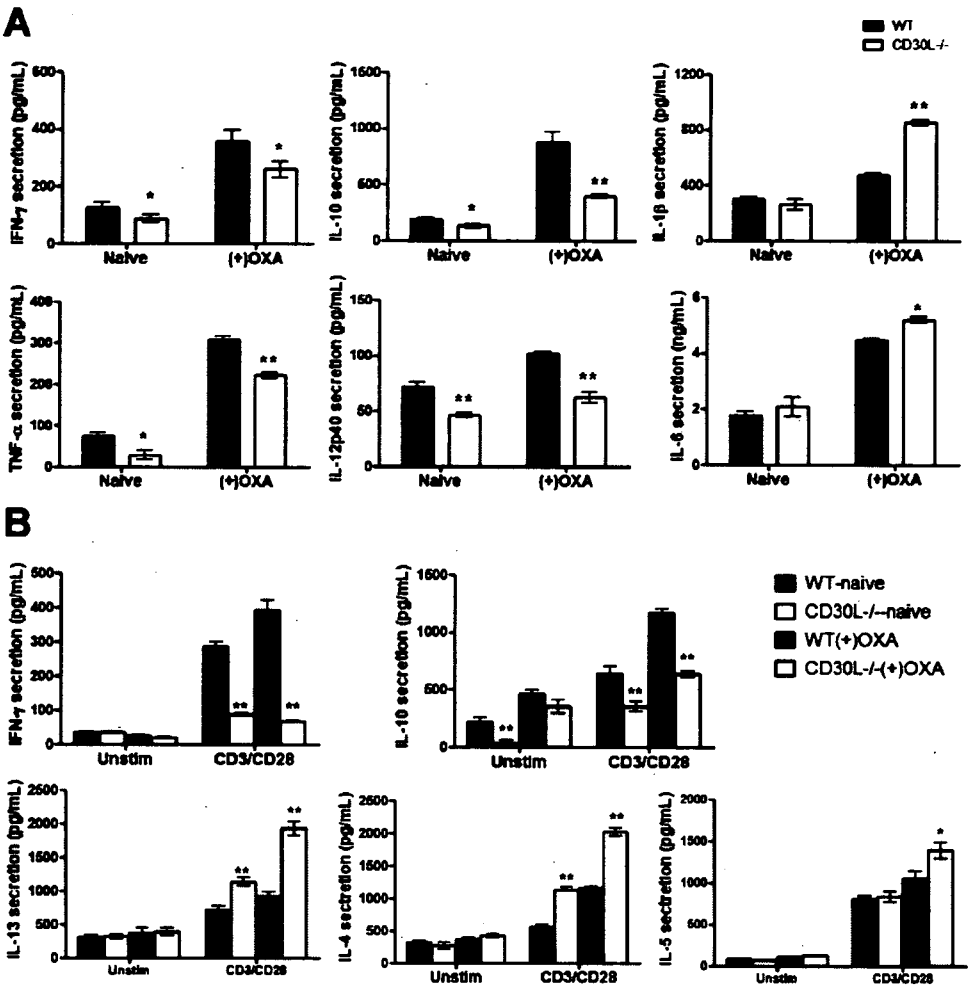

Figure 2. Cytokine production of LP cells in large intestines in OXA-inducted colitis after 24 hours cultured without any stimulation (*A*) and 48 hours cultured with anti-CD3/CD28 mAbs (*B*). Each *column* and *vertical bar* indicates means ± SD for 5 mice of each group. Data of a representative experiment are shown from 3 independent experiments. Statistically significant differences are shown (*$P < .05$ or **$P < .01$).

CD30LKO mice upon stimulation with anti-CD3/anti-CD28 mAbs, (Figure 4*D*, *$P < .05$ or **$P < .01$). IL-13 production was not affected in CD30LKO mice by anti-IL-4 mAb treatment. Similarly, anti-IL-4 mAb treatment ameliorated OXA-induced colitis in WT mice (see Supplemental Figure 3 online at www.gastrojournal.org). Thus, these results suggest that IL-4 is involved in the pathogenesis of OXA-induced colitis in WT and CD30LKO mice.

In Vivo Treatment With Agonistic Anti-CD30 mAb Ameliorates OXA-Induced Colitis in CD30LKO and WT Mice

To elucidate the roles of CD30L/CD30 signaling in OXA-induced colitis, we examined the effect of in vivo administration of agonistic anti-CD30 mAb (CD30.1)[18] on OXA-induced colitis in CD30L KO mice. Mice were injected IP with anti-CD30 mAb or control hamster IgG1 24 hours before 1% OXA administration for survival rate or 0.8 % OXA treatment for weight loss. In vivo injection of anti-CD30 mAb significantly protected against OXA-induced colitis in CD30LKO mice as assessed by both survival rate and weight loss (Figure 5*A*, *$P < .05$ or **$P < .01$). These results suggest that CD30 signaling is important for controlling OXA-induced colitis. The secretion of IL-4 and IL-13 were significantly lower, but the levels of IL-10 and IFN-γ were significantly higher in anti-CD30 mAb- and OXA-treated CD30LKO mice than in control antibody and OXA-treated CD30LKO mice upon stimulation with anti-CD3/anti-CD28 mAbs (Figure 5*B*, *$P < .05$ or **$P < .01$). Thus, these results suggest that stimulating reagent for CD30 signaling may be useful to control OXA-induced colitis.

