

CD30 Ligand/CD30 Interaction Is Involved in Pathogenesis of Inflammatory Bowel Disease

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Abstract

Background and Aims Although CD30 has long been recognized as an important marker in many lymphomas of diverse origin, and as an activation molecule on B and T cells, its primary function has remained obscure. Soluble CD30 (sCD30) is released from CD30 on the cell membrane by enzymatic cleavage. This study investigated the role of CD30 ligand (CD30L)/CD30 signals in intestinal mucosal damage.

Methods Serum sCD30 in patients with ulcerative colitis (UC) and Crohn's disease (CD) and healthy individuals was assessed. A model of enteritis induced by anti-CD30 monoclonal antibody injection was studied in wild-type mice and in CD30L knockout mice.

Results Increased sCD30 was observed in UC and CD patients, and the level was correlated with disease activity in both conditions. In a murine model of enteritis, histological intestinal damage was significantly reduced in CD30L knockout mice with decreased Th1 and Th17 cytokine levels. Moreover, blocking of CD30L/CD30 signals by CD30-immunoglobulin (CD30-Ig) resulted in reduced inflammation.

Conclusions Increased sCD30 expression correlating with disease activity suggested that CD30L/CD30 signals play an important role in pathogenesis of UC and CD. CD30L/CD30 pathway acts as an accelerator of enteritis in a murine disease model. Successful blockade of enteritis by

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CD30-Ig suggests a potential tool for future therapy of inflammatory bowel diseases.

Keywords CD30 · CD30 ligand · Inflammatory bowel disease · Ulcerative colitis · Crohn's disease

Introduction

CD30 and CD30 ligand (CD30L) are glycoproteins that belong to the tumor necrosis factor receptor (TNFR) and tumor necrosis factor (TNF) superfamily, respectively [1, 2]. CD30 is expressed by activated lymphoid and lymph node cells in the parafollicular area, decidua, and in endometrial cells with deciduoid changes. The extracellular part of membrane-bound CD30 can be proteolytically cleaved by the action of a zinc metalloprotease [3], which produces a soluble form of CD30 (sCD30) with a molecular mass of 85/90 kDa. Elevated levels of sCD30 have been observed in patients with various diseases such as systemic lupus erythematosus, rheumatoid arthritis and human immunodeficiency virus-1 infection [4]. The source of sCD30 is assumed to be CD30-expressing cells. Although its function remains to be clarified, sCD30 can bind to CD30L and block the interaction between CD30 and its ligand. CD30L/CD30 signaling is thought to augment T and B cell differentiation and proliferation, and negative selection of T cells in the thymus [2, 5–9]. We have previously reported that triggering of CD30 signals in a lymphoma cell line can down-modulate lymphocyte effector function and proliferation, while directing the cells to lymph nodes and increasing their susceptibility to certain apoptotic signals [10]. We have constructed CD30L knockout mice (CD30LKO), and have found that CD30L/CD30 signals play potent roles in regulation of CD4⁺ T-cell-mediated graft-versus-host disease [11], and in the differentiation of long-lived central memory CD8⁺ T cells following infection [12].

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is of unknown etiology. Recent reports have suggested that chronic inflammation occurs as a consequence of aberrant intestinal immune responses to the bacterial microflora in genetically susceptible individuals [13]. It is believed that T cells, especially T helper (Th) cells, play a crucial role in disease development. Some reports have suggested that Th cells in the intestinal mucosa in CD are polarized to Th1 and to Th2 in UC [13]. In addition, recent evidence has suggested that a new Th subset, Th17, is implicated in the pathogenesis of CD and UC [14, 15]. As regards the involvement of CD30L/CD30 signaling in IBD, serum sCD30 concentration is elevated in UC but not in CD [16]. We have reported previously

[17] that an experimental colitis model induced by oxazolone enema was worsened in CD30LKO mice with increased expression of Th2 cytokines including interleukin (IL)-4 and IL-13. On the contrary, CD30LKO mice were resistant to another experimental colitis by trinitrobenzene sulfonic acid (TNBS) enema, with significant reduction in Th1 cytokine, interferon (IFN)- γ expression. The latter showed the involvement of CD30L/CD30 signaling in Th1-mediated colitis and is inconsistent with the former, which suggests that CD30L/CD30 signals play a role in Th2 responses in UC [16]. We thus considered that many issues remained to be clarified about the role of CD30L/CD30 pathways in the pathogenesis of IBD. To address such issues, we investigated the expression of sCD30 in IBD patients and the effect of inhibiting CD30L/CD30 signals in a murine experimental model of enteritis.