We further examined the effect of in vivo treatment with agonistic anti-CD30 mAb on OXA-induced colitis in WT mice. Although in vivo treatment with anti-CD30 mAb 1 day after 1.5% OXA administration did not affect

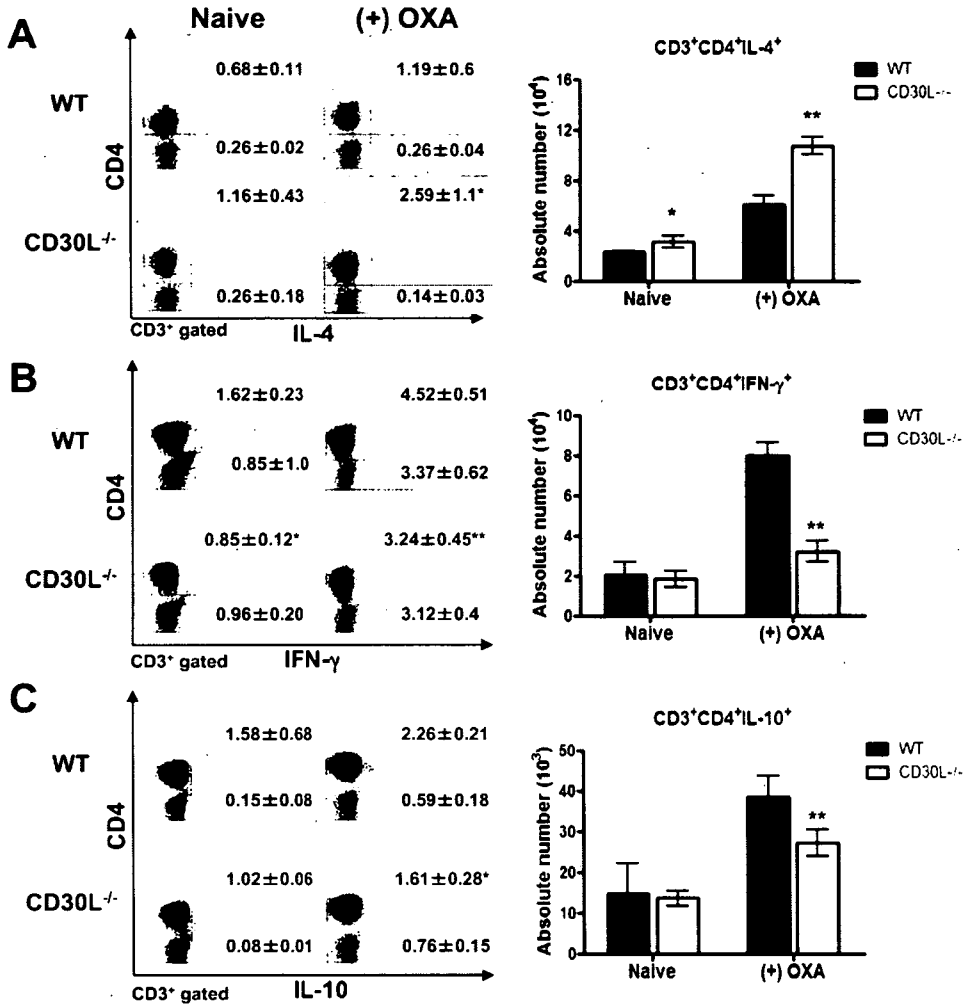

Figure 3. Intracellular cytokine expression by LP T cells from OXA-treated CD30L$^{-/-}$ mice. LP T cells from mice treated with 0.8% OXA 4 days previously were cultured with PMA plus ionomycin and analyzed for the expression of CD4 and IL-4 (*A*), IFN-γ (*B*), or IL-10 (*C*) by intracellular staining. The absolute number of each subset was calculated by multiplying the total number of LP T cells by the percentage of each subset. *Values* of each *column* and *vertical bar* indicate means ± SD for 5 mice within each group. Representative data are shown from 3 independent experiments. Statistically significant differences are shown (*$P < .05$ or **$P < .01$).

OXA-induced colitis, the treatment 1 day before or at the same time as OXA administration significantly extended the survival period and decreased weight loss of WT mice with OXA-induced colitis (Figure 5C, **$P < .01$). Thus, anti-CD30 mAb may be useful as a novel biologic therapy for UC.

CD30LKO Mice Are Resistant to TNBS-Induced Acute Colitis

TNBS-induced acute colitis is thought to be a Th1 cell-mediated inflammation.[11-13] To examine the role of CD30L in development of TNBS-induced acute colitis in mice, we next examined TNBS-induced acute colitis with a Th1-like response in CD30LKO mice with C57BL/6 background. The CD30L was expressed mainly on a part of CD4$^+$ T cells from freshly isolated LPL in naïve C57BL/6 mice and mice treated with TNBS 7 days previously (see Supplemental Figure 1*A* online at www.gastrojournal.org). The CD30 expression on CD4$^+$ T cells from LPL was detected only when the LPLs were cultured in vitro and the level of CD30$^+$ in CD4$^+$ cells were significantly increased in mice with TNBS-induced colitis than naive mice (see Supplemental Figure 1C online at www.gastrojournal.org, *$P < .05$).

As shown in Figure 6*A* and *B*, TNBS-induced acute colitis was attenuated in CD30LKO mice as indicated by survival rate and weight loss (*$P < .05$). Macroscopic inspection showed a significantly longer colon in CD30LKO mice than in WT mice on day 7 after TNBS administration (Figure 6C, *$P < .05$). The histologic score of the colon was significantly lower in CD30LKO mice than in WT mice (Figure 6*D*, **$P < .01$).