Methods

Patients

All patients in this study were admitted to Kyushu University Hospital, Saiseikai Fukuoka General Hospital, National Fukuoka-Higashi Medical Center, or Harasanshin Hospital between 2003 and 2004. The protocol was approved by the institutional ethical committee at all institutions. Written informed consent was obtained from all patients. We obtained peripheral blood samples from 25 UC patients, 16 CD patients, and 12 hospital employees as control subjects. Nine UC and five CD patients were subjected to examination before and after medical treatment. For diagnosis of UC, all patients underwent colonoscopy and a pathological examination of a colonic biopsy sample, to rule out CD, ischemic colitis, or infectious colitis. The disease activity was scored according to the Rachmilewitz Clinical Activity Index [18]. CD patients underwent colonoscopy and/or X-ray examination of the small intestine, and a diagnosis was made based on the presence of typical longitudinal ulcers and/or a cobblestone appearance, and in some cases, the detection of granuloma by histological examination. UC, ischemic colitis, Behçet's disease, and infectious enterocolitis were all ruled out.

Mice

BALB/cN Sea mice were purchased from Kyudo (Saga, Japan). CD30LKO mice on a BALB/c background were obtained by backcrossing CD30LKO mice with wild-type (WT) BALB/c mice for more than nine generations [11].

Genotypes of CD30LKO mice were screened by PCR as described previously [11]. All mice were 8–12 weeks of age, housed in a pathogen-free facility, and handled according to the recommended guidelines of the Animal Centre of Kyushu University.

Reagents

Anti-mouse CD3 monoclonal antibody (mAb) (145-2C11) was purchased from R&D Systems (Minneapolis, MN, USA), or purified from culture supernatant of a hybridoma purchased from American Type Culture Collection (Manassas, VA, USA). To produce recombinant protein CD30-immunoglobulin (CD30-Ig), murine CD30 cDNA was truncated at the extracellular domain next to the transmembrane segment (nucleotide 906) and fused to the hinge region of murine IgG1, as described previously [6].

sCD30

sCD30 concentrations in human serum were determined by a specific ELISA kit purchased from Bender Med Systems (Burlingame, CA, USA). sCD30 concentration in mouse serum was determined by a specific ELISA kit (GT, Minneapolis, MN, USA).

Experimental Enteritis Model of Anti-CD3 mAb Injection

Anti-CD3 induced enteritis is a well-established experimental model of T-cell-dependent enteritis in mice [19]. The toxic effects of anti-CD3 antibody are caused by activated CD4⁺ and CD8⁺ effector cells in the gut mucosa. WT and CD30LKO mice were injected i.p. with 100 µg anti-CD3 mAb in 500 µl PBS. After 2, 4, 24, and 72 h, peripheral blood was obtained and analyzed. Body weight was measured every day after injection of anti-CD3 mAb. Mice injected with anti-CD3 mAb were killed 24 h after injection, and the terminal ileum was removed. The tissue sections were stained with hematoxylin and eosin (H&E) and examined by optical microscopy. The occurrence of apoptosis in the ileal section was assessed by TUNEL staining using the in situ Apoptosis Detection Kit (Takara Bio, Shiga, Japan).

Cytokine Measurement

Peripheral blood was collected from mice at 2 or 4 h after the injection of anti-CD3 antibodies. Sera were separated by centrifugation (4 °C, 200 × g, 20 min). TNF-α, IFN-γ, IL-4, and IL-17 concentrations in mouse serum were determined with specific ELISA kits (GT).

Statistical Analysis

Student's *t* test was used for statistical comparisons between two groups. *p* < 0.05 was considered statistically significant.

Results

Expression of CD30 in IBD

We analyzed sera obtained from 25 UC and 16 CD patients, and 12 healthy volunteers, whose demographics are summarized in Table 1. The levels of sCD30 in UC and CD patients were significantly higher than those in healthy subjects (Fig. 1a). To investigate the relationship between disease activity and sCD30 expression, levels of sCD30 measured before and after treatment were compared in nine UC and five CD patients (Fig. 1b). Disease activity was reduced in all patients after treatment; similarly, serum levels of sCD30 decreased after treatment in all but one patient with UC. sCD30 levels and clinical scores in UC and CD were assessed and were found to be significantly correlated (Fig. 1c). sCD30 is thought to be generated as a consequence of shedding of CD30 from the membrane of CD30-expressing cells; therefore, these results suggested increased CD30 expression in IBD in association with disease activity.

CD30LKO Mice Are Resistant to Anti-CD3 Antibody Enteritis

To validate a role for CD30 signals in mucosal immunity, we studied anti-CD3-induced enteritis in WT and

Table 1 Patient demographics

	Healthy controls	UC	CD
Number	12	25	16
Male:female	6:6	15:10	10:6
Age (years; mean)	33.6	36.5	36.8
Affected area		Pancolitis: 19	Without colonic lesion: 5
		Left-sided: 5	With colonic lesion: 11
		Proctitis: 1	
Treatment		5-ASA: 22	5-ASA: 10
		Steroid: 12	Steroid: 2
		Apheresis: 7	Infliximab: 1

ASA 5-aminosalicylic acid

Fig. 1 Expression of sCD30 in serum of IBD patients. **a** Concentration of sCD30 in serum obtained from 12 healthy controls and 25 UC and 16 CD patients was analyzed by sCD30-specific ELISA. *Open circles* represent medians of each group. **b** Concentrations of serum sCD30 before and after treatment were compared in nine UC and five CD patients. *Open circles* represent medians of each group. **c** Correlations of sCD30 and clinical scores in UC and CD are shown. * $p < 0.05$, CAI clinical activity index, CDAI Crohn's disease activity index

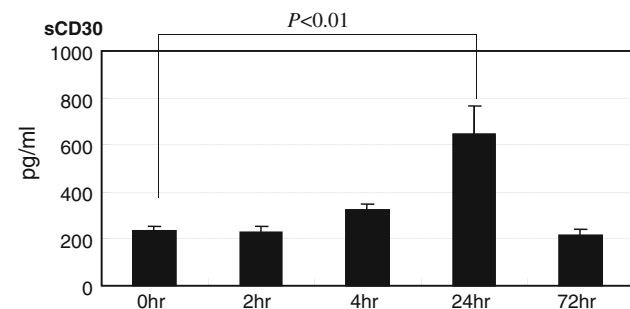
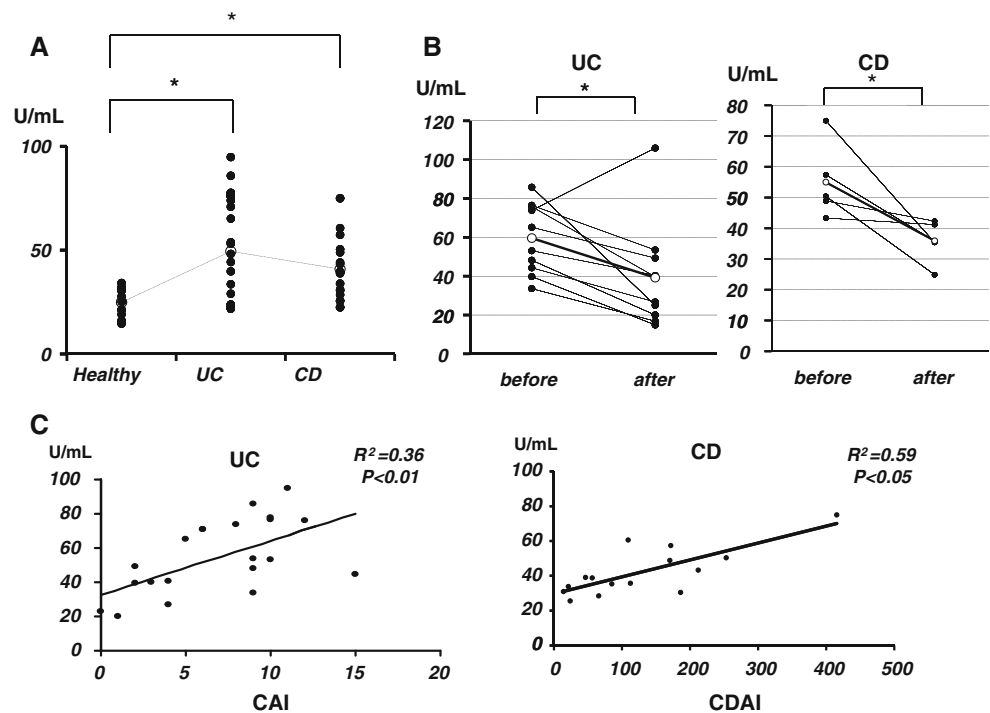