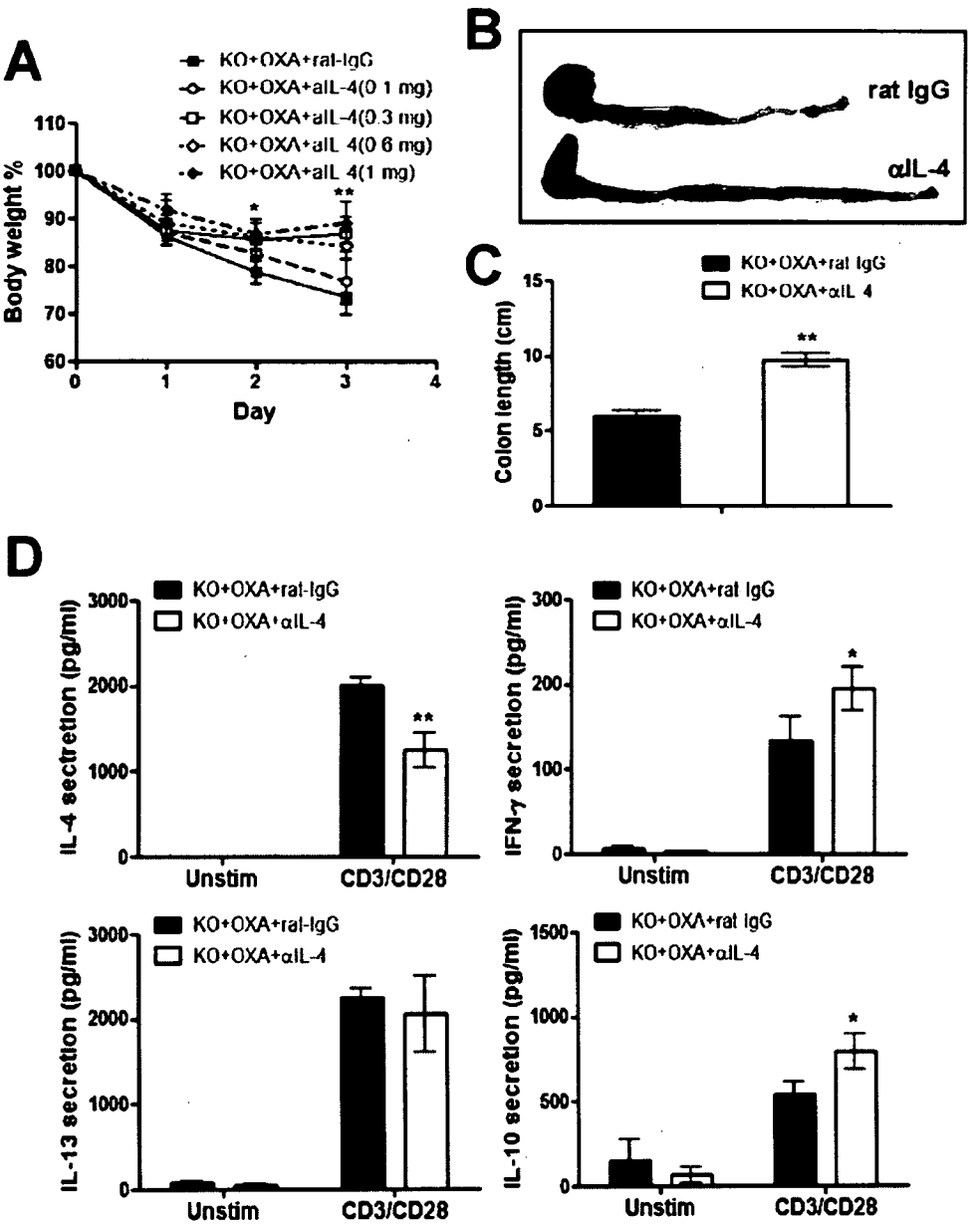

Figure 4. Effects of in vivo treatment with anti-IL-4 mAb on OXA-induced colitis. CD30L$^{-/-}$ mice were treated with indicated doses of anti-IL-4 or control rat IgG at the time of colitis induction with OXA. The body weight (*A*) and macroscopic appearance of colons (*B*), colon length (*C*), or cytokine production (*D*) on day 3 after OXA and 0.3 mg of anti-IL-4 mAb administration. Each *column* and *vertical bar* indicates means ± SD for 5 mice of each group. Data of a representative experiment are shown from 3 independent experiments. Statistically significant differences are shown (*$P < .05$ or **$P < .01$).

To determine whether CD30 signaling is involved in TNBS-induced acute colitis, we examined the effect of in vivo administration of agonistic anti-CD30 mAb to CD30LKO mice with TNBS-induced colitis. Anti-CD30 mAb aggravated TNBS-induced colitis in CD30LKO mice as assessed by both survival rate and body weight (Figure 6*E*, *$P < .05$ or **$P < .01$). Thus, these results indicate that CD30L/CD30 signaling is involved in development of TNBS-induced acute colitis.