Fig. 2 sCD30 expression in an anti-CD3-induced enteritis model. Time-course of sCD30 levels in mouse serum detected by ELISA at 0, 2, 4, 24, and 72 h after injection of anti-CD3. Serum sCD30 at 24 h after injection was significantly higher than that at 0 h

CD30LKO mice. In this model, the systemic administration of anti-CD3 induces small bowel inflammation and causes diarrhea, piloerection, and changes in overall mobility [19]. We analyzed levels of serum sCD30 in WT mice by ELISA after injection of anti-CD3 antibody. Serum sCD30 levels increased after anti-CD3 antibody treatment and reached a peak at 24 h (Fig. 2), which suggested upregulation of CD30 expression.

To examine the impact of the absence of CD30 signals on anti-CD3-induced enteritis, changes in body weight of WT and CD30LKO mice were measured daily after administration of anti-CD3 (Fig. 3a). Body weight loss in CD30LKO mice was significantly less than that in WT mice at days 2, 4, 5, 6, and 7, which indicated that the absence of CD30 signals diminished anti-CD3-induced

enteritis. Differences in the histopathology of enteritis induced by anti-CD3 in WT and CD30LKO mice were assessed at 24 h after injection. The absence of CD30L was associated with diminished anti-CD3-induced inflammation in the terminal ileum (Fig. 3b), with inhibition of reduction in the villous/crypt length ratio (Fig. 3c). Apoptotic bodies detected by TUNEL staining were counted in five villous crypt units of each group of WT and CD30LKO mice (Fig. 3d). The average number of apoptotic bodies in CD30LKO was significantly lower than that in WT mice (Fig. 3e). In addition, serum levels of IFN- γ (Fig. 4a), TNF- α (Fig. 4b), and IL-17 (Fig. 4c) in CD30LKO were significantly lower than those in WT mice. The level of IL-4 was not significantly altered (Fig. 4d). These results indicated that inflammation of the intestine induced by anti-CD3 antibody was reduced in the absence of CD30L/CD30 signals.

Recombinant Fusion Protein CD30-Ig Suppresses Anti-CD3-induced Enteritis

Recombinant fusion protein (CD30-Ig) contained the extracellular domain of CD30 fused to the hinge, CH2, and CH3 domains of murine IgG1, as described previously [6]. CD30-Ig has appropriate binding activity for the CD30L and blocks CD30L/CD30 signals. One hundred micrograms of CD30-Ig was administered to WT mice i.p. 1 h before and after injection of anti-CD3 mAb. The terminal ileum was removed from mice 24 h later, and tissue sections were stained with H&E and examined by optical