Cytokine Production by LP Cells of CD30LKO Mice With TNBS-Induced Acute Colitis

The levels of IFN-γ, TNF-α, IL-1β, IL-6, and IL-12p40 production by LP cells without stimulation were significantly lower but IL-10 secretion was higher in CD30LKO mice than in WT mice on day 7 after TNBS administration (Figure 7*A*, * $P < .05$ or **$P < .01$). The secretion of IL-4, IL-13, and IL-10 was significantly higher

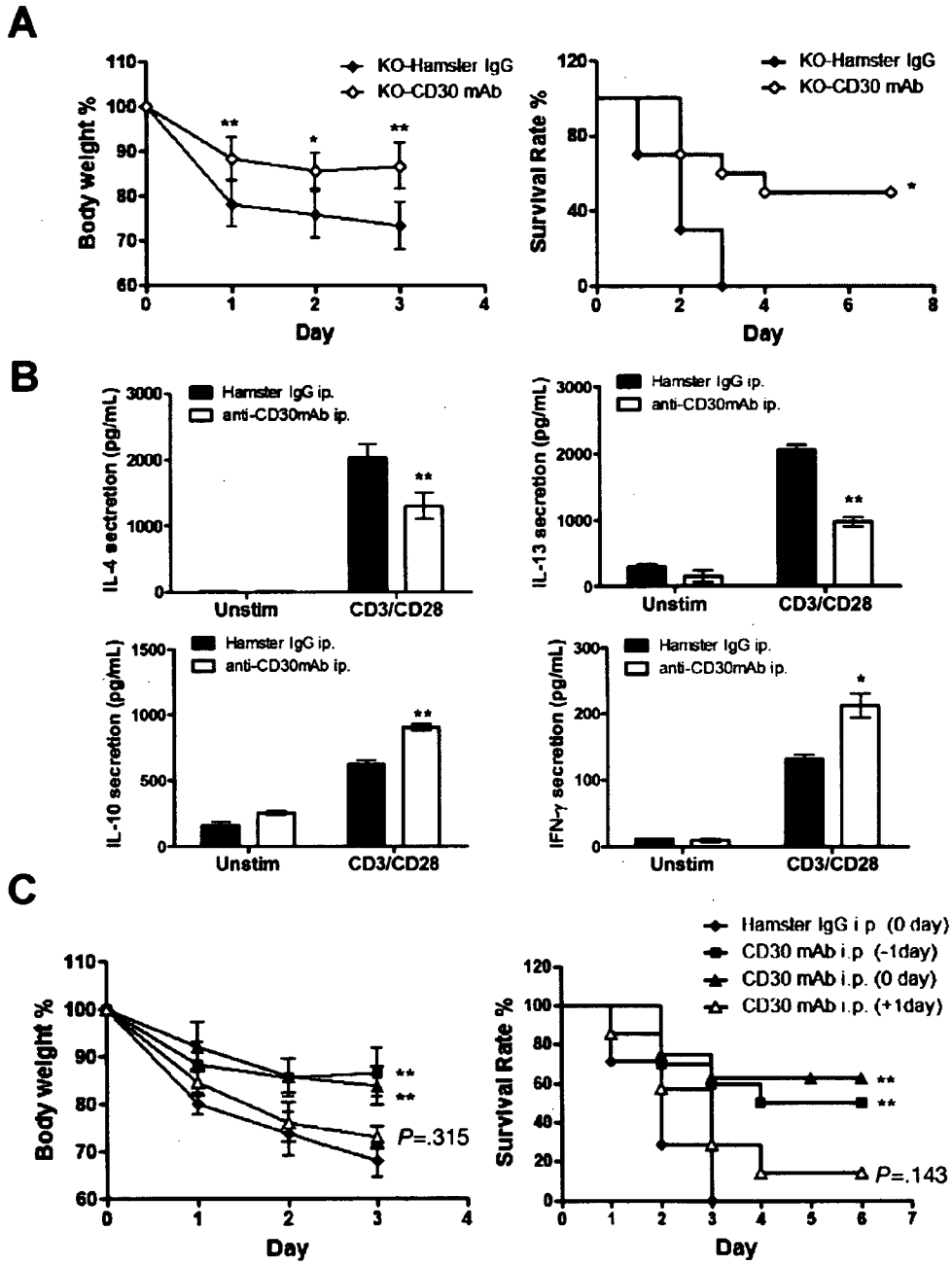

Figure 5. Effects of in vivo treatment with agonistic anti-CD30 mAb on OXA-induced colitis. (*A*) Anti-CD30 mAb (100 μg/head, clone CD30.1) or hamster IgG1 was injected intraperitoneally into CD30L$^{-/-}$ mice on day 1 before 1% OXA administration, and then weight loss and survival rates were monitored daily. (*B*) LP T cells of anti-CD30 mAb and 0.8% OXA-treated CD30L$^{-/-}$ mice were cultured coating with or without anti-CD3/CD28 mAbs, and cytokines secretion were assayed by ELISA on day 4 after OXA treated. (*C*) Anti-CD30 mAb or hamster IgG1 was injected intraperitoneally into WT mice on day −1, day 0, or day 1 after 1.5% OXA treated, and weight loss and survival rates were monitored daily. Each *column* and *vertical bar* indicates means ± SD for 6–10 mice of each group obtained from a representative experiment in 3 independent experiments. Statistically significant differences from control hamster IgG1- and OXA-treated mice are shown ($^*P < .05$ or $^{**}P < .01$).

but the level of IFN-γ was significantly lower in TNBS-treated CD30LKO mice than in TNBS-treated WT mice upon stimulation with anti-CD3/CD28 mAbs (Figure 7*B*, $^*P < .05$ or $P < .01$). These results suggest that CD30L signaling is involved in development of TNBS-induced acute colitis in association with Th1-like response.