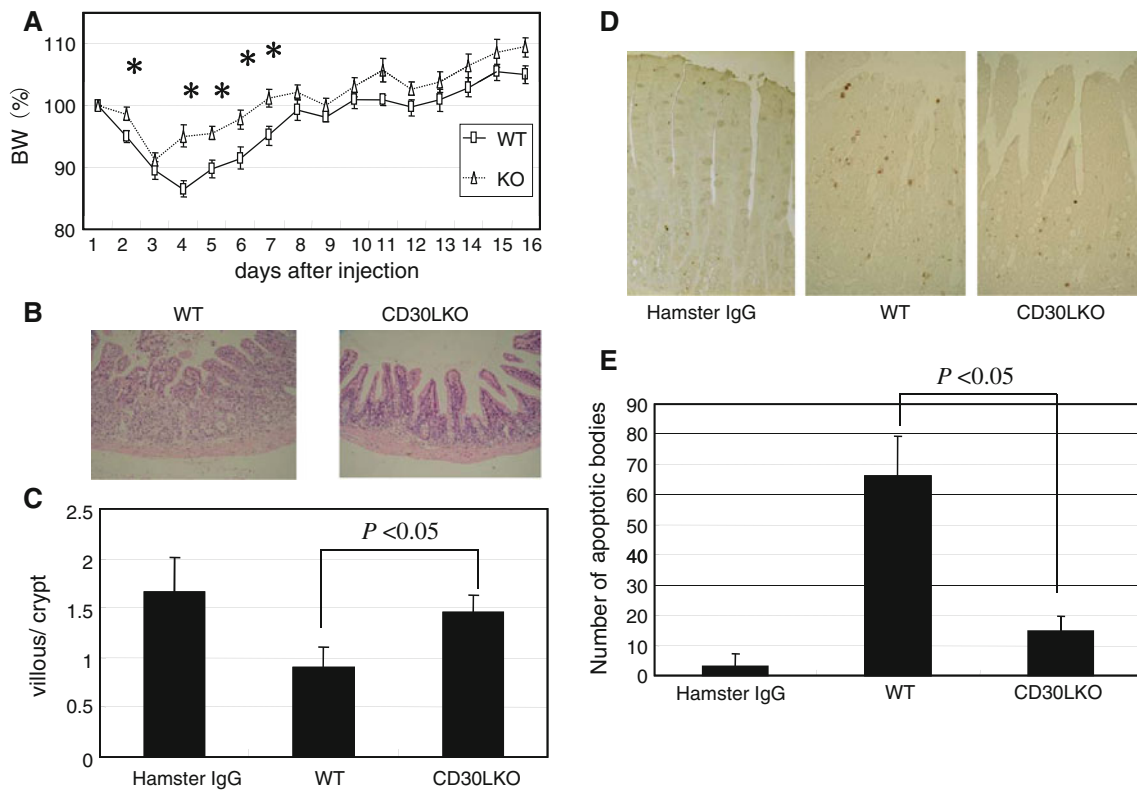


Fig. 3 CD30LKO mice were resistant to anti-CD3-induced enteritis. **a** Body weight changes in WT and CD30LKO mice were measured every day after injection of anti-CD3 mAb. The body weight losses in CD30LKO mice were less than those in WT mice at days 2, 4, 5, 6, and 7 (average of five mice), $*p < 0.05$. **b** Histological findings of anti-CD3-induced enteritis in WT and CD30LKO mice. Terminal ileum was removed from mice injected with anti-CD3 mAb at 24 h

after injection. Tissue sections were stained with H&E and examined by optical microscopy. **c** Five villous crypt units were measured in each mouse. The average villous/crypt length ratio was calculated from three mice in each group. **d** Terminal ileum was removed 24 h after injection, and apoptotic bodies were detected by TUNEL staining. **e** The number of apoptotic bodies was counted in five villous crypt units of each group of mice

Fig. 4 Serum IFN- γ was measured in WT and CD30LKO mice at 4 h after anti-CD3 injection (a). Serum IFN- γ was higher in WT than CD30LKO mice. Serum TNF- α (b), IL-17 (c), and IL-4 (d) were measured in WT and CD30LKO mice at 2 h after anti-CD3 injection. N.S. not significant