Discussion

In the present study, we found that OXA-induced colitis was exacerbated in CD30LKO mice, of which CD4$^+$ T cells in the LP of large intestine produced higher levels of Th2-type cytokines but less IFN-γ than those in

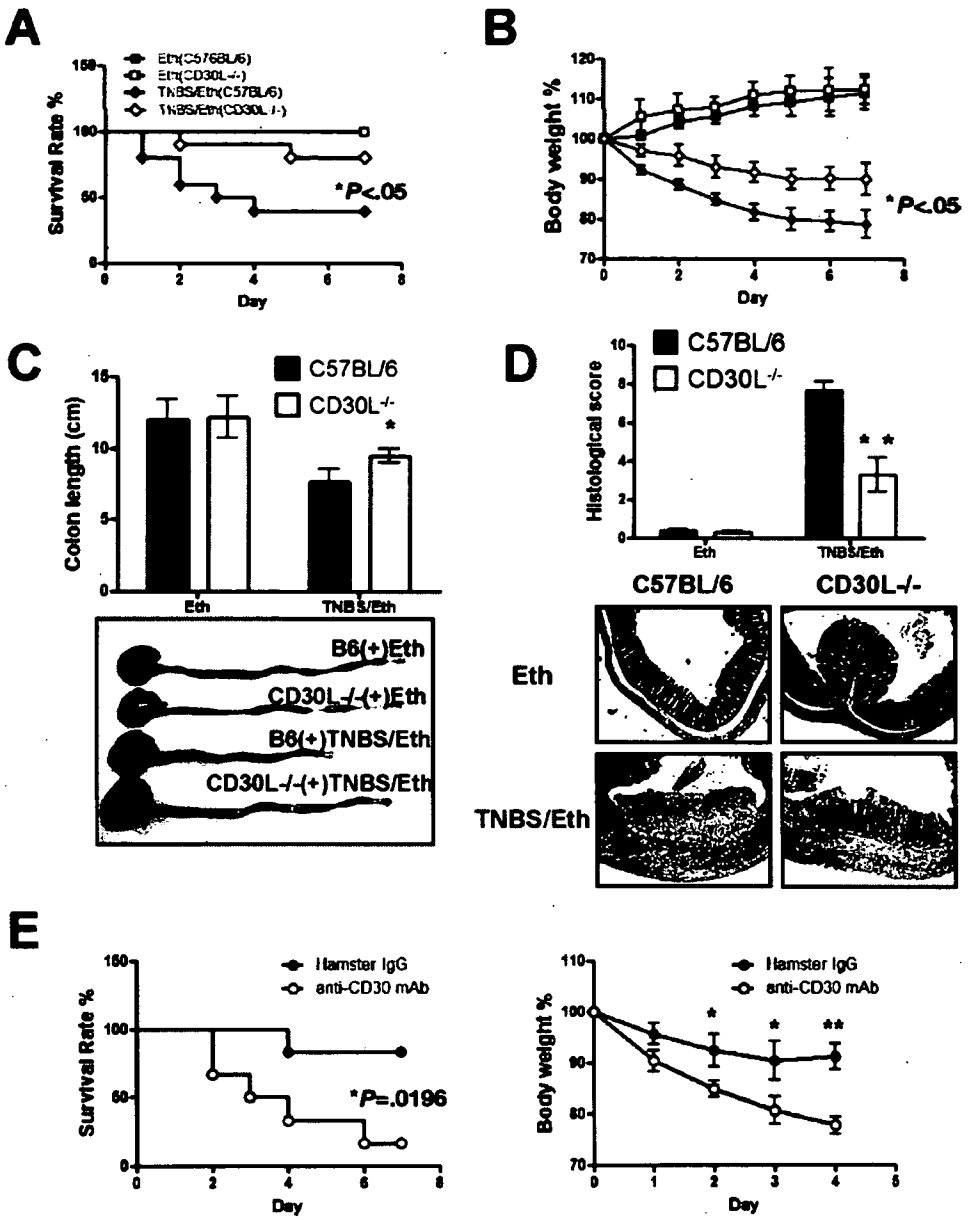

Figure 6. Susceptibility of CD30L$^{-/-}$mice to TNBS-induced acute colitis. CD30L$^{-/-}$ mice were administered 3 mg TNBS/mouse, and, thereafter, survival rate (*A*) and body weight (*B*) were monitored every day. (*C*) Macroscopic changes of colons and colon length and (*D*) histologic analysis and score were analyzed on day 7 after TNBS treatment. (*E*) In vivo administered with anti-CD30 mAb to CD30L$^{-/-}$ mice with TNBS induced colitis, and then survival rate and body weight were monitored every day. Data shown represent mean values ± SD of 10 mice of each group obtained from 3 independent experiments. Statistically significant differences are shown (*$P < .05$ or **$P < .01$).

WT mice. However, CD30LKO mice are resistant to TNBS-induced colitis with impaired IFN-γ production in LP T cells of the colon. Thus, Th1-like immunity characterized by IFN-γ production was impaired, whereas Th2-like immunity capable of producing IL-4 and IL-13 was enhanced in both murine experimentally induced colitis models in CD30LKO mice. Stimulation with CD30 signaling by agonistic anti-CD30 mAb' increased Th1-like response in the mucosa of colons and ameliorated the course of OXA-induced colitis but aggravated TNBS-induced acute colitis in CD30LKO mice. These results proved that CD30 signaling via CD30L played a role in controlling colitis by deviating the balance of Th1/Th2 to Th1 response in colon.