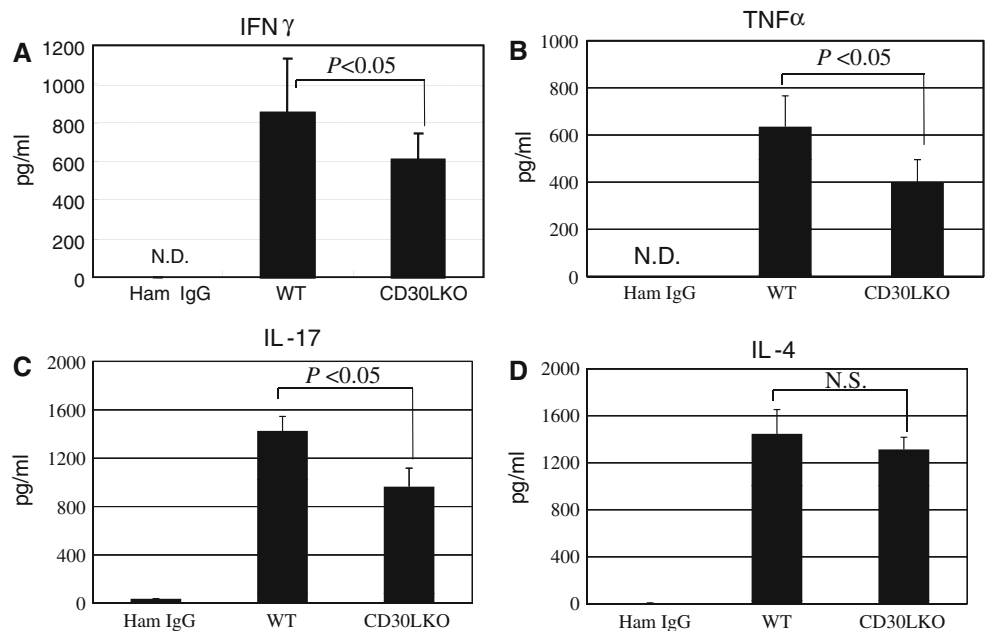
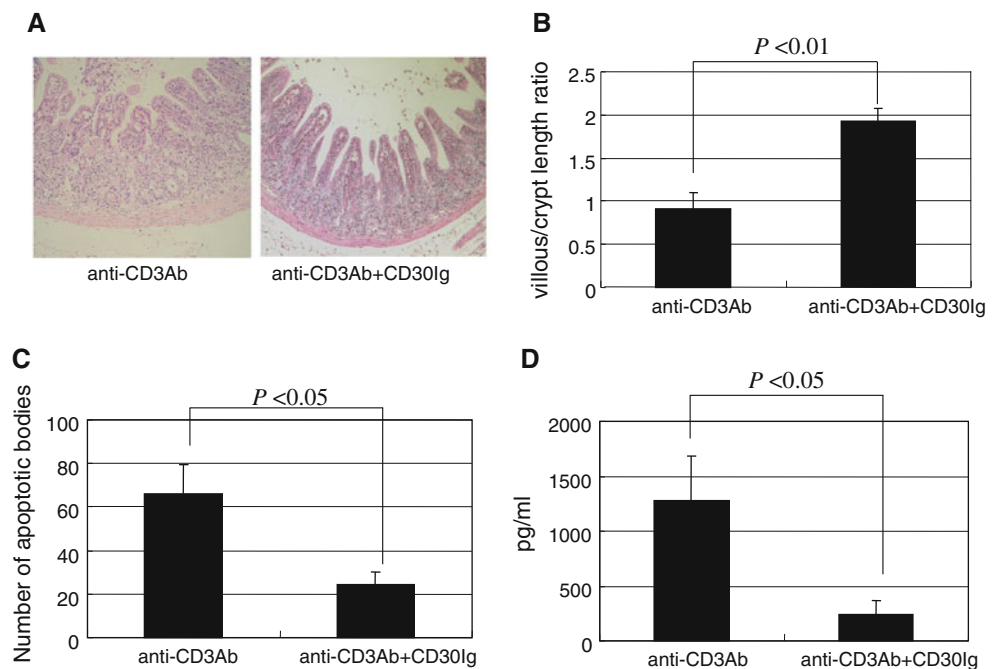


Fig. 5 Recombinant fusion protein CD30-Ig suppressed anti-CD3-induced enteritis. **a** CD30-Ig (100 μ g) was injected i.p. 1 h before and after injection of anti-CD3 mAb. Terminal ileum was removed from mice at 24 h after injection of anti-CD3 mAb. Tissue sections were stained with H&E and examined by optical microscopy. **b** Five villous crypt units were measured in each mouse. The average villous/crypt length ratio was calculated from three mice in each group. **c** The number of apoptotic bodies was counted in five villous crypt units of each group of mice. **d** Serum IFN- γ was measured at 4 h after anti-CD3 antibody injection and was lower in mice with CD30-Ig injection



microscopy (Fig. 5a). Enteritis was significantly reduced in mice that received CD30-Ig. Similar to the experiment with CD30LKO, villous crypt units were measured and villous/crypt length ratio was significantly greater in CD30-Ig-treated mice than in controls (Fig. 5b). Furthermore, the number of apoptotic bodies and the level of serum IFN- γ were also diminished in mice that received CD30-Ig (Fig. 5c, d).