Th1 cells inhibit the proliferation of Th2 cells, and Th2 cells shut down IFN-γ production by Th1 cells, indicating that Th1 and Th2 cells are mutually regulated.[36,37] Therefore, it is most likely that the Th1 response producing IFN-γ is selectively suppressed in the mucosa of the colon of CD30LKO mice, resulting in the dominant

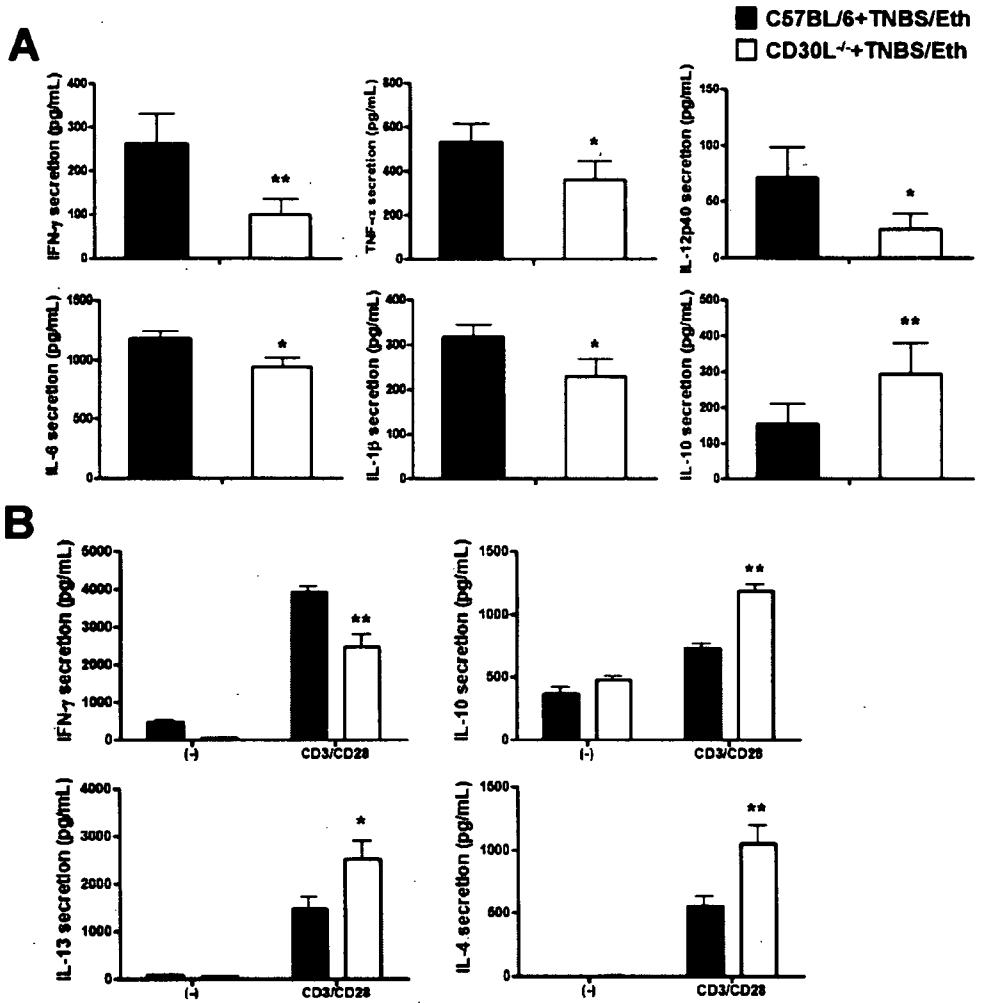

Figure 7. Cytokine production of LP cells in TNBS-induced colitis and effects of in vivo treatment with agonistic anti-CD30 mAb on TNBS-induced colitis in CD30LKO mice. (*A*) Cytokine production of LP cells in TNBS-induced colitis after culture without any stimulation. (*B*) Cytokine production of LP T cells in TNBS-induced colitis after culture with anti-CD3/CD28 mAbs. Culture supernatants were analyzed for concentrations of cytokines by specific ELISA. Each *column* and *vertical bar* indicates means ± SD for 5 mice of each group. Data of a representative experiment are shown from 3 independent experiments. Statistically significant differences are shown ($^*P < .05$ or $^{**}P < .01$).

Th2 responses. As a consequence, OXA-induced colitis was exaggerated but TNBS-induced colitis was ameliorated in CD30LKO mice. CD30L is expressed on activated T cells irrespective of Th1 and Th2 types, macrophages, DC, and B cells.[19-22] On the other hand, CD30 has been reported to be preferentially expressed by effector and memory Th cells but not by macrophage/DC or B cells.[23-26] We showed here that CD30L was expressed mainly by a part of freshly isolated $CD4^+$ T cells but not apparently by B cells or DC/macrophages in the colon of naïve mice. Although CD30 expression was not detected on freshly isolated $CD4^+$ T cells in the colon of naive mice and mice with colitis, it was apparent after in vitro culture with or without anti-CD3 mAb stimulation. It is notable that the numbers of $CD30^+$ T cells in $CD4^+$ population of the colon were significantly increased after induction of colitis by OXA or TNBS. These results suggest that CD30/CD30L signaling executed by $CD30^+$ T cell to $CD30L^+$ T cell interaction may at least partly responsible for Th responses in the colon.

It has recently been reported that both UC in humans and OXA-induced colitis in mice are at least partly mediated by CD1d-restricted NKT cells producing IL-13.[17,38] Furthermore, both Th1 and Th2 pathways have been implicated in the pathogenesis of OXA-induced colitis.[39] We found in our study no difference in the number of $DX5^+CD3^+$ T cells in the LP of the colon between CD30LKO and WT mice. The experiment with anti-IL-4 mAb treatment revealed the involvement of IL-4 in OXA-colitis in CD30LKO and WT mice. CD30L/CD30 signaling may be involved in the shift of $CD4^+$ Th1/Th2 balance to Th1 type. Blazar et al have recently reported that the homing of alloreactive $CD4^+$ T cells to the gastrointestinal tract was inhibited in CD30LKO recipi-

ents, leading to reduced mortality and lower weight loss in graft vs host disease.[40] CD30L/CD30 signaling is reported to be involved in chemokine receptor expression.[32,41] Therefore, it is also possible that migration of $CD4^+$ Th1 cells to the colon may be selectively impaired in CD30LKO mice. Further experiments are required to elucidate these possibilities.

It is noted that IL-10 production by $CD4^+CD25^+Foxp3^-$ T cells corresponding to T-regulatory type1 (Tr1) cells were fewer in the LP of CD30LKO mice. T cells capable of producing IL-10 in the intestine are termed *Tr1 cells*, which are able to prevent the development of experimentally induced colitis when transferred in vivo.[42–44] Therefore, it is also speculated that CD30L/CD30 signaling plays a role in induction of Tr1 cells producing IL-10, which may contribute to attenuate the development of colitis. However, IL-10 production was impaired in CD30LKO mice with OXA-induced colitis, whereas it was augmented in CD30LKO mice with TNBS-induced colitis. Considering all of the data, it appears that CD30L plays a critical role in deviating $CD30^+$ Th cells to Th1 cells in the colon, which may regulate the development of both OXA-induced and TNBS-induced acute colitis.

It is notable in our study that therapeutic application of the agonistic anti-CD30 mAb ameliorated OXA-induced colitis in WT mice. Furthermore, attenuation of colitis induced by TNBS in CD30LKO mice suggests that neutralizing anti-CD30 mAb is potentially useful for control of TNBS-induced colitis. Taken together, our study suggests that Abs against CD30L/CD30 could be a novel biologic therapy for IBD.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi:10.1053/j.gastro.2007.11.004.