Discussion

The role of CD30L/CD30 signals in mucosal immunity has not received much attention. Giacomelli et al. [16] have reported elevated levels of sCD30 in UC patients. On the contrary, in CD patients, the levels of sCD30 were not significantly higher than those of normal controls. They concluded that, as CD30-expressing cells produce Th2 cytokines, these differences come from the Th2 immune response in UC. Likewise, Elewaut et al. [20] have reported that lymphocytes obtained from UC patients overexpress CD30 when compared to those from CD patients. These studies have indicated that CD30L/CD30 signals play a more important role in UC than in CD. However, after the discovery of Th17 cells, Th reactions in human disorders need to be considered in a new axis of Th1/Th2/Th17 rather than a classical Th1/Th2 paradigm. Therefore, the involvement of Th2 reactions in the pathogenesis of UC needs to be re-evaluated. We thus considered that many issues remained to be investigated on the role of CD30L/CD30 signaling in IBD and conducted this current study.

Contrary to a previous report, we found sCD30 levels were elevated in both CD and UC patients, and were correlated with disease activity. To clarify the reason for these contradictions, analysis of larger numbers of patients is needed. Nevertheless, our results suggest that CD30L/CD30 signals play some role in the development of intestinal inflammation in IBD.

Anti-CD3 antibody enteritis is induced purely by activated T cells. It is a useful murine enteritis model for discerning the role of T cells in enteritis. Merger et al. [19] have reported that apoptosis and mucosal injury are significantly reduced in perforin knockout mice, and a further significant reduction is observed in mice with perforin and Fas ligand (FasL) knockout. Both FasL and CD30L are members of the TNF superfamily. We have reported previously that the CD30 signal induces perforin expression [10]. Thus, we used the anti-CD3-induced enteritis model to analyze CD30L/CD30 signals in CD30LKO mice. As expected, serum sCD30 increased at a peak of 24 h after anti-CD3 antibody injection. sCD30 recovered to normal levels at 72 h after injection of anti-CD3, with recovery of intestinal mucosa indicating that sCD30 level reflected severity of inflammation. These results suggest that reduced sCD30 after treatment in IBD patients is also caused by convergence of disease activity, and is not simply caused by immunosuppressants.

Recently, we have reported that oxazolone and TNBS induce colitis in CD30LKO mice [17]. We have found that CD30LKO mice are susceptible to oxazolone-induced colitis but are resistant to TNBS-induced acute colitis, and have concluded that CD30L plays a crucial role in

deviating CD30⁺ Th cells to Th1 cells in the colon [17]. In anti-CD3-induced enteritis, body weight loss, histological appearance, and serum levels of IFN- γ and IL-17 were significantly suppressed in CD30LKO mice, which suggests that CD30L/CD30 signals deviate Th cells not only to Th1 but also to Th17 phenotype. In addition, CD30-Ig fusion protein, which blocks CD30L, significantly decreased disease activity of anti-CD3-induced enteritis in WT mice, which ruled out other genetic effects due to CD30L deletion in CD30LKO mice. Compared to the agonistic anti-CD30 mAb used in the former study [17], CD30-Ig fusion protein blocked CD30L/CD30 signals and was shown to be suitable for reduction of enteritis *in vivo*. These results suggest that blocking agent of CD30L/CD30 signaling, such as CD30-Ig or antagonistic anti-CD30 antibodies could be new therapeutic tools for IBD.

In this study, the decrease in weight changes in CD30LKO mice in comparison with WT mice was relatively small and did not correlate with the marked differences in histology and the number of apoptotic cells. However, it correlated well with relatively small differences in cytokine productions between CD30LKO and WT mice. It was therefore suggested that weight loss in this model may be due to the augmented expression of inflammatory cytokines as well as intestinal tissue damage itself. Improvement of inflammation seemed less efficient by the blocking of CD30-signal in CD30LKO mice than in the CD30-Ig-treated mice, especially in inflammatory cytokine production. This might be because of the redundancy of CD30L/CD30-signaling. It is well known that TNF/TNFR-signaling is frequently redundant. It is, therefore, reasonable to consider that another signal was augmented to overcome the absence of CD30-signaling in CD30LKO mice. Such mechanism does not work in CD30-Ig-treated mice.

In summary, we showed that expression of sCD30 was elevated in patients with UC and CD, suggesting the involvement of CD30L/CD30 signaling in the pathogenesis of IBD. Using a murine enteritis model and recombinant protein CD30-Ig, we clarified that CD30L/CD30 signals play an important role in induction of enteritis in mice. We also introduced a new strategy for treatment of IBD and other diseases in which CD30L/CD30 signals are involved.

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Conflict of interest The authors have no conflicts of interest to declare.

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