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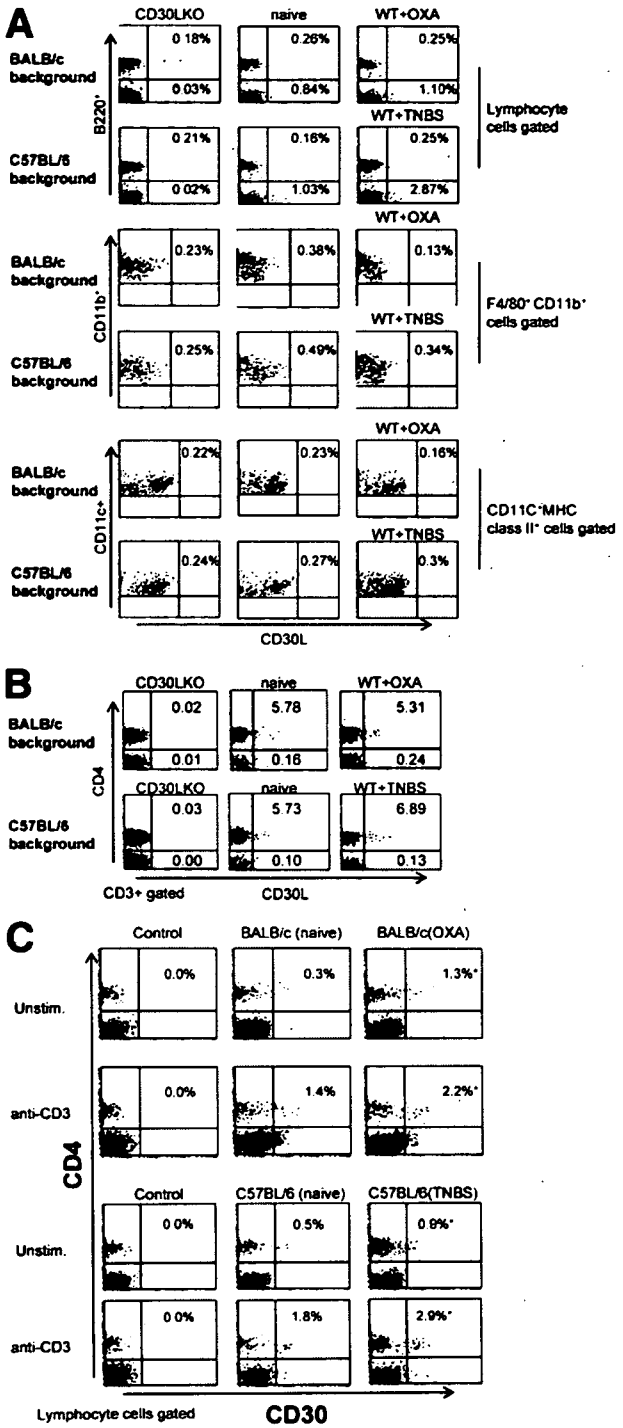
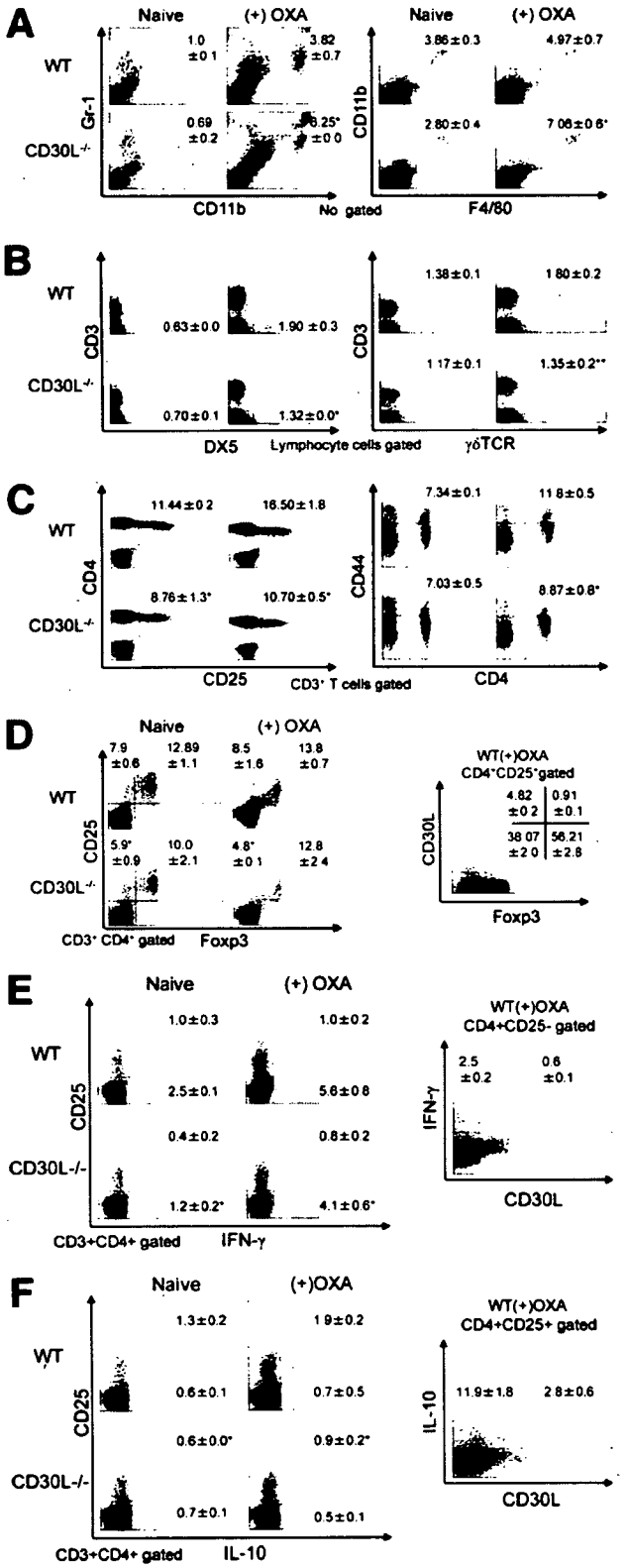


Supplementary Figure 1. Flow cytometry analysis of CD30L or CD30 expression on LP cells in the colon from naive and mice with colitis. (*A*) CD30L expression on laminal propria (LP) cells in the large intestine from naive CD30LKO mice (negative control) and naive WT mice or mice with colitis. Representative staining of various cell surface molecules on LP cells in the colon from mice before and on day 4 after OXA-administration or day 7 after TNBS-treatment. The results are presented as typical profiles after an analysis gate had been set on lymphocyte cells. F4/80$^+$CD11b$^+$ and CD11c$^+$MHC class II$^+$, (*B*) CD3$^+$. (*C*) LP cells of BALB/c or C57BL/6 mice before and after induction of colitis by 0.8% OXA or 3 mg TNBS were obtained and cultured in 96-well plate stimulation with or without 10 μg/mL anti-CD3 mAb. These cells were collected after 24 hr cultured and then analyzed by flow cytometry for CD30 expression with PE-CD30 and isotype control hamster IgG1. Values of each column and *vertical bar* indicate means ± SD for 5 mice within each group. Representative data are shown from 3 independent experiments. Statistically significant differences are shown (*$P < .05$).



Supplementary Figure 2. Flow cytometry analysis of LP cells in the large intestine from naive and OXA-treated CD30L$^{-/-}$ mice. Representative staining of various cell surface molecules on LP cells in the colon from mice before and on day 4 after 0.8% OXA administration. The results are presented as typical profiles after an analysis gate had been set on no gate (*A*), lymphocytes (*B*), CD3$^+$ cells (*C*), CD3$^-$CD4$^-$ cells (*D*) and CD4$^+$CD25$^-$ cells. Intracellular cytokine expression by LP T cells from OXA-treated WT mice or CD30L$^{-/-}$ mice. LP T cells from naive or mice treated with 0.8% OXA 4 days previously were cultured with PMA plus ionomycin and analyzed for the expression of CD4, CD25, CD30L, and IFN-γ (*E*) or IL-10 (*F*) by intracellular staining. The absolute number of each subset was calculated by multiplying the total number of LP T cells by the percentage of each subset. Values of each *column* and *vertical bar* indicate means ± SD for 5 mice within each group. Representative data are shown from 3 independent experiments. Statistically significant differences are shown (*$P < .05$ or **$P < .01$